Peter Pietschmann
Editor

Principles of Osteoimmunology

Molecular Mechanisms and Clinical Applications

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Title image legend
Epifluorescence image of multinucleated osteoclasts and precursor cells derived from murine bone marrow cells after eight days of culture. Osteoclast differentiation was induced by medium supplementation with receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). Cells were stained for nuclei, the calcitonin receptor, α-tubulin and the precursor cell specific F4/80 macrophage marker. The colours of the image were artistically enhanced. The image was captured by M. Schepelmann as part of the project discussed in the chapter "Towards the automated detection and characterization of osteoclasts in microscopic images", Heindl et al., in this book.

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Preface

Osteoimmunology is a rapidly developing research field on the crosstalk between bone and the immune system. Examples of such immune-bone interactions are pathogenic mechanisms of bone diseases that are caused by or related to altered immune reactions. The English term "osteoimmunology" was first used in 2000 by Arron and Choi in a comment in Nature (430: 535). Nevertheless, the concept that osteoclasts, multinucleated bone resorbing cells, develop from the monocyte-macrophage lineage dates back to the 1920s. In the 1980s proinflammatory cytokines such as interleukin-1 or TNF-alpha were shown to stimulate bone degradation. The discovery of the RANK/RANKL/osteoprotegerin system and the development of an antibody-based targeted therapy for osteoporosis and other bone diseases have significantly increased the momentum of osteoimmunology.

The purpose of this book is to give an introduction to the emerging field of osteoimmunology to scientists and clinicians working in immunology, pathophysiology and osteology. The book is organized into 11 chapters. The first chapters give an introduction to cell and molecular biology of bone and the immune system, including methodological issues such as automated cell detection and bone markers. Dedicated chapters also describe effects of vitamin D on the immune system and immunological aspects of biomechanics. The next chapters deal with molecular mechanisms and the clinical presentation of osteoimmune diseases such as osteoporosis and rheumatoid arthritis as well as preclinical and clinical data on the treatment of bone diseases by RANKL inhibition. The final chapter describes osteoimmunological aspects of periodontal diseases.

I am very thankful to all authors who contributed to this book for their valuable time, expertise and effort. Moreover, I would like to acknowledge the great help and dedication of the staff from SpringerWienNewYork, in particular Mag. Angelika Heller and Dr. Amrei Strehl. Special thanks also to Maria Steiner and Birgit Schwarz for their continuous support of this book project.

I am convinced that our readers will enjoy the book as much as I enjoyed editing it.

Peter Pietschmann August 2011

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Basics of Bone Biology

1

Martina Rauner, Nicola Stein, Lorenz C. Hofbauer

1.1 Introduction to Bone

1.1.1 Bone Function and Structure

Bone is the major constituent of the skeleton which is a hallmark of all higher vertebrates. Besides the protection of internal organs and the support of body structures, the most important functions of bone are to serve as an attachment site for muscles allowing locomotion and provide a cavity for hematopoiesis in the bone marrow (Mendez-Ferrer et al. 2010; Zaidi 2007). Moreover, bone has a central role in mineral homeostasis as it functions as a reservoir for inorganic ions that can be mobilized rapidly on metabolic demand.

Although bone is often considered an inert, static material, it is a highly organized, living tissue that undergoes constant remodeling. Different cell lineages have emerged to serve distinct skeletal functions. While cells from the hematopoietic lineage, such as osteoclasts, break down bone tissue to remove old and damaged bone, or release calcium to maintain calcium homeostasis, cells from the mesenchymal lineage, including chondroblasts, fibroblasts and osteoblasts construct and later remodel bone tissue (Jiang et al. 2002). Osteoblasts produce the organic components of the extracellular matrix, which mainly includes type I collagen (approximately 95 %), but also non-collagenous proteins (i. e. osteocalcin, osteopontin, osteonectin, bone sialoprotein) and proteoglycans. The inorganic matrix predominantly contains calcium and phosphorus, appearing as hydroxyapatite crystals ($[3Ca_3(PO_4)_2](OH)_2$), and is deposited into the collagenous matrix. This complex organization confers rigidity and strength to the skeleton while maintaining a high degree of elasticity.

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Two types of osseous tissues are found in all bones: cortical or compact bone and trabecular or cancellous bone, sometimes also referred to as spongy bone (Fig. 1). Cortical bone is mainly found in the shafts of long bones (diaphyses) and is made of numerous overlapping cylindrical units termed Haversian systems or osteons. The central Haversian canal, containing the blood vessel and nerves, is surrounded by densely packed collagen fibrils which are formed into concentric lamellae. Osteocytes, terminally differentiated osteoblasts, are located between concentric lamellae and are connected to each other via canaliculi, allowing the exchange of nutrients and metabolic waste and the sensation of mechanical stress. Volkmann's canals are responsible for the conjunction of blood vessels from the inner and outer bone surfaces to the vessels of the Haversian canals. The dense organization of cortical bone thus provides maximum strength and load-bearing capacity by being highly resistant to bending and torsion. Cancellous bone, on the other side, is predominantly found at the ends of long bones (epiphyses) as well as in flat bones and in vertebral bodies where force may be applied at variable angles. It is composed of a meshwork of trabeculae, thereby reducing skeletal weight without compromising strength. This particular construction also establishes a vast surface area. Considering that bone remodeling only takes place at bone surfaces, cancellous bone is quick to render metabolic activities but also disproportionally susceptible to damage when net bone loss occurs.

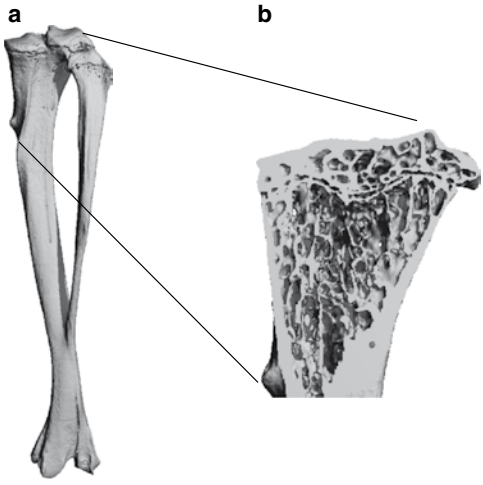

Fig. 1 Illustration of compact and cancellous bone. (**a**) Whole tibial structure obtained by micro-computed tomography. 12 microns isotropic spatial resolution. (**b**) Lateral view of the tibia. The trabecular meshwork structure is clearly visible at the epiphysis. Cortical bone is found at the shaft. Source: Peter Varga and Phillipe Zysset, Technical University of Vienna, Austria

1.1.2 Ossification Processes

Ossification occurs either intramembranously or endochondrally. During skeletal development, flat bones (e. g. calvariae) and some irregular bones are formed by intramembranous ossification where bony tissue directly forms from the connective tissue without an intermediate cartilage stage (Blair et al. 2008). Within this process, mesenchymal stem cells (MSCs) condense into highly vascularized sheets of primitive connective tissue at sites of eventual bone formation. Certain MSCs group together and differentiate into osteoblasts that deposit extracellular matrix (osteoid) which is subsequently mineralized to form the bone matrix. These small aggregates of bone tissue, termed bone spicules, continuously expand with new MSCs lining on the surface, differentiating into osteoblasts and secreting extracellular matrix. Once they become embedded by the secreted mineralized matrix, osteoblasts terminally differentiate into osteocytes. As the bone spicules grow and interconnect with others, a trabecular network of woven bone – also referred to as primary spongiosa – is formed. Although woven bone forms quickly with the collagen fibres being randomly organized, it is structurally weak. Thus, it is soon replaced by a more solid lamellar bone, which is composed of a highly organized collagen structure (Frost and Jee, 1994). Several collagen fibers align in the same layer, and several such concentric layers stacked in alternating orientations finally constitute a bone unit called osteon (Parfitt 1988). This highly sophisticated organization confers strength and resistance to torsion forces to lamellar bone. However, the complex architecture and orderly deposition of collagen fibers requires more time and restricts the formation of osteoid to 1–2 μm per day. Besides the creation of woven bone in fetal bone development, it may also occur in adults after fractures or in patients with Paget's disease (Parfitt 1994).

In contrast to flat and irregular bones, bones of the vertebral column, pelvis, and extremities develop by endochondral ossification. Thereby, hyaline cartilage devoid of blood vessels is first formed and then replaced by bone matrix starting at the primary ossification center. During embryonic development chondrocytes congregate to a cartilaginous model that alleges the shape of the future bone and after the local enlargement of chondrocytes (hypertrophy) endochondral bone formation is initiated in the middle of the shaft at the primary ossification center. The perichondrium, which surrounds the cartilage model, becomes invaded with blood vessels and then is called periosteum (Stanka et al. 1991; Streeten and Brandi 1990; Trueta and Buhr 1963). The periosteum contains layers of MSCs that differentiate into osteoblasts during development, when the bone increases its width (appositional growth), or after fractures, when new bone formation is required. In addition to its important function to supply nutrients via the blood vessels, the periosteum contains nociceptor nerve endings that allow the sensation of pain (Fortier and Nixon 1997; Grubb, 2004; Jimenez-Andrade et al. 2010).

The growth plates are characterized by the orderly proliferation and maturation of chondrocytes in longitudinal columns, forming stratified zones of reserve, proliferative, maturing, and hypertrophic cartilage (Poole et al. 1991). Hypertrophic chondrocytes secrete large amounts of a specialized extracellular matrix rich in col-

lagen type X and alkaline phosphatase, which becomes calcified. After the calcification of the collagenous matrix, hypertrophic chondrocytes start producing matrix metalloproteinase 13, which is crucial for the subsequent degradation of the cartilage matrix, and undergo apoptosis (Stickens et al. 2004). By doing so, transverse septa of cartilage matrix surrounding them are broken down, leaving vertical septa largely intact, but allowing the entry of capillaries and invading cells of the ossification front. These cells mainly include cells of the mesenchymal (osteoblast precursors and stromal cells) and hematopoietic lineages (osteoclast precursors and other hematopoietic lineages that constitute the bone marrow). After osteoblast precursor cells have migrated to the surface of remnant cartilage spicules, they differentiate into fully mature osteoblasts and deposit a predominantly type I collagen-containing extracellular matrix (osteoid), which subsequently becomes mineralized into the mature bone matrix. The ossification continues towards the ends of the bones, where the further elongation of long bones occurs in the growth plates of the metaphysis. Finally, the trabecular bone in the diaphysis is broken down by osteoclasts to open up the medullary cavity.

The same process applies to the secondary ossification center, located in the epiphysis, except that the trabecular bone is retained (Alini et al. 1996). The length of bones increases until the early twenties through a process similar to endochondral ossification (Riggs et al. 1999). The cartilage in the epiphyseal plate proliferates constantly and is continuously replaced by bone matrix until the skeleton has reached maturity and the epiphyseal plate has become almost completely ossified. The articular cartilage remains uncalcified and covers the ends of the long bones. Due to its incredibly low coefficient of friction, coupled with its ability to bear very large compressive loads, articular cartilage is ideally suited for placement in joints, such as the knee and hip.

1.2 Bone Remodeling

During a person's life-time, continuously changing functional demands require permanent adaptation of the bone structure and microarchitecture. Wolff has observed this principle of functional adaptation already over 100 years ago (Wolff 1892). The process of where "form follows function" occurs in conditions of disuse (as during immobility, space flights, or long-term bed rest), overloading (weight gain), growth, and after fracture healing, and consists of two activities, namely, bone formation and bone resorption (Sommerfeldt and Rubin 2001; Frost 1990). While these processes are locally separated in modeling (Frost 1990), bone remodeling is characterized by the spatial and temporal coupling of bone formation by osteoblasts and bone resorption by osteoclasts (Rodan and Martin 1981). The so called basic multicellular unit (BMU) is covered by a canopy of cells that creates a bone remodeling compartment (BRC). While the nature of the canopy cells remains under debate, evidence in humans suggests that it is bone-lining cells, generating a unique microenvironment to facilitate coupled osteoclastic bone resorption and osteoblastic synthesis

(Andersen et al. 2009). Interestingly, the action of BMUs slightly differs in cortical (endocortical as well as intracortical surfaces) and trabecular bone. While in cortical bone the BMU forms a cylindrical tunnel of about 2,000 µm long and 200 µm wide and the BMU burrows through the bone with a speed of 20–40 µm/day, the remodeling process in trabecular bone is mainly a surface event reaching a depth of approximately 50 µm. With a speed of 25 µm/day, active remodeling sites of BMUs in trabecular bone cover areas of varying sizes ranging from about 100 – 1,000 µm². In general, approximately 5–25 % of bone surface is undergoing bone remodeling (Parfitt 1994; Raisz 1988), thereby restoring microdamages and ensuring mechanical integrity as well as regulating the release of calcium and phosphorus, while maintaining the global bone morphology.

An active BMU performs one bone remodeling cycle that occurs over several weeks and includes four main processes: activation, resorption, reversal and formation (Parfitt 1988). While the process of bone resorption is usually accomplished within 2–3 weeks, the new synthesis of bone requires around 2–3 months. The remodeling cycle is initiated by the detection of signals that induce the activation of the quiescent bone surface, which is covered with bone lining cells. These signals may be provided through osteocytes that sense mechanical strain or are affected by structural damage, which severs the processes of osteocytes in their canaliculi and leads to osteocyte apoptosis (Aguirre et al. 2006; Bonewald 2007; Hazenberg et al. 2006; Verborgt et al. 2002). Alternatively, hormone actions (e. g. estrogen or parathyroid hormone (PTH)) due to more systemic changes in homeostasis or effects of corticosteroids on bone cells may negatively alter osteocyte biology. Current research points towards an intricate communication between osteocytes, which sense bone damage deep within the osteon or hemiosteons, and lining cells on the bone surface, which receive signals through the long processes of osteocytes, and communicate the health status of the bone to the marrow environment to initiate the establishment of a BRC (Hauge et al. 2001). Osteocyte apoptosis may also contribute to the recruitment of osteoclast precursor cells by diminishing the osteocytic secretion of factors that usually inhibit osteoclast formation, such as transforming growth factor-β (TGF-β) (Heino et al. 2002). *In vivo* evidence indicates that osteocyte apoptosis precedes osteoclast formation as osteocyte apoptosis occurs within three days of immobilization and is followed within two weeks by osteoclastogenesis (Aguirre et al. 2006). Although the process of osteoclast precursor attraction is not fully understood yet, osteoblast-secreted products including monocyte chemoattractant protein-1 (MCP-1) and the osteoclast differentiating factor receptor activator of NF-kB ligand (RANKL) may play an important role (Li et al. 2007). After osteoclast precursor cells are recruited to the activated surface they fuse to form mature, bone resorbing osteoclasts (Vaananen and Horton 1995). The osteoclasts attach to the surface and form a ruffled border at the bone/osteoclast surface that is completely surrounded by a sealing zone. Thereby, osteoclasts create an isolated acidic microenvironment in order to dissolve the inorganic matrix and degrade the organic matrix with specific enzymes (Teitelbaum 2000). As bone resorption subsides and a resorption pit with a demineralized collagen matrix remains, osteoclasts disappear and mononuclear cells of undetermined lineage remove the colla-

gen remnants and prepare the surface for bone formation. This phase is called reversal. Currently, there is a debate about whether the reversal cell is of hematopoietic or mesenchymal origin. Recent evidence suggests that this cell type may be a resident macrophage of the bone termed osteomacs (Pettit et al. 2008). These cells are positive for the macrophage markers F4/80+ and CD68, but negative for the osteoclast marker tartrate-resistant acid phosphatase (TRAP), and are found throughout the periosteum and endosteum. Moreover, these cells have been shown to produce MMPs, which are required for matrix degradation, as well as TGF-β and ephrin B2, which may promote osteoblast recruitment, differentiation, and/or activation of bone lining cells (Chang et al. 2008; Compagni et al. 2003). Thus, these cells would be the ideal coupling agents of bone resorption and formation. However, further research is needed to clarify the nature of the reversal cells. After the reversal phase, the bone remodeling cycle is finished with the synthesis and deposition of bone matrix by osteoblasts until an equal amount of bone is reproduced. Also in this case, the mechanisms that terminate bone formation are not known, but may be mediated by signals from osteocytes that have become embedded in the mature bone matrix. Finally, bone lining cells build a canopy covering the surface, keeping the material dormant until the next cycle (Fig. 2).

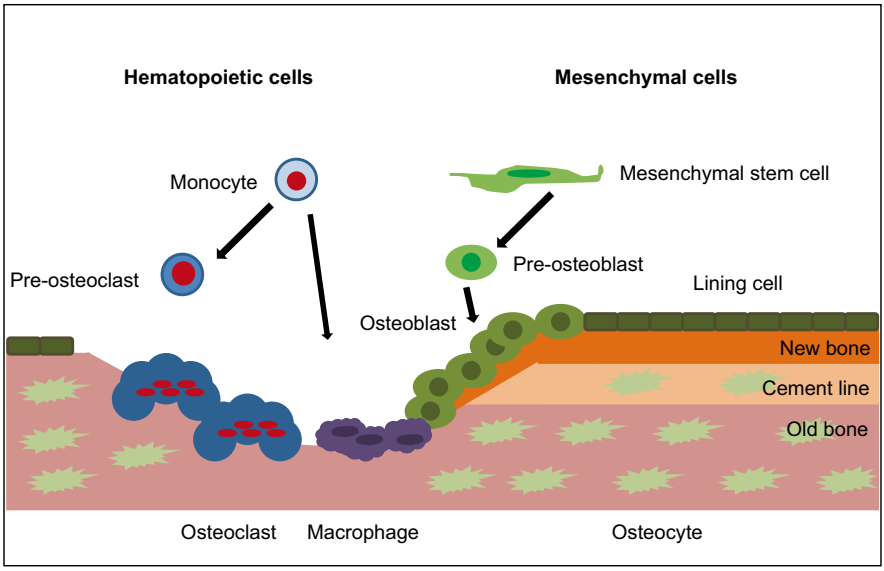

Fig. 2 Bone remodeling. Monocytes from the hematopoietic lineage differentiate into osteoclasts, which resorb old and damaged bone tissue. Macrophages, which also originate from the hematopoietic lineage, contribute to the initiation of bone remodeling and attract osteoblast precursors that mature to bone-forming osteoblasts at the bone surface. After filling the resorption lacunae, osteoblasts become embedded by the bone matrix and turn into osteocytes. Quiescent lining cells remain at the bone surface

1.3 Key Players of Bone Remodeling

1.3.1 Cells of the Osteoblast Lineage – Osteoblasts, Osteocytes, Bone Lining Cells

Osteoblasts are derived from MSCs and their primary function is to synthesize the organic collagenous matrix and orchestrate its mineralization by producing bone matrix proteins including osteocalcin, osteopontin and bone sialoprotein, and providing optimal environmental conditions for crystal formation (Ducy et al. 2000). Due to their active protein machinery, osteoblasts have a prominent golgi apparatus and endoplasmatic reticulum. As mentioned earlier, osteoblasts are also the main producers of RANKL and its decoy receptor osteoprotegerin (OPG), and are therefore critically involved in regulating osteoclastogenesis (see also 1.4.2). Fully differentiated osteoblasts that are surrounded by mineralized bone tissue are called osteocytes and act as mechanosensors in bone tissue (Paic et al. 2009). They are the most numerous cells within the bone tissue and scattered evenly through the matrix. With their flattened morphology and long processes, they form a sensory network which allows the detection of abnormal strain situations such as generated by microcracks (Hirao et al. 2007; Martin and Seeman 2008). By communicating these signals to bone lining cells (the second terminally differentiated osteoblast cell type) or secrete factors that recruit osteoclasts, osteocytes initiate the repair of damaged bone. Other emerging roles of osteoblast lineage cells include the maintenance of hematopoietic stem cell (HSC) niches and HSC homing, as well as acting as non-professional antigen-presenting cells in conditions of inflammation (Fleming et al. 2008; Jung et al. 2007; Mendez-Ferrer et al. 2010; Ruiz et al. 2003; Schrum et al. 2003; Skjodt et al. 1989). While the capacity to stimulate effector cells of the immune system may only be relevant under pathophysiological conditions, the osteoblast-driven maintenance of the stem cell niche is of critical importance for the homeostasis of hematopoiesis. Experiments in mice have shown that the number of long-term repopulating HSCs increases or decreases in parallel with *in vivo* osteoblast stimulation by PTH or osteoblast ablation using a mouse genetic approach (Visnjic et al. 2004). Although the underlying signaling events are not fully understood yet, several mechanisms such as the selective expression of signaling molecules (i. e. jagged, G-protein Gsα), adhesion molecules (i. e. integrins, N-cadherin), and components of the ECM (i. e. proteoglycans) may determine the long-term repopulating ability of HSCs and their ability to home into the bone marrow.

MSCs give rise to a variety of cells including osteoblasts, adipocytes, chondrocytes, and myoblasts (Pittenger et al. 1999). The MSC goes through several progressive steps in generating progeny with progressively more limited differentiation capacities until the differentiated end-stage cell is able to express distinct functional markers and morphological traits. Typical osteoblast markers include alkaline phosphatase (ALP) and type I collagen, as well as various non-collagenous proteins such as osteocalcin, osteopontin or bone sialoprotein. However, cells of the osteoblastic lineage also selectively express proteins at distinct differentiation stages, such as

RANKL in immature osteoblasts or osteocalcin and sclerostin in fully mature osteoblasts or osteocytes.

Osteoblasts express receptors for various hormones including PTH, 1,25-dihydroxyvitamin D_3, estrogen, glucocorticoids, and leptin, which are involved in the regulation of osteoblast differentiation (see 1.4.1). Furthermore, osteoblasts are regulated by multiple local factors including bone morphogenetic proteins (2, 4, 6, and 7) (Shore et al. 2006; Storm and Kingsley 1999; Wu et al. 2003; Wutzl et al. 2010), growth factors (transforming growth factor-β, epidermal growth factor, insulin-like growth factor) (Canalis 2009), Sonic and Indian hedgehogs (Guan et al. 2009; Maeda et al. 2007), as well as members of the Wnt family in a paracrine and autocrine fashion (Bodine and Komm 2006). Because the Wnt signaling pathway is of such critical importance for bone mass maintenance, it will be discussed here in more detail.

Wnt signaling is highly conserved throughout evolution among a variety of species and plays an important role in regulating cellular processes, such as proliferation, differentiation, cell survival and motility (van Amerongen and Nusse 2009). Wnt signaling further plays a key role in embryonic development and maintenance of tissue homeostasis, including bone. Wnt proteins are cystein-rich glycoproteins that act on target cells by binding to the seven-span transmembrane receptor protein Frizzled (FZD), and low-density lipoprotein receptor-related proteins 5 and 6 (LRP 5/6). In bone, various components of this pathway have been shown to positively or negatively regulate osteoblast differentiation (Bodine and Komm 2006). Evidence that the Wnt/β-catenin pathway is involved in bone mass homeostasis has been provided by observations of mutations in the LRP5 gene, in which gain-of-function mutations led to a high bone mass phenotype in humans and mice, and loss-of-function mutations led to low bone mass phenotypes (Boyden et al. 2002; Gong et al. 2001; Van Wesenbeeck et al. 2003).

Wnt signaling comprises several pathways, that are usually divided into the canonical or β-catenin-dependent pathway and non-canonical or β-catenin-independent pathways. As the canonical Wnt pathway seems to be critical for bone mass maintenance, only this pathway will be presented in this chapter. In the absence of Wnt ligands, cytoplasmic levels of β-catenin are kept low through the continuous ubiquitin-proteasome-mediated degradation of β-catenin, which is regulated by a multiprotein complex containing axin, adenomatous polyposis coli, glycogen synthase kinase 3β (GSK3β), and casein kinase 1α (CK1α). The canonical pathway is activated upon binding of Wnt proteins to a receptor complex consisting of FZD and its co-receptor, LRP5 or LRP6. Disheveled (Dvl) is then phosphorylated by CK1α and in turn induces the formation of another protein complex consisting of Dvl, Frat1, axin as well as LRP5/6 and FZD. This interaction ultimately leads to the inhibition of GSK3β and results in the stabilization of β-catenin, which then translocates into the nucleus to join T cell factor (TCF)/lymphoid enhancer binding factor (LEF) and other factors to induce the transcription of Wnt target genes (Clevers 2006). Wnt signaling is regulated at various levels such as through the presence or absence of multiple Wnt ligands, co-receptors, intracellular signaling molecules, and transcription factors. Furthermore, it is tightly regulated by a series of extracellular inhibitors including members of the secreted frizzled-related protein (sFRP) family and Wnt inhibitory

factor that bind to Wnt ligands, as well as dickkopfs (Dkks) and sclerostin, both binding LRP5/6 (Semenov et al. 2005; Tian et al. 2003). In both cases, these interactions lead to the blockade of Wnt ligands binding to FZD receptors. Many Wnt inhibitors have been proposed as therapeutic targets for increasing bone mass by applying neutralizing antibodies, whereas sclerostin may be of particular interest due to its specific expression solely in osteocytes (Keller and Kneissel 2005; Paszty et al. 2010).

At a transcriptional level, osteoblast differentiation is induced by the master transcription factor runx2 (runt-related transcription factor 2, also called core binding factor 1, Cbfa1) and several signaling pathways converge to increase runx2 expression. Runx2-deficient mice have no osteoblasts and thus only contain a cartilage-like skeleton (Harada et al. 1999; Miller et al. 2002). Intriguingly, although runx2 supports osteogenic differentiation, it inhibits osteoblast maturation into osteocytes, keeping osteoblasts in an immature state (Lian et al. 2006). Runx2 expression is induced by BMPs, TGFβ1, Indian hedgehog, members of the Wnt pathway and is tightly regulated by various post-translational modifications as well as co-repressors, such as Twist and menin-1, and co-activations, such as TAZ. Even though runx2 is regarded as the master transcription factor for osteoblasts, other transcription factors also participate in the regulation of osteoblast differentiation, including osterix (also called specificity protein 7, sp7) (Kim et al. 2006a), β-catenin (Krishnan et al. 2006), dlx3 and dlx5 (distal-less homeobox) (Harris et al. 2003), msx2 (homeobox factor) (Liu et al. 1999; Satokata et al. 2000), ATF4 (activating transcription factor 4) (Tozum et al. 2004), as well as NFATc1 (nuclear factor of activated T cells c1) (Koga et al. 2005). Many of these factors control osteoblast differentiation at very specific locations such as dlx proteins in the skull.

After osteoblasts have fully matured and deposited a mineralized matrix surrounding them, they become osteocytes, which serve functions different from matrix deposition. As mentioned above, osteocytes are evenly located throughout the bone tissue and produce a dense network by connecting each other via gap junctions on their processes. In this respect, connexion-43 seems to play a critical role in the formation of hemichannels which allow an extensive communication between two osteocytes (Plotkin et al. 2002, 2008). Mice made osteocyte-depleted exhibit enhanced bone fragility, intracortical porosity, and microfractures, indicating the crucial function of osteocytes to maintain bone integrity (Tatsumi et al. 2007). Also several other studies have shown that loss of osteocyte viability is related to bone loss (Aguirre et al. 2006; Teti and Zallone 2009; Weinstein et al. 2000). Besides mechanosensation, osteocytes express several mineralization inhibitors including fetuin-A, dentin matrix protein-1, phex, and the Wnt inhibitor sclerostin, which allows them to control the amount and quality of the bone matrix (Coen et al. 2009; Liu et al. 2009; Poole et al. 2005). Dentin matrix protein-1-deficient mice, for example, show impaired osteocyte maturation, increased fibroblast-growth factor-23 expression, and severe abnormalities of bone mineralization (Feng et al. 2006). Of note, also glucocorticoids have the potential to increase the expression of mineralization inhibitors, thereby compromising bone quality and bone strength (Yao et al. 2008).

1.3.2 Cells of the Osteoclast Lineage – Osteomacs and Osteoclasts

Tissue-resident macrophages, also referred to as osteomacs, and osteoclasts both derive from the hematopoietic monocytic lineage. The concept of osteomacs has only recently been developed due to thorough observations of periosteal and endosteal tissues and the bone remodeling compartment (Pettit et al. 2008). Pettit et al. determined that osteomacs constitute about one sixth of the total cells within osteal tissues and span a network along bone surfaces with their stellate morphology. Due to their abundance and widespread location, it is likely that osteomacs contribute to immune surveillance in the bone marrow compartment and react quickly to inflammatory stimuli. Osteomacs are distinguishable from osteoclasts by the expression of the murine macrophage marker F4/80, which is not present on osteoclasts, and by being negative for osteoclast-specific markers such as TRAP (Chang et al. 2008). As they are also located at the bone remodeling compartment, it has been suggested that osteomacs participate in the reversal phase of bone remodeling and closely interact with osteoblasts through the production of osteoblast-stimulating factors, such as bone morphogenetic protein-2 or transforming growth factor-β.

Osteoclasts are tissue-specific giant polykaryons (up to 100 μm in diameter) derived from the monocyte/macrophage hematopoietic lineage and are the only cells capable of breaking down large amounts of mineralized bone, dentine and calcified cartilage (Teitelbaum 2000). Bone resorption is a crucial step in bone remodeling which is necessary for healthy bone homeostasis, thereby, repairing microdamages and adapting to new mechanical loads and altered metabolic conditions. Bone remodeling starts with the retraction of bone lining cells uncovering bone tissue and attracting mononuclear precursors to the bone surface. The earliest step in osteoclastogenesis is the determination of the stem cell precursor to the osteoclastic lineage following the induction of PU.1 (Tondravi et al. 1997). Soon thereafter, precursors express the M-CSF receptor, *c-fms*, and after activation with the ligand, proliferation is induced. The next determination step towards a mature osteoclast is the expression of receptor activator of NFkB (RANK). The presence of its ligand, RANKL, is essential for the formation and fusion of multinucleated cells. Mice lacking either RANKL or RANK have no osteoclasts and suffer severe osteopetrosis (for more detail see 1.4.2) (Anderson et al. 1997; Dougall et al. 1999; Kong et al. 1999b; Lacey et al., 1998; Yasuda et al. 1998). RANK signaling activates several transcription factors that are essential for osteoclastogenesis including activated protein-1, NFkB, or nuclear factor of activated T cells (NFAT). In the osteoclast, most signals converge to induce the activity of NFATc1. This is also proven genetically, as embryonic precursors lacking NFATc1 fail to become osteoclasts (Takayanagi et al. 2002). Importantly, NFATc1 is indispensible and sufficient for osteoclastogenesis, as its overexpression yields osteoclasts even in the absence of RANK signaling (Matsuo et al. 2004).

Although several down-stream effectors of RANK signaling induce NFATc1 expression, the mechanisms that induce NFATc1 in a calcium-dependent way have only recently been identified. Therein, immunoreceptor tyrosine-based activation

motifs (ITAMs)-containing adaptor molecules, such as DAP (DNAX-activating protein) 12 and Fc common receptor γ chain (FcRγ) have been shown to be indispensable for osteoclastogenesis as mice deficient for both receptors are severely osteopetrotic (Koga et al. 2004; Mocsai et al. 2004). The activation of phospholipase-Cγ, Syk, and Tec kinases has been shown to be required for the activation of calcineurin-dependent calcium (Faccio et al. 2005; Mocsai et al. 2004; Wada et al. 2005). Paired immunoglobulin-like receptor-A (PIR-A) and osteoclast-associated receptor (OSCAR) have been found to associate with FcRγ (Kim et al. 2002), whereas triggering receptor expressed on myeloid cells-2 (TREM-2) and signal-regulatory protein-b1 (SIRP-β1) bind to DAP12. These signals are considered to act as co-stimulatory signals for RANKL in osteoclast precursors, since those signals alone are not able to induce osteoclastogenesis (Koga et al. 2004).

Mature osteoclasts express several specific proteins including TRAP, cathepsin K, calcitonin receptor (CTR), and integrin receptors (Teitelbaum 2000, 2003). Via integrins, osteoclasts attach very tightly to the matrix (sealing zone), thereby, creating an isolated lacuna (Howship's lacuna) able to maintain an acidic environment necessary for matrix dissolution (Mimura et al. 1994; Miyauchi et al. 1991). At least four integrin receptors are expressed in osteoclasts, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_2\beta_1$ and $\alpha_v\beta_1$ binding to various extracellular matrix proteins such as vitronectin, collagen, osteopontin and BSP. After attachment, intracellular rearrangements lead to the polarization of the cell borders, whereas the sealing zone is adjacent to the baso-lateral domain and the ruffled border, respectively. At the opposite side of the ruffled border emerges the functional secretory domain. The ruffled border and the functional secretory domain are connected to each other via microtubules on which exocytotic vesicle traffic has been observed, suggesting the secretion of resorbed material into the extracellular space (Vaananen and Horton 1995). In addition to the development of distinct membrane domains, the cytoskeleton undergoes organizational changes, creating a dense actin-ring in osteoclasts preparing for resorption (Silver et al., 1988). This process has been shown to be greatly dependent on Rho-GTPases, which require the mevalonate pathway for isoprenylation and activation (Chellaiah 2006). Of note, bisphosphonates have been shown to block osteoclast activity by inhibiting farnesyl diphosphate synthase, a critical enzyme in the mevalonate pathway.

The resorption of bone matrix takes place in the resorption lacuna. The ruffled border is formed by the fusion of cytoplasmic acidic vacuoles, thereby releasing acid into the resorption lacuna and initiating rapid dissolution of the hydroxyapatite crystals (Blair et al.,1989; Teti et al. 1989). Furthermore, ATPases, located in the ruffled border, additionally transport protons into the Howship's lacuna (Li et al. 1999; Mattsson et al. 1994). The protons are supplied by the reaction of water and carbon dioxide catalyzed by the enzyme carbonic anhydrase II resulting in the formation of protons and HCO_3^-. Whereas H^+ is pumped into the resorption lacuna, HCO_3^- is transported into the extracellular space via HCO_3/Cl exchangers. The imported chloride ions are also pumped into the resorption lacuna to form hydrochloric acid with a pH as low as 4, which is capable of dissolving the mineralized matrix (Silver et al. 1988). The organic matrix is degraded by various enzymes, including

TRAP, cathepsin K and matrix MMP-9. Cathepsin K is a lysosomal cysteine proteinase capable of degrading type I collagen (Gelb et al. 1996). Although osteoclasts form in cathepsin K-deficient mice, build a ruffled border and are able to mobilize bone mineral, they are unable to efficiently degrade the collagen matrix and thus resorb bone (Saftig et al. 1998). Furthermore, active osteoclasts express high levels of matrix metalloproteinases such as TRAP and MMP-9 (Okada et al. 1995; Wucherpfennig et al. 1994). Using electron microscopy Okada and colleagues were able to show that MMP-9 degraded collagen into fragments, suggesting the involvement of MMP-9 in the resorption process. Stronger evidence is provided by mice lacking MMP-9, which are severely osteopetrotic and have difficulties in the endochondral ossification process, as the collagen matrix is only insufficiently being broken down (Engsig et al. 2000).

After the resorption of bone tissue, osteoclasts die by apoptosis and are quickly removed by phagocytes (Teitelbaum and Ross 2003). At present, little is known about the molecular mechanisms that terminate osteoclast resorption and initiate osteoclast apoptosis *in vivo*. Nevertheless, targeting osteoclasts for apoptosis, such as by using bisphosphonates or also the more obsolete therapy with estrogen and progestin, has, until recently, been the predominant approach to prevent bone destruction in conditions of bone loss such as post-menopausal osteoporosis, therapy-induced or cancer-related bone loss.

1.4 Regulation of Bone Remodeling

1.4.1 Hormones

Bone formation and resorption, as well as the cell machinery that performs those tasks, are under the subtle control of various hormones, whereas the most extensively studies ones are estrogens and androgens, parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3, and glucocorticoids, due to their common use as anti-inflammatory drugs. These major endocrine regulators will be discussed in more detail. However, it should be noted that bone homeostasis is also regulated by other hormones such as calcitonin (Huebner et al. 2008), leptin (Karsenty and Ducy 2006), and hormones of the anterior pituitary gland (follicle-stimulating hormone, thyroid-stimulating hormone, and adrenocorticotropic hormones) (Imam et al. 2009).

PTH is a peptide hormone and one of the most important regulators of calcium ion homeostasis (Kronenberg 2006; Lanske et al. 1999). PTH is produced and secreted by C cells in the parathyroid gland in response to low blood calcium levels and acts on the kidney, bone and intestine to maintain blood calcium concentrations. In bone, PTH stimulates the production of interleukin-6 and RANKL by osteoblasts and stromal cells, thereby promoting the differentiation, activation and survival of osteoclasts (Dai et al. 2006; Greenfield et al. 1993). Thus, PTH as well as PTHrP (PTH-related protein) promote bone resorption and consequently

the release of calcium (Lanske et al. 1999; Pollock et al. 1996). However, it should be noted that an intermittent exposure to PTH has bone anabolic effects mainly by increasing osteoblast functions, and is thus currently the only approved anabolic treatment option in the treatment of postmenopausal osteoporosis (Bilezikian and Kurland 2001).

Calcitriol (1α,25-dihydroxyvitamin D_3), the active hormonal form of vitamin D, is a steroid hormone either ingested from the diet or synthesized in the skin from 7-dehydrocholesterol through exposure to sunlight (Webb and Holick 1988). Its importance for the development and maintenance of the mineralized skeleton was demonstrated in studies using vitamin D receptor or 1α(OH)ase knock-out mice (Dardenne et al. 2001; Panda et al. 2004). The mineralization defect was normalized after a high-calcium, high-phosphate and high-lactose diet (rescue diet) was administered. However, the administration of only $1,25(OH)_2D_3$ to 1α(OH)ase knock-out mice was not sufficient to normalize the impaired mineralization if hypocalcemia was not corrected (Panda et al. 2004). Moreover, vitamin D-deficient mice showed an increase in osteoblast number, bone formation and bone volume as well as increased serum ALP levels. Additionally, osteoclast numbers were decreased due to a decreased production of RANKL and an enhanced production of OPG (Kitazawa et al. 2003).

Besides PTH and calcitriol, which mainly regulate calcium homeostasis, estrogens and androgens are sex steroids with profound effects on bone. In contrast to PTH and 1,25-dihydroxyvitamin D_3, they enhance bone formation and inhibit bone resorption (Carani et al. 1997; Khosla et al. 2001; Leder et al. 2003). Lack of estrogen as well as testosterone inevitably leads to an increased bone turn-over rate with a simultaneous increase in osteoclastic bone resorption as well as osteoblastic bone formation (Eghbali-Fatourechi et al. 2003; Khosla and Riggs 2003; Weitzmann et al. 2002). However, the net effect of estrogen deficiency is bone loss as a result of an increased production of RANKL and a decreased production of OPG in osteoblastic cells as well as an increase in the secretion of pro-inflammatory and pro-resorptive cytokines in lymphocytes such as IL-1, IL-6 and tumor necrosis factor-alpha (TNF-α) (Jilka et al. 1992, 1995; Tanaka et al. 1993). Although clinical trials have shown that hormone replacement therapy decreased the incidence of major osteoporotic fractures (Cauley et al. 1995; Orwoll et al. 1996), serious side effects including cardiovascular disease and cancer have occurred and therefore other medications are now used in the treatment of osteoporosis (e. g. selective estrogen receptor modulators such as raloxifene) (Riggs and Hartmann 2003).

Bone cells also contain glucocorticoid receptors that confer responsiveness to endogenously produced and exogenously administered glucocorticoids. While active forms of endogenous glucocorticoids such as cortisol are necessary for bone development, glucocorticoid excess is detrimental to many metabolic systems including bone. Studies in mice lacking 11β-hydroxysteroid dehydrogenase-2, an enzyme that inactivates active glucocorticoids, showed that endogenous glucocorticoids are necessary to prime MSCs to the osteoblastic lineage (Eijken et al. 2005; Hamidouche et al. 2008; Sher et al. 2004; Zhang et al. 2008) and support osteoblastogenesis. This is also recapitulated in human osteoblast cultures, which require

physiological amounts of glucocorticoids to differentiate into fully mature, mineralizing osteoblasts (Rauner et al. 2010). Moreover, when the glucocorticoid receptor was specifically deleted in osteoclasts glucocorticoids enhanced the lifetime of osteoclasts, but at the same time inhibited bone resorption by disrupting the osteoclastic cytoskeleton (Kim et al. 2006a). In contrast to these bone-anabolic physiological effects of glucocorticoids, the prolonged exposure to synthetic glucocorticoids, such as required to treat inflammation or organ rejection, results in severe bone loss already within the first months of administration. The pathophysiology of glucocorticoid-induced bone loss includes the transient hyperactivation of osteoclasts due to an increased RANKL/OPG ratio in osteoblasts (Hofbauer et al. 1999, 2009) and a severely inhibited osteoblast function, mediated by the suppression of critical pro-osteoblastic factors such as runx2, Wnt, and BMP signaling, as well as the induction of mineralization inhibitors such as dentin matrix protein-1 or phex (O'Brien et al. 2004; Rauner et al. 2010; Wang et al. 2005, 2008; Yao et al. 2008). Animal studies suggest that glucocorticoid-induced osteoporosis may be successfully prevented administering bisphosphonates, PTH, or denosumab (Hofbauer et al. 2009; Yao et al. 2008). Analogous to selective estrogen receptor modulators, selective glucocorticoid receptor modulators have also been developed that display an improved benefit/risk ratio, but need to be verified in human studies.

1.4.2 RANKL/OPG

Although interactions between osteoblasts and osteoclasts have already been observed in the 1980s by Rodan and colleagues, it took another 15 years to identify the two main negotiators in osteoblast-osteoclast communication, RANKL and OPG (Anderson et al. 1997; Kong et al. 1999b; Lacey et al. 1998; Yasuda et al. 1998) (Fig. 3). Today, the high efforts invested in understanding and characterizing the RANKL/RANK/OPG system have led to detailed knowledge of the pathogenesis of metabolic bone diseases and have already contributed to the development of inno-

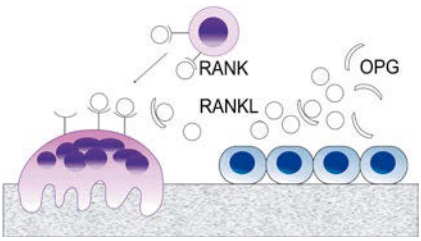

Fig. 3 RANK/RANKL/OPG system. RANK is expressed on mononuclear osteoclast precursor cells (*pink*). Upon binding of RANKL (*circles*), produced by osteoblasts (*blue*), osteoclast differentiation is induced. RANKL/RANK signaling is also active in mature osteoclasts (*pink*) to promote resorption activity and prolong survival. OPG (half circle), which is also produced by osteoblasts, is a soluble decoy receptor for RANKL and can thereby prevent binding of RANKL to RANK and thus the induction of osteoclastogenesis

vative therapeutic drugs that are now in clinical use (human anti-RANKL antibody, denosumab, Prolia) (Cummings et al. 2009; Smith et al. 2009).

As mentioned earlier, bone formation and bone resorption are coupled processes in remodeling. Often, dysregulations favoring osteoclastogenesis are responsible for the development of metabolic bone diseases, such as osteoporosis, Paget's disease, rheumatoid arthritis or osteoarthritis. The discovery of RANKL and its receptors RANK and OPG has finally highlighted the molecular processes in osteoclastogenesis, raising the possibility to inhibit the development of osteoclasts, rescuing bone from exorbitant resorption.

In 1997 Simonet et al. discovered a protein which exposed an osteopetrotic phenotype when overexpressed in transgenic mice (Simonet et al. 1997). Investigating even further, they found that this protein was secreted by preosteoblasts/stromal cells and was capable to inhibit osteoclast development and activation. Due to its bone-protective effects they named it osteoprotegerin (OPG). OPG belongs to the TNF receptor superfamily, although it lacks a transmembrane and cytoplasmic domain. OPG is expressed on a variety of tissues, including lung, heart, kidney, liver, stomach, intestine, brain, spinal cord, thyroid gland and bone, indicating multiple possible functions. The most prominent role of OPG has been assigned to bone protection. However, recent investigations have also proposed important functions of OPG in endothelial cell survival (Holen et al. 2005; Malyankar et al. 2000) and vascular calcification (Bucay et al. 1998; Al-Fakhri et al. 2005; Rasmussen et al. 2006).

After the identification of OPG followed the discovery of RANKL, which does not only have a huge repertoire of names (TRANCE: TNF-related activation-induced cytokine; ODF: osteoclast differentiating factor; OPGL: osteoprotegerin ligand; TNFSF11: TNF superfamily member 11), but also many facets regarding its structure, function and appearance in tissues. The names originated from the four discoverers, each one having used different approaches to identify the protein. They either searched for a ligand for OPG (Yasuda et al. 1998), screened for apoptosis-regulating genes in T cell hybridomas (Kong et al. 1999b), or found RANKL to induce osteoclastogenesis (Lacey et al. 1998) and enhance the life span of dendritic cells (Anderson et al. 1997). Kartsogiannis and colleagues detected RANKL protein and mRNA expression in a variety of tissues, including bone, brain, heart, kidney, liver, lung, intestine, skeletal muscle, mammary tissue, placenta, spleen, thymus and testis (Kartsogiannis et al. 1999). This extensive distribution of RANKL throughout the body already indicates its multiple functions, whereas the most important one is dedicated to the regulation of bone remodeling. RANKL knock-out mice reveal a severe osteopetrotic phenotype due to the absence of osteoclasts. Furthermore, defects in tooth eruption, lymph node genesis, mammary gland and lymphocyte development were reported, as well as disturbances in T cell/dendritic cell interactions and thermoregulation (Kong et al. 1999a; Martin and Gillespie 2001). RANKL is a member of the TNF superfamily and is mainly expressed in preosteoblasts/stromal cells as well as activated T cells. It exists in three isoforms: RANKL1 and RANKL2 are type II transmembrane proteins, whereas RANKL2 encodes for a shorter intracellular domain. RANKL3 is a soluble protein, supposed to be cleaved by TACE (TNFα-converting enzyme, a metalloprotease) from the transmembrane

form. Other isoforms of RANKL are likely to exist, for Kong and colleagues mentioned a primary secreted form of RANKL in activated T cells (Kong et al. 1999a) and other groups found diverse post-translational modifications on the N-terminus (Dossing and Stern 2005).

The third participant in the bone remodeling regulatory system is RANK and belongs to the TNFR superfamily like OPG (Anderson et al. 1997). RANK represents a type I transmembrane protein and is expressed in tissues as ubiquitously as RANKL, though, most commonly found in osteoclasts and dendritic cells (Hofbauer and Heufelder 2001). RANK deficient mice show similar phenotypes to those of RANKL knock-out mice, including lack of tooth eruption, osteopetrosis and missing lymph nodes (Dougall et al. 1999). The RANK-signaling cascade is initiated when RANKL binds to the extracellular domain of RANK which passes the signal along to TRAF6 (TNF receptor-associated factor 6). TRAF6 has various downstream mediators, including the transcription factors NFκB, NFATc1 (nuclear factor of activated T cells) and AP-1 (activator protein-1) as well as the cascades of mitogen-activated protein kinases (MAPK), such as p38 stress kinase, JNK (c-Jun N-terminal kinase) and ERK (extracellular signal regulated kinase) (Koga et al. 2004; Rauner et al. 2007).

The expression of OPG and RANKL is highly inducible by various systemic and local factors. Among others, estrogen, bone morphogenetic protein-2, INF-γ, and TGF-β positively regulate OPG, whereas PTH, $1,25(OH)_2$ vitamin D_3, glucocorticoids, prostaglandin E_2, IL-6, IL-8 and IL-11 enhance the expression of RANKL (summarized in Khosla (2001) and Leibbrandt and Penninger (2009)).

1.4.3 Cytokines and Chemokines

Bone remodeling is also critically regulated by various cytokines and chemokines, not only in pathopyhsiological conditions, but also within physiological bone remodeling. Many pro-inflammatory cytokines including TNF-α, interleukin (IL)-1, IL-6, IL-7, IL-11, IL-15, and IL-17 create bone loss either by increasing osteoclast generation and activation or by inducing RANKL expression by the osteoblasts. On the other hand, IL-4, IL-5, IL-10, IL-12, IL-13, IL-18, and interferon (IFN)-α, IFN-β and IFN-γ are inhibitors of osteoclastogenesis by blocking RANKL signaling, either directly or indirectly (reviewed in Lorenzo et al. (2008) and, Sipos et al. (2008)). Interestingly, IL-1 directly stimulates TRAF6 expression on the osteoclast, thereby potentiating RANK signaling, whereas IFN-γ is known to down-regulate TRAF6 by targeting it for proteosomal degradation, thereby aborting osteoclast formation (Takayanagi et al. 2000). TGF-β is described to activate both, directly suppressing osteoclastogenesis or inducing osteoclastogenesis via suppressor of cytokine signaling 3 (SOCS3) (Lovibond et al. 2003; Ruan et al. 2010).

In contrast to osteoclasts, little is known about the effects of cytokines on osteoblasts. TNFα, IL-1, and IFNγ were shown to inhibit osteoblast differentiation and block collagen synthesis (Canalis 1986; Centrella et al. 1992; Gilbert et al. 2000; Kuno et al. 1994). IL-6 and the IL-6 receptor were shown to be produced by

osteoblasts and stromal cells, but the effects on osteoblastogenesis remain unclear (Franchimont et al. 1997a,b). IL-4 has been reported to be a chemoattractant for osteoblasts and to directly stimulate the proliferation of osteoblasts (Ura et al. 2000). However, it has an inhibitory effect on osteoblast differentiation. Accordingly, IL-4-overexpressing mice exhibited a decrease in bone formation and decreased differentiated osteoblasts on their bone surface (Jilka et al. 1998).

So far, only little is known about the regulation of bone mass by chemokines. However, Binder et al. reported on a critical role of the C-C chemokine receptor 2 (CCR2) in normal and pathological bone mass maintenance by regulating osteoclastogenesis (Binder et al. 2009). In this study, CCR2, the receptor for several monocyte chemoattractant proteins, was found to induce the expression of RANK in osteoclast precursor cells using the NFκB and ERK signaling pathways, thereby making them more susceptible to RANKL-induced osteoclastogenesis. Osteoblast differentiation and activity, on the other hand, were not affected. Thus, chemokines and chemokine receptors as well as cytokines are likely to play an important role in the maintenance of bone mass, but their definitive functions still remain to be determined in more detail.

1.5 Concluding Remarks

Bone is a highly dynamic tissue that undergoes constant remodeling to repair structural damage or adapt to changing functional demands. Osteoblasts, osteocytes, and osteoclasts intensively communicate with each other to coordinate the remodeling process, and their functions are tightly regulated by various systemic and local factors such as e. g. hormones or cytokines. Local factors may be produced by bone cells themselves to act in an autocrine manner or by other cell types (i. e. immune cells, vascular cells) that also participate in the regulatory process. Due to the increasing knowledge of cellular and molecular mechanisms of bone remodeling, efficient therapies have already been developed (bisphosphonates, PTH, denosumab) and will continue to develop (i. e. anti-sclerostin antibodies) to encounter exacerbated bone loss that occurs with aging, estrogen-deficiency, or malignant and inflammatory disease.

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Towards the Automated Detection and Characterization of Osteoclasts in Microscopic Images

2

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2.1 Introduction

Microscopes have been used for a long time to observe biological samples. However, measurements of tissue and cell-related parameters were conducted by human observers and were consequently ad hoc, not reproducible and restricted to small sample numbers. Since computers have become increasingly powerful, classic life-sciences now routinely take advantage of new opportunities to link microscopes and computers. Automated image-segmentation in large numbers of digital images allows recognition of cell and tissue structures via computer algorithms, and subsequent linear measurements of cellular parameters. Nevertheless, they also come with technical challenges linked to memory, computational resources, disk space and sensor limitations as well as new software algorithm approaches for image processing.

Nowadays state-of-the-art hardware allows applying versatile machine-learning based approaches to huge stacks of images, which would not have been feasible just a few years ago. In the context of bone research, an application for automated image-segmentation is the automated quantification of osteoclasts in culture. Such culture models using either murine or human osteoclasts offer the possibility to study parameters such as osteoclast formation from their mesenchymal precursors, osteoclast differentiation, maturation and apoptosis. Advanced molecular imaging of osteoclasts allows the study of pathological processes and to elucidate the effects of osteoclast-targeted therapies for diseases in which excess bone resorption is a crucial pathological process. This includes osteoporosis, rheumatoid arthritis, as well as bone tumors such as giant cells tumors, osteosarcomas, and bone metastases.

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Quantification of osteoclasts in culture is currently performed manually. Therefore, a lot of useful information, such as the number of precursor cells, cell areas, number of nuclei per cell, or protein levels in each cell class, cannot be assessed reliably. Particularly the cell associated protein levels are of great interest as the protein pattern may vary greatly between osteoclasts of different stages of development, in healthy conditions as well as in diseased tissue.

It is possible that these limitations prevent the detection of small-scale correlations between changes of cell-associated parameters and certain pathological states. In contrast, when employing a computerized quantification method, all of these above-mentioned limitations can be overcome. Additionally, automated analysis of samples allows for very large scale experiments, which would not be possible with manual counting since it is restricted in accuracy and time. The osteoclast detection scenario clarifies the benefits of reproducibility, efficiency and the generation of observer-independent measurements, which are valuable assets of state-of-the-art image-processing systems.

In this chapter, we describe the steps needed to build an algorithm for murine osteoclast detection starting from fixed and immunofluorescence-labeled cultures of isolated and *in vitro* differentiated murine osteoclasts. Since they are based on standard procedures, the culture conditions, markers for osteoclast detection as well as the applied immunofluorescence labeling protocol are only briefly described in Sect. 2.2 Information about digital image formats and slide-based microscopy follow. Software packages for image processing are discussed and valuable tools are mentioned for handling the workflow.

The chapter continues with evaluation of the generated ground-truth data and algorithm results. Measurements are introduced and examples are given on how to apply them in real-life scenarios, where our developed osteoclast-detection algorithm is used as an example of a typical image-processing algorithm. The described steps are not programming language specific and can be implemented in the framework of the reader's choice. That section, however, does not cover implementation details, as these are beyond the scope of this work and should be read in existing books on microscopy and digital-image processing e. g. (Burger and Burge 2008; González and Woods 2008; Wu et al. 2008). Many of the image-processing problems mentioned in these paragraphs are so-called *ill defined* problems – e. g. segmentation (Martin et al. 2001) – meaning that there is no unambiguous gold-standard to compare these algorithms to. Furthermore, the question of how to compare a developed algorithm to the performance of human experts still remains an important open research problem. To illustrate this difficulty, Sect. 2.5 covers human intuition and limitations in perception and vision that have an influence on the development and evaluation of image analysis algorithms. In the last part of this chapter, we describe the advantages of our new osteoclast detection system in detail and show possible applications.

In summary, the purpose of this chapter is to introduce biologists and medical scientists to image processing by introducing a versatile, automated approach to quantify various parameters in osteoclast cultures. We hope that we can thereby raise the audience's awareness and interest in the possibilities and limitations of this new, powerful technology.

2.2 Methods

2.2.1 Culture Conditions for Isolated Murine Osteoclasts

The culture conditions for osteoclasts suitable for an automatic detection method were developed from standard protocols (Akatsu et al. 1992), but different culture parameters were evaluated.

Mice (Mus musculus) were sacrificed by neck dislocation following asphyxiation. Tibiae and femura were prepared; the caps of the bones were cut off and the bones were rinsed with 10 ml pre-heated (37 °C) minimum essential medium containing antibiotics and antifungals. The cells were diluted to 2×10^6 cells per ml and osteoclast formation-stimulating additives (25 ng/ml RANKL, 15 ng/ml M-CSF) were added. 1 ml of cell suspension was added per well (each containing a sterile glass coverslip) of a 24-well culture plate. Cultures were maintained at 95 % relative humidity/5 % CO_2 in an incubator for 8 days. Change of medium was performed every second day.

2.2.2 Staining Protocol

At the beginning of the development of an algorithm, characteristic features of the target structure or cell need to be defined by biological experts, preferably in a customer requirement specification (CRS). In the case of osteoclasts, the biological experts defined two important criteria to identify mature osteoclasts, viz. *criterion 1*: the amount of nuclei per cell (≥3) (Andersson and Marks 1989), and *criterion 2*: a low to undetectable expression of the macrophage-antigen F4/80 (van de Wijngaert

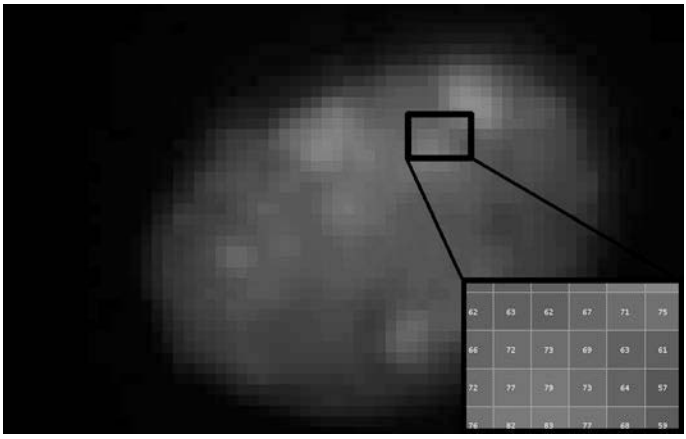

Fig. 1 This figure illustrates the digital representation of a captured image derived from epifluorescence microscopy. The small insert on the *right* shows the grey level value of the selected pixel (eight bit image: a value between 0 and 255)

et al. 1987). The expression of the latter marker is lost due to differentiation from precursor cells to osteoclasts. Therefore, the staining protocol included: (1) labeling of the nuclei with DAPI, (2) staining of the cells with an antibody directed against F4/80 macrophage marker (eBioscience), probed with Alexa Fluor 568 (Invitrogen Molecular Probes), and (3) in order to make all cells (osteoclasts and precursors alike) "visible", using one antibody against the membrane-bound calcitonin receptor (Acris) and one antibody against the cytoskeleton component α-tubulin (SigmaAldrich), both probed with Alexa Fluor 647 (Invitrogen Molecular Probes®). Using such staining protocol, in the acquired images (Fig. 2), osteoclasts will appear white (due to the lack of F4/80 staining), while precursor cells will be red or pink.

The mature cells were fixated using a 4 % formaldehyde solution and the remaining aldehyde groups were quenched with 50 mM NH4Cl. The cells were incubated with blocking/permeabilization buffer (0.5 % Triton X-100 + 1 % bovine serum albumine in phosphate-buffered saline, PBS) for 60 min. After this period, the primary antibodies were applied directly in the culture plate at a dilution of 1 : 1000 in blocking buffer (parallel approach) for 60 min. After washing with PBS, the secondary antibodies were applied in the dark at a dilution of 1 : 1000 in blocking buffer (parallel approach) for 30 min. To stain the nuclei, the cells were incubated for 15 min with DAPI (1 ug/ml) in aqua bidistillata. The cells, grown on coverslips, were finally mounted on conventional microscope slides using Fluoromount G (Southern Biotech).

Images of the stained cells were acquired using an automated Axio Imager epifluorescence microscope (Zeiss) equipped with TissueFAXS™ hard- and software (TissueGnostics GmbH, Austria) using a 40x Neofluar 1.4 (oil) objective. We refer to following sections for special considerations about the acquisition process.

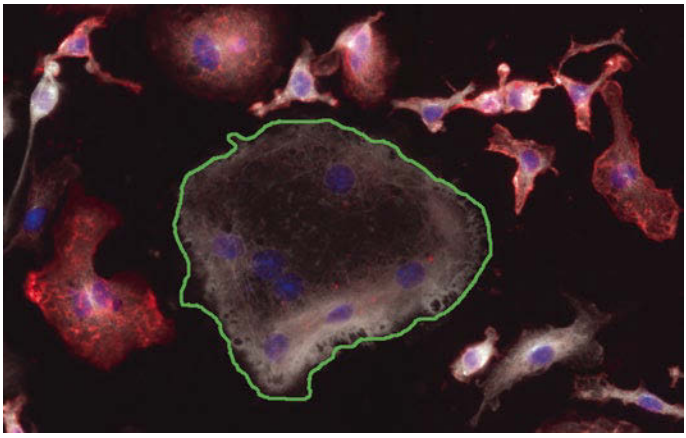

Fig. 2 This image shows a sample markup (*green*) of one immunofluorescence-labeled osteoclast cell in culture. The perimeter of the target cell is created on a separate layer so that the original content of the image is not modified. This additional layer can later be extracted and processed by computer-based algorithms

2.2.3 Digital Images

Before algorithm development, images have to be acquired and stored. The digitalization of specimens requires a suitable representation of the captured light by an electronic sensor that can be further processed by the computer. This sensor is built of rectangular elements that capture the incoming light. Exposure time controls the amount of light that is able to hit the sensor elements. Finally, quantification transforms these values to a limited set of intensities that can be further processed by the computer. Typically ranges of 256 ($=2^8=8$ Bit), 4096 ($=2^{12}=12$ Bit) or 65536 ($=2^{16}=16$ Bit) are used to represent these intensity values. An example of an 8-bit image with a corresponding gray-level matrix is shown in Fig. 1.

2.2.4 Automated Slide-based Microscopy

Sampling of images from large-scale experiments necessitates automation of the acquisition process. A suitable microscope with a fully motorized stage has to be used to automatically acquire images. Several slides can be mounted on this stage which is located beneath the objective. During acquisition the stage moves the slides in such a way that the camera captures each region of interest as a set of overlapping fields of view (FOVs). For this image acquisition we use the TissueFAXS™ system (TissueGnostics GmbH, Austria) which offers a convenient workflow to acquire up to eight slides automatically. This is a requirement for further statistical analysis because we want to analyze global effects of various compounds on the growth and formation of osteoclasts in culture. If these cultures contain huge cells, in this case osteoclasts, then the stitching (tiling together of the single FOVs to one big image) of acquired regions is essential for further steps, because these cells may have been split at the border of a FOV. Automatic stitching-algorithms have two main components: finding the best alignment for two neighboring images and finding the best alignment taking all neighboring images into account. TissueFAXS already includes stitching during and after acquisition. External tools like AutoPano[1] or Hugin[2] are also able to stitch adjacent images. In the course of the algorithm development phase, we also devised our own stitching algorithm. Vertical and horizontal shifts (alignment errors) were calculated by cross-correlation with respect to each adjacent FOV (Rankov et al. 2005). Such alignment errors are not completely avoidable due to mechanical restrictions of the stage.

2.2.5 Software for Image Processing

Common tools like GNU Image Manipulation Program (GIMP) or Adobe Photoshop are not suitable for algorithm development due to lack of a powerful and fast scripting language. High performance algorithms made image software-tools like

[1] http://www.autopano.net/en/

[2] http://hugin.sourceforge.net/

OpenCV, ImageJ, and Matlab very popular: They offer special image-processing toolboxes that are optimized for high throughput while still being relatively easy to use for rapid prototyping. Commercial state-of-the-art solutions like TissueQuest[3] offer a broad range of features to analyze images but are usually based on the "one nucleus = one cell" paradigm.

Depending on the desired task, one needs to pick the right framework. For rapid prototyping Matlab and ImageJ are good choices. The disadvantage of Matlab and ImageJ is that they are slow compared to OpenCV, so if speed is an issue, algorithms have to be developed or ported to OpenCV. In case of an analysis of regular single cells, TissueQuest (Steiner et al. 2000), is a good option. Its graphical user interface offers the user powerful options to efficiently configure cell analysis and combines this with an easy to use user-interface. Coupled with TissueFAXS, acquisition and analysis are possible in a homogeneous workflow and CE-marked analysis environment for use in research as well as *in vitro*-diagnostics. An Open Source alternative, which requires only basic knowledge of image processing, is Cell Profiler[4]. This suite offers a framework to build versatile *pipes* (algorithms) for various biological applications. Many predefined pipes for various applications can be downloaded from the online forum and are free of charge.

Already at this early decision stage of development, generation of ground-truth markups should be started so that there is a continuous flow of new marked-up data. This dataset is then used to evaluate the newly developed algorithms, train machine-learning systems and optimize parameters throughout the whole development process.

2.3 Evaluation of Expert Markups and Developed Image-processing Algorithms

As mentioned above, after and during development, the performance of the newly implemented algorithms needs to be measured in an unbiased way. For this purpose, visual inspection is mostly used. Unfortunately, this step is error-prone and highly dependent on the observer who in many cases is still a computer scientist rather than a qualified biological researcher. A better method for this evaluation is to let biological domain-experts do markups of so called ground-truth data from original images. This can be done using any graphics-editing program like GIMP or Adobe Photoshop. The idea of this markup is to point out those objects of interest (e. g. cells, tissue structures) that should be detected by the algorithm solely by using the images provided without any additional information. The number of markups needed differs from project to project. In case of the osteoclast detection algorithm, about 100 FOVs were manually marked-up for osteoclasts. It is essential to draw these markups on a second "layer" (like a

[3] http://www.tissuegnostics.com

[4] www.cellprofiler.org

transparency film on top of the original image) of the target image so that no information from the original image is lost. These markups can then be automatically compared to automatically generated masks by the algorithm, ensuring that the newly developed tool and the tissue experts produce comparable results. Trainable machine-learning classifiers like support-vector machines, neural networks or logistic regression need ground-truth data as an input to build their decision model. Before starting to develop an algorithm, agreement between different human experts has to be confirmed. If this is very low, meaning that there is no consensus between the different human experts, even the best detection algorithm cannot succeed and therefore development should be postponed until an agreement between human experts has been achieved. The quality of the ground-truth data can be increased if several human experts provide markups of the same set of images. It is very important that the markups done by humans are performed independently of other experts and of the algorithm developer so that afterwards a reliable ground truth can be obtained. The observations of different experts can be compared by computing the correlation between the markups. In some cases pixel-level (meaning that the experts actually drew exactly the same lines around the cells) scoring is not very reasonable, therefore evaluation on a higher level of abstraction, e. g. object-based evaluation, is preferable. This approach counts the number of detected objects and compares it with those that are found in the ground-truth data. Overlap between detected objects can be used to compute a rough place agreement beyond the number of detected objects. A substantial disagreement in the object number or placement indicates a discrepancy between the experts' opinions and should be investigated before further steps are taken in the development of the system.

In both cases (pixel-/object-based evaluation) having more than two human experts can generate a more objective ground-truth by performing a majority-voting. As the name suggests, majority voting selects those pixels/objects (e. g. osteoclasts) that are marked up by the majority of the human experts.

The next step is the evaluation of the algorithm output. In various scenarios (e. g. segmentation), algorithms have a vast set of parameters. Optimizing them manually is an impossible task because it would mean evaluating several hundreds of thousands of images by hand. Therefore *exhaustive parameter-optimization* techniques are applied that compare their output with previously created ground-truth data. This technique runs the algorithm with a large set of possible parameter combinations and returns those that have the highest agreement compared to the human experts' ground-truth data. Care must be taken to prevent "overfitting" i. e. choosing seemingly optimal parameters, which are only performing well due to chance and only on the images that were used for parameter-optimization. Therefore, not all data should be used for this technique – some data should be held back for a final evaluation of the best parameter settings.

Now follows the step that compares the optimal algorithm output with the experts' markups. One question that arises is how to compute a score of agreement between the human experts and the algorithm. On pixel level we can calculate the

F-score to rank different algorithm output-masks. The F-score (Chinchor and Sundheim 1993) is composed of *precision* and *recall* which are computed as follows:

$$\text{Precision} = \frac{\text{TP}}{\text{TP} + \text{FP}}$$

$$\text{Recall} = \frac{\text{TP}}{\text{TP} + \text{FN}}$$

TP represents the true positive pixels, those that were marked up by both the expert and the algorithm whereas *TN* stands for pixels that were not marked by either of them. If the pixel was only detected by the algorithm then it is called a false positive (FP). Analogous, pixels that were not assigned as belonging to the object of interest by the algorithm, but were marked up by the expert are called false negative (FN). With these measures we can now finally define the balanced F-score as:

$$F_\beta = (1 + \beta^2) \cdot \frac{\text{Precision} \cdot \text{Recall}}{\beta^2 \cdot \text{Precision} + \text{Recall}}$$

The parameter β determines the importance for higher precision and depends on the specific task. In case of evenly weighted *recall* and *precision,* β is set to 1, which is a reasonable default.

Besides a pixel- and object-based comparison, there are other methods which may be more suitable for specific scenarios. An extensive survey of segmentation methods was published in Zhang (1996). Segmentation is a well-studied topic in computer vision but still a challenging task. New evaluation methods were published recently in Zhang (2001) and Smochina et al. (2010).

Putting these discussed points into the practical example of osteoclast detection, an example markup of a target cell can be seen in Fig. 2 (the green line indicates the manually drawn perimeter of an osteoclast). Since the number of osteoclasts is the desired output of the algorithm, an object-based evaluation is more suitable than a pixel-based one. In this case it is less important whether a specific pixel belongs to the osteoclast or to the background as long as the number and general location of the objects (= osteoclasts) agrees with the ground-truth data. As mentioned above, to obtain a general detection algorithm that does not only model the specific knowledge of one expert, more than one expert with a biological background and experience with osteoclast cultures should be used. In our case, two experts marked up osteoclasts in different cultures and only those that have been marked up by both human experts where considered as *real* osteoclasts. We evaluated the object-level agreement between these two experts in 7 different regions (about 70 FOVs) of osteoclast cultures (Fig. 3). The mean agreement between the two experts was 70 ± 17 %. This shows that even though the manual markup of osteoclasts appears simple in theory, in "the real world", the opinions of the human experts as to what qualifies as an osteoclast and what does not can be quite incongruent. Reasons why human-based classification may be error-prone were published in Baak (1991) and are discussed in Sect. 2.5.

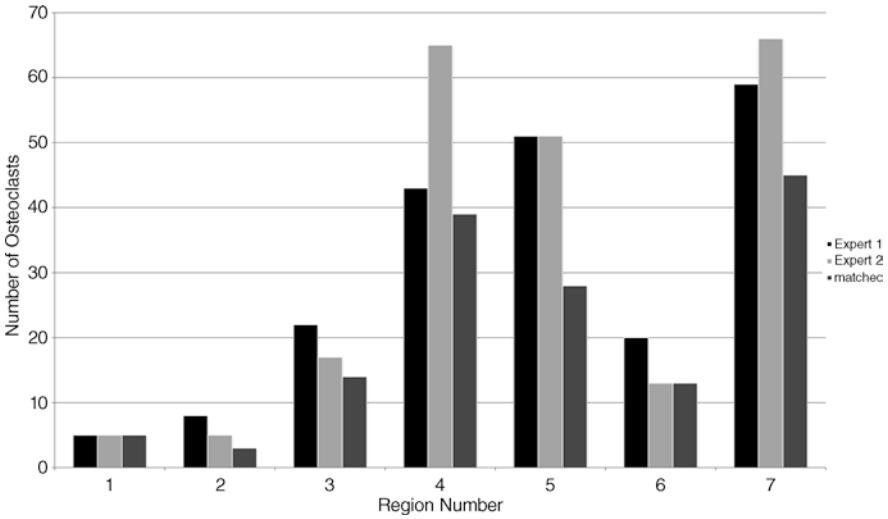

Fig. 3 This chart exemplifies the consensus between two human experts. It shows the number of osteoclasts (y-axis) detected in seven acquired regions from different cultures (*x-axis*). In each region, the first and the second column represent the osteoclast number detected by each expert, whereas the third column shows the number of osteoclasts detected congruently by both experts

2.4 How to Design an Image-processing Algorithm Based on Osteoclast Detection in Culture

In this chapter we present our algorithm for osteoclast detection in culture, starting with the already acquired and stitched images. It follows the general scheme of algorithms for image processing (Fig. 4). Most of the existing published imaging-processing algorithms were derived from this or a similar workflow. Before designing an algorithm, criteria to distinguish between objects of interests (osteoclast) and the remaining objects (precursors) have to be specified. The detailed staining protocol can be found in Sect. 2.2.2. Osteoclasts are defined as multinucleated cells with at least three nuclei (Criterion 1). Additionally, they should not exhibit significant expression levels of F4/80 macrophage marker that identifies only the osteoclast precursor cells (Criterion 2). Such biological criteria should be written down in a document called *customer requirement specification* (CRS) to prevent unexpected results due to misinterpretation by the algorithm developer. The following sections refer to the respective steps of Fig. 4 (1. Correction of illumination, 2. Segmentation, 3. Postprocessing, 4. Labeling).

2.4.1 Correction of Illumination

The first step of the detection algorithm is very critical because all further steps will be based on the image(s) generated by this step. Misalignment due to shifts in

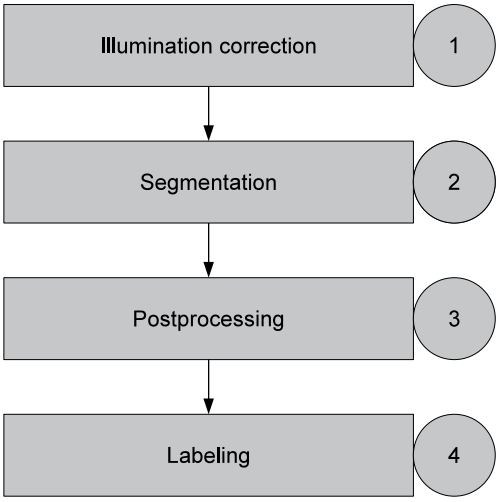

Fig. 4 This flowchart demonstrates the general scheme of image-processing algorithms. Four major processing steps must be distinguished

camera setup, lamps or condenser causes uneven illumination (Figs. 5a, 5b). To compensate for the introduced bias, an illumination correction function can be computed (Wu et al. 2008). It represents a special image illumination image that contains the overall pattern of illumination in all future acquired images. Various publications deal with this process such as Zhu et al. (2003) and Ljosa and Carpenter (2009). If the approximation of the illumination function fails, adaptive thresholding can be applied, which is discussed in the next section.

2.4.2 Segmentation

Step 2 splits the pixels in two groups, those belonging to the foreground (cells, including osteoclasts) and those belonging to the background (Fig. 6a). This separation can be achieved by applying intelligent thresholding. Examples are the classical triangle algorithm (Zack et al. 1977) or machine-learning based classifiers with appropriate features (Kapelner et al. 2007). Most of these image-segmentation techniques operate on histograms. A histogram is a discrete distribution function of the image's intensity values. It counts the number of gray values that pertain to each of the single categories/bins (intensity values, 0 to 255 in case of an 8-bit image). The triangle algorithm obtains a threshold to distinguish between background ($<$ threshold) and foreground ($\geq$ threshold) by computing two local maxima of this histogram. Now these two peaks are connected and the maximum distance (an orthogonal vector to the line connecting the two peaks) between the line and the

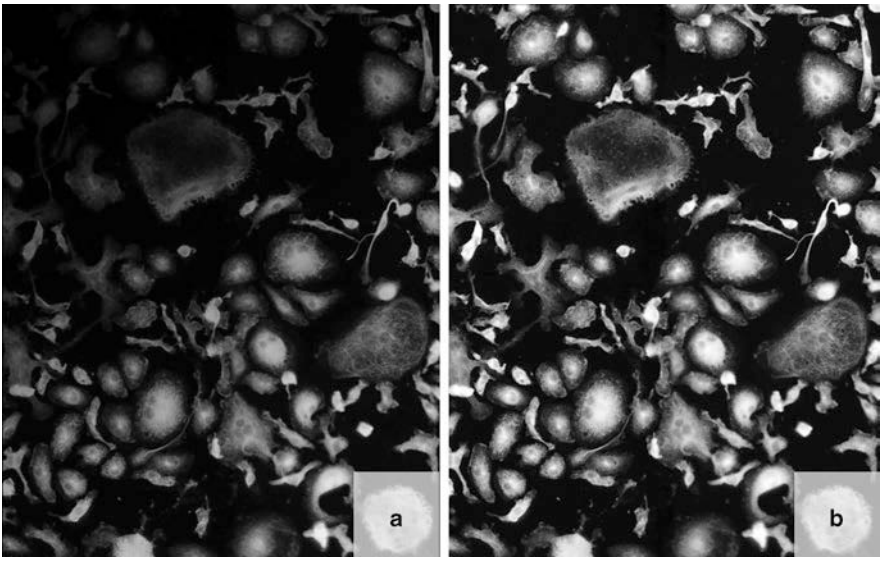

Fig. 5 This figure illustrates the effect of a post-acquisition illumination-correction (processing step 1) on images obtained by epifluorescence microscopy. In the original image (**a**), an illumination-gradient is clearly visible from the left upper corner to the right lower corner. This gradient is cleared after application of illumination-correction (**b**)

histogram is computed. The intensity value indicated by the maximum distance is the threshold value. Everything greater or equal (brighter) is considered as foreground. Every value beneath this threshold (darker) is classified as background. The machine-learning based methods are more complex and require extensive knowledge in learning theory. Therefore the interested reader should refer to the publication mentioned above (Kapelner et al. 2007).

If illumination correction (step 1) did not produce an acceptable result, adaptive thresholding can be used to partition images into foreground and background as well. Compared to a single threshold like in the triangle approach, this is a more sophisticated process that chooses different thresholds for each pixel of the image. Because of this method of operation, this is sometimes also called *local* or *dynamic* thresholding (Shapiro and Stockman 2001; Burger and Burge 2008; González and Woods 2008).

For our osteoclast detection algorithm, we used adaptive thresholding (Liu et al. 2002).

2.4.3 Postprocessing

Step 3 implements a clean-up step which removes artifacts (Fig.s 6a, 6b) and unwanted cells such as osteoclast precursor cells. This is often achieved by binary operations such as area opening or the computation of morphological features that can be used to distinguish between the object of interest and other objects. Area opening removes all objects with an area smaller than a chosen threshold. If this

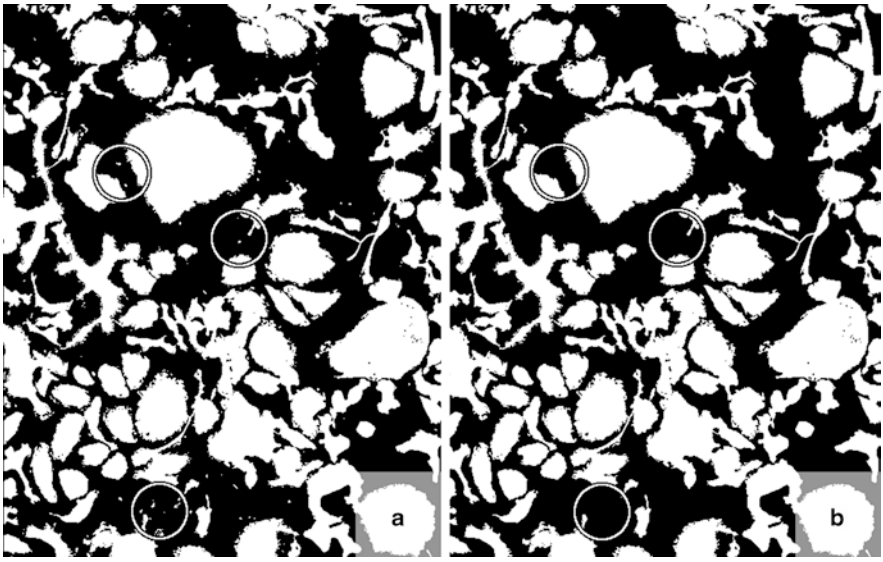

Fig. 6 This figure illustrates the results of (**a**) segmentation (processing step 2) and (**b**) post-processing (processing step 3) on images obtained by epifluorescence microscopy. In (**a**), the output image of processing step 1 (see Fig. 5b) has been subjected to automated image-segmentation. White structures represent identified objects. This segmentation may introduce artifacts visible as small white blobs that do not belong to actual structures. *Gray circles* indicate example areas where these artifacts are visible. Their removal in the post-processing step results in image (**b**)

condition is not specific enough, morphological features such as eccentricity, solidity, convex hull etc. can be used to distinguish between the desired cell and unwanted artifacts. In case of our developed osteoclast detection algorithm, we have also used features from specific staining. To eliminate non-osteoclast cells, we make use of Criterion 1 (< 3 nuclei) and 2 (F4/80 staining).

2.4.4 Labeling

Step 4 performs a labeling of the remaining cells (Fig. 7a, 7b) (Samet and Tamminen 1988). This is done to directly pinpoint each single cell on the image so that further measurements can be computed by directly addressing the single cells.

As a side note, the image context is also of great importance during feature calculation. Fig. 8a and 8c show two osteoclasts. Reasoning from the example at the top, a feature such as the distance between the nuclei may perfectly identify the target cell. Unfortunately, in the same region another osteoclast can be found (Fig. 8c) where the distance is much larger than in the upper example. So if one were to try and detect osteoclasts only in the DAPI channel, this might produce false positives or negatives. This is why we introduced the additional immunofluorescence-stainings of the microtubules, the calcitonin-receptor and the F4/80 macrophage-marker to detect the whole cells (Fig. 8b and 8d).

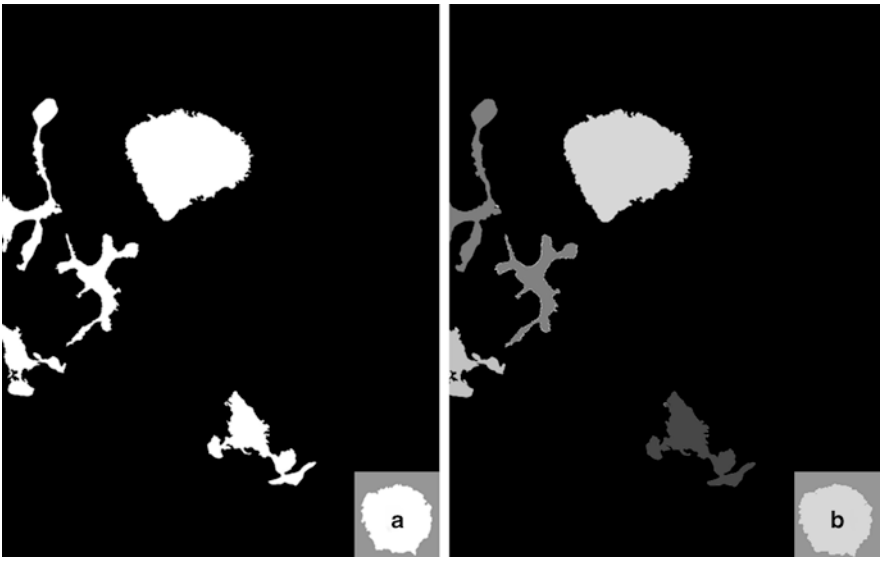

Fig. 7 This figure shows the labeling (processing step 4) of images obtained by epifluorescence microscopy. In image (**a**), an enlarged area of the output image of processing step 3 (see Fig. 6b) is shown. In image (**b**), the labeling of the segmented image is illustrated. For demonstration purposes, each object is labeled in a different shade of gray. Internally, this would be represented by assigning a unique number to each object

Having computed all these features, we can finally start to create statistics and draw conclusions about disease-related morphological or staining intensity-based (equates to associated protein levels, e. g. receptors) differences.

One recent application of this osteoclast detection system was presented in Heindl et al. (2010). We tested the influence of the hormone melatonin and the polyphenols piceatannol and resveratrol on osteoclast growth and formation. Preliminary results indicate that the osteoclast formation is almost abolished with physiological doses of melatonin, while polyphenols at μM concentrations seem to inhibit in vitro cell growth in general (Schepelmann et al. 2010).

2.5 Common Pitfalls

Although high-end microscopy technologies open up new ways to examine tissue and cell culture samples, they also require detailed knowledge of biology, optics and computer science. This includes appropriate sampling of the tissues, optimized cell-culture conditions, and furthermore well chosen fixation and staining protocols. It also includes the selection of the best microscope objective for acquisition of the specific experiment (Pearson 2007). This section does not treat these kinds of problems, but focuses instead on those that occur during image acquisition and evalua-

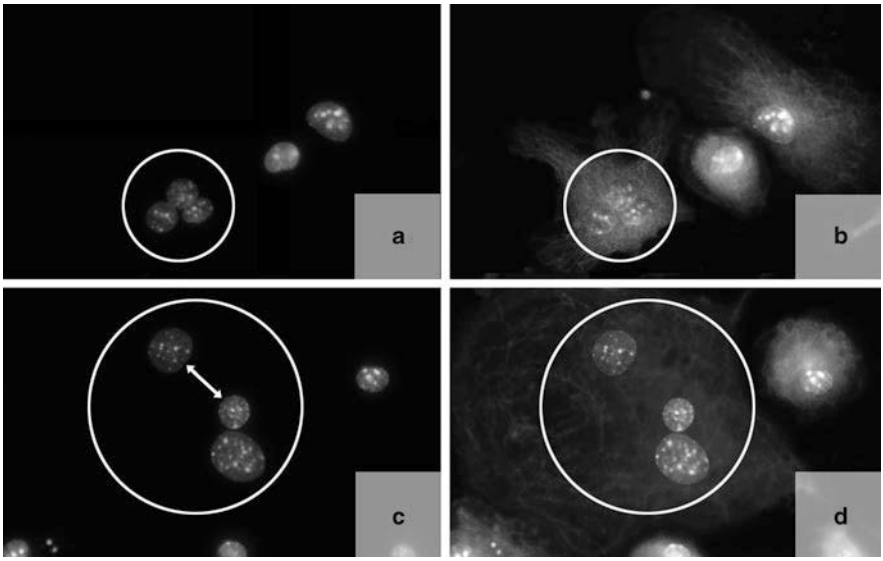

Fig. 8 The importance of context is illustrated in this figure. Figures (**a/b**) and (**c/d**) show two different image details of immunofluorescence-labeled osteoclast cultures. White circles indicate osteoclasts. During the staining process, cell nuclei are stained with DAPI (**a/c**). Additionally, microtubules and a membrane receptor are immunofluorescence-labeled to visualize cell bodies and cell borders (**b/d**). An important criterion of mature osteoclasts is to have three or more nuclei. An algorithm that operates only on the nuclei (DAPI channel) would probably miss the osteoclast in (**c**) due to the larger distance between the nuclei in contrast to (**a**). Taking the additional staining of cell bodies and borders into account, the same nuclei in (**b**) and (**d**) are now allocated to one cell and consequently these cells are identified as osteoclasts

tion. We discuss pitfalls such as uneven illumination, uneven staining, touching clumps of nuclei and psychological aspects resulting in fallacies due to Gestalt laws. For basics about microscopy and optics we refer to Spector and Goldman (2006).

2.5.1 Imaging-based Errors

2.5.1.1 Illumination

To perform a quantitative measurement, each step of image acquisition has to be as exact as possible. Noise is often a result of a misaligned light source and increases the error rate in all following algorithmic steps. Therefore, having an evenly illuminated image is of great importance. To achieve this goal, calibration slides can be used to align the light source properly before recording microscopy images. Applying an illumination-correction function (as discussed in Sect. 2.4.1) afterwards is a measure of last resort since it modifies the intensity values of the image. If this is done in the channel containing the target protein, the researcher has to consider that she/he may have created artificial (= false) staining due to the applied correction function. Using these results for further statistical analysis is problematic and may lead

to wrong conclusions. The most common optical problem, non-aligned condensers, causes an uneven staining that is almost impossible to repair with image-processing techniques in later phases. A priori detection and elimination of illumination gradients before recording images saves a lot of work and increases the significance of quantitative biological results which are derived from the acquired images.

2.5.1.2 Acquisition Parameters

Acquiring the same sample on different instruments or with different settings on the same instrument often results in images looking different. Depending on the settings and the experience of the user who controls the microscope, image quality may change. Currently, there is no standard (Yagi and Gilbertson 2005) so the comparability between various acquisition systems is impossible. An example illustrating the same FOV with different acquisition settings on the same instrument is shown in Fig. 9. The first image (9a) of this figure is in focus and has balanced color values as should be expected. Fig. 9b is out of focus; consequently segmentation will be tricky because borders are blurred. To prevent out-of-focus images, techniques such as so-called *extended focus* (Abrahamsson et al. 2006) can be used: we acquire

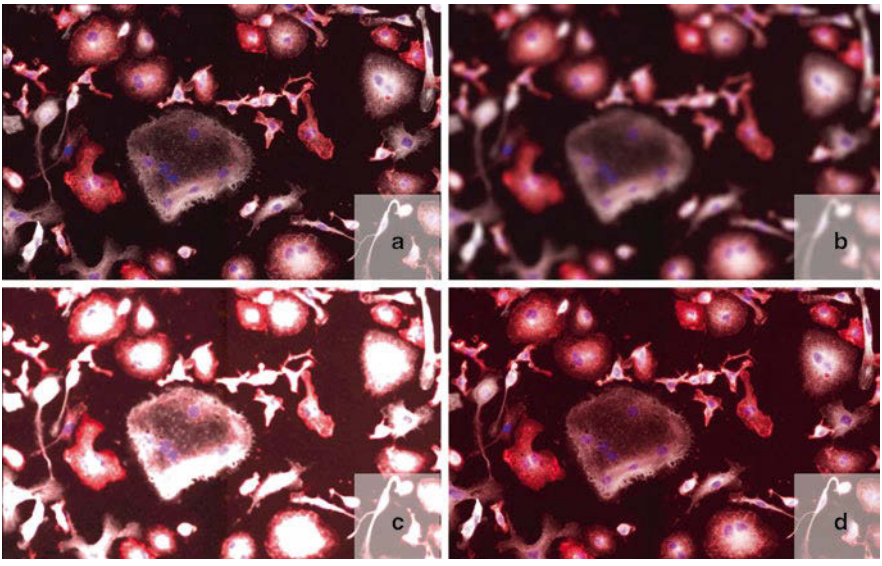

Fig. 9 This figure illustrates the effect of acquisition conditions in image processing. The same image derived from an immunofluorescence-labeled osteoclast culture has been acquired with four different settings (**a–d**). Image (**a**) represents an ideal acquisition, showing the cells in focus with balanced color values. In image (**b**), cells are out of focus and their borders are no longer clearly visible. Image (**c**) is overexposed (exposure time set too long). Saturated areas make further quantitative analysis impossible. Finally, image (**d**) has a significantly increased red value that may mislead the observer. Note: These images have been processed for demonstration purposes

a stack of images of the same FOV on five different focal planes. A subsequent fusing step merges all images per stack and takes only those parts that are in focus on each plane. As a result, the amount of unfocused cells is reduced to a minimum. Figure 9c shows an overexposed FOV. Saturated areas are visible as white spots and prevent useful feature computation. An increased red level is illustrated in Fig. 9d. This may mislead the observer to draw incorrect conclusions about the expression of a certain marker.

2.5.1.3 File Formats

Another pitfall can be the file format used to store the image. Lossless formats should always be preferred to prevent quantification artifacts. A good choice for storing high-quality images would be the Portable Network Graphics (PNG) (Fig. 10a). This format can be read by a variety of tools and supports lossless compression. Besides PNG, the tagged image file format (TIFF) is popular for storing acquired images. Compared to PNG, it offers a wide range of storage options, which is also the drawback of this format. It cannot be guaranteed that other applications which offer TIFF support are able to read and correctly interpret the chosen TIFF settings. The most commonly used Joint Photographic Experts Group (JPEG) graphics format has to be avoided (Fig. 10b). The intention of its compression is to remove details that are not visible to the human eye. Obviously, this alters intensity values and can limit the applicability of machine-learning and image-processing techniques, as well as quantification of biological features.

For osteoclast detection we selected PNG due to the portability of the format to different operating systems like Windows, Mac OSX, and Linux, and since preliminary experiments indicated about 30 % less disc space usage than when using compressed TIFF images.

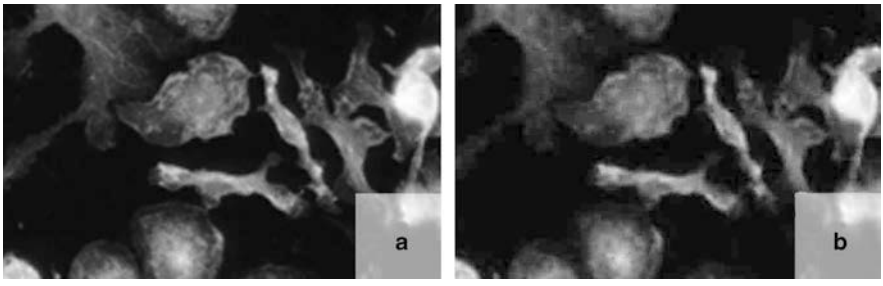

Fig. 10 The importance of file formats in image processing is illustrated in this figure. Images (**a**,**b**) represent the same image detail derived from an immunofluorescence-labeled osteoclast culture. While image (**a**) was stored as PNG (with lossless compression), image (**b**) was saved as JPEG. JPEG may reduce image quality dramatically and may introduce rectangular artifacts that could affect machine-learning classification

2.5.2 Errors Related to the Gestalt Laws

The origin of the term Gestalt (the essence of an entity's complex form) goes back to Ernst Mach in 1886 (Mach 1886). Since that time Gestalt laws are extensively examined in psychology. Examples of these laws are grouping of objects based on proximity, similarity, closure, good continuation and connectedness. A summary published in Scientific American describes and illustrates these Gestalt laws comprehensively (Rock and Palmer 1990). Especially when ground-truth data are created, Gestalt laws play an important role. Human recognition of shapes is still a research field with many open questions. One way to reduce the effect of Gestalt laws is to make people aware of it: Training them with examples can increase the output quality of ground-truth data, thus improving detection accuracy of the algorithm. As a result, interdisciplinary projects should be preferred to avoid such imprinted pitfalls.

Up to this day, there are scenarios where image processing cannot compete with human intuition. One frequently found example is the problem of touching clumps of nuclei (Moffat et al. 2006). Figure 11a exemplifies a case where image processing may fail, although the nuclei can be intuitively separated by a human (Fig. 11b) and different human observers are remarkably consistent in where they separate given nuclei. Analyses that require identification of single nuclei fail if no proper segmentation of these clumps is available. There are several approaches to dividing clumps in single nuclei in culture and tissues (Rogojanu et al. 2010) such as applying watershed algorithms (Malpica et al. 1997), cutting of nuclei along the angles following their morphological shape (Cloppet and Boucher 2010), level-set based processes (Xiong et al. 2006) or dynamic programming that try to model the human expertise (Nandy et al. 2007). However, each of these approaches has scenarios where it fails, so currently there is no computational algorithm available that can handle all different cases of clumps.

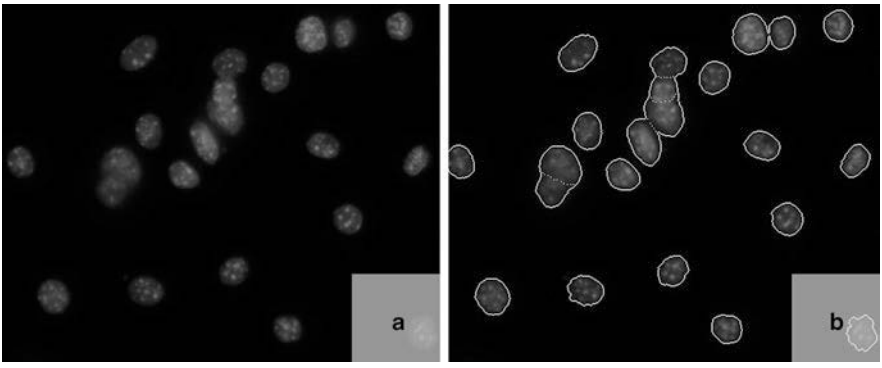

Fig. 11 This figure illustrates the limitations of automated image-segmentation in comparison to human object-recognition. Figure (**a**) shows an image detail of an osteoclast culture, where cell nuclei are labeled with DAPI. Clusters of nuclei, i. e. overlapping nuclei are visible. The result of a segmentation of these nuclei is indicated in image (**b**). The detected perimeters derived from automated image-segmentation are drawn as *solid white lines*, while the *dotted lines* represent the additional separation of the overlapping nuclei as a human would intuitionally draw them

Trying to model this method is tricky due to the fact that it is not yet known how the human brain recognizes objects (Liu et al. 2009). Figure 12 illustrates an idealized decision-making process of a human. In real life this process is assumed to be less structured and contains trained template-recognition, which is believed to be present in the human subconscious and to be instantly available (Lennert and Stein 1981; Baak 1991) – an example would be recognition of numbers or letters by literate humans. In contrast to the human brain, slight variations in size, staining, orientation, illumination are not accounted for by the computer and therefore result in poor recognition performance. Currently, image processing uses features (e. g. curvature, intensity changes, homogenous textures, edges etc.) that seem to be too different and too "weak" to model the human performance of perception.

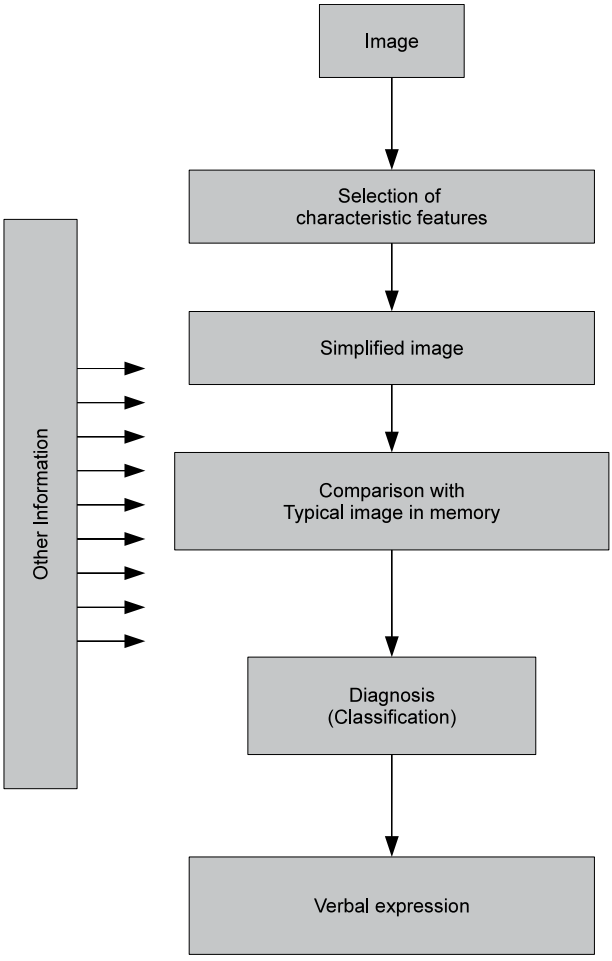

Fig. 12 This flowchart shows an assumed human diagnostic-process of an idealized decision-making situation. Modified after Baak (1991)

Nevertheless, visual perception is not the only source of error: verbal expression differs from expert to expert. An example would be the size of osteoclasts. One human expert may assume that a "huge" osteoclast is one with up to six nuclei whereas for another expert, "huge" osteoclast means more than 16 nuclei. Obviously, development of an algorithm requires background knowledge of the target cell structure and texture. Ideally, the computer scientist obtains this information during interviews with the biological expert and together they create a CRS at the beginning of the project instead of relying on verbally communicated information only. Quantitative evaluation is given by ground-truth data which should agree with explicit knowledge given in the CRS, and over the course of the project the CRS should be regularly discussed and possibly adapted. The interpretation of the given features to classify the target cells varies depending on the final knowledge of the algorithm engineer. This was shown for pathologists in Livesey et al. (1978) and Pool et al. (1979). However, nowadays when pathological interpretations are quantified by computer-based evaluation, computer scientists take the place of the pathologists by developing tools to support their diagnoses. Clearly, they face the same problems with less medical and biological experience and additionally they interpret the data based on their technical background which may lead to problems of overgeneralization.

2.5.3 Benefits of Automated Osteoclast Detection

Despite these pitfalls, automated segmentation and analysis of osteoclasts has significant advantages. Large amounts of data can be processed – we have processed regions up to 10×10 FOVs (respectively $13{,}920 \times 10{,}240$ pixels) and the only limits for further scaling up are the acquisition time of the images and the computer's processing and memory capacity. Our computer-based evaluation is faster by about two orders of magnitude compared to a trained human expert. Additionally, the result after manual quantification is just the number of osteoclasts whereas our algorithm yields many more informative measures, such as total cell number, total and relative numbers of osteoclasts and their precursor cells, total area of all cells, total and relative area of osteoclasts and their precursor cells, numbers of nuclei, quantification of associated proteins and many morphological and statistical features. Furthermore, it is highly unlikely that a single human quantification on large regions is reproducible or consistent if compared to another human expert (due to fatigue and different interpretations by different humans). However, reapplying an algorithm to the same set of images always yields an identical result.

2.6 Conclusion

Applying image processing and machine-learning techniques to biological and medical images can improve the quality of research and diagnostics dramatically.

Automated analysis produces consistent quantitative measures. Small differences not visible to the human eye, but possibly linked to states of disease, can be detected. Currently applied visual inspection normally produces an overall score rather than measuring each cell. The human mind also cannot keep track of the multiple informative measures of cells or tissue and is generally less able to integrate many weak predictive measures. It should also be mentioned that machine-based analysis is more efficient after development and can operate 24 h a day, 7 days a week.

One domain that especially benefits from such systems is bone research. Osteoclasts quantification is currently done manually, so that large-scale experiments cannot be conducted. The intra- and inter-variability between experts is normally very high, in contrast to an automated system that detects and quantifies these cells, improves the quality of the result and is always consistent. Applying the algorithm to several hundreds of slides is then feasible compared to manual counting that would take months and yield far less information.

However, legal issues have to be considered before such algorithms can successfully be applied to standard diagnosis in hospitals, and the results of automated quantifications are dependent on a number of parameters like correct staining and acquisition. Image processing will never replace human experts completely because the final diagnosis and interpretation is still up to human expertise, but it can relieve the scientists or physicians from a huge amount of repetitive work and at the same time increase the significance of the obtainable results.

2.7 Acknowledgements

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An Introduction to the Immune System 3

Veronika Lang and Georg Schett

3.1 Innate and Adaptive Immunity

The term osteoimmunology describes the molecular and cellular crosstalk between the skeletal and immune systems. Before referring to these immune – skeletal interactions, we introduce the two main branches of the immune system, the innate and the adaptive immunity. The immune system developed during evolution to protect the organism against invading infectious agents such as bacteria, fungi and viral agents. In principle, the immune system is divided into two major branches that differ from each other in preferentially involved cell types and the mechanisms of action of the immune response. However, innate and adaptive immunity are also closely linked and synergized to ensure best immune defence against microbes:

On the one hand, innate immunity constitutes an old defence system, which has been highly conserved over millions of years among different species. The innate immunity can be characterized as ancient, rapid, non-specific, invariant and constant, with minimal expansion and limited diversity. Highly conserved molecular patterns such as lipopolyscaccharide, sugar moieties such as mannans and glycans, which are so-called pathogen associated molecular patterns (PAMPs) are recognized by germ line-encoded receptors on a distinct subset of leukocytes. The cells that participate in the innate immunity do not undergo clonal expansion. Therefore, these strategies are suitable for the quick recognition of structurally conserved patterns and an immediate defence against the offending agents. A further characteristic aspect is that there is no immunological memory with innate immune responses.

On the other hand, there is the adaptive immunity that evolved to form a defence that is specifically fitting to the particular pathogen. As a consequence, it requires

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some time to ensure building-up highly specific responses to the infectious agent. Another feature of the adaptive immunity is the development of an immunological memory that ensures rapid and highly specific responses after a second encounter with the same pathogen.

Beside the efficient protection against potential danger caused by various microorganisms, it is very important that the immune response is not directed against the host himself with consequent damage or destruction of the body's own tissues and organs ("horror autotoxicus"). This so-called self/non-self-discrimination is the essential feature of the immune system that protects against developing autoimmunity. Here we want to give an overview on the types of cells and pathways of this defence system that creates both innate and adaptive immunity.

3.2 The Innate Immune Response

3.2.1 Effector Cells in Innate Immunity

The distinctive features of innate immunity commonly refer to a broadly distributed variety of myeloid and lymphoid cells that can exert rapid effector functions through a limited repertoire of germline-encoded receptors. These effector cells can be characterized by the expression of glycoprotein surface molecules, the "cluster of differentiation" (CD) molecules, which are determined by their binding of specific monoclonal antibodies. In particular, the types of cells that are capable of the innate immune response are neutrophils, monocytes, macrophages, natural killer (NK) cells, eosinophils, basophils and mast cells. All these cells except the NK cells are derived from the common myeloid progenitor, the stem cell that also gives rise to megacaryocytes and erythrocytes. As there is a morphological difference between these aforementioned lineages, these cells also differ in their immunological function.

3.2.1.1 Neutrophils

Neutrophils represent the first line of defense as they are attracted by chemotactic factors and accumulate at the site, where a potentially noxious agent is recognized for the first time. As an initial action neutrophils take up the microorganims by phagocytosis and destroy the pathogen in cytoplasmic vacuoles by producing oxygen species and lytic enzymes. Beside the quick destruction of the invading agent, neutrophils also produce chemokines and cytokines such as TNFα to attract more effector cells such as macrophages to the site of the pathogenic invasion. Therefore, neutrophils are also of key importance for further steps of immune activation. Another task of neutrophils is that they are involved in tissue regeneration after elimination of the pathogen by producing cytokines that promote cell proliferation.

3.2.1.2 Monocytes, Macrophages and Dendritic Cells

Monocytes represent approximately 10 % of leukocytes in human blood. Primarily, monocytes function as phagocytes that scavenge toxic components and pathogens. By means of phagocytosis they are enabled to quickly remove bacteria or virus-infected cells. Possibly dangerous structures are recognized by pathogen recognition receptors such as toll-like receptors that bind particular molecular patterns. These receptors are described below in more detail. Beside their ability to destroy microbes, monocytes have the function to remove apoptotic cells and cellular debris. Thus, monocytes play a crucial role for cellular homeostasis and may prevent the development of autoimmunity. In accordance, it is known that a defective removal of apoptotic cells contributes to the pathogenesis of autoimmune disorders like systemic lupus erythematosus. Monocytes also play a pivotal role in inflammatory conditions, as they are a major source for pro-inflammatory cytokines such as TNFα. Based on their phagocytic activity and their pattern of cytokines expression, monocytes are further subdivided in three different subsets: The major population are the CD14+CD16-CCR2^{high}CX3CR1^{low} monocytes that represent up to 90 % of human monocytes and are characterized by production of IL-10 after LPS stimulation *in vitro*. Other subsets are the CD16+ monocytes, which can be divided into two subclasses: The CD16+CD14+ CD64+CD32+ positive cells function as phagocytes and produce TNFα and IL-1 after LPS-stimulation. In contrast, the CD16+CD14^{low} monocytes lack phagocytic activity and do not produce cytokines in response to LPS. Macrophages are derived from monocytes that have left the blood circulation and emigrate to target tissues.

Depending on their localisation in different organs, macrophages are given distinct names: for example, liver-resident macrophages are called Kupffer cells, macrophages in the central nervous system are named microglia, and histiocytes are macrophages embedded in the connective tissue. Macrophages are characterized by surface expression of CD11b, CD14, CD68, F4/80 (mice)/EMR1 (human), lysozyme M and MAC-1/MAC-3. Like neutrophils or monocytes, macrophages also serve as phagocytes. They appear to be mobilized shortly after the recruitment of neutrophils. However, they persist much longer than the neutrophils at sites of chronic inflammation. Macrophages kill bacteria by the production of nitric oxide. Additionally, macrophages play a role in linking of innate and adaptive immune responses as they produce cytokines like IL-12 or INFγ that direct adaptive immune responses to the TH1-type.

Depending on the co-stimulatory signals, macrophages are polarized into two groups and have broadly been classified as M1 and M2 macrophages. M2 macrophages can further be divided into M2a, M2b and M2c macrophages. These two major subsets of macrophages differ in terms of their receptors, cytokine and chemokine expression, as well as effector function. Whereas M1 macrophages are microbicidal and inflammatory, M2 macrophages are immune modulators and are poorly microbicidal. The classically activated M1 macrophages produce cytokines such as IFNγ, IL-6, IL-12 or TNF, which are potent proinflammatory mediators. Alternatively, activated M2 macrophages are induced by IL-4. IL-10 or IL-13 and

act anti-inflammatory by production of IL-10 and TGF-β, which antagonize pro-inflammatory cytokines. Thus, macrophage activation can be either pro-inflammatory or anti-inflammatory.

A further important crosslink to the adaptive immune system is that macrophages serve as antigen-presenting cells. After phagocytosis by a macrophage, the antigen is processed by proteolysis. Particles of the pathogen are presented on the surface of the macrophage on MHC class II molecules, which – different from MHC class I molecules – are only expressed by professional antigen-presenting cells. The presentation of the antigen in a processed form is required to activate T-dependent responses in adaptive immunity. This issue is described in more detail below.

The most potent antigen-presenting cells are the dendritic cells (DCs) that can be found in most tissues of the body with a maximum in secondary lymphoid organs. There are two kinds of DCs: The so-called classical, myeloid DCs (mDCs) are enabled to phagocyte antigens and present the processed particle on MHC class II molecules to permit antigen recognition by T cells. The mDCs recognize pathogens commonly via the "toll-like" receptors TLR 2 and TLR 4 and drive the immune response by the production of IL-12 that activates both NK and T cells. Besides these conventional dendritic cells, which are monocyte-derived, a second type of dendritic cells is known, viz. the plasmacytoid DCs (pDCs). These cells are designated according to their morphology and are believed to be derived from lymphoid progenitors. pDCs detect DNA and RNA from viruses by "toll-like" receptors TLR 7 and TLR 9, and respond to pathogen encounter by the secretion of inflammatory chemokines and the interferons INF-α and INF-β.

3.2.1.3 Natural Killer Cells

Natural killer (NK) cells are defined morphologically as large granular lymphocytes. Comparable to T- and B cells they originate from the common lymphoid progenitor. Human NK cells are characterized by the surface expression of CD16 and CD56 and the lack of CD3 or a T cell- or B cell receptor. NK cells kill viral-infected cells by cytolysis. The lack of MHC class I molecules on the surface of a virus-infected or a tumor cell, a condition called "missing self", a viral strategy to evade other mechanisms of defence such as apoptosis-induction by CD8+ cytotoxic T cells, inactivates mechanisms that prevent NK cells from killing their target. Further, preceding stimulation of NK cells by activating ligand-receptors interaction is required. These receptors bind soluble ligands such as cytokines like IL-1 or IL-2, cell surface molecules or chemokines. Additionally, NK cells are involved in immunoregulatory mechanisms, and inflammatory processes as they are able to produce cytokines such as IFN-γ or IL-10. NK cells play a crucial role to sustain self-tolerance while efficiently destroying virus-infected or malignant derived cells. Recently it was discovered that NK cells can develop an immunological memory. Therefore, their position is attributed both to the innate and the adaptive immunity.

3.2.2 Pathogen Recognition in Innate Immune Responses by "Toll-Like"-Receptors

Toll was first discovered in *Drosophila melanogaster* where it was found to play a key role in embryonic development and the immune response against fungi. Ten human "toll-like" receptors (TLR) have been identified to date. TLRs recognise pathogen-associated molecular patterns (PAMP), which mostly are conserved products of microbial metabolism produced by bacteria, but not by the host. Ligands of TLRs are for example LPS for TLR-4, peptidoglycans for TLR-2, flagellin for TLR-5 or unmethylated CpG DNA for TLR-9. These receptors are found on phagocytes such as macrophages, neutrophils or dendritic cells and allow to quickly distinguish between self and infectious non-self. Structural properties of all TLRs are the N-terminal leucine-rich repeats and the cytoplasmic Toll/IL-1 receptor homology domain (TIR). Upon ligand-recognition TLRs drive the expression of genes that are involved in host defence, such as antimicrobial proteins, proinflammatory cytokines, chemokines, co-stimulatory and MHC molecules. There are different downstream signal cascades depending on the adaptor molecules that are recruited to the TIR. One important intracellular signalling pathway is the activation of NFκB that promotes the expression of proinflammatory cytokines such as TNFα or IL-1.

3.2.3 Humoral Effectors of the Innate Immunity

3.2.3.1 The Complement System

The complement system consists of over 25 plasma and cell surface proteins and is an important effector mechanism that links both the innate and the adaptive immunity. The main function of the complement system is to mark invading pathogens to have them recognized for phagocytosis by, for example, macrophages, a process called opsonisation. The second aim is to attract other effectors of the innate immunity to the site of pathogen encounter by release of cleavage products that are produced during complement activation and act as chemoattractants. The third way of action is that complement activation can directly promote cell destruction by forming the so-called membrane attack complex (MAC) and inducing lysis of the cell by increased membrane permeability. There are three different ways of complement activation: The classical pathway requires stepwise proteolytic cleavage of complement components and is mediated by antibody-antigen complexes. The alternative way of complement activation is triggered by microbial structures that neutralize inhibitors of spontaneous complement activation and does not require antigen-antibody interaction. The common final path of both modes of activation is the formation of the MAC that promotes removal of viral-infected cells or microbes, respectively. During the formation of the MAC the complement component C3a is produced which is a potent chemokine and efficiently attracts immune cells to the site of inflammation. The third activation pathway is called the lectin pathway of complement activation because it is started by the contact with mannan-containing

microbial cell walls or by complexes of microorganisms and host pentraxins and ficolins.

The mechanisms of complement-mediated cell destruction potently drive local inflammation by recruiting cells of both the innate and adaptive immunity to the place of pathogen encounter. Therefore, the complement system can be seen as a means of crosstalk between the two branches of the immune system. This feature can also be applied to different kinds of cytokines that represent a kind of communication between the different effectors of the immune system.

3.2.3.2 Cytokines

Cytokines are glycoprotein or glycopeptide molecules that are produced by a variety of cells including almost all cells, which are involved in the adaptive or innate immunity. Cytokines act as humoral mediators that regulate cellular growth and differentiation, drive inflammatory processes and direct the course of inflammation and defence strategies against pathogens. There are five main groups of cytokines: the interleukins (IL), the interferons (INF), the tumor necrosis factors (TNF), the colony-stimulating factors (CSF), and the chemokines. Members of all of these five groups are significantly involved in regulation of both the innate and the adaptive immunity.

Interleukins are named after their discovery as they are secreted by leukocytes as a means of communication. There are thirty-seven Ills known to date with a combination of shared and separate functions. Almost all mechanisms of action in both branches of immunity are directed by ILs, as these cytokines are produced by nearly all involved cells such as T- and B cells, monocytes, macrophages, NK cells and DCs, to only name the major participating subsets. The role of different ILs for the two kinds of T-helper (TH)-mediated cell response is described below in more detail. INFs are mainly produced as response to viral infection and activate macrophages, NK cells and T-lymphocytes. Members of the TNF family are mostly secreted by macrophages. TNFα plays a pivotal role in promoting inflammation and macrophage activation. Chemokines serve as attracting agents for different kinds of immune cells to the site of inflammation or pathogen encounter. There are three different types of chemokines related to the localisation of a cysteine-residue in their amino-terminal. Chemokines serve as ligands for the corresponding chemokine-receptors, which are expressed by various subsets of effectors in innate and adaptive immunity. In summary, the cytokines regulate a concerted action of the two different branches of immunity to ensure an efficient defence against antigens.

3.3 The Adaptive Immune System

The aim of the adaptive immunity is to generate a response that is perfectly fitting to the particular antigen. This process takes time by necessity, but has the advantage of simultaneously establishing an immunological memory. After re-exposure

to the antigen the immunological memory ensures an immediate reaction against the invading agent. B- and T cells are the key players in adaptive immunity, but they are also dependent on the support of the effectors of innate immune response. After antigen encounter B cells differentiate and mature helped by TH-cells (T-Helper cells) to produce specific antibodies of high affinity. Cytotoxic T cells constitute the defence against intracellular pathogens. Adaptive immunity is dominated by the interplay of B- and T cells. Therefore these two lymphocyte-subsets are described in more detail.

3.3.1 B Cells

3.3.1.1 Early B Cell Development

B cells are named after the organ, in which they develop, the Fabricius' Bursa in birds or the bone marrow in mammals. Both B- and T cells are derived from the common lymphoid progenitor. Depending on co-stimulatory signals the lymphocytes developing from these stem cells are committed either to the B cell lineage or the T cell lineage. As mentioned, development from the progenitor to the mature B cell takes place in the bone marrow, supported by bone marrow stromal cells that produce cytokines and deliver co-stimulatory signals allowing the survival of B cells. This process is divided into several stages of maturation with the aim to generate B cells with a functional B cell receptor, which is a membrane bound immunoglobulin (Ig)-molecule. The formation of the Ig is a highly complex process that requires somatic reassembly and hypermutation of the encoding genes. Principally, Ig molecules are composed of a heavy and a light chain and have antigen-recognising variable as well as constant regions that also define the Ig subclass. Antibody diversity is generated through the rearrangement of the V, D and J segments of the Ig heavy and light chain genes. Only one allele in each B cell becomes successfully rearranged, a phenomenon called allelic exclusion. Thereby the "one B cell one specificity concept" is guaranteed, which means that one particular antigen can only be recognised by one specialised B cell.

As a first step of B cell maturation the stem cell differentiates to the pro-B cell stage that is characterized by the rearrangement of heavy chain genes. If the rearrangement is successfully completed, the cell expresses the pre-B cell receptor (BCR), a membrane bound μ heavy chain molecule, and is then defined as pre B cell. Signalling via the pre-BCR and the IL-7 receptor are critical for the further development to immature B cells: after the successful rearrangement of the Ig light chain the B cell expresses the BCR, a membrane-bound IgM molecule, and is called immature B cell. If the B cell fails at any stage of this developmental process, it is removed by the induction of apoptosis. To guarantee immune tolerance there are two steps of selection that prevent the generation of auto-reactive B cells. In the first step (positive selection) only those B cells whose BCR can strongly interact with MHC II molecules receive survival signals. Next, B cells that recognize auto-antigens by their BCR are eliminated by apoptosis. This latter process is called negative selection. After completing positive and negative selection the B cell additionally

expresses surface IgD and becomes a mature B cell. Mature B cells leave the bone marrow and migrate to secondary lymphoid organs such as lymph nodes or the MALT (mucosa associated lymphatic tissue) where they wait for antigen encounter.

3.3.1.2 B Cell Development to Antibody-Forming Plasma Cells and B Cell Memory in T Cell Dependent Responses

B cells are able to recognize native structures of antigens by their BCR. In T cell dependent immune responses to protein antigens the B cell requires co-stimulatory signals from a CD4+-TH-cell that already had contact with the same antigen. After recognition by the BCR the antigen is processed intracellularly and presented on a MHC class II molecule to the specialized, so-called cognate T cell. Additional co-stimulatory ligand-receptor-interactions between the B- and the TH-cell are required to promote further B cell differentiation, such as the interaction between B7 on the surface of the B cell with CD28/CTLA4 on the T cell. Another important co-stimulatory signal is delivered by the interaction of CD40 on the B cell with CD40L/CD154 on the T cell. This interplay between the B- and T cell takes place in so-called germinal centres (GC). GCs are the area of lymphoid follicles in lymph nodes and the spleen where the B cells clonally proliferate and mature to antibody-secreting plasma cells or memory B cells. These developing B cells, called germinal centre B cells, are supported by follicular dendritic cells that present antigen-antibody complexes on their surface, which stimulate B cell growth and differentiation. During the clonal expansion, genes encoding for the BCR undergo an extremely high rate of somatic mutation to generate a BCR that interacts highly selectively with its target. Thereby a B cell clone is selected that recognizes the antigen with the highest specificity. The less-specific B cells lack further stimulatory signals and die by apoptosis. The apoptotic cells are removed by macrophages in the GC. Next the selected clone divides several times and differentiates further into plasmablasts, plasma cells and memory B cells.

Plasmablasts produce antibodies to a greater extent than B cells, but are still able to produce antigens and to interact with T cells. In contrast, plasma cells are only capable of antibody production. Plasma cells secrete an enormous mass of Ig molecules and are the body's "antibody producing machinery". Contrary to their precursors, plasma cells do not express MHC class II or surface Ig molecules anymore. The class of the secreted Ig molecules depends on the co-stimulatory signals (i. e. cytokines) the B cell received during differentiation by the T cell. This means that there might be a class switch from IgM antibodies that are primarily produced in the antibody response, to another Ig subtype. The lifespan of the plasma cell can be limited to a few days, but there are also plasma cells that migrate to bone marrow and survive for a lifetime. These long-lived plasma cells ensure immediate antibody-production after re-encounter with the same pathogen.

Another developmental pathway of the selected B cell clone is the differentiation to a memory B cell. Human memory B cells can be identified by the expression of CD27, whereas CD27 is not found on their murine equivalent. They usually express

IgG, IgA, or IgE on their surface, although some also display IgM. They generally lack IgD. Memory B cells migrate actively between the blood and secondary lymphoid organs and have a lifespan up to decades. As a hallmark, memory B cells are able to directly differentiate to high affinity-plasma cells after antigen re-encounter without the preceding process of affinity maturation in a GC. In contrast to the primary response, class-switched antibodies are produced in the secondary response to a pathogen. The ability to immediately respond to a pathogen is due to the already hyper-mutated Ig V region. The generation of an immunological memory is characteristic of T-dependent responses, although it is believed that it may also arise in response to T-independent antigens.

3.3.1.3 T Cell Independent Immune Responses

Antigens that activate B cells without any help from T cell are called T cell-independent (TI) antigens. As a common feature these antigens have highly repetitive structures that activate B cells by BCR cross-linking. For example, such structures are found in bacterial cell wall components. There are two kinds of TI antigens: TI type 1 (TI-1) antigens such as LPS also activate immature B cells, whereas TI-2 antigens such as bacterial polysaccharides of gram negative bacteria like *pneumococci* or *meningococci* only activate mature B cells. A specialized B cell subset, the splenic marginal zone (MZ) B cells, is capable of TI-2 responses. These B cells are named after their localisation in the splenic marginal zone, which lies between the red and the white pulp and represents a filter station of the blood. However, humans lack a histologically distinguishable MZ in the spleen, but also have MZ B cells, which are re-circulating in contrast to their murine equivalents. MZ B cells are characterized as IgM+CD21highCD23low. After antigen recognition they differentiate into IgM-secreting plasmablasts within hours and ensure the first-line defence against pathogens. The MZ B cell- derived plasmablasts produce IgM in great number, and in the later phase of the response antibodies of the IgG subclass are also secreted. The loss of MZ B cells in splenectomised individuals explains why they are at increased risk of developing septic complications after infection with gram-negative bacteria.

3.3.1.4 Ig Subclasses

Ig molecules have a variety of functions: The simplest form of action is that antibodies neutralize the antigen and prevent it from causing damage to the body. Further, Igs contribute to the recognition of pathogens and their elimination by initializing various cellular and humoral effector mechanisms. Antibody-tagged microbes can be recognized and eliminated by phagocytes. Another feature is that cell surface-bound antibodies activate the classical pathway of the complement system.

There are five different Ig subclasses: IgM, IgG, IgA, IgE and IgD. The IgG-subclass is further subdivided into IgG1 to IgG4. The Ig molecule is structurally similar to a "Y". It integrates four separate polypeptides, two light (L) and two heavy (H)

chains that are covalently joined by disulfide-bonds. The protease papain cleaves the Ig molecule into two antigen-binding fragments (Fab) and the crystalline fragment (Fc). The Fab consists of the light chain and the part of the heavy chain that carries the antigen-binding domain. The Fc fragment consists of the constant C region of the heavy chains that determines the antibody-subclass. L chains are composed of a variable (V) region, joining (J), and a constant (C) region. There are two classes of L chains in mammals, κ and λ, determined by the V and J region. There are five different kinds of H chains μ, γ, α, ε and δ corresponding to the Ig-subclasses IgM., IgG, IgA, IgE and IgD. H chains are similarly a combination of a V, a diversity (D), a J, and a C region. The V domain of the L and the H chain work together as antigen-recognition site; the constant domains form the Fc-fragment and represent the complement-binding region. Further, the Fc-fragments of pathogen-bound antibodies are ligands for the corresponding Fc-receptors on phagocytes and facilitate antigen-recognition. This process of marking the pathogen with antibodies is called opsonisation.

IgM molecules are found as membrane-bound monomers on B cells. In their secreted form they occur as high-molecular pentamers that are connected by a J chain. IgM is the first Ig subclass to be produced in adaptive immune responses. In addition, the pentameric IgM is the most potent activator of the complement system. Antibodies of the IgG subtype represent the majority of serum Ig with a proportion of 75 %. IgG is the predominant antibody-subtype produced during memory responses, but it is also secreted to a small extent in the primary adaptive immune response. As the only antibody-subclass, IgG antibodies are able to cross the placental barrier and give passive immunity to the foetus. IgA antibodies occur as monomers in the blood. However, these antibodies are mainly secreted in a dimeric form into different body spaces. Therefore, IgA play a pivotal role for the mucosal immune system in the gut and the lung as these antibodies form a protective layer against invading pathogens on the mucosal surfaces. IgA is also found in the breast milk contributing to the passive immunity of the newborn. Antibodies of the IgE subtypes are associated with atopic conditions such as allergic asthma bronchiale and are crucial for the defence against eukaryotic parasites such as helminths. This subclass has the lowest serum-concentration. IgD is predominantly expressed on mature B cells and is only found in low levels in the serum.

3.3.2 T Cells

3.3.2.1 T Cell Subsets and Development

The second key player in adaptive immunity is the T cell, named after the thymus, the place where they differentiate. Depending on the composition of their T cell receptor (TCR), T cells can be subdivided into two classes, the αβ T cells and the γδ T cells. The TCR is composed of an αβ or a γδ heterodimer that is non-covalently associated with the CD3 complex. CD3 is a pan-T cell marker and is importantly involved in TCR-signal transduction. Additional accessory molecules are CD4 and CD8 that determine the interaction with MHC I or MHC II molecules and the func-

tional specificity. The αβ T cells represent the bulk of T cells in lymphoid organs and they are restricted to the response to antigens that are either presented on MHC I or II molecules. In contrast, the γδ T cells constitute only 2 % of T cells. γδ T cells are generally not MHC-restricted and are mostly found in the gut mucosa where they seem to be involved in microbial surveillance. Further, T cells can be subdivided according to their antigenic specificity and their effector functions into CD4+ TH-cells and CD8+ cytotoxic T cells. Similar to the antibody-response driven by B cells, memory is also established in the T cell mediated adaptive immunity. Memory T cells are both of the CD4+ and the CD8+ subtype and rapidly achieve effector functions after re-encounter with the pathogen.

The T cell progenitors are derived from the common lymphoid progenitor in the bone marrow and enter the thymus to differentiate to the mature T cells, supported by antigen-presenting cells of the thymic microenvironment. T cell development can be divided into three steps: the first stage is the CD4-CD8- double-negative; the second is the CD4+CD8+ double-positive, and the last the CD4+ or CD8+ single-positive stadium. The fate of CD4 or CD8-expression is driven by the property of the TCR to better interact with MHC II or I molecules, respectively. Comparable to B cells, the T cells are positively and negatively selected during their developmental process to guarantee immunological tolerance and prevent autoimmunity.

3.3.2.2 CD4+ Effector T Cells

TH-cells, also known as effector T cells, are important for steering the adaptive immune response. Based on their functional specificity TH cells can further be subdivided to different populations, the TH1-, TH2-, Treg- and TH17-cells. TH1-cells produce the signature cytokine IFNγ along with proinflammatory cytokines as TNFα and TNFβ, to stimulate innate and T cell mediated immune responses. The most important function of TH1-cells is to promote cellular immunity that is mediated by CD8+ T cells that possess direct cytolytic activity to eliminate obligate intracellular pathogens. IL-12 drives the differentiation to the TH1-specifity. Self-reactive TH1-cells are related to autoimmune disorders such as insulin-dependent diabetes mellitus or rheumatoid arthritis. TH2-cells are characterized by the production of IL-4, IL-5, IL-9, Il-10 and IL-13. The TH-2 mediated response promotes the humoral immunity and is crucial for the defence against extracellular pathogens. IL-4 essentially directs the TH2 response. An aberrant TH2 response is implicated in atopic conditions such as allergic asthma bronchiale.

A newly defined subset of TH-cells are the IL-17 producing TH17-cells that play a critical role in the induction and propagation of autoimmune conditions such as multiple sclerosis, rheumatoid arthritis, psoriasis or inflammatory bowel disease. TH17-development is driven by TGFβ, IL-6, IL-21 and IL-22. STAT3 (signal transducer and activator of transcription 3) and RORα (retinoic-acid-receptor-related orphan receptors alpha) are also critical for TH17-differentiation. CD4+CD25+FoxP3+ regulatory Treg-cells can either differentiate in the thymus or are derived from naive T cells after stimulation with TGFβ. Treg-cells are characterized by the expression of the transcription factor FoxP3 (Forkhead Box Protein-P3),

which mainly regulates T cell function and directs the expression of surface molecules and cytokines. In contrast to the other TH-subsets, Treg-cells are immunosuppressive. Importantly, Treg-cells sustain self-tolerance and prevent auto-reactivity. It is suggested that Treg-cells set a limit in immune responses against infections and regenerate homeostasis after pathogen elimination. Disrupted Treg-function is associated with a variety of autoimmune disorders.

3.3.2.3 Cytotoxic CD8+T Cells

Cytotoxic CD8+ T cells recognize antigens that are presented on MHC class I molecules. MHC I molecules are found on nearly all cells of the body and are loaded with peptides that are produced from cytosolic proteins by proteasomal degradation. Therefore, CD8+ T cells are capable of defending against intracellular pathogens such as viruses or obligate-intracellular bacteria such as *Listeria monocytogenes*. The majority of CD8+ cytotoxic T cells are resident in lymphoid tissues. Before activation, co-stimulatory signals by an antigen-presenting cell (APC) are required. Cytotoxic T cells possess a variety of effector functions. On the one hand, CD8+ T cells produce cytokines such as TNFα or IFNγ, which are crucial for the defence against microbes. On the other hand, CD8+ T cells have potent cytotoxic activity: the target cell is killed by either apoptosis-induction through FAS-FAS-ligand interaction or by the granule- exocytosis pathway. FAS-FAS ligand interaction initiates apoptosis by the release of cytochrome c out of mitochondria and consecutive caspase-activation. Caspases are cysteine-proteases that drive apoptosis in a multi-step enzymatic process. The granules contain the pore-forming protein perforin, which increases cell-permeability by damaging the membrane and facilitates the entry of other granule enzymes. One of these is the granzymes, a family of serin-proteases that contribute to apoptosis-induction by caspase-activation. In addition to the defence against microbial pathogens CD8+ T cells are also crucial for the elimination of tumor cells. Similar to the antibody response, immunological memory is also established during cellular immune responses. Auto-reactive CD8+ T cells are involved in the pathogenesis of various autoimmune disorders such as insulin-dependent diabetes mellitus, systemic lupus erythematosus, or ANCA-associated vasculitides. Further, CD8+ T cells contribute to allograft rejection and graft-versus-host disease in transplantation medicine.

3.4 Conclusion

Bone is a tissue with several pivotal functions for the organism: it stabilizes the body's structure, stores calcium and harbours the hematopoietic system. There are many relations between the skeletal and the immune systems: Osteoclasts that resorb bone and play a crucial role in inflammatory conditions are derived from monocytes or dendritic cells. Bone-forming osteoblasts also act as regulators of the hematopoietic niche that gives rise to all cells of both the myeloid and the lymphoid lineage. Further, it is well known that multiple factors that regulate immune cells, including cytokines, chemokines, and growth factors, also control osteoblast and osteoclast activity. It is well established that immune cells and cytokines contribute to the loss of bone mass observed in osteoporosis or in inflammatory diseases such as rheumatoid arthritis or inflammatory bowel disease. Cytokines that are secreted by activated immune cells in inflammatory conditions drive increased bone turnover and skeletal pathology. Hence, an improved understanding of osteoimmunology might be the key to develop new therapies for inflammatory disease, involving both bone and the immune system.

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Effects of Vitamin D in the Immune System

4

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The term "vitamin" for vitamin D reflects the original finding that plants contain a substance able to restore bone metabolism in patients suffering from rickets. Later on, endogenous production of a related molecule in animal and man was recognized, dependent on sufficient sun exposure of the skin and increasing its activity 10^5 times after further renal metabolism.

This was the discovery of the "hormone" vitamin D_3. In the last century, it was discovered that cells outside the renal system were also capable of generating the most active metabolite of vitamin D_3, namely $1\alpha,25(OH)_2D_3$, and that this was independent of the hormonal regulatory pathways responsible for homeostasis in bone metabolism. Today we are aware of a large number of different cells and tissues able to generate and metabolize $1\alpha,25(OH)_2D_3$ and, as a counterpart, many cell types have been identified as targets for this molecule. In many ways the properties of this part of the vitamin D family classify it as a mediator with some characteristics of cytokines – except for its steroid nature – with paracrine, autocrine and, only under specific conditions, endocrine functions. Its integral role in the innate and adaptive host defense system is well established now, and in part there is interference as well as independence between the osteological and the immunological branch of the family.

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4.1 Vitamin D

In this section, a brief summary of the metabolism and activity of vitamin D is presented; for more detailed information the reader is referred elsewhere (Norman 2008; Holick 2008).

The term vitamin D describes a group of secosteroids originally believed to act as essential fat-soluble hormones on bone and mineral homeostasis. Most prominent members of the vitamin D group are vitamin D_2, ergocalciferol, and vitamin D_3, cholecalciferol. While vitamin D_2 derives from plant ergosterol, vitamin D_3 can be produced in the human skin from cholesterol or additionally be provided by nutritional intake of animal products. Since it was originally believed that vitamin D is essential for health but could not be produced by the organism itself, it was classified among fat-soluble vitamins. All vitamin D hormones are provitamins, gaining their biological activity by rupture of their ring b and are then called "secosteroids".

4.1.1 Vitamin D Metabolism

In the human organism, vitamin D_3 has the greatest significance. vitamin D_3 is produced from cholesterol in the skin by radiant sun energy: Ultraviolet B mediates the conversion from 7-dehydrocholesterol to the instable previtamin D_3, which is stabilized by spontaneous, heat-dependent isomerisation to the vitamin D_3. The further classical metabolizing pathway involves two hydroxylation steps taking place in the liver and in the kidneys. Firstly, the parent vitamin D_3 is hydroxylated to 25(OH)D_3 by hepatocytes, a reaction catalyzed by the 25-hydroxylase (CYP2R1) (Baeke et al. 2010a). A very important event in regulation of bone and mineral homeostasis is the conversion from 25(OH)D_3 to the biologically active form of vitamin D_3, 1α,25(OH)$_2D_3$ or 24R,25(OH)$_2D_3$. This step is mediated by the enzyme 1α-hydroxylase (CYP27B1) produced by the kidney renal proximal tubulus cells and is under tight control by the endocrine system. Most data in publications and within this chapter refer to effects of the active metabolite 1,25(OH)$_2D_3$. In accordance with other authors we use the terms "vitamin D_3" or "calcitriol" as synonyms for 1,25(OH)$_2D_3$.

Vitamin D_3 and metabolites are transported in the blood by the vitamin D binding protein (DBP), a member of the albumin family. DBP not only serves as transporter, but also as an important vitamin D_3 reservoir, since only free amounts of 1,25(OH)$_2D_3$ are crucial for determination of biologic activity.

Inactivation of 1,25(OH)$_2D_3$ is promoted by a further hydroxylation step. 1,25(OH)$_2D_3$ itself promotes its own degradation by induction of the 24-hydroxylase (CYP24A1), an enzyme expressed in most tissues, this mechanism probably serving as an internal feedback loop. Metabolites are secreted into the bile and are partly reabsorbed via the enterohepatic circulation (Bringhurst et al. 2008; Norman 2008).

4.1.2 Vitamin D Signal Transduction

In target tissues, biological functions of vitamin D are realized via binding to the vitamin D receptors (VDR). As to date, two different receptor types are known. The classical pathway comprises the nuclear VDR, a classical nuclear steroid receptor mediating biological activities within hours. In addition, rapid actions of vitamin D taking only seconds to minutes are known. These are mediated by VDR receptors located in the cell membrane. Throughout this chapter, if not mentioned otherwise, we will name the nuclear VDR simply as VDR, since this receptor type is not only crucial for bone and calcium homeostasis but also for immune function regulated by vitamin D (Norman 2008).

4.1.3 The VDR

The VDR is a classical nuclear hormone receptor belonging to the steroid receptor superfamily, such as nuclear receptors for estradiol, glucocorticoids and vitamin A metabolites. After intracellular binding of its key ligand $1,25(OH)_2D_3$, the VDR mainly forms heterodimers with the retinoid X receptor (RXR). The DNA-binding domain of the VDR consists of two zinc-finger domains and the ligand-VDR-RXR complex can bind to vitamin D-responsive elements within promoters of vitamin D responsive genes in order to regulate mRNA-transcription and, consequently, physiological actions of vitamin D are also modulated by co-activators or co-repressors.

In contrast to the nuclear VDR, membrane receptors are responsible for rapid actions, such as regulation of calcium absorption in the intestine known as "transcaltachia". These receptors are located in flask-shaped plasma membrane invaginations called "caveolae". Second messenger systems others than the mechanism involved in signal transduction for the nuclear receptors participate in these processes: Signal transduction via phospholipase C, proteinkinase C, G-protein, phosphoinositide 3-kinase, voltage-gated calcium or chloride channels are assumed. The existence of a crosstalk between the nuclear and membrane vitamin D receptor pathways via the RAF/MAP kinases network has also been reported (Norman 2008).

A summary of classic vitamin D actions is given in Fig. 1.

4.1.4 Classic Effects of Vitamin D

The effects of vitamin D regarding bone and calcium homeostasis are well established. Regulation of serum calcium and serum phosphate levels is under tight control. Since the first step in hydroxylation in the liver is poorly regulated and almost the total amount of the parent vitamin D_3 is converted to $25(OH)D_3$, it is seen as a good indicator for evaluation of the vitamin D_3 status of an individual (Peterlik and Cross 2006; Baeke et al. 2010a). The real key player in this tightly regulated process is the 1α-hydroxylase, the enzyme catalyzing the second hydroxylation step.

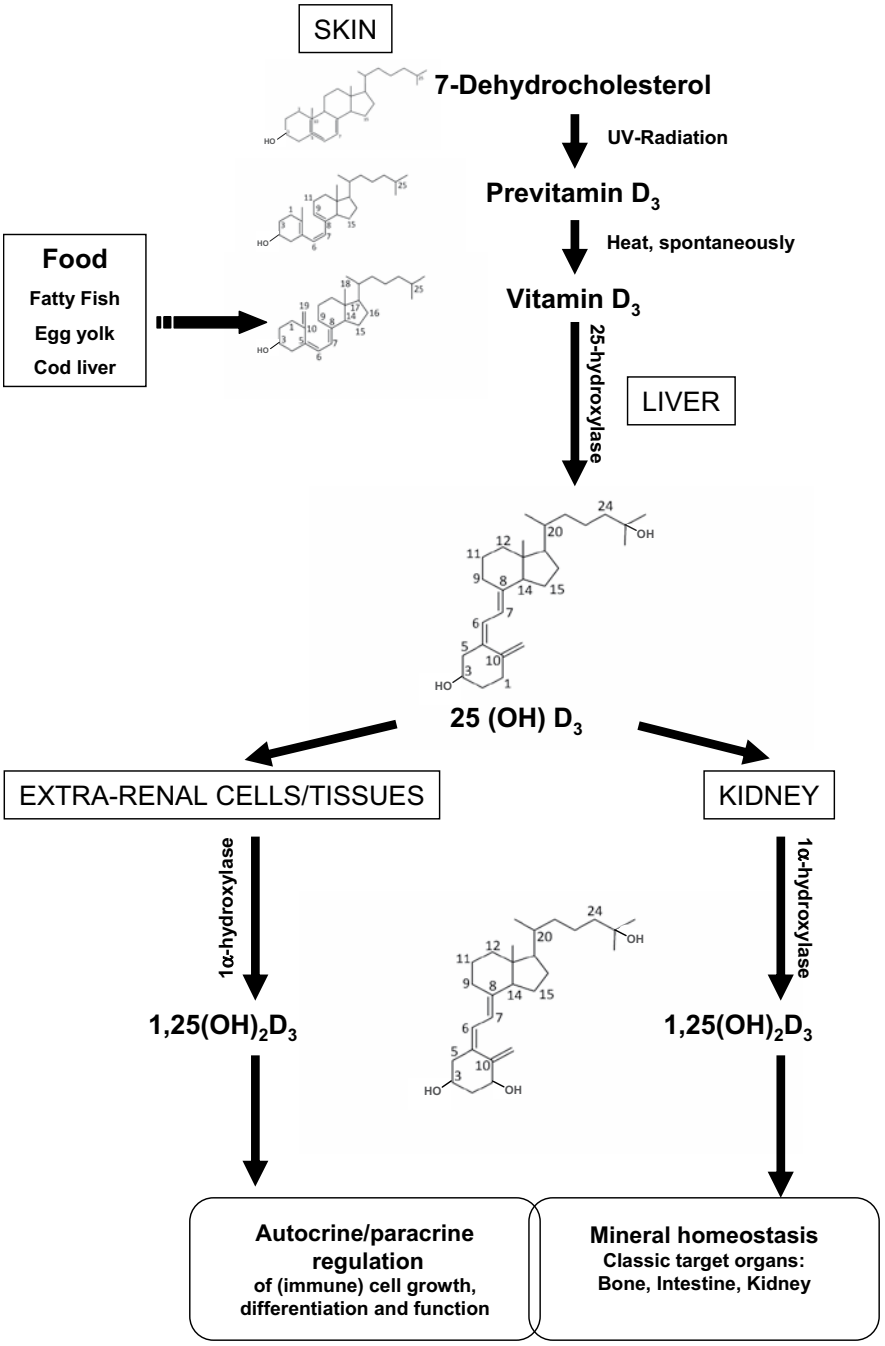

Fig. 1 Summary of classic vitamin D actions (modified from Norman 2008)

Expression of 1α-hydroxylase is strongly influenced by serum calcium and phosphate levels, parathyroid hormone (PTH) and $1,25(OH)_2D_3$ itself (Baeke et al. 2010a). Decrease in serum calcium levels stimulates PTH secretion from the parathyroid glands. PTH triggers phosphate secretion in the kidneys, thus resulting in decreased serum phosphate levels. Decrease in serum phosphate stimulates conversion from $25(OH)D_3$ to $1,25(OH)_2D_3$ in the kidneys. $1,25(OH)_2D_3$ elevates the serum calcium levels by facilitating calcium absorption in the intestine and decreases renal calcium excretion. Furthermore, the maturation of osteoclasts allows calcium mobilization from bone to blood (Herold 2008; Norman 2008). $1,25(OH)_2D_3$ itself induces its own degradation by induction of the 24-hydroxylase (CYP24A1), thus probably serving as an internal feedback loop (Norman 2008).

4.1.5 Additional Effects

Effects of vitamin D are not limited to its significant role in bone and calcium homeostasis. Many tissue and cell types express the VDR (as shown in Table 1) (Holick 2008). Furthermore, the key enzyme 1α-hydroxylase is expressed by various cell types and is controlled differently from the renal enzyme (Holick 2008; Peterlik and Cross 2006). There is strong evidence that $1,25(OH)_2D_3$ operates not only in an endocrine, but also in a paracrine fashion, thus being important for regulation of normal cell differentiation and proliferation in many biological systems. Vitamin D_3 is known to enhance cell differentiation and inhibit cell proliferation. Therefore, adequate vitamin D_3 levels can be considered crucial for normal immune function and normal cell regulation, including inhibition of cancerogenesis (Peterlik and Cross 2006).

4.2 Vitamin D and the Immune Cells

For detailed information on the Immune System the reader is referred to the section "Immunology" (see the Chapter by Lang und Schett) in this book. The following only refers to basic immunology where essential for understanding.

Intersection of vitamin D with the immune system has been discovered more than 25 years ago. Clinical observations indicated that patients suffering from the granulomatous disease sarcoidosis showed elevated serum calcium levels and it could be demonstrated that excessive local $1,25\ (OH)_2D_3$ production in macrophages situated in granulomas was responsible for this (Adams et al. 1983). Furthermore, it was shown that the VDR receptor is expressed by almost all cells of the immune system (see Table 1). While antigen presenting cells like dendritic cells, macrophages and monocytes show a constitutive expression of the VDR, resting T and B lymphocytes are able to upregulate VDR expression upon activation (Provvedini et al. 1983, 1989; Holick 2008) (also see Table 1). Moreover, the key enzymes

of $1,25(OH)_2D_3$ production (1α-hydroxylase) and degradation (24-hydroxylase) are also expressed by immune cells. Macrophages and dendritic cells show expression of the 1α-hydroxylase identical to the renal form, but activity of this key enzyme in immune cells is differently regulated compared to the tight endocrine control mechanisms of calcium and bone homeostasis. Local production of $1,25(OH)D_3$ is strongly dependent on the amount of $25(OH)D_3$ available and predominantly under control of immune signals such as IFN γ, LPS (lipopolysaccharide) and viral infections (Baeke et al. 2010a; van Etten and Mathieu 2005). Furthermore, 1α-hydroxylase activity of immune cells seems to lack a direct negative feedback control, which explains the occurrence of hypercalcemia in granulomatous diseases such as sarcoidosis and tuberculosis, where massive local production of $1,25(OH)_2D_3$ is mediated by macrophages situated in the granulomas (Adams et al. 1983). In situ production of $1,25(OH)_2D_3$, makes high local levels possible, far exceeding those of serum and allowing a paracrine modulation of immune function virtually independent of systemic serum levels (Baeke et al. 2010a).

Table 1 Extra-renal 1α-hydroxylase and VDR expression in various cell types and tissues (modified from Peterlik and Cross (2005) and Holick (2008)

Extra-renal tissues or cells expressing 1α-hydroxylase	VDR expression in non calcemic tissues/ cells
Monocytes/macrophages	Thymus
Osteoblasts	Lymphocytes
Endothelial cells	Monocytes
Synovial cells	Epidermis
Pancreas	Hair follicles
Prostatic gland	Dermis
Ovary	Melanocytes
Uterus	Myocytes
Breast	Cardiac muscle
Large intestine	Pituitary gland
	Pancreas
	Prostatic gland
	Gonads
	Placenta
	Breast
	Stomach
	Brain

4.2.1 Innate Immunity

Historically, there were early signs that vitamin D deficiency could affect the immune function. Already in the 17th to 19th century physicians observed that children with rickets were more likely to develop pneumonia or to be infected with tuberculosis. In the 19th century, "sun-light" was a well accepted therapy option for tuberculosis as well as vitamin-D-enriched diets e. g. cod liver oil (Chesney 2010; Holick 2008). Today it is a well supported fact, that vitamin D_3 is an enhancer and stimulator of the innate immune response and that vitamin D_3 deficiency influences innate immune functions (Chesney 2010; Mora et al. 2008).

Major components of innate immune responses are monocytes and macrophages. vitamin D_3 seems to have a pro-differentiating effect on monocytes gaining a macrophage like phenotype. Furthermore, expression of Fc receptors is modulated, and antigen processing, chemotaxis, phagocytic capacity, tumor cell cytotoxicity as well as antimicrobial activity is enhanced. Production of IL-1β and TNF α, induced by a variety of stimulators, is decreased in vitamin D_3-treated mature monocytes, although a positive effect of vitamin D on IL-1β and TNF α production has been described for immature monocytic cells. (Chesney 2010; Baeke et al. 2010a; Adams et al. 2007b; Boltz-Nitulescu et al. 1995; Zarrabeitia et al. 1992; Almerighi et al. 2009).

Macrophages are able to rapidly recognize certain "danger" signals on microbes, the highly conserved pathogen-associated molecular patterns (PAMPS) by means of so-called pattern recognition receptors. The most prominent subgroup of pattern recognition receptors are the Toll-like-receptors (TLR) with many of their signalling pathways leading to activation of the transcription factor NFkappaB, consequently enhancing production of pro-inflammatory cytokines such as IL-1 and TNFα (Akira et al. 2006; Chesney 2010, also see Chapter Immunology; Adams et al. 2007b).

Binding of PAMPS to the TLR2/1 on macrophages induces not only expression of VDR, a process probably mediated by IL-15 (Baeke et al. 2010a; Krutzik et al. 2008) but also the 1α-hydroxylase. This enzyme catalyzes local conversion from available $25(OH)D_3$ to $1,25(OH)_2D_3$. After binding of $1,25(OH)_2D_3$ to the VDR, production of fundamental antimicrobial peptides (AMPs), such as cathelicidin with its active form LL-37, is induced. Cathelicidin, localised in autosomes, seems to be crucial in the defence against *Mycobacterium tuberculosis*, since it induces autophagy and therefore promotes killing of intracellular pathogens (Liu et al. 2006). Liu et al (2006) provided evidence for human TLR induction of cathelicidin on a DNA level. Furthermore, they observed that addition of sera from African Americans, known to have lower vitamin D_3 serum levels due to skin pigmentation and to be more susceptible to tuberculosis, to monocyte cultures resulted in weaker TLR dependent induction of cathelicidin mRNA when compared to addition of sera obtained from a Caucasian cohort. After supplementation of $25(OH)D_3$, a recovery of TLR induced cathelicidin production could be detected. In a subsequent study, the authors could show a raise of cathelicidin production in vivo induced by TLR in monocytes upon vitamin D_3 supplementation and therefore provide evidence that

vitamin D_3 is crucial for maintenance of localized innate immune functions (Adams et al. 2009; Hewison et al. 2010).

On the monocyte surface, expression of CD14, the TLR2 co-receptor, is strongly boosted by VDR (Miller and Gallo 2010; Spittler et al. 1997; Do et al. 2008). Interestingly, at the same time surface expression of TLR2 as well as TLR4 on monocyte and macrophage is diminished by vitamin D_3 (Sadeghi et al. 2006). It is hypothesized that TLR downregulation leading to hypo-responsiveness to PAMPS not only prevents cellular over-stimulation and oxidative stress but also limits an inflammatory response (Baeke et al. 2010a). It is postulated that elevated TLR expression is associated with several chronic inflammatory diseases. One study conducted with patients suffering from Behçet Disease showed higher expression of TLR 2 and 4 on monocytes compared to healthy controls and remarkably, this alteration was even found to be inversely associated with 25(OH)D_3 serum levels (Do et al. 2008).

Vitamin D effects on cathelicidin production and surface receptor expression are summarized in Fig. 2.

Vitamin D_3 signalling is also essential for induction of defensin β 4, an antimicrobial peptide used in defense against intracellular invaders. Transcription of the defensin β 4 gene may be initiated via convergence of the VDR and IL-1β pathways (Chesney 2010; Liu et al. 2009).

Data on regulation of production of the inducible nitric oxide synthase (iNOS) by vitamin D_3 are conflicting (Baeke et al. 2010a). Enhancement (Rockett et al. 1998)

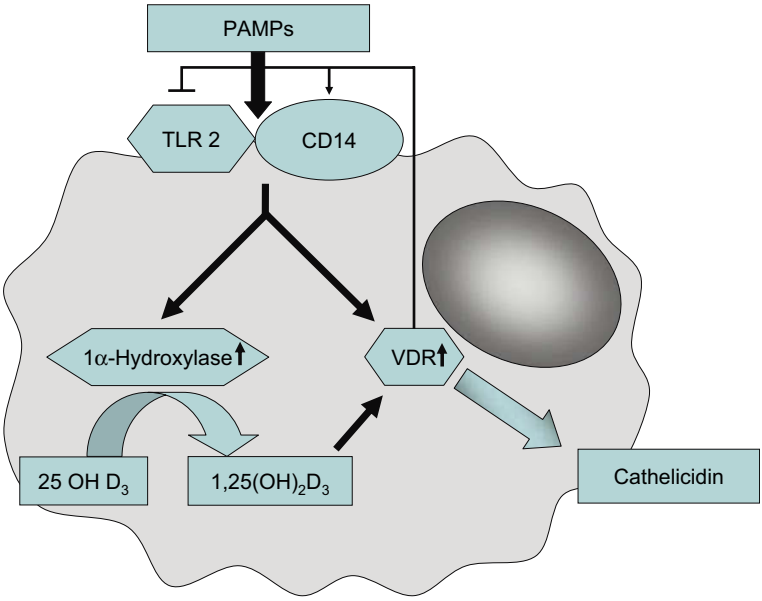

Fig. 2 Regulation of innate immune reaction by Vitamin D_3 following infection (e. g. *Mycobacterium tuberculosis*); For further explanation see text. PAMPS: pathogen-associated molecular patterns; TLR: Toll-like-receptors; VDR: Vitamin D receptor; (modified from Chesney 2010 and Miller and Gallo 2010)

as well as suppression of production (Chang et al. 2004; Pedersen et al. 2007) by vitamin D_3 has been described. NOS is an enzyme important for the generation of the reactive oxygen species nitric oxide (NO). At least in rodents, NO participates in the oxidative burst, an important mechanism of monocytes and macrophages in microbe killing (Baeke et al. 2010a). NO production in human monocytes or macrophages seems to be far less pronounced and relevance of iNOS for the oxidative burst in humans has still to be elucidated (Chang et al. 2004). Nevertheless, Chang et al. (2004) could show that PBMC from tuberculosis patients showing high vitamin D_3 serum levels released less NO. When mouse macrophages were exposed to vitamin D_3 at increasing concentrations, a dose-dependent decline in iNOS expression was observed. The authors hypothesize that this may protect cells from oxidative injury, since NO metabolites and LDH (lactate dehydrogenase) were diminished at the same time.

4.2.2 Antigen Presenting Cells (APC): Dendritic Cells and Monocytes/Macrophages

The dendritic cell (DC) is regarded as the most potent APC and therefore essential for priming of naïve T cells. Exposing DC to vitamin D_3 in vitro inhibits their differentiation and maturation process and their antigen-presenting capacity is severely hampered. Expression of MHC II, co-stimulatory molecules (CD40, 80, 86) and other DC maturation surface markers such as CD1a and CD83 is diminished, while CD14, a monocytic marker and co-receptor of TLR2, is persistent (Baeke et al. 2010a, van Etten and Mathieu 2005). IL-12 production, the key cytokine for initiation of the Th1 pathway, is suppressed, as well as expression of IL-23, a cytokine essential for Th17 differentiation. Increased production of the immunosuppressive cytokine IL-10, also necessary for regulatory T cell generation, and CCL22, a chemokine able to attract CCR4+ Foxp3+CD4+CD25+ regulatory T cells (Penna et al. 2007; dÁmbrosio 2006) can be observed. Furthermore, up-regulation of the inhibitory immunoglobulin-like transcript 3 (ILT-3), an inhibitory receptor that wield negative control on APC activation, is observed even though not crucial for Treg induction (Penna et al. 2005; Unger et al. 2009). Additionally, DC treated with vitamin D_3 are capable of antigen specific Treg induction and express high levels of programmed death-1 ligand, a member of the co-stimulatory B7 family also negatively affecting T cell response and probably important in tolerance induction (Unger et al. 2009). In summary, exposure of differentiating myeloid (but not plasmacytoid) DC to vitamin D_3 results in production of tolerogenic DC with reduced capacity to prime and activate T cells (Adorini et al. 2004; Penna et al. 2007), exerting their immunosuppressive effect not only by suppression of the Th1 and Th17 pathway but also by induction of T cells with regulatory properties (Tregs) (Adorini et al. 2004; Penna et al. 2005; Baeke et al. 2010a). While expression of the 1α-hydroxlase and therefore local production of $1,25(OH)_2D_3$ increase with the differentiation stage of DCs, VDR expression is diminished in maturing DC, thus reducing the autocrine effect. As a consequence, initiation of the T cell response is permitted and high

local concentration of vitamin D_3 might prevent further maturation of other DC and excessive stimulation of T cells in a paracrine fashion (Hewison et al. 2003, 2010).

Regarding monocytes, the immune-modulatory effects of vitamin D_3 on antigen presentation and T cell stimulation is considered similar to the DC: expression of MHC II, co-stimulatory molecules (CD40, 80, 86) is reduced (Baeke et al. 2010a; van Etten and Mathieu 2005; Spittler et al. 1997) and the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and IL-12, as well as of M-CSF (Baeke et al. 2010a; van Etten and Mathieu 2005; Boltz-Nitulescu et al. 1995) is diminished. The influence of vitamin D_3 on TNF α production – subsequently triggered by a variety of different stimulators – seems to be dependent on the differentiation status of monocytic cells with increased production in immature cells, such as cell lines and bone marrow cells, and a decrease in more mature cells, such as peripheral blood monocytes (Hakim et al. 2003). Vitamin D_3 also affects Fc-receptor expression on monocytes, leading to downregulation of FcγRI/CD64, FcγRII/CD32, FcγRIII/CD16 and FcεRII/CD23 and stimulates the expression of FcαR/CD89 (Boltz-Nitulescu et al. 1995). Furthermore, vitamin D_3 exerts several effects on monocytes, reflecting their differentiaton to macrophages (Kreutz and Andreesen 1990).

In several cell types, the 24-hydroxylase, responsible for degradation of the active $1,25(OH)_2D_3$, is inducible by $1,25(OH)_2D_3$, thus providing negative feedback control. In monocytes, expression of the 24-hydroxylase seems to depend on their maturation and activation. Undifferentiated/resting monocytes show inducible expression of the 24-hydroxylase upon exposure to vitamin D_3, while activated macrophages are resistent to $1,25(OH)_2D_3$ linked to 24-hydroxylase induction. This could be explained by the observation that infection-induced INFγ production activates STAT 1 α (Signal Transducers and Activators of Transcription 1 α) which negatively regulates the expression of the 24-hydroxylase (van Etten and Mathieu 2005; Chesney 2010). Upregulation of 1α-hydroxylase with subsequent (local) production of $1,25(OH)_2D_3$, accompanied by the lack of its negative feedback 24-hydroxylase eventually may even result in systemic hypercalcemia as observed in sarcoidosis.

4.2.3 T Cells

CD4+T cells have important modulating functions in adaptive immunity (also see chapter Immunology). In the 1980s, Mosmann and Coffman initially described two different effector T cell subsets, the Th1 and Th2 cells (Mosmann and Coffman 1989). It was believed that after antigen presentation to a naïve T cell, immune responses either take the Th1 or the Th2 pathway, dependent on the respective pathogen involved and the local cytokine environment present. Several years ago, the classical Th1/Th2 dichotomy has been expanded by the identification of further CD4+subsets (also see chapter immunology). On the one hand, a heterogeneous group of T cells with regulatory properties necessary for maintenance of self tolerance and with limiting immune reactions was identified and termed regulatory T cells (Tregs). On the other hand, a T cell subset, characterized by its key cytokine IL-17, which was discovered in murine disease models of chronic (auto) inflammatory conditions and later on verified in humans, was designated Th17 sub-

set (Annunziato and Romagnani 2009a). Another subset, described by our group and highly inducible by vitamin D_3, is characterized by production of IL-6 and was therefore denominated "Th6"(Azizi-Semrad et al. 2010; Willheim et al. 1999; Thien et al. 2005; Pichler et al. 2002). Very recently, the Th9, which is also an assumed new T-helper cell subset acting as a key player in autoimmune conditions, has been characterized. Whether the latter two subsets picture independent T-helper cell subsets or may be interpreted as implication of the plasticity of the Th system still remains to be elucidated (Veldhoen et al. 2009).

Relatively few data exist about functional subsets of CD8 T cells (Tc) and many of them only refer to the murine system. There are, however, reports about equivalents to Th1-, Th2- and Th17-cytokine production patterns in CD8 T lymphocytes. These consistently have been termed Tc1, Tc2 and Tc17, and essentially develop under the same conditions as their CD4+ counterparts (Seder et al. 1992; Kelso et al. 1991; Salgame et al. 1991; Byun et al. 1994; Croft et al. 1994; Le Gros and Erard 1994; Kemeny et al. 1994; Noble et al. 1995; Hamada et al. 2009; Huber et al. 2009). Effects of vitamin D_3 on in vitro differentiation of human CD8+ T lymphocytes have been described as very similar to the differentiation of Th1, Th2 and Th6 (Thien et al. 2005).

4.2.4 Th1/Th2 Cells

Th1 cells are characterized by production of their signature cytokine IFNγ and are therefore crucial in the defence against intracellular pathogens by means of macrophage activation, while Th2 cells, characterized by production of IL-4, IL-5 and IL-13, cooperate with B cells in immunoglobulin production (also see chapter Immunology) (Annunziato et Romagnani 2009b). Excessive and pathologic reactions of these distinctive patterns present themselves as the Th1 mediated subgroup of autoimmune diseases at one end of the spectrum and as Th2 mediated allergic conditions at the other end. The decisive role of the local cytokine milieu for Th-differentiation has been recognized for the first time in connection with the hypothesized dichotomy of Th1 and Th2 lineage: whereas IL-12 is a strong inductor of the Th1 response whilst inhibiting the Th2 pathway, IL-4 is an essential cytokine for initiation of the Th2 pathway (Demeure et al. 1994; O'Garra and Murphy 1994; Seder et al. 1994; Thien et al. 2005).

Naïve T cells are able to express the VDR upon activation (Baeke et al. 2010b) and vitamin D_3 can affect T cells in two ways: T cells can act as a direct target (Boonstra et al. 2001; Thien et al. 2005; Mahon et al. 2003; Baeke et al. 2010b) or can be influenced by modulation of APC differentiation and their cytokine production (see above Innate Immunity), possibly hampering antigen presentation to naïve T cells, leading to inhibition of T cell priming and differentiation. Direct effects of vitamin D_3 on T cells have already been verified on gene level and over 102 target genes for vitamin D_3 in CD4+ cells have been described (Mahon et al. 2003).

Vitamin D_3 effectively inhibits T cell proliferation (Bikle 2009; Rigby et al. 1984), partly mediated by inhibition of IL-2 production. IL-2, a cytokine secreted by activated T cells, operates in an autocrine fashion and is an indispensable growth and

survival factor for T cells (Smith 1998; Smith and Popmihajlov 2008; van Etten and Mathieu 2005). Administration of vitamin D_3 to human or murine T cell cultures inhibits IL-2 production directly (Tsoukas et al. 1984; Müller et al. 1993; Dimeloe et al. 2010; van Etten and Mathieu 2005; Takeuchi et al. 1998; Rigby et al. 1984), resulting in reduced proliferation and differentiation capacity of T cells.

It is well established that vitamin D_3 potently inhibits the induction of the Th1 pathway (van Etten et al. 2005; Boonstra et al. 2001; Willheim et al. 1999; Pichler et al. 2002.; Thien et al. 2005), leading to the assumption that local adequate vitamin D_3 levels could prevent Th1 mediated autoimmunity (Cutolo et al. 2007). Th1 cells are characterized by production of their signature cytokine IFNγ, which is a necessary feedback signal for further activation of macrophages in defence against intracellular pathogens such as *Mycobacteria, Listeria* or *Leishmania* (Cantorna et al. 2008; van Etten and Mathieu 2005, also see chapter Immunology). It has been shown, that vitamin D_3 significantly inhibits the differentiation of (IFNγ producing) Th1 cells (Müller et al. 1996; Willheim et al. 1999; Pichler et al. 2002.; Thien et al. 2005) and the IFNγ gene, containing a VDRE, was found to be a direct target of vitamin D_3 (Dimeloe et al. 2010; Cippitelli and Santoni 1998). In fact, vitamin D_3 seems to inhibit especially the IL-12 induced IFN γ production while the constitutive IFNγ production is only slightly affected (Thien et al. 2005).

While these results from murine and human in vitro studies unequivocally substantiate the suppression of Th1 cytokines, data on Th2 differentiation seem to be contradictory. In an APC-free murine culture system it was shown that in vitro administration of vitamin D_3 to naïve T cells inhibited the Th1 pathway while enhancing Th2 differentiation. Frequency of T cells showing Th2 cytokine expression (IL-4, IL-5 and IL-10) was increased and expression of the Th2 associated transcription factors GATA-3 and c-maf was upregulated. Abrogation of these effects by neutralization of IL-4 suggested that they were all mediated by IL-4 (Boonstra et al. 2001). Other in vitro studies, in the murine as well as the human system, showed no response or even suppression of the Th2 lineage by calcitriol (Dimeloe et al. 2010; Pichler et al. 2002; Staeva-Viera and Freedman 2002). Interestingly, one study using a murine culture system showed a suppression of Th2 induction when vitamin D_3 was present during in vitro differentiation and polarization of naïve T cells (i. e. addition of IL-4 and anti-IL-12) while in already activated T cells vitamin D_3 had no effect on IL-4 production (Staeva-Viera and Freedman 2002).

Another in vitro study using human adult peripheral blood mononuclear cells showed a small reduction of IL-2 positive human T cells and an increased proportion of T cells positive for IL-4 and especially for IL-13 on a single cell level (Willheim et al. 1999). A subsequent study showed that vitamin D_3 only slightly enhances the constitutive IL-4 production in Th2 cells. Nevertheless, when vitamin D_3 was administered in combination with IL-4, a strong co-stimulatory effect on the production of the Th 2 cytokines IL-4 and IL-13 could be observed (Thien et al. 2005).

A possible explanation for the conflicting results regarding Th2 differentiation was recently presented by Dimeloe et al (2010). They reviewed in vitro vitamin D_3 levels in several studies and hypothesized that low in vitro concentrations of vitamin D_3 (10^{-7}M to 1^{-9}M) induced suppression of the Th1 lineage as well as the Th2

pattern, while high concentrations of vitamin D_3 (1x 10^{-6}M) might enhance IL-5 and IL-13 production (Jirapongsananuruk et al. 2000). This seems to corroborate data from epidemiological studies: Hyppönen et al. (2009) found a significant but non-linear positive correlation of serum 25-OH vitamin D levels with serum IgE concentration. In this large British cohort study both subjects showing very low and very high 25-OH vitamin D_3 serum levels had increased total and specific IgE concentrations. Such a U-shaped correlation might predispose both of them for atopy and allergy, conditions at the Th2 end of the T-helper cell spectrum.

Nevertheless, in studies applying exactly the same "low" vitamin D_3 concentrations (10^{-7}M to 1^{-9}M) in vitro, different effects could also be observed: a complete suppression of Th1 and Th2 cytokine induction was found in human cord blood which exclusively contained naïve T cells (Pichler et al. 2002), while in adult blood Th2 cytokine production was enhanced (Willheim et al. 1999; Thien et al. 2005). Different effects of vitamin D_3 on the naïve T cell population compared to the memory T cell subset have already been shown, showing the CD45RA+ (naïve) subset to be less susceptible to inhibitory effects of vitamin D_3 (Müller and Bendtzen 1992, 1996).

As a constant result, independent from suppression or induction of IL-4 producing T cells, a CD4+ T cell population characterized by production of IL-6 is stimulated by in vitro vitamin D_3 administration to human PBMC (Willheim et al. 1999). A strong co-stimulatory effect of IL-4 and vitamin D could be observed, and in adult T cells this is accompagnied by co-expression of IL-4, as has been demonstrated on a single cell level, whereas naïve IL-6+ T lymphocytes remain negative for IL-4 (Pichler et al. 2002; Thien et al 2005). A distinct IL-6 producing subset could also be detected in the peripheral blood at a low proportion, and since typical transcription factors of the Th2 (GATA-3) and Treg (FoxP3) lineage were not found, the subset was termed "Th6" (Azizi-Semrad et al. 2010). The function of this subset still remains to be elucidated, since peripheral mononuclear cells other than T cells have the capacity to produce great amounts of IL-6 and therefore are eminent for systemic effects. It can be hypothesized that the importance of the Th6 might lay in a paracrine (similar to other cytokines) modulation of immune function, since IL-6 is the cytokine decisive for initiation of the pro-inflammatory Th17 or a tolerance inducing Treg pathway (Workman et al. 2009). The consistent induction of IL-6 in T cells may be a crucial effect of vitamin D, at least in humans, and increase of IL-4 production (or IL-4 producing T cells) might be a mutable concomitant circumstance.

4.2.5 Tregs

Regulatory T cells are a heterogeneous group of CD4+ T cells important for maintenance of immunological tolerance, prevention of autoimmune disease and limitation of inflammation due to their highly suppressive capacity. The group of regulatory T cells can be divided into 2 subpopulations according to their origin: firstly, the natural Tregs (nTregs), characterized by expression of their master transcription

factor FOXp3 and high constitutive expression of the IL-2 receptor α chain, CD25, derive from the thymus. The second subset can be induced in the periphery, and is therefore called i(nducible)Tregs and can be further subdivided into the Tr1 subset, characterized by production of IL-10 as its signature cytokine and the inducible FOXp3+ subset. Regulatory T cells exert their suppressive function by secretion of the anti-inflammatory cytokines IL-10, TGFβ, as well as by consumption of the T cell survival factor IL-2. They also modulate APC function by upregulation of CTLA-4, a surface protein expressed by activated T cells, leading to down-regulation of co-stimulatory molecules (CD80 and CD86) on APCs and in consequence limiting T cell activation (Dimeloe et al. 2010; Robinson et al. 2009).

Similar to other cells of the adaptive immune system, vitamin D can either directly exert its influence on the regulatory subset, or modulate APC function, the latter resulting in induction of a tolerogenic DC subset (for details see above) and thereby modulating T cell response. The effect of vitamin D on the generation of tolerogenic myeloid DC is already described in the upper section of this chapter. These DC are characterized by secretion of large amounts of IL-10, which leads to Treg induction (Penna et al. 2005, 2007; Adorini et al. 2004). Several studies report also on direct effects of vitamin D on Tregs: Barrat et al. (2002) conducted an in vitro study on human CD4+ T cells in absence of APC and reported on a strong synergistic effect of vitamin D_3 and dexamethason on developement of an IL-10 producing subset staining negative for IL-4, IL-5 or IFNγ. In this in vitro generated murine antigen specific IL-10 producing T cells could prevent experimental autoimmune encephalitis (an animal model for multiple sclerosis), when transferred into mice. A subsequent study of the same group demonstrated that vitamin D and IL-10 restored the generation of an IL-10 producing regulatory CD4+ T cell subsets in PBMC obtained from patients with glucocorticoid resistent asthma, a condition characterized by impaired induction of IL-10 positive T cells (Xystrakis et al. 2006). The group showed in a following in vitro study that vitamin D_3 alone also induces an IL-10 positive Treg subset showing enhanced expression of TLR9. Induction of this subset could also be demonstrated in vivo after oral administration of calcitriol in healthy volunteers. Binding of TLR 9 agonists resulted in a loss of regulatory function characterized by a decrease in IL-10 production in vitro. The authors hypothesized that this mechanism might permit an adequate immune reaction in the course of infections (Urry et al. 2009). Another group demonstrated a direct strong synergistic effect of vitamin D and IL-2 on human iTreg generation in vitro. After isolation of CD4+CD25-T cells from human PBMC and following stimulation with $1,25(OH)_2D_3$ in combination with IL-2, strong upregulation of FOXp3 and CTLA-4 was observed. At the same time, a reduction in production of the pro-inflammatory cytokines IL-17, IL-23 and IFNγ could be detected (Jeffery et al. 2009).

Taken together with the fact that vitamin D has been shown to mediate transplant tolerance and prevent development of autoimmune disease due to Treg induction in animal models (Adorini and Penna 2008; Gregori et al. 2001), it can be concluded that vitamin D is vital for maintenance of self-tolerance and prevention of immune mediated disease.

4.2.6 Th17

The Th17 are characterized by expression of their signature cytokine IL-17, an important mediator in host defence by attracting neutrophils and macrophages to the site of inflammation. Moreover, Th17 cells are supposed to play a pathogenic role in induction, enhancement and maintenance of various auto-reactive disorders and chronic inflammatory conditions, formerly believed to be Th1 mediated. This has been already confirmed in animal disease models such as collagen induced arthritis or experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis. Th17 seem also to play a role in psoriasis or inflammatory bowel diseases. The Th17 were firstly characterized in mice and following studies in humans unveiled that human Th17 might differ from their murine counterpart. In both species, RORγT or its human homolog RORC, respectively, was recognized as master transcription factor and in both, human and murine Th17, CCR6 was identified as a major chemokine receptor. The transcription factors RORα and STAT-3 are found in murine Th17, while in humans, co-expression of the Th1 master transcription factor T-bet and RORC can be detected. Primarily discovered in humans, a remarkable proportion of IL-17+CD4+ cells produce IFNγ additionally to IL-17 (Th17/Th1 cells). Moreover, Th17 exhibit a potential plasticity to Th1, which might be associated with their pathogenic role in autoimmune disorders or chronic inflammation. Murine Th17 originate from naïve T cells after stimulation with IL-1β, TGFβ and IL-6 and are expanded by IL-21 and IL-23, while human Th17 seem to derive from CD4+CD161+ precursor T cells in the thymus and probably differentiate under exposure to IL1β and IL-23 to Th17. Especially the role of TGFβ in induction of human Th17 is controversially appraised (Annunziato et Romagnani 2009a,b).

Beside vitamin D mediated modulation of APC function, which consequently affects T cell priming and therefore Th17 differentiation, several in vitro studies reported on direct effects of vitamin D_3 on Th17 induction and expansion. In a murine in vitro study it was found that vitamin D_3 suppresses the development of CD4+ IL-17 and IL-22+ T cells in an APC free culture under Th17 polarizing conditions. Although augmentation of an IL-10 producing subset could be observed at the same time, Th17 suppression could not be attributed to IL-10 signalling, unlike the situation in Th9 (see below). The authors of this study ascribed this inhibitory effect of vitamin D on Th17 partly to suppression of the Th17 transcription factors (Palmer et al. 2011). However, another murine in vitro study suggested a post-transcriptional inhibition, since at least at physiological concentrations vitamin D only affected Th17 cytokine production at protein and not at transcriptional level (Chang SH et al. 2010). Another in vivo study conducted in mice could show that orally administered vitamin D inhibited the onset of EAE and diminished the frequency of murine Th17. Migration of Th17 to the central nervous system is regarded as crucial for initiation of EAE and is mediated by the CCR6 and MIP3α/CCL20 axis. Since the authors observed not only a decreased CCR6 expression in Th17 in response to vitamin D, but also a reduced migration capacity in vitro, they concluded that vitamin D might negatively affect migration of pathogenic Th17 to the central nervous system (Chang JH et al. 2010).

In human studies similar results were obtained: it could be shown that the development of Th17 and the IL-17 production in CD4+memory T cells is suppressed in a dose-dependent manner and a strong synergistic effect of vitamin D and all-trans retinoid acid could be observed. The authors also reported on suppression of the master Th17 transcription factor RORγT and enhancement of FOXp3 (Ikeda et al. 2010). Another study examined effects of vitamin D on PBMC obtained from therapy-naive patients with early rheumatoid arthritis (RA). After stimulation, PBMC from RA patients showed a higher production of IL-17 when compared to healthy controls. When vitamin D was administrated in vitro, a suppressive effect on IL-17 production in these PBMC could be detected and additionally a strong synergistic effect of vitamin D and dexamethason was observed. Since memory T cells are regarded as the main source for IL-17 in PBMC, the authors assessed IL-17 and IL-22 production in isolated CD4+CD45RO+T cells obtained from RA patients and found increased frequencies positive for these cytokines. Consecutively, in vitro administration of vitamin D diminished TNF α, IL-22 and IL-17 levels in the memory T cell subset (Colin et al. 2010).

Regarding these studies it can be concluded that vitamin D might prevent and ameliorate Th17-mediated autoimmune conditions by suppressing not only development but also expansion and cytokine production of this Th subset.

4.2.7 Th9 Cells

Very recently, a new CD4+T cell subset characterized by IL-9 production has been described. It could be shown, that TGFß and IL-4 murine and human Th9 differentiation in vitro in mice (Veldhoen et al. 2008; Dardalhon et al. 2008, Wong et al. 2010), while IL-10 exerts an inhibitory effect on Th9 induction (Palmer et al. 2011). Th9 cells have been shown to promote experimental colitis and experimental autoimmune encephalitis (EAE) (Jäger et al 2009; Dardalhon et al. 2008) and are therefore proposed to enhance chronic inflammatory conditions. Regarding vitamin D effects on Th9 development, there is only one study available so far: Palmer et al. (2011) could show that vitamin D_3 inhibits murine Th9 differentiation in vitro. Since abolishment of the IL-10 signalling pathway restored Th9 differentiation by blocking the cytokine or the receptor, the authors concluded that vitamin D exerts its inhibitory effect on Th9 initiation by induction of an IL-10 positive subset in the T cell culture. It was hypothesized that by this mechanism vitamin D might contribute to prevention of autoimmune diseases.

4.2.8 B Cells

Similar to T cells, B cells are able to express the VDR as well as the 1α-hydroxylase upon activation (Chen et al. 2007; Heine et al. 2008). Already more than 20 years ago it was observed that $1,25(OH)_2D_3$ potently inhibited IgG and IgM production in human activated PBMC in a dose dependent manner (Lemire et al. 1984). However, since then the research focus in vitamin D mediated effects has been rather laid on

CD4+ T cells, highlighting the significance of impairment of T cell or APC function on B cell differentiation and Ig production (Müller et al. 1991). Nevertheless, there has been strong evidence of direct effects of vitamin D on B cells. This was clearly demonstrated by an early in vitro study on B cell Ig production by using EBV infected B cells, since EBV induced activation and Ig production are independent from T cells (Provvedini et al. 1986). Another in vitro study showed that vitamin D inhibits proliferation and Ig production in B cells only when administered before their differentiation into Ig producing cells (Iho et al. 1986).

More than two decades later, Chen et al (2007) provided evidence that vitamin D can affect B cells directly by inhibiting proliferation of activated B cells, plasma cell generation, Ig secretion and class-switch memory cell generation. Moreover, they demonstrated that VDR is expressed constitutively in resting B cells and can be upregulated upon stimulation. In the same study the authors observed that patients suffering from systemic lupus erythematodes (SLE), an autoimmune disease going along with B cell dysregulation and excessive Ig production, showed lower serum levels of 25 OH D_3 and 1,25$(OH)_2D_3$ than healthy controls. Regarding this finding, however, it has to be noticed that SLE frequently involves dermal lesions, and the possible role of sun avoidance by the patients has not been evaluated. The authors postulated that vitamin D might be an important factor for maintenance of B cell homeostasis (Chen et al. 2007).

4.2.9 NKT Cells

Natural Killer T cells (NKT) were first identified in mice as a T cell subset sharing some features with NK cells: NKT were found to express an α/β T cell receptor (TCR) with a restricted repertoire as well as CD161 (human) /NK1.1 (mouse), a receptor normally expressed by NK cells. Although NKT are regarded as T lymphocytes, they are not restricted to MHC dependent peptide antigen presentation, but recognize glykolipid structures on exogenous and endogenous ligands presented by CD1d. By gaining more information on NKT over time, the description as T cells with NK markers seemed to be insufficient, not matching real conditions. Various subsets have been identified and classification appears difficult and inconsistent in literature. NKT might be assigned to a specific subset by their specific TCR chains, CD161, CD4 and CD8 expression as well as reactivity to α-galactosylceramid (αGalCer), a synthetic glykolipid resulting in strong activation of NKT cells. Two main types of NKT subsets have been described: The Type 1 "classic" NKT express an invariant Vα14- Jα18 (mouse) or Vα24-Jα18 (human) rearranged α chain in their TCR and are therefore also called invariant (i) NKT. The Type 2 "non-classic" NKT are able to express diverse TCR. Upon stimulation NKT cells have the capacity to produce large amounts of different cytokines including those that are assigned to Th1 (IFN-γ) and Th2 (IL-4) cells. NKT might bridge innate and adaptive immunity since they respond rapidly, a feature of innate immune responses, to their specific antigen with secretion of polarizing cytokines determining adaptive immunity pathways. NKT seem to cover a broad spectrum of functions in the immune system

by contributing to host defence against pathogens, tumor surveillance and prevention of autoimmune conditions (Balato et al. 2009; Godfrey et al. 2004).

Although T cell function is modulated by vitamin D, normal T cell development does not require vitamin D or the VDR expression (Cantorna 2011). Remarkably, the situation is different for NKT. In VDR KO mice not only thymic and peripheral numbers of NKT were reduced, but also effector functions of remaining NKT were impaired (Yu and Cantorna 2008). NKT cells remained at an earlier maturation level, lacking expression of NK1.1 and the transcription factor T-bet. Moreover, expression of CD1d in thymocytes was reduced, thus impairing selection of NKT in the thymus (Yu and Cantorna 2008). In a subsequent study it was shown that vitamin D deficiency in utero resulted in reduced numbers of iNKT cells. This was caused by apoptosis of iNKT precursors (Yu and Cantorna 2011) and could not be corrected by later supplement of vitamin D. Since it is suggested that reduced NKT numbers can contribute to autoimmunity, sufficient vitamin D supply seems a critical factor for maintenance of self tolerance (Cantorna et al. 2011).

4.2.10 Summary of Vitamin D_3 Effects on Immune Cells

The spectrum of effects of vitamin D has increased in parallel with the discovery of (functional) subsets of cells involved in innate and specific immunity. In a complex network of regulatory and effector mechanisms vitamin D seems to be integrated with multiple points of application, revealing a subtle modulatory mode of action rather than the previously supposed immuno-suppression (also see Fig. 3). From the available literature enhancement of innate defense efficiency (e. g. monocyte function) and control or balance of specific immune reactions (e. g. via dendritic cells, NKT or Treg) can be subsumed as major tasks of vitamin D. Thereby vitamin D obviously exerts an important role at the level of cell development and differentiation, but also in a negative feedback mode in already initiated and ongoing inflammatory processes. In this respect, conclusions about the role of vitamin D drawn from the various studies have to be critically evaluated: the point of time within the life span of a specific cell, the phase of an (immunological) process, and, last but not least, the age of the individual seems to play a decisive role for the overall impact of activity or, as a rather frequent phenomenon, deficiency of vitamin D.

4.3 Pathophysiological Aspects of Vitamin D_3

4.3.1 Vitamin D Deficiency

Hypovitaminosis of vitamin D_3 is a widespread phenomenon. The major source of vitamin D is UV-dependent production in the skin (see above), while the contribution of dietary intake appears negligible, at least in countries where dairy products are not fortified. Naturally, due to UV-exposition, vitamin D_3 levels are affected by geographic distribution in terms of latitude and altitude. Altered life-styles includ-

ing reduced outdoor activity favours vitamin D_3 deficiency as well as use of sun blockers and skin cancer awareness (Ascherio et al. 2010; Peterlik and Cross 2005; Cantorna and Mahon 2004).

Serum vitamin D_3 levels and as a consequence deficiency have been primarily defined with regard to maintenance of calcium and phosphate homeostasis and

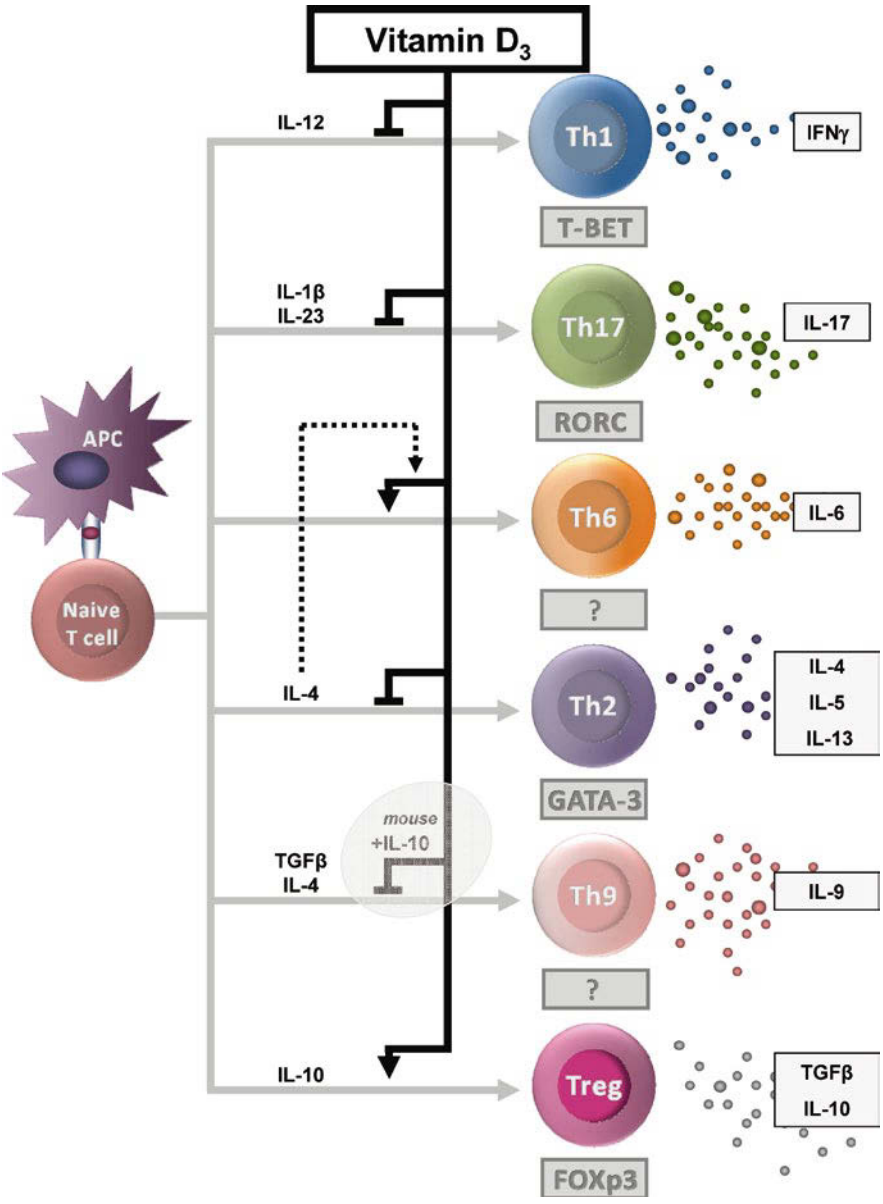

Fig. 3 Summary of immunomodulatory effects of Vitamin D_3 on T cell response

a cut-off could be determined by rise of the parathyroid hormone level (Klaushofer 2010). The most commonly measured parameter in vitamin D_3 metabolism, $25(OH)D_3$, reflects the general supply of the organism, while the $1,25(OH)_2D_3$ level represents the systemic level of its active metabolite, mainly achieved by further hydroxylation in the kidneys. Serum levels of $25(OH)D_3$ at 20nmol/l seem to be efficient in prevention of rickets in children, but much higher levels are supposed to be necessary for general health maintenance (Dimeloe et al. 2010). Optimal levels are currently a matter of debate and recommendations for $25(OH)D_3$ vary from 30 to 75nmol/l, although 50–75nmol/l are considered desirable (Klaushofer 2010). Definition of sufficient levels in regard of bone and calcium homeostasis is less demanding than establishing a serum threshold for maintenance of physiologic immune functions. Bone mineral density and fracture risk assessment are well measurable and useful clinical parameters as control for optimal vitamin D_3 levels in a patient's long term outcome regarding bone health (Klaushofer 2010). On the contrary, it is the local production of the active metabolite $1,25(OH)_2D_3$ in an immunological micro-environment which is crucial for regulatory actions exerted by vitamin D_3. Although sufficient systemic levels of $25(OH)D_3$ are required, local levels of the active metabolite are predominantly dependent on 1α-hydroxylase activity in immune cells, a process beyond control of the strictly regulated calcium and phosphate feed-back loop. Moreover, since the amount of local production quantitatively affects vitamin D_3 levels only as an exception, local vitamin D_3 levels are probably poorly reflected in systemic serum levels and definition of optimal local levels for immunological health maintenance appears difficult.

4.3.2 Vitamin D_3 in Health and Disease

Since it is well demonstrated that vitamin D_3 is important for maintenance of immunological homeostasis, it can be concluded that vitamin D deficiency consequently leads to a range of disorders. As for research, two different approaches can be chosen: On the one hand, a row of epidemiological studies showed a correlation between vitamin D deficiency or supplementation and several health disorders. On the other hand, a mechanistic approach was based on the hypothesis that a deficiency of vitamin D_3, which in previous studies was found to suppress the Th1 pathway, might favour occurrence of Th1 mediated autoimmune diseases acting as "environmental factor." On the other hand, since it was believed, that vitamin D shifts the immunologic balance towards a Th2 pattern, influence of vitamin D_3 on allergy development was also investigated. In order to confirm these considerations, several animal disease models were employed.

In several epidemiological studies measurable parameters such as vitamin D_3 supply, rickets prophylaxis, serum vitamin D_3 levels and occurrence of immune mediated disorders were correlated. In the first large epidemiological study on vitamin D_3 in a large Finish birth-cohort study, Hyppönen et al. (2001) observed a reduced risk for (autoimmune mediated) Type 1 Diabetes under vitamin D_3 supplementation for rickets prevention.

A further epidemiological study by Hyppönen et al. raised the question that infant supplementation with vitamin D_3 in order to prevent rickets might be associated with an increased risk for allergic conditions in later life (Hyppönen et al. 2004).

4.3.3 Vitamin D_3 and Allergic Conditions

Allergy can be considered as an excessive reaction of the immune system towards a normally harmless antigen. The often very casually used term "allergy" mostly refers to a hypersensitivity reaction (Type I according to the classification by Gell and Coombs), characterized by a pathologic shift towards a Th2 pattern with excessive IgE production. After "sensitization", the first contact with the respective allergen, specific IgE production is initiated and mast cells are loaded with IgE. Re-exposition to the allergen leads to cross-linking of the membrane-bound IgE molecules by their specific antigen and de-granulation of vasoactive mediators such as histamine and leukotriens takes its course. A late component of the allergic response comprises an inflow of Th2 cells as well as of eosinophils and causes further tissue damage (Dimeloe et al. 2010). Allergy clinically manifests itself as rhino-conjuncivitis, asthma or anaphylaxis. The also often used term "atopy" refers to a genetic predisposition to allergy and a bias to produce excessive IgE as reaction to specific antigens. Atopy can presents itself as atopic dermatitis, allergic rhino-conjuncivitis or allergic asthma (Fritsch 2009; Herold 2008).

Since it has originally been assumed that in adaptive immune responses vitamin D_3 shifts the balance towards a Th2 pattern by inhibition of the Th1 pathway, the question has arisen whether vitamin D_3 supplementation might favour development of allergies. A large epidemiological Finish birth cohort study showed a possible association between vitamin D supplementation (as prevention for rickets) in infancy and later allergic conditions (Hyppönen et al. 2004). Contrasting this observation, more recent epidemiological studies suggest a preventive effect of vitamin D_3 in pathogenesis of allergic asthma, a chronic obstructive airway disease. Vitamin D_3 seems to be already essential for normal fetal lung development, maturation and surfactant production (Litonjua 2009). Two epidemiological studies observed a negative correlation between vitamin D_3 intake during pregnancy and risk for asthma in the offspring (Erkkola et al. 2009; Camargo et al. 2007). Additionally, the study by Erkkola et al. (2009) also reports on a decreased risk for allergic rhinitis. The finding by Hyppönen et al. (2009) that both very low and very high 25-OH vitamin D_3 serum levels are associated with increased average and specific IgE concentrations might suggest a predisposition for atopy and allergy, but in this study clinical manifestation was not assessed (also see above). A recently published large U.S. survey also reported on an association of deficient 25(OH)D_3 levels and higher prevalence of allergic sensitization by measurement of specific IgE levels. Remarkably, the correlation was observed in children and adolescents, but could not be defined that clearly in the adult population (Sharief et al. 2011).

Taken together, these results obtained from epidemiological studies raise the question whether a specific time frame in life might be susceptible to an altered vita-

min D_3 status. On a cellular level, a bias towards allergy caused by vitamin D supplementation during infancy could not be substantiated: in human PBMC obtained from cord blood vitamin D showed a virtually complete inhibition of Th2 differentiation (Pichler et al. 2002, see above).

Furthermore, several studies showed induction of an IL-10 producing regulatory T cell subset upon vitamin D_3 exposure (see above). Regulatory T cell subsets are capable of limiting potentially detrimental immune reactions as observed in allergic conditions and have been shown to protect against asthma in several studies (Robinson 2009; Dimeloe et al. 2010; Xystrakis et al. 2006). vitamin D_3 alone, as well as in combination with glucocorticoids seems to be a strong enhancer of IL-10 production in T cells. Glucocorticoids are considered a first-line therapy in asthma and their effects are probably mediated by suppression of Th2 cytokines as well as induction of an IL-10 producing T cell subset. In patients suffering from glucocorticoid resistant asthma, CD4+ T cells fail to produce IL-10. This can be overcome by in vitro administration of vitamin D_3. Moreover, in a pilot study, when glucosteroid-resistant asthmatic patients were orally supplemented with vitamin D_3, responsiveness to steroids was ameliorated, shown by increase of IL-10 production in vitro (Xystrakis et al. 2006).

Additionally, research of vitamin D_3 actions on B cells, the cellular source of IgE production, could show that in vitro vitamin D_3 inhibits IgE production stimulated by anti-CD40 mAb plus IL-4 (Heine et al. 2002; Hartmann et al. 2011). In a subsequent study, it was shown that Il-10 production in activated B cells was enhanced by vitamin D_3. IL-10, a suppressive cytokine produced by many immune cells including APC and T cells (see above) enhances an Ig class switch to IgA , IgG and IgM (Heine et al. 2008).

As a conclusion, recent studies suggest a potentially protective role of vitamin D_3 in allergic conditions. Moreover, it was even suggested that vitamin D analogs might be applied in future allergy therapy and vitamin D_3 might enhance responsiveness to actually applied anti-inflammatory drugs (Hartmann et al. 2011; Xystrakis et al. 2006).

4.3.4 Vitamin D_3 and Autoimmunity

Since various in vitro studies showed that vitamin D_3 suppresses the Th1 pathway, it was also concluded that deficiency might cause the onset or exacerbation of formally believed Th1 mediated autoimmune diseases. The pathophysiological concept of autoimmune diseases mediated by a shift towards a Th1 pattern has been renewed by the discovery of new CD4+T cell subsets (also see above). Th17, a subset capable of entertaining chronic inflammatory conditions and Tregs, vital for maintenance of self-tolerance have contributed among them to a far better understanding of autoimmune reactions. In several autoimmune-mediated diseases suppressive function of Tregs seems to be compromised (Costantino et al. 2008). The role of vitamin D_3 in the prevention of autoimmune conditions has already been extensively defined on a cellular level by its suppressive capacity on the Th1 pathway. Effects of vitamin D_3 on

the more recently described subsets also fit well into this concept by inhibition of the Th17 lineage and induction or functional restoration of the regulatory T cell subset.

Regarding autoimmune diseases, mechanisms of origin and onset still remain to be fully elucidated. Gender-related differences to the advantage of females have been observed. Specific HLA-constellations might predispose and serve as genetic factor, but additionally mostly unidentified environmental factors seem to be crucial at the onset (Fritsch 2009). The role of vitamin D_3 deficiency is discussed in various immune-mediated diseases with multiple sclerosis (MS), typ I diabetes, psoriasis, rheumatoid arthritis, SLE, inflammatory bowel diseases, sclerodermia, pre-eclampsia, Morbus Behçet and autoimmune iridocyclitis among them (Hyppönen 2005; Do et al. 2008; Adorini and Penna 2005; Kriegel et al. 2011). Various epidemiological studies indicate that vitamin D_3 deficiency could be related to a higher prevalence in several autoimmune disorders. However, the vulnerable period in life remains to be elucidated when sufficient vitamin D_3 supply is crucial to prevent auto-reactive disorders. In several epidemiological studies decreased serum levels for 25(OH)D_3 or 1,25(OH)D_3 were observed before the onset or in the course of the respective autoimmune disease. This was also a reason to hypothesize that there might be a correlation between vitamin D_3 deficiency and development of autoimmune conditions. Nevertheless, a reverse causation has also to be taken into account, since an altered vitamin D_3 status might be the consequence of the disease and not the cause (Kriegel et al. 2011).

VDR polymorphisms have been attributed to an increased risk for development of various autoimmune diseases in humans, including Hashimoto's thyroiditis, inflammatory bowel disease, Graves' disease, RA, SLE, primary biliary cirrhosis, autoimmune hepatitis, Addison's disease, vitiligo, celiac disease, and type I diabetes, as well as MS. Unfortunately, not all of the detected polymorphisms associated with autoimmune diseases have known functional consequences and not all of these associations have been reproduced (Kriegel et al. 2011; Cantorna et Mahon 2004).

4.3.4.1 Multiple Sclerosis

MS is a chronic inflammatory autoimmune disease characterized by de-myelination in the central nervous system leading to severe neurologic consequences and disability. In addition to genetic susceptibility, exposure to not yet fully identified environmental factors is required for the onset. Apart from Epstein-Barr-Virus infection and smoking, vitamin D deficiency is suspected as one of these major environmental factors. The prevalence of MS is associated with a remarkable geographical profile: MS onset is less frequent at the equator and increases with either North or South latitude. Furthermore, living at higher altitudes, which is associated with higher UV-exposure, negatively influences MS prevalence. Climatic factors with diminished occurrence in very sunny regions also contribute to the risk of MS. Sun exposure at a younger age seems to be especially protective. Migration from a high latitude/high prevalence region to a lower latitude/low prevalence region during the first two decades seems to be beneficial and vice versa, a fact underlining the crucial role of environmental factors in MS onset. Furthermore, a correlation between

low vitamin D_3 serum levels and onset of MS has been described in several studies. The time of birth, with a higher risk of developing MS for the spring-born (with a peak in May) and lower risk for autumn (with a peak in November) may reflect the vitamin D_3 supply of the mother in pregnancy. Additionally, dietary factors affecting vitamin D_3 supply also seem to contribute, since a lower prevalence in populations with high consumption of fatty fish has been described (Ascherio et al. 2010; Pierrot-Deseilligny et Souberbielle 2010; Smolders et al. 2008).

MS belongs to the group of Th1-mediated autoimmune-diseases (see above). Therefore, it could be concluded that adequate vitamin D_3 supply might prevent MS or ameliorate the disease by suppression of the Th1 lineage. The experimental allergic encephalomyelitis (EAE) mouse model, achieved by vaccination with spinal cord homogenates containing myelin proteins, is commonly used in MS animal studies (Arnson et al. 2007; Smolders et al. 2008). In several studies, administration of $1,25(OH)_2D_3$ before EAE induction avoided its onset and even administration after immunization prevented the start or at least led to a milder course of the disease. In mice already suffering from EAE vitamin D_3 administration ameliorated the disease and inhibited progression (Smolders et al. 2008). Since it has been discovered that Th17 substantially contribute to Th1 mediated autoimmune-diseases as a pro-inflammatory subset, effects of vitamin D_3 on Th17 obtained from EAE mice have also been examined. It could be shown that application of vitamin D_3 not only inhibited Th17 induction but also down-regulated CCR6, a chemokine receptor necessary for migration and entry to the central nervous system (Chang JH et al. 2010). Data on vitamin D_3 mediated effects on Tregs, other important players in autoimmune diseases, appears conflicting. Since it has been shown in several studies that vitamin D_3 enhances the regulatory T cell compartment (see above), the same effect in MS could be expected. Nevertheless, Chang et al reported a suppressive effect of vitamin D_3 on Treg in vitro induction in T cells obtained from EAE mice. In a human study a correlation of serum $25(OH)D_3$ levels in MS patients and suppressive capability of Tregs was found, while an association of vitamin D_3 status and Treg numbers could not be observed. Due to a decline in the IFNγ/IL-4 ratio in patients with high $25(OH)D_3$ serum levels, the authors concluded that vitamin D_3 might skew the balance towards a Th2 phenotype. Taken together, they suggest a shift towards a less inflammatory T cell cytokine profile by vitamin D_3 (Smolders et al. 2009, 2010).

4.3.4.2 Type I Diabetes Mellitus (DM I) and Vitamin D_3

Type 1 Diabetes Mellitus is a chronic autoimmune disease characterized by destruction of pancreatic islet β-cells, the source of endogenous insulin. The progression from insulitis to the clinical onset of DM I occurs usually during childhood and adolescence, at a time point when almost 90 % of the β cells are already destroyed. Typical feature of the insulin deficiency is hyperglycemia, and a severe keto-acidosis may present itself as life-threatening first clinical manifestation. Chronic damage to blood vessels due to hyperglycaemia presents as retinopathy, coronary heart disease,

stroke and renal failure as a long term prospective. Similar to other "Th1"-mediated autoimmune diseases the pathogenesis of DM I is considered to be triggered by environmental factors acting upon a genetic background (Takiishi et al. 2010; Herold 2008). Several retrospective studies propose vitamin D_3 deficiency as an environmental factor. As to prevalence, a striking geographic distribution similar to MS has been described, emphasizing the role of environmental factors in – autoimmune – disease onset (Szodoray et al. 2008; Takiishi et al. 2010; Hyppönen 2010; Mathieu et Badenhoop 2005). In 2001, Hyppönen et al. published a Finish birth-cohort study on a significantly reduced risk for DM I when participants had been supplemented with vitamin D during the first year of their life. A large multi-centre study had also observed this protective effect of vitamin D_3 supplementation in infancy (Anonymous 1999). Furthermore, the risk to develop DM I in later life was found to be increased in children suffering from rickets (Hyppönen et al. 2001). Moreover, further epidemiological studies showed that vitamin D_3 obtained from food or cod-liver intake during pregnancy also reduced the risk of DM I in the offspring (Fronczak et al. 2003; Stene and Joner 2003).

The non-obese diabetic mouse (NOD) model is predominantly used in animal studies examining vitamin D_3 effects in regard to DM I in vivo. Corresponding to other animal autoimmune disease models, treatment with therapeutic doses of vitamin D_3 or analogues resulted in prevention or delay of development of insulitis or clinically manifested diabetes. Since therapeutic doses of vitamin D_3 were required, hypercalcemia and bone decalcification were observed, leading to a search for effective less-calcemic alternatives (Arnson et al. 2007; Takiishi et al. 2010).

4.3.4.3 Inflammatory Bowel Diseases (IBD)

IBD are chronic recurring inflammatory diseases of the gastrointestinal tract. Crohn's disease and ulcerative colitis are two distinct forms of the IBD. These immune-mediated diseases are characterized by an excessive Th1 reaction to mostly bacterial antigens (Szodoray et al. 2008; Peterlik and Cross 2005; Cantorna et al. 2000). As for other autoimmune mediated diseases, an encounter of genetic susceptibility and environmental factors is necessary for IBD onset, and vitamin D deficiency might contribute to its development. Similar to MS and DM I, a specific geographic pattern in its prevalence can be observed (Szodorary et al. 2008). IL-10 knockout mice serve as one of the experimental IBD models, since mice spontaneously develop a disorder similar to human IBD, due to an uncontrolled immune reaction towards the intestinal flora. It was shown that vitamin D_3 deficiency aggravated symptoms in the IL-10 KO mice, while supplementation ameliorated the condition caused by the disease (Cantorna et al. 2000). In two other different experimental IBD models, lack of the VDR led to an agressive course of the disease (Froicu et al. 2003). vitamin D_3 deficiency is common in IBD and a recent study also reported a correlation between $25(OH)D_3$ serum levels and disease activity. Whether vitamin D_3 deficiency is a reason for the onset or a consequence of the local inflammation of the gastrointestinal tract still remains to be elucidated (Ulitsky et al. 2011).

4.3.4.4 Vitamin D_3 and Rheumatoid Arthritis (RA)

RA is an immune-mediated disease characterized by erosive arthritis and joint destruction, potentially leading to disability (Szodaray et al. 2008; Adorini and Penna 2008). As in other immune-mediated diseases, the genetic background, in combination with mostly unidentified environmental factors, is responsible for the onset of RA. Regarding the geographic distribution, the prevalence of RA was found to be higher in the north of Europe than in the southern part. Patients suffering from RA have been reported to show decreased vitamin D_3 serum levels. Moreover, lower vitamin D_3 serum levels have also been correlated with higher disease activity in RA (Cutolo et al. 2007). In one epidemiological survey conducted in elderly women, a negative correlation between dietary or supplementary vitamin D_3 intake and risk for RA could be observed (Merlino et al. 2004). Nevertheless, this study relied solely on a questionnaire on food intake, and serum 25(OH)D_3 levels, respectively sun exposition, were not assessed or taken into account. Another study measured vitamin D_3 serum levels without detecting a correlation, thus not corroborating the data from Merlino et al. (Nielen et al. 2006).

Effects of vitamin D_3 on RA were also examined in animal disease models. After vitamin D_3 supplementation, Cantorna et al. (1998) observed amelioration of symptoms or even prevention of disease progression in murine Lyme arthritis and collagen-induced arthritis. This effect was also achieved in rats with collagen induced arthritis treated with a vitamin D_3 analogue (Larsson et al. 1998).

4.3.5 Vitamin D_3 in Therapy

As extensively outlined above, vitamin D_3 acts as a modulator on immune functions by shifting the balance within the T cell compartments and influencing adaptive immunity. Therefore it could be expected that vitamin D_3 might be applied as therapy in various diseases. Unfortunately, therapeutic doses cause severe side effects in form of hypercalcemia, hypocalciuria, nephrocalcinosis and increased bone resorption (van Etten and Mathieu 2005). Efforts have been laid on development of low-calcemic analogues with enhanced immunomodulatory effects. So far, vitamin D_3 and VDR agonists have been already successfully applied in vitro and in various animal disease models, but there is still a lack of large clinical studies in humans, probably still due to fear of calcemic side effects.

In humans, vitamin D_3 and analogues are currently applied only topically, as a first line therapy in psoriasis, a chronic inflammatory immune-mediated disease of the skin. vitamin D_3 efficacy on psoriasis can be attributed to its pro-differentiating and proliferation-inhibitory effect on keratinocytes as well as its immune modulatory capacity to reduce inflammation (Adorini 2005; Fritsch 2009; Miller and Gallo 2010).

In order to avoid or ameliorate side effects, several strategies have been proposed: Since a number of synthetic analogues, while being less calcemic, still cause

severe bone demineralisation, a combination with bone resorption inhibitors was suggested. The most promising strategy would probably be the administration of vitamin D in conjunction with other immune-suppressive or immuno-modulatory agents. Synergistic effects with other immuno-suppressive drugs have been observed in several studies. Administration of vitamin D_3 analogues in combination with immuno-suppressive agents could save the amounts needed on both sides and therefore generally reduce side effects in treatment of autoimmune disorders, graft rejection or allergy (van Etten and Mathieu 2005).

4.3.6 Conclusion and Perspective

An increasing number of publications suggests a possible role of vitamin D in (auto-)immune diseases, the majority of them focusing on the – rather frequent – deficiency of vitamin D. Experimental data partially substantiate the conclusions drawn from epidemiological studies. However, there remains a hiatus between in vitro results on vitamin D effects, systemic vitamin D levels measured in individuals and epidemiological data on incidence and course of the disease. As discussed in some of the papers reviewed, there is no definite end-to-end concept of how a specific concentration/deficiency of vitamin D may lead to autoimmunity, and a reverse causation (an altered vitamin D_3 status being the consequence and not the cause of the disease) cannot be unequivocally excluded.

There are two major drawbacks in this context: firstly, the mechanism of autoimmunity itself is still a matter of debate, so whatever effect of vitamin D on the immune system is discussed, causality for development of autoimmune disease cannot be directly evaluated. Secondly, concentrations of the active metabolite $1\alpha25(OH)D_3$ in different compartments and tissues are reflected only very roughly by systemic serum levels of $25(OH)D_3$ or $1\alpha25(OH)D_3$. Low levels of $25(OH)D_3$, which is also a prerequisite for extrarenal $1\alpha25(OH)D_3$ production, are most likely associated with local vitamin D deficiency, relevant for interference with the immune system. However, the actual local concentration of $1\alpha25(OH)D_3$ cannot be directly measured.

The value of animal models may be limited, since results are not always clearcut, and their interpretation as accurate image of the human system has to be viewed critically.

However, as mentioned by several authors, improvement of the vitamin D status, in a natural way or by supplementation of vitamin D is cheap and easy, and bone metabolism benefits also from ameliorating the widespread deficiency.

The therapeutic application of higher doses of vitamin D_3 is based on promising concepts, but is still hampered by side effects, and has not become clinical routine so far. Whenever the systemic effects can be successfully separated from bone metabolism, either by the production of less calcemic analogues or by concentrating the active metabolite on the area of interest, a wide field of applications becomes reality.

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Osteoimmunological Aspects of Biomechanics

5

Katharina Kerschan-Schindl, Gerold Ebenbichler

5.1 Introduction

Osteoporosis and fragility fractures are a major health concern, especially in industrialised countries. Osteoporosis per se and osteoporosis-related complications may impair functioning and health and, thus, lead to an inferior quality of life. Moreover, skeletal health has a profound financial and social impact.

Different endogenous and exogenous factors which interfere with bone health have been identified. Among these, physical activity that relates to regular mechanical bone loading seems to be one of the major factors controlling bone mass and the prevention of osteoporotic fractures. Moreover, there is an interaction between bone homeostasis and the immune system which may be modified by regular physical activity. Bone and immune cells share a common site of origin, the bone marrow. They are supposed to influence each other not only during maturation; osteoclasts and immune cells have a number of regulatory molecules in common, including cytokines, receptors, signalling molecules, and transcription factors, which influence each other.

The aim of this chapter is to review the impact of both bone loading and muscle activity on bone remodelling, thereby elucidating the immunological communication pathways intended to modulate osteoblast and osteoclast activity.

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5.2 Mechanical Loading and Bone Formation

Galileo already documented that the shape of bone would be related to loading. Later, in the 19th century Julius Wolff's work developed a more educated understanding of bone mechanical biology that considered functional adaptation of bone structure and mass at the tissue level. Nowadays we know that the cells of the skeleton are responsible for bone modelling and remodelling.

The initial peak bone mass is highly dependent on genetic factors. Nevertheless, lifestyle factors like calcium intake and physical activity influence peak bone mass and bone remodelling. While disuse and inactivity reduce bone mass (Zerwekh et al. 1998), strenuous exercise increases it (Courteix et al. 1998). The earlier a child starts with physical activity, the greater is the benefit on bone mass (Kontulainenen et al. 2002). Such modulations of bone formation/loss are not restricted to young people, but may also hold true for the elderly population. Both, elderly males and females who performed an exercise program regularly demonstrated improvements of bone mineral density values (Bemben and Bemben 2010; Kemmler et al. 2010). Although it has not been proven yet, it is speculated that the degree of bone response is dependent on the type of exercise. Relevant factors would be magnitude of load, number of repetitions/loading cycles, and the rate of strain (for a review see Rubin et al. 2006).

Bone remodelling is a balanced process that is mainly determined by its mechanical demands. It is estimated that approximately one quarter of cancellous bone and 3–4 % of cortical bone are renewed every year. People who lead a sedentary life style deprive their bones from sufficient mechanical stimulation. Because of an uncoupling of bone formation and bone resorption their bone mass and structure will degrade towards an osteopenic/ osteoporotic fragile bone. In contrast an active lifestyle that regularly overloads bone increases bone mass and structure leading to strong fracture resistant bones. It has been shown that the strength of a bone needs to be greater than the maximum to which it is likely to be exposed during normal activity if it is not to fail during the extremes of normal use (Currey 1979). In humans, the limb bones can usually withstand loads that deform them by 3–4 times the amount they are deformed by peak physiological activity before they fracture (Biewener et al. 1993).

5.2.1 Mechanotransduction

Alterations in bone remodelling are sensitive to changes in magnitude, the number of loading cycles, distribution of the loading, and the rate of strain (see Rubin et al. 2006). During vigorous activities, peak strain magnitudes range from 2000 to 3500 microstrain in animal experiments (Rubin and Lanyon 1984) and up to 2000 microstrain in humans (Burr et al. 1996). The peak strain of about 2000 microstrain that may be induced during locomotion is thought to cause microdamage to bone material and, thus, stimulate osteoclasts and osteoblasts to remove and then replace damaged tissue (Burr et al. 1985). Surprisingly, low strain (5 microstrain) and high

frequencies of 30 Hz during 20 min daily revealed a significant increase in bone mineral density in sheep hind limbs after one year (Rubin et al. 2001). Thus, both low and high strains seem to be crucial to the maintenance of bone structure that measured bone loading during everyday life activities in humans. In one of these studies very low strains of less than 10 microstrain prevailed whereas large strains above 1000 microstrain occurred only occasionally (Fritton et al. 2000).

There is evidence that most cells in the body are able to sense their mechanical environment. Mechanical forces are experienced by osteoprogenitor cells which are not only present in the bone marrow but also in soft mesenchymal tissues subjected to mechanical strain. Dependant on the magnitude of mechanical stress osteoprogenitors differentiate or transdifferentiate into osteoblast-like cells that express characteristic proteins and can form bone matrix (Aubin 1998). However, most important targets of skeletal loading seem to be the osteocytes (Cowin et al. 1991; Bacabac et al. 2004). They are distributed three-dimensionally throughout the bone and connected to each other via their cytoplasmatic processes within canaliculi in trabecular and cortical bone. The mechanisms involved in cell activation as a result of mechanical loading are not yet completely understood.

Physical activity loads the muscular-skeletal system, thereby causing processes of converting mechanical forces into biochemical signals. Such process is known as *mechanotransduction* (Rubin et al. 2006). Bone loading generates shifts of interstitial fluid out of regions with highly compressive strains and shifts back when the load is removed (Burger and Klein-Nulend 1999). Mechanotransduction mediates the release of various pathways that mediate the bone remodelling process via mutually interacting osteoblasts and osteoclasts (*sensing*). Different mechanisms of mechanotransduction have been described: 1. deformation of the hard tissue with strain across the cell substrate, 2. pressure within the intramedullary cavity and within the cortices with transient pressure waves, 3. *shear* forces through canaliculi which cause drag over cells, and 4. dynamic electric fields as interstitial fluid flows past charged bone crystals (Fig. 1).

The mechanosensitivity of osteocytes and, thus, the activation of osteoblasts was supposed to depend on this strain-energy density (SED) rate (Huiskes et al. 2000). The shear stress induced by interstitial fluid shifts is supposed to be in the order

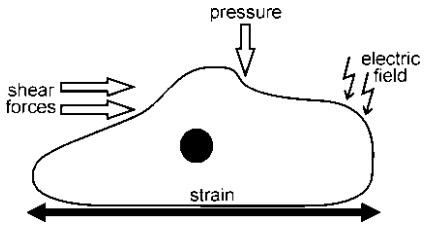

Fig. 1 Skeletal loading generates strain across the cell, pressure in the intramedullary cavity and within the cortices, shear forces through canaliculi, and dynamic electric fields (modified from Rubin et al. 2006)

of 0.8 to 3 Pa (Weinbaum et al. 1994). Additionally, high frequency (> 30 Hz), low magnitude loads (< 1MPa) were efficient to elicit cellular response in bone (Xie et al. 2006). As compared to continuous hydrostatic pressure applied to bone, only intermittent compressive forces were shown to increase bone formation (Klein-Nulend et al. 1987). In most of the everyday life activities like walking or doing sports, bone loading is cyclic rather than static.

5.2.2 Sensing Mechanisms and Signalling Pathways Activated by Bone Loading

During the bone loading process there are different pathways by which mechanical signals may be sensed by cells of the appendicular skeleton (Fig. 2). Cells that may convert the mechanical signal into biomechanical language include osteoblasts, osteoclasts, lining cells on the bone surfaces, osteocytes within the calcified matrix, and mesenchymal precursors within the bone marrow.

In response to mechanical forces a total of four different osteocyte-mediated *sensing and signalling mechanisms* have been identified. These are 1) the activation of ion channels, 2) the production of integrins, 3) the activation of gap junctions and hemichannels, and 4) an unclear role of primary cilia (Fig. 3).

Ad 1) Three different *ion channels*, the gadolinium-sensitive cation channels (Rawlinson et al. 1996), the L-type voltage-dependent channels (Miyauchi et al. 2000), and the α, ß, and γ units of epithelium sodium channels (ENaC; Mikuni-Takagaki 1999) have been described to be strain-responsive. Additionally, three potassium channels were identified in MLO-Y4 cells, an osteocyte-like cell line (Gu et al. 2001). Their role in mechanotransduction is not yet defined.

Ad 2) A variety of intracellular signaling pathways are activated in osteocytes in an *integrin*-dependant manner. Integrins are membrane spanning proteins that

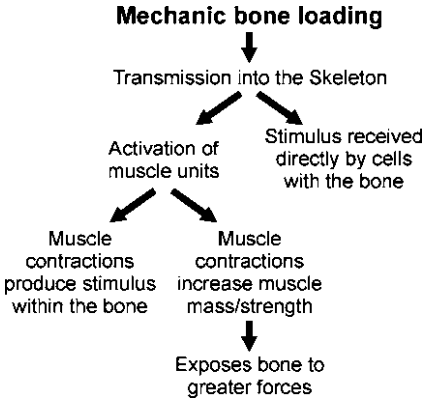

Fig. 2 Three different pathways by which mechanical signals may be sensed by cells within a bone (modified from Judex and Rubin 2010)

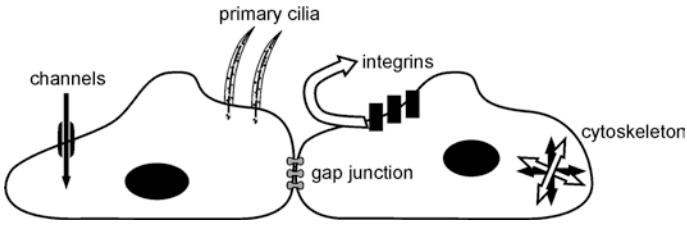

Fig. 3 Intracellular signals are activated by different mechanosensors

couple the cell to its extracellular environment. Functional integrins are heterogenous dimers made of α and ß subunits. Ligation of α and ß3 integrin enhances calcium influx in mechanically stretched osteocytes (Miyauchi et al. 2006). Osteocyte apoptosis is prevented by integrin engagement leading to FAK (focal adhesion kinase) activation, Src kinase activity, and phosphorylation of the adaptor protein Shc, and, thus, ERK1/2 activation as well as an interplay with actin filaments and microtubules (Plotkin et al. 2005). Cytoskeleton-mediated signalling in response to fluid flow could occur due to drag forces on the pericellular matrix (You et al. 2001) as tethering elements, presumably composed of integrins connect the canalicular wall and pericellular matrix to the osteocyte cytoskeleton. Expression of E11, an osteocyte-selective protein, is increased by mechanical loading in regions of potential bone remodelling, suggesting dendrite elongation (Zhang et al. 2006).

Ad 3) Osteocytes are connected to one another and to surface osteoblasts via *gap junctions* (Yellowley et al. 2000). Gap junctions are formed when connexins (Cx), especially the protein Cx 43, join to form an intercellular channel allowing the direct exchange of small molecules; Cx also form *hemichannels* that function independently of gap junctions. Studies are conflicting but fluid flow-induced release of ATP and PGE_2 may be mediated in part by hemichannels.

Ad 4) Independently of any channels *primary cilia* may also play a role in anabolic signalling in bone cells (Malone et al. 2007). They are supposed to deflect during fluid flow and possibly interact with extracellular matrix proteins, and thus, integrins on the primary cilium could translate deformations of the extracellular matrix into intracellular signals, amplifying mechanical stimuli.

5.2.3 Cytokine Response as a Signalling Pathway Activated by Bone Loading

These multiple intracellular signalling pathways are activated after application of force. Under physiological mechanical stimuli osteocytes prevent bone resorption by changing the RANKL/OPG ratio (You et al. 2008). Wnt ("wingless in Drosophila") gene expression is increased in osteocytes subjected to fluid flow (Santos et al. 2009). This regulatory pathway mediated by load-induced bone adaptation seems to depend on the low density lipoprotein receptor-related protein (LRP 5/6)

and frizzled transmembrane proteins as well as the gene ß-catenin and the protein sclerostin, which inhibits bone formation by blocking WNT signalling (Robinson et al. 2006).

Osteocytes exposed to fluid flow also express osteopontin and PGE_2 – both of them are important in the process of bone remodelling (Mc Garry et al. 2005). The activity-induced inhibition of osteoclast formation is at least partially dependent on the activation of the nitric oxide (NO) pathway in the osteocyte (Tan et al. 2007; Vezeridis et al. 2006). It is noteworthy that the intracellular signalling pathways activated after application of force still have a limited effect on osteoprogenitor differentiation and osteocyte functions still.

Whereas direct loading of the bone produces cytokines involved in bone remodelling, systemic factors may further contribute to this process. Muscle activity directly stimulates mechanically induced bone remodelling through traction and bending forces. On the other hand, muscle has been identified as a major source of cytokine production (myokines) that may facilitate bone metabolism via systemic effects.

5.3 Muscular Exercise and Bone Integrity

5.3.1 Activity, Inflammation and Bone Loss

A relationship between physical activity per se and bone formation has been suggested several centuries ago. Muscle activity seems to be especially beneficial to bone integrity in patients who have a risk of increased bone loss due to sterile, low grade chronic systemic inflammations. Systemic low grade inflammation is defined as a 2- to 4-fold elevation of circulating pro- and anti-inflammatory cytokines, naturally occurring cytokine antagonists and acute phase proteins, as well as minor increases in counts of neurtophiles and natural killer cells (Brüünsgaard et al. 2003). Both lack of physical activity and low grade inflammation was linked to various chronic diseases like obesity, type 2 diabetes, or cardiovascular diseases (Booth et al. 2002; Hu et al. 2004; Orsini et al. 2008). For example, the development of type 2 diabetes and insulin resistance is closely correlated with immune cell infiltration and inflammation in white adipose tissue (Hotamisligil 2006) and regular, moderate exercise may reduce systemic inflammation (Gleeson 2007).

The impact of inflammatory diseases on bone metabolism is described elsewhere in detail. In brief, the coupling of osteoclasts and osteoblasts is severely disturbed. Both local and systemic inflammation release cytokines like TNFα that may directly activate osteoclast differentiation and activity and thereby lead to bone destruction. Interleukin-6 has a special role; it positively influences osteoclast differentiation by inducing the expression of RANKL on osteoblasts and also directly suppresses osteoclast differentiation via an inhibition of the RANK/RANKL pathway (Yoshitake et al. 2008).

Although the systemic mediators of these beneficial exercise effects are unclear, several candidate mechanisms have been identified. Regular muscular activity increases epinephrine, cortisol, growth hormone, and other factors that have immune modulatory effects and may all influence bone metabolism. The detailed interactions are not a subject of this chapter and will be described elsewhere. However, recent research that has focused on cytokine secretion with the intention to reduce inflammation has shown that this seems a likely candidate to directly improve the coupling of osteoclasts and osteoblasts in a way that bone integrity will be improved.

5.3.2 Anti-inflammatory Effect of Exercise

Recent findings have demonstrated that muscle training induces increases in the systemic level of a number of cytokines with anti-inflammatory properties which differ significantly from cytokines produced in an acute inflammation. Following an acute inflammation the cytokine cascade consists of the following candidates: TNF-α, IL-1β, IL 6, IL 1 receptor antagonist (IL-1ra), soluble TNF-α receptors (sTNF-R), and IL-10. IL-1ra inhibits IL-1 signal transduction, and sTNF-R represents the naturally occurring inhibitors of TNF-α (Pedersen and Febbraio 2008).

As opposed to an acute inflammation, acute bouts of exercise that are not as highly strenuous as marathon running, do not increase TNF- α and IL-1 β levels and IL-6 is usually the first cytokine present in the circulation (Petersen and Pedersen 2005) (Fig. 4). The basal plasma IL-6 concentrations may increase up to 100 fold and depend on the duration and intensity of exercise (Fischer 2006). Its appearance precedes that of other cytokines. Exercise induced IL-6 does not only seem released from contracting muscles but also from sources outside the muscle that were stressed during exercise (Fischer et al. 2004). Using the microdialysis method, Achilles tendons revealed high concentrations of IL-6 following a running exercise (Langberg et al. 2002). Furthermore, chronic effects of exercise may differ from those of acute exercise as plasma levels of IL-6 remained unchanged during 1 year of training intervention (Rokling-Andersen et al. 2007). During and following exercise, the high circulating levels of IL-6 are followed by an increase in IL-1ra and IL-10, and sTNF-R. It was further shown that IL-1ra and IL 10 can be induced by IL-6 (Steensberg et al. 2003).

Until now several proteins secreted by skeletal muscle cells have been identified as reviewed by Pedersen (2006), with the potential to act as hormones, either locally within the muscle tissue or by targeting other distant organs. Such proteins have been termed myokines within the context of skeletal muscle physiology, although they are not exclusively secreted by muscle cells. Muscle contraction may induce acute release of myokines like calprotectin (Mortensen et al. 2008), IL-15, IL-6, and IL 7 which may pertain to homeostatic control under various physiological conditions (Haugen et al. 2010).

Both type I and II muscle fibers seem to express the myokine IL-6, with some studies suggesting that IL-6 expression was more prominent in type 1 fibers

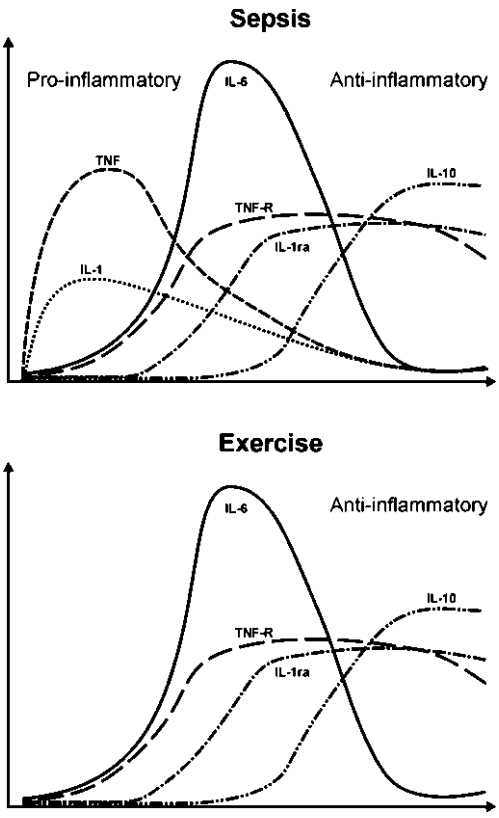

Fig. 4 In sepsis, the cytokine cascade within the first few hours consists of TNF-α, IL-1ß, IL-6, IL-1ra, TNF-R, and IL-10. Exercise leads to a different cytokine response that does not include TNF-α and IL-1ß but shows a marked increase in IL-6, followed by an increase in IL-1ra, TNF-R, and IL-10 (modified from Petersen and Pedersen 2005)

(Plomgaard et al. 2005), whereas others demonstrated a higher IL-6 expression in type 2 fibers (Hiscock et al. 2004). IL-6 from muscle was suggested to be a signal indicating that muscle glycogen stores are reaching critically low levels and that the active muscles' reliance on blood glucose as a source of energy is on the increase (Pedersen and Febbraio 2008). As the increase of IL-6 is followed by an increased level of the anti-inflammatory IL-10 and IL-1 ra, it has repeatedly been suggested that IL-6 produced by muscles could be critically involved in mediating the anti-inflammatory environment (Pedersen 2009) which subsequently acts both locally and systemically.

During non-strenuous exercise IL-8 seems to play a role in the exercise induced angiogenesis. One study that measured the arteriovenous concentration difference across non exhausting concentric bicycle and knee extension exercises, observed a transient net release of IL-8 with its peak being between 6 and 9 h after exercise that did not lead to increased plasma IL-8 levels (Akerstrom et al. 2005). During over-

strenuous exercise, however, IL-8 seems to exhibit a systemic angiogenetic effect. In one recent study, 29 male healthy subjects performed 45 min of downhill running. 3 and 24 h after exercise, mRNA expression of IL-6, IL 8 and COX-2 was significantly increased as compared to baseline, whereas pro-inflammatory cytokines like TNF-α or IL-1 β remained more or less unchanged (Buford et al. 2009). As muscle soreness was significantly correlated with IL-8 24 h after the exercise, the authors suggested that IL-8 transcription would play a role in inhibiting post exercise muscle soreness through the regulation of angiogenesis.

IL-15 has recently been discovered as growth factor that acts independently from IGF-1 and induces an accumulation of myosine heavy chain proteins in differentiated myotubes (Furmanczyk et al. 2003). In contrast to IGF-1, the muscle hypertrophic effect of IL-15 is independent from proliferation or differentiation of skeletal myoblasts (Quinn et al. 2005). The regulatory role of muscle contraction in relative to IL-15 remains unclear. Whereas in human training studies IL-15 levels were unchanged after several hours of running (Nieman et al. 2003; Ostrowski et al.1998), others found clearly increased IL-15 levels after acute resistance exercise (Riechman et al. 2004) and mainly in skeletal muscles dominated by type 2 fibers (Nielsen et al. 2007).

5.4 Therapeutic Exercise: Possible Non-mechanical Effects on Bone

Whereas the mechanical stimulation of bone is probably the main source for stimulating bone metabolism, several pathways are known to modulate bone metabolism. Among these, several proinflammatory cytokines such as IL-1ß, IL-6, and TNF α promote osteoclastogenesis and bone resorption in synergy with RANK. TNF α is the key inflammatory cytokine that directly and indirectly promotes inflammation associated osteoporosis (Teitelbaum 2007); it may activate the fully differentiated osteoclast independently of RANK signalling (Fuller et al. 2002). IL-1 for instance may enhance osteoclastogenesis only in the presence of permissive levels of RANKL and mediates a substantial component of TNF α's osteoclastogenic effect in the bone marrow stromal cells and osteoclast precursors (Wei et al. 2005). IL-6 may exhibit a positive and negative effect on osteoblast and osteoclast differentiation. IL-6 knockout mice were significantly protected from joint inflammation and destruction in a mouse model of arthritis (Cuzzocrea et al. 2003). They were further protected from bone loss through estrogen depletion as observed in postmenopausal osteoporosis (Cuzzocrea et al. 2003). Thus, the systemic effects of myokines could be likely candidates to facilitate bone integrity both in healthy and osteoporotic bones.

Pure increases of IL-6 without increases in TNFα or IL-1 and others would induce a coupling between osteoblasts and osteoclasts that is intended to apposite bone. Thereby, IL-6 would directly suppress the differentiation of osteoclast progenitors. In the presence of IL-6RX, IL-6 evokes discordant responses for bone homeostasis by both inducing bone formation and increasing the osteoclast sup-

porting activity in osteoblasts. It was demonstrated that IL-6 and IL-11 exerted a direct inhibition of osteoclast formation (Duplomb et al. 2007; Yoshitake et al. 2008). Thereby the gp-130 signalling pathway up-regulates RANK expression on osteoblasts but down-regulates this pathway in progenitor osteoclasts. Likewise, IL-6 would be able to directly induce anti-inflammatory cytokines like IL-1ra and IL 10 that would diminish the osteoclast stimulating effects of TNFα and IL-1 (Steensberg et al. 2003) (Fig. 5).

The impact of non-mechanical effects in addition to the mechanic effects of exercise on bone integrity has been examined in several studies. Premenopausal women for instance, who were engaged in 8 months of running versus weight lifting exercise revealed similar increases in spine bone mineral density (BMD), whereas only the strength training group experienced relevant increases in muscle mass (Snow-Harter et al. 1992). Similarly in postmenopausal women, a 9 months impact versus non-impact exercise program led to similar increases in lumbar spine BMD, but muscle mass increases differed significantly between groups (Kohrt et al. 1997). Such data do not support the notion that strength training increases muscle mass only, but not endurance training (Evans 2004). Furthermore, these studies indirectly support the hypothesis that in addition to the mechanical effects of exercise, the non-mechanical effects seem to contribute to bone integrity in a relevant way.

These non-mechanical effects may be dose dependent. Athletes who participate in highly exhausting sports like cycling, long distance running or swimming have usually a low BMD (Kohrt et al. 2009), which may be explained by low grade inflammation and partial deficiency of the immune system that is associated with over-strenuous exercise. The negative effect of running on bone metabolism and the pro-inflammatory status has been shown in rats (Sipos et al. 2008). Marathon running is also supposed to induce inflammation (Hikida et al. 1983). Ultra-endurance

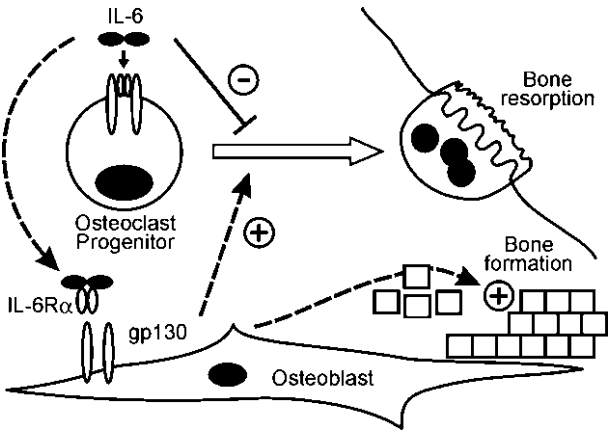

Fig. 5 Schematic diagram: effects of IL-6 on osteoclast precursors. IL-6 directly suppresses the differentiation of osteoclast progenitors; in the presence of IL-6Rα, Il-6 induces both bone formation and increases osteoclast-supporting activity of osteoblasts (modified from Yoshitake et al. 2008)

runners do not only frequently suffer from rhabdomyolysis (Skenderi et al. 2006), but such a Spartathlon (246 km) also leads to an uncoupling of bone metabolism with increased bone resorption, and suppressed bone formation (Kerschan-Schindl et al. 2009). However, it is noteworthy to mention that solely exposing the skeleton to muscle loads generated during daily activities and exercises (astronaut exercises on average approximately 2 h per day) is minimally capable or incapable of suppressing bone loss associated with removal of gravitational loading (Judex and Carlson 2009).

5.5 Summary

Osteoporosis is a systemic disease that is associated with increased morbidity, mortality, and health care costs. Osteoclasts and osteoblasts are the main regulators of bone homeostasis, However, the key role of the immune system has received little attention so far. Both mechanical and non-mechanical factors activate bone cells via immunological pathways. The mechanical effects of bone loading are mediated by mechanotransduction. Although the exact mechanosensory mechanisms involved in osteoprogenitor differentiation and osteocyte function remain partly undiscovered, several sensing and signaling pathways have been identified: 1) the activation of ion channels, 2) the production of integrins, 3) the activation of gap junctions and hemichannels, and 4) an unclear role of primary cilia. Under physiological mechanical stimuli osteocytes prevent bone resorption by changing the RANKL/OPG ratio. Non-mechanical sources of bone remodelling may be circulating cytokines that are produced by activated muscles (myokines). They may diminish osteoclast differentiation by inducing the expression of RANKL on osteoblasts and/or by directly suppressing osteoclast differentiation via an inhibition of the RANK/RANKL pathway.

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Utility of the Determination of Biomarkers of Bone Metabolism

6

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6.1 Introduction

Biochemical serum markers of bone metabolism are substances involved in all processes of bone activity. Systemic dispersion is the basis for their biochemical measurements. Both serum/plasma and urine measurements are widely used for clinical parameters of bone turnover, but accuracy and usability have favoured blood analyses over the years. This review will therefore focus on serum/plasma parameters of bone metabolism and their clinical applications and give hints on further applications within biological material.

Targets of biochemical analysis of bone metabolism are enzymes and proteins or their respective metabolites produced during bone formation and degradation. However, direct regulators of osteoblast and/or osteoclast cell function and activity might represent additional parameters of interest, since they may very well reflect dynamic processes in bone and adjacent tissue.

The usefulness of serum markers of bone metabolism has been widely recognized during the past decades, but increased significantly with the technical improvement of biochemical methods and the knowledge of new compounds in bone metabolism. Many of these markers have been established until now, but only few of them have entered daily clinical routine applications. The reason for this increasing interest is the possibility of characterization and diagnosis of metabolic bone diseases, but also the improvement of therapy decisions and therapy monitoring by biomarkers.

Regarding their potential, why do these biomarkers not appear in international diagnostic and therapeutic recommendations?

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Firstly, physical measurements of bone, e. g. by dual energy x-ray (DXA) absorptiometry, have led to believe in a precise golden standard measurement method for osteoporosis diagnosis and its follow-up. A long-term standardizing process for equipment, interpretation and reference measurements as well as a low number of technical types and a high number of international publications on the technology have guaranteed its place in clinical routine. The World Health Organization's (WHO) definition of osteoporosis is based on DXA-criteria, but this clearly overestimates the importance of bone density measurements in view of newer more holistic criteria.

Secondly, a broad spectrum of biomarkers of bone metabolism has been established, but laboratory methods may not be directly comparable and are often poorly standardized. Biomarker studies may therefore not be comparable with others and will not be suitable to appear in general recommendations.

Thirdly, general feasibility of laboratory methods per se might be poor. Specialized institutions such as academic laboratories might have more experience, equipment and standardized methods, as well as specialists and interpretation tools than smaller labs or point-of-care testers.

Fourthly, preclinical conditions are crucial for many of these tests. Therefore, long transportation times for biological material or special patient conditions (e. g. time of the day or nutritional requirements) are not feasible or difficult to achieve in general settings. The general implementation of biomarkers of bone metabolism may therefore be delayed with regard to its importance. However, the implementation might be accomplished more easily in central facilities, where biomarkers of bone metabolism are already increasingly used.

Fifthly, economical problems in recent years have forced communities to stabilize or even reduce their health care costs. Even if biomarker measurements tend to be cheaper and more accurate than densimetric or tomographic measurements, new biomarkers on the way of their establishment might be prone to be postponed with regard to traditional methods.

This chapter aims to describe current types of bone biomarkers and the pros and cons of their use in bone diseases. Interpretation, standardization and future aspects will be discussed.

6.2 Overview of Bone Biomarkers

6.2.1 Calciotropic Hormones

Bone biomarkers cannot be interpreted without a view on the individual calciotropic hormones. Given the fact that more than 70 % of the general population tends to have decreased vitamin D levels (Holick 2007), bone metabolism has to be seen in the light of a neglected problem of possible osteomalacia, with wide-ranging consequences.

6.2.1.1 Parathyroid Hormone (PTH)

A long history of analytical attempts for parathyroid hormone exists, starting in 1909, when MacCallum and Voegtlin noted that removal of the parathyroid glands quickly resulted in rapidly falling blood calcium levels (MacCallum and Voegtlin 1909). Subsequent work in the 1920s to purify the hormone established its central role in calcium homeostasis.

Parathyroid hormone (PTH) or parathyrin is secreted by the parathyroid glands as a polypeptide containing 84 amino acids. It increases the concentration of calcium in the blood by acting upon parathyroid hormone receptors in many parts of the body. PTH half-life is approximately four minutes. It has a molecular mass of 9.4 kDa. PTH is stocked in secretory granules that fuse with the cellular membrane and release PTH in response to a decrease in the extracellular ionized-calcium concentration. Most of PTH is secreted in its intact form, 1–84, although it can also be secreted as N-terminal truncated fragments or C-terminal fragments after intracellular degradation.

However, identification of different types of PTH fragments and several generations of PTH assays may be responsible for the great variability of PTH values and the difficulties of implementing international recommendations such as those of the National Kidney Foundation/Kidney Disease Outcomes Quality Initiative (Ureña and Torres 2006). Calibration attempts are currently on the way, as the generalized use of a unique, true, and accurate intact 1–84 PTH assay is not available yet.

Usability/utility: The measurement of PTH plays an important role in daily clinical routine, not only for endocrinologists and nephrologists but also for a variety of other disciplines. Technical development and standardization should be set up with the executive laboratory. Second- and third-generation PTH assays can be aligned by specific factors depending on the assays used (Souberbielle et al. 2010). This will minimize the problem of inter-method variability of PTH measurements, but – depending on the type of disease – problems with standardization and reference populations remain to be resolved.

6.2.1.1.1 Parathyroid Hormone-related Protein (PTH-rP)

Parathyroid hormone-related protein (PTH-rP) is structurally related to PTH and seems to play a physiological role in lactation, possibly as a hormone for the mobilization and/or transfer of calcium to the milk. PTH and PTH-rP bind to the same G-protein coupled receptor. It is occasionally secreted by cancer cells (breast cancer, certain types of lung cancer). Cancer-derived PTH-rP appears to play a critical role in bone invasion by tumor cells, mediated by osteoclasts. In bone, PTH-rP regulates enchondral bone development by maintaining the endochondral growth plate at a constant width (Fritchie et al. 2009).

Usability/utility: PTH-rP testing is more appropriately performed after assessment of PTH. If PTH is not low or low normal, testing for PTH-rP is usually uninformative. The clinical use of PTH-rP is restricted to validated assays and might be a diagnostic challenge for experienced laboratories.

6.2.1.2 Vitamin D

6.2.1.2.1 25(OH)Vitamin D

Vitamin D, a secosteroid, is structurally similar to steroids such as testosterone, cholesterol, and cortisol. The molecular weight of vitamin D2 (calciferol) is 396.6, of vitamin D3 (cholecalciferol) 384.6. Vitamin D2 originates from irradiation at about 260 nm of ergosterol, while vitamin D3 is formed by irradiation of a provitamin molecule (7-dehydrocholesterol) present in the skin and gut-lining cells.

The role of vitamin D in maintaining bone health has been known for decades. Recently, however, the fact that many tissues express vitamin D receptors and are regulated by vitamin D has accentuated the need for valuable vitamin D serum measurements. In addition, 25(OH)vitamin D levels rather than the more locally acting $1,25(OH)_2$vitamin D have been associated with an important role in many diseases, including the development of cancer, autoimmune diseases, cardiovascular diseases, diabetes, and infections. Vitamin D deficiency, defined as serum 25(OH)vitamin D levels < 30 ng/mL, is very common in our population (Holick 2007).

Measurement of 25(OH)vitamin D is widely used for assessing vitamin D status. For higher analytical throughput, traditional solvent extraction of samples has been replaced by immunological methods. The Vitamin **D** **E**xternal **Q**uality **A**ssessment **S**cheme (DEQAS) has revealed method-related differences in 25(OH)vitamin D results, raising concerns about the comparability and accuracy of different assays (Carter 2011).

Usability/Utility: The analytical evaluation of 25(OH)vitamin D assays between different methods (e. g. radioimmunoassays, ELISAs or mass spectrometry (MS) is currently on the way. However, most of the existing assays are able to produce sufficently reliable results for a clinical estimation of 25(OH)vitamin D levels and may therefore monitor not only the initial but also the follow-up values during vitamin D supplementation.

6.2.1.2.2 $1,25(OH)_2$Vitamin D

Calcitriol, also called 1,25-dihydroxycholecalciferol or $1,25(OH)_2$vitamin D3, is a hormonally highly active form of vitamin D with three hydroxyl groups. It increases the level of calcium in the blood by increasing the uptake of calcium from the gut into the blood, by decreasing the transfer of calcium from the blood to the urine by the kidneys, and by increasing the release of calcium into the blood from the bone. In turn, $1,25(OH)_2$vitamin D declines very late in vitamin D deficiency. Therefore the measurement of 25(OH)vitamin D better reflects body vitamin D storage. Decreases in $1,25(OH)_2$vitamin D are associated with kidney impairment, already with slightly decreased renal function or very rarely with 1α-hydroxylase defects. High levels of $1,25(OH)_2$vitamin D have been found in sarcoidosis or idiopathic hypercalciuria. Both of them can lead to hypercalcemia as well as other causes like paraneoplastic syndromes.

Assays often involve partial purification and extraction e. g. by using coated silica cartridges to remove other vitamin D metabolites followed by high pressure liquid chromatography, radioimmunoassay, ELISA or MS technologies. Therefore, the routine measurement is often limited to central lab facilities.

Utility: The action of $1,25(OH)_2$vitamin D on a cellular level depends on local production which has paracrine and autocrine effects in many tissues. Serum measurements of $1,25(OH)_2$vitamin D may therefore not reflect local production, and may not be representative of the cellular levels of the hormone. However, in some patients e. g. with chronic kidney disease and impairment of 1α-hydroxylase activity, sarcoidosis or hypercalciuria syndromes, $1,25(OH)_2$ vitamin D determination can help to characterize the actual vitamin D status and needs.

6.2.2 Collagen and Collagen Products

Collagen is a ubiquitous element of human tissue. More than 90 % of the organic matrix of bone is constituted of collagen type I. Its biochemical composition of small, non-steric amino acids (e. g. glycin) facilitates the development of long chains, named helices, which are linked by lysin and hydroxylysin to form the well-known triple structures. Both renal and hepatic functions are crucial in the interpretation of serum collagen product levels, because both interfere with formation, storage and degradation of collagens and their metabolites.

6.2.2.1 Serum Crosslaps (CTX)

Serum crosslaps (CTX), also called crosslinks or crosslinked C-telopeptides of type I collagen, are linear fragments of collagen with a "cross-link" between these fragments at the aspartic acid position. Antibodies of the respective assays are selective for either ß-aspartic acid in the mature, degraded bone or a -aspartic acid in newly formed bone. The latter has been used in a urine-based measurement to detect bone metastases in breast cancer or Paget's disease (Reid et al. 2005). However, serum assays have not yet been fully established and analyses are therefore restricted to the probably more blurred urine substrate.

The former, more abundant ß-crosslaps (ß-CTX) are important degradation products of bone metabolism in clinical routine. These ß-CTX – in the narrow sense "CTX" – measurements have been established to date in more than 2000 scientific publications including recent studies on therapy outcomes in osteoporosis (Bauer et al. 2006; Blumsohn et al. 2010) and the management of bisphosphonate-induced osteonecrosis of the jaw (Lazarovici et al. 2010).

The interpretation of CTX measurements has been widely introduced in diagnosis and therapy of osteoporosis. Most frequent findings are marked decreases of CTX by antiresorptive treatment within a very short onset time of hours to days

– depending on the application route and the drug used. Increases of CTX can be found in osteoanabolic treated patients, but these increases are less pronounced than found in formation marker measurements. e.g. PINP (Obermayer-Pietsch et al. 2008). However, circadian and nutrition-dependent rhythms of CTX have to be taken into account (Bjarnason et al. 2002, Bieglmeier et al. 2009). The world-wide use of CTX biomarkers of bone metabolism has been recognized and will drive a global laboratory standardization protocol by international bone research societies starting in 2011.

6.2.2.2 PINP, PICP and ICTP

PICP (100 kDa) and PINP (53 kDa) are both propeptides of the C- or N-terminal ends of procollagen type I, respectively. Both peptides are produced in equimolar amounts during collagen synthesis and reflect therefore the process of collagen formation.

The involvement of ADAMTS-2 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif), an extracellular matrix metalloproteinase that cleaves PINP, is of increasing interest in collagen metabolism. Its activity is blocked by TIMP-3 (tissue inhibitor of matrix metalloproteinase) (Wang et al. 2006). During degradation, collagen products are bound by a scavenger receptor and incorporated into liver cells. Serum levels are therefore dependent on liver function.

The degradation of more mature collagen I material results in a variety of fragments. As specific proteinases cleave collagen I in a specific way, these fragments can be attributed to different degradation processes. Cathepsin K from osteoclast bone cleavage produces crosslaps; matrix metalloproteinases from mononuclear cells tend to produce other fragments, such as ICTP (carboxyterminal telopeptide of type I collagen, Garnero et al. 2003). Further proteolysis results in smaller fragments with characteristic cross-links such as pyridinolines and deoxypyridinolines. Final fragments are amino acids such as hydroxyprolin, hydroxylysin and glycosylated molecules.

6.2.2.3 Pyridinoline and Deoxypyridinoline

Pyridinoline (PYD) and deoxypyridinoline (DPD) are covalent pyridinium cross-links also produced from the breakdown of collagen during bone resorption. They are released into the circulation and excreted into urine. Both can be detected by RIA and ELISA, methods that allow clinical application and have a better sensitivity compared to the earlier high-performance liquid chromatography (HPLC) methods. However, their clinical relevance has declined during recent years.

The diagnostic and therapeutic relevance of collagen bone biomarkers is defined only by its origin from mature collagen I. This specificity can be documented by a combination of their primary structure and the modifications during formation, maturation and ageing of the bone matrix.

During bone resorption, collagen markers will normally be elevated, but suppressed during and some time after antiresorptive therapy. This can be widely used for therapy monitoring e. g. during bisphosphonate therapy. Bone formation or osteoanabolic therapy will produce higher levels of collagen serum products.

Due to the scattering of collagen serum markers, all changes in individual serum levels should refer to a "least significant change" of about 50 % of the initial values (Bieglmayer et al. 2006).

Usability/utility: The clinical use of collagen markers ranges from diagnosis of high bone turnover to therapy monitoring of antiresorptive drugs. Despite a huge number of studies using collagen products as biomarkers of bone metabolism, the heterogeneity of these analytes and their diverse and complex measurement methods has hindered a more sophisticated use of a wide range of collagen markers that could support a more differentiated interpretation of bone metabolism. However, CTX and PINP have evolved to most established parameters of bone resorption (CTX) and formation (PINP) and are on the way to international standardization processes.

6.2.3 TRAP 5b

Tartrate resistant acid phosphatase type 5b is a monomeric metalloenzyme protein present in osteoclasts, activated macrophages and dendritic cells. It has a molecular weight of approximately 35 kDa, a basic isoelectric point (7.6–9.5), and optimal activity in acidic conditions. This enzyme is specific for osteoclasts and is activated by RANKL (Liu et al. 2003). TRAP is synthesized as a proenzyme and activated by proteolytic cleavage and reduction. The enzyme has two subunits linked by disulfide bonds. It is distinguished from other mammalian acid phosphatases by its resistance to inhibition by tartrate, its molecular weight and characteristic purple colour. Two closely related isoforms of type 5 TRAP (5a and 5b) can be distinguished. Serum TRAP 5a has an activity 10-fold less specific compared to TRAP 5b. The difference in specific activity and pH optima allow immunoassays to be constructed to provide a high degree of selectivity for TRAP 5b.

In osteoclasts, TRAP is localized within the ruffled border area, the lysosomes, the Golgi cisternae and vesicles. Since the enzyme is not influenced by kidney function, serum levels reflect bone turnover also in renal osteodystrophy (Fahrleitner-Pammer et al. 2008).

Proposed functions of TRAP include osteopontin/bone sialoprotein dephosphorylation, generation of reactive oxygen species, iron transport, cell growth and differentiation. There is some evidence that its activation is induced by cathepsin K by cleaving a small peptide in a side chain (Ljusberg et al. 2005). Its physiological role is not fully understood, but the high phosphatase activity could be related to collagen turnover (Roberts et al. 2007). TRAP 5b is inactivated by the loss of the iron ion in the active centre of the molecule and it is then proteolyzed. This is important, because up to 90 % of the circulating molecule might be inactivated fragments, secreted through liver and kidneys. It is of importance that laboratory-

measured TRAP 5b activity does not always correspond to the biological activity of osteoclasts but that it reflects the total number of osteoclasts. Serum also contains TRAP 5a activity, primarily derived from inflammatory macrophages (Chao et al. 2010).

Usability/utility: In the management of patients with severe kidney disease, and probably of patients with bone metastases, the determination of serum TRAP 5b will overcome the problems of other biomarkers by providing reliable results. However, substrate stability and the need for frozen transportation may limit its use to central facilities.

6.2.4 ALP and Bone ALP

Alkaline phosphatase (ALP) and bone alkaline phosphatase (bone ALP or bALP) belong to the membrane-associated alkaline phosphate enzymes present in all tissues throughout the entire body with a molecular weight of about 140 kDa. The isoforms are generated by the same gene locus, but differ due to their extent of glycosylation. There are about fifteen related isoforms of alkaline phosphatases, mainly occurring in the liver, bile duct, kidney, placenta, and bone, the latter being generated by osteoblasts during bone formation. Normally, bone and liver isoforms contribute to ALP activity in the circulation in approximately equal amounts.

A proposed function of this form of the enzyme is matrix mineralization during osteoblast growth. However, mice that lack a functional form of this enzyme show relatively normal skeletal development. This enzyme has been linked directly to hypophosphatasia, a disorder characterized by hypercalcemia including skeletal defects (Whyte 2010). The character of this disorder varies depending on specific AP mutations determining age of onset and severity of symptoms.

ALP is linked to the cell membrane by inositolphosphate – the circulating form is therefore a cleaved part of the original molecule. However, ALP can be secreted by exocytosis directly from osteoblasts. Anorganic pyrophosphate is known to inhibit mineralization and might also be able to preserve vessels from calcification. A direct link to vascular calcification has been seen in osteoporotic patients, where ALP levels directly correlated with aortic calcification (Iba et al. 2004).

Usability/utility: In general, total ALP assays are suitable for the assessment of bone turnover in patients with normal liver function, but bone ALP reflects greater specificity for osteoblast function. Assay techniques are widely used and reflect bone formation. Its increase in osteomalacia requires a sufficient knowledge of vitamin D/PTH status of the patient.

6.2.5 Osteocalcin

Osteocalcin, also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP), is the most frequent non-collagenous protein found in bone and dentin and comprises 49 amino acids. Osteocalcin synthesis has been shown to be regu-

lated by vitamin D and K. For instance, carboxylation of the three glutamine residues is dependent on vitamin K and crucial for its affinity to hydroxyapatite. Vitamin K deficiency may thus cause higher amounts of uncarboxylated osteocalcin, as it is the case in normal individuals (Rennenberg et al. 2010). Osteocalcin is mainly bound in bone matrix, but 20–30 % may be released systemically. During osteoblast development, osteocalcin is a late marker after ALP and collagen type I.

The function of osteocalcin is not entirely understood, but it is of central importance for bone remodelling as has been shown from knockout experiments and other in vivo tests, both in osteoclasts and osteoblasts.

Osteocalcin has been one of the first important biomarkers of bone metabolism. Circulating osteocalcin is heterogeneous and partly fragmented. Many of these fragments, mainly released during bone resorption, are not detected by current commercial assays.

Based on two decades of research, recent new discoveries have linked osteocalcin and glucose metabolism as well as leptin. Crosstalk between bone and adipose tissue is a new window of importance for osteocalcin and might be intensified in the near future (Kanazawa et al. 2010).

Usability/utility: Even though its assignment to bone formation or bone resorption is either-or, the analysis of osteocalcin has been part of many of the diagnostic and therapeutic osteoporosis studies. It has been routinely observed that higher serum osteocalcin levels are relatively well correlated with increases in bone mineral density (BMD) during treatment with anabolic bone formation drugs for osteoporosis.

6.2.6 RANKL/OPG

The RANKL/OPG system has been described in other chapters of this book. In brief, osteoprotegerin (OPG) is a member of the tumor necrosis factor (TNF) receptor superfamily. It is a basic glycoprotein comprising 401 amino acid residues arranged into seven structural domains. It is found as either a 60 kDa monomer or as a 120 kDa dimer linked by disulfide bonds. Osteoprotegerin inhibits the differentiation of osteoclast precursors, related to monocyte/macrophage cells and derived from granulocyte/macrophage-forming colony units and also regulates the resorption of osteoclasts in vitro and in vivo. Osteoprotegerin is a RANK homolog, and acts by binding to RANKL on osteoblast/stromal cells, thus blocking the RANKL-RANK ligand interaction between these cells and osteoclast precursors. This inhibits the differentiation of the osteoclast precursor into a mature osteoclast.

RANK (Receptor Activator for Nuclear Factor κ B) is a type I membrane protein that is expressed on the surface of osteoclasts and is involved in their activation upon ligand binding. RANK is also expressed on dendritic cells and facilitates immune signalling.

RANKL (Receptor Activator for Nuclear Factor κ B Ligand) is found on the surface of osteoblasts, but also stromal cells and T cells.

The potential of the OPG/RANKL determinations has been investigated in several studies. A large prospective population study in 2004 on fracture risk and OPG/RANKL quotients showed an association with higher risk for low trauma fractures and low RANKL levels independent of age, sex, menopausal status and OPG levels (Schett et al. 2004). This was attributed to suppressed bone turnover with accumulation of microdamage and reduced bone quality. However, these results have not been confirmed by further studies. By contrast, low OPG levels have been associated with prevalent vertebral fractures in postmenopausal osteoporosis patients and transplant recipients. However, there are also studies with no differences of OPG levels between patients with or without fractures and recent studies showing an association between high fracture risk and high OPG levels (Li et al. 2009), possibly related to immunological diseases.

Usability/utility: At the moment, there is no clear answer whether OPG or RANKL determinations might be of further help in the diagnosis and risk determination of osteoporosis. This could be related to the major role of OPG/RANKL, not only in bone metabolism but also in immune regulation. However, OPG/RANKL determinations might be useful in special clinical conditions.

6.2.7 New Markers

With the discovery of several new pathways in bone metabolism, a number of interesting substances were identified as promising new candidates for bone serum biomarkers.

6.2.7.1 Cathepsin K

Cathepsin K is a lysosomal cysteine protease, a member of the peptidase C1 protein family, which has an optimal enzymatic activity in acidic conditions. It is synthesized as a proenzyme with a molecular weight of 37 kDa, and transformed into the mature, active form with a molecular weight of ~ 27 kDa. Its origin are osteoclasts, but it may also be found in bone giant cell tumors. The enzyme's ability to catabolize elastin, collagen, and gelatin allows it to break down bone and cartilage. Cathepsin K expression is stimulated by inflammatory cytokines and is associated with the initiation of the osteoclastic brush border, earlier than the appearance of TRAP during the bone resorption process. Its catabolic activity is regulated by Cystatin D, an endogenous inhibitor of cysteine proteases (Holzer et al. 2005).

Usability/utility: Cathepsin K inhibitors, such as odanacatib, show great potential in the treatment of osteoporosis. The use of cathepsin K in clinical routine measurements will depend on the outcome of current studies and on a possible relationship to indication and monitoring in osteoporosis patients as well as in patients with other forms of metabolic bone disease.

6.2.7.2 Matrix Metalloproteinases – MMPs

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases belonging to a family of at least 28 secreted or transmembrane proteases. They are collectively capable of processing and degrading various proteins, but also a number of bioactive molecules (Egeblad and Werb 2002). In addition, they are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands, and chemokine/cytokine in/activation. MMPs also play a major role in cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defence.

The major difference to other endopeptidases is their dependence on metal ions as cofactors, their ability to degrade extracellular matrix, and their specific evolutionary highly conserved DNA sequence. The role of MMPs in bone metabolism is far from being entirely understood, but will probably play an important role in rheumatic diseases and therapy assignment (Pelletier et al. 2010).

Usability/utility: MMPs have potential effects on the expansion of bone metastasis but are also considered important in rheumatoid and degenerative arthritis. The usability of these parameters in clinical routine has to be further evaluated in the future.

6.2.7.3 Bone Sialoprotein

Bone sialoprotein (BSP), a glycopeptide with high sialic acid content, is a component of mineralized tissues formed by osteoblasts and osteoclasts. Native BSP has a molecular weight varying between 33 to 80 kDa. It is a significant component of the bone extracellular matrix and has been suggested to constitute approximately 8 % of all non-collagenous proteins in the bone. BSP-1, also called osteopontin, is a cell-binding sialoprotein or integrin-binding sialoprotein and belongs to the "SIBLINGs" ("**s**mall **i**ntegrin **b**inding **li**gand **N**-linked **g**lycoprotein") family. It has been demonstrated that BSP is extensively modified post-translationally, which makes the protein highly heterogeneous.

The amount of BSP in bone and dentin is roughly equal. However, the function of BSP in these mineralized tissues is not known. One possibility is that BSP acts as a nucleus for the formation of the first apatite crystals. As the apatite forms along the collagen fibres within the extracellular matrix, BSP could then help direct, redirect or inhibit the crystal growth. However, BSP also supports osteoclast formation by RANKL and inhibits their apoptosis. Therefore, circulating levels of BSP might correlate to bone metastasis formation. Additional roles of BSP are MMP-2 activation, angiogenesis, and protection from complement-mediated cell lysis.

Usability/utility: Bone sialoprotein has been found to be of value in assessing bone metastases in cancer as well as in some special conditions in metabolic bone disease. These promising results must still be confirmed in large trials. However, combinations of markers have at times helped in assessing cancer stages and lytic bone disease and in monitoring specific treatment modalities.

6.2.7.4 Osteonectin (SPARC)

Osteonectin (also referred to as secreted acidic cysteine-rich protein, SPARC) is an acidic glycoprotein of bone with a molecular weight of 43 kDa that binds calcium protein at the N-terminal acidic region. It is secreted by osteoblasts during bone formation, initiates mineralization and promotes mineral crystal formation. Osteonectin also shows affinity for collagen in addition to bone mineral calcium. It is a secreted extracellular matrix glycoprotein that plays a vital role in bone mineralization, cell-matrix interactions, and collagen binding.

Osteonectin shows context-specific effects, but generally inhibits adhesion, spreading and proliferation, and promotes collagen matrix formation. It increases the production and activity of matrix metalloproteinases, a function important to cancer cells invading bone. In endothelial cells, osteonectin disrupts focal adhesions and binds and sequesters PDGF and VEGF. Osteonectin is abundantly expressed in bone, where it promotes osteoblast differentiation and inhibits adipogenesis. Additional functions of osteonectin have been described as beneficial to tumor cells including angiogenesis, proliferation and migration. An exciting new observation is the association of osteonectin derived from adipose tissue with insulin resistance and diabetic retinopathy, nephropathy as well as adipose tissue fibrosis (Kos and Wilding 2010).

Usability/utility: Overexpression of osteonectin has been reported in many human cancers such as breast, prostate and colon cancer and therefore might be of increasing interest for the search of bone metastases, but also for osteoblast function in metabolic bone diseases and for insulin regulation in obesity.

6.2.7.5 Phosphatonins – FGF23

Fibroblast growth factor 23 (FGF23), encoded by the FGF23 gene, is a member of the fibroblast growth factor family which is responsible for phosphate metabolism. The FGF23 gene is located on chromosome 12 and is composed of three exons. Mutations in FGF23 render the protein resistant to proteolytic cleavage. They lead to increased activity of FGF23 and to renal phosphate loss as found in the human disease autosomal dominant hypophosphataemic rickets (Ramon et al. 2010) Some types of tumors also overproduce FGF23, causing tumor-induced osteomalacia. Loss of FGF23 activity has been shown to increase phosphate levels and develop tumor calcinosis.

The study of several renal phosphate-wasting disorders resulted in the identification of four factors with the predicted characteristics of phosphatonins, namely fibroblast growth factor 23 (FGF23), secreted frizzled related protein-4 (sFRP-4), matrix extracellular phosphoglycoprotein (MEPE), and FGF7. Increased serum phosphate, vitamin D, and probably PTH levels stimulate FGF23 production by bone (Ramon et al. 2010). During vitamin D replacement therapy, decreased FGF23 concentrations decline further and may again have a favourable impact on bone

mineralization by counterregulatory effects on phosphate homeostasis (Uzum et al. 2010).

Usability/utility: The usefulness of FGF23 in clinical settings is not only restricted to the differential diagnosis of tumors and hereditary forms of hypo- and hyperphosphatemia. Several studies on phosphate therapy showed a tight reaction of FGF23 levels on phosphate interventions. The determination of this phosphatonin might therefore be useful in the follow-up of patients with renal diseases as well as for oncological patients with newly diagnosed disturbances of the electrolyte balance.

6.2.7.6 Wnt associated Proteins and Receptors

The Wnt signaling pathway includes many receptors, agonists and antagonists and has been elucidated during the past years. This signalling system contributes to bone formation and mechanoreception.

6.2.7.6.1 Secreted Frizzled-related Proteins

Secreted frizzled-related proteins (SFRP1 SFRP2, SFRP3, SFRP4, SFRP5) with a molecular weight of about 35 kDa are members of the SFRP family that contain a cysteine-rich domain homologous to the putative Wnt-binding site of frizzled proteins. SFRPs act as soluble modulators of Wnt signalling. Each SFRP is ~300 amino acids in length and contains a cysteine-rich domain that shares 30–50 % of sequence homology with frizzled (Fz) receptors. SFRPs are able to bind Wnt proteins and Fz receptors in the extracellular compartment. The interaction between SFRPs and Wnt proteins prevents the latter from binding the Fz receptors. SFRPs are also able to downregulate Wnt signalling by the formation of an inhibitory complex with the frizzled receptors. The Wnt pathway plays a key role in embryonic development, cell differentiation and cell proliferation. It has been shown that the deregulation of this critical developmental pathway occurs in several human tumor entities.

Usability/utility: Due to their frequent involvement in breast cancer, SFRPs have been investigated as bone markers in bone metastasis and metabolic bone disease. However, the clinical use of SFRP determination might increase with some more specific knowledge on its importance in diagnosis of metabolic changes or during therapy regimens.

6.2.7.6.2 Dickkopf-1

Dickkopf-1 (DKK1) is another analyte of the Wnt signalling cascade that has been developed to characterize bone metabolism and is encoded by the DKK1 gene. It is a

secreted protein with two cysteine rich regions involved in embryonic development through its inhibition of the Wnt signalling pathway.

Besides a negative correlation with bone mass (Macdonald et al. 2007), elevated levels of DKK1 in bone marrow, plasma and peripheral blood have been shown to be associated with the presence of osteolytic bone lesions in patients with multiple myeloma. The regulation of canonical Wnt signalling by DKK-1 may act as a molecular switch mediating the transition from an osteolytic to an osteoblastic response. Therefore, blocking DKK-1 activity may prove to be a relevant therapeutic target in the prevention of bone metastasis (Hall and Keller 2006).

Usability/utility: DKK-1 determination is based on several available kits and might be of help for bone metastasis detection. Further research and clinical studies will increase the potential of this biomarker.

6.2.7.7 Sclerostin

Sclerostin, the product of the SOST gene, was originally believed to be a non-classical inhibitor of bone morphogenetic protein (BMP), but has finally been identified as binding to LRP5/6 receptors and inhibiting the Wnt signalling pathway. It is produced by osteocytes. Wnt activation under these circumstances is antagonistic to bone formation. Sclerostin may be of increasing importance as a marker of osteocytes, accounting for the majority of the mature bone cells, although neglected over the past years. Mature osteocytes are sensors for mechanical loading of bone; they inhibit osteoclasts and stimulate osteoblasts (Bonewald 2007). Many new results link osteocytes with osteoblastic cell contacts and microfractures (Taylor et al. 2007).

The currently investigated anti-sclerostin antibody for the treatment of osteoporosis co-developed by Amgen and UCB introduces a new focus of therapy for the future and may require some sclerostin determination. The reaction of sclerostin levels has been recently investigated in several trials. Whereas bisphosphonates had no substantial effects due to a general non-activation of osteocytes, estrogen replacement therapy (Mirza et al. 2010) and PTH osteoanabolic therapy (Drake et al. 2010) induced significant changes in sclerostin levels.

Usability/utility: Sclerostin inhibitors are currently developed as a new generation of medication for osteoporosis. The clinical usefulness of sclerostin measurements will depend on further investigations on diagnostic and monitoring applications for metabolic and oncologic bone diseases.

6.3 Analytics

6.3.1 Preanalytics

The analytical process starts with the selection and preparation of the patients and comprises blood drawing, the performance and validation of the analytical measurement, as well as the presentation and interpretation of the gained data.

Many of the recent tests have required extensive work for specificity and reproducibility of the results under controlled conditions. However, the main problem might lie far ahead of the controlling of laboratory techniques.

A main source of variability is the preanalytical phase, where patient conditions are very difficult to standardize. It is therefore strictly recommended to use biomarkers known to have a robust performance on their way from the patient to analysis and validation.

Some clinical conditions are crucial for the choice of bone biomarkers: renal elimination has been shown for collagen products, crosslaps and osteocalcin. They had been characterized in urine and tests had been routinely used, mainly before serum measurements were made available. Although many publications have used urinary assays, these determinations have been replaced by serum analyses. Firstly, the provision of material was sometimes difficult or not scheduled at the right time. Secondly, 24 h sampling or even more spot urine measurements were prone to mistakes made by patients or staff. Thirdly, interfering admixture by nutrition or medication may have raised problems.

6.3.1.1 Circadian Rhythms

A clear circadian rhythm has been documented both for PTH and biomarkers of bone formation and degradation. Amplitudes of these undulations are different for each marker. The maximum level of most of these biomarkers is reached during sleep. Blood drawing should therefore always be restricted to the same hours in the morning after an (at least) overnight fast.

6.3.1.2 Nutrition

The dependence of biomarkers of bone metabolism on dietary influences has been known for some years. In an initial study, serum crosslaps levels varied during the day with a maximum at about 05:00 in the morning and a minimum of about 14:00 in the afternoon. The variation had a magnitude of about 40 % around the 24 h mean and was independent of menopause, bone mass, and bed rest (Qvist et al. 2002). In a cross-over study bone resorption was reduced by intake of glucose, fat, and protein and counteracted by fasting, independent of age and gender. Both exogenous and endogenous insulin stimulation tests induced a decrease in bone resorption by

50 %, but this was modest when compared with the reduction observed during food intake (Bjarnason et al. 2002). Blood drawing should therefore always be carried out under the same conditions, preferentially in the morning after an overnight fast.

There is a controversial discussion on an additional role of dietary protein in bone health. A very recent publication shows an additional modest beneficial long-term effect of protein rich nutrition on bone markers and bone density (Jesudason and Clifton 2011).

6.3.1.3 Stability

Reports on the stability of bone biomarkers have been controversial and general statements are difficult. Most of the analytes – except for small well-defined molecules – are very heterogeneous substances and may even differ between each other. Epitope specificity is therefore often rare and might be subject to change due to sample-taking, transportation, and storage.

Cross-reactions between epitopes and antibodies might further influence the stability of the results.

For longer storage, some specific publications describe collagen products such as P1NP and serum crosslaps as stable in serum for at least 48 h at room temperature, seven days at 2–8 °C, and frozen for at least six months at –20 to –80 °C (Lomeo and Bolner 2000). In summary, storage of serum and urine samples for bone metabolism markers at 2–8 °C can be delayed for at least one week; for long-term storage, (deep) freezing of the sample provides molecular stability for several months for most analytes.

6.3.2 Interpretation

Problems with the analysis per se and with the interpretation due to environmental factors are inherent issues not only of bone biomarkers.

6.3.2.1 Disturbances during Analysis

Many of the presented bone biomarkers can be measured by commercially available assays. Most of them use common technologies such as radioimmunoassays or ELISAs. However, problems with divergent epitope structure such as in collagen metabolites, interference of other molecules or haemolysis, degradation prior to measurement as in enzymes like TRAP 5b, and many other influences may contribute to results that may not be reproducible or prone to frequent scattering. Experienced labs have therefore introduced validation and standardization steps during implementation and routine assay performance. Special standard operating procedures

and a clear documentation for all analytical steps should therefore be obligatory in all labs performing bone marker analyses.

6.3.2.2 Therapeutic Influences

As stated in the sections on changes during bone therapy, bone biomarkers are very much influenced by all kinds of osteotropic therapies. Whereas bone resorption markers decrease significantly during antiresorptive medication, biomarkers of bone formation and resorption may greatly increase during osteoanabolic therapy, even after prior antiresorptive treatment. Interpretation of lab values has therefore to take into account both therapy regimens and the interval between medication intake and blood drawing.

6.3.2.3 Lack of Standardization

Many of the biomarker tests currently used have been validated by their customers and should again be optimized in the respective user lab. However, there are significant differences in bone biomarker results between labs and between research publications. International standardization procedures are on the way for serum crosslaps, PINP and 25(OH)vitamin D measurements.

Some of these complications may also originate from the use of different marker units in different countries. 25(OH)vitamin D is an example with the use of ng/ml in Europe and the use of nmol/ml in the US (conversion: ng/ml × 2,496 = nmol/l). These problems have long been recognized and many institutions are currently working on more general recommendations and standardizations for the most frequently used biomarkers, e. g. again 25(OH)vitamin D for assay and normal value standardization.

6.3.2.4 Age and Gender

Biomarkers are prone to change, especially due to age. As the skeleton evolves and reacts to the age-specific requirements, bone biomarkers may be elevated during phases of growth and decreased during phases of "rest". The differences in female and male skeletons and disease incidences have been known for decades or a priori, and should be characterized separately, except for several regulators such as PTH and 25(OH)vitamin D.

The problem of confining "normal" serum biomarker values includes the availability of higher numbers of disease-free, medication-free, gender-equated and age-distributed persons who have to be phenotyped for as many bone characteristics and concomitant variables as possible. These conditions are the reason why only relatively small, gender- or age-controlled groups have been investigated for most of

the bone biomarker analytes. New efforts and a complex biobanking system as well as international consortia are on the way to solve these issues.

6.3.2.5 Reference Ranges

The most intensively studied group of persons using bone biomarkers are postmenopausal women, being the first group of interest for the diagnosis and treatment of osteoporosis. Premenopausal and male subjects have been identified more recently as diagnostic and therapeutic targets.

Normal values for these age and gender groups have now been established for most of the current parameters, but not for the new generation of more specific bone assays. Children and their normal values are of special interest to researchers and pediatricians, but studies on healthy children are difficult to conduct (Rauchenzauner et al. 2007). However, normal values should be evaluated in the given population and environment wherever possible to be used for routine applications.

6.3.2.6 Representation of Bone Marker Dynamics

Biochemical markers of bone turnover reflect resorptive and reconstructive effects on the skeleton. Although elevated markers are commonly interpreted as a sign of an increased turnover rate, the balance between bone resorption and formation is mostly neglected. Two methods of standardization have been invented: Firstly, the uncoupling index (the difference of *z*-scores from resorption and formation markers in urine and serum), which estimates balance and not turnover rate (Eastell et al. 1993). Secondly, more recently, a bone marker plot (Bieglmaier and Kudlacek 2009) based on the combined analysis of serum formation and resorption markers was established, assuming a skewed distribution of the biomarkers. The graphic method is designed to report both on individual- and on group-specific changes in bone metabolism reflected by bone markers.

6.4 Utility of the Determination of Serum Markers of Bone Metabolism

6.4.1 Fracture Prediction

High bone remodelling rates have been associated with an increased risk of fractures. Large epidemiologic studies have demonstrated that bone turnover is an independent contributor to fracture risk. Combining a bone biomarker with bone density showed an additive effect on fracture risk e. g. in the OFELY (Os des Femmes de Lyon) and the EPIDOS (Epidémiologie de l'Ostéoporose) study (Garnero et al. 2000). In the OFELY Study, women in the highest quartile of bone biomarkers had

a twofold higher risk of fractures compared with women with low markers of bone turnover.

6.4.2 Therapy Monitoring

By using and interpreting bone turnover rates, the biological action of therapies can be assessed, e. g. the therapeutic efficacy of antiresorptive agents. Bone biomarkers rapidly decrease during bisphosphonate therapy in postmenopausal women, and these changes are associated with increased bone mass and/or fracture rate reduction.

Nonetheless, the notion that fracture risk reduction is associated with a decrease in bone biomarkers during therapy is supported by all studies on oral or intravenous bisphosphonates. Similar results exist for alendronate, risedronate, ibandronate, and more recent data also demonstrate that zoledronic acid, administered once annually for three years, resulted in a reduction in vertebral fractures accompanied by reduction in CTX, bone ALP, and PINP levels (Black et al. 2007). Likewise, similar correlations have been reported for other antiresorptive agents.

An opposite reaction is seen during osteoanabolic therapy by the increase of bone formation markers like PINP and bALP, but also by the increase of bone resorption biomarkers e. g. teriparatide (Obermayer-Pietsch et al. 2008). The changes in bone biomarkers are seen as early as during the first days till the first month after the initiation of therapy (Blumsohn et al. 2011) and are not dependent on prior antiresorptive therapy.

6.4.3 Compliance and Adherence to Therapy

Patients may not always be compliant to therapy regimens, especially over longer periods of treatment as requested in bone therapy. Compliance and adherence to treatment is a potentially useful application of bone biomarkers, particularly in the case of bone resorption inhibitors. Adherence to oral medications is notoriously poor and represents one of the major challenges of reducing the incidence of fractures in the elderly. Bisphosphonates must be taken following a strict dosing procedure, however, the introduction of weekly and monthly oral formulations has only slightly improved adherence or persistence. Thus, bone biomarkers could be useful in identifying a less-than-expected suppression of bone turnover, which may suggest either persistence failure (i. e., the patient has discontinued treatment) or that the patient has not been fully compliant with the dosing regimen (Seibel 2006). Such findings could then be discussed with the patient and corrective measures implemented.

Alternatively, inadequate suppression of bone biomarkers might indicate poor intestinal absorption of oral bisphosphonates. Unfortunately, these highly polar compounds are poorly absorbed by the intestine (Clowes et al. 2004), and low bioavailability may be more frequent than generally thought, especially in elderly

patients. This problem might well be a frequent cause of "treatment failure" of oral bisphosphonates. In such cases, a change in treatment modality, e. g. switching from oral to parenteral delivery or changing to a different agent or class of medication, should be considered.

6.4.4 Selection of Pharmacological Therapy

Based on a profile of bone biomarkers, therapy decisions may be facilitated: patients with accelerated bone turnover tend to lose bone at a faster rate than those with normal turnover; therefore, they should be the best candidates for antiresorptive therapy. For instance, greater reduction in non-vertebral fractures was observed in subjects with high PINP levels after alendronate treatment (Bauer et al. 2006). In turn, low-turnover bone disease has to be excluded from antiresorptive treatments and might benefit more from osteoanabolic therapy.

6.5 Future Prospects

Bone biomarkers open a big spectrum of disease research in the field of bones. New pathways and a challenging number of new biomarkers have been developed recently. Together with new therapeutic applications, a more precise and personalized diagnosis and therapy is therefore possible.

At present, bone biomarkers are not widely used in the clinical setting. Methodological improvements may ultimately make their use more common beyond the clinical trial environment and combine them with currently used morphological methods.

As discussed in this chapter, bone biomarkers have a variety of potential clinical applications based on their rapid response to treatment, their value in monitoring compliance to medications, and in guiding precise and reliable therapeutic decisions.

The burden of an ageing society will per se increase the need and knowledge for diseases of the skeletal system. Cheap and reliable techniques of bone characterization will therefore help to ensure public health.

In summary, bone biomarkers represent a tool to further intensify the implementation of personalized medicine with the focus of medical diagnostics and therapy on individual persons, as we would all want to claim for ourselves.

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Osteoporosis: Pathophysiology and Clinical Aspects

7

Peter Mikosch

7.1 Osteoporosis, a Worldwide Disease with High Economic Burden

Osteoporosis has become a major health issue over the last years due to the steadily increasing life expectancy. Beyond the age of 50 years more than 50 percent of women and 13 percent of men will sustain an osteoporosis-related fracture (1). In line with the demographic development, fractures of the humerus, wrist, or hip will occur noticeably more often during the next four decades (2). The number of patients with hip fractures will increase to 170 % of present-day numbers, and in the age group over 80 years to 250 % (2). In the United States over 10 million have been diagnosed with osteoporosis (1) causing direct medical costs of 17 billion dollars (3, 4). In Germany 7.8 million (6.5 million women) were affected by osteoporosis in 2003 (5). At least one clinical fracture was present in 4.3 % of these patients leading to direct costs of 5.4 billion Euro, although only 21.7 % of the patients were treated with anti-osteoporotic drugs as shown in a recent study from Germany (5). Considering only osteoporosis-attributable hip fractures, 108,341 occurred in Germany in 2002 resulting in costs of almost 3 billion Euro, which will more than double according to estimations in 2050 (6). These already tremendous costs of health care linked to osteoporosis are further alarming, as a care gap with under-diagnosis and under-treatment of the entity has been stressed in several studies (7–13). Improvements in diagnostic strategies, the diagnostic work up in the context of interdisciplinary settings, are warranted in order to optimize the management and care of patients with osteoporosis. The basis of such an aim has to be set up with a better and broader understanding of the pathophysiology, clinical presentation, interactions with other disorders, and the currently available therapeutic possibilities.

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Before discussing the pathophysiological mechanisms on the cellular and intracellular level, a short description of the clinical presentation of patients with osteoporosis and the development of bone mass during lifetime will be presented in order to show the connection between clinical appearance of osteoporosis and its underlying cellular mechanisms.

7.2 Balance of Bone Remodeling During Lifetime and the Development of Bone Fragility

Bones and the skeleton resemble a static organ giving shape to the human body and protection to certain organs such as the brain, heart and lungs. It is also a niche for mesenchymal and hematopoetic progenitors with the bone marrow being the place of blood production. However, bone is a highly vivid and dynamic organ being constantly moulded, shaped and repaired in order to meet the changing demands throughout life. This process of constant restructuring is termed remodeling, which is a coupled process involving bone resorption by osteoclasts and new bone formation by osteoblasts (14). The action of osteoblasts and osteoclasts in bone remodeling resembles a delicately balanced process that ensures the continual replacement of old bone, weakened by microfractures, with new bone. Failure to reach peak bone mass or the uncoupling of remodeling can result in bone fragility. Bone formation exceeds bone resorption during the years of the growing skeleton in childhood and early adolescence, finally reaching peak bone mass at the age of 30–40 years. In the following years the balance between bone formation and bone resorption changes towards dominance of bone resorption over bone formation, leading to continuous loss of bone tissue. This loss of bone tissue and also its decline in means of quality and structural integrity with increasing age lead to a state of increased bone fragility.

7.3 Clinical Presentation of Patients with Osteoporosis

In its current definition osteoporosis is characterized as a disorder of the skeleton with a decrease in bone mass and structural deterioration of bone tissue, leading to bone fragility and increased susceptibility to fractures of the hip, spine and wrist (15). After achieving peak bone mass at the age of 30–40 years, patients start to steadily loose bone due to the imbalance of bone formation and bone resorption in favor of bone resorption. This process of bone loss is accelerated in women with the onset of menopause. Initially patients will not face any symptoms as long as loss of bone mass, deterioration of microarcitecture and the decline of quality in bone material will be limited. With ongoing age, however, the stability of bone will deteriorate to such a level that even without severe traumatic events microcracks within bone will not be fully repaired anymore. The morphological changes associated

with osteoporosis start within the central skeleton. The consequences are a slow but steady deformity and loss of height of the vertebrae, which are part of the weight bearing skeleton. The first regions of the vertebral column affected by these changes are the lower parts of the thoracic spine and the upper part of the lumbar spine. The kyphosis of the thoracic vertebral column increases, leading to an forward curving of the body and in severe cases to a clearly visible hump ("Dowager's hump"). In addition, patients are facing a progressive loss of height. The increased kyphosis of the thoracic spine and the loss of height are the first clinical signs of osteoporosis. At this stage patients may develop back pain which may be due to the changed static situation. Beside a slow and steady loss of vertebral height, the first vertebral fractures may also emerge, followed by severe local back pain. In progressive stages patients have lost several centimeters of height and usually present severe kyphosis of the thoracic spine. In addition, secondary to these changes, the arms of the patients seem to be elongated in relation to the patient's height and the abdomen and lungs are compressed, leading to a protruding abdomen and eventually to shortness of breath and pulmonary symptoms of restrictive lung disease. With ongoing age the progressive loss of bone mass and bone quality will make the bone so fragile that simple falls during daily life will lead to fractures, most commonly hip fractures but also to fractures of the shoulders, the upper- or the forearms. In the elderly, hip fractures are most common and will cause increased morbidity and mortality. Elderly people who have sustained a hip fracture will fully recover only in a small percentage, most face smaller or larger impairments and losses of abilities after a hip fracture. About 20 percent will die within one year after a hip fracture. Very severe cases may even face spontaneous fracture without a fall or any trauma. The deformity of the vertebral column and chronic back pain severely impairs the quality of life of the patients. Patients will often become dependent on assistance in order to perform the daily life activities. Furthermore, anxiety to fall often leads to possible social retraction and isolation, as patients will not be able or willing to leave their homes anymore. Thus, in this respect osteoporosis then becomes not only a disease affecting the bone, but actually affects the life of the patient by severely impairing his mobility, independence, and mental state (Fig.1).

7.4 An Approach to the Mechanisms Leading to Osteoporosis

The quantitative loss of bone mass and qualitative structural changes of bone leading to osteoporosis are based on complex metabolic changes during life. Estrogen deficiency is the major cause of osteoporosis. However, also other hormonal or inflammatory processes may cause bone loss. Bone tissue and bone cells on the one hand and also cells of the bone marrow on the other hand are closely linked with each other, not only in space but also by physiological and pathophysiological interaction. Hormones, cytokines and a range of inflammatory mediators all interfere with each other and exert a range of effects in the various organs. Osteoimmunology

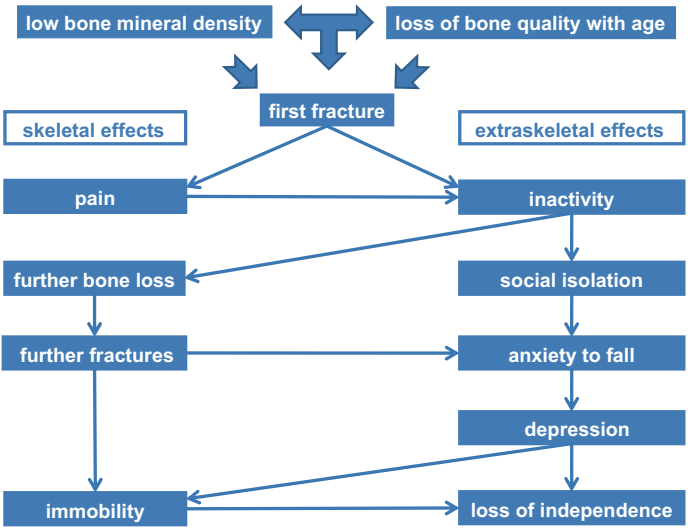

Fig. 1 Osteoporosis as a cascade of events affecting not only the skeleton but also activity, social contacts, independence and the mental status of the patient

investigates the complex connections and interactions between immune cells, immune system cytokines and chemokines on the one hand, and bone cells involved in the bone remodeling process on the other hand (16). The common origin of bone and immune stem cells is the key to understand this system and the physiology of bone loss. Knowledge in this rapidly growing field may also facilitate the translation from basic scientific knowledge on bone biology to an improved understanding of different bone disorders, including osteoporosis, paradontal disease and rheumatoid arthritis. Furthermore, it also provides the basis for the development and application of targeted therapies of the future (17, 18).

7.5 Osteoblasts and Osteoclasts: Cellular Promoters of Bone Formation and Degradation

Bone-resorbing cells (osteoclasts) and cells of the immune system both originate in the bone marrow from hematopoetic cells (Fig. 2). Bone-forming cells (osteoblasts) are of mesenchymal origin derived from pluripotent mesenchymal stem cells (MSC), which can also give rise to chondrocytes, myoblasts, tenocytes, neurons and adipocytes (16). During osteoblast differentiation, MSC express increased amounts of phenotypic markers (e. g. alkaline phosphatase, osteocalcin), receptors for bone morphogenetic proteins (BMP) and the Wnt receptors low-density lipoprotein

receptor related proteins (LRP) 5 and 6. Through the activation of these receptors the progentitor cells are differentiated into osteoblasts with the capacity of bone formation (19).

The counterparts of the bone forming osteoblasts are the osteoclasts, which origin and develop from precursors of the mononuclear monocyte-macrophage cell line (Fig. 2). Osteoclasts resemble highly specialized multinucleated cells being exclusively enabled to resorbe bone. Osteoclasts precursors at or close to the bone surface are induced to differentiate into mature osteoclasts after stimulation by macrophage colony-stimulating factor (M-CSF) and receptor for activated nuclear kappa B (RANK) ligand (RANKL). Osteoclasts then attach to bone surface, mediated by integrines. A sealing zone, which is formed by rearrangement of the actin cytoskeleton, occludes the bone surface and the outer rim of the attaching osteoclast, forming a separated resorption pit with the underlying bone surface afterwards. During this process the osteoclasts undergo a structural change into a polarized cell shape. The basal area of the osteclast enlarges by forming a "ruffled border". The osteoclast can release hydroxigen iones through the H+ ATPase into the resorption pit. Proteases, like tartrate-resistant acid phosphatase (TRAP), are then excreted via endocytoplasmatic transport into the resorption pit where they mobilize the hydroxyl apatite crystals. In a next step cathepsin K is secreted by the osteoclast to degrade the organic bone matrix within the acidified fluid. Cathepsin K plays a major role in the proteolytic process of matrix degeneration. The products resorbed

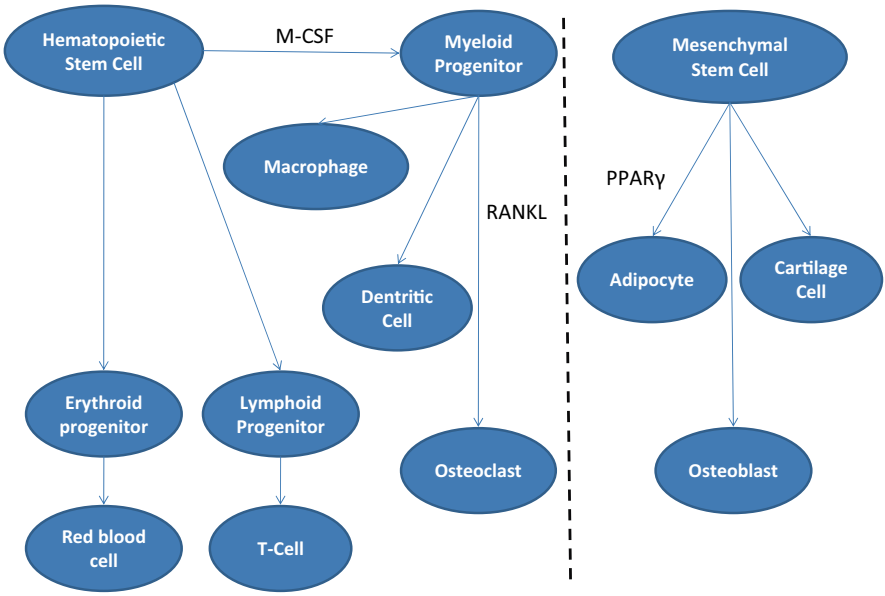

Fig. 2 Lineage of osteoclast and osteoblast development

from the bone, mainly calcium, phosphate and fragments of the organic matrix, are taken up by the osteoclast and released into the circulation via its apical surface. Osteoclasts are of essential importance for the homeostasis of calcium and phosphate, and their activity is tightly controlled via different modes including activation by parathyroid hormones (14, 20).

7.6 Osteoblasts and Stromal Cells: the Connection with the Hematopoietic System

The vast majority of hematopoiesis is located in the bone marrow. Proliferation and differentiation of hematopoietic stem cells (HSC) is controlled by "stromal" cells. The nature of these mesenchymal cells and their mode of action still lacks explanation. However, recently it has been pointed out that the stromal cells responsible for HSC regulation in the bone marrow are most likely osteoblasts (21). It could be shown that osteoblastic cells produced high levels of Notch ligand jagged 1 and supported an increase in HSC under the influence of parathyroid hormone (PTH) (22). This regulatory component of osteoblastic cells on the hematopoietic stem cell niche through Notch activation may be the background for the positive action of

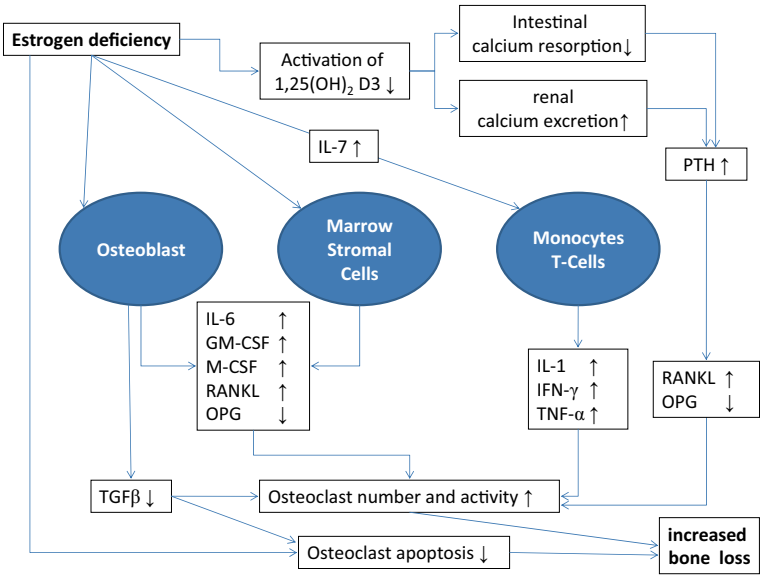

Fig. 3 Direct and indirect effects of estrogen deficiency in modulating bone metabolism

recombinant PTH in osteoporosis therapy. Furthermore, through this interaction of PTH with the HSC niche, possible therapeutic options for anemia and immunosuppresion are now under investigation.

7.7 Osteoblasts and Adipocytes

Both adipocytes and osteoblasts originate from pluripotent MSC. In the process of aging negative effects on osteoblast production and activity evolve, whereas the development and production of adipocytes is enhanced. Adipocytes are rare in the hematopoetic bone marrow of neonatal mammals, but with increasing age adipocyte number and size increase constantly, transforming the initially red bone marrow into yellow fatty marrow. Intra- and extracellular signals influence the development of the undifferentiated MSC. Runx2 (runt-related transcriptional factor) is essential for the development into the osteoblast linage, whereas PPARγ2 (peroxime proliferator-activated receptor gamma 2) induces differentiation into adipocytes. PPARγ2 concomitantly induces the differentiation of adipocytes and inhibits the differentiation of osteoblasts (23). This makes PPARγ2 a key regulator of the differentiation of osteoblasts. Ectopic expression of recombinant PPARγ2 on osteoblasts suppressed irreversibly runx2 expression and the osteoblast phenotype, transforming the osteoblasts in terminally differentiated adipocytes (24).

7.8 Osteoclastogenesis and Monocyte Macrophage Interaction

Osteoclasts are derived from the mononuclear monocyte-macrophage cell line, which also generate immune cells, including dendritic cells. Osteoclasts derive from the myeloid-monocyte branch of hematopoetic cells, thus sharing the same precursors as macrophages and myeloid dentritic cells. Depending on the micro-environement to which the precursor cells are exposed, a further development specific to the different cell lines will emerge. The multipotential myeloid progenitor cell population is defined by the surface marker C-Kit and it also expresses the panmyeloid marker CD11b, whereas c-Fms, which is the tyrosin kinase receptor for M-CSF necessary for cell differentiation into osteoclasts, is not expressed. Through interaction of the CD11b+ monocytic precursor cells with stem cell factor (SCF) the cells become c-Fms positive (25). C-Fms is a key determinant in the development of cells in the monocyte-macrophage lineage (14). The presence of M-CSF transforms the early-stage precursor cells to late-stage precursors by increased CD11b expression and upregulation of the expression of receptor-activator of NFkB (RANK). RANK ligand (RANKL) will then bind to RANK, inducing a cascade of signalling events which leads to osteoclast formation (25). RANK signalling is mediated via TRAF6 (TNF receptor associated factor 6) in osteoclast precursors. TRAF6 is by itself essen-

tial in the regulation of further downstream factors regulating the expression of specific genes necessary for osteoclast differentiation and activation (NFkB, alkaline phosphate-1 mediated by JNK pathway, TGF-β-inducable kinase TAK1, p38 stress kinase) (26). NFkB and c-Fos activation induce the transcriptional factor NFATc1, leading to the expression of genes such as TRAP, cathepsin K, and dentritic-cell-specific transmembrane protein (DC-STAMP), which are essential for osteoclast formation and function. DC-STAMP is able to induce IL-4 and has been found in osteoclasts, myeloid dentritic cells, and macrophages (27).

7.9 The RANK/RANKL/OPG System – Key Regulator of Bone Homeostasis

Bone homeostasis is regulated by a delicate balance between osteoblastic bone formation and osteoclastic bone resorption. Although estrogen is the key sex hormone governing bone homeostasis, the primary regulator of bone remodeling is now being recognized as the RANK/RANKL/OPG system. Osteoclastogenesis is controlled by the ratio of receptor activator of NF-kappaB ligand (RANKL) relative to its decoy receptor, osteoprotegerin (OPG). During normal bone remodeling, marrow stromal cells and osteoblasts produce RANKL, which binds to the transmembrane receptor RANK on osteoclast precursors and induces differentiation and activation as pointed out above (Fig. 2). This occurs through the transcription factor, nuclear-factor kappa B (NFkB), which is responsible not only for activating osteoclastogenesis but also the body's inflammatory response. Both osteoclast differentiation and the inflammatory process occur via regulation of interleukin-6 (IL-6).

The major role that cytokines play in bone remodeling is demonstrated by the fact that receptors for the proinflammatory cytokines IL-1, IL-6, and tumor necrosis factor-alpha (TNF-α) are present on both osteoclast precursor cells and mature osteoclasts. Estrogen exhibits its nuclear regulatory effects by inhibiting IL-6 activation of NFkB during bone remodeling. Osteoblasts also produce OPG, a soluble decoy receptor that blocks RANKL and maintains control of the remodeling process. OPG is vital to the success of the RANK/RANKL/OPG system of bone homeostasis.

This system determines the success or failure of bone homeostasis. The different inflammatory cytokines, which act in the development of osteoporosis, are in fact essential for adequate bone remodeling. For example, TNF-α-mediated cartilage and bone degradation was IL-1 dependent in a murine arthritis model (28) and systemic inflammatory bone resorption is fully dependent on IL-1 (29).

At this point the important interplays between osteoblasts and osteoclasts and hormones also needs to be highlighted with the RANK/RANKL/OPG system being the connecting link between these players. RANKL, which is essential for osteoclast differentiation and activation, is produced by osteoblasts under the influence of vitamin D, parathyroid hormone and estrogen (20, 30). Detailed information on the RANK/RANKL/OPG system will be given in a separate chapter (see chapter "Basics of Bone Biology").

7.10 Dendritic Cell Interaction with Bone Cells

Dendritic cells are derived like osteoclasts from the monocyte/macrophage lineage. Dendritic cells develop after exposure to macrophage colony-stimulating factor (M-CSF) and IL-4 (31). Dendritic cells are responsible for antigen presentation with selective stimulation of T cells and B cell, thereafter leading to a specific immune response. A direct involvement of dendritic cells in osteoclastogenesis is suspected, as dendritic cells can transdifferentiate into osteoclasts in vitro (32). Dendritic cells may also be involved in bone loss due to inflammation (33) where they interact with T cells. This is thought to be based on the fact that dendritic cells express RANK and that they mediate an osteoimmunological interaction indirectly by activating T cells to produce RANKL, which then induces osteoclastogenesis (34).

7.11 T Cells and Osteoporosis

Activated T cells can either stimulate or suppress the formation of bone-resorbing osteoclasts. T-helper cells 1 (Th1) produce IFN-γ and Th2 IL-4, which are cytokines suppressing osteoclastogenesis. Imbalance in the Th1/Th2 adaptive immune response initiated by antigenic stress may play a role in specific cases of osteoporosis. With T cell activation now known to have a major role in RANKL-induced osteoclastogenesis, more research is needed to determine whether early maturational and/or chronic immunological stressing agents contribute to excessive bone loss in later years. In order to resolve this discrepancy further research was promoted in order to find T-helper cell populations, which may only exert proinflammatory stimuli towards osteoclastogenesis. This means that these T-helper cells should express low levels of IL-4 and IFN-γ, but should express TNF-α and RANKL. These criteria were met by a subpopulation of T-helper cells expressing IL-17 (35, 36).

In a recent study (37) on TallyHo/JngJ (TH) mice, a polygenic model of type II diabetes, the spontaneous development of osteoporotic features, possibly mediated by IL-17, was observed. Bone mineral density (BMD) was decreased in male TH mice, which displayed hyperglycemia. The bone formation markers osteocalcin (OC) and OPG were decreased, whereas bone resorption markers such as IL-6 and RANKL were significantly elevated in the bone marrow and blood. Furthermore, RANKL expression was increased in $CD4^+$ T cells of TH mice upon T cell receptor stimulation due to enhanced IL-17 production. IL-17 production in $CD4^+$ T cells was directly promoted by treatment with leptin while IFN-γ production was not. Blockade of IFN-γ further increased RANKL expression and IL-17 production in TH-$CD4^+$ T cells. These results indicate that increased leptin in TH mice may act in conjunction with IL-6 to stimulate IL-17 production in $CD4^+$ T cells and induce RANKL-mediated osteoclastogenesis (37). Meanwhile, infiltrating Th17 cells have been identified also in rheumatoid arthritis (RA) and periodontitis, highlighting

the pathological role of the immune system in these inflammatory disorders (38, 39). An interesting connection with 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) on the immunological processes mediated via Th17 cells was shown in patients with RA, thus highlighting the immunomodulating capacity of vitamin D (40).

Mononuclear cells and $CD4^+CD45RO^+$ (memory) and $CD4^+CD45RO^-$ (naive) T cells from treatment-naive patients with early RA were stimulated with anti-CD3/anti-CD28 in the absence or presence of various concentrations of $1,25(OH)_2D_3$, dexamethasone, and a combination of both. The presence of $1,25(OH)_2D_3$ reduced IL-17A and IFN-γ levels and increased IL-4 levels. In addition, $1,25(OH)_2D_3$ had favorable effects on TNF-α : IL-4 and IL-17A : IL-4 ratios and prevented the unfavorable effects of dexamethasone on these ratios. Enhanced percentages of IL-17A- and IL-22-expressing $CD4^+$ T cells and IL-17A-expressing memory T cells were observed in mononuclear cells from treatment-naive patients with early RA, as compared with healthy controls. $1,25(OH)_2D_3$, in contrast to dexametasone, directly modulated human Th17 polarization, accompanied by suppression of IL-17A, IL-17F, TNF-α, and IL-22 production by memory T cells (40).

Inflammatory bowel diseases alter bone metabolism and are frequently associated with osteopenia, osteoporosis, and increased risk of fractures. Although several mechanisms may contribute to skeletal abnormalities in patients with inflammatory bowel diseases, inflammation and inflammatory mediators such as TNF, IL-1β, and IL-6 may be the most critical (41). In addition to limited nutrient absorption, high antigen load from food allergies or intestinal microbial overgrowth may also contribute to bone loss. Mature osteoclasts gain access to bone surfaces only after mononucleated preosteoclasts have travelled from the circulatory system to the bone, possibly through mechanisms involving transendothelial migration (42). The gut-associated lymphoid tissue normally provides an immunological barrier against disease. When this barrier becomes compromised by endothelial hyperpermeability secondary to chronic inflammation, food allergy or bacterial overgrowth, nutrient absorption is reduced, and a loss of oral tolerance can initiate a gastrointestinal-immunological stress factor to the bone remodeling process (42).

RANKL not only regulates the function of osteoclasts but also dendritic cells (professional antigen-presenting cells). In chronic inflammation, RANKL promotes dendritic cell survival and the expression of proinflammatory cytokines (43). As the gut is overrun by pathogens, professional antigen-presenting cells, through the activation of toll-like receptors and C-type lectin receptors, are no longer able to silence immune activation (44) and release proinflammatory cytokines that activate T cells and reduce Tregs. This antigenic stress leads to a Th1-dominant, cell mediated immune system with increased RANKL, reduced IFN-γ, and a possible uncoupling of bone remodeling (45, 46).

Toll-like receptors: A healthy gut flora maintains a reduced production of gut-related proinflammatory cytokines. Toll-like receptors are transmembrane receptors found on macrophages, dendritic cells, and some epithelial cells. These receptors exhibit an integral role in the maintenance of oral tolerance. They recognize the molecular patterns of bacteria and cause an inflammatory, destructive response to pathologic microbes and a tolerogenic response to commensal bacteria. An exam-

ple of how a disease-related genetic polymorphism can be influenced through the reduction of metabolic stress-inducing factors can be seen in the case of toll-like receptors and IL-1 receptors. In theory, as the cytoplasmic portion of the toll-like receptor is similar to that of the IL-1 receptor antagonist gene, it could be susceptible to an increased diversion or "switch" of cells from the monocyte-macrophage cell line to form osteoclasts. A reduction of antigen load and oxidative stress independent of the cause (e. g. insulin/glucose imbalance, toxicity, or gut pathogenic microflora), could reduce proinflammatory cytokine-induced chronic inflammation and T cell activation.

Involution of the thymus gland and the start of bone loss: Reduced oral tolerance may be a factor in the coincidence between thymus gland involution (and subsequent reduction of naive T cells) and the beginning of bone loss in humans in their mid-30s. Although BMD does not usually decrease significantly until menopause, accelerated bone loss can commence at an earlier age for some individuals. Reduced numbers of naive T cells from chronic systemic inflammation or antigen overload from the gut leads to oligoclonal T cell expansion and increased T cell senescence (47). Senescence reduces a T cell's ability to produce IFN-γ and is a sign of immune aging (48).

The primordial thymus developed as a bud on the immature digestive tract, providing embryological evidence of the uniquely co-dependent and interrelated functions of the thymus gland and the gastrointestinal tract (49). As an infant grows, the function of the thymus is to relieve the gut of its primordial function of lymphopoisis (49). With involution of the thymus, the adult gastrointestinal tract remains the source of the least 75 percent of the body's immune cells (50). Therefore, it is in the gut that an adult's immune health is maintained or lost. As an individual ages, antigen load often increases and oral tolerance decreases, leading to reduced levels of IL-2 (necessary for the T cell proliferation and differentiation into activated effector cells) and IFN-γ, and ultimately to the greater cache of RANKL-expressing (and thus osteoclast-activating) memory cells harbored in the bone marrow.

7.12 B Cells and their Influence on Bone Cells

In comparison to T cells the link between B cells and osteology has been sparsely investigated so far, but B cells also seem to play a crucial role in the regulation of bone turnover. The source of OPG has in general been attributed to osteoblasts, but it could be demonstrated that B-lymphoid lineage cells are also a major source of endogenous RANKL in bone marrow and support osteoclast differentiation in vitro (51, 52). In addition, B-lymphoid lineage cells in earlier developmental stages may hold a potential to differentiate into osteoclasts when stimulated with M-CSF and soluble RANKL in vitro. Thus, B-lymphoid lineage cells may participate in osteoclastogenesis in two ways: they 1) express RANKL to support osteoclast differentiation, and 2) function themselves as osteoclast progenitors (52). In a recent study the extent of B cell involvement on OPG production was evaluated. With the use

of immunomagnetic isolation of bone marrow B cells and B cell precursor populations, and quantification of their OPG production by enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcriptase-polymerase chain reaction (RT-PCR), cells of the B lineage were found to be responsible for 64 % of total bone marrow OPG production, with 45 % derived from mature B cells (51). B cell knockout mice were found to be consistently osteoporotic and deficient in bone marrow OPG, phenomena rescued by B cell reconstitution (51). In another recent study by Breuil et al. (53) changes of different B lymphocyte populations, related to bone mineral density (BMD) and fractures, were evaluated in postmenopausal osteoporosis. Postmenopausal women with osteoporosis had lower numbers of $CD19^+$, CD19+/$CD27^+$, $CD19^+/CD27^+/CD5^-/CD38^+$ and $CD19^+/CD27^+/RANK^+$, $CD4^+/CD27^+/CD45RA^-/RANK^+$, and $CD4^+/CD27^+/CD45RA^-/CD28^+$ as compared to healthy controls and were positively correlated to BMD. In addition, in postmenopausal women with osteoporosis $CD4^+$ secreted less IFN-γ and B lymphocytes but more GM-CSF under basal conditions. GM-CSF was positively correlated to fracture rate and negatively to BMD. These results suggest a possible role of IFN-γ in the pathophysiology of osteoporosis based on changes in B-lymphocyte populations (53).

At the gene expression level an in vivo genome-wide expression study on human B cells in relation to osteoporosis seems to confirm the significant role of B cells in the etiology of osteoporosis (54). Real-time RT-PCR showed differential expression of eight genes, including estrogen receptor 1 (ESR1) and mitogen activated protein kinase 3 (MAPK3). It was assumed that downregulation of ESR1 and MAPK3 in B cells regulates cytokine expression, causing increased osteoclastogenesis or decreased osteoblastogenesis. These results highlight the significance of B cells in the etiology of osteoporosis (54).

7.13 Hormonal Influences on Bone Remodeling

The RANK/RANKL/OPG system is essential for the bone remodeling process and bone homeostasis and is regulated by different factors, which will be presented in the following section. Relevant hormone influences include estrogen, testosterone, parathyroid hormone and thyroid hormone.

7.14 Estrogen

Estrogen has a critical role in the skeletal preservation of both women (55) and men (56). In women estrogen deficiency appears to be the major determinant in the development of osteoporosis, whereas in men it has to be regarded as one factor beside others (e. g. level of testosterone, sex hormone binding globulin). During three to four years after the onset of menopause due to estrogen deficiency a rapid

loss of bone can be observed, in particular trabecular bone. During this early postmenopausal period the estrogen-deprived skeleton exhibits histological features of accelerated bone remodeling with abundant osteoclasts and resorption bays. Markers of bone turnover are frequently elevated during this period reflecting the accelerated remodeling process. The anti-resorptive effects of estrogen on osteoclasts are based on the regulatory effects of estrogen on the OPG/RANK/RANKL system. Estrogen shows direct and indirect effects on the skeleton (Fig. 3). The direct effects of estrogen on bone are mediated via estrogen receptors on osteoblasts and osteoclasts (57–59). Estrogen by itself induces OPG production in osteoblasts. Due to the anti-resorptive effect of OPG in the RANK/RANKL/OPG system, estrogen exerts osteoprotective effects, whereas estrogen deficiency leads to decreased OPG with increased formation and activation of osteoclasts (60).

The indirect effects of estrogen (Fig. 3) on bone are caused by different cells including marrow stromal cells and cells of the immune system. These also have estrogen receptors, but exert influences on bone indirectly by upregulation of OPG due to extrogen exposure. Estrogen induces a downregulation of cytokines which are involved in osteoclast formation, such as IL-1, TNF-α produced by monocytes, IL-6 and GM-CSF produced by stromal cells and osteoblasts. Through this downregulation of cytokines by estrogen the expression of RANKL on bone marrow cells is suppressed, whereas in estrogen deficiency the lack of the suppressive effect on RANKL will lead to accelerated maturation and activation of osteoclasts (61). The upregulation of RANKL in estrogen deficiency is also mediated by T cell activation beside the cytokines IL-1, IL-6, TNF-α. Beyond the downregulation of estrogen on the inflammatory immune response also the upregulation of immunoglobulin production has to be mentioned. Thus, estrogen exerts a dichotomous impact on the immune system, modulating an immune response.

In addition, estrogen acts to maintain the appropriate ratio between bone-forming osteoblasts and bone-resorbing osteoclasts, in part through the induction of osteoclast apoptosis, which is mediated via increased levels of TGF-β. In addition, a direct estrogen effect on the extent of osteoclast apoptosis is also demonstrated. Recent studies have suggested a role for Fas ligand (FasL) in estrogen-induced osteoclast apoptosis by an autocrine mechanism involving only osteoclasts. Recently, a paracrine mechanism has been described in which estrogen affects osteoclast survival through the upregulation of FasL in osteoblasts (but not osteoclasts), leading to the apoptosis of pre-osteoclasts (62). A cell-type-specific hormone-inducible enhancer located 86 kb downstream of the FasL gene has been characterized as the target of estrogen receptor-alpha induction of FasL expression in osteoblasts. In addition, tamoxifen and raloxifene, two selective estrogen receptor modulators that have protective effects in bone, induce apoptosis in pre-osteoclasts by the same osteoblast-dependent mechanism. These results demonstrate that estrogen protects bone by inducing a paracrine signal originating in osteoblasts leading to the death of pre-osteoclasts and offer an important new target for the prevention and treatment of osteoporosis (62).

Furthermore, estrogen has extraskeletal effects with influence on calcium homeostasis (Fig. 3). In estrogen deficiency an increased renal calcium excretion

and decreased intestinal calcium absorption can be observed (63–65). This leads to a negative calcium balance which needs to be compensated by mobilization of calcium deposited in bone by upregulation of PTH. In addition, bone sensitivity to PTH exposure is increased in estrogen deficiency (66). Beside the compensatory upregulation of PTH in order to maintain calcium homeostasis, estrogen also seems to have a direct PTH suppressive effect (67). Estrogen also exerts an influence on vitamin D metabolism, as estrogen therapy could increase not only intestinal calcium absorption but also serum vitamin D levels in postmenopausal women (68).

7.15 Testosterone

Androgens are C-19 steroids secreted from the testes in men and the adrenals in both men and women. Testosterone (T) is the most important androgen in men. About 95 % of the amount of testosterone is secreted by the testis. In the adrenal cortex weakly active androgens are produced such as dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEA-S), and androstenedione. T acts via the androgen receptor (AR) after conversion by the enzyme 5αreductase to the more potent 5α-dihydrotestosterone (DHT) in peripheral tissues. Furthermore, T can also interact with the estrogen receptor α (ERα) and β (ERβ) after being converted into 17β-estradiol (E2) by the P450 aromatase enzyme. In relation to the relative expression of P450 aromatase and 5α-reductase, androgens may preferentially activate the AR or the ERs. AR and ERs are both expressed in bone tissue, thus local metabolism of androgens may have a significant relevance in bone metabolism.

7.16 Parathyroid Hormone

Estrogen deficiency induces elevated IL-6 levels which cause increases of PTH. Estrogen deficiency is also associated with decreased intestinal calcium absorption and an increased renal calcium excretion due to reduced levels of $1,25(OH)_2$ D3, the most potent vitamin D metabolite. The sensitivity of PTH towards bone is also increased with estrogen deficiency. Finally, with increasing age vitamin D deficiency occurs in most individuals due to impaired skin, gastrointestinal and renal function in connection with the vitamin D metabolism. Vitamin D deficiency gives further rise to increased PTH levels.

7.17 Thyroid Hormone

The hypothalamic-pituitary-thyroid axis plays a key role in skeletal development, acquisition of peak bone mass and regulation of adult bone turnover. An euthy-

roid status is essential for maintenance of optimal bone mineralization and strength. In population studies, hypothyroidism and hyperthyroidism have both been associated with an increased risk of fracture. Furthermore, recent studies in healthy euthyroid post-menopausal women indicate that thyroid status in the upper normal range is also associated with low bone mineral density and an increased risk of non-vertebral fracture. Studies in mutant mice have demonstrated that thyroid hormone receptor α is the major mediator of T3 action in bone and that thyroid hormones exert anabolic actions during growth but have catabolic effects on the adult skeleton. Nevertheless, TSH has also been proposed to be a direct negative regulator of bone turnover, although the relative importance of T3 and TSH actions in the skeleton has yet to be clarified.

7.18 Activation of the Immune System in Osteoporosis

However, beside the endocrine effects on osteoblasts and osteoclasts, further influences on bone were discovered, which led to a broader understanding of the development of osteoporosis. In this respect osteoimmunology could demonstrate that activated lymphocytes also contribute to imbalances in bone remodeling leading to osteoporosis. This perspective changes the picture of osteoporosis as a solely endocrine disorder mainly due to estrogen deficiency, but also to a disease caused by inflammatory processes (69). Furthermore, bone cells, influenced by immune cells themselves, thereafter produce a changed spectrum of cytokines with effects on the immune system. Therefore, the exchange between the immune system and bone cells is of complex nature and bidirectional (70). The communication between the immune system and bone has to be divided into interactions between immune cells and osteoclasts on the one hand and osteblasts on the other.

With reduced estrogen levels and/or chronic or recurrent immune activation from either systemic or gastrointestinal origin, there may be a reduction in the body's natural ability to limit the production of RANKL. Associated with estrogen deficiency there is a progressive proinflammatory status with increased production of IL-1, IL-6, and TNF-α in postmenopausal women. This proinflammatory status is also seen with aging in general and is thus also entitled as inflammaging (71). Special subsets of T cells seem to be involved in this process (e. g. $CD8^+$ $CD57^+$ subsets), which produce TNF-α and increase in women with osteoporotic hip fractures (72). Estrogen deficiency also causes increased IL-7 production, which induces T cell activation. Associated to this T cell activation there is also an increased production of interferon (IFN)-γ and TNF-α by T cells (73, 74). IFN-γ upregulates major histocompatibility complex (MHC) class II molecules on antigen presenting cells (e. g. bone marrow macrophages and dentritic cells), which causes further T cell activation. Interestingly, although T cell production of IFN-γ is higher in elderly women as compared to young women, in elderly men no increase of IFN-γ production could be seen (75). Such gender-specific differences may be part of the explanation why osteoporosis is more frequent in women than men and also exerts rapid

bone loss during early postmenopause. These gender differences also underline the pathogenic influence of proinflammatory processes in the pathogenesis of osteoporosis.

Activated T cells produce RANKL, thus being able to target RANK on osteoclast progenitors, and TNF-α, resulting in increased osteoclast activation through a "switch-like" diversion of osteoprogenitor-cell differentiation away from monocyte-macrophage-cell development towards osteoclastogenesis. In this respect osteoclastic activity, induced by proinflammatory cytokines and activated T cell-induced RANKL, is thought to be modulated by the action of IFN-γ on tumor necrosis factor receptor-associated factor 6 (TRAF-6). TRAF-6 is a RANK adaptor protein that mediates NFkB activation. However, the role of IFN-γ on bone metabolism seems to be variable as the modulating capacity of IFNγ on RANKL is influenced by both vitamin D and estrogen. Under normal conditions without estrogen deficiency IFN-γ seems to be anti-osteoclastogenic (76), whereas in the context of estrogen deficiency it has a pro-osteoclastogenic effect (77).

7.19 Oxidative Stress in Aging

Aging leads not only to a reduction in sex-hormone production, but also to an increase in the general level of proinflammatory cytokines and diminution of the immune system function. This also seems to be mediated, at least in part, by the accumulation of reactive oxygen species (ROS). In ovariectomized mice, estrogen deficiency led to an accumulation of ROS within the bone marrow causing a proinflammatory status with increased levels of TNF-α produced by activated T cells. This upregulation was mediated via the co-stimulatory molecule CD80 on dentritic cells (78). In vivo, free radicals have been shown to increase bone resorption, and oxidative stress reduces BMD in humans. These environmental and/or age-related catabolic stress-inducing factors contribute to normal bone loss. But when there is a chronic, elevated antigenic load or excessive oxidative stress, which increases proinflammatory cytokine-induced RANKL, the activation of this "switch" in osteoprogenitor-cell differentiation may, independent of age, adversely affect the balance of bone remodeling. It is in this abnormal state that chronic immune activation may alter IFN-γ modulating capacity. When estrogen is deficient, causing RANKL levels to increase, the body's natural ability to limit the transcription factors TRAF-6 and NFkB may be reduced and IFN-γ may exert a pro-osteoclastogenic effect. This uncoupling of the remodeling process results in bone loss. In studies using mice, chronic antigenic load with T cell activation and production of ROS must be present for the low estrogen levels to cause bone loss. It appears that reducing antigenic load and oxidative stress may be equally important as estrogen in maintaining bone health.

7.20 Oral Tolerance

Oral tolerance and bone health: Oral tolerance, the muted immunological response to harmless gut antigens, depends on the presence of commensal microorganisms and an intact healhty gut wall. Epithelial cell integrity is maintained by the presence of beneficial organisms such as *Lactobacillus* and Bifidobacteria that do not elict an inflammatory response. When the normal gastrointestinal flora is maintained, immunological self-tolerance through the activation of T-regulatory cells (Treg) favors a non-inflammatory Th2 dominant response to gut microbes. Pathological bacterial or fungal overgrowth causes inflammation and increased gut permeability that reduces oral tolerance. Focus on the traditional osteo-endocrine explanation for bone homeostasis fails to acknowledge the important role of the immune system in remodeling and the possible role of oral tolerance in maintaining bone health. It is now understood that a high systemic antigen load of bacterial or viral origin and/or a loss of oral tolerance due to pathologic microbial overgrowth (long suspected as major contributing factors in other chronic degenerative diseases) may also contribute to the pathogenesis of bone loss.

Estrogen normally helps to preserve bone by enhancing macrophage production of transforming growth factor ß (TGF^{-}) and limiting $CD4^{+}$ T cell activation. Reduced levels of estrogen result in an increase in antigen-presenting cells and a reduction in TGF-ß and Treg. This leads to T cell activation and production of proinflammatory cytokines and RANKL, which stimulates osteoclastogenesis. By improving gut health and oral tolerance, antigen presentation to T cells is reduced, TGF-ß production is maintained, Tregs are enhanced, and RANKL-induced osteoclastogenesis is limited, even with reduced levels of estrogen.

7.21 Diagnosis of Osteoporosis and Aspects of Osteoimmunology in Clinical Medicine

Diagnosis of osteoporosis is initially based on the evaluation of medical history, including assessment of risk factors for osteoporosis and physical presentation of the patient. X-rays are the primarily used imaging method to diagnose fractures, although in particular within the central skeleton, including the vertebral column, chest, and pelvis, fractures may be frequently overseen by X-rays. For occult fractures other imaging methods such as computed tomography, magnetic resonance imaging, or bone scintigraphy may add in as diagnostic tools for optimized fracture detection. Imaging methods including X-rays are also of importance in the detection of other bone pathologies such as malignant bone tumors, metastases, hyperostotic bone disorders (e. g. Paget's disease) or degenerative processes. However, osteopenia can be seen by X-ray only after progressive bone loss, thus not allowing early diagnosis of osteoporosis with this diagnostic tool. Osteodensitometry (DXA) allows a quantification of bone loss and measurements are classified as normal, osteopenia

and osteoporosis based on the amount of bone lost as compared to healthy persons with peak bone mass. Laboratory parameters are also used in the diagnostic evaluation of osteoporosis. In clinical routine the aim of laboratory parameters primarily aims at evaluating the function of central organs such as the liver or the kidneys, which also have a part in metabolic pathways associated with bone. The evaluation of endocrine laboratory parameters allows to estimate the function of endocrine axes with influence on bone. Furthermore, relevant pathological conditions such as malignant myeloma, which can also be the cause of osteoporosis, should be clarified by laboratory assessment. In recent years a range of bone formation and degradation markers also found their way to clinical practice giving the clinician an insight into the activity of bone turnover. Other parameters, although of high importance for the regulation of bone metabolism (e. g. OPG, RANKL), specific processes (e. g. Cathepsin K) or signal transduction (e. g. cytokines), are frequently determined only for study purposes and do not play a role in clinical practice. In this light of clinical medicine, diagnostic evaluation of the osteoimmunological interplay remains superficial. Only major determinants such as hormonal deficits (estrogen or testosterone deficiency, hypothyroidism) or hormonal excess (hyperthyroidism, Cushing syndrome) will be potentially measurable.

7.22 Causes of Osteoporosis from a Clinical Perspective – Postmenopausal Osteoporosis and Secondary Osteoporosis

As outlined above, the mechanisms and interactions of immune cells, cytokines and hormonal influences on bone cells are complex and interact with each other in a complex network that leads to a balanced formation and resorption of bone tissue under healthy conditions. From a clinical point of view these interactions on the cellular level will neither be diagnosed nor will they have any impact on the clinical management on a case by case basis. However, the perception that different endocrine disorders or inflammatory processes have an important impact on the pathogenesis of bone loss and its clinical management has to be highlighted. It is the clinicians aim to evaluate, diagnose and set them into context with pathological processes of the bone. In this respect the clinician has to see the patient's problems not in an organ-focused perspective, but rather in a holistic way in order to percept the full range of possible pathological influences in bone disease. In this respect a short survey of relevant clinical disorders and its connections to bone pathophysiology and connections to osteoimmunology will be given. Other causes such as drugs, lifestyle factors, diseases with complex backgrounds of osteoporosis development such as anorexia nervosa or less frequent causes such as organ transplantations are listed in Table 1. However, the table does not provide a full range of all known causes of osteoporosis; for further detailed information on different disorders causing osteoporosis the reader is referred to the literature.

7.23 Osteoporosis and Disorders with Chronic Inflammation – Osteoimmunology as a Link

The assessment of different inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, celiac disease, cystic fibrosis and chronic obstructive pulmonary disease is of relevance when diagnosing osteoporosis, as these inflammatory disorders have been associated to pathological bone resorption. As mentioned, the link between osteoclast, M-CSF and pro-inflammatory cytokines, especially TNF-α and IL-1, explain the association between inflammation and osteoporosis. Other TNF-related cytokines such as RANK, RANKL, and OPG are also important mediators in inflammatory processes and are critically involved in the pathophysiology of bone loss. These diseases are therefore related to osteoporosis and high fracture risk, independently of other risk factors common to inflammatory diseases such as reduced physical activity, poor nutritional status, hypovitaminosis D, decrease in calcium intake and glucocorticoid treatment.

7.24 Endocrine Disorders

Different endocrine axes including the gonads (estrogen, testosterone), thyroid gland (L-thyroxine), and parathyroid gland (parathyroid hormone) play a crucial role in bone metabolism and were described above in their actions on bone. Hormonal deficiencies, such as estrogen deficiency due to menopause or ovariectomy, hypothyroidism or hormonal excesses such as hyperthyroidism, Cushing syndrome, and hyperprolactemia will influence bone homeostasis. Also other endocrine disorders such as diabetes mellitus were reported to be associated with osteoporosis.

7.25 Intestinal Disorders and Liver Diseases

Pathologies of the intestine and the liver currently represent a still underestimated field of causes for osteoporosis (79). A broad range of pathologies including Crohn's disease, colitis ulcerosa, celiac disease, pancreatitis, hepatitis, postgastrectomy may lead to osteoporosis (79). The pathophysiological mechanisms are diverse and include reduced calcium absorption, pathologies of the vitamin D metabolism, poor digestion, malabsorption or reduced production of proteins. In inflammatory bowel disease inflammatory cytokines such as serum IL-1b, TNF-α, IL-6, and IL-1 are increased, promoting negative effects on bone turnover (80). In a recent study (81) fracture risks for different frequent gastrointestinal disorders associated with osteoporosis were evaluated: The odds of fracture (odds ratio, 95 % confidence interval) compared with controls, adjusted for age, gender, and race were: chronic

pancreatitis 2.4 (2.1, 2.9); Crohn's disease 1.7 (1.5, 2.0); gastrectomy 2.5 (1.5, 4.1); cirrhosis 2.6 (2.4, 2.7); and celiac disease 2.7 (2.1, 3.4) (81). These gastrointestinal disorders thus have to be considered as relevant pathological states regarding the development of osteoporosis and should therefore be included into the diagnostic evaluation of osteoporosis.

7.26 Renal Disorders

Renal disease can lead to complex pathologies of bone known as renal osteodystrophy. These pathologies include pathological extraosseous calcifications, osteomalacia and fractures due to osteopenia and osteoporosis. Parathyroid hormone is also frequently increased thus adding aspects of hyperparathyroid induced bone disease.

7.27 Treatment

The therapy of osteoporosis is first of all based in risk reduction and change of lifestyle as far as necessary. This includes cessation of smoking and alcohol intake, continued physical activity, calcium-enriched food and the elimination of other disorders with potential deletious effects on bone (e. g. hyperthyroidism). Furthermore, in particular in the elderly, risks or disorders leading to falls have to be also assessed and eliminated. This means that therapy of osteoporosis can become a very complex venture in order to meet all demands of treatment and in some circumstances therapeutic interventions will be aimed not only at bone but also at other organs (e. g. prescription of glasses in case of falls and impaired vision, implantation of a heart pacemeaker in case of falls due to rhythmological problems), thus not showing a direct connection to treatment of osteoporosis. In case of fractures diverse surgical interventions are nowadays available including fracture stabilisation by ostesynthesis or vertebroplasty and kyphoplasty, in order to keep immobilisation as short as possible and to allow rapid remobilisation of the fractured patients. However, specific drug therapy also plays a key role in the management of osteoporosis. The aim of drug therapy in osteoporosis is to reduce fracture risk. This is achieved by influencing bone metabolism in such a way that bone formation exceeds bone resorption, leading in the long term to a net bone gain and greater stability of bone structure. Common to all drug regimens in osteoporosis therapy is the consequent application of calcium and vitamin D. According to current guidelines 1000 mg of calcium and at least 800 units of vitamin D should be applied. Specific osteoporosis therapies can be divided into therapies influencing bone resorption and bone formation. Therapies such as estrogen, selective estrogen receptor modulators (SERMs), bisphosphonates, calcitonin, and denosumab influence bone metabolism by decreasing osteoclast activity. On the other hand, parathyroid hormone (1–84) and teriparatide (1–34) are potent osteoanabolic agents which have shown to lead

to significant formation of new bone and bone structure, if intermittently applied via subcutaneous route. Strontium-ranelate shows a dual action on bone by both stimulation of osteoblasts and inhibition of osteoclasts, also leading to formation of new bone. All mentioned drugs for osteoporosis have demonstrated fracture reduction for vertebral fractures, some for hip fractures and other skeletal sites in large multicenter studies.

Considering osteoimmunology, questions arise in which way these drugs may influence bone metabolism on the level of signalling, cytokine expression, and the interplay with the immune system.

7.28 Hormone Replacement Therapy

As mentioned above, estrogen limits bone loss through its effects on osteblast and osteoclast activity. Since estrogen deficiency in menopause is a major cause of osteoporosis in women, substitution of the estrogen deficit may postpone the development of osteoporosis. The interactions between the different bone cells and cells of the bone marrow, including stromal cells and cells of the immune system, their interactions and cytokine expressions effected by estrogen and also its immunomudulatory capacity have been outlined above.

7.29 Selective Estrogen Receptor Modulators (SERMs)

The anti-resorptive effect of raloxifene might be mediated by changes in several cytokines involved in the bone remodeling process. Serum OPG levels in postmenopausal women significantly increased after treatment with raloxifen (78). On the contrary, in another study postmenopausal women were treated with 60 mg raloxifen, but serum levels of OPG significantly decreased after three and six months of therapy and returned to basal levels after one year of treatment (82). There was a significant decrease of RANKL levels and OPG/RANKL ratio after one year of raloxifene treatment. According to the authors of the study this might be due to a reduced number of osteoblasts (82). On the whole, current results support the hypothesis that raloxifene may inhibit osteoclast activity, at least by partly modulating the OPG-RANKL system (83). It could also be demonstrated that raloxifene reduced IL-6 (84, 85), TNF-α (85), and TGF-β1 (84) and, as pointed out above, SERMs may also induce apoptosis in pre-osteoclasts (62).

LY117018, a raloxifene analogue, can significantly inhibit the generation of osteoclasts in vitro at concentrations between 10^{-12} M and 10^{-9} M and stimulate osteogenic differentiation at concentrations of 10^{-14} to 10^{-7} M. No influence on the proliferation and transcription of RANKL and osteoprotegerin was observed. TNF-α production was suppressed by LY117018, which may add to its anti-osteoclastogenic effect (86). The effects on estrogen-deficiency osteoporosis of ormeloxi-

fen, another SERM, were studied in retired breeding female rats (87). Ormeloxifen like estradiol demonstrated its inhibition of estrogen-deficiency osteoporosis effects via inhibition of osteoclastogenesis, apoptosis of osteoclasts and up-regulation of TGF beta-3 expression. Raloxifene – though effective in inhibiting osteoclastogenesis – induced no apoptosis at any concentrations. Thus, raloxifen appears to have a different mechanism of action than ormeloxifene and estradiol (87).

7.30 Bisphosphonates

Bisphosphonates are the drugs most widely used in treating osteoporosis. Bisphosphonates act by binding to the hydroxyapatite in bone tissue. This inhibits the activity of mature osteoclasts, induces both reduced osteoclastogenesis and increased apoptosis of osteoclasts. Aside from these known effects of bisphosphonates, the most drug-sensitive steps have not been determined so far. Risedronate inhibited osteoclast differentiation in co-culture of bone marrow cells (BMCs) and osteoblasts and suppressed RANKL-mediated osteoclast differentiation from bone marrow-derived macrophages (BMMs) in a dose-dependent manner without toxicity. Risedronate significantly inhibited expression of c-Fos and nuclear factor of activated T cells (NFAT) c1 induced by RANKL (88). Risedronate significantly reduced the number and degree of differentiation of osteoclast precursors, osteoclast formation, and their vitality and activity after three months (89). Furthermore, the levels of RANKL and TNF were reduced in cultures and of TNF and OPG in serum (89). In a further study on postmenopausal women (90), risedronate significantly decreased serum levels of RANKL and IL-1beta and the level of OPG significantly increased after three and six months, but no significant difference was found in TNF-α level (90). In cases of bone turnover, markers of bone resorption and formation significantly decreased after six months (90). A significant reduction of bone resorption markers was observed after three months of alendronate (91), whereas no significant reduction in the number of osteoclast precursors, osteoclast formation and viability, and cytokine levels was present at that time (91). After one year of alendronate treatment, reduced osteoclast precursors, osteoclast formation, and serum RANKL were present. This supports the fact that bisphosphonates mainly act on mature bone resorbing osteoclasts in the short term, whereas its long-term administration diminishes their formation by reducing their precursors and serum RANKL (88, 89, 91, 92).

Interactions with the immune system were outlined in a recent study (93) with a significant increase in mRNA expression and serum levels of IL-6, TNF-alpha and IFN-gamma shortly after pamidronate infusion. Furthermore, a notable rise in serum C-reactive protein (CRP) was observed over three days. In this respect, an intermittent large dose of aminobisphosphonate has an impact on the immunological level, causing acute inflammation. The acute-phase response (APR) is the most frequent side effect after the first dose of intravenous nitrogen-containing bisphosphonates. It has been demonstrated in vitro that nitrogen-containing bisphospho-

nates stimulate γδ T cell proliferation and production of cytokines and that vitamin D is able to modulate these. Levels of 25(OH)D were negatively correlated with post-dose body temperature and CRP (94). An exponential increase in fever and CRP has been found with 25(OH)D levels lower than 30 ng/mL and body temperature less than 37 degrees C, whereas normal CRP was associated with 25(OH)D levels above 40 ng/mL (94). The association between post-N-BPs APR and 25(OH)D suggests an interplay among nitrogen-containing bisphosphonates, 25(OH)D, and the immune system. These results (93, 94) may explain the acute phase response often seen in patients after receiving nitrogen-containing bisphosphonates for the first time.

7.31 Denosumab

Denosumab (RANKL-specific monoclonal antibody) is a specific monoclonal antibody directed against RANKL. By binding to RANKL, denosumab prevents RANKL from binding to its receptor, resulting in a decrease in bone resorption due to reduction in the formation, activity, and survival of osteoclasts. The antiresorptive effect of a single 60-mg injection of denosumab substantially exceeds the effects of alendronate (70 mg weekly) (95, 96). Denosumab increased BMD at all measured skeletal sites and decreased concentrations of bone turnover markers compared with a placebo at 24 months (96). At the lumbar spine, BMD increases with denosumab in the range of 4.13 % to 8.89 %. BMD changes with denosumab 30 mg every 3 mo and > or = 60 mg every 6 mo were similar to, or in some cases greater than, the use of alendronate (96). The role of denosumab in the treatment of osteoporosis is described in more detail in the chapter "RANKL inhibition: clinical data".

7.32 Parathyroid Hormone

Subcutaneous intermittent therapy with teriparatide (rhPTH 1–34) is an established anabolic therapy in osteoporosis. It could be demonstated that rhPTH 1–34 leads to a rapid and significant increase of RANKL within one month, with a persistent increase during the *following time* of therapy. On the contrary, OPG was suppressed by rhPTH 1–34 after six months. OPG normalized to baseline after discontinuation of the rhPTH 1–34 treatment. Furthermore, the cytokines IL-6, IL-6sR are upregulated by rhPTH 1–34. Thus, rhPTH 1–34 controls bone remodeling primarily through a modulation of the OPG/RANKL/RANK system. Immature cells of the osteoblast lineage are stimulated by rhPTH 1–34, leading to an increase of RANKL, which may bind to its receptor RANK. As OPG is also produced by osteblasts this RANKL-RANK interaction is counteracted by OPG by preventing the binding of RANKL to its RANK receptor. It seems that rhPTh initially stimulates osteoblast maturation and function, which then induces an osteoclast activation combined with a shift in the balance of bone formation and bone resorption in favor of bone

formation. Interestingly, when in a small uncontrolled study rhPTH 1–34 was combined with the bisphosphonate risedronate, OPG levels remained unchanged while RANKL decreased gradually after three and six months of therapy with risedronate and rhPTH 1–34 (92). Thus, when two bone active drugs are given, a further modulation of the OPG/RANKL/RANK system and bone metabolism is induced, which seems to be dissimilar to the effects of the substances when applied singly.

7.33 Strontium-Ranelate

In a study with ovariectomized goats strontium (Sr) was co-administered with calcium to investigate the effects of Sr on cytokine expression and cell activities (70). Serum Sr levels increased 6- and 10-fold in the Ca + 24 mg/kg/day Sr and Ca + 40 mg/kg/day Sr groups, respectively. In bone Sr increased four- and sixfold in these two groups and Sr-Ca co-administration considerably increased bone mineral apposition rate. The expression of IGF-1 and runt-related transcription factor 2 (Runx2) was significantly upregulated within the Ca + 40 mg/kg/day Sr treatment group; TNF-α expression was significantly downregulated in the Ca + 40 mg/kg/day Sr group. This indicates that Sr-Ca co-administration increases osteogenic gene expression and stimulates new bone formation.

Strontium (Sr)-ranelate reduces fracture risk in postmenopausal women with osteoporosis. Sr-ranelate has been proposed as an agonist of the calcium-sensing

Table 1 Overwiew of clinical disorders causing osteoporosis

endocrine disorders	**complex causes of osteoporosis**	**drugs**
hyperthyroilism	chronic hepatitis	glucocorticoids
prolactemia	chronic exocrine pancreatitis	L-thyroxine
estrogene deficiency	malabsorption	aromatase inhibitors
testosterone deficiency	celiac disease	herparine
hyperparathyroidism	lactose intolerance	protone pump inhibitors
growth hormone deficiency	postgastrectomy	SSRIs[2]
diabetes mellitus	anorexia nervosa	anticonvultans
	renal insufficiency	glitazone
inflammatory disorders	mastocytosis	cytostatics
rheumatoid arthritis	multiple myeloma	
Crohn's disease	AIDS[1]	**life style**
colitis ulcerosa	Gaucher disease	inactivity
		alcoholism
rare genetic disorders	**transplantation**	smoking

[1]AIDS – Acquired Immune Deficiency Syndrome
[2] SSRIs – Selective Serotonin Reuptake Inhibitors

receptor (CaSR), thus revealing effects on OPG and RANKL expression and cell replication. In postmenopausal women Sr-ranelate increased mRNA and protein levels of OPG and suppressed those of RANKL. Sr-ranelate also stimulated osteoblast replication and differentiation and increased cell survival under stress. Knocking down CaSR suppressed the Sr-ranelate-induced stimulation of OPG mRNA, the reduction of RANKL mRNA and an increase in replication, indicating the involvement of CaSR in these responses (97).

7.34 Other Therapies Including Upcoming Therapeutic Options

Concerning other therapeutic options fluoride and calcitonin have to be briefly mentioned. Both substances were used in the past for the treatment of osteoporosis. However, because of questionable treatment effects (calcitonin), a narrow therapeutic window with increased bone mass but not bone strength (fluoride), and the lack of trials showing fracture reduction with these drugs, both were in the meantime outpaced by other more potent drugs as presented above. Concerning osteoimmonological aspects, neither fluor nor calcitonin therapy revealed any effects on the concentrations of IGF-1, IGF-2 and TGF-β1 in bone matrix extracts from osteoporotic patients (98). However, it could be shown that calcitonin prevented apoptosis of osteocytes and osteoblasts, thus being the possible mechanism of action for its anti-osteoporotic effects (99).

Today, further new therapies are in the stage of pre- or (already) clinical testing. In comparison to the currently available drugs, which either interact at the hormonal level via hormone receptors (estrogen, SERMs, parathyroid hormone) or by deposition of specific molecules within bone altering bone metabolism (bisphosphonates, Sr-ranelate), these new drugs now aim to interact in a specific way with the players of bone metabolism. One example is the Cathepsin K-inhibitor odanacatib, which has been shown to interfere specifically with the activation of bone resorbing processes in active osteoclasts.

7.35 Conclusion

Bone cells interact at different points with cells of the immune system, including adipocytes. The interactions are modulated by hormonal influences setting up complex mechanisms regulating and influencing bone metabolism. Our understanding of this complex interplay is constantly increasing. The improved understanding of these modes of action already has led to the development of new drugs which specifically interact at certain points of bone metabolism. In this respect the field of osteoimmunology gains a new dimension, as its complex view on all aspects of bone metabolism gives us not only better insights into bone and bone metabolism at the

level of basic research, but also becomes a motor in the development of new therapeutic strategies. With the translational shift of basic research results in osteology and osteoimmunology into clinically applicable treatments, further improvements in the fight of osteoporosis seem to become available.

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Rheumatoid Arthritis and Ankylosing Spondylitis

8

Douglas H. N. White

Introduction

Recent years have seen an extraordinary growth in our knowledge and understanding of the pathogenesis of inflammatory arthritis that has been reflected in the development of new therapies and changing clinical practice. This chapter will review two of the most common forms of inflammatory arthritis, rheumatoid arthritis and ankylosing spondylitis and explore their epidemiology, pathogenesis, clinical features and treatment.

8.1 Rheumatoid Arthritis

Historical Perspective

Rheumatoid arthritis (RA) has been recognised in Europe since the 17th century, with Sydenham publishing the first case report in 1676. The artwork of the Dutch painter, Peter Paul Rubens (1577–1640) is thought by some to show evidence of hand deformities that can occur in RA (Appelboom et al. 1981). Interestingly however, the typical erosive changes of RA have not been found within the European fossil record, yet the characteristic joint damage found with gout, osteoarthritis and ankylosing spondylitis has been well documented (Aceves-Avila et al. 1998). Similar evaluation of skeletal remains from indigenous North Americans has shown these characteristic changes and in these areas, prevalence of RA remains remark-

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ably high at around 5% (Rothschild et al. 1988). This has led to speculation that perhaps RA was brought from the New World back to the Old World by returning explorers. There is however no direct evidence supporting this hypothesis at the current time.

8.1.1 Epidemiology

Rheumatoid arthritis has a worldwide distribution affecting all ethnic groups and although all ages can be affected, the peak incidence is between the 4th and 6th decades with females being affected 2–4 times more commonly than males. The gender ratio becomes less pronounced with increasing age. Prevalence varies considerably but published work suggests that ~0.5–1% of European and North American adults are affected with rates being lower in Southern Europe than Northern Europe and highest in native North America (Alamanos et al. 2006).

Several authors have suggested that RA appears to be becoming less common and less severe and there is evidence that the incidence of extra-articular features is declining (Turesson and Matteson 2009). The change in incidence appears to have begun before the advent of aggressive disease management strategies and remains unexplained.

8.1.2 Pathogenesis

Insights into the pathogenesis of RA have been gained through the study of affected tissues, genetic studies and more modern molecular approaches. Nevertheless, despite the growth in our understanding of the mechanisms underlying RA it is not yet possible to unite the different elements into a comprehensive explanation of the heterogeneous phenotype.

As we shall see, RA has features of both T cell activation with the formation of rheumatoid nodules and also B cell activation with autoantibody production. Indeed, microscopic examination of synovial tissue from inflamed joints shows evidence of a dense but non-specific infiltration of inflammatory cells including neutrophils, B cells, T cells, macrophages and mast cells.

The inflammatory response is coordinated by a complex cytokine network with macrophages being the key secretors of pro-inflammatory cytokines in the inflamed rheumatoid synovium. It is now known that the cellular basis of the inflammatory response changes as the disease progresses with T cells playing an important role in the early stages of disease (Raza et al. 2005).

Inflammation within the synovium results in the formation of a destructive pannus that may lead to the erosion of bone and consequent deformity and functional impairment. Recent work, to be discussed shortly, has suggested that bone oedema on MRI scan can predict future erosion, suggesting that the process of erosion may begin within the bone.

8.1.2.1 Cellular and Molecular Mechanisms

The first clues to the autoimmune nature of RA came from the discovery of a "rheumatoid factor" in the serum of affected patients by Waaler in 1938. Subsequently rediscovered by Rose in 1948, it was to be another nine years until this rheumatoid factor was characterised as an antibody that binds to the Fc portion of immunoglobulin (Franklin et al. 1957). Whilst rheumatoid factor is most commonly IgM directed against the Fc portion of IgG, it can also exist in IgA and IgG subtypes.

A proposed mechanism of action of rheumatoid factor was put forward in 1973 by Zvaifler in which immune complexes were formed that subsequently fixed complement and released chemoattractant factors to recruit neutrophils and other inflammatory cells to the synovium (Zvaifler 1973). Whilst there is considerable evidence to support this hypothesis, one of the key arguments against it is that rheumatoid factors are found in up to 15 % of the healthy older population and in those with other autoimmune diseases, infection and malignancy without joint involvement.

Subsequently, numerous other auto-antibodies have been detected in the sera of patients with rheumatoid arthritis including anti-perinuclear factor and anti-keratin antibodies. Characterisation of these antibodies revealed that they were binding to citrullinated filaggrin (Girbal-Neuhauser et al. 1999), with citrullinated epitopes on fibrinogen and vimentin also acting as targets. Citrullination is a post-translational modification of the amino acid arginine and the process is thought to have a natural role in apoptosis. The modification is carried out by the enzyme peptidyl arginine deaminase (PAD) in the presence of relatively high calcium concentrations. Commercial assays are now available for anti-citrullinated peptide antibodies (ACPA) which are found in 60–70 % of people with RA and rarely in other diseases. These antibodies can be present for up to two decades before symptoms develop (Jørgensen et al. 2008).

The abundance of Th1 cytokines such as IFN-γ and the relative lack of the Th2 cytokines IL-4, IL-5 and IL-12, supported the hypothesis that RA was primarily a Th1 disease. However, in recent years, our understanding has changed with the discovery of a new subclass of regulatory T cell that produces IL-17, the Th17 cell. Production of IL-17 by these cells is driven by IL-23 which shares a common subunit with IL-12. Increased concentrations of IL-17 and IL-23 are found in the sera of patients with RA compared to controls with osteoarthritis supporting their role in the disease. In addition, mice deficient in IL-23 are resistant to developing arthritis in the collagen induced arthritis model. The Th17 cells produce TNF-α, IL-6, IL-17, IL-22 and GM-CSF, cytokines known to be important in the inflammatory response. IL-17 is an important stimulator of further cytokine production including IL-1β, IL-6, IL-23, IL-8, GM-CSF, G-CSF, VEGF and COX-2 thereby amplifying the immune response (Furuzawa-Carballeda et al. 2007; Lundy et al. 2007). IL-17 and Th17 cells are therefore becoming an important area of research and are offering new therapeutic targets.

The role of the activated macrophage in the synovium of affected joints is crucial to the maintenance of chronic inflammation. In addition to interaction with T cells

and fibroblasts, the macrophage is a potent effector cell that produces pro-inflammatory cytokines, expresses toll-like receptors (TLRs) and is involved in antigen processing and presentation. They also have phagocytic ability and are involved in tissue remodelling. Interestingly, the macrophage also responds to estrogen concentrations, such that high concentration as in pregnancy inhibit IL-1 secretion (Cutolo and Lahita 2005). This may be responsible in part for the improvement many women experience during their pregnancies.

The presence of auto-antibodies, together with the formation of germinal centre like structures in the synovium of affected joints and the good therapeutic response to B cell depletion suggests B cell dysfunction is also important in pathogenesis. B cells have several roles in both humoral and innate arms of the immune system including antigen presentation and antibody and cytokine production. The link between humoral and innate responses is evidenced by the expression of TLRs on B cells These receptors can bind to hypomethylated CpG sequences in bacterial or mitochondrial DNA, single stranded RNA or bacterial cell wall components and it has been shown that mitochondrial DNA from apoptotic synovial cells can stimulate potentially auto-reactive B cells through this mechanism (Leadbetter et al. 2002).

Central to the development of autoimmunity is the breakdown of tolerance. Given that the majority of patients develop RA at an age when thymic function has severely declined or ceased, the defect is more likely to be with peripheral tolerance rather than central tolerance. The precipitating event in RA is not yet known but the T cell repertoire in those with RA is altered in a number of respects from those without RA including evidence of early senescence as evidenced by reduced telomere lengths (Colmegna et al. 2008). In addition, the T cell repertoire appears to be reduced by a factor of 10 in those with RA compared to controls without RA (Wagner et al. 1998). Since the proliferation of naive T cells is dependent on antigenic stimulation, over time, this will lead to the development of a peripheral T cell repertoire with an increasing affinity for self. Thus, it is hypothesised that defective thymic selection coupled with peripheral selection over time predisposes the susceptible individual to the development of anto-immunity.

8.1.2.2 Genetics

Concordance rates in monozygotic twins indicate that approximately 50 % of the variation in prevalence of RA is genetic and that 30 % of this is attributable to the HLA-DR locus. Experiments first performed in 1969 identified a region on chromosome 6, now known to code for genes within the major histocompatibility complex (MHC). Further work has mapped the linked region precisely to the third hypervariable region of the HLA-DRβ chain (Nepom 1989). The precise amino acid sequence between positions 70 and 74 appears to be particularly important as variations in the sequence both increase and decrease the risk of ACPA positive RA. The amino acid sequence DERAA appears in ~30 % of healthy controls but in only 15 % of patients with RA and tends to be associated with less erosive disease when present whereas the sequence QKRAA, QRAAA or RRRAA appears to increase the

risk of ACPA positive RA. Thus, it appears that the amino acids in position 70 and 71 modulate the T cell response such that the amino acids arginine (R), glutamine (Q) or leucine (K) increases the risk and alanine (A) or glutamic acid (E) are protective (van der Helm-van Mil 2005). Work continues to establish exactly how these differences confer variable risk. This sequence is common to several HLA-DR alleles including DR*0101, DR*0102, DR*0401, DR*0404, DR*0405, DR*0408, DR*1001 and DR*1402 and has been termed the "shared epitope" by Gregersen et al. (Gregersen et al. 1987). Individuals who are heterozygous for one of these alleles tend to have more severe, erosive disease, and the effect is further enhanced by homozygosity (Weyand et al. 1992).

Further work on the HLA region has found an association between the HLA-DR3 locus and ACPA negative RA (van der Helm-van Mil et al. 2007). The precise mechanism by which genes at this locus influence disease is not known, although it is conceivable that DR3 polymorphisms could predispose to production of an as yet unidentified antibody.

Genetic factors independent of the HLA region have also been identified. The C > T single nucleotide polymorphism at position 1858, causing a mis-sense mutation in the protein tyrosine phosphatase (PTPN22) gene has been linked to ACPA positive RA (Wesoly et al. 2005). The polymorphism has been validated in Canadian, North American and European populations but does not appear to exist in Asians. Protein tyrosine phosphatase exerts a negative feedback regulation in T cell receptor signalling; it binds to the regulatory kinase, Csk and this complex is responsible for the dephosphorylation of the Lck protein at position 394 and its phosphorylation at position 505, thereby terminating the T cell receptor signal. The C>T 1858 polymorphism appears to directly modify the phosphorylase activity or affect the binding of PTPN22 to Csk (Cloutier and Veillette 1999). Interestingly, the polymorphism has also been found in a number of other autoimmune diseases including type I diabetes, Graves' disease, SLE, JIA and vitiligo and appears to be a gain of function mutation that is hypothesised to impair thymic selection of auto-reactive T cells (Bottini et al. 2006). The genetic factors above are neither necessary nor sufficient for the development of RA, however they do indicate that these pathways are important for disease susceptibility in individual patients.

8.1.2.3 Environment

Genetic factors alone are unable to account for the susceptibility to RA and attention has focussed on an environmental trigger for the disease. Pathogens including Epstein Barr virus, Parvovirus B19 and mycobacteria have been investigated, but to date, pathogen-derived antigens have not been discovered and evidence for molecular mimicry is lacking.

The most consistent environmental factor is cigarette smoking where there appears to be a relationship between the number of pack-years of cigarettes consumed and the risk of developing RA, which can be as much as 21-fold above non-smokers (Klareskog et al. 2006, 2007). Importantly, smoking increases the risk for

ACPA positive RA but not ACPA negative RA and the risk is further increased in those who possess the shared epitope. There are two hypotheses that might explain the link between smoking and ACPA positive RA. Firstly, the detection of citrullinated peptide in the alveolar fluid of smokers has led to the hypothesis that smoking induces apoptosis in the lung, generating citrullinated peptides that are then recognised more strongly by those with the shared epitope who then develop RA. Secondly, smoking is known to increase the levels of tetrachlorodibenzo-P-dioxin (TCDD) which has been shown to up-regulate IL1β, IL-6 and IL-8 production through binding to the arylhydrocarbon receptor. Recently, work on the pharmacokinetics of methotrexate, the drug used most commonly to treat RA, has shown that smoking also reduces intracellular methotrexate polyglutamate levels, which may account, in part, for the worse outcome in smokers (Stamp et al. 2009).

Other environmental factors that have received attention include coffee and alcohol consumption, periodontitis, exposure to mineral oils and body mass index however these factors are not as consistent as cigarette smoking and should be interpreted with caution. Additionally, pregnancy does appear to be a risk factor in as many as 12 % of females developing RA do so within 1 year of pregnancy.

8.1.2.4 Mechanisms of Bone Erosion

It has been appreciated for some time that the quality of bone in RA is adversely influenced by the inflammatory response. This is evident as peri-articular osteopenia and well as generalised osteoporosis. The increased risk of fracture is most marked in the spine (RR 2.4) and at the hip (RR 1.8) and this risk is increased substantially by the use of glucocorticoids (van Staa et al. 2006).

In RA, there appears to be a coupling between the processes of inflammation and bone erosion. TNF-α is capable of binding to two receptors, designated TNFR1 or p55 and TNFR2 or p75, the former appears to have greater activity as it is directly coupled to a death domain that can induce apoptosis. Both receptors for TNF-α, but mostly TNFR1, are found on osteoclast precursors, osteoclasts as well as on osteoblasts. These cells are also capable of secreting TNF-α in response to external stimulation.

The osteoclast is the key cell type involved in the destruction of bone within the inflamed rheumatoid joint. Osteoclast differentiation required the cytokines macrophage colony stimulating factor (M-CSF) and the receptor activator of NF-κB (RANKL). M-CSF promotes proliferation and survival of the monocyte lineage through activation of a tyrosine kinase (*cFms*) and RANKL, through binding to its receptor, RANK, leads to activation of the transcriptions factors NFATc1, AP-1 and NF-κB required for osteoclast differentiation. Since RANKL is part of the TNF superfamily and RANK shares many of the TNF-α signalling properties, it is conceivable that the elevated levels of TNF-α found in inflammatory arthritis contribute to the increased osteoclast differentiation (Abu-Amer et al. 2000; Kobayashi et al. 2000). This is strengthened by the finding that the *in vitro* differentiation of osteoclast precursors lacking RANK could be driven by TNF-α and TGF-β (Kim

et al. 2005). It would appear, however, that *in vivo* osteoclast differentiation required IL-1 as TNF-α alone is unable to activate the transcription factor TRAF-6, a necessary condition for the formation of the actin ring structure required for bone resorption (Nakamura et al. 2002). The central role of TNF-α in the regulation of bone metabolism is presented in Fig. 1.

In RA, the source of RANKL is mainly from stromal cells including fibroblasts and osteoblasts, stimulated in turn, by the action of the inflammatory cytokines, IL-1, IL-6, TNF-α and IL-17. In addition, RANKL is produced by CD4+ regulatory T cells recruited to the inflamed synovium. The effects of these cytokines on osteo-

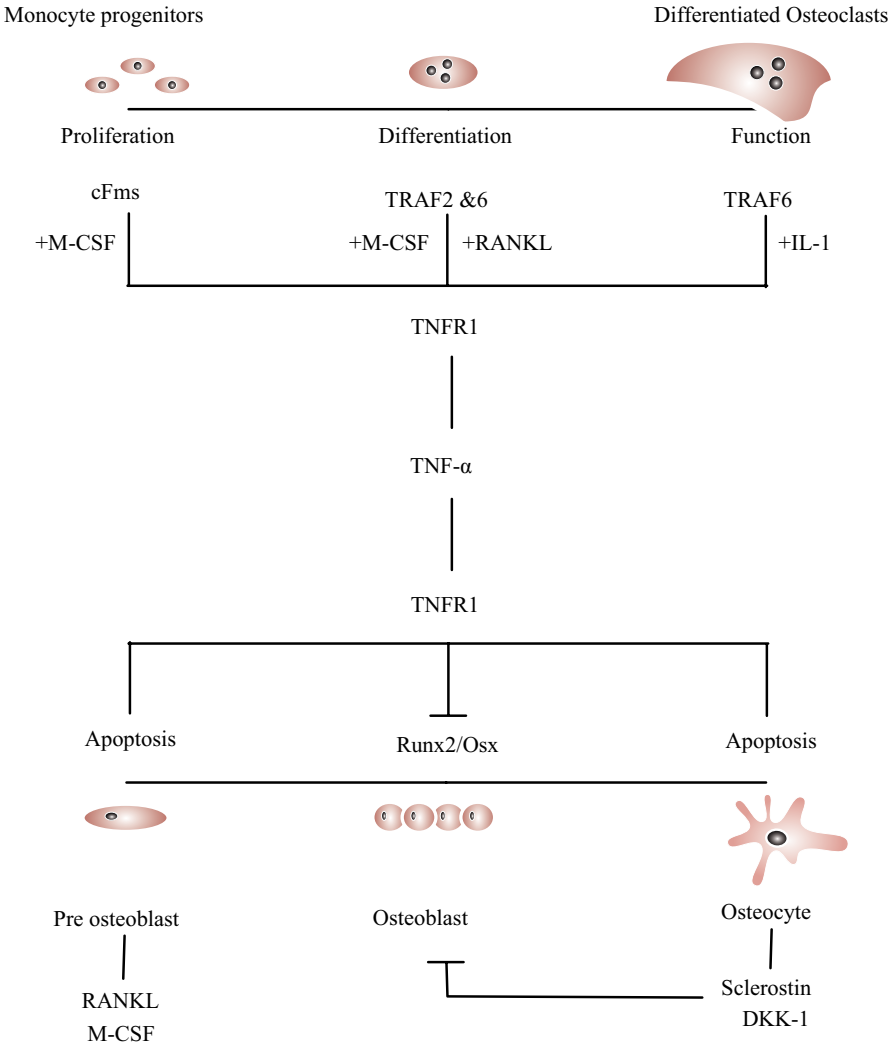

Fig. 1 The central role of TNF-α in bone metabolism. Legend: Adapted from David and Schett (2010)

clast progenitors is amplified by up-regulation of RANK through the direct action if IL-1β and TNF-α on the progenitor cells.

The process of erosion results as a consequence of both increased bone resorption as well as reduced bone healing. This suggests alterations in osteoblast function and indeed, TNF-α is a potent inhibitor of osteoblastogenesis through inhibition of the transcription factors Runx2 and Osterix (Lu et al. 2006). Additionally, TNF-α inhibits Wnt signalling, a major pathway regulating osteoblast differentiation (Baron et al. 2006). Members of the Wnt family of cytokines bind to complex membrane bound receptors incorporating the Frizzled protein and LRP5 and 6. TNF-α is able to interfere with this process at multiple levels by inducing secretable Frizzled related proteins and by production of Dikkopf-1 and sclerostin that interfere with binding of LRP5 and 6 to the Frizzled receptor (Diarra et al. 2007).

In summary, the effects of systemic inflammation are to enhance bone resorption through osteoclast differentiation and activation and to impair bone healing by inhibition of osteoblast differentiation. Our increased understanding of the molecular mechanisms of bone erosion and repair has opened up novel therapeutic targets to prevent bone erosion in RA. The results of a 12-month study of denosumab, a monoclonal antibody directed against RANKL in RA have shown reduced evidence of erosive damage on MRI scan as early as 6 months after treatment (Cohen et al. 2008). Consistent with its mechanism of action, this antibody has no effect on measures of disease activity in RA.

8.1.3 Diagnosis and Presentation

Unfortunately, there is no single diagnostic test for RA and the diagnosis therefore requires an appropriate constellation of clinical findings, blood investigations and imaging. A collaboration between the American College of Rheumatology (ACR) and EULAR recently released the 2010 classification criteria for RA (Table 1). These replace the 1987 criteria which had a poor sensitivity in early disease.

Presentation may be preceded by a period of non-specific malaise with widespread aches and pains and fatigue. Symptoms can evolve slowly or start suddenly and can affect all joints from the beginning or spread to affect different joints as disease progresses. Classically, the patient will describe symptoms with an "inflammatory rhythm" where their symptoms are worst early in the mornings or overnight and improve during the course of the day.

Disease is not confined solely to the joints and there are well described extra-articular features (Table 2). The incidence of cardiovascular disease and lymphoma is increased in RA, indeed, the major cause of premature mortality in RA is cardiovascular disease. The incidence of extra-articular features appears to be falling, possibly as a consequence of earlier diagnosis and more aggressive initial management however the decline appears to have begun before these management changes were well established.

Table 1 The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA

	Criterion	Score
A	Joint Involvement	
	1 large joint	0
	2–10 large joints	1
	1–3 small joints (with or without large joint involvement)	2
	4–10 small joints (with or without large joint involvement)	3
	>10 joints (including at least 1 small joint)	5
B	Serology	
	Negative RF and negative ACPA	0
	Low-positive RF or low-positive ACPA	2
	High positive RF or high positive ACPA	3
C	Acute phase reactants	
	Normal CRP and ESR	0
	Abnormal CRP or abnormal ESR	1
D	Duration of symptoms	
	< 6weeks	0
	> 6weeks	1

The new criteria should not be applied if the symptoms are explained by another disease, joint involvement includes either tenderness or swelling and can include imaging assessment. RA is defined as a score of > 6/10

Table 2 Extra-articular Manifestations of RA

Organ System	Extra-articular Manifestation
Eye	Scleritis, episcleritis and scleromalacia perforans, keratoconjunctivits sicca
Skin	Rheumatoid nodules, vasculitis, pyoderma gangrenosum, panniculitis
Lung	Pulmonary fibrosis, rheumatoid nodules, pleural effusion
Haematological	Anaemia, Felty syndrome
Nervous system	Vasculitis, peripheral neuropathy, atlanto-axial subluxation
Kidney	Amyloidosis
Cardiovascular	Accelerated atherosclerosis, pericarditis

8.1.4 Treatment

The management of RA requires attention to both pharmacologic and non-pharmacologic aspects and is best undertaken as part of an inter-disciplinary team, drawing upon the expertise of the nurse specialist, physiotherapist, occupational therapist, orthotist and podiatrist as necessary.

As mentioned above, the course of RA is highly variable and to date, prognostic models have not been particularly useful in predicting those who will develop severe disease. Nevertheless, the following poor prognostic factors have been identified: rheumatoid factor (RF) positivity, the presence of anti-CCP antibodies, smoking, HLA-DRB genotype, low socioeconomic status and bone oedema on MRI (Manfredsdottir et al. 2006; Kaltenhauser et al. 2007; Harrison et al. 2005; Hetland et al. 2009; Sanmarti et al. 2007). In those with aggressive disease, damage to articular structures occurs early in the disease process: erosions were detected in 12.8 % of patients after a median of 8 weeks in one study (Machold et al. 2002). This provides the impetus for early aggressive treatment.

Pharmacological management of RA can be thought of in terms of agents to relieve symptoms and agents to suppress the underlying disease. Many patients find benefit from the use of non-steroidal anti-inflammatory drugs which can be helpful for relief of pain and stiffness. They are however associated with potential side-effects of gastric irritation, nephrotoxicity and increased cardiovascular risk making their long term use undesirable. Similarly, simple pain-killers such a paracetamol alone or in combination with weak opioids can be useful for some patients. It is important to remind the patient that although these medications can help some of their symptoms they have no effect on the progression of erosive disease, and this is the quality shared by the disease modifying anti-rheumatic drugs (DMARDs) discussed next.

Over the past 15 years, the armament of DMARDs has grown with the development of biological therapies that target specific molecules and it is now necessary to think about these drugs as being split into two classes: conventional small molecules and biological therapy (Table 3).

As a consequence of our growing understanding of disease pathogenesis and our enlarging therapeutic armamentarium, the treatment paradigm for RA has also changed over recent years. The traditional approach of beginning with simple pain killers and then cautiously introducing and up titrating the dose of a single disease modifying drug has been replaced by a more intensive use of DMARD therapy from early in disease. Treatment frequently has to be tailored according to patient wishes, previous drug reactions or allergies, interactions with other medications, or contraindication because of existing comorbidity.

Intervention with early combination DMARD therapy has been shown to have beneficial effects on disease progression independent of treatment in later years, suggesting that there is a "window of opportunity" in which the disease process can be altered (Boers et al. 1997; Möttönen et al. 1999). In addition, it has been calculated that the risk of under-treatment is 5–6 times the risk of overtreatment if all patients are treated aggressively from the outset, providing justification for an aggressive approach in early RA and this is now the norm in most centres (de Vries-Bouwstra et al. 2006).

Combination DMARD therapy was pioneered by James O'Dell using combinations of methotrexate, sulphasalazine and hydroxychloroquine (O'Dell et al. 2002). Other combinations have been trialled and apart from the combination of methotrexate and azathioprine, which is not associated with increased efficacy, this approach appears safe and effective in the management of RA. Recommendations from the European League Against Rheumatism (EULAR) have advised that the efficacy of

Table 3 Disease modifying anti-rheumatic drugs currently available for the management of RA

	Typical Dosing	Proposed Mechanism of Action	Common Side-Effects
Conventional DMARDs			
Methotrexate (MTX)	o, s/c max 30 mg/ week	Inhibition of folate metabolism Inhibition of Adenosine release	Myelotoxicity, hepatotoxicity
Sulphasalazine (SSZ)	o, 2000–3000 mg daily	Uncertain	Myelotoxicity, hepatotoxicity
Hydroxychloroquine (HCQ)	o, 200–400 mg daily	Alteration of lysosomal pH	Ocular toxicity
Azathioprine (AZA)	o, 150–200 mg daily	Anti-metabolite	Myelotoxicity, hepatotoxicity
Gold (IMG)	im, 50 mg 3–4 weeks	Uncertain	Myelotoxicity, nephrotoxicity
Cyclosporine A (CSA)	o, 200–400 mg daily	Inhibition of IL-2 signal transduction	Nephrotoxicity
Leflunomide (LEF)	o, 10–20 mg daily	Inhibition of pyrimidine synthesis	Myelotoxicity, hepatotoxicity, Gastrointestinal upset
Biological DMARDs			
Etanercept	s/c, 50 mg weekly	Soluble TNF receptor	Infection TB reactivation, drug-induced lupus
Adalimumab	s/c, 40 mg every 2 weeks	Humanised anti-TNF antibody	
Infliximab	iv, 3 mg/kg 0,2,6 then 8-weekly	Chimeric anti-TNF antibody	
Golimumab	s/c 50 mg once monthly	Fully human anti-TNF antibody	
Rituximab	iv, 1000 mg on day 0 and 14 then 6-monthly	B cell depletion, targets CD-20	Infusion reactions
Anakinra	s/c, 100 mg daily	IL-1 receptor antagonist	Injection site reactions
Abatacept	iv, weeks 0,2,4 then 4 weekly	CTLA4-Ig, blocks co-stimulation	Headache, nausea, infection
Tocilizumab (TCZ)	iv, 8 mg/kg infused monthly	Monoclonal antibody against IL-6	Heptotoxicity, neutropenia, abnormal lipid profiles

o – oral, s/c – subcutaneous, im – intramuscular, iv – intravenous

methotrexate and leflunomide appears superior to other conventional DMARDs, but this combination may also be associated with increased hepatotoxicity (Gaujoux-Viala et al. 2010).

The evolution of biological therapies has provided insight into the pathogenesis of RA as well as opening a new era in its treatment. There are numerous agents available, targeting TNF-α, IL-1, IL-6, CTLA-4 and CD-20 with new targets under investigation including the phase III trials of small molecule inhibitors of the JAK kinase signalling pathway.

Peculiar to the inhibitors of TNF-α, is the propensity for TB reactivation that arises because of the pivotal role of TNF-α in the formation of palisading gramulomas around the tuberculous bacilli. This necessitates careful screening before treatment, usually with a combination of chest radiograph, Mantoux and interferon release assay. Rates of infection with other agents appears to be increased, particularly in the first 12 months. Most authorities would suggest the use of anti-TNF therapy as initial biological treatment unless contraindicated as a consequence of recent malignancy, active sepsis or hypersensitivity.

These agents are costly but efficacious and their efficacy is enhanced by using them in combination with methotrexate or leflunomide (Nam et al. 2010). Recent evidence suggests that the addition of a biologic agent to those patients who have had an inadequate response to methotrexate at 3 months is superior to combination standard DMARD therapy. This may be through synergistic action or because the conventional DMARD prevents formation of neutralising antibodies.

Whichever regime is chosen by the treating clinician, the aim should be to adjust treatment, within the bounds of tolerability to the patient, in order to achieve the best possible disease control. The impact of disease in RA can be assessed by looking at the three domains of disease activity, disability and structural damage. There are a number of well-validated tools in routine use to facilitate this process including the DAS, DAS28, CDAI, SDAI (Table 4) and ACR response criteria for disease activity, the Health Assessment Questionnaire (HAQ) and SF36 for disability and the Sharp-van der Heidje method for assessing joint space narrowing and erosion on plain radiograph. Full details of these measures is beyond the scope of this text, however, important targets are presented in Table 4.

Table 4 Measures of Disease Activity in RA

	Formula	Moderate Activity	Low Activity	Remission
DAS	$0.54 \cdot \sqrt{RAI} + 0.065 \cdot SJC + 0.33 \cdot \ln(ESR) + 0.0072 \cdot VAS$	3.7	2.4	1.6
DAS28	$0.56 \cdot \sqrt{TJC} + 0.039 \cdot SJC + 0.72 \cdot \ln(ESR) + 0.0013 \cdot VAS$	5.1	3.2	2.6
SDAI	$TJC + SJC + VAS + PhysicianVAS + CRP$	26	11	3.3
CDAI	$TJC + SJC + VAS + PhysicianVAS$	22	10	2.8

Variations on the DAS and DAS28 formulae are available for use with the CRP and without the VAS component. For the SDAI, CRP should be measured in mg/l. DAS – disease activity score, SDAI, simplified disease activity index, CDAI – clinical disease activity index, TJC – tender joint count, SJC – swollen joint count, RAI – Ritchie articular index, ESR – erythrocyte sedimentation rate, CRP – C reactive protein, VAS – visual analogue scale

The advent of ultrasonography and magnetic resonance imaging has shown us that although, synovitis may be undetectable on clinical examination, disease activity can still be detected on these modalities. Ultrasonography in particular, is being used routinely in many centres to assist with diagnosis and monitoring as well as to guide procedures such as joint injection.

8.1.5 Conclusion

Our growing understanding of RA suggests that this heterogeneous disease is most probably the result of numerous perturbations in the immune system presenting with a similar phenotype. Although our understanding of the pathogenesis of this complex disease is incomplete, the basic science research is being translated into clinical practice with the development of more effective biologic therapies. Additionally, the evolution of imaging modalities is enabling the clinician to monitor disease activity more closely than ever before. This progress has resulted in improved patient outcomes as a result of earlier diagnosis and more intensive management from an early stage of the disease.

8.2 Ankylosing Spondylitis

Ankylosing Spondylitis (AS) is the prototype of the wider group of conditions that we now call spondylarthroarthritis. Together with reactive arthritis, enteropathic arthritis, psoriatic arthritis, the enthesitis-related subtype of juvenile idiopathic arthritis and undifferentiated spondyloarthropathy, these conditions share common clinical features and pathogenesis that shall be described in the following paragraphs.

8.2.1 Epidemiology

Several studies in different populations have found an annual incidence of 7 cases per 100,000 population and that this appears to have remained constant over the last 55 years, with males affected approximately three times more commonly than females (Gabriel and Michaud 2009). Although the mean age of onset is 24–26 years, approximately 15 % experience disease onset in childhood; onset after age 45 is rare.

The incidence of AS follows closely that of the HLA-B27 allele around the world, and as such, is highest in Native North Americans and lowest in Australian Aborigines and Africans (Lau et al. 1998). In white European populations, the frequency of the HLA-B27 gene ranges from 26 % in the Lapps of northern Norway to 4 % in Southern Europeans.

8.2.2 Pathogenesis

It has been clear for many years that like RA, AS is influenced by genetic, environmental and immunologic factors. Despite huge progress into our understanding of these areas, it is not yet possible to link them into a unified theory of pathogenesis.

8.2.2.1 Pathology and Cellular Mechanisms

The pathology of AS differs from RA, in two key factors. Firstly, in AS, the enthesis is the site of major histologic changes which progress in an orderly sequence, beginning with a destructive enthesopathy followed by a healing process with new bone formation linking deeper bone to the ligament and ultimately, resulting in bony ankylosis (Ball 1971). Vertebral changes typically begin with an erosive lesion at the anterior corner of the annulus fibrosus. Secondly, the healing process results in increased bone formation which is laid down initially as cancellous bone which is then remodelled into mature lamellar bone creating the typical syndesmophytes that are seen on plain radiography of the spine.

The pattern of joint involvement is different from that seen in RA with sacroiliitis, presenting as buttock pain, being a hall-mark clinical feature of AS and other spondyloarthritides. Changes seen on plain radiology of these joints are most prominent towards the inferior aspects of the joints. Once disease has been longstanding, there is encasement of the joints as a result of ossification of the capsule, often surrounding small islands of intact articular cartilage. It is important to bear in mind that even in the population without AS, there can be progressive fusion of the sacroiliac joint as a result of osteoarthritis; changes are more marked on the iliac side and towards the superior aspect of the joints in osteoarthritis.

It has only recently become possible to obtain histological specimens from the hip, zygoapophyseal and sacroiliac joints of patients with active disease and investigators have found infiltrates of T cells, B cells, macrophages and osteoclasts as well as cells involved in angiogenesis. Synovitis is less common in AS than in RA and can be distinguished by the greater proportion of M2 regulatory CD163+ macrophages in AS. Expression of TNF-α and TGF-β mRNA is increased and recent advances in treatment with therapies blocking TNF-α have shown that this cytokine is responsible for the pain, fatigue, swelling and stiffness (François et al. 2006). Nevertheless, neither the cell stimulating TNF-α production, nor its target cell have yet been identified. Since a proportion of patients fail to achieve a complete remission with anti-TNF-α therapy, it is clear that there are still questions to be answered in this area.

Additionally, the target antigen driving the immune response has yet to be identified however, a T cell response against a proteoglycan link protein has been demonstrated in humans with AS (Mikecz et al. 1988), In the Balb/c mouse, a clinical picture similar to AS can be induced by immunization with fetal human cartilage creating a humoral and cellular response against aggrecan (Glant et al. 1987).

8.2.2.2 Genetic and Immunologic Factors

It has long been appreciated that AS is a heritable disease with genetic factors being responsible for ~ 90 % of the susceptibility. Insight into the genetic basis of AS began with the recognition of familial clustering of cases in the 1950s and 60s. Additional evidence has come from twin studies; if a condition were entirely genetically determined, one would anticipate 100 % concordance between monozygotic twins however, for AS, only 63 % concordance is seen suggesting involvement of non-genetic factors to be discussed shortly (Jarvinen 1995).

The discovery of the association if HLA-B27 with AS was made contemporaneously by two groups (Schlosstein et al. 1973; Brewerton et al. 1973). Development of the B27 transgenic rat in the 1990s confirmed the direct involvement of this molecule in the disease process (Taurog and Hammer 1995). To date, approximately 60 different subtypes of HLA-B27 have been identified that have most probably evolved from the B*2705 allele. The common alleles, B*2702, B*2704, B*2705, B*2707 are associated with axial disease. Interestingly, in the Sardinian population, B*2709 and, in the Southeast Asian population, B*2706 are much less common in those with AS and axial disease (Khan 2000).

There are currently three hypotheses explaining the possible role of HLA-B27 in the pathogenesis of AS. Firstly, it is known that the HLA-B27 molecule does not behave like other HLA class I molecules in that it is capable of homo-dimerising in the absence of β2-microglobulin – a process termed misfolding. It is postulated that this stimulates NK (natural killer) and T cells through interaction with cell-surface receptors in the leukocyte immunoglobulin like receptor (LILR) family (Kollnberger et al. 2004). Secondly, the unfolded protein response hypothesis holds that a reduced rate of folding of the HLA-B27 molecule in the endoplasmic reticulum triggers an intracellular signalling response that may in turn lead to IL-23 release. Evidence supporting this unfolded protein response has been found in synovial biopsies from patients with AS and it is known that the ERAP1 gene product is important in processing the antigenic peptide required during the folding process (Dong et al. 2008). Finally, the molecular mimicry hypothesis suggests that HLA-B27 preferentially binds to a self antigen that resembles a microbial peptide. This is supported by the presence of T cells that recognise self antigens in vivo (Atagunduz et al. 2005).

The finding that the risk of developing AS was 16 fold higher in first degree relatives of those who are HLA B27 positive compared to those who are HLA-B27 positive in the general population suggested that although HLA-B27 was important, there were likely to be additional genetic factors to be identified (van der Linden et al. 1984).

Attempts to identify other genetic factors based on a candidate gene approach have proved largely unsuccessful however, work progressed with the advent of whole genome scanning techniques and, to date, there have been three published genome scans in AS including one in the Han Chinese population (Laval et al. 2001; Brown et al. 2003; Gu et al. 2004; Zhang et al. 2004). Seven loci have been identified and validated by this procedure, these include: HLA-B27, ERAP-1 (ARTS-1), IL-23R, IL1R2, ANTXR2 (CMG2), the other two loci do not encode gene sequences. The population attributable risks for HLA-B27, ARTS-1 and IL23R are 90 %, 26 % and 1 %

respectively (Burton et al. 2007). The association with IL-23R has been replicated in Spanish and Canadian cohorts. Other candidate genes include TNF-α and CYP2D6.

ERAP-1 is known to be involved in the processing of HLA-B27 molecules as well as in the shedding of receptors for TNF-α, IL-1 and IL-6 from the cell surface. It is conceivable therefore that defective ERAP-1 function could lead to defective cytokine regulation. IL-23R is expressed on Th17 cells, which, in turn, secrete IL-17, a a potent pro-inflammatory cytokine that leads to the release of IL-1, TNF-α. This cell type is being subject to increasing scrutiny in spondyloarthritis as well as in RA and may offer a new therapeutic target in the future. The role of ANTXR2 is still unknown.

8.2.2.3 Environmental Factors

Since the presence of the genetic factors identified to date are neither necessary nor sufficient to initiate disease, investigators have been searching for an environmental trigger. The observation that a syndrome of arthritis, anterior uveitis and urethritis can develop after specific infections (*Campylobacter, Shigella, Salmonella, Yersinia* and *Chlamydia* species) is evidence that infection can be an initiating event for these disorders. Further work with the HLA-B27 transgenic mouse has shown that it only develops AS if it is exposed to environmental pathogens in the animal house. Indeed, intestinal colonization with *Bacteroides* species appears to be sufficient to initiate disease in the susceptible host (Rath et al. 1996).

Antibodies against *Klebsiella* have shown an association with human AS, suggesting that there may be a role for this agent in initiating or maintaining disease activity (Rashid and Ebringer 2007). The association is attractive in that is seeks to explain the association between the gastrointestinal tract and AS. A small open label trial of moxifloxacin has shown promise however more work is clearly required in this area.

A final factor that has been investigated is mechanical stress at the enthesis; it is hypothesised that this leads to downstream events that ultimately result in inflammation, erosion and bone formation (Benjamin et al. 2007).

8.2.2.4 Mechanisms of Bone Erosion and Formation in AS

As in RA, bone quality is reduced in AS, and the incidence of spinal osteoporotic fractures is increased (Magrey and Khan 2010). In contrast to RA, evidence from the randomised trials of anti-TNF therapy in AS suggests that these agents do not prevent bone erosion or new bone formation (van der Heijde et al. 2009). The analysis of histological specimens from inflamed tissues shows heavy expression the enzymes cathepsin K and matrix metalloproteinase-1 (MMP1) by the invading mononuclear cells in those with AS (Neidhart et al. 2009). In contrast, those with

RA demonstrate over-expression of RANKL and MMP3 suggesting that the pathways involved in bone metabolism are different.

Evidence from animal model suggests involvement of the RANKL-OPG axis in AS (Rauner et al. 2009). The principal mechanism of bone formation at the enthesis appears to be through endochondral ossification. Members of the bone morphogenic protein (BMP) family of growth factors influence chondrocyte development and are important early in the process. Reduced expression of the negative regulator, sclerostin has been shown in AS suggesting that this pathway may be important *in vivo* (Appel et al. 2009). Later stages of bone development are influenced by the Wnt signalling pathway through intra-cellular accumulation of β-catenin which ultimately leads to osteoblast differentiation. Reduced levels of an inhibitor of this pathway, DKK-1, have been found in AS and it is postulated that this may be important in syndesmophyte formation (Daoussis et al. 2010).

Work in this area is in its infancy, nevertheless it provides a tantalizing glimpse of pathways that may underlie the bony changes that characterise AS.

8.2.3 Diagnostic and Classification Criteria

There are two sets of classification criteria that can be used to stratify groups of patients, those from the European Group for the Study of Spondyloarthropathies (ESSG) and the Amor criteria. The Amor criteria show a trend towards higher sensitivity and specificity than the ESSG criteria, mainly because the ESSG criteria will only detect patients suffering from axial disease or peripheral arthritis whereas the Amor criteria allow the diagnosis to be made in the absence of axial disease or peripheral arthritis if other extra-articular symptoms are present.

The diagnostic criteria for AS were developed in Rome in 1960, and subsequently underwent modification in New York in 1966 and again in 1984 (Table 5) (Goie The et al. 1985). It should be borne in mind that ankylosing spondylitis, and spondyloarthropathy in general, encompass more than is included in these criteria; entities such as seronegative oligoarthritis or dactylitis have not been recorded in many epidemiologic studies because of the lack of an acceptable definition.

For the individual patient, diagnosis is often delayed because of late presentation; some patients present with features other than axial disease which can mislead the unwary and plain radiographic changes of sacroiliitis take several years to become evident (Kidd and Cawley 1988). It is for this reason that MRI is becoming increasingly important in the early diagnosis of inflammatory spinal disease where changes are evident at a much earlier stage.

8.2.4 Presentation

The classical feature of AS is inflammatory spinal pain. Usually felt in the lower back or buttocks, the pain is typified by its rhythm which is worst overnight or early in the mornings. Many patients report prolonged stiffness making it difficult for them to

dress. The stiffness and pain tend to improve over the course of the day and almost always with exercise or anti-inflammatory medications. Some patients notice that their symptoms wax and wane over time, with flare-ups followed by periods of little or no symptoms.

If extra-axial arthritis is present, it is typically found in the large joints in the lower limbs, in an asymmetrical manner. Enthesitis is a frequent feature and most often affects the Achilles tendon, plantar fascia or common extensor origin at the lateral humeral epicondyle. Dactylitis may also be seen as the swelling of an entire digit so that it takes on a sausage shape.

Extra-articular features include anterior uveitis, which can affect up to 25–40 % of patients with AS and is typically unilateral. In adults, this presents as a painful red eye and requires urgent ophthalmological review and management to reduce the chances of complications such at cataract, posterior synechiae and visual loss. The majority of cases in children are asymptomatic and regular screening is warranted in this population.

The relationship between AS and spinal osteoporosis has been well recognised and spinal fracture may account for some of the exaggerated kyphosis seen in many patients with longstanding disease (Ghozlani et al. 2009). Fracture of the rigid spine can occur with minimal trauma and is usually longitudinal across a syndesmophyte rather than across a vertebral body.

A second well recognised association is between inflammatory intestinal lesions and AS. Colonoscopic evaluation has found sub-clinical lesions in 20–70 % of cases and it was this association that led to the routine use of sulphasalazine for the treatment of AS. The corollary of this is that approximately 30 % of patients with inflammatory bowel disease have axial disease; 10–20 % having sacroiliitis alone, 7–12 % having spondylitis and 10 % having features indistinguishable from classical AS.

Other features seldom seen in practice are secondary amyloidosis due to deposition of serum amyloid A protein, apical lung fibrosis, aortitis and cardiac conduction defects due to fibrosis of the conduction system. Rarely, in longstanding axial disease, cauda equina syndrome can develop and arachnoid diverticulae are described.

It should be evident from the foregoing discussion of clinical features that a careful examination can reward the examiner with plentiful signs. Of particular importance is assessment of spinal motion and this is carried out to best advantage with a physiotherapist so that intervention can be directed appropriately. The modified Schober's test is performed to assess the range of lumbar flexion, lateral lumbar

Table 5 Modified New York Criteria for the Diagnosis of AS

Low back pain for at least 3 months improved by exercise and not relieved by rest
Limitation in spinal motion in sagittal and frontal plains
Chest expansion reduced compared to normal values for age and gender
Unilateral sacroiliitis (grade 3–4) or bilateral sacroiliitis (grade 2–4)
AS present if the imaging criteria and one of the other three criteria are met

bending is assessed by finger-floor distance and the extent of cervical lordosis by a tragus-wall distance. This latter measure being preferred over the occiput-wall distance as the tragus lies closer to the centre of rotation of the skull on the atlas and is relatively unaffected by the degree of neck flexion and extension. Chest expansion should also be included in routine measurement.

Imaging has always been an important factor in making the diagnosis, reflecting the presence of sacroiliitis in the diagnostic and classification criteria. As mentioned above, the characteristic findings are within the axial skeleton where early changes include sacroiliitis which can be detected on MRI or CT in the early stages, or visualised on plain radiograph once disease has been present for several years. Family studies have suggested that a mean of 9 years is required for plain radiographic changes to become apparent after changes are first detected on MRI. "Bright corners" or Romanus lesions may be seen on plain radiography or MRI in the antero-superior or antero-inferior corners of the vertebral bodies reflecting marginal erosion with reactive sclerotic change. Eventually, this leads to squaring of the vertebral body and finally to ossification of the superficial layers of the annulus fibrosus and longitudinal ligaments forming the typical, end-stage bamboo spine of AS. Some may develop single or multi-level spondylodiscitits (Andersson lesions), which can be mistaken for septic discitis or osteomyelitis.

Factors predicting poor prognosis include hip involvement, ESR > 30 mm/hr, lack of response to non-steroidal anti-inflammatory drugs, reduced spinal motion, dactylitis, oligoarticular arthritis, age of onset less than 16 and the presence of inflammatory bowel disease, psoriasis or urethritis (Amor et al. 1994).

8.2.4.1 Assessment and Monitoring of Disease Activity

It is an unfortunate fact that about 40 % of patients with clinically and radiologically active axial disease do not reflect this with a rise in their serum inflammatory markers nevertheless, the ESR and CRP are commonly monitored as they have prognostic implications and in some countries, a sufficient rise in these markers is required for funding of anti-TNF therapy.

Numerous tools have therefore been developed to facilitate both the assessment of activity at one point in time and the change in disease activity over time. The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) is a validated six item questionnaire that asks patients to mark the severity of their pain, stiffness and fatigue on visual analogue scales. A score of 4 or greater is consistent with active diseases and a change of 50 % is considered significant for assessing the efficacy of interventions. Functional limitation can be quantified with numerous composite measures such as the BASFI (Bath Ankylosing Spondylitis Functional Index), HAQ or WOMAC scores. In practice, the BASFI is used most commonly and consists of a ten item questionnaire on activities of daily living.

Primarily for use in clinical trials, there are response criteria to assess the efficacy of interventions in AS. The Ankylosing Spondylitis Assessment Score (ASAS 20)

response criteria are fulfilled if there is a 20 % reduction on patient global assessment, function (as measured on the BASFI), pain and inflammation (Anderson et al. 2001).

8.2.5 Treatment

The natural history of ankylosing spondylitis is that it leads to progressive spinal ankylosis with increasing pain and functional limitation. Even in the early stages, if there is prominent involvement of the cervical spine, many patients have problems driving due to loss of cervical rotation.

The wide array of potential symptoms and sites of disease involvement means that the treatment has to be tailored to the individual with the overall aims being to reduce pain, maintain mobility and function and possibly modifying the underlying disease process.

Patient education is a principle component of any effective management strategy. Group education programs are offered in some centres and although comparison of these is not possible because of the heterogeneous nature of the different groups, many believe that they promote coping with the emotional consequences of the disease and help patients participate in the management. The role of education is acknowledged in the EULAR practice recommendations as a cornerstone to effective management (Zochling et al. 2006).

For axial disease, non-steroidal anti-inflammatory agents (NSAIDs) and physical therapy should be considered first line interventions. The importance of early and regular physical therapy in AS is hard to understate; for many years, it was the sole management for the condition. There is evidence from a well-conducted but small multicentre trial that physical therapy in addition to medical management resulted in better spinal mobility, chest expansion and work capacity than medical management alone (Ince et al. 2006). The best form of physical therapy is still debated, however multimodal programs encouraging stretching and aerobic exercise seem appropriate for most, others with specific issues may merit tailored programs.

Approximately 70–80 % of patients with AS will derive benefit from NSAIDs compared to only 15 % of those with non-inflammatory low back pain. Analysis of the data from randomised trials of NSAID use in AS has suggested that all patients received maximum benefit within 2 weeks and this would therefore seem an appropriate length of treatment trial. Approximately 10–60 % will develop minor gastrointestinal side effects including epigastric pain and nausea but serious side effects such a gastrointestinal bleeding occur in 2–4 % treated for 12 months. The risk of serious adverse events rises with age, comorbidity and concomitant use of other anti-inflammatory agents, Agents selective for the COX-2 isoenzyme have less gastrointestinal toxicity, however, this has to be balanced with the well documented rise in cardiovascular events with these agents. It has even been suggested that regular use of NSAIDs – taken daily rather than as needed – can retard radiographic progression however further work is required to confirm these findings.

The above measures will control symptoms adequately in approximately 70 % of patients, however, in those with ongoing symptoms, high inflammatory markers, high score on the BASDAI, or evidence from imaging that disease is progressing, further treatment is required.

There is now widespread recognition that conventional DMARDs such as sulphasalazine or methotrexate are not effective for the control of axial disease however, the inhibitors of TNF-α, infliximab, adalimumab and etanercept have demonstrated remarkable efficacy in the treatment of axial and extra-axial disease in AS. To date, nine randomised controlled studies have been conducted showing that improvement can be seen as early as 2 weeks, is maximal after 12 weeks and is sustained over several years (van den Bosch et al. 2001; Braun et al. 2002; Gorman et al. 2002; Brandt et al. 2003; Davis et al. 2003; Calin et al. 2004; van der Heijde et al. 2005, 2006; Lambert et al. 2007). In addition, these agents are associated with improvement in quality of life, reduced sick leave, improved work productivity and reduction in inflammation on MRI. Even patients with complete spinal ankylosis report symptomatic benefit from these agents. Nevertheless, no reduction in radiographic spinal disease has yet been demonstrated and it is therefore important that patients continue physical therapy.

These biologic agents appear to be of similar efficacy for treating axial disease with best responses seen in younger patients with high inflammatory markers and active disease on MRI. Anecdotally however, adalimumab and etanercept are given in fixed dosage regimens and for those larger patients and those with very active disease, response may be improved with infliximab as the dose can be adjusted to body weight. In addition, anterior uveitis can develop in those receiving etanercept and so this agent may not be preferred in patients where this is a prominent feature.

For extra-axial disease, the anti-TNF agents show excellent efficacy however, prior to their introduction, studies had focussed on the use of sulphasalazine as a consequence of the link with inflammatory bowel disease. To date, ten randomised, double-blind studies including two multi-centre studies, have been conducted assessing the efficacy of this agent in peripheral arthritis and finding it to be of modest efficacy (Dougados et al. 1995; Clegg et al. 1996). A subsequent meta-analysis of four trials concluded that the duration and severity of morning stiffness, pain severity and patient global reached statistical significance. There is very little evidence for the use of methotrexate or leflunomide in the management of AS. Similarly, there is a very limited role for systemic corticosteroids in AS but intra-articular steroids, given either into a peripheral joint or in to the sacroiliac joint can provide substantial symptomatic benefit.

Approximately 30 % of patients with AS will not respond to treatment with anti-TNF therapy, or will experience side-effects leading to its discontinuation. Trials are underway using anti-IL-6, anti-IL-23 and targeted B cell therapy. There is evidence from one small study that the intravenous bisphosphonate, pamidronate, given in a dose of 60 mg once monthly improved symptoms (Maksymovych et al. 2002). Although it is not helpful in the presence of peripheral joint synovitis, it also has the advantage of treating the well-documented association of AS with osteoporosis.

8.2.6 Conclusions

Our understanding of the genetic, environmental and immunologic components is progressing rapidly and will hopefully offer new insights into the disease as well as provide new therapeutic targets. Compared to RA, AS and spondyloarthritis have been a somewhat neglected partner. However, in the era of biological therapy, there is now an effective treatment for what can be destructive and disabling conditions.

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RANKL Inhibition: Preclinical Data

9

Wolfgang Sipos

9.1 Animal Models of Osteoporosis

Osteoporosis is a complex systemic disease (Pietschmann et al. 2008). Therefore, researchers have to rely on rodent and large animal models for the development of new anti-osteoporotic therapeutics. Rodent models are well established and have been widely used in osteoporosis research. However, the US Food and Drug Administration also demands the use of large animal models besides rats in preclinical testing of anti-osteoporotic substances, with an experimental time frame of 12 months when using rats and 16 months when using larger species. According to FDA regulations, valid animal models have to develop an osteoporotic phenotype either spontaneously or after ovariectomy (OVX) (FDA 1994). In order to give an idea of the different biomechanical forces on the skeletons of small vs large animal species, Fig. 1 shows the skeletons of a rat, a sheep, and a miniature pig. There are several ways of inducing osteopenia or osteoporotic phenotypes in mammals, such as *OPG* gene knock-out, systemic RANKL administration, prolonged glucocorticoid administration, age-related osteoporosis, dietary calcium shortage, OVX, and combinations. All these manipulations have specific advantages and disadvantages. In this chapter, we will focus on rodent and artiodactyl models, but largely spare canine as well as primate models, as these are to be used less frequently due to ethical reasons.

To date, a variety of transgenic mouse strains exists, but there are only limited genetically modified large animal species. *OPG*$^{-/-}$ mice suffer from severe osteoporosis associated with a high incidence of fractures and vertebral deformities but lack unwanted side effects on immune function, which can be observed in *RANKL*$^{-/-}$ mice (Bucay et al. 1998; Kong et al. 1999; Yun et al. 2001). Systemic RANKL admin-

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istration leads to increased osteoclast numbers and to an osteoporotic phenotype in mice (Lacey et al. 1998). Prolonged glucocorticoid exposure by subcutaneously implanted slow-release prednisolon pellets (2.1 mg/kg/d) is another reasonable means of inducing osteoporosis in rodents (Weinstein et al. 1998). Wang et al. (2001) demonstrated an age-related decline of bone mass in the axial and appendicular skeleton and a decrease in bone formation in the spine of male Sprague-Dawley rats. Pietschmann et al. (2007) described similar changes, i. e. markedly reduced cancellous bone mineral density (BMD), bone volume (BV), and trabecular number (Tb.N.), in the proximal tibiae of aged male rats. Additionally, bone formation as well as osteoclastogenesis appeared decreased, which was substantiated by lower insulin-like growth factor 1 (IGF-1), osteocalcin, and RANKL serum levels. Bone densitiy and bone strength are reduced in young growing rats by dietary calcium restriction in the range of 0.3–2.5 g Ca^{2+}/kg diet (Thomas et al. 1988; Persson et al. 1993, Talbott et al. 1998). In the growing pig model, calcium restriction (0.1–0.4 %) for one month also leads to increased plasma PTH, calcitriol, alkaline phosphatase (ALP), propeptide of type 1 procollagen (P1CP), and hydroxyproline titers, which are associated with osteoporotic changes of metacarpals (Eklou-Kalonji et al. 1999).

Although calcium shortage itself may induce osteopenia to some extent, it is usually combined with OVX, which may be considered the main model of postmenopausal and thus estrogen-deficiency induced osteoporosis. In this context it is essential to be aware of species-specific differences in sexual endocrinology. Whereas women (and macaques) have a sexual cycle of 28 days, sows experience spontaneous ovulations as well but with a cycle length of 21 days. With regard to sheep there are seasonal and aseasonal breeds with a mean cycle length of 17 days. The seasonal rhythm is regulated by the epiphyseal melatonine secretion, which is significantly higher in winter ("short day breeders"). However, the mainly used merinos are an aseasonal breed. Another feature, which has to be kept in mind, is extragonadal estrogen synthesis, which may cause a rebound effect in ovariectomized sheep in terms of lowering BMD change differences when compared to sham animals starting from four months after OVX (Sigrist et al. 2007). Also, ovariectomized sows likely experience extragonadal estrogen synthesis to some extent (Sipos et al. 2011). Female dogs are seasonally monestric, usually in March and September. Heat includes proestrus, estrus, and early metestrus and takes three weeks. Estradiol increases only during this very short period to titers between 18 and 50 pg/ml during proestrus and decreases already after start of estrus to the detection limit of 5 pg/ml (Sipos 1997). Rats have sexual cycles of four to five days, whereas mice experience inducible ovulations. Furthermore, the occurrence of a natural menopause is not demonstrated in all model animals, except for macaques.

In general, estrogen deficiency induced by OVX leads to an osteoporotic phenotype in rodents as well as diverse ungulates and can be aggravated by additional glucocorticoid administration or calcium shortage. In the rat, rapid loss of cancellous bone mass and strength occurs following OVX, which after a while reaches a steady level of bone mass with an increase in the rate of bone turnover (Wronski et al. 1986, 1988, 1989, 1990, 1992; Kalu 1991). The proximal tibial metaphysis, the

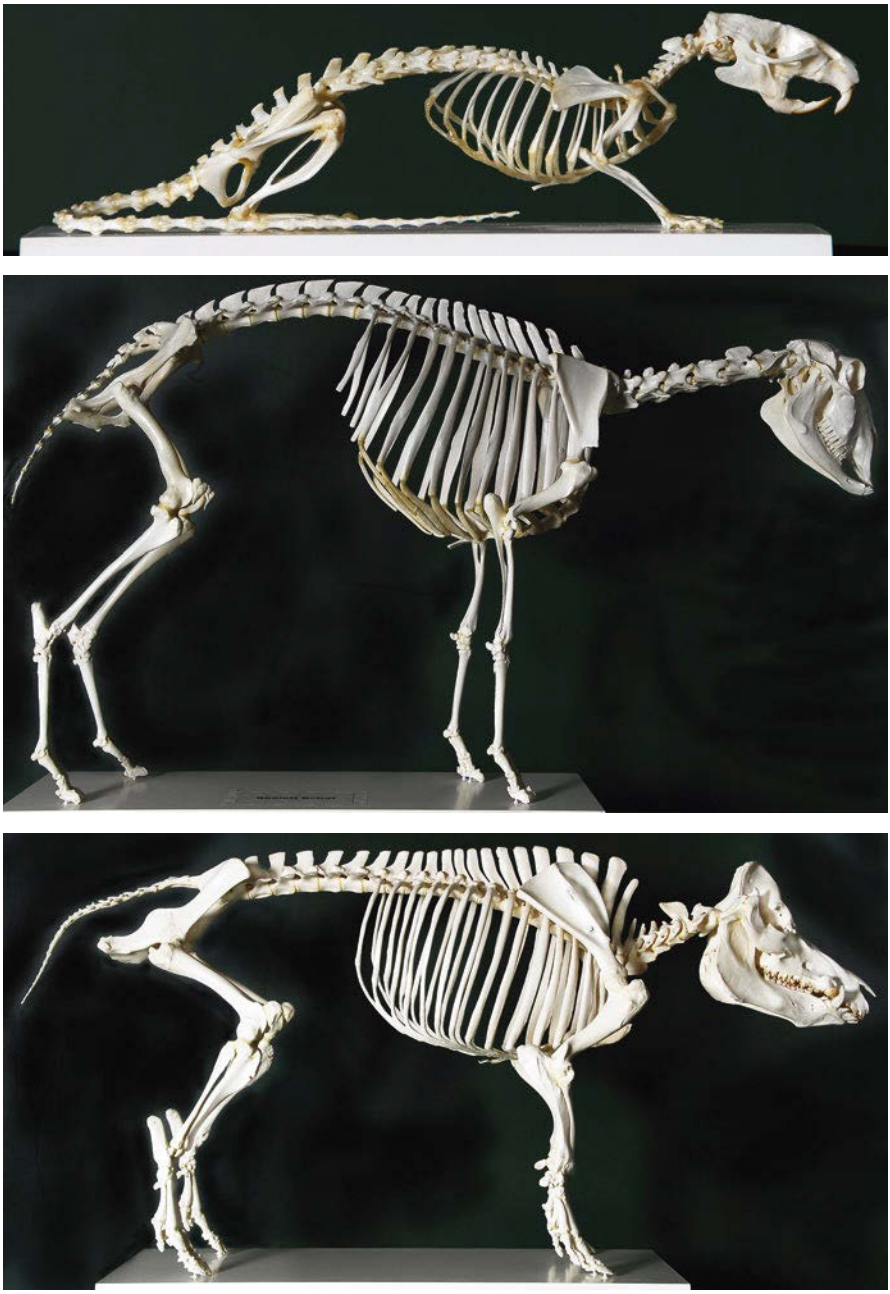

Fig.1 Skeletons of a rat, a sheep, and a miniature pig. Differences between species concerning the mechanical impact on the axial and the appendicular skeletons are resulting not only from large differences of body mass, but also from the different position of extremities in relation to the body and different functions of the vertebral column in ensuring stability of thorax and especially abdomen (photos with kind permission of G. Weissengruber, Vienna)

lumbar vertebral bodies, and the femoral neck are affected by cancellous bone loss within one month after OVX, whereas OVX-induced bone loss does not occur in trabecular bone of long bone epiphyses, the distal tibial metaphysis, and the caudal vertebrae (Ma et al. 1994; Li et al. 1996; Westerlind et al. 1997; Miyakoshi et al. 1999). The situation of cortical bone in the ovariectomized rat is complex, as increased bone resorption taking place at the endosteum of the diaphysis of long bones is antagonized by stimulated periosteal bone growth (Turner et al. 1987; Miller et al. 1991; Aerssens et al. 1996). Thus, enlargement of the marrow cavity is the most sensitive index of cortical bone loss in this species. As the rat, like most other experimental animal models, does not experience naturally occurring pathological fractures, bone strength has to be tested mechanically. Significant loss of vertebral and femoral neck bone strength occurs three months after OVX (Mosekilde et al. 1991; Mosekilde et al. 1993a; Peng et al. 1994; Sogaard et al. 1994; Jiang et al. 1997; Yoshitake et al. 1999). Reduction of dietary calcium from 0.4 to 0.2 % in ovariectomized rats results in a further decrease in bone density and mechanical properties (Shahnazari et al. 2009).

Alternative rodent osteoporosis models include the Botox-induced and the LPS-induced bone loss models (Grimston et al. 2007; Ochi et al. 2010). In the former, mice are injected intramuscularly with 2.0 U Botox per 100 g body weight in the quadriceps, hamstrings, and posterior calf muscles of one hind limb. Maximum limb dysfunction occurs by days 2–3 after Botox injection without gender differences. By 3–4 weeks post injection, full activity is restored. Bone loss in the injected limb is rapid and profound with the difference to the non-injected limb reaching significance by week two. At 12 weeks trabecular BV in the injected limb is substantially reduced. Also, cortical thickness is lowered. In the endotoxin model, male adult mice are subcutaneously injected with 20 mg/kg LPS. LPS treatment reduces BV/TV for approximately 40 % in the proximal region of tibial bones by 48 h post treatment. This LPS-induced enhancement of osteoclastogenesis can be blocked by OPG administration giving evidence of the importance of RANKL signalling in this setting, but neither LPS nor OPG affect osteoclastogenesis in *TNFR1*$^{-/-}$ mice. Interestingly, osteoblast surface is remarkably reduced in these mice as a result of enhanced osteoblast apoptosis due to TRAIL-mediated signalling, which triggers apoptosis of primary osteoblasts only when the TNFR1 signal is ablated in vitro.

There is also a bulk of literature dealing with the ovine species and its suitability as a postmenopausal osteoporosis model. Compact bone of growing sheep is predominantly plexiform. A well developed Haversian system consisting of secondary osteons is not developed until an age of seven to nine years (Turner 2002; Pearce et al. 2007). Seasonal fluctuations in bone metabolism complicate the interpretation of data. It should also be mentioned that biochemical bone metabolism markers are regarded as having only limited informative value in the ovine model (Gerlach 2002). Due to anatomical peculiarities of ovine vertebrae, biomechanical parameters fail to correlate with BMD of these bone sites, but do so with femoral BMD. Anatomical features of the iliac crest seem to be close to the corresponding human bone site (Ito et al. 1998). Although sheep do not experience a natural menopause, decreasing bone mass in aging sheep might make this species a potential model for age-related osteoporosis (Turner et al. 1993). OVX alone is not as efficient as in

other species, whereas glucocorticoid treatment induces an osteoporotic phenotype comparable to the one in other species including humans (Chavassieux et al. 1993; Hornby et al. 1995). BMD of L5 and distal radius as evaluated by DXA is significantly changed over six months, and the one of L4 one year after OVX, whereas the proximal parts of femur, humerus, and tibia do not exhibit changes to that extent (Turner et al. 1995). However, MacLeay et al. (2004) were not able to detect areal BMD changes in lumbar vertebrae in ovariectomized sheep three months after surgery. Another study showed a significantly decreased femoral but not lumbar vertebral BMD as well as significant effects on cortical bone parameters by six months after OVX (Chavassieux et al. 2001). Interesting and seemingly inconsistent with the likelihood of extragonadal estrogen synthesis is the fact that sheep experience significant microarchitectural changes in vertebral cancellous bone (decreased BV/TV by approximately 30 %, trabecular thickness (Tb.Th.) by 13 %, and increased trabecular separation (Tb.Sp.) by 46 %) two years after OVX; they also show histomorphometric changes (i. e. significantly increased osteoclast numbers) already three months after surgery (Giavaresi et al. 2001; Pogoda et al. 2006).

Combining OVX with profound dietary calcium restriction (1.5 g Ca^{2+} and 100 IU $VitD_3$/d instead of the physiological daily need for 4–5 g Ca^{2+} and 1.000 IU $VitD_3$) and weekly doses of 120 to 200 mg methylprednisolone for seven months in 7–9 years old ewes led to an approximately 35–40 % reduction of BMD of spongiosa of radius and tibia without affecting the corticalis (Lill et al. 2002). Lumbar vertebral bodies exhibited a decrease in BMD of 13 %. Also, all analysed histomorphological parameters of iliac crest as well as BV/TV of L4, and Tb.Th., BV/TV, and bone surface (BS/BV) of femoral head were significantly altered and corresponded well with biomechanical data. Although the high decrease of BMD of approximately 4 SD is remarkable in this model, the increased susceptibility for opportunistic infections and the draw-backs concerning osteoimmunological effects due to the prolonged exposure to glucocorticoids have to be considered. Another study showed the advantages of combining OVX and glucocorticoid administration over combining OVX and calcium restriction with a higher decrease of BMD of distal radius, distal tibia, and calcaneus (spongiosa by 25 % and corticalis by 17 % in the former group and 10 and 5 % in the latter) (Lill et al. 2000). Combining all three measures led to the most pronounced reductions (60 and 25 %). Age-dependency of osteoporosis-inducibility in sheep is still a matter of debate, but there is some consensus that inducing measures should last at least seven months and the time span between OVX and lactation should be over a year due to increased intestinal calcium resorption (Hornby et al. 1995; Lill et al. 2002).

Pigs seem to have some advantages over sheep concerning their suitability as biomedical large model species because of several anatomical and physiological reasons. Although there are a lot of similarities of diverse porcine organ systems to their human orthologs, the pig's usefulness as an osteological model species is still not entirely clear. However, porcine femoral compact bone is predominantly plexiform, but is converted to well developed osteonal bone earlier than in sheep (Mori et al. 2005). Peak bone mass is obtained with an age of two to three years. Ovariectomized and calcium restricted (0.3 % Ca^{2+}) multiparous conventional sows

do not appear to be suitable models for osteoporosis research, most probably due to their extremely high mean areal BMD of 1.5 g/cm² as measured at the femoral neck, which seemingly reduces bone plasticity (Sipos et al. 2011). On the contrary, growing pigs aged two months have a mean areal BMD of 0.56 g/cm² as measured by DXA (Sipos et al. 2012). Therefore, the main body of investigations in this area of research was performed using growing miniature pigs, which, however, might not appropriately reflect the situation of the postmenopausal osteoporotic woman due to their juvenile age. On the other hand, miniature pigs achieve sexual maturity earlier than conventional pigs and thus OVX may induce the desired phenotype earlier than in conventional sows. Mosekilde et al. (1993b) successfully established a miniature pig bone loss model. OVX in 10 months old miniature pigs resulted in a 6 % decrease in BMD, 15 % in BV, and 13 % in Tr.N., and an increase of 15 % in Tr.Sp. after six months, whereas OVX in combination with a mild nutritive calcium shortage (0.75 % Ca^{2+}), which had already been started at an age of four months, led to a 10 % reduction in vertebral BMD and significant increases in final erosion depth and vertebral marrow star volume. A study investigating multiparous sows being fed a standard diet (1.5 Ca^{2+}) showed a significant increase in plasma PTH, calcitriol and AP levels over a time span of one year after OVX (Scholz-Ahrens et al. 1996). This could not be reproduced by our group in a similar setting (Sipos et al. 2011). However, Scholz-Ahrens et al. (1996) also did not observe significant bone morphometric changes.

Glucocorticoid treatment alone seems to be sufficient to induce osteoporosis in the porcine model (Scholz-Ahrens et al. 2007). In adult (30 months old) primiparous Göttingen miniature pigs an osteoporotic phenotype could be induced by daily oral treatment with 1 mg/kg of prednisolone for two months and a reduction to 0.5 mg/kg thereafter until the end of the experiment, which was after 8 months in the short-term group and 15 months in the long-term group. In the short term, glucocorticoids reduced BMD at the lumbar spine by 48 mg/cm³ from baseline, whereas in the control group reduction was 12 mg/cm³. These changes were also evidenced by plasma BAP levels, which decreased significantly in the glucocorticoid group. In the long term, the loss of BMD became more pronounced and bone mineral content, Tb.Th., and mechanical stability tended to be lower compared to the control group. There was a negative association between the cumulative dose of glucocorticoids and BMD, which could be traced back to impaired osteoblastogenesis. Other authors used juvenile miniature pigs (Ikeda et al. 2003). They treated 8 month old Göttingen miniature pigs subcutaneously with prednisolone at a dosage of 0.5 mg/kg 5 days/week for 26 weeks. Glucocorticoid treatment significantly reduced bone formation markers (serum osteocalcin, urinary type-1 collagen N-telopeptide) levels at 13 weeks and thereafter also serum BAP levels relative to baseline. At 26 weeks, the longitudinal axis of the lumbar vertebral bone and length of femur were smaller in the glucocorticoid group compared to the control group. The same applied to the BMD of the femur, but not L2, as measured by DXA. Age-dependent increases in trabecular bone were also reduced by glucocorticoids. L2 and femora of these animals were also tested mechanically and prednisolone was shown to significantly reduce the ultimate load and maximum absorption energy of

both sites. Further regression analyses revealed that bone minerals, bone structure, and chemical markers correlated with mechanical properties of L2 and mid-femur. It was concluded that prednisolone reduced systemic bone formation and resorption and suppressed the age-dependent increases in bone minerals, structure, and mechanical properties of L2 and mid-femur.

9.2 Animal Models of Rheumatoid Arthritis

Like osteoporosis RA is a very complex pathological condition. Whereas it is still speculative for osteoporosis, rheumatoid arthritis is clearly identified as being a chronic inflammatory and autoimmune disease, although the autoantigen(s) is (are) not entirely known. Nevertheless, there are several models, which reflect the pathophysiological situation satisfactorily on the focal (including redness, joint swelling, cartilage and bone destruction) as well as the systemic level with upregulated proinflammatory cytokines. In the following, the most frequently used models will be presented in short: the adjuvant-induced arthritis (AIA), the collagen-induced arthritis (CIA), and the TNF-α transgenic mouse. Contrary to biomedical osteoporosis research, only rodent models are used in rheumatoid arthritis research. Classically, AIA is induced by an intradermal injection of heat-killed *Mycobacterium tuberculosis* in paraffin oil at the base of the tail followed by a consecutive injection into one knee joint. The clinical onset is nine days following the second injection as indicated by hind paw swelling and locomotory difficulties (Feige et al. 2000). In a modified protocol, mice are immunized intradermally at the base of the tail and four footpads with 100 mg of methylated BSA (mBSA) emulsified in an equal volume of complete Freund's adjuvant (Ohshima et al. 1998). Additionally, mice are intraperitoneally injected with *Bordetella pertussis*. This procedure is repeated seven days later. On day 21, 100 mg of mBSA in 10 ml of saline is injected into one knee joint. As a control, the same volume of saline is injected into the contralateral one. The acute phase of AIA lasts for one week starting after the booster injection and is followed by a chronic phase. Mice usually are sacrificed 35 days after the first immunization.

CIA is elicited by an intradermal injection or intravenous infusion of heterologous type II collagen emulsified in a 1 : 1 ratio in incomplete Freund's adjuvant (Cremer et al. 1983; Stolina et al. 2009b). Following other regimens, CIA is induced after intradermal immunization with collagen emulsified in complete Freund's adjuvant, followed three weeks later by a booster dose of collagen emulsified in incomplete Freund's adjuvant. Disease susceptibility is strongly linked to the MHC class II haplotype. CIA susceptible mice such as DBA/1, B10.Q and B10.III are the most commonly used strains for the CIA model. Mice should be young (approx. eight weeks) and healthy, and in many settings male mice are preferred because they develop disease earlier than females. CIA is consistently induced 16–35 days after immunization in 90–100 % of male mice and in 60–100 % of females using bovine collagen. Notably, progression of the two models, AIA and CIA, is mediated by distinct

immunopathogenic mechanisms (Cremer et al. 1983). With respect to the dominant pro-arthritic cytokines, AIA is driven mainly by TNF-α (Stolina et al. 2009a), while CIA is provoked mainly by IL-1 (Stolina et al. 2008). Of importance for a systemic pro-inflammatory disease is that both CIA and AIA are characterized by a systemic upregulation of acute-phase proteins, IL-1β, IL-8, CCL2, and RANKL, whereas TNF-α, IL-17, and PGE_2 are elevated exclusively in clinical AIA. In contrast, Sarkar et al. (2009) found in their CIA model that joint inflammation was associated with a higher ratio of systemic IL-17/IFN-γ. Neutralization of IFN-γ accelerated the course of CIA and was associated with increased IL-17 levels in serum and joints. The authors concluded that the absolute level of IL-17 is not the only determinant of joint inflammation. Instead, the balance of Th1, Th2, and Th17 cytokines is suggested to control the immune events leading to joint inflammation.

More recently, a serum transfer arthritis model has been generated. This model is based on T cells expressing a single autoreactive TCR recognizing glucose-6-phosphate isomerase (G6PI). These cells escape negative selection in mice bearing a specific MHC class II allele, IAg7. In the periphery, these T cells promote a breach in B cell tolerance and high levels of anti-G6PI antibodies are produced, leading to a destructive and erosive arthritis similar to that seen in human RA. The adoptive transfer of serum from these mice results in peripheral joint swelling in most recipient strains. Early events that trigger paw swelling in the serum transfer model include signalling through FcRs. Further analysis demonstrated that $Fc\gamma RII^{-/-}$ mice manifested accelerated arthritis whereas $Fc\gamma RIII^{-/-}$ mice experienced a more slowly progressing arthritis. In the K/BxN serum transfer model of arthritis, there is a clinically apparent acute phase, which is modulated by FcγRII and FcγRIII, and a subacute component, which results in bone erosion, even in the absence of FcγR signalling (Corr and Crain 2002).

The TNF-α transgenic mouse (Keffer et al. 1991) allows deregulated human *TNF-α* gene expression. These mice develop a chronic inflammatory and destructive polyarthritis within six weeks after birth (Redlich et al. 2002).

9.3 Animal Models of Cancer-associated Osteolytic Lesions

Many aggressively growing malign entities metastasize into bone causing osteolytic lesions, which rely on the RANKL-mediated osteoclastogenesis-promoting nature of these tumor cells. Amongst the clinically most relevant bone-affecting tumors are the sexual steroid controlled mammary and prostate cancers as well as multiple myeloma. In rodent mammary cancer models the human breast cancer cell line MDA-231 is usually injected into the left ventricle of female athymic BALB/c nude mice aged 4–8 weeks under general anaesthesia (Mbalaviele et al. 1996; Morony et al. 2001). Transplanted mice develop a profound cachexia. Multiple osteolytic lesions with highly active osteoclasts are evident in the bones of the proximal and distal extremities within one month after tumor inoculation.

More sophisticated data than those produced by radiography can be acquired by means of *in vivo* whole-body bioluminescence imaging (BLI). This technique demands the application of luciferase (*Luc*)-gene transduced cells of interest. Visibility is induced by a preceding intraperitoneal injection of luciferin (Canon et al. 2008). For example, BLI has been applied in the 4T1 model. The 4T1 orthotopic breast cancer model has been extensively utilized to examine the efficacy of a series of bisphosphonate compounds for the treatment of breast cancer bone metastases (Yoneda et al. 2000). This model is characterized by the occurrence of bone metastases in nearly 100 % of animals. Histological examination reveals the occurrence of profound osteoclastic bone resorption and luciferase activity assays confirm tumor burden (Reinholz et al. 2010). In the 4T1/Luc model, washed 4T1 *Luc*-transduced mouse mammary cancer cells are suspended in sterile PBS and subcutaneously injected into mammary fatpads of 4–5 week old syngeneic female BALB/c mice. Primary mammary tumors form approximately one week after cell inoculation and metastases to lung and liver develop within two weeks. Metastases to bone, adrenals, kidneys, spleen, and heart occur by three weeks post-inoculation. Mice typically succumb four weeks after tumor cell injection.

Prostate cancer (PC) bone metastases can also be induced by intracardial tumor cell infusions into male nude mice (Miller et al. 2008). *Luc*-transduced cells were found to develop a pattern of bioluminescence consistent with tumor metastatic foci in bone, with the highest concentrations in hind limbs and mandible as early as 3–5 days after intracardial injection. Another route for administration of PC cells is the intratibial injection (Ignatiski et al. 2008). Here, the hind limb is shaved, the knee cap is located and cells are percutaneously injected in a volume of 50 ml into the marrow cavity via the tibial crest.

Xenograft models are also utilized for inducing multiple myeloma-associated bone metastases. The KAS-6/1-MIP-1α mouse model may serve as an example (Reinholz et al. 2010). Herein, genetically engineered KAS-6/1 myeloma cells carrying the osteoclast activating factor MIP-1α are injected into female SCID mice. Bone loss occurs within two weeks, hind-limb paralysis occurs within two months, and mice typically die one week later.

Other tumor cell types used for generating osteolytic bone metastases include human A431 epidermoid carcinoma cells and murine colon adenocarcinoma-26 cells. A431/Luc cells were found to cause osteolytic lesions in the hind limbs after 20 days and Colon-26 cells colonize the skeleton and cause significant localized bone destruction in syngeneic 7–8 week old BALB/c DBA/2 mice within twelve days after intracardial tumor inoculation (Morony et al. 2001; Canon et al. 2010).

9.4 RANKL Blockade in Animal Models of Osteoporosis

The following subchapters focus on RANKL blockade in rodent and non-primate large animal models. First, the effects of RANKL blockade have been investigated in models of postmenopausal osteoporosis. Fewer preclinical data are available for this

treatment option of male osteoporosis. Min et al. (2000) demonstrated the protective and osteoporosis-reversing effects of treatment of eight weeks old *OPG*$^{-/-}$ mice with high intravenous doses of 50 mg/kg of recombinant human (rh) OPG three times per week for four weeks. The advantage of rhOPG, which is fused to an Fc fragment, is its sustained serum half-life enabling a prolonged antiresorptive activity. Capparelli et al. (2003) treated male, ten weeks old Sprague-Dawley rats with a single intravenous bolus injection of 5 mg/kg rhOPG. Maximum rhOPG concentrations were seen within 12 hrs after injection and coincided with significant elevations of serum PTH levels, which normalized 24 hrs later. Although a remarkable decline in rhOPG serum levels started at day 10, rhOPG serum concentrations remained at measurable levels throughout the 30 day study. Suppression of osteoclastic bone resorption started within 24 hrs after treatment. Significant gains in tibial cancellous BV were evident within five days. Femoral BMD increased between days 10 and 20. The significant decrease of osteoclast surface of 95 % in the rhOPG group was paralleled by a 35 % decrease in the serum bone resorption marker TRAP5b. Repeated rhOPG treatment (five times 2 mg/kg within two weeks) further led to an increase of bone fracture strength at the femur mid-diaphysis in three-point bending by 30 % without affecting elastic or maximum strength in young male Sprague-Dawley rats (Ross et al. 2001). At the femoral neck, rhOPG significantly increased elastic (45 %), maximum (15 %), and fracture (35 %) strengths. Additionally, rhOPG treatment significantly increased whole bone dry mass (25 %), mineral mass (30 %), organic mass (17 %), and percent mineralization (4 %). Overall, rhOPG augments mineralization and strength indices in the rat femur with its effects on strength being more pronounced in the femoral neck than at the mid-diaphysis.

Ominsky et al. (2008) treated ovariectomized rats aged three months with 10 mg/kg rhOPG twice weekly. OVX was associated with significantly higher serum RANKL titers, increased osteoclast surface, and reduced areal and volumetric BMD. OPG markedly reduced osteoclast surface and serum TRAP5b while completely preventing OVX-associated bone loss in the lumbar vertebrae, distal femur, and femoral neck. μCT analyses showed that trabecular compartments in rhOPG-treated OVX rats had a significantly greater BV fraction, volumetric BMD, bone area, Tb.Th., and Tb.N. Additionally, rhOPG improved the cortical area in lumbar vertebrae and femoral neck to levels that were significantly greater than in OVX or sham controls. Also, bone strength was increased in OVX rats by rhOPG-treatment.

Clinical development of rhOPG was discontinued in favour of denosumab. Thus, preclinical experiments demanded a rodent model in which this fully humanized monoclonal antibody that specifically inhibits primate and human RANKL would be effective. Preliminary experiments showed that denosumab did not suppress bone resorption in normal mice or rats, but prevented the resorptive response in mice challenged with a human RANKL fragment encoded primarily by the fifth exon of the RANKL gene. Therefore, exon 5 from murine RANKL was replaced by its human ortholog. The resulting huRANKL mice exclusively express chimeric (human/murine) RANKL that maintains bone resorption at slightly reduced levels when compared to wild type controls (Kostenuik et al. 2009). In these mice denosumab reduced bone resorption, increased cortical and cancellous bone mass, and

improved trabecular microarchitecture. Hofbauer et al. (2009) analyzed the bone protective effects of denosumab (10 mg/kg subcutaneously twice weekly over four weeks) in glucocorticoid-treated male, eight month old homozygous huRANKL knock-in mice. They showed that prednisolone treatment induced loss of vertebral and femoral volumetric BMD, which was associated with suppressed vertebral bone formation and increased bone resorption as evidenced by increases in the number of osteoclasts, TRAP5b protein in bone extracts, serum levels of TRAP5b, and urinary excretion of deoxypyridinoline. More detailed analysis showed that glucocorticoid-induced bone loss was most pronounced in the cortical and subcortical compartment in the distal femoral metaphysis, whereas trabecular BMD remained unchanged by prednisolone treatment. Denosumab prevented prednisolone-induced loss of total BMD at the spine and the distal femur. Additionally, biomechanical compression tests of lumbar vertebrae revealed a detrimental effect of prednisolone on bone strength that could also be prevented by denosumab.

In analogy to rhOPG and denosumab as RANKL antagonists, Kim et al. (2009) developed a cell-permeable inhibitor termed RANK receptor inhibitor (RRI), which targets a cytoplasmic motif of RANK. The RRI peptide blocked RANKL-induced osteoclast formation from murine bone marrow-derived macrophages. Furthermore, RRI inhibited the resorptive function of osteoclasts, induced osteoclast apoptosis, and protected against OVX-induced bone loss in mice. As RANK blockade may theoretically impair the immune system, the authors also determined whether the RRI peptide interferes with phagocytosis or dendritic cell differentiation. Both immune functions were not affected.

Li et al. (2009) tested the effects of rhOPG treatment on bone biology of orchiectomized (ORX) rats. Whereas serum testosterone declined within two weeks after surgery, no changes in serum RANKL could be observed. In contrast, there was an increase in RANKL in bone marrow plasma, which correlated positively with marrow plasma TRAP5b. RANKL inhibition was induced by treating ORX rats twice weekly for six weeks subcutaneously with 10 mg/kg rhOPG. Whereas vehicle-treated ORX rats showed significant deficits in BMD of femur and tibia as well as lower trabecular BV in the distal femur, rhOPG treatment increased femoral and tibial BMD and trabecular BV to levels that significantly exceeded values for ORX or sham controls. Histologically, rhOPG treatment reduced trabecular osteoclast surfaces in ORX rats by 99 %. μCT of lumbar vertebrae from rhOPG-treated ORX rats demonstrated significantly greater cortical and trabecular bone volume and density compared to ORX-vehicle controls. These data are encouraging for RANKL inhibition as a strategy for preventing bone loss associated with androgen ablation or deficiency.

Data on effects of rhOPG treatment in large animal models are limited. The homology between a porcine RANKL-specific sequence and the corresponding human RANKL sequence was found to be 79 %. Also, RANKL is upregulated during *in vitro* osteoclastogenesis and expressed by a variety of different cell types including bone marrow stromal cells and immunocytes in pigs (Sipos et al. 2005). Sipos et al. (2012) analyzed the effects of a single intravenous rhOPG bolus of 5 mg/kg in pigs aged two months. Peak rhOPG levels were seen at day 5, but markedly declined

thereafter. TRAP5b levels decreased to 50 % of baseline levels in the OPG group between days 5 and 10, whereas they slightly increased in the control animals. Notably, BAP levels paralleled TRAP5b levels in the rhOPG-treated pigs. At termination of the experiment at day 20, μCT analysis showed a significantly higher connectivity density in proximal femur, proximal tibia, and L4 as well as BMD of femur, hip, and tibia. Interestingly, Tb.Th. (femur, hip, L4) was significantly lower in the rhOPG-treated pigs, but Tb.N. (femur) was higher and a trend towards lower Tb.Sp. was evident in the tibia. In summary, rhOPG treatment inhibited osteoclast function as evidenced by TRAP5b decrease and led to a more arborescent architecture and higher mineralization in the weight-bearing skeleton. These data show that not only anatomical but also microstructural and biochemical bone parameters may differ in detail with regard to their responsiveness to RANKL inhibition between large mammal species such as humans and pigs, and also between these species and rodents with their distinct biomechanical forces on the axial as well as the appendicular skeleton.

9.5 RANKL Blockade in Animal Models of Rheumatoid Arthritis

Antirheumatic drugs exhibit pronounced adverse side effects. Therefore, efforts aim to establish more specific anti-inflammatory and osteoclastogenesis-inhibitory therapeutic regimens including RANKL blockade. First, rhOPG monotherapy was tested for its efficacy to ameliorate or abolish bone destructive processes of RA. In their study of male Lewis AIA rats treated with subcutaneously administered rhOPG, Campagnuolo et al. (2002) found that rhOPG provided dose- and schedule dependent preservation of BMD and periarticular bone while essentially eliminating intralesional osteoclasts. Dosages > 2.5 mg/kg/d preserved or enhanced BMD and prevented all erosions. OPG treatment was also successful in preventing loss of cartilage matrix proteoglycans and was shown to be most effective when initiated early in the course of the disease. However, signs of inflammation could not be affected by rhOPG treatment. In another study based on TNF-α transgenic mice, the anti-inflammatory as well as anti-osteoclastogenic potential of infliximab, an anti-TNF-α monoclonal antibody, was compared to rhOPG and pamidronate treatment (Redlich et al. 2002). As may be concluded from the preceeding study, clinical improvement was achieved only in the infliximab group. Radiographic analyses revealed a significant retardation of joint damage in animals treated with rhOPG (55 % reduction of erosions), pamidronate (50 % reduction), a combined therapy of rhOPG and pamidronate (64 % reduction), and with infliximab (66 % reduction). These data show that rhOPG alone or in combination with bisphosphonates may be an effective therapeutic tool for the prevention of inflammatory bone destruction. Stolina et al. (2009b) also investigated Lewis rats with established AIA or CIA. Rats were treated with pegsunercept (a TNF-α inhibitor), anakinra (an IL-1 receptor antagonist), or rhOPG. Anti-TNF-α treatment ameliorated paw swelling in both

models and reduced ankle BMD loss in AIA rats. Anti-IL-1 treatment decreased paw swelling in CIA rats and reduced ankle BMD loss in both models. Both anti-TNF-α and anti-IL-1 applications reduced systemic markers of inflammation as well as, at least in part, systemic RANKL, but failed to prevent vertebral BMD loss in either model. OPG reduced TRAP5b by over 90 % and consequently also BMD loss in ankles and vertebrae in both models, but as anticipated had no effect on paw swelling.

The former studies demonstrated that rat AIA and CIA feature bone loss and systemic increases in TNF-α, IL-1β, and RANKL. Anti-cytokine therapies targeting inflammatory cytokines consistently reduce inflammation in these models, but systemic bone loss often persists. On the other hand, RANKL inhibition consistently prevents bone loss without reducing joint inflammation. Logically, RANKL inhibition has to be further combined with anti-inflammatory, most preferably site-specific drugs. Oelzner et al. (2010) treated AIA rats with dexamethasone (0.25 mg/kg/d, i. p.), rhOPG (2.5 mg/kg/d, i. p.), or a combination of both at regular intervals for three weeks. As expected, dexamethasone monotherapy substantially suppressed joint swelling without inhibiting bone loss of the secondary spongiosa, whereas rhOPG monotherapy showed no anti-inflammatory effect. Interestingly, rhOPG monotherapy failed to inhibit AIA-induced bone loss, whereas the combination of dexamethasone and rhOPG produced an anti-inflammatory effect and resulted in inhibition of periarticular and axial bone loss. Thus, the principle of combining an anti-inflammatory drug with RANKL inhibition may prove an effective bone-saving therapy in RA.

9.6 RANKL Blockade in Animal Models of Cancer-associated Osteolytic Lesions

RANKL is one of the key mediators of malignant bone resorption. Thus, RANKL inhibition prevents primary tumor or metastasis-induced osteolysis and decreases skeletal tumor burden. Morony et al. (2001) were among the first, who tested the ability of rhOPG to inhibit tumor-induced osteoclastogenesis, osteolysis, and skeletal tumor burden in two animal models. In one model they induced lytic bone lesions by transfecting mice with mouse colon adenocarcinoma (Colon-26) cells and found that treatment with rhOPG decreased the number and area of radiographically evident lytic lesions with high efficacy depending on the dose used. This therapeutic approach was also effective in nude mice transplanted with human MDA-231 breast cancer cells, completely preventing radiographic osteolytic lesions. Histologically, rhOPG decreased skeletal tumor burden by 75 % and completely eradicated MDA-231 tumor-associated osteoclasts. In both models, rhOPG had no effect on tumor metastases in soft tissue organs. The MDA-231 model for investigating the effects of RANKL inhibition in order to reduce bone tumor burden was also used by other authors, who confirmed the above findings. Investigating this model, Canon et al. (2008) found that RANKL protein levels were significantly higher in

tumor-bearing bones than in tumor-free ones. They monitored the anti-tumor efficacy of RANKL inhibition by rhOPG on MDA-231 cells in a temporal manner using bioluminescence imaging. One mechanism by which RANKL inhibition reduced tumor burden appeared to be indirect through increasing tumor cell apoptosis as measured by active caspase-3. In this model, treatment with rhOPG resulted in an overall improvement in survival rate. Zheng et al. (2007) combined the anti-tumor effects of rhOPG and ibandronate, again using the MDA-231 mouse model. Ten days after intratibial tumor cell injection (when the tumors were evident radiologically), mice were treated with rhOPG (1 mg/kg/d), ibandronate (160 μg/kg/d), or a combination for one week and the effects of each treatment on lytic lesions, tumor cell growth, cell apoptosis, and proliferation were measured. Compared to vehicle controls, treatment with all regimens prevented the expansion of osteolytic bone lesions at a similar rate (2.3 % increase in the mean vs 232.5 % increase in sham animals). Treatment with all regimens also produced similar reductions in tumor area (mean 51.3 %) as well as a similar increase in cancer cell apoptosis (339.7 %) and decrease in cancer cell proliferation (59.7 %).

Inhibition of prostate cancer bone metastases is also subject of rodent model-based research. For treatment of metastatic hormone-refractory prostate cancer docetaxel is a well-established medication. However, the side effects associated with docetaxel treatment can be severe, resulting in discontinuation of therapy. Thus, efforts are ongoing to identify an adjuvant therapy to allow lower doses of docetaxel. As advanced prostate carcinomas are typically accompanied by skeletal metastases, targeting RANKL may be a promising option. A combined treatment regimen based on RANKL inhibition and docetaxel decreased establishment and progression of prostate carcinoma growth in bone in murine models (Ignatoski et al. 2008). The combination of RANKL inhibition and docetaxel reduced tumor burden in bone to a greater extent than either treatment alone and increased median survival time by 16.7 % (Miller et al. 2008).

A recent study investigated whether reduction of osteolysis by RANKL inhibition could enhance the anti-tumor effects of an anti-EGFR antibody (panitumumab) in a novel murine model of human A431 epidermoid carcinoma bone metastasis by bioluminescence imaging (Canon et al. 2010). As shown by earlier studies, these authors also found that RANKL inhibition by rhOPG treatment resulted in a reduction in tumor progression in bone sites and in tumor-induced osteolysis. The anti-tumor efficacy of panitumumab could be increased by rhOPG. The combination completely blocked tumor-induced bone breakdown. These studies demonstrate an additive effect of RANKL inhibition to the first-line agent in various murine bone metastases settings and may lead to sanguine novel therapeutic options in the fight against tumor-associated bone diseases.

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RANKL Inhibition: Clinical Data

10

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The following chapter gives an overview of the clinical data of RANKL-inhibition by denosumab in conditions of bone loss in postmenopausal osteoporosis (PO), rheumatoid arthritis (RA), and malignant bone disease (MBD).

Poor bone quality with reduced bone strength in localized as well as generalized form is associated with an increased incidence of fractures, high health care costs, and increased mortality. Additionally an increased need of painkillers due to malignant bone pain, painful joint dysfunction due to insufficient fracture healing are also associated with a wide spectrum of secondary disease conditions. Medical reduction and prevention of bone loss is therefore expected to be crucial in therapy of PO, RA and MBD.

RANKL is known as a major activator of osteoclast differentiation and activation, causing enhanced bone loss. Several data underline the prominent role of RANKL in conditions of severe bone loss and an increased RANKL-OPG ratio has been implicated in the pathogenesis of PO, RA and MBD. Therefore RANKL is supposed to be the most promising target for therapeutic intervention. The chimeric OPG-Fc fusion protein initially synthesized to antagonize RANKL failed in clinical practice because of neutralizing antibodies being formed against OPG after administration of the fusion protein and its potential cross-reactivity with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), thereby increasing the amount of unwanted side effects (Bekker et al. 2001; Emery et al. 1998). These inconveniences are avoided by the availability of denosumab, a fully human monoclonal anti-RANKL antibody of the immune globulin type IgG_2, which binds with high affinity to RANKL and shows a higher specificity and superior pharmacokinetic properties compared to OPG-Fc (Bekker et al. 2004). The bioavailability of denosumab is about 61 % after subcutaneous (s. c.) injection with a distribution comparable to plasma

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volume and a clearance by the reticulo-endothelial system (Body et al. 2006). With injection of 60 mg denosumab s. c. every six months, the medium time to maximum concentration is achieved in about 26 days after the first dose. The long duration of denosumab activity is caused by the combination of a long half-life and a very potent antiresorptive effect at early stages of osteoclast differentiation. No neutralizing antibodies have been found in the clinical trials with denosumab.

The trade name for denosumab in the accepted indications by the FDA is Prolia by Amgen. The BLA for Prolia for the treatment and prevention of osteoporosis in postmenopausal women and treatment and prevention of bone loss in women and men receiving hormone therapy for either breast cancer or prostate cancer was accepted by the FDA in February 2009. In these indications the drug is administered twice yearly subcutaneously hormone-ablative at a 60 mg dose. The trade name is only for these indications and may not apply for other indications of denosumab.

Since July 2011 denosumab is accepted by the EMA for prevention of bone complications in patients with bone metastasis originating from a solid tumor. This indication does not include manifestations of the multiple myeloma. The trade name is Xgeva by Amgen. Its application is subcutaneously every four weeks at a dose of 120 mg.

A large study program on a wide spectrum of bone diseases, including several types of osteoporosis, rheumatoid arthritis and bone metastases is currently ongoing (www.clinicaltrials.gov).

10.1 Denosumab in Postmenopausal Bone Loss and Osteoporosis

10.1.1 Denosumab in Postmenopausal Women with Low Bone Mass

Low bone mass and osteoporosis are highly prevalent in the elderly population and lead to fragility fractures. Especially postmenopausal women are affected by osteoporosis and with an aging population the prevalence of osteoporosis is expected to steadily increase in the near future (Burge et al. 2007; Melton et al. 1994; Ray et al. 1997). Osteoporosis is an emerging medical and socioeconomic threat by increasing the propensity of fractures due to the systemic impairment of bone mass, strength, and microarchitecture leading to high fragility of the skeleton (NIH Consensus Development Panel 2001). A patient with osteoporosis has a lifetime fracture risk of about 40 %. Fractures most commonly occur in the spine, the hip, or the wrist, but also other sites of fractures occur i. e. at the humerus and the ribs. An osteoporotic fracture means loss of mobility and autonomy not only during the phase of bone healing but often for the whole remaining lifetime, and therefore it represents a major loss in life quality. Additionally osteoporotic fractures require hospitalization and subsequently enhance the risk of loss of organ function and death by hospi-

tal-acquired infections, thromboembolic disease, and other medical complications (Center et al. 1999).

The therapies of osteoporosis can be divided into two classes: anti-resorptive drugs, which slow down bone resorption, and anabolic drugs, which stimulate bone formation. The largest class of antiresorptive drugs are bisphosphonates, which have a high affinity for bone and a long safety record. They have the largest market share because of their low costs and accreditations for a broad spectrum of different types of osteoporosis and bone loss. Based on current data denosumab is expected to be an attractive alternative in comparison to other anti-resorptive drugs such as raloxifene and strontium ranelate and also bisphosphonates themselves. While direct comparative denosumab-studies with fracture endpoints are not yet available, evidence from completed trials with established surrogates suggests that denosumab may be more effective than the most potent amino-bisphosphonate, zoledronic acid. Denosumab is characterized by full reversibility in targeting RANKL and is not incorporated into the bone mineral. Denosumab lacks gastrointestinal side effects and the convenient biannual subcutaneous administration may translate into an improved long-term adherence. Moreover, it is a potential drug in case of impaired renal function (glomerular filtration rate < 30 ml/min) as it is not eliminated by the kidneys. However, isolated cases of ONJ have also been reported and data suggest that denosumab shares the side effect of bisphosphonates in increasing the risk of osteonecrosis of the jaw.

The efficacy and safety of denosumab in postmenopausal women with low BMD was evaluated in a phase II randomized placebo-controlled study of 412 women with a lumbar spine T-score of –1.8 to –4.0 or total hip or femoral neck T-score of –1.8 to –3.5 (McClung et al. 2006). They were randomized for treatment with denosumab 6, 14 or 30 s. c. every three months, 14, 60, 100 or 210 mg every six months, open-label alendronate 70 mg weekly p. o. or placebo. The primary endpoint was the percentage change in lumbar spine BMD at 12 months compared to baseline. Other endpoints included the percentage change from baseline in BMD at the total hip, femoral neck, distal one-third radius, and assessment of bone turnover with serum C-telopeptide (CTX), urinary N-telopeptide (NTX), and bone-specific alkaline phosphatase (BSAP).

Under treatment with denosumab the lumbar spine BMD increased significantly by 3.0–6.7 %, dose- and dosing-interval dependent at 12 months after the start of administration. The BMD increase at other skeletal sites was also significant, but smaller. 30 mg denosumab s. c. 3-monthly and 60 mg denosumab 6-monthly both achieved a greater increase in BMD at the total hip and distal third radius compared to alendronate.

Serum levels of bone turnover markers (CTX, NTX, BSAP) decreased under denosumab in a dose-dependent, rapid, sustained and reversible manner. The maximum therapeutic benefit with minimal exposure dose was achieved with 60 mg denosumab every six months and 30 mg every three months. Adverse events and serious adverse events (SAEs) were similar in the treatment groups, except for a higher frequency of dyspepsia under therapy with open-label alendronate. Special evaluation of the immune function in a subset of denosumab-treated patients

showed no clinically meaningful changes in cell counts and percentage of white blood cells and subtypes in one year (Cohen et al. 2005). An extended increase of BMD and suppression of bone turnover markers was achieved after 24 months treatment with denosumab (Lewiecki et al. 2007). Also the advantage of denosumab over alendronate to gain BMD response sustained at 24 months. The adverse effects continued to be nearly similar in all groups. Nevertheless, SAEs of infection (6 cases, 1.9 %) were only seen in the denosumab groups, amongst which two cases of diverticulitis and three of pneumonia.

No neutralizing antibodies to denosumab were observed in the first 24 months of treatment. The third time interval of the study was an extension for again 24 months of the denosumab-treated patients with reassignment on the basis of the randomization groups at enrolment (Miller et al. 2008). Patients that had been treated with denosumab 6 or 14 mg 3-monthly and 14, 60 and 100 mg 6-monthly s. c. were switched to denosumab 60 s. c. 6-monthly. Patients that had been treated with 210 mg denosumab 6-monthly were switched to placebo and the group treated with denosumab 30 mg 3-monthly received placebo for 12 months and after that re-treatment with denosumab 60 mg 6-monthly for the last 12 months. The alendronate-group discontinued their treatment and received no additional drug therapy. The placebo group was maintained for the additional 24 months.

Patients receiving continued denosumab treatment gained further increase of BMD at the lumbar spine (9.4–11.8 %) and total hip (4.0 – 6.1 %) compared to baseline and showed consistent suppression of bone turnover markers during the time of study. In patients who discontinued denosumab treatment the BMD decreased about 6.6 % at the lumbar spine and 5.3 % at the total hip within the 12 months of treatment-discontinuation. During retreatment with denosumab the following 12 months after discontinuation the BMD increased again about 9.0 % at the lumbar spine and 3.9 % at the total hip compared to baseline, which is an increase similar to the initial treatment. Also, levels of bone turnover markers increased with discontinuation of denosumab and decreased again after retreatment. The decrease in BMD at the lumbar spine during the 48 months after discontinuation of alendronate was modest, the BMD at the total hip and distal one-third-radius showed a greater decrease. The levels of bone turnover markers increased after interruption of alendronate but remained below the baseline at 48 months. SAEs occurred more frequently in the denosumab (17.8 %, 56/314) and in the alendronate (17.4 %, 8/46) group than in the placebo group (10.9 %, 5/46). The incidence of malignancies did not differ between the treatment groups. Although the overall incidence of infections was similar between the treatment groups, only patients receiving denosumab treatment acquired infections requiring hospitalization (3.2 %, 10/314). All infections had been documented as common community acquired infections. All infections responded appropriately to standard antibiotic therapy and no opportunistic infections had been reported.

Based on these promising data of the advantage of denosumab compared to alendronate in postmenopausal women, phase III studies were initiated. Two phase III studies evaluated the effect in postmenopausal women with low BMD, one compared to placebo (DEFEND: Bone et al. 2008) and one compared to alendronate (DECIDE: Brown et al. 2009).

The DEFEND-trial (DEnosumab FortifiEs boNe Density) is the pivotal trial for registration of denosumab for the prevention of PO. It is a 2-year phase III trial in 332 postmenopausal women with a lumbar spine T-score between –1.0 and –2.5 (mean -1.61). The participants received either denosumab 60 mg s. c. 6-monthly or placebo. The primary endpoint was the percentage change of lumbar spine BMD measured with DXA from baseline at 24 months compared to placebo. The percentage change from baseline in BMD at the total hip, femoral neck, distal one-third radius, and total body at 24 months were defined as other endpoints, as well the percentage change from baseline in trabecular, cortical, and total volumetric BMD measured by quantitative computed tomography (QCT) at the distal radius at 24 months, the percentage change from baseline in bone turnover markers (serum CTX-I and tartrate-resistant acid phosphatase-5b [TRAP-5b], and intact N-terminal propeptide of type 1 procollagen [P1NP]) at 24 months, the proportion of patients with BMD gains greater than 0 % at the spine and total hip, and also parameters of hip structural analysis (HSA) by DXA.

Under treatment with denosumab a significant increase at the lumbar spine compared to placebo (6.5 % vs. –0.6 %, $p < 0.0001$) at 24 months was observed. As well the increase of BMD at the total hip, distal third radius and total body was significant ($p < 0.0001$, each vs. placebo). The volumetric BMD at the distal radius showed an increase under denosumab compared to placebo ($p < 0.01$), level of CTX-1, TRAP-5b and P1NP decreased significantly under denosumab and HAS parameter showed an improvement under denosumab compared to placebo. The incidence of adverse events did not differ between the groups.

The DECIDE trial (Determining Efficacy: Comparison of Initiating Denosumab vs. alendronate) compared denosumab to alendronate. This 1-year phase III trial was a double-blind, double-dummy, non-inferiority trial. 1189 women with postmenopausal low BMD (lumbar spine or total hip T-score of –2.0 or less) were randomized to receive denosumab 60 mg 6-monthly s. c. and oral placebo weekly or alendronate 70 mg weekly and placebo s. c. 6-monthly. The primary endpoint was the effect of denosumab compared to alendronate after 12 months demonstrated by the percentage change from baseline of the total hip BMD. The increase of total hip BMD after 12 months treatment with denosumab was significantly greater compared to BMD in patients treated with alendronate (denosumab 3.5 % vs. alendronate 2.6 %, $p < 0.0001$). The advantage of denosumab in increasing BMD was consistent at all other measured skeletal sites (treatment difference 0.6 % at the femoral neck, 1.0 % at the trochanter, 1.1 % at the lumbar spine, and 0.6 % at the distal one-third radius ($p < 0.0002$) for all sites). The suppression of bone turnover markers was satisfactorily greater under denosumab than under alendronate. The pattern and frequency of adverse events was similar in both treatmentgroups.

10.1.2 Denosumab in Postmenopausal Osteoporosis

The DECIDE trial for the postmenopausal osteoporosis was correlated with the 1-year phase III double-blind, active-controlled, double-dummy trial STAND (Study of Transitioning from AleNdronate to Denosumab; Kendler et al. 2008).

Participants were 504 postmenopausal women, aged 55 and older, with a lumbar spine or total hip T-score of –2.0 to –4.0 who had been previously treated with alendronate. The primary endpoint was the change in BMD compared to baseline at total hip, after a switch of treatment from a 12-month alendronate treatment to a 12-month denosumab treatment, as compared to continued treatment with alendronate. The study design allowed testing of the primary endpoint for superiority if non-inferiority was demonstrated. At 12 months the BMD in the group who switched treatment to denosumab showed a significantly greater increase than the group with continued alendronate treatment at the total hip, lumbar spine, and distal one-third radius. The incidence of adverse events remained similar.

The pivotal trial for registration for the treatment of postmenopausal osteoporosis was the FREEDOM trial, a 3-year international phase III study in 7,868 women with osteoporosis (Cummings et al. 2008, 2009). The mean age was 72.3 years and the mean baseline T-score was 2.8 at the lumbar spine. The women were randomized to receive either denosumab 60 mg 6-monthly s. c. or placebo 6-monthly s. c. for three years. Both groups received 1 g elemental calcium and 400–800 IUs of vitamin D daily. The primary endpoint to evaluate the efficacy of denosumab was the incidence of new vertebral fractures at 36 months. The time to first hip or other non-vertebral fractures was defined as secondary endpoint. About 83 % of patients completed the 36-month study. At the time of entry in the study approximately 23 % of the randomized patients had a minimum of one prevalent vertebral fracture. In the three years of the study, 2.3 % of the patients under treatment with denosumab and 7.2 % of the patients receiving the placebo had a new vertebral fracture. The reduction of fracture incidence by denosumab was 68 % and significant ($p < 0.0001$). Splitting the trial in one-year-periods the reduction of fracture incidence under denosumab remained similar (61 %, 78 % and 65 %, $p < 0.0001$). The incidence of

Table 1 A multicenter, international, 3-year-, phase-III-study in postmenopausal women. The effect of denosumab compared to placebo on the incidence of new fractures at different sites, on BMD and on the serum level of bone turnover marker (sCTX, P1NP)

New fracture	Denosumab	Placebo	Significance	Relative reduction of fracture incidence
Vertebral	2.3 %	7.2 %	$p < 0.001$	– 68 %
Non-vertebral	6.5 %	8.0 %	$p = 0.01$	– 20 %
Hip	0.7 %	1.2 %	$p = 0.04$	– 40 %

Denosumab effect on*	1 month treatment **	6 month, before 2nd dose **	36 month treatment **
BMD lumbar spine			+ 9.2 %
BMD total hip			+ 6.0 %
sCTX	–86 %	–72 %	–72 %
P1NP	–18 %	–50 %	–76 %

* compared to placebo ** treatment with denosumab 60 mg

other non-vertebral fractures was also reduced under denosumab treatment, the reduction by 20 % was significant (p = 0.011) (6.5 vs. 8.0 % under placebo). Although hip fractures were rare, the reduction of incidence by denosumab was significant (40 %: 1.2 % vs. 0.7 %, p = 0.036). Consistent with the reduction of fracture incidence was a greater increase of BMD at the lumbar spine under treatment with denosumab than in the placebo-receiving group. The increase was 9 % over the three years and 6 % at the total hip (both significant at $p < 0.0001$). The incidence and types of adverse events and SAEs were similar in the denosumab and placebo groups, including serious infections and neoplasms.

10.2 Denosumab in Rheumatoid Arthritis

The manifestation at the skeleton in rheumatoid arthritis (RA) is characterized by an imbalance of bone resorption and formation caused by local and systemic effects of the inflammation process. Advanced RA is characterized by loss of bone mass with focal joint erosions, and periarticular and systemic osteoporosis with destruction of joint architecture, leading to immobilization and impairment of physical activity as well as to an increased risk of fracture. At the early stage of disease the affected joints show inflammation of the synovial tissue that occurs with activation und proliferation of synoviocytes and infiltration of the synovial tissue with different cell types known as participants in inflammation processes. A "pannus" develops, invades and destroys the cartilage of the joint, causing bone erosion. This process is accompanied by periarticular bone loss (Lacativa and Farias 2010; Viswanathan and Sylvester 2008). Expression of RANKL critically mediates the bone resorption process in RA with structural damage of the skeleton (Crotti et al. 2002; Haugeberg 2008; Haugeberg et al. 2003; Lacey et al. 1998; Schett et al. 2005).

As a matter of fact, levels of serum markers of bone resorption increase in active RA and correlate positively with the disease activity (Seriolo et al. 2002). The common use of glucocorticoids in active inflammation further increases the risk of fracture (van Staa et al. 2006). Also older women with other risk factors for development of osteoporosis represent a significant population of RA patients (Symmons et al. 2006).

In this context several phases II and III trials are currently investigating the safety and efficacy of denosumab in patients with rheumatoid arthritis and manifest bone loss.

The effects of denosumab on the structural damage in 277 patients with RA and treated with a stable dose methotrexate were evaluated in a 12-months, multicenter, randomized, double-blind, placebo-controlled phase II clinical trial (Cohen et al. 2008). For participation in the study, the RA had to be diagnosed for at least 24 weeks according to the American College of Rheumatology 1987 criteria (Arnett et al. 1988); they had to have at least six swollen joints (66-joint count, excluding the distal interphalangeal joints); the RA had to attained the state of a bone-erosive disease, defined as at least three definite erosions on x-ray of the hands and feet or both a CRP of minimum 2.0 mg/dl and the presence of CCP-antibodies. The patients had

to receive a stable dose of methotrexate (7.5–25 mg/week) for at least eight weeks. Criteria for exclusion were a glucocorticoid-therapy with a dose above 15 mg/d or equivalent, scheduled surgery or joint replacement in the hands, wrists, or feet, pregnancy or use of a biologic agent for RA or leflunomide within eight weeks prior to study randomization.

The participants were divided into three groups, one receiving denosumab 60 mg, one denosumab 180 mg and one a matched placebo, each administered in the beginning and again after six months s. c. The primary endpoint for efficacy was the change in MRI erosion score of the hand and the wrist from baseline to six months, the scoring for bone erosions was accorded to a variation of the RA MRI (developed by OMERACT (McQueen et al. 2003)). Secondary endpoints were the change in modified Sharp score (van der Heijde 2000) from baseline to 12 months assessed by radiographs of the hands, wrists, and feet at baseline, after six and after 12 months, respectively, and percentage change in BMD at 12 months measured by DXA of lumbar spine and hip at baseline, after 6 and after 12 months respectively. Additionally blood and urine samples for bone and cartilage biochemical markers had been taken.

Under treatment with denosumab 180 mg the progression in MRI erosion score was significantly lower compared to placebo-treated patients (mean change 0.06 compared to mean change 1.75, $p = 0.007$). Under 60 mg denosumab a slightly but not significant lower increase of MRI-erosion-score was noted compared to placebo (mean change 0.13, $p = 0.118$).

A significant effect of denosumab compared to the placebo and measured by the modified Sharp erosion score was seen after 6 months at a dose of 180 mg denosumab ($p = 0.019$) and after 12 months at both 60 and 180 mg denosumab ($p = 0.012$ and $p = 0.007$). Additionally treatment with denosumab caused a sustained suppression of bone turnover marker and an increase in BMD compared to baseline.

An effect on the joint space narrowing and RA disease activity itself could not be evidenced. Rates of adverse events and SAEs did not differ between the three groups. Neutralizing antibodies had not been detected.

Post-hoc analysis (Sharp et al. 2010) was based on digitized radiographs to assess the effect of denosumab on the cortical bone thickness of the metacarpal shaft, a parameter for bone erosion in progressive RA (Hoff et al. 2009; Goldring 2009). The images had been taken at one, six and 12 months. 218 of the enrolled patients who received therapy were analyzed. The baseline median values in cortical bone thickness for each of the metacarpal bones did not differ between the groups. At six months patients treated with denosumab had significantly less metacarpal bone loss compared to placebo ($p < 0.05$ 60 mg, $p = 0.09$ 180 mg). At 12 months denosumab-treated patients showed a significantly smaller decrease in metacarpal bone thickness compared to placebo.

A post-hoc analysis described the effects of denosumab on BMD and bone turnover markers in patients with RA (Dore et al. 2010). To determine a potential influence of denosumab effects by other treatments effecting bone metabolism, the changes in BMD and bone turnover markers have been evaluated according to concurrent glucocorticoid or bisphosphonate administration and compared to results

of patients not receiving those agents. The analyses also examined the correlation between BMD changes and baseline values of bone turnover markers.

Patients using ≥ 2.5 mg/d glucocorticoids (prednisone or equivalent) for ≥ 90 days during the study represented the glucocorticoid subgroup; patients receiving bisphosphonates for any length of time during the study were included in the bisphosphonate group.

The glucocorticoid-receiving group included more men and the patients were slightly younger than the control group. They received a median dose of 5 mg prednisone at the beginning and 4 mg at the end of the study. The patients in the bisphosphonate group were a little older than the non-receiving patients. The disease characteristics and mean RA duration of 11 years did not differ between the groups. Also the BMD T-scores were similar in both groups and within the normal range. The median levels of bone turnover markers differed and were lower in patients receiving bisphosphonates compared to those who did not (sCTx-I, 0.2 ng/ml vs 0.40 ng/ml; P1NP, 26.4 – 35.6 µg/l vs 43.0 – 46.4 µg/l).

Analyses at 6 and 12 months showed a significant increase in BMD in denosumab-treated patients compared to placebo at the lumbar spine and hip ($p < 0.001$), and a similar increase of BMD in denosumab-treated patients at the spine and total hip in the glucocorticoid-group and bisphosphonate group. Levels of sCTx-I and P1NP were rapidly and consistently reduced by denosumab in all groups. The correlation between P1NP baseline level and BMD increase over the course of treatment (regression correlation coefficient of baseline P1NP: 0.05, 95 % CI 0.02 to 0.07, $p < 0.0001$, at lumbar spine; regression correlation coefficient of baseline P1NP: 0.02, 95 % CI 0.00 to 0.04, $p = 0.03$ at total hip). There was no correlation or difference in clinical fracture incidence and type, indication for anti-TNF-rescue treatment or rates, and types of adverse events between the different treatment groups. Importantly, no cases of ONJ were reported.

Use of glucocorticoids at a dose of ≤ 15 mg/d appeared not to alter the denosumab-effect on BMD in this study. The bisphosphonate-receiving patients showed a lower baseline level of sCTx-I compared to the other groups. The increase of BMD under denosumab treatment was higher compared to bisphosphonates alone, an effect already documented in other phase III study of postmenopausal osteoporosis (Kendler et al. 2008).

The results suggest denosumab as a new therapeutic option for preventing bone loss in RA, even additionally after bisphosphonate treatment. Caution should be warranted due to the low group sizes.

10.3 Denosumab in Metastatic Bone Disease, Multiple Myeloma and Primary Bone Tumor

10.3.1 Denosumab in Metastatic Bone Disease

There are several types of cancer which metastasize into bone in their advance stage. 75 % of patients with advanced prostate and breast cancer and more than 95 % of patients with multiple myeloma develop bone metastases and suffer from subsequent complications, including bone pain, hypocalcemia, pathological fractures at metastases-affected sites of bone, and spinal cord compression, which lead to the need for radiation, surgery, and additional stay in the hospital (Coleman 1997, 2001). Additionally, high doses of painkillers are the current standard of treatment in this state of disease followed by a systemic therapy with chemotherapeutics, and local radiation or surgery to bone. Bisphosphonates are used for maintaining skeletal integrity as well as reducing the pain by inhibiting bone metabolism (Berenson et al. 2002; Brody and Mancini 2002; Hillner et al. 2003). However, there are limitations in bisphosphonate therapy leading to contra-indication in patients with impaired renal function or gastrointestinal problems. Also some patients do not respond to bisphosphonates, as shown by data based on measurement of marker of bone turnover (urinary N-telopeptide uNTx). Despite intravenous bisphosphonate therapy, about 20 % of the patients have a persistently increased bone turnover and therewith an increased risk for fractures, progression of bone lesions and death (Coleman et al. 2005).

Levels of bone turnover marker, including uNTx, sCTX, P1NP, osteocalcin, BSAP, and TRAP-5b are reflecting the increase of bone turnover in all types of osteoblastic and osteolytic lesions and the consequent increase in malignant bone processes such as metastases or primary bone tumors (Brown et al. 2003, 2005; Costa et al. 2002).

Bone destruction in osteoblastic and osteolytic metastasis is mediated by activated osteoclasts. Their activation is induced by an increased RANKL and decreased OPG expression by osteoblasts (Demers et al. 2000; Lacey et al. 1998) and it is suggested that tumor cells induce osteoclasts by expression of RANKL (Roodman 2001; Fizazi et al. 2003). Activated bone-resorbing osteoclasts release growth factors from the extracellular matrix, which subsequently stimulate tumor growth and further bone destruction, a process that has been termed vicious cycle (Roodman 2004; Yang et al. 2001). RANKL, as the target of treatment in metastatic bone disease, is suggested to be effective in reducing bone destruction and tumor growth as well. Denosumab as specific antibody to RANKL is consequently an auspicious drug.

A randomized, double-blind, double-dummy multicenter study evaluated the efficacy and safety of denosumab therapy in patients with bone manifestations of breast cancer or multiple myeloma (Body et al. 2006). This small numbered-study evaluated 29 patients with breast cancer and 25 patients with multiple myeloma who had radiologically confirmed osteolytic or mixed bone lesions. Four different dosages of denosumab s. c. (0.1, 0.3, 1.0 and 3.0 mg/kg BW) and pamidronate i. v. 90 mg have been compared in their effect over a time period of 84 days. The sup-

pression of bone erosion was measured by the decrease of serum levels of s-NTX. The baseline level of NTX was higher in patients with breast cancer than in patients with myeloma. The median percentage of skeletal involvement was similar in both groups. The duration of the suppression of the bone resorption by denosumab showed to be dose-dependent. In patients receiving 0.1 mg/kg denosumab, the levels of NTX in urine and serum decreased within 24 h after application of denosumab, but returned to baseline values after 21 days. Patients receiving 1.0 or 3.0 mg/kg showed a suppression of NTX levels throughout the whole duration of the study (84 days). The effect of pamidronate was delayed compared to denosumab with a significant decrease of uNTX levels at day 3. In this group, the level rose again after 28 days. The pattern of effects was similar in patients with breast cancer and with multiple myeloma. Changes in sNTX were not as clear as uNTX.

A randomized open label multicentre phase II trial evaluated the benefit of denosumab after i. v. bisphosphonate therapy regarding continuation of this therapy (zoledronic acid or pamidronate). Included were patients with bone metastases of breast, prostate or other cancers, who had at least one histologically confirmed bone metastasis and uNTx levels higher than 50 nmol/l/Cr despite therapy with bisphosphonates. The participants were divided in groups by the origin of their metastases and level of their uNTX and after that randomized to discontinue the previous treatment and receive s. c. denosumab 180 mg every four weeks, or every 12 weeks or continue the treatment with i. v. bisphosphonates every four weeks. The end point of suppression of uNTx level lower than 50 nmol/l/Cr at 13 weeks was achieved by 71 % in the denosumab group and only by 29 % in the bisphosphonate group ($p < 0.001$). Also the proportion of patients who maintained the uNTx level below the mark of 50 nmol/l/Cr was higher among the denosumab treated patients. At week 25 64 % of the denosumab group compared to 37 % in the bisphosphonate group achieved the end point ($p < 0.01$). The effect of denosumab was consistent across tumor types and baseline uNTX levels. The median time for gaining the endpoint was nine days for denosumab and 65 days for bisphosphonates. Denosumab also suppressed other markers of bone turnover with a sustained duration to 25 weeks. SRE's occurred in 8 % in the denosumab group and in 17 % in the bisphosphonate group, AEs were similar (Fizazi et al. 2009).

Denosumab and bisphosphonate effects have also been evaluated in larger study groups such as published by Lipton et al. (2008), who compared denosumab to zoledronic acid, pamidronate and ibandronate in 225 bisphosphonate-naive women with breast cancer metastases in bone. They were randomized to receive i. v. bisphosphonate 4-weekly-i.v., s. c. denosumab 4-weekly (30, 120 or 180 mg) or 12-weekly (60 or 180 mg) open labeled. Next to the primary endpoint of percentage change in uNTX/Cr to baseline at 13 weeks, the percentage change in uNTX/Cr in week 25, the percentage of patients reaching a 65 % reduction in uNTX/Cr, and SRE as further endpoints had been evaluated. The uNTX/Cr level was suppressed by all dosages of denosumab within 2 weeks, and the effect lasted until week 25. Denosumab-receiving patients had a suppression of 73 % (13 weeks) to 75 % (25 weeks) of baseline uNTX compared to 79 % (13 weeks) and 71 % (25 weeks) suppression in bisphosphonate-receiving patients. Bisphosphonate and denosumab achieved a

similar amount of patients who reached the 65 % reduction of uNTX from baseline (46 % bisphosphonates to 52 % denosumab) and also the number of patients experiencing SRE was similar (12 % in denosumab-treated patients, 16 % in bisphosphonate-treated patients). AEs were reported by 95 % of patients in each group. Most often, these were referred to the advanced state of cancer in the study group. Denosumab-receiving patients complained mostly about nausea, vomiting and diarrhea, whereas the bisphosphonate-treated patients complained mostly about arthralgia, asthenia, nausea and flu-like symptoms. Denosumab seemed to reduce bone metastases-related risk similar to bisphosphonates and presented a safety profile, which fit in the expectations of AEs in a population with advanced malignant disease receiving systemic therapy.

Furthermore, denosumab has been compared to zoledronic acid (ZA) in a double-placebo controlled study with 2,000 patients with metastasized breast cancer. The patients were randomized to receive denosumab 120 mg or zoledronic acid 4 mg to evaluate the safety with the first SRE as primary endpoint. The study lasted 34 months. Denosumab delayed the occurrence of the first SRE significantly compared to ZA (HR 0.82, 95 %, CI 0.71–0.95, $p = 0.01$). The median time to first SRE in ZA was 806 days. Myalgia, arthralgia, and renal complications occurred in denosumab-treated group to a lesser extent than in the ZA-treated group. Osteonecrosis of the jaw occurred in 2 % in denosumab-treated patients and in 1.4 % in ZA-treated patients ($p = 0.39$). There were no differences in overall survive and time to disease progression (Stopeck et al. 2009).

Another study in patients with bone-metastasized solid tumors excluding breast and prostate cancer or multiple myeloma evaluated 1,776 patients and randomized them to denosumab 120 mg or ZA 4 mg. Again the time point of first SRE was in ZA prior to denosumab-treated patients with median time of 16.3 months to 20.6 months ($p = 0.06$). The profile of adverse events was similar as in the previously described study (Henry et al. 2009).

Focusing on women with breast cancer, a subgroup of these patients is of special interest for a new drug to prevent bone resorption. Postmenopausal women who have an hormone receptor-positive breast cancer nearly all receive an adjuvant aromatase inhibitor therapy to reduce the risk of tumor recurrence and increase survival (Thürlimann et al. 2005; Coombes et al. 2004; Group TAT 2006; Howell et al. 2005). While inhibiting the aromatization of the androgenic hormone precursor into estrogens, this intervention increases RANKL-dependent osteoclast activation and bone resorption and as a result increases the risk of fracture in these patients (Coates et al. 2007; Coleman et al. 2007; Eastell and Hannon 2005; Eastell et al. 2008). A phase III placebo-controlled study evaluated the effect of denosumab on BMD in 252 women (age ≥18 years), who underwent adjuvant aromatase inhibitor therapy and showed a low bone mass but no osteoporosis. After stratification by the duration of aromatase inhibitor therapy (longer versus shorter than 6 months) they randomly receive either denosumab 60 mg every 6 months or placebo. In addition all patients received supplementary calcium and vitamin D. The primary endpoint of the study was the percentage change of BMD at the lumbar spine at 12 months. The denosumab-treated

patients had a significant ($p < 0.0001$ each) increase in BMD by 5.5 % at 12 and 7.6 % at 24 months compared to the placebo-receiving group. The improvement in BMD started one month after the first application of denosumab and was not influenced by the previous duration of aromatase-inhibitor therapy. Secondary endpoint also showed an increase of BMD at other sites of the skeleton and a decrease of bone turnover marker under treatment with denosumab. The adverse events did not differ in incidence between the groups. The observations in this study demonstrate the potential benefit of adjuvant denosumab-treatment in drug-dependent bone loss in women obtaining aromatase-inhibitor for breast cancer (Ellis et al. 2008).

Subgroup-analyses evaluated the factors that might influence the BMD at the observed skeletal sites in these patients. The focus was on duration and type of the aromatase inhibitor, tamoxifen use, age, time since menopause, body mass index, and t-score. The baseline characteristics were well balanced in both treatment groups. Although the results of this analysis should be interpreted with caution because of small subgroup size, they show a consistent effect of denosumab in all subgroups (Ellis et al. 2009).

Taken together, denosumab presents itself as effective in reducing BMD with a promising SRE-profile and administration technique.

10.3.2 Denosumab in Multiple Myeloma

About 1 % of all cancers are multiple myeloma (Ries et al. 2007). Bone destruction by this bone marrow malignancy is one of the devastating consequences of multiple myeloma and since nearly all patients with multiple myeloma eventually relapse or develop resistance to therapy, preservation of bone stability and minimization of pain-causing osteolysis are important goals. The severity of bone destruction correlates with the progression of the disease and the known mechanisms of tumor growth and bone destruction suggest a vicious circle of osteoclastogenesis and osteolysis and the stimulation of tumor cell growth mediated by paracrine mechanisms or by cell-cell-interactions (Pearse et al. 2001; Terpos et al. 2003; Yaccoby et al. 2002, 2004). With an increased RANKL/OPG ratio, denosumab is expected to be a promising drug for both inhibition of bone destruction and slowing the progress of the malignant process itself.

A proof of concept study was designed as a single arm trial to evaluate the effects of denosumab on the M protein level in patients with relapsed or plateau-phase multiple myeloma. 96 (–1) patients (53 relapsed, 43 (–1) plateau-phase) were included and suggested to receive a minimum of six 28-day cycles of denosumab treatment. Previous treatment with bisphosphonates was 34 % in relapsed and 51 % in the pp-patients. 68 % discontinued the study because of progression of the cancer.

Denosumab suppressed bone resorption in both groups, represented by lowered levels of sCTx compared to baseline. A reduction of serum M levels in the defined range has not been seen in the relapse-group. However, stable disease was observed in 21 % of the patients. The progression free survival was 2.7 months. Also the M

protein level did not drop in the range in the plateau-group, 46 % patients showed stable disease. The median progression free survival was 8.0 months. Moreover, no significant reduction of the M protein was seen and an impressive amount of the 21 % patients who started with a progressive disease maintained a stable state for a maximum of 16.5 months and 46 % of plateau-phase multiple myeloma were in a stable state for maximum 18.5 months. Also the majority experienced less pain under treatment, although it depended on the dimension of pain in the beginning and a higher life quality in the domains of physical, functional, social/family, and emotional well-being (Vij et al. 2009).

Phase I ad II clinical trials present denosumab as effective in reducing bone resorption in patients with multiple myeloma or breast cancer. Biochemical markers of bone resorption (uNTX, sCTx) decreased under treatment with denosumab in a dose-dependent manner (Body et al. 2006; Lipton et al. 2007).

10.3.3 Denosumab in Prostate Cancer

The most common cancer in men is prostate cancer (Parkin et al. 2005). The standard therapy is dependent on the state of the disease. Nearly all metastasized prostate cancers go along with metastatic bone destruction. The standard therapy for metastasized prostate cancer is androgen ablation caused by ether bilateral orchiectomy or GnRH agonists. GnRH agonists are also used in the non-metastasized state of prostate cancer (Heidenreich et al. 2005; Loblaw et al. 2007; Sharifi et al. 2005). The deprivation of endogenous androgens improves the disease-free and

Table 2 Randomized, multicenter, 2-year, phase-III-study in men receiving androgen-deprivation therapy for prostate cancer. Effect of denosumab compared to placebo on BMD at different sites, new fracture and serum-level of bone turnover marker

Denosumab effect on	% Compared to placebo	Significance
BMD lumbar spine	+ 5.6	$p < 0.001$
BMD total hip	+ 4.8	$p < 0.001$
BMD femoral neck	+ 3.9	$p < 0.001$
BMD distal 1/3 radius	+ 5.5	$p < 0.001$
BMD whole body	+ 4.0	$p < 0.001$

Effect on (36 month)	Denosumab	Placebo	Significance
New vertebral fracture	1.5 %	3.6 %	$p = 0.006$
Any fracture	5.2 %	7.2 %	$p = 0.10$
sCTX	– 45 %	– 13 %	
P1NP	– 61 %	– 18 %	
TRAP5b	– 33 %	– 8%	

overall survival and is also used in patients with recurrently increasing PSA after initial therapy. The unwanted side effects of this therapy are an increase in bone resorption and therewith reduction of bone density and increasing fracture risk (Higano 2008; Michaelson et al. 2004; Shahinian et al. 2005; Smith et al. 2001, 2005). The effect on bone is dependent on dose of GnRH agonists and duration of therapy (Shahinian et al. 2005; Smith et al. 2005) and is an important factor of therapy-associated mortality. Bisphosphonates and selective estrogen receptor modulators have already been tried as inhibitors of therapy-associated bone loss, but as they reduce bone resorption, no effect on the heightened fracture incidence was demonstrated (Greenspan et al. 2007; Michaelson et al. 2007; Smith et al. 2001, 2003, 2008). Because of its data-based effect in increasing bone density by reduction of bone loss in postmenopausal women and in women receiving aromatase inhibitor therapy for breast cancer, denosumab is an excellent candidate for use in prostate cancer patients with therapy-induced bone loss.

The randomized phase III trial published by Smith et al. (2009) yielded the expected data for accreditation of denosumab for use in men to treat bone loss caused by androgen deprivation. The double-blind multicentre study randomized 1,468 men undergoing androgen-deprivation therapy for non-metastatic, hormone-sensitive prostate cancer to receive either 60 mg denosumab s. c. 6 monthly or placebo. The patients were stratified according to age and duration of androgen-deprivation therapy and were instructed to take at least a minimum amount of supplementary vitamin D and calcium. 912 patients (62.1 %) completed the study. Primary endpoint was the percent change in BMD at 24 months. Also the percent change in BMD at the femoral neck and at the total hip at 24 months and the percent change in BMD at 36 months at the three sites were assessed as secondary end points, as well as the incidence of new vertebral fractures at 36 months, the time to first clinical fracture, and safety events.

At all measured skeletal sites denosumab increased BMD significantly compared to placebo. A significant difference between both groups was seen at one month and was maintained throughout the study. The effect of denosumab was documented in all subgroups. With the increase of BMD the incidence of new vertebral fractures decreased about 62 % under treatment with denosumab compared to placebo. In addition, the levels of markers of bone resorption decreased significantly in the denosumab group compared to placebo. Adverse events were 87 % for both groups, whereas cataracts developed only in the denosumab group. New primary carcinomas were seen in both groups with an incidence of 5 % each. Discontinuation of treatment because of adverse events was similar in both groups (6.7 % denosumab, 6.5 % placebo). Serious adverse events occurred in 34.6 % of the denosumab-treated group and 30.6 % in the placebo group. Infection-related SREs occurred in 5.9 % under denosumab treatment and in 4.6 % under placebo-treatment, cardiovascular events were nearly similar in both groups and also deathrates (6 % each). None of the deaths in the denosumab group was considered to be treatment-related. In both groups no delays of healing, no cases of osteonecrosis of the jaw, or detection of neutralizing antibodies were reported.

In conclusion, denosumab achieved an increase in bone density at all skeletal sites, reduced the incidence of new vertebral fractures in men receiving androgen-deprivation therapy by biannual administration, and showed an acceptable safety profile. Ongoing trials now examine the ability of denosumab (1) to prevent bone metastases in castration-resistant prostate carcinoma and (2) to prevent SREs in patients with such carcinoma including bone metastases.

10.3.4 Denosumab in Giant Cell Tumor

The giant cell tumor (GCT) is a primary osteolytic tumor of the bone. Other names are giant cell myeloma or osteoclastoma. It is a very rare tumor and typically occurs in skeletally mature individuals, more frequently in women than in men. The tumor is characterized by multinucleated giant cells, osteoclast-like cells and is originally a benign tumor and shows a slow development. When turning to malignancy (5–10 %) metastasis in the lung may occur. In 50 % it is localized at the knee or the long bones. The tumor causes lytic lesions at the epiphyseal site of the bone and grows to the articular surface. Patients usually suffer from pain and limited joint function by decreased joint space and bone and joint destruction. Nervous impairment caused by narrowing of the spinal space can sometimes occur. The tumor can be asymptomatic until the first pathologic fracture. The usual treatment is the curettage of the tumor. Patients who cannot undergo surgery are treated with radiation. After treatment the recurrence of the tumor or a malignant transformation by radiation are possible. 80 % of the GCT cannot be treated by surgery (Bullough et al. 2002; Mendenhall et al. 2006; Szendroi et al. 2003; Szendroi 2004; Zheng et al. 2001).

As the giant tumor cells and their precursors express RANKL (Roux et al. 2002), denosumab is evaluated by its effect of reducing fracture risk, tumor recurrence after surgery and tumor growth by inhibiting its aggressive osteolytic activity. An open-label phase II study presents the data of denosumab effects in patients with unresectable or recurrent GCT (Thomas et al. 2010). 37 patients were included into the study, all with recurrent or unresectable GCT. They received denosumab 120 mg monthly (28 days) with a loading dose on day 8 and 15 in the first month. Primary endpoint was the tumor response with elimination of 90 % of giant cells or no radiological progression up to week 25. In addition, all patients received daily calcium and vitamin D and were not allowed to additional treatment of the GCT. The denosumab administration was discontinued when tumor progressed without any benefit, the tumor was resected, or the patient decided so. All of the 20 patients who were histologically assessable for efficacy and ten of the 15 patients who were radiologically assessable for efficacy showed a tumor response at week 25 (86 % of evaluated patients). In histology nearly all patients showed a nearly complete removal of giant cells. The radiologically-assessed patients showed a reduction in FGD uptake in FDG-PET. The reduction in FDG-uptake was positively correlated to histologically assessed new bone formation and increased bone mass measured by CT and DXA-bone-scan. Markers of bone resorption (uNTX, sCTX) decreased rapidly and substantially in about 28 days after the first dose of denosumab. Addi-

tionally to these visible effects of denosumab, 84 % of the patients experienced reduction in pain or improvement in mobility, and 29 % had bone repair. Adverse events had been reported in 33 patients (of 37), mostly as pain in the extremity or back pain and headache. No SRE was reported to be treatment-related and only a single more severe adverse event without pregnancy-related elevation of βHCG was documented in one patient, caused by high-grade sarcoma.

In the evaluation of the efficacy and safety of denosumab the study is limited by its small sample size, its design, the selected group, and the short duration, although the study is ongoing for evaluation of survival. Additional studies are needed to free the effect of denosumab of potential confounders. Nevertheless, this study underlines the potential of denosumab in inhibiting malignant bone destruction.

In conclusion denosumab is a promising drug for preventing bone loss. The suppression of bone resorption and the increase of BMD are present early after first administration. The effect is sustained under continuation of treatment and reversible after stopping denosumab administration. The 6-monthly s. c. dose of 60 mg denosumab is efficient to reduce the risk of pathological fractures in women with postmenopausal osteoporosis. The effect on BMD and bone turnover of denosumab is mostly greater than bisphosphonates, although not all bisphosphonates have been compared to denosumab. In active erosive RA and in addition to methotrexate, denosumab increases the positive effect of preventing further bone erosion and bone turnover. Additional treatment with denosumab in malignant disease with bone manifestations is supposed to help to reduce primary and secondary bone loss.

The safety profile is acceptable with less gastrointestinal side effects than bisphosphonates. Incidence of infections or malignancies did not differ compared to placebo in most studies. Further clinical trials are needed, especially for long-term safety.

10.4 Usage of Denosumab, Safety and Precautions

During therapy with denosumab additional administration of calcium and vitamin D is crucial, especially because of documented loss of serum calcium under treatment with denosumab. The risk of hypocalcaemia is increased in patients with impaired renal function. Therefore control of serum calcium levels under treatment with denosumab is suggested and as well the adequate substitution of calcium in patients with hypocalcaemia before the start of treatment.

Among acquired infections under treatment with denosumab, special attention should be given to infections of the subcutaneous tissue and should lead to consultation of a doctor.

The frequently monitored osteonecrosis of the jaw under treatment with bisphosphonates may also occur under treatment with denosumab, usually with dosages above 60 mg/month and in patients with progressive malignant diseases. Risk factors include additional therapy with chemotherapeutics, anti-angiogenic biologicals, corticosteroids and radiotherapy, bad care of tooth and oral health and co-

Table 3 Documented side effects of denosumab at a dose of 60 mg 6-monthly s.c. in use for the treatment and prevention of osteoporosis in postmenopausal women and treatment and prevention of bone loss in women and men receiving hormone therapy for either breast cancer or prostate cancer

Organ system/class of disease	Frequency	Side effects
Infections	≥1/100, <1/10 ≥1/100, <1/10 ≥1/1000, <1/100 ≥1/1000, <1/100 ≥1/1000, <1/100	Urinary tract Upper respiratory tract Diverticulitis Subcoutanous tissue ear
Metabolism	< 1/10000	hypocalcaemia
Nerval system	≥1/100, <1/10	affection of nervus ischiadicus
Eye	≥1/100, <1/10	cataract
Gastrointestinum	≥1/100, <1/10	obstipation
Cutis and subcutis	≥1/100, <1/10 ≥1/1000, <1/100	skin rash eczema
Skeletal muscles, soft tissue, bone	≥1/100, <1/10	limb pain

morbidities like anemia, systemic and local infections, and coagulopathy. A status evaluation of dental health, meticulous dental hygiene, and preventive treatment should take place before treatment with denosumab.

Treatment with denosumab is not recommendable in pregnancy because of possible influence in fetal immune system development (http://www.amgen.com/patients/products_prolia.html).

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Osteoimmunological Aspects of Periodontal Diseases

11

Kristina Bertl, Peter Pietschmann, Michael Matejka

11.1 Introduction

Inflammatory processes in the proximity of bone tissue have been shown to modify bone metabolism. Under homeostatic conditions bone resorption by osteoclasts is closely followed by approximately the same amount of bone formation by osteoblasts, known as the coupling of bone formation and resorption (Parfitt 1982). Yet, inflammatory cytokines may disturb this delicate equilibrium by enhancing differentiation and activation of osteoclasts. The occurring bone loss can more likely be attributed to increased bone resorption than reduced bone formation and the osteoclasts are considered to be the principal responsible cells (Biancu et al. 1995; Bromley and Woolley 1984). Such inflammatory osteolysis is observed in rheumatoid arthritis, osteomyelitis, bone surrounding a loosened joint prosthesis or marginal and apical periodontitis.

Periodontitis is a chronic infectious inflammatory disease. The presence of bacteria in a biofilm on the root surface of the teeth triggers an immune response of the innate as well as of the adaptive immunity. Yet, the biofilm provides a convenient and rather safe environment for the bacteria and thus they are well protected against the host's defense mechanisms. First clinical signs appear as reversible gingivitis with the typical "cardinal signs of inflammation": erythema, edema, heat, pain and more or less loss of function. Depending on several influencing factors (e. g. amount of bacteria, type of bacteria or host susceptibility), the equilibrium between the immune system and the bacterial invasion, which is more or less present in gingivitis, might be disturbed. Next, the inflammatory process expands into the proximity of alveolar bone (Fig. 1a, b) where differentiation and activation of osteoclasts is promoted. The resulting uncoupling of bone formation and resorption leads to increased alveolar

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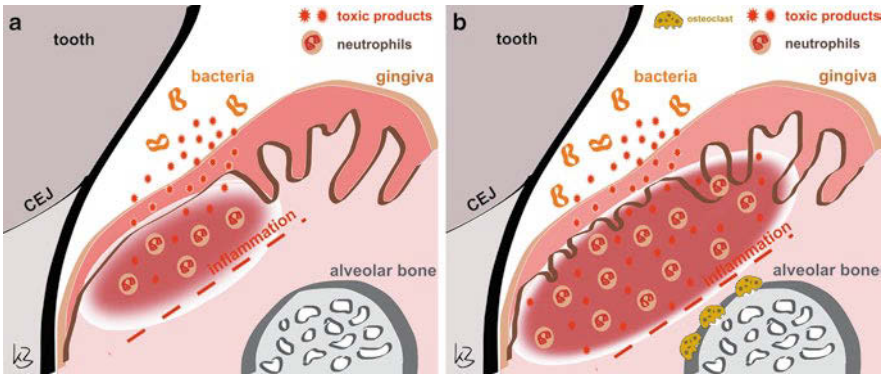

Fig. 1 (A) Inflammatory reaction is restricted to gingiva; (B) inflammatory process reaches proximity of the alveolar bone and induces osteoclastogenesis and alveolar bone loss. Modified by Graves et al. (2010) and Schröder and Lindhe (1981). CEJ: cemento enamel junction

bone loss, which is the cardinal sign of periodontitis and might ultimately lead to tooth loss. Consequently, the immune system, which is trying to stop the bacterial invasion, is responsible for the destruction of soft and mineralized periodontal tissue. The presence of the bacteria is "only" required for disease initiation, but not sufficient for development (Graves 2008; Graves et al. 2008). This interaction and communication between immune and bone cells displays the role of osteoimmunology in the pathogenesis of periodontitis. With a prevalence of 52 % of moderate and 20 % of severe periodontitis in patients aged 35 to 44, which is even increasing in higher age cohorts (Holtfreter et al. 2010), periodontitis is one of the most important causes of tooth loss and the most prevalent form of bone pathology. In contrast to most other diseases in the field of osteoimmunology, such as osteoporosis or rheumatoid arthritis, the immune system has a dual role in the pathology of periodontitis. On the one hand, it is the destructive part, whose intense response is responsible for tissue degradation. On the other hand, it is necessary to control the infection and the bacterial invasion adopting a protective attitude. Cells, which are present at periodontal active lesions, their mediators and enzymes, their protective as well as destructive potentials and the interaction of the immune and musculoskeletal system are discussed in the following section.

11.2 Anatomy of the Periodontium

The human integument provides a barrier against external influences. A leakage or gap in this barrier offers a weak point for infections. The teeth, penetrating the oral mucosa, are such a weak point, especially as the oral cavity is the habitat of over 500 different bacterial species. Yet, the anatomy and function of the tissue surrounding and supporting the teeth, the periodontium, is well adapted to resist most of those

external influences. The term "periodontium" is assembled from the Greek terms "peri-" (around) and "-odons" (tooth). It is composed of four main structures: gingiva, periodontal ligament, alveolar bone and root cementum. Although those four components are distinct in their structure, they act as a single unit and influence each other in healthy and diseased conditions. The gingiva mainly protects the underlying tissues from outside factors. The other three compounds of the periodontium are responsible for the support necessary to maintain function of teeth and are considered as the "attachment apparatus". The root cementum is histologically a part of the tooth, but is functionally integrated into the periodontium. Together with the bone lining the tooth socket (alveolar bone) the cementum incorporates the fibers of the periodontal ligament (Newman et al. 2006).

The extensive blood supply of the periodontium and the gingival crevicular fluid provide two important defense mechanisms. The gingival crevicular fluid, also termed sulcular fluid, is regarded as an inflammatory exudate, whose amount clearly increases at inflamed sites. It is diffusing through the basement membrane and the junctional epithelium into the sulcus. The junctional epithelium provides the attachment via hemidesmosomes to the tooth surface and forms a barrier against bacterial invasion itself. The gingival crevicular fluid contains enzymes, antibodies as well as neutrophils and displays the first defense mechanism against the bacterial attack (Newman et al. 2006; Pollanen et al. 2003).

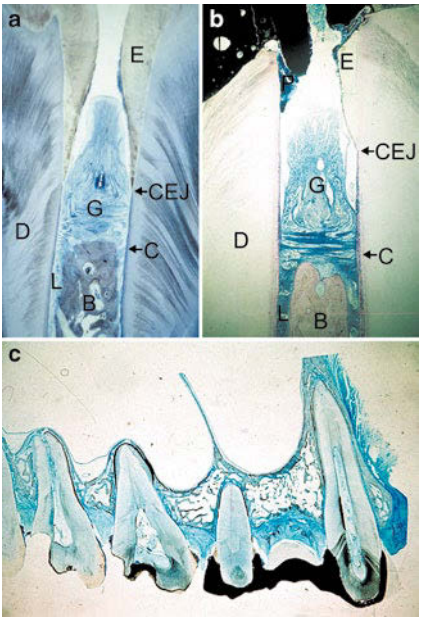

Fig. 2 Thin ground sections of two adjacent teeth and alveolar bone in a periodontally healthy (A) and diseased (B) condition. (C) Thin ground section of the upper jaw. Staining: toluidin blue. Abbreviatons: B, alveolar bone; C, root cement; CEJ, cemento-enamel junction; D, dentin; E, enamel; G, gingival fibers; L, periodontal ligament; P, plaque (due to overlapping crown). By courtesy of late Prof. Dr. mult. K. Donath

11.3 Etiology and Classification of Periodontal Diseases

Periodontitis is a chronic infectious inflammatory disease affecting the tooth-supporting structures. It is caused by a bacterial biofilm, which is mainly consisting of gram-negative anaerobic or microaerophilic bacteria (e. g. Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola or Aggregatibacter actinomycetemcomitans). This biofilm accumulates on the surface of teeth, first in supragingival and later on in subgingival regions. Microbial components are perceived as "danger signals" by cells of the innate immune system and their recognition initiates influx of neutrophils and expression of an array of inflammatory cytokines. Parts of the microorganisms, like lipopolysaccharide (LPS), bacterial DNA or peptidoglycan, are recognized by leukocytes and resident cells via toll-like receptors (TLR). Activation of TLR induces an intracellular signal pathway, the expression of transcription factors and subsequently the release of cytokines and chemokines. TLR-2 and -4 have been shown to be primarily responsible for sensing periodontal pathogens, like Aggregatibacter actinomycetemcomitans or Porphyromonas gingivalis (Kikkert et al. 2007). Peptidoglycans as well as atypical LPS from Porphyromonas gingivalis activate TLR-2, LPS from gram-negative bacteria activate TLR-4 and Aggregatibacter actinomycetemcomitans is able to activate both receptors (Akira and Takeda 2004; Ford et al. 2010). Although, the absence of TLR-2 and -4 may reduce the amount of alveolar bone loss in experimental periodontitis due to reduced inflammatory reaction, they are indispensable for controlling bacterial infection (Gelani et al. 2009; Nakamura et al. 2008). The excessive inflammatory response causes detachment of the junctional epithelium and its conversion into a pocket epithelium, loss of connective tissue and alveolar bone, and accumulation of chronic inflammatory cells. If these processes are left untreated, it leads definitely to the loss of the tooth itself. At early lesions mainly macrophages and T cells are present, suggesting a T helper (Th)1 cell response, while they are replaced by B and plasma cells in advanced lesions, suggesting a Th2 cell response. The extent, speed and severity of tissue destruction mainly depend on the intensity of the inflammatory response of the host and far less on the bacterial attack, which is primarily responsible for the disease initiation (Bartold et al. 2010; Brook 2003; Ford et al. 2010; Newman et al. 2006; Stabholz et al. 2010). Several risk factors, such as host specific, genetic or environmental factors (e. g. stress, smoking, poor oral hygiene or diabetes mellitus), are well known. This interaction of bacterial attack, immune response and risk factors was summarized by Page and Kornman (1997) and is illustrated below in Fig. 3.

The current classification system of periodontal diseases was established in 1999 at the International Workshop for the Classification of Periodontal Diseases and Conditions (Armitage 1999):

1. Gingival Diseases
2. Chronic Periodontitis
3. Aggressive Periodontitis
4. Periodontitis as a Manifestation of Systemic Diseases
5. Necrotizing Periodontal Diseases

6. Abscesses of the Periodontium
7. Periodontitis Associated with Endodontic Lesions
8. Developmental or Aquired Deformities and Conditions

This chapter will mainly discuss the aggressive and chronic form of periodontal disease. Patients affected are suffering from inflammation induced alveolar bone loss. The clinical differences and diagnostic criteria of aggressive and chronic periodontitis are given below. The biochemical and histological characteristics as well as the pathological process and the involved mediators and cells are more or less the same between the two disease entities. Yet, the onset, magnitude, extent and speed present rather clear differences. "Periodontitis as a Manifestation of Systemic Diseases" will be discussed later in this chapter.

Aggressive periodontitis is characterized by rapid attachment loss and bone destruction, which is inconsistent with the amount of microbial deposits. The affected patients are otherwise typically clinically healthy except for periodontitis and familial aggregation as well as phagocyte abnormalities and hyper-responsive macrophages are characteristical. Localized and generalized aggressive periodontitis are considered to be two different diseases. Localized aggressive periodontitis has a circumpubertal onset, a huge serum antibody response to infecting agents and at least two permanently affected teeth (first molars and/or incisors with interproximal attachment loss), but not more than two affected teeth other than first molars or incisors. Generalized aggressive periodontitis usually affects persons up to 30 years of age, but patients may be older. They present a poor serum antibody response to infecting agents, a pronounced episodic destruction of attachment and alveolar bone and a generalized interproximal attachment loss affecting at least three permanent teeth other than first molars or incisors (Armitage 1999; Armitage and Cullinan 2010; Tonetti and Mombelli 1999).

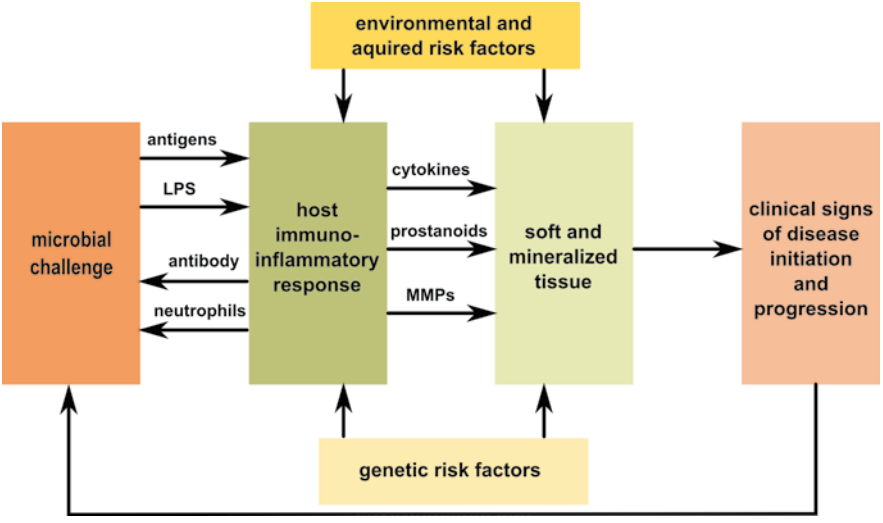

Fig. 3 Pathogenesis of periodontal disease. Modified from Page and Kornman (1997)

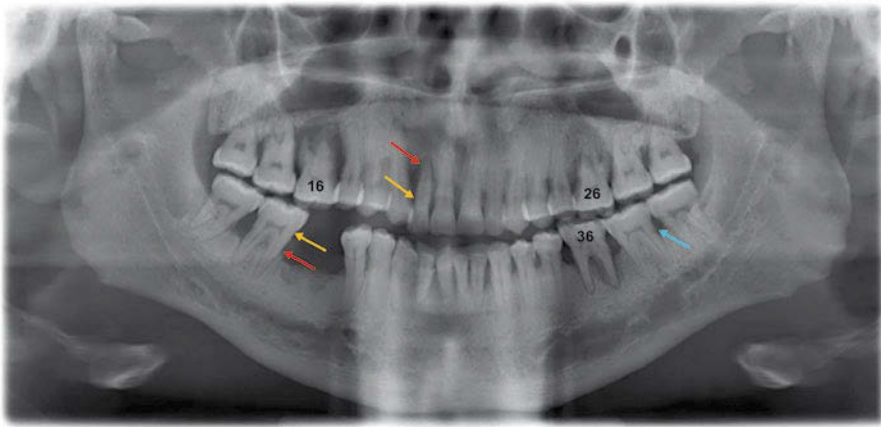

Fig. 4 Panoramic radiograph of a 24-year old man with severe generalized aggressive periodontitis. *Yellow arrows* are marking height of healthy alveolar bone, *red arrows* are marking the actual bone level of this patient. The *light blue arrow* is marking concrement, which is a rare finding in aggressive periodontitis. Severe bone loss is present around the first molars (16, 26 and 36–46 is already missing)

Chronic periodontitis, the most frequently occurring form of periodontitis, is normally prevalent in adults, but it may occur at a younger age as well. It is characterized by a consistence of the amount of destruction with the present local factors, the presence of subgingival calculus, a variable microbial pattern and a slow to moderate rate of progression with possible periods of rapid cycles. Localized and generalized chronic periodontitis are regarded as the same disease with a slightly different manifestation. The differentiation depends on the amount of affected sites, whether less or more than 30 % are afflicted (Armitage 1999; Armitage and Cullinan 2010; Flemmig 1999).

11.4 Skeletal Aspects

11.4.1 RANKL-RANK-OPG-System

Pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 or tumor necrosis factor-alpha (TNF-α), are elevated in tissues affected by periodontal disease. By now, an interaction of those cytokines with the receptor activator of nuclear factor "kappa-light-chain-enhancer" of the activated B cells ligand (RANKL)/receptor activator of NF-κB (RANK)/Osteoprotegerin (OPG) system is well described in the current literature. Increased amounts of IL-1 and TNF-α cause increased levels of RANKL, subsequently increasing osteoclastogenesis (Wei et al. 2005). Their inhibition exhibited promising results, including reduced bone resorption (Firestein 2003; Graves 2008). RANK activation by RANKL, a member of the TNF

family, is necessary for osteoclast formation, differentiation and activity. RANK is expressed on the surface of osteoclast precursors and the activation of RANK initiates their differentiation and maturation. This activation can be decreased by the soluble counterpart OPG. OPG acts as a decoy receptor by binding to RANKL and inhibiting the interaction between RANKL and RANK. It is expressed by gingival fibroblasts, periodontal ligament cells or epithelial cells. Consequently, a strict balance of this system is necessary to maintain equilibrium between bone resorption and bone formation and sustainment of structural integrity and calcium metabolism. Not unexpectedly in periodontitis, elevated levels of RANKL and/or decreased levels of OPG are detectable. This generates a RANKL/OPG ratio, which favours bone resorption (Bartold et al. 2010; Cochran 2008; Crotti et al. 2003). The RANKL/OPG ratio may differ between aggressive and chronic periodontitis. Both clinical forms present higher levels of RANKL in the gingival tissue. Yet, in the samples of patients suffering from the chronic form the level of OPG is higher than in those patients with aggressive periodontitis (Garlet et al. 2004). Similar differences were reported in the expression level of RANKL and OPG in tissue samples of gingivitis (without active bone resorption) and tissue samples of active periodontal lesions (with alveolar bone loss). Elevated levels of RANKL indicate an active periodontal lesion (Menezes et al. 2008). The results of human studies match the results of experimental periodontitis. In mice the expression level of RANKL correlated with the level of pro-inflammatory cytokines (IL-1ß, TNF-α, interferon-gamma (IFN-γ)) in a time period of active bone loss (Garlet et al. 2006). Osteoblasts, dendritic cells, B and T cells and resident cells, like periodontal ligament and gingival fibroblasts, have to be considered as important sources for RANKL in periodontal lesions (Belibasakis et al. 2007; Kawai et al. 2006). Regarding these findings, it is not surprising that in experimental periodontitis therapeutic intervention by administering OPG to block RANKL is able to prevent bone loss and could be regarded as a possible therapeutic strategy against alveolar bone loss (Jin et al. 2007; Teng et al. 2000).

11.4.2 Osteoclasts

Osteoclasts are multinucleated monocyte/macrophage lineage cells of hematopoietic origin and the only bone-resorbing cells. Osteoclast differentiation and activation is regulated by the RANKL/RANK/OPG system and the macrophage colony-stimulating factor (M-CSF) (Fuller et al. 1998; Lacey et al. 1998; Quinn et al. 1998; Yasuda et al. 1998). RANKL induces osteoclastogenesis by binding to its receptor RANK, which is expressed on osteoclast precursors. M-CSF promotes this step by stimulating proliferation of RANK expression on osteoclast precursors. The activation of RANK by RANKL is essential for the process of osteoclastogenesis, but can be prevented by a previous binding of OPG to RANKL (Boyle et al. 2003). In vitro mature osteoclasts can be differentiated from peripheral blood mononuclear cells cultured in a M-CSF and RANKL supplemented medium. In particular, they are mainly derived from the monocyte fraction (Bar-Shavit 2008; Faust et al. 1999; Massey and Flanagan 1999).

To date, it is still unclear why some patients remain in the stage of gingivitis for years, while others are progressing to periodontitis with alveolar bone loss. Discrepancies in peripheral osteoclastogenesis might be able to contribute to the progress of disease. If peripheral blood mononuclear cells contain a relatively mature proportion of osteoclast precursors, those would need less triggering at the inflammatory sites to finally differentiate into active mature osteoclasts. Spontaneous formation of osteoclasts (without stimulation with M-CSF and/or RANKL) out of peripheral blood mononuclear cells was detected from patients with chronic periodontitis, but is strictly dependent on the presence of T cells. T cell depleted PBMC cultures require the addition of M-CSF and RANKL to form osteoclasts. The directly isolated T cells overexpress RANKL and TNF-α and the addition of anti-RANKL and anti-TNF-α antibodies inhibit spontaneous osteoclast formation in a dose-dependent manner. In conclusion, in patients with periodontal disease the priming of the osteoclast precursors may already take place in peripheral blood (Brunetti et al. 2005; Tjoa et al. 2008). Directly at the periodontal lesion, cell-to-cell contact between periodontal ligament fibroblasts and osteoclast precursors seems to increase the expression level of osteoclastogenesis related genes (RANKL, RANK, TNF-α and IL-1ß) and consequently the formation of osteoclasts (Bloemen et al. 2010).

11.4.3 Osteoblasts

Many studies have demonstrated the influence of inflammatory cells on osteoclast precursors and osteoclastogenesis. However, less information is available on the effects on osteoblasts and bone formation. Yet, at least in vitro results are accumulating, that LPS from periodontal pathogens seems to impair osteoblastic cell differentiation and consequently bone formation simultaneously with the stimulation of bone resorption (Kadono et al. 1999; Roberts et al. 2008; Wang et al. 2010). TNF-α plays a role in the osteogenic part as well. LPS-induced inhibition of osteogenesis seems to be mediated at least partially via TNF-α (Tomomatsu et al. 2009b). Additionally, the activation of TLR on osteoblasts induces the release of factors such as RANKL and TNF-α, which are known to upregulate the process of osteoclastogenesis (Bar-Shavit 2008).

Further, not only osteoblasts themselves are impaired within the inflammatory process. Periodontal ligament cells (PLC) are located between the alveolar bone and the cementum and are essential for the maintenance and regeneration of periodontal tissue. PLC are considered to be multipotential cells and a possible source of osteoblasts. Their osteoblast-like properties have been shown in several studies as well as their ability to affect the RANKL/RANK/OPG system (Kook et al. 2009; Lee et al. 2009; Rodrigues et al. 2007). Recently, it could be demonstrated that, within the inflammatory reaction, PLC are impaired to differentiate into osteoblasts and thus bone formation and regeneration seems to be reduced (Joseph et al. 2010).

11.4.4 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of calcium- and zinc-dependent proteases, which are usually balanced by their inhibitors, tissue inhibitors of metalloproteinases (TIMPs). They are expressed in healthy periodontal tissue to control the physiological turnover of the extracellular matrix. The presence of inflammatory cytokines disregulates the MMP/TIMP system and leads to higher levels of MMPs and lower levels of TIMPs. This imbalance leads to an increased degradation of periodontal tissue. This phenomenon plays an important role in the progress of periodontal disease and is a possibe target for an adjuvant therapeutic approach (Giannobile 2008; Verstappen and Von den Hoff 2006).

11.5 Immunological Aspects

11.5.1 Monocytes/Macrophages

Monocytes, myeloid cells of the hematopoietic system, are leaving the bone marrow in a rather immature state already after two days. They are able to further differentiate into macrophages after leaving the blood and entering the tissue. Macrophages are typical phagocyting cells, which kill the pathogens through production of antimicrobial factors, such as myeloperoxidase, inducible nitric oxide synthase (iNOS) and nitric oxide (NO). Further, they ingest, process and present antigens to T cells (Newman et al. 2006).

The role of monocytes as osteoclast precursors in periodontal disease was already mentioned before. In addition to neutrophils, macrophages are the main phagocyting cells and a source of pro-inflammatory cytokines, such as TNF-α or IL-1, which promote the degradation of soft and mineralized periodontal tissue. Patients with periodontal disease may present a hyper-reactive phenotype of mononuclear cells, which release increased amounts of pro-inflammatory cytokines (Gustafsson et al. 2006; Newman et al. 2006).

11.5.2 Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes, differentiate more or less completely in the bone marrow before entering the blood flow. This enables them to produce an immediate response. They are the most abundant leukocytes in the blood, accounting for about two thirds of all blood leukocytes. Neutrophils leave the blood flow following a chemical gradient across the cell body and migrate into regions with higher concentrations (chemotaxis). Typical chemotactic factors for the neutrophils are IL-8, complement factor C5a or IFN-γ (Newman et al. 2006).

Neutrophils enter the periodontal tissue within the terminal blood circulation until they migrate through the endothelium following a chemotactic gradient. Sub-

sequently, neutrophils are able to enter the gingival crevicular fluid as an important part of the first defense. They phagocyte foreign microorganisms, upregulate the inflammatory reaction and form a "defense wall". The understanding of the role of neutrophils in the pathogenesis of periodontal disease has changed over time. They have been regarded as hypo-reactive as well as hyper-reactive. Hypo-reactive neutrophils weaken the first defense mechanism and the bacterial attack may overwhelm the residual host immunity. Neutrophil function can be impaired by defects in chemotaxis, transendothelial migration or phagocytosis. On the other hand, the intense response of hyper-reactive neutrophils not only degrades the invaders; it also causes collateral inflammatory tissue damage by an excessive activity, release of toxic products and elevated oxidative burst (Gustafsson et al. 2006; Newman et al. 2006; Ryder 2010; Shaddox et al. 2010). Defects in transendothelial migration or reduced amounts of neutrophils (e. g. due to cyclic neutropenia) may provoke periodontal attachment loss similar to aggressive periodontitis and will be discussed later (Ryder 2010).

11.5.3 B Cells

The function of B cells, lymphoid cells, is to recognize antigens using a high-affinity receptor, to process and present antigens (e. g. to T cells). In an inflammatory situation this may lead to clonal expansion and antibody secretion. Primarily, B cells express immunoglobulin (Ig) M, and after secondary antigen exposure high-affinity antibodies of the appropriate isotype can be produced. For example, neutrophils are only able to control the periodontal pathogen Aggregatibacter actinomycetemcomitans after opsonization of the microorganism by an antibody of the IgG isotype. In this case the other opsonizing factors, such as complement, LPS-binding protein or any other Ig isotype, are not effective (Newman et al. 2006).

B cells contribute to periodontal bone loss via expression of RANKL in response to periodontal pathogens. B and T cells make up the predominant mononuclear cell types in periodontally diseased gingival tissue and are considered as an important source of RANKL (Cochran 2008; Han et al. 2009; Kawai et al. 2006). Studies on animals have demonstrated the important role of B cells in the process of periodontal tissue destruction. B cell deletion leads to a remarkably reduced alveolar bone loss (Baker et al. 2009). Further, B cells in the absence of T cells are still able to promote periodontal lesions. This can be attributed to the high expression of RANKL by B cells (Han et al. 2006). Yet, the presence of B cells may not be irreplaceable. A study using mice reported that T cells, in the absence of B cells, are also able to further promote LPS-induced alveolar bone loss (Yamaguchi et al. 2008). Beside the increased production of RANKL, autoreactive B cells have been detected in periodontal tissue, which produce autoantibodies against components, such as collagen or keratin (Donati et al. 2009; Koutouzis et al. 2009). This displays the destructive role of B cells in the progression of periodontitis. Yet, B cells have a protective role too. Opsonization of periodontal pathogens by antibodies produced by B cells facilitates phagocytosis by neutrophils or macrophages or activates the complement sys-

tem (Guentsch et al. 2009; Teng 2006a). This is confirmed by an increased alveolar bone loss after antibody depletion of B cells in experimentally induced periodontitis (Graves 2008). Interestingly, mice severely affected by a combined immunodeficiency (complete deletion of B and T cells) experience less bone loss after being challenged with Porphyromonas gingivalis. These results indicate that lymphocytes may not be essential to protect the host against the periodontal pathogen, but that they even contribute to the alveolar bone loss as part of the immune system (Baker et al. 1999).

11.5.4 T Cells

T cells, a part of the adaptive immunity, use a low-affinity receptor for recognition of various antigens. T cells can be subdivided based on the expression of co-receptors (CD4 or CD8). CD8+ T cells are mainly cytotoxic cells, which control intracellular antigens (e. g. viruses). CD4+ T cells, the relevant T cell population for periodontal disease, initiate the immune response to occurring antigens. After further differentiation they can be divided into subpopulations (Mosmann et al. 1991). High IL-12 levels favour Th1 cell development and low IL-12 level favour Th2 cell development. Th1 and 2 subsets are separated based on their cytokine expression pattern. The Th1 cell subpopulation, which is characteristically cellular and pro-inflammatory, mainly expresses IL-2, IFN-γ, IL-12 and TNF-ß. Th2 cells, which tend to have anti-inflammatory attributes and support the humoral immunity, predominantly release IL-4, -5, -6 and -10 (Murphy and Reiner 2002; Newman et al. 2006).

Recently Th17 and regulatory T cells (Treg) have been found to play major roles in periodontal disease, beside the established positions of Th1 and 2 cells. CD8+ T cells are not directly involved in the process of periodontal tissue destruction (Cardoso et al. 2008; Gaffen and Hajishengallis 2008; Teng 2006b).

IFN-γ, the main cytokine expressed by Th1 cells, strongly increases the activity of phagocytes and induces expression of inflammatory cytokines and chemokines. High levels of IFN-γ were shown in periodontal lesions and increase with the severity of periodontal disease (Garlet et al. 2003; Honda et al. 2006). Similar results were reported in animal studies (Teng et al. 2005). On the contrary, IFN-γ seems to inhibit osteoclastogenesis in vitro (Ji et al. 2009). It seems now established that the proinflammatory effects of IFN-γ in vivo, such as the elevated expression of IL-1ß, TNF-α and RANKL, overbalance the direct anti-osteoclastogenic effects of IFN-γ, which were reported in vitro. Consequently, IFN-γ is considered to be a strong pro-inflammatory cytokine in vivo, which is enhancing osteoclastogenesis and alveolar bone loss (Gao et al. 2007; Garlet et al. 2008). Similarly to IFN-γ, contrary results have been reported on IL-12, another typical cytokine for Th1 cells, and its role in periodontal disease. Reduced levels of IL-12 have been reported for periodontal diseased tissues, while another study reported reduced levels of IL-12 in the gingival crevicular fluid after periodontal therapy (Johnson and Serio 2005; Thunell et al. 2010). Regarding the contradictory results on cytokines expressed by Th1 cells (IL-12 and IFN-γ) further studies are required to determine the exact role

and position of Th1 cells in the pathogenesis of periodontal disease, especially in aggressive cases.

IL-4 is the characteristic cytokine typically expressed by Th2 cells and promotes Th2 cell differentiation. IL-4 and IL-6, also expressed by Th2 cells, promote B cell activation, differentiation and antibody production (Appay et al. 2008; Cronstein 2007). It is assumed, that the number of B cells exceeds the number of T cells in periodontal lesions. The cytokine expression of Th2 cells favours B cell development and a strong Th2 cell response may lead to a high number of RANKL producing B cells in periodontally diseased tissue (Gemmell et al. 2002; Kawai et al. 2006). Beside this osteoclastogenesis promoting ability, IL-4 also has suppressive and anti-inflammatory properties. IL-4 is able to inhibit the expression of IFN-γ and to promote the expression of the anti-inflammatory cytokine IL-10 (Appay et al. 2008; Jarnicki and Fallon 2003). Further, IL-4 was shown to elevate the levels of TIMPs and OPG and simultaneously inhibit the production of MMPs and RANKL (Ihn et al. 2002; Saidenberg-Kermanac'h et al. 2004). Accordingly, the levels of IL-4 are lower in the gingival crevicular fluid of periodontally diseased patients when compared to the healthy control group (Pradeep et al. 2008). The Th2 cells might have a protective function via those functions of IL-4. Another protective role may be the Th2 cell induced humoral immunity. A longitudinal human study showed lower serum levels of IgG antibodies against Aggregatibacter actinomycetemcomitans or Porphyromonas gingivalis in patients with active periodontal lesions compared to patients being stable in a maintenance program (Rams et al. 2006). This overview on the available literature on the role of Th2 cells and their cytokines in periodontal disease underlines the controversary roles and the still open questions of this topic.

Th17 cells are CD4+ cells, which are expressing IL-6 and -17. Th17 cell differentiation is dependent on the presence of IL-23, which is expressed by monocytes. The presence of Th17 cells and IL-17 in periodontally diseased tissue was demonstrated recently. On the one hand, IL-17 is a pro-inflammatory cytokine, which is enhancing osteoclastogenesis. It seems to amplify the pro-inflammatory loop of IL-1ß, IL-6 and TNF-α expression and to increase the level of RANKL and MMPs (Cardoso et al. 2008; Ford et al. 2010; Kotake et al. 1999; Sato et al. 2006). On the other hand, IL-17 has a role in the defense mechanism against periodontal pathogens. The IL-17 receptor knockout mice present increased alveolar bone loss induced by Porphyromonas gingivalis (Yu et al. 2008). IL-17 seems to play an important role in the mobilisation and activation of neutrophils after pathogenic attack (Yu et al. 2007). Additionally, IL-17 seems to improve the responsiveness of TLR in human gingival epithelial cells (Beklen et al. 2009). This demonstrates the role of IL-17 in the recognition of a bacterial attack as well as in the proper response to it.

The simultaneous activity of Th1, 2 and 17 cells is unlikely, as those cells normally inhibit each other (Kelchtermans et al. 2009). The exact role of each Th cell subpopulation in periodontal disease needs further investigation. One limiting factor in studying this topic is the missing clinical parameter that displays active bone resorption at the moment of sample collection. Longitudinal studies with simultaneous determination of multiple cytokines may help to solve this problem.

The Treg cells represent a protective T cell subset, which are also present in periodontal tissue. The protective property of the Treg cells and their inhibiting influence on the progression of periodontal disease is mediated via the expression of IL-10 or transforming growth factor-beta (TGF-ß) (Cardoso et al. 2008). TGF-ß is an immunosuppressive factor, which is able to suppress pro-inflammatory cytokines, such as IL-1ß or TNF-α, as well as MMPs. A negative correlation between levels of TGF-ß and RANKL was shown in periodontally diseased tissue (Steinsvoll et al. 1999). The anti-inflammatory ability of IL-10 will be described in detail below. The mediating and protective role of Treg cells was confirmed in experimental periodontitis and their anti-inflammatory property does not influence defense mechanisms against bacterial attack (Garlet et al. 2010). Altogether, this demonstrates the anti-inflammatory and anti-resoptive properties of Treg cells.

In conclusion, Th 1 and 17 cells are pro-inflammatory and definitely play a destructive role in the degradation of periodontal tissue. On the other hand, the cytokine expression of these T cells is necessary in the defense pathway against the periodontal pathogens. They recruit and activate phagocyting cells. The role of the Th2 and B cells in the pathogenesis of periodontal disease is also controversial. IL-10 and Treg cells attenuate the periodontal infection.

11.5.5 Dendritic Cells

Peripheral dendrictic cells are professional antigen processing and presenting cells (Wolff 1972). After incorporating an antigen they transport it to the lymph nodes in order to conduct one of their main duties, i. e. to prime naive T cells. Langerhans cells are dendritic cells located above the basal layer of epithelial cells, e. g. in the skin or oral mucosa. Studies on Langerhans cells in the gingiva of periodontally diseased patients resulted in controversial findings, ranging from decreased to increased levels compared to periodontally healthy individuals (Ford et al. 2010; Newman et al. 2006).

Immature dendritic cells, which express RANK and the receptor for M-CSF (cFMS/CSF1R – colony stimulating factor 1 receptor), represent another source of osteoclast precursors. In the presence of M-CSF and RANKL they differentiate into osteoclasts and contribute directly to bone loss in inflammatory conditions. In the absence of RANKL they differentiate into mature dendritic cells and help to control the inflammation and bacterial challenge, but lose their osteoclastogenic potential (Liu et al. 2010). Further, RANKL influences the interaction between dendritic cells and T cells. RANKL augments the stimulation of naive T cells by dendritic cells (Anderson et al. 1997).

11.5.6 Complement System

The complement system is supporting the immune system through its ability to identify, opsonize or destruct complex pathogens through a membrane attack, or

to attract other immune cells. Binding of complement factors to foreign substances enables the immune system to detect those substances, for which they do not possess receptors (Newman et al. 2006).

The complement system may indirectly induce alveolar bone loss. The membrane attack complex (C5b-9) can lead to activation of phospholipase A2, release of arachidonic acid and especially synthesis of prostaglandin E2, which is a very potent well-known osteolytic substance (Klein and Raisz 1970). Further, the complement receptor 3, which is expressed on neutrophils or monocytes, is strongly activated by Porphyromonas gingivalis. This increases the recruitment of phagocytes into periodontal tissue and the expression of the main pro-inflammatory cytokines (IL-1ß, IL-6 and TNF-α). Together this promotes alveolar bone loss. The blockade of the complement receptor 3 in experimental periodontitis inhibits the induction of alveolar bone loss (Hajishengallis 2010).

11.6 Pro- and Anti-Inflammatory Mediators

11.6.1 IL-1, IL-6 and TNF-α

IL-1, IL-6 and TNF-α are well known to play a major role in peridontal disease and to trigger the inflammatory reaction and inflammation induced alveolar bone loss (e. g. by upregulating the expression of RANKL). They are expressed by cells of the periodontium, such as fibroblasts or epithelial cells, as well as by cells of the immune system, such as neutrophils or macrophages. One upregulates the other's production and vice versa. They stimulate the production of chemotactic factors and other pro-inflammatory mediators (e. g. prostaglandins), enhance osteoclastogenesis and matrix degradation by inducing MMPs, and induce apoptosis of matrix producing cells, which impairs the regenerative potential. The application of antagonists to these cytokines in experimental periodontitis significantly reduces inflammatory reactions and tissue destruction. Increased levels were detected in the gingival crevicular fluid as well as in the periodontal tissue and a positive correlation was reported with bone and tissue resorption markers, such as RANKL and MMPs (Cochran 2008; Garlet et al. 2006; Graves 2008; Graves and Cochran 2003). The role of those three cytokines in the process of inflammatory alveolar bone loss seems to be complementary, as the inhibition of all three enables an even more effective inhibition of the osteoclastic process (Graves 2008; Zwerina et al. 2004). Although RANKL is the essential ingredient for osteoclastogenesis, TNF-α induced osteoclast development and bone resorption occurs even in the absence of RANKL. Further, TNF-α not only increases bone resorption, but also reduces the repair by bone formation or rebuilding of connective tissue. In vitro, the proliferation of osteoblasts and the differentiation of osteoblast precursors, such as periodontal ligament cells, is significantly inhibited by TNF-α (Bartold et al. 2010; Graves and Cochran 2003; Graves et al. 2010; Lacey et al. 2009; Tomomatsu et al. 2009a).

While IL-1 and TNF-α are clearly destructive cytokines, IL-6 – besides its pro-inflammatory effects – may have a protective or regulatory function. IL-6 was shown to induce IL-1 receptor antagonist and TNF-α soluble receptor and therefore possibly regulates the production of IL-1 and TNF-α. This may provide a certain balance in the upregulation of the main pro-inflammatory cytokines (Irwin and Myrillas 1998).

11.6.2 Nitric Oxide

NO, previously known as "endothelial-derived relaxing factor", is synthesized during conversion of L-arginine to L-citrulline by NO-synthases (NOS). The endothelial and neuronal NOS are constitutively expressed whereas the inducible isoform (iNOS) is expressed on pro-inflammatory stimuli, such as cytokines (IFN-γ and TNF-α) or bacterial products. NO has an important role in vascular regulation, platelet aggregation and regulation of mineralized tissue function as well as in the pathogenesis of inflammatory diseases (Ugar-Cankal and Ozmeric 2006). In bone iNOS seems mainly to influence bone resorption. Animal studies investigating iNOS knockout mice reported a higher bone mineral density in the femur of the knockout mice compared to the wild type mice and a lower number of osteoclasts. The increase in bone mineral density was attributed to an increase in density and thickness of the cortical bone, while the trabecular structure was unaffected. The authors confirmed their results in vitro and explained the reduced bone resorption by a decreased expression of tumor necrosis factor receptor-associated factor-6 (TRAF-6), which is a major adapting molecule linking RANKL to osteoclastogenesis in bone marrow cultures of the iNOS knockout mice after M-CSF and RANKL treatment (Gyurko et al. 2005).

In periodontal diseases NO has a protective and destructive role and a controversial influence on osteoclastogenesis. The expression of iNOS is increased by inflammatory stimuli in neutrophils and macrophages as well as by cells of the gingival tissue (Alayan et al. 2006; Daghigh et al. 2002; Matejka et al. 1998). On the one hand, NO is part of the defense mechanism and helps to control the bacterial attack. Experimental periodontitis in iNOS knockout mice show an elevated inflammatory reaction compared to wild type mice. An increased amount of neutrophils migrated into the periodontal tissue. This suggests NO as an important part for an effective immune response to bacterial challenge (Alayan et al. 2006; Fukada et al. 2008). A clinical study demonstrating reduced amounts of NO2-, which is the stable metabolite of NO, in the saliva of patients with severe periodontal disease underlines this assumption (Aurer et al. 2001). Further, NO regulates osteoclast formation and activity. iNOS knockout mice show increased expression of RANK and reduced expression of OPG, which is displayed in increased alveolar bone loss, suggesting a bone-protective role of NO (Alayan et al. 2006; Fukada et al. 2008). On the other hand, an excessive expression of iNOS and its product NO may have destructive properties for the host tissue. Elevated levels of iNOS in periodontally diseased tissue have been reported (Lappin et al. 2000; Matejka et al. 1998). Excessive amounts of NO are toxic to various cells, such as fibroblasts or epithelial cells, and NO is

assumed to increase MMPs and reduce TIMPs (Brennan et al. 2003; Nguyen et al. 1992, Ugar-Cankal and Ozmeric 2006). In contrast to the above-mentioned studies on experimental periodontitis in mice, it was reported that Prophyromonas gingivalis failed to induce alveolar bone loss in iNOS knockout mice compared to wild type mice, suggesting an important role of NO in the development of osteoclasts (Gyurko et al. 2005). Further, a mediating role of NO in the process of osteoclastogenesis was reported as an autocrine negative feedback stimulated by RANKL (Zheng et al. 2006).

11.6.3 Lipid Mediators

Arachidonic acid, released by phospholipase A2 from membrane phospholipids, is further processed to either pro- or anti-inflammatory mediators. The pro-inflammatory mediators, like prostaglandins and leukotrienes, are well known and discussed in the literature. They induce recruitment of neutrophils, such as leukotriene B4, or osteclastogenesis and bone resorption, such as prostaglandin E2. Elevated levels in the gingival crevicular fluid of diseased patients have been detected (Offenbacher et al. 1986; Zhong et al. 2007). The anti-inflammatory lipid mediators, such as lipoxins, resolvins or protectins, are of interest due to their pro-resolving and anti-inflammatory properties (Garlet 2010; Graves et al. 2010). Pro-resolution is an actively regulated program in contrast to the passive determination of inflammation. Lipoxin A4, derived from arachidonic acid, is described to inhibit the expression of pro-inflammatory cytokines (IL-1ß, TNF-α) and to attenuate inflammatory reactions provoked by the periodontal pathogen Porphyromonas gingivalis (Van Dyke and Serhan 2003). Similar anti-inflammatory effects were described for protectin D1. Protectin D1 reduces the recruitment of neutrophils and T cells and the expression of pro-inflammatory cytokines, and promotes T cell apoptosis (Serhan et al. 2008). The recently discovered resolvin E1, derived from omega-3 eicosapentaenoic acid, strongly reduces experimental periodontitis and by binding to a distinct side seems to be more effective than the longer known lipoxin. Resolvin seems to regulate migration of neutrophils and to attenuate leukotriene B4-dependent pro-inflammatory signals. Consequently, the application of agonists of pro-resolution should be considered as a new therapeutic approach. Additionally, they are missing the unwanted side effects of the traditional anti-inflammatory approaches, like nonsteroidal anti-inflammatory drugs (Hasturk et al. 2006, 2007).

11.6.4 IL-10

Besides the huge amount of pro-inflammatory cytokines, IL-10 is one of the controlling and anti-inflammatory cytokines, which is expressed in periodontal tissue. The importance of IL-10 was demonstrated in IL-10 knockout mice, which presented a remarkably higher susceptibility against periodontal pathogens (Sasaki

et al. 2004). IL-10 seems to directly interfere with pro-inflammatory cytokines, such as IL-1, -17 or IFN-γ, and to restrict their pro-inflammatory activity (Jovanovic et al. 1998; Naundorf et al. 2009). Besides the interference with pro-inflammatory cytokines, IL-10 directly upregulates TIMPs and OPG, which reduces the soft and mineralized periodontal tissue destruction and attenuates disease severity (Garlet

Table 1. The protective and destructive properties of the innate and adaptive immune system

cell type	protective	destructive
monocytes/ macrophages	phagocyting pathogens	release of pro-inflammatory cytokines causing tissue degradation
	processing and presenting antigens to T cells	
neutrophils	"defense wall" against pathogens	collateral tissue damage (release of toxic products, elevated oxidative burst)
B cells	Ig production (IgG) (e. g. for opsonization or activation of complement system)	important source of RANKL autoantibodies against periodontal tissue components
Th1 cells	IFN-γ increases phagocyte activity	IFN-γ increases expression of pro-inflammatory cytokines
	in vitro: IFN-γ inhibits osteoclastogenesis	in vivo: increased expression of pro-inflammatory cytokines by IFN-γ overbalances direct anti-osteclastogenic abilities
	IL-12 inhibits osteoclast formation	IL-12 favours Th1 cell development and IFN-γ production
Th2 cells	IL-4 and IL-6 promote B cell (induced humoral immunity)	strong Th2 response may favour RANKL expressing B cells
	IL-4 elevates TIMPs and OPG	
	IL-4 inhibits production of MMPs and RANKL	
	IL-4 promotes IL-10 expression	
	IL-4 inhibits IFN-γ expression	
Th17 cells	IL-17 mobilizes neutrophils	IL-17 increases expression of other pro-inflammatory cytokines
		IL-17 increases expression of RANKL and MMPs
Treg cells	IL-10 and TGF-ß act anti-inflammatory	
	IL-10 upregulates TIMPs and OPG	
dendritic cells	professional antigen processing cells	immature dendritic cells can differentiate into osteoclasts

et al. 2004; Zhang and Teng 2006). Recently, the interplay of IL-10 with anti-resorption markers (TIMPs and OPG) was shown in patients with periodontitis and a polymorphism, which reduces IL-10 mRNA transcription. The reduced transcription of IL-10 was accompanied by a reduced transcription of TIMP-3 and OPG (Claudino et al. 2008). Moreover, IL-10 seems to have not only an inhibitory effect on pro-inflammatory and resorption parameters, but also a direct positive effect on bone formation. IL-10 knockout mice presented a reduced expression of osteoblast and osteocyte markers. The changes were independent of microbial or inflammatory influences (Claudino et al. 2010).

11.7 Periodontitis in Immunologically Compromised Patients

On the one hand, a strong response of the immune system increases the amount of tissue destruction by excessive upregulation of the inflammatory reaction and release of toxic products. On the other hand, an impairment of the immune system weakens the periodontal defense mechanisms. In particular, an impairment of the neutrophils causes severe problems for the host to control the bacterial invasion. This can result in a severe periodontal disease with extremely fast loss of alveolar bone already in the early childhood. The deciduous as well as the permanent dentition can be affected. The current Classification System has regarded this as an own chapter "Periodontitis as a Manifestation of Systemic Diseases" (Armitage 1999):

1. Hematologic disorders
   a. Acquired neutropenia
   b. Leukemias
   c. Other
2. Genetic Disorders
   a. Familial and cyclic neutropenia
   b. Down syndrome
   c. Leukocyte adhesion deficiency syndromes
   d. Papillon-Lefèvre syndrome
   e. Chédiak-Higashi syndrome
   f. Histiocytosis syndrome
   g. Glycogen storage disease
   h. Infantile genetic agranulocytosis
   i. Cohen syndrome
   j. Ehlers-Danlos syndrome (types IV and VIII AD)
   k. Hypophosphatasia
   l. Other
3. Not otherwise specified

11.8 Aggressive Versus Chronic Periodontitis

As stated above, the biochemical and histological characteristics as well as the pathological process and the involved mediators and cells of the periodontal lesion are more or less the same between aggressive and chronic periodontitis. Consequently, the question remains: what causes the differences in the onset, magnitude, extent or speed between the two disease entities?

Periodontal diseases are definitely no mono-infections. They include a consortium of multiple microorganisms. Differences in the composition of the subgingival biofilm or in the present strains and clones of certain pathogens between the different disease entities may cause differences in the intensity of the immune response. Many periodontally healthy individuals harbor periodontal pathogens, but the immune system and the pathogens seem to tolerate each other, which characterizes the periodontal pathogens as commensal opportunistic (Armitage 2010).

A modulating factor could be a co-infection by certain viruses, like cytomegalovirus. This co-infection may disturb the homeostatic balance between the host and the microorganisms of the biofilm. Development of localized aggressive periodontitis may be favoured by an early infection with cytomegalovirus during root formation. This possibly causes a malformation and its reactivation during puberty (Slots 2010).

Concerning immunological features between aggressive and chronic periodontitis no real differences are identifiable at the moment. Attention has been drawn to TLR or anti-microbial substances, such as ß-defensins (Ford et al. 2010).

Environmental risk factors such as smoking, stress or poor oral hygiene or systemic disease, such as diabetes mellitus, are influencing factors in periodontoal disease. At the moment, there seems to be no huge differences between the two disease entities. Nevertheless- as opposed to chronic periodontitis – poor oral hygiene is not correlated with the occurrence of localized aggressive periodontitis. Genetic risk factors seem to be present especially for aggressive periodontitis. A positive family aggregation is one of the main diagnostic criteria. However, familial aggregation cannot be detected in each case of aggressive periodontitis and a genetic base cannot be excluded for chronic periodontitis either (Stabholz et al. 2010).

The association of aggressive periodontitis with a "dysfunction" of neutrophils has been extensively investigated. In the past, hypo-reactive neutrophils have been assumed to be a reason for the development of aggressive periodontitis. This has changed over time into the hypothesis of hyper-reactive neutrophils. Reduced chemotaxis, transendothelial migration and phagocyting ability have been presented as reasons for hypo-reactivity. However, changes observed till now were not conclusive and may not be able to induce the increased tissue destruction alone. Therefore, increased adhesion ability, enzyme release and elevated oxidative burst as signs for hyper-reactivity have been discussed more recently. Altogether there is still an open debate, whether those changes are inherent or induced by inflammation and whether they are unique for aggressive periodontitis (Ryder 2010).

As stated above, the pathological process seems to be "more or less" the same, although small differences may account for the discrepancy. For instance, the RANKL/OPG ratio was reported to differ between aggressive and chronic periodontitis. Both clinical forms presented higher levels of RANKL in the gingival tissue. Yet, in the samples of patients with aggressive periodontitis the level of OPG was lower and the RANKL/OPG ratio higher than in patients with chronic periodontitis, favouring bone loss (Garlet et al. 2004). All studies on possible differences between aggressive and chronic periodontitis have the common denominator that the overlapping case definition impedes the investigation of this topic and makes the drawing of firm conclusions difficult.

11.9 Periodontal Therapy

The aim of periodontal therapy is the reduction of bacterial infection by mechanical removal of infectious agents of the root surface (scaling and root planning) in combination with strict oral hygiene instructions. This can be combined with local or systemic antibiotic therapy or local anti-infectious therapy (e. g. chlorhexidine digluconate). If this conservative therapy does not provide long-term stability, resective surgical therapy is an option. Optionally, if the morphology of the bone defect is promising, regenerative surgical therapy should be considered. Additionally, periodic maintenance treatment is essential for long-term stability and prevention of recurrence of disease. Ironically, although knowing that the bacterial invasion is "only" necessary for disease initiation, but not sufficient for disease progression, the therapy mainly focuses on fighting the bacterial part and much less on modulating the host's response. Regarding recent studies on the interaction of immune and bone cells in periodontal disease activity, new targets for therapeutic intervention should be considered and a selection of them are discussed below and are summarized in Table 2.

11.9.1 New Therapeutic Aspects

In contrast to osteoporosis or rheumatoid arthritis, for periodontal diseases there are two main concerns with anti-inflammatory or bone resorption inhibitory therapeutic approaches, such as non-steroidal anti-inflammatory drugs or glucocorticoids. On the one hand, periodontitis is a localized disease. Although systemic influences during active periodontal disease are reported and systemic diseases, such as diabetes mellitus can worsen in combination with untreated periodontitis, the disease itself is restricted to the oral cavity. Therefore, the partly severe and unwanted side effects may not outbalance the additional positive effects to conservative periodontal therapy. On the other hand, the blockade of parts of the immune system may have the side effect that the host response to bacterial invasion is decreased to a limit that the bacterial invasion gets out of control of the immune system.

The destructive properties of TNF-α suggest the use of TNF-α-antagonists, which are approved and accepted in the treatment of rheumatoid arthritis. Possible advantages in the treatment of periodontitis were already examined. The results described lower periodontal indices and lower levels of TNF-α in the gingival crevicular fluid in the test group (Mayer et al. 2009). At the moment, it has to be considered that the blockade of TNF-α may impair pathogen clearance, which is unfavourable in periodontal diseases. TNF-α is an important cytokine for the control of the bacterial attack. In TNF knockout mice an increased load of the pathogen Aggregatibacter actinomycetemcomitans and elevated levels of inflammatory markers were detected (Garlet et al. 2007). Similar problems appeared for IFN-γ knockout mice, which also presented a severe impairment of the defence against the periodontal pathogen Aggregatibacter actinomycetemcomitans (Garlet et al. 2008). Both knockout models (TNF-α and IFN-γ) share the reduced activation and migration of phagocytes, such as neutrophils or macrophages, into the periodontal diseased tissue and present a clearly reduced phagocyting activity. The application of TNF-α-antagonists in the treatment of periodontitis needs further research to evaluate their true efficacy relative to their expense and possible side effects.

Pro-inflammatory interleukins, such as IL-1, -6, -12 or -17, have also been approached as a possible target. Nevertheless, the effect of antibodies against those interleukins has been hardly examined for periodontal disease. The possible advantage of an anti-IL-1-therapy was shown in a non-human primate periodontitis model. The results demonstrated reduced signs of inflammation and reduced destruction of soft and mineralized periodontal tissue (Delima et al. 2002). Further studies on this topic are warranted.

The pro-resolving and anti-inflammatory properties of lipid mediators, such as lipoxins, resolvins or protectins, have already been discussed above and present a promising option, which needs to be further investigated.

Antagonists to interleukins or TNF-α mainly target the reduction of the inflammation and only indirectly a reduction of bone resorption. According to our present knowledge a direct interference might to be favourable. One logical target is the RANKL/RANK/OPG system. So far the databases for periodontitis are restricted to animal studies. The results of experimental periodontitis showed that therapeutic intervention by administering OPG or analogues to OPG (e. g. Denosumab) seems to be able to prevent bone loss in periodontal disease by blocking the binding of RANKL to RANK (Jin et al. 2007; Lin et al. 2010; Teng et al. 2000). Nevertheless, it has to be considered that long-lasting systemic application of a RANKL inhibitor as treatment for chronic diseases may have severe side effects. In focus is the immune system, as RANK is not only expressed on osteoclasts and their precursors, but also on monocytes/macrophages and dendritic cells (Ferrari-Lacraz and Ferrari 2010, Kong et al. 1999). Additionally, possible unwanted side effects on systemic physiologic bone turnover should be considered. Therefore, a modulation of this system in chronic diseases, especially in rather localised diseases, such as periodontitis, may be more favourable than the blocking of the whole system.

Bisphosphonates inhibit osteoclast activity and consequently reduce bone resorption. They are used in the treatment of osteoporosis or tumor-associated

osteolysis. Studies examining a possible positive effect for periodontal diseases have shown only modest improvements. The clinical significance is questionable and the risk of osteonecrosis of the jaw as a possible side effect has to be regarded (Bartold et al. 2010).

Cathepsin K is expressed in osteoclasts and plays an important role in bone resorption, being able to degrade type I as well as type II collagen. Elevated levels of cathepsin K have been detected in the gingival crevicular fluid of periodontally diseased patients and they correlate with the levels of RANKL. In a nonhuman primate model of postmenopausal bone loss, inhibition of cathepsin K resulted in a remarkably reduced bone resorption. Data on a possible advantage for periodontitis are missing so far (Bartold et al. 2010; Stroup et al. 2001).

Statins are widely used to control lipid metabolism by reducing serum cholesterol levels and are claimed to have anti-inflammatory properties too. Simvastatin is the most common used statin. The immunomodulatory, anti-inflammatory and possible anti-resorptive effects of simvastatin seem to provide possible advantages in the therapy of periodontitis, but those effects may depend on the inflammatory status of the subject and further studies are necessary (Bartold et al. 2010; Saxlin et al. 2009).

Deficiency of vitamin D is associated with bone loss and increased loss of calcium from the bone. Due to its immunomodulatory ability and its role in bone homeostasis Vitamin D and calcium supplementation may be a useful adjuvant for periodontal therapy. Possible improvements of the periodontal parameters have been described (Hildebolt 2005; Miley et al. 2009).

Another option, although not really on the cutting edge anymore, is to modulate the host immunity by an adjunctive therapy with a subantimicrobial dose of doxycycline (20 mg doxycycline twice daily). Doxycycline is known to downregulate the activity of MMPs, which are one of the main destructive enzymes in periodontal disease. A statistically and clinically significant improvement of periodontal parameters has been reported (Preshaw et al. 2004).

Inhibitors against mitogen-activated protein kinases, which are intracellular molecules involved in signal transduction during inflammation, have shown promising results as well. Inflammatory reactions and alveolar bone loss was reduced in experimental periodontitis (Kirkwood et al. 2007; Rogers et al. 2007).

The role of Wnt proteins in regulating cell differentiation and function extends on bone tissue too. Secreted frizzled-related protein-1 influences osteoblast and osteocyte apoptosis and negatively regulates Wnt signalling. Elevated levels of secreted frizzled-related protein-1 have been described in experimental periodontitis as well as reduced severity of the experimental periodontitis after supplementation of an antibody to secreted frizzled-related protein-1 (Li and Amar 2007). Further studies are necessary to prove the safety and efficiency of this approach.

Based on the destructive and pro-resorptive role of iNOS, iNOS inhibitors have been investigated as a possible therapeutic approach. So far the results are limited to in vitro and animal studies. Yet, iNOS inhibitors, like mercaptoethyl guanidine, presented promising results. In vitro they reduced NO production in human gingival fibroblasts (Daghigh et al. 2002) and in experimental periodontitis they reduced alveolar bone loss (Di Paola et al. 2004; Lohinai et al. 1998).

Table 2 Adjunctive therapeutic approaches in the treatment of periodontal diseases

therapeutic		mode of action	possible negative side effects
target	**agent**		
TNF-α	TNF-α-antagonists (Mayer et al. 2009)	anti-inflammatory and anti-resorptive	impairment of the pathogen clearance? (Garlet et al. 2007)
IL-1	IL-1-antagonists (Delima et al. 2002)	anti-inflammatory and anti-resorptive	–––
---	lipid mediators (lipoxin, resolvin, protectin) (Hasturk et al. 2006, 2007; Serhan et al. 2008; Van Dyke and Serhan 2003)	pro-resolving and anti-inflammatory	–––
RANKL/ RANK/OPG system	OPG / OPG-analogues (Jin et al. 2007; Lin et al. 2010; Teng et al. 2000)	blockade of binding RANKL to RANK	impairment of the immune system or the systemic physiological bone turnover (Ferrari-Lacraz and Ferrari 2010, Kong et al. 1999)
---	bisphosphonates (Bartold et al. 2010)	reduced osteoclast activity and bone turnover	osteonecrosis of the jaw (Bartold et al. 2010)
cathepsin K	cathepsin K inhibitors (Stroup et al. 2001)	reduce bone resorption	–––
---	Statins (simvastatin) (Saxlin et al. 2009)	anti-inflammatory and anti-resorptive	–––
---	Vitamin D and calcium supplementation (Hildebolt 2005; Miley et al. 2009)	immunomodulatory and regulator of bone homeostasis	–––
MMPs	subantimicrobial dose doxycycline (Preshaw et al. 2004)	inhibit MMPs	–––
mitogen-activated protein kinases	inhibitors of mitogen-activated protein kinases (Kirkwood et al. 2007, Rogers et al. 2007)	anti-inflammatory and anti-resorptive	–––
Wnt pathway/secreted frizzled related protein-1	inhibitors of secreted frizzled related protein-1 (Li and Amar 2007)	reduce bone loss	–––
iNOS	iNOS inhibtors (mercaptoethyl guanidine) (Di Paola et al. 2004; Lohinai et al. 1998)	NO production is reduced	impairment of the pathogen clearance?

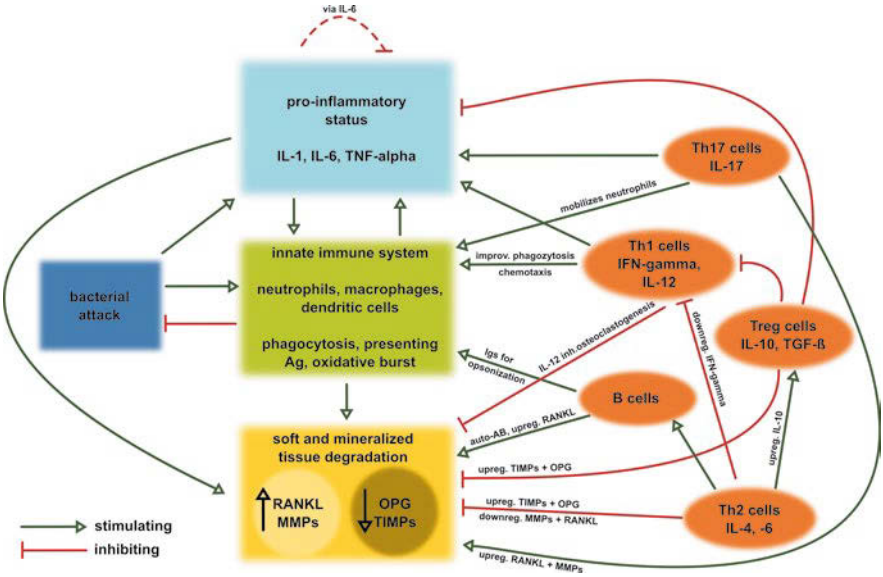

Fig. 5 Response and interaction of the innate and adaptive immune system due to bacterial challenge and effect on the soft and mineralized tissue. Ag: antigen, AB: antibody

Returning to the bacterial aspect, numerous efforts have been performed to test active or passive immunization targeting a wide array of periodontal pathogens. Most promising results are reported for Porphyromonas gingivalis. However, it has to be considered that the majority of studies are still animal-based studies and the results could not be incorporated as a "complete vaccine" against periodontitis in human so far (Dhingra and Vandana 2010; Sharma et al. 2007).

11.10 Summary

Periodontal disease is a chronic infectious disease. The immune system driven by the bacterial attack causes a loss of soft and mineralized periodontal tissue. The mechanisms are summarized below in Fig. 5 and display the role of osteoimmunology in the pathogenesis of periodontitis. Compared to other diseases, such as osteoporosis or rheumatoid arthritis, the immune system has a unique dual-role in periodontal disease. It is initiated by periodontal pathogens and its upregulation to fight

and control the bacterial attack causes collateral damage, yet with a reduced immune system, the bacterial invasion may overwhelm the host. This dual role is reflected in the role and duties of the involved cells and mediators. Interestingly, it seems that the intensity of the immune response is not the relevant factor for the periodon-

tal pathogen control, but the tissue destruction depends on the magnitude of the host's immune response. A higher intensity of the immune response results in an increased tissue destruction (Trombone et al. 2009). One main limiting factor in the examination of this topic is the lack of a clinical parameter that displays active bone resorption at the time point of sample collection. Regarding the interaction of the immune and the bone cells new therapeutic approaches should be considered as adjunctive possibilities to the well established and proven removal of the biofilm.

Abbreviations

IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible NOS
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MMP	matrix metalloproteinase
NO	nitric oxide
NOS	NO-synthases
OPG	osteoprotegerin
PLC	periodontal ligament cells
RANK	receptor activator of nuclear factor "kappa-light-chain-enhancer" of activated B cells
RANKL	receptor activator of nuclear factor "kappa-light-chain-enhancer" of activated B cells ligand
TGF-ß	transforming growth factor-beta
Th cells	T helper cells
TIMP	tissue inhibitor of metalloproteinases
Treg	cellsregulatory T cells
TLR	Toll like receptors
TNF-α	tumor necrosis factor-alpha
TRAF	tumor necrosis factor receptor-associated factor

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