Vascular calcification, atherosclerosis and bone loss (osteoporosis): new pathophysiological mechanisms and future perspectives for pharmacological therapy

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Vascular calcification or ectopic mineralization in blood vessels is an active, cell-regulated process, increasingly recognized as a general cardiovascular risk factor. Ectopic artery mineralization is frequently accompanied by decreased bone mineral density or disturbed bone turnover and development of the osteoporosis. The latest data support the correlation of osteoporosis and atherosclerosis, indicating the parallel progression of two tissue destruction processes with increased fatal and non-fatal coronary events, as well as a higher fracture risk. Patients with osteoporosis, have a higher risk of cardiovascular diseases than subjects with normal bone. Many proteins responsible for bone formation and resorption have been identified in the arterial wall. Vascular calcification includes mostly osteogenic and, to a lesser extent chondrogenic differentiation of osteoblasts and osteoclast-like cells. It has been shown that many of the regulators of bone formation and resorption some bone structural proteins, such as osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL) are also expressed in the atherosclerotic plaque. When RANKL binds to RANK, osteoclasts are activated and bone resorption occurs and processes of vascular calcification become also activated. OPG, protein homologue to receptor activator of nuclear factor-κB (RANK), can bind to RANKL, blocking the binding of RANKL to RANK, that results in inhibition of differentiation of preosteoclasts to mature osteoclasts, lower osteoclast capacity for resorption of bone mineral matrix, and development vascular calcification. The latest data supports that cathepsin K, a cysteine protease, can efficiently degrade type I and II collagen, both of which are major matrix components of the bone and atherosclerotic plaque. These findings further underscore the potential of cathepsin K as a target for novel molecules to treat osteoporosis and atherosclerosis. Thus, the discovery of the cytokine RANKL-RANK-OPG system and significant role of the cathepsin K in the process of bone remodeling, vascular calcification and atherosclerosis has made progress in understanding the mechanisms of disease development and possibly to develop new dual therapies. New therapies for osteoporosis and atherosclerosis that may potentially improve or augment existing treatments include the recently approved anti-receptor activator of NF-κB-ligand monoclonal antibody fms (denosumab) and the cathepsin K inhibitor odanacatib, presently in the late stage of clinical development.

Key words: atherosclerosis, osteoporosis, common mechanisms, RANKL-RANK-OPG system, cathepsin K, denosumab, odanacatib

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Introduction

Cardiovascular disease and osteoporosis are public health problems with several epidemiological links and important economic consequences [1–7]. Recent studies have demonstrated that cardiovascular disease and mortality are associated with reduced bone mineral density and bone fracture (Fig. 1) [8–11]. Cardiovascular disease and osteoporosis might be related to each other in terms of pathogenesis and therapeutic agents [12]. Osteoporosis is a progressive systemic skeletal disorder characterized by low bone mineral density (BMD), deterioration of the microarchitecture of bone tissue, and increased risk for fracture [5, 6]. Osteoporosis is becoming an escalating problem worldwide due to an increase in life expectancy and therefore in the ageing of population. Currently it is estimated that over 200 million people worldwide suffer from this disease [7, 8]. An estimated prevalence in Europe is expected to rise to 12 million of persons with osteoporosis older 50 years of age by the year 2010 and to nearly 14 million by the year 2020. Cardiovascular diseases (CVD) are the most frequent cause of premature death in modern industrialized countries, accounting for 4.35 million deaths each year in Europe, and 35% of all deaths in the United Kingdom and the United States [13]. The World Health Organization (WHO) estimates there will be about 20 million CVD deaths in 2015, accounting for 30 percent of all deaths worldwide [14].

Vascular calcification is an independent risk factor for CVD. Calcification of any artery or cardiac valve increases the risk cardiovascular events and mortality three- to fourfold and is accepted as a predictor of coronary heart disease [15]. Vascular calcification reduces arterial elasticity resulting in substantial morbidity and mortality from hypertension, aortic stenosis, cardiac hypertrophy, myocardial infarction and lower limb ischemia [16, 17]. In the recent years, several studies have indicated important roles for RANK-RANKL-OPG cytokine system and proteasome cathepsins in the atherogenesis [18, 19] and osteoporosis [20, 21]. Cathepsin K inhibitors, which block the effects of cathepsin on bone resorption, and the role of cathepsins in atherogenesis attracted attention to investigation of the therapeutic potential of cathepsin inhibitors on the initiation and/or progression of atherosclerosis and osteoporosis [22, 23, 24]. The fact that these drugs act both on osteoporosis and on vascular calcification suggests that these diseases share common pathophysiological pathways. In this review, we will focus on recent evidence for new mechanisms regulating vascular calcification, including the potential role of the RANK-RANKL-OPG axis and cathepsin K. Finally, we review potential treatments that are under investigation for preventing and/or regression of vascular calcification and osteoporosis.

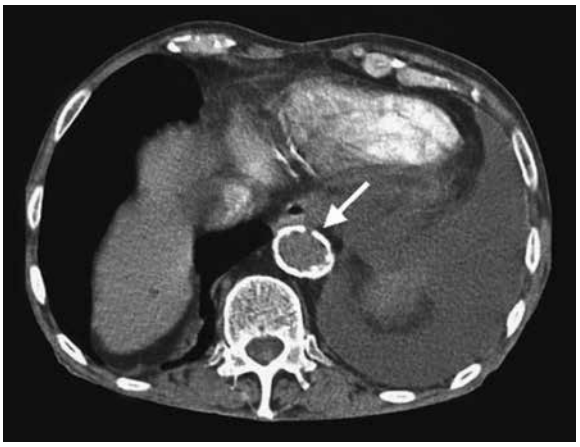

Fig. 1. The osteoporosis / arterial calcification syndrome. Computerized tomography demonstrating severe aortic calcification (arrow) in a 71-year-old man with an osteoporotic hip fracture (T-score by DEXA: -3.1 at the spine and -2.6 at the proximal femur). His risk profile includes type 2 diabetes mellitus, arterial hypertension and a 60-pack-year history of cigarette smoking

Cellular and molecular pathophysiology of osteoporosis

Osteoporosis results from an imbalance between bone resorption and bone formation favoring bone resorption. The major cell types responsible for these two processes are osteoblasts and osteoclasts.

Cells involved in bone remodeling: osteoblasts and bone formation

Bone is a dynamic tissue that undergoes life-long adaption to attain and preserve skeletal size, shape and structural integrity and regulate mineral homeostasis. Two processes, remodeling and modeling, underpin the development and maintenance of the skeletal system. Bone modeling is responsible for growth and mechanically induced adaption of bone; it requires that the process of bone formation and bone resorption, while globally coordinated, occur independently at distinct anatomical locations [25, 26]. This tightly coordinated event requires the synchronized activities of multiple cellular participants to ensure bone resorption and formation occur sequentially at the same anatomical location to preserve bone mass. Bone remodeling is a physiological process that maintains the integrity of the skeleton by removing old bone and replacing it with a young matrix. Two principle cell types are found in bone, the osteoclast and the osteoblast, which are the major effectors in the bone matrix turnover [27, 28]. Osteoblasts and osteoclasts dictate skeletal mass, structure, and

strength via their respective roles in resorbing and forming bone. Osteoblasts are specialized mesenchymal-derived cells whose function is the deposition and maintenance of skeletal tissue. Osteoblasts derive from pluripotent mesenchymal stem cells (MSC) that prior to osteoblast commitment can also differentiate into other mesenchymal cells line ages, such as fibroblasts, chondrocytes, myoblasts and bone marrow stromal cells, including adipocytes, depending on the activated signaling transcription pathways [29]. Thus, understanding the mechanisms that control the differentiation of osteoblastic cells from MSC is one of the fundamental areas of research in bone biology. Several specific transcription factors are responsible for the commitment of pluripotent MSC into the osteoblast cell lineage [30]. Lineage specific gene expression is ultimately under the control of research of bone biology. Several specific transcription factors are responsible for the commitment of pluripotent MSC into the osteoblast cell lineage [31]. Lineage specific gene expression is ultimately under the control of transcription factors that act to regulate specific gene expression. They act as the key switching mechanisms to induce gene transcription. Considerable progress has been made in identifying those transcription factors which act as "master switches" during commitment of multipotent cells to specific lineages. A major breakthrough in understanding genetic regulation of osteoblast differentiation was made with the identification of the role of the transcription factor core binding factor-1 (Cbfa-1 / runt-related transcription factor-2 (RUNX-2)) [32, 33]. Cbfa-1/RUNX-2 expression is an absolute requirement for osteoblast differentiation. In Cbfa-1 knockout mice, there is a normal cartilaginous skeleton seen but a complete absence of bone formation [34, 35]. Cbfa-1/RUNX-2 has been known to interact directly with the osteocalcin promoter to induce its expression [36]. However, an additional transcription factor, Osterix, which is a downstream target for Cbfa-1/RUNX-2, has also been shown to be an absolute requirement for normal osteoblast differentiation in knockout mice experiments [37]. More recent studies have shown the existence of distinct isoforms of Cbfa-1, which may have subtly different roles during normal tissue formation, including regulation of cartilage expression, in addition to bone. Another is the runt-related gene that plays an important role in the commitment of multipotent MSC to the osteoblastic lineage and for osteoblast differentiation at an early stage is RUNX-2. Cbfa-1/RUNX-2 is involved in the production of bone matrix proteins [38], as it is able to upregulate the expression of major bone matrix protein genes, such as those of type I collagen, osteopontin, bone sialoprotein and osteocalcin leading to an increase of immature osteoblasts from MSC and the immature osteoblasts from immature bone [39].

Osteoblast commitment, differentiation and growth are controlled by several local and systemic factors that can also act in a paracrine and/or autocrine way and that can regulate the activity of specific transcription factor [40]. Huge advances have been made in the understanding of cellular and molecular control of bone formation in the past decade. The establishment of *in vitro* models of osteoblast differentiation and formation has been essential for determining the effects of specific growth factors and growth factor-induced transcription factors on osteogenesis. Osteoblasts play a crucial role in the process of bone formation, in the induction and regulation of extracellular matrix mineralization and in the control of bone remodeling [41, 42]. During bone formation, mature osteoblasts synthesize and secrete type I collagen (which represents the greater part of the organic extracellular bone matrix) and various non-collagen proteins, such as osteocalcin, osteopontin and bone sialoprotein (which exert various essential functions, including regulation of bone turnover, control of bone mineral deposition and regulation of bone cell activity). Osteocalcin (Gla) is a vitamin-K-dependent osteoblast-specific protein whose synthesis is enhanced by 1.25 OH vitamin D_3 and reflects metabolic cellular activity. Of the *de novo* synthesized osteocalcin, 60–90% is incorporated into the bone matrix where it binds to hydroxyapatite during matrix mineralization. Osteopontin (OPN) is a phosphorylated acidic glycoprotein that is present in large amounts in immature bones. OPN is synthesized by osteoblast but is expressed by other cellular types, such as chondrocytes; it is involved in various physiological and pathological events. Bone sialoprotein I is a glycosylated, phosphorylated and sulfated protein that promotes hydroxyapatite crystal nucleation and osteoblast differentiation [43]. This has been confirmed by the observation that bone sialoprotein-knockout mice present hypomineralized bone, a reduction in the size of their long bones and aberrant levels of osteoblast markers [44]. Osteoblasts also synthesize cytokine interleukin (IL)-1 and IL-6, which control bone cells in an autocrine and/or paracrine manner. Various *in vitro* studies of human and murine osteoblastic cell lines suggest that IL-1 can affect proliferation, collagen and osteocalcin synthesis and alkaline phosphatase (Alp) production [45, 46]. Osteoblasts express receptors for various hormones including parathyroid hormone (PTH) [47], 1.25 (OH) 2D3 [48], estrogens [49], which are involved in the regulation of osteoblast differentiation and activity. Vitamin D_3 is able to modulate

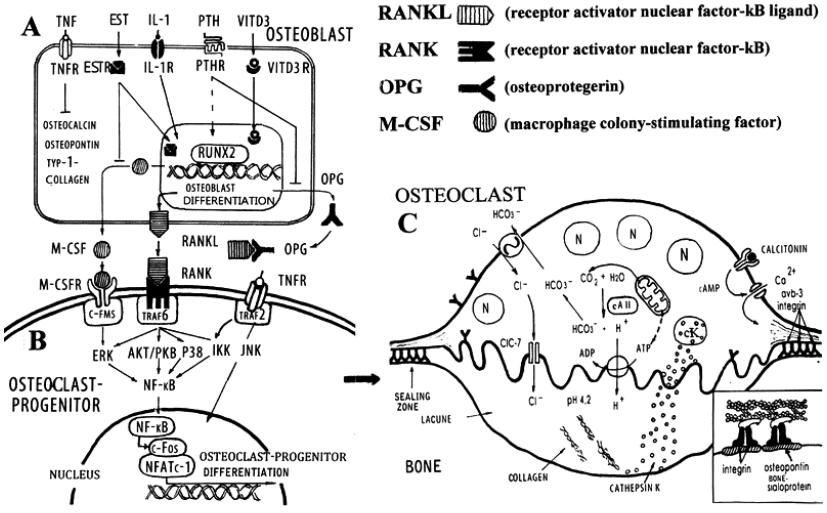

Fig. 2. RANKL-RANK-OPG system and regulation of osteoclast precursor by osteoblast (**A**, **B**) and mechanisms of osteoclastic bone resorption (**C**). Under physiologic condition, RANKL produced by osteoclasts binds to its receptor RANK on the surface of osteoclast precursors and recruits the adaptor protein TRAF6, leading to NF-κB activation and translocation to the nucleus. NF-κB increases c-Fos expression and c-Fos interacts with NFATc1 to trigger the transcription of osteoclastogenic genes. OPG inhibits the initiation of the process by binding to RANKL. The mechanisms of osteoclastic bone resorption (**C**): several transport systems including the H^+-ATPase proton pump, Cl^-/HCO_3 exchanger and chloride channel are responsible for the acidification in the osteoclastic resorption lacune. The osteoclast attaches to bone, which promts formation of a convoluted ruffled membrane and a resorptive microenvironment beneath the cell. Hydrochloric acid, the product of a vacuolar-type H^+-ATPase and charge-coupled CL channel concentrated in the ruffled membrane, is secreted, resulting in mineral dissolution. Vesicles containing acidic collagenolytic enzymes in the form of cathepsins K fuse with the bone-apposed membrane, leading to matrix degradation. Intracellular pH balance is maintained by a passive Cl^-/HCO_3- exchanger on the contra-resorptive surface of the cell. In the right corner: this figure summarized current information and hypotheses on the regulating role of $\alpha_v\beta_3$-integrin in osteoclast formation, adhesion, polarization and migration. The natural ligand for $\alpha_v\beta_3$-integrin is not known; however, osteopontin and bone sialoprotein are two RGD (arginine-glycine-aspartate) containing proteins which could potentially be ligands. See text for more details; TNF and TNFR – tumor necrosis factor-α and its receptor; EST and ESTR – estrogen and its receptor; IL-1 and IL-1R – interleukin-1 and its receptor; PTH and PTHR – parathyroid hormone and its receptor; VitD3 and VitD3R – 1,25-dihydroxyvitamin D3 and its receptor; RUNX2 – runt-related transcription factor 2; OPG – osteoprotegerin; RANK – receptor activator nuclear factor-κB; RANKL – receptor activator nuclear factor-κB ligand; TRAF2 and TRAF6 – tumor necrosis factor receptor-associated factor 2 and 6; NFATc-1 – nuclear factor of activated T cells; M-CSF and M-CSFR – macrophagal colony stimulating factor-1 and its receptor; C-FMS – colony stimulating factor-1 receptor; c-Fos – transcription factor; ERK – extracellular signal-regulated kinase; AKT/PKB – serine / threonine protein kinase B; P38 – mitogen-activated protein kinase; IKK – inhibitor kappa B kinase; JNK – Jun N-terminal kinase; NF-κB – nuclear factor kappa B; cAMP – cyclic adenosine monophosphate; ATP – adenosine triphoaphate; ADP – adenosine diphosphate; CIC-7 – chloride channel; cAII – carbonic anhydrase II; cK – cathepsin K

the metabolic activity of osteoblasts through the activation of a series of vitamin D-responsive genes that reflect a more mature osteoblast phenotype.

Control of bone remodeling by osteoblasts: the role of RANKL-RANK-OPG system in the osteoclast development

In the recent years it has become evident that osteoblasts have a global role in orchestrating the bone remodeling process. Their function is not restricted solely to bone formation, but it is now firmly established that they are responsible for initiating bone resorption. In cellular terms, apart from forming the mineral and organic extracellular compartment of bone, the osteoblast provides the essential and sufficient stimuli that control the behavior of the osteoclast, an event that occurs via cell-cell interaction. The bone resorption cascade involves a series of steps directed towards the removal of both the mineral and organic constituents of the bone matrix by osteoclasts, aided by osteoblasts. The role of the osteoclast as a major resorbing cell and its structure and biochemical properties have been well characterized [50]. The first stage involves the recruitment and dissemination of osteoclast progenitors to the bone. Progenitor cells are recruited from the haemopoietic tissue such as bone marrow and splenic tissue to the bone via the bloodstream. They proliferate and differentiate into osteoclasts through a mechanism involving cell-to-cell interaction with osteoblast stromal cells. Osteoclast formation from osteoclast precursors is regulated mostly by osteoblastic cells during normal bone remodeling. Osteoblastic cells in the bone marrow express two cytokines that are required for osteoclast progenitor differentiation into osteoclasts: RANKL and osteoprotegerin (OPG) [27, 51] (Fig. 2A). The discoveries of the RANKL and OPG have revolutionized our understanding of the process underlying osteoclast formation and activation [51, 52]. RANKL and OPG potently stimulate and inhibit, respectively, osteoclast differentiation.

Receptor activator of nuclear factor-κB ligand (RANKL)

RANKL, similar to OPG, belongs to the TNF super family. It is a homotrimeric glycoprotein consisting of 316 aminoacids which exists as a transmembrane protein and in a soluble form. Most of the factors that stimulate osteoclasts' formation and activity induce RANKL expression by osteoblastic and stromal cells. RANKL is also expressed in activated T-lymphocytes, lymph nodes, thymus, mammary glands, lungs, spleen and bone marrow [53]. It is considered to be a dendritic-cell stimulator; that was a reason to propose its first name TRANCE – TNF-related activation-induced cytokine. RANKL acts as a survival factor for dendritic cells and for mature T-cells through regulation of their proliferation. While OPG presents as a soluble bone protector, RANKL is considered to be stimulator of bone resorption. It is a pro-resorptive factor because of induction of osteoclasts' differentiation and activation of mature osteoclasts. In contrast to OPG deficient mice, RANKL transgenic mice exhibit marked osteoporosis, while mice disrupted for RANKL are strongly osteopetrotic, with a total absence of mature

osteoclasts [53]. RANKL production is stimulated by IL-1, IL-6, IL-11, IL-17, TNF-α, vitamin D3, calcium, PTH, glucocorticoids, prostaglandin E2 and immunosuppressive drugs [53, 54]. Its production is downregulated by TGF-β [55]. RANKL stimulates RANK and in presence of stimulating factors (e.g. M-CSF) may lead to initiation of osteoclastogenesis, i.e. development of multinucleated bone-resorptive osteoclasts from monocytes' precursors [56]. It also promotes the resorptive activity and survival of mature osteoclasts. RANKL is a membrane-bound factor that is produced by osteoblasts and stromal cells in response to a variety of signals such as PTH, tumor necrosis factor (TNF)-α and IL-1. RANKL bind to the cytoplasmic membrane receptor RANK (receptor activator of NF-κB), which is a member of the TNF receptor super family and subsequently induces both osteoclast differentiation and activation. OPG is a soluble decoy receptor for RANKL and can inhibit its effects, thereby preventing osteoclast development and subsequent bone resorption [53, 57]. Overexpression of OPG in transgenic mice results in osteopetrosis and, conversely, OPG deficient mice exhibit severe osteoporosis. Many of the same agents that stimulate RANKL expression (including PTH, IL-1, prostaglandin E) also inhibit OPG expression [48, 58], which enhances osteoclastogenesis even further. While fibroblast growth factor-2 induces RANKL expression by osteoblasts, it also inhibits osteoclast differentiation directly by interfering with the action of macrophagal colony stimulating factor (M-CSF) [56]. In contrast to the stimulatory effects of the agents described above, estrogens inhibit the production of RANKL by osteoblasts [49]. Transforming growth factor (TGF)-β also strongly suppresses RANKL expression by osteoblasts, whereas it stimulates OPG expression [55]. Administration of RANKL to mice causes osteoporosis, whereas disruption of the RANKL gene in mice leads to severe osteopetrosis, impaired tooth eruption, and the absence of osteoclasts [59, 60]. Membrane bound M-CSF is also a critical early modulator in the differentiation of osteoclasts [56]. M-CSF binds to c-fms on the surface of osteoclast precursors, and this event enhances their proliferation and survival. M-CSF enhances the survival of monocyte stem cells, thereby permitting them to respond to direct inducers of differentiation such as RANKL. A combination of M-CSF and RANKL is sufficient for human, mouse, and rat multinucleated osteoclast formation *in vitro* [61]. Although RANKL is critical for osteoclast formation and activation, a series of complementary studies has revealed a number of additional gene products necessary for osteoclastogenesis and a variety of hormones and cytokines that modulate osteoclast formation [27, 53]. Deletion of the genes for M-CSF, c-fos, RANK and NF-κB results in absent osteoclast formation that confirms their requirement for osteoclastogenesis. Osteoclasts are formed in mice with deleted genes for TRAF6 and the c-fos; however, these osteoclasts exhibit defects in bone resorption resulting in osteopetrosis [62]. Interestingly, another TRAF6 knockout mice exhibit defective osteoclastogenesis. TRAF6 activates the MAP kinase cascade, and eventually activates JNK, JKK and N-κB have been directly implicated in the response to RANKL [62, 63]. Different domains of TRAF6 modulate both the initial differentiation and subsequent maturation of osteoclasts by activating various kinase cascades. RANKL also activates NF-κB in osteoclasts, in large part via TRAF stimulation of Ik kinase (IKK) to phosphorylate IkB, which then dissociates from NF-κB, and permits NF-κB translocation into the nucleus and subsequent binding to NF-κB responsive genes. TNF-α also acts to induce osteoclast formation and activation in concert with RANKL via the TNF receptor and TRAF2/6 and subsequently to activate NF-κB signaling [62].

Receptor activator of nuclear factor-κB (RANK)

RANK, another member of TNF receptor superfamily, is a homotrimeric transmembrane protein consisting of 616 aminoacids. It is expressed on osteoclasts' precursors, mature osteoclasts, dendritic cells, mammary glands, and some cancer cells including breast and prostate cancers that have very high bone metastatic potential [64, 65]. After binding its ligand RANKL, RANK assembles into functional trimeric receptor. This trimerisation is required to generate multiple intracellular signals that regulate cell differentiation, function and survival, among the others – those of mature and functional osteoclasts.

Osteoprotegerin (OPG)

Osteoprotegerin, called "bone protector", belongs to the TNF receptor's family. It is a soluble glycoprotein (has no transmembrane domain) consisting of 380 aminoacids and seven domains. It is expressed in many types of cells like osteoblasts, heart, kidney, liver, spleen and bone marrow [66, 67]. In bone tissue OPG is produced by osteoblasts, while in the vessels by endothelial (EC) and vascular smooth muscle (VSMCs) cells. Molecules that upregulate OPG synthesis by osteoblasts are: IL-1α, IL-6, IL-11, IL-17, IL-18, TNF-α, TNF-β, bone morphogenic protein (BMP-2), calcium, vitamin D3, estrogens, angiotensin II and platelet derived growth factor (PDGF) [68]. In contrary, PTH, glucocorticoids, prostaglandin E2, immunosuppressant drugs, peroxisome proliferators activated receptor (PPAR-γ) and basic fibroblast

growth factor (bFGF) downregulate OPG production [52, 64, 68, 69]. OPG has been identified as a cytokine that increases mineral density and volume of bone tissue by decreasing the number of active osteoclasts. Overexpression of OPG in transgenic mice results in severe osteopetrosis, characterized by increased bone turnover and inhibition of osteoclastogenesis [16, 70]. OPG-deficient mice develop osteoporosis because of unopposed actions of RANKL to stimulate osteoclastic cells formation, activity and survival [52, 71]. Based on the presence of renal artery and aortic medial calcification in osteoprotegerin deficient mice, OPG also appears to protect large vessels from media calcification [72]. Osteoprotegerin functionally acts as a decoy receptor that blocks interaction between the receptor activator of nuclear factor-κB (RANK) and its ligand (RANKL), thereby inhibiting osteoclasts' differentiation, as well as theirs activity, and prevents bone loss. OPG has also been shown to bind TNF-related apoptosis inducing ligand (TRAIL/Apo2L), another member of TNF ligand super family. In this way it inhibits the induction of human osteoclasts apoptosis by preventing TRAIL from binding to its receptors [73, 74]. OPG is also involved in efficient antibody response and B-cell maturation [75]. In endothelial cells OPG is physically associated with von Willebrand factor (vWF), a glycoprotein involved in primary hemostasis and also an important marker of endothelial injury. OPG and vWF are rapidly secreted in response to inflammatory stimuli that can prove the OPG role in vascular injury, inflammation and hemostasis [76]. Because OPG is able to bind vWF reductase, thrombospondin-1 (TSP-1), it may also play a role in regulation of thrombus formation [77].

OPG/RANK/RANKL interaction

RANKL acts through its receptor RANK. After binding, RANKL induces intracellular signals that regulate differentiation, function and survival of osteoclasts. OPG secreted by osteoblastic lineage cells acts as a decoy receptor binding RANKL and preventing RANKL interaction with RANK. This results in inhibition of osteoclastic differentiation and consequently leads to decreased bone resorption. Additionally, OPG modulate the RANKL half-life, and in its turn, RANKL controls the bioavailability of OPG, its internalization and degradation [78]. Because OPG directly counter all RANKL-mediated actions through RANK, RANKL/OPG ratio is an important determinant of bone mass and skeleton integrity. Imbalances in the RANKL/OPG ratio or RANK signaling underlie the pathophysiology of many skeletal disorders with excessive bone loss, excessive bone formation, or diseases with disordered bone remodeling [79]. It has also been shown that this system is involved in regulation of immune system and development of vascular calcification [80].

Osteoclast and bone resorption

The development of an *in vitro* bone resorption model using isolated primary osteoclasts and mineralized bone matrix as a substrate almost twenty years ago provided an excellent system for detailed cell biological studies of bone resorption [81, 82]. Although this model has several limitations in attempts to study the whole physiological cascade of bone resorption, it provides an excellent tool for detailed studies of cellular mechanisms involved in the destruction of mineralized bone matrix. The sequence of cellular events needed for bone resorption is called the "resorption cycle". Resorption requires cellular activation, such as migration of the osteoclast to the resorption site, its attachment to the bone, polarization and formation of new membrane domains, dissolution of hydroxyapatite, degradation of organic matrix, removal of degradation products from the resorption lacuna, and finally either apoptosis of the osteoclasts or their return to the non-resorbing stage (Fig. 2C). The term "resorption cycle" covers neither the differentiation pathway nor the cellular activities needed for the fusion of mononuclear precursor to form the multinuclear mature osteoclast. It should not be mistaken for the more widely used term "remodeling cycle", which is used to describe the bone remodeling at the tissue level that involves the activities of various cell types. After migration of the osteoclast to the resorption site, a specific membrane domain, the sealing zone, is formed beneath the osteoclast. The plasma membrane attaches tightly to the bone matrix and seals the resorption site from its environment. The molecular interaction between the plasma membrane and the bone matrix at the sealing zone is still unknown. Several lines of evidence have shown, however, that integrins play an important role in early phases of the resorption cycle [83]. At least four different integrins are expressed in osteoclasts: $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_2\beta_1$ and $\alpha_v\beta_1$ [84]. The role of $\alpha_v\beta_3$ has received much attention, because antibodies against $\alpha_v\beta_3$, as well as argynine-glycine-aspartic acid (RGD)-containing peptides such as echistation and kistrin, are defective inhibitors of bone resorption both *in vitro* and *in vivo*. $\alpha_v\beta_3$ is highly expressed in osteoclasts and is found in the plasma membrane and in various intracellular vacuoles. However, the exact function of $\alpha_v\beta_3$ in resorbing osteoclasts remains unknown; the integrin could play a role both in adhesion and migration of osteoclasts and in endocytosis of resorption products. The latter possibility is supported by the observation that high amounts of $\alpha_v\beta_3$ are

present at the ruffled border and by recent data from receptor-binding assays showing that denaturated type I collagen has high affinity to $\alpha_v\beta_3$ [42, 85]. Some authors have suggested that $\alpha_v\beta_3$ integrin also mediates the attachment of the sealing zone to the bone matrix [85]. Previous ultrastructural studies indicated that resorbing osteoclasts were highly polarized cells [86]. Current data suggest that resorbing osteoclasts contain not only the sealing zone but also at least three other specialized membrane domains: a ruffled border, a functional secretary domain and a basolateral membrane [85, 87]. As the osteoclast is preparing to resorb bone, it attaches to the bone matrix through the sealing zone and forms another specific membrane domain, the ruffled border. The ruffled border is a resorbing organelle, and it is formed by fusion of intracellular acidic vesicles with the region of plasma membrane facing the bone [87]. During this fusion process much internal membrane is transformed and creates long, finger-like projections that penetrate the bone matrix. The characteristics of the ruffled border do not match those of any other plasma membrane domains described. Although facing the extracellular matrix, it has several features that are typical of the late endosomal membranes. Several late endosomal markers, such as CIC-7, V-type H-ATPase, are densely concentrated at the ruffled border [88, 89]. The main physiological function of osteoclasts is to degrade the mineralized bone matrix. This involves dissolution of crystalline hydroxyapatite and proteolytic cleavage of the organic matrix, which is rich in collagen. Before proteolytic enzymes can reach and degrade collagenous bone matrix, tightly packed hydroxyapatite crystals must be dissolved. It is now generally accepted that the dissolution of mineral occurs by targeted secretion of HCl through the ruffled border into the resorption lacuna. This is an extracellular space between the ruffled border membrane and the bone matrix, which is sealed from the extracellular fluid by the sealing zone. The low pH in the resorption lacuna is achieved by the action of ATP-consuming vacuolar proton pumps both at the ruffled border membrane and in intracellular vacuoles. Osteoclasts attach to bone and form a circumferential sealing zone that isolates the bone resorption compartment from the extracellular space. Osteoclast plasma membrane within the sealing zone develops into the ruffled border. The observation that NH_4Cl reversibly inhibits bone resorption by osteoclasts indicates that the resorption compartment is acidic and that the sealing zone does not permit the entry of H^+ and NH_4^+. The osteoclast cytoplasm is rich in carbonic anhydrase II (CA II) [90, 91], providing a continuous supply of protons and bicarbonate. Protons are transported across this membrane into the bone resorption compartment by vacuolar-type H^+-ATPase (V-type ATPase) [92, 93]. Chloride ions passively follow the protons through conductive anion channels. The combined activities of the proton pump and chloride channel acidify the resorption compartment and alkalinize the cytoplasm. Bicarbonate exits the cell into the extracellular space in exchange for chloride via a basolateral electroneutral anion exchanger, correcting the cytoplasmic alkalinization and compensating for cytoplasmic chloride loss. The net result of these coordinated transport activities is the transcellular movement of HCl into the bone resorption compartment. This model predicts that both the ruffled border proton pump and chloride channel play key roles in bone resorption. The proton pump provides the proton-motive force necessary to generate a pH gradient. However, the pump is electrogenic. The chloride channel shot-circuits the electrogenic pump and allows maximal proton transport. It follows that limitation of the chloride conductance could inhibit acid transport independently of the intrinsic activity of the proton pump. Similarly to a current model for regulation of the pH of some intracellular organelles, regulation of the anion conductance rather than proton pump activity could be the key point at which the rate of osteoclast acid transport, and hence bone resorption, is governed. Thus, molecular characterization of the ruffled border chloride channel may provide insight into regulation of osteoclast bone resorption and could define a pharmacological target for the treatment of metabolic bone disease [94]. The osteoclast proton pump is sensitive to bafilomycin A1, which also effectively inhibits bone resorption both *in vitro* and *in vivo*. The recent finding that vacuolar ATPase at the ruffled border contains cells specific subunits has further encouraged development of resorption inhibitors that inhibit the osteoclast proton pump. Protons for the proton pump are produced by cytoplasmic carbonic anhydrase II, high levels of which are synthesized in osteoclasts. In order to generate protons, the presence of CA II is essential. It catalyzes the conversion of H_2O and CO_2 into H_2CO_3, which then is ionized into H^+ and HCO_3^- [94, 95]. A mutation in CA II can cause osteopetrosis due to non-functional osteoclasts [45]. The HCO_3^- ions are exchanged for Cl^- through an anion exchanger, a membrane transporter protein AE2 located in the basolateral membrane, leading to continued influx of Cl^- for acidification of the resorption lacuna [96, 97]. After solubilization of the mineral phase, several proteolytic enzymes degrade the organic bone matrix, although the detailed sequence of events at the resorption lacuna is still obscure. Two major classes of proteolytic

enzymes, lysosomal cysteine proteinases and matrix metalloproteinases (MMPs) have been studied most extensively. Osteoclasts produce proteases, of which cysteine proteinase cathepsin K has been proven to be the most important [98], aiding to the degradation of the organic bone matrix. Eleven different types have been described (B, C, F, H, K, L and other) with cathepsin K being the most important with respect to bone remodeling, since it is a protease with intense collagenase activity, especially with respect to acid pH, which is essential to dissolve calcic hydroxyapatite, the main mineral component of bone. It degrades the two types of collagen, I and II and is predominantly expressed in osteoclasts [99, 100]. Cathepsin K gives rise to specific degradation products-like C terminal cross-linking telopeptide of type I collagen (CTXI), which can be used for measurements of bone resorption [100]. The role of cathepsin K in bone resorption was determined using evidence from an autosomal recessive osteochondrodysplasia named pycnodysostosis, a very rare disease characterized by high BMD, acroosteolysis of the distal phalanxes, shot stature, and cranial deformaties with late closing of the fontanelles [98, 101]. Studies in mice submitted to nonfunctional mutations of cathepsin have given rise to different models of osteopetrosis [102]. The bone matrix is resorbed, during which, MMP activity is known to give rise to a specific degradation fragment, C-terminal telopeptide of type I collagen (ICTP) [103]. After matrix degradation, the degradation products are removed from the resorption lacuna through a transcytotic vascular pathway from the ruffled border to the functional secretory domain, where they are liberated into the extracellular space. Quantitative data are still missing, but clearly large amounts of degraded extracellular material must be transported through the resorbing cell, given that the volume of the resorption pit can easily exceed the volume of the entre cell. The extent to which the degradation of collagen and other matrix components take place outside the cell and the extent to which it takes place in the cellular transcytotic compartments are not known. Recent results have suggested that tartrate-resistant acid phosphatase (TRAP), a widely used osteoclast marker, is localized in the transcytotic vesicles of resorbing osteoclasts, and that it can generate highly destructive reactive oxygen species able to destroy collagen [103]. This activity, together with the co-localization of TRAP and collagen fragments in transcytotic vesicles, suggests that TRAP functions in further destruction of matrix-degradation products in the transcytotic vesicles. The observed mild osteopetrosis in TRAP-knockout mice supports this hypothesis [100, 103, 104].

Cellular and molecular aspects of vascular calcification

Vascular calcification often occurs with atherosclerosis and various cardiovascular diseases. Vascular calcification can be categorized into four main types according to its location: atherosclerotic intimal calcification, medial calcification (Mönckeberg's sclerosis), cardiac valve calcification and calcific uremic arteriolopathy [105, 106]. Histologically, calcified deposits may be amorphic, chondromorphic or osteomorphic in structure, and may be characterized as metastatic or dystrophic. Medial calcification, also termed Mönckeberg's sclerosis, occurs in the tunica media of blood vessels. It is a characteristic feature of generalized arterial calcification of infancy [107, 108], chronic kidney disease and diabetes [109, 110], and is associated with increased cardiovascular mortality and the risk of amputation [13, 111, 112, 113]. Medial calcification occurs independently of atherosclerotic calcification and is a process similar to intramembranous bone formation, with no cartilaginous precursor required [114, 115]. Calcium deposition can be observed throughout most of the medial thickness in the early stage of disease. At later stages of the disease, the media is filled with circumferential rings of minerals. In some cases, osteocytes and bone trabeculae can also be observed. Atherosclerotic calcification of the intima is the formation of plaques within the intimal layer of large vessels, and underlies coronary artery disease and cerebrovascular disease, the most common forms of life-threatening cardiovascular disorders [116, 117]. Atherosclerosis can be induced by chronic inflammation and lipid deposition, with dyslipidemia frequently linked to the severity of calcium deposition [118, 119]. Atherosclerotic calcification is the most common form of calcific vasculopathy and occurs as early as in the second life decade just after fatty streak formation [114, 120]. Small aggregates of crystalline calcium can be detected in the developing lesions, and in adults in their fourties greater lesion areas may be calcified. The degree of calcification correlates with the extent of atherosclerosis, with age and hypertension as dominant risk factors for systemic calcified atherosclerosis [121]. Although valuable research has defined many key factors and cell types involved, surprising new insights continue to arise that deepen our understanding and suggest novel research directions or strategies for clinical intervention in vascular calcification. One emerging area in vascular biology involves the RANKL-RANK-OPG system, recently discovered to be critical regulators of skeletal tissue. Overall, the RANKL-RANK-OPG system may mediate important and complex links between the bone and vascular systems. Thus, these molecules may play a central role in

regulating the development of vascular calcification coincident with declines in bone mineralization with osteoporosis [122, 123].

Regulation of vascular calcification by osteoclast regulatory factors RANKL-RANK-OPG

Like most biological processes, vascular calcification is actively regulated by the networks that involve positive and negative regulators, temporal expression or activation of modulators, and multiple amplification or suppressive feedback loops that orchestrate cell recruitment, differentiation, function, survival, and interactions with other cells or matrix molecules [124, 125]. Mounting evidence suggests that RANK, RANKL, and OPG may participate in multiple aspects of these processes governing vascular calcification.

The OPG-RANKL-RANK axis is undoubtedly of central importance in regulating immune and skeletal tissue. Recent data revealed that the vascular system is also involved in this axis [75, 126]. Vascular endothelial cells are leading coordinators of inflammatory response, and immune-mediated mechanisms are involved in an abundance of vascular diseases, including vascular calcification. Moreover, vascular calcification may involve differentiation of osteogenic cells from VSMC or calcifying vascular cells, expression of multiple ossification-related molecules, formation of calcified structures resembling bone, and attendance of T-cells, macrophages and endothelial cells, which may constitute the source or target of OPG-RANKL-RANK actions. It seems possible that the OPG-RANKL-RANK axis exerts an important role in the vascular system through immunobiologic and osteogenetic mechanisms. Growing evidence suggests that the triad of RANK-RANKL-OPG, key proteins involved in bone metabolism, may be important players in vascular calcification. OPG, RANKL and RANK are present in atherosclerotic plaques and valve disease, and their relative expression levels are different depending on the stage of the disease [127]. Vascular calcification, a degenerative process considered in the past to be a passive procedure, has now been suggested to be related to ossification. Many proteins responsible for bone formation have been identified on the arterial wall. The RANKL-RANK-OPG axis, responsible for ossification and bone mineralization, seems to play a major role in vasculature and atherosclerosis. The concept of an active role of RANKL-RANK-OPG system in vascular pathophysiology is intriguing and is gaining increasing support from both epidemiological and basic research [76, 128]. RANK-RANKL-OPG is a pathway of interest and yet with undefined role in progression of vascular calcification in clinical settings. It implies the interaction between the receptor activator of nuclear factor κB (RANK) with its ligand (RANKL) and the inhibitory function of OPG (as a decoy) on this interaction. OPG reduces the activity of the nuclear factor κB, a transcription factor that regulates immune-mediated genes, and is important for inflammatory activity, innate immunity, and cellular differentiation [129, 130]. RANKL is expressed in osteoblasts, smooth muscle cells, T lymphocytes, and stromal cells, while OPG is expressed in these cells besides the endothelium. Regarding bone remodelling (reabsorption), activation of RANK by RANKL transforms preosteoclasts into their mature form (Fig. 3) [131]. This interaction is directly inhibited by OPG. However, the role of osteoclasts in vascular calcification and in bone reabsorption in the vessel is still undetermined. On the other hand, it is known that the RANK-RANKL-OPG axis participates in various stages of a complex atherosclerotic inflammatory cascade (Fig. 3) RANKL is expressed in smooth muscle cells and in T lymphocytes, modulating dendritic cells maturation and inhibiting apoptosis. This activity may be inhibited by the action of OPG on RANKL. OPG, on the other hand, is stimulated by multiple inflammatory mediators, such as IL-1, TNF-α, TGF-β, and IFN-γ. These mediators increase endothelial OPG synthesis. Consequently, OPG stimulates adhesion molecules expression and leukocyte infiltration in the vessel wall. This process promotes the expression of RANKL, and the proliferation of smooth muscle cells [131, 132]. Subsequently, RANKL directly stimulates osteogenic differentiation of these cells, or, indirectly promotes osteogenesis by increasing TNF-α secretion in monocytes [130, 133] or via BMPs [134]. The presence of RANKL in combination with OPG (particularly with a reduced OPG/RANKL ratio) increases metalloproteinase activity, which plays an important role in atherosclerotic plaque erosion and rupture [75, 131, 135].

RANKL/RANK and vascular calcification

The receptor activator of nuclear factor-κB ligand (RANKL) is a member of the tumor necrosis factor family important in bone remodeling. Recent evidence suggests that calcification in the vessel wall is equivalent to mechanisms mediating bone formation. RANKL is highly expressed by T cells and endothelial cells (ECs), but not by PTH or VD3; which elevate RANKL in bone OB/stromal cells, and up regulated by inflammatory cytokines [123, 127, 131]. Cell response to RANKL depends on the level of expression of its receptor RANK, but also on the presence of its decoy receptor, OPG [123]. Immune mechanisms involving activated T cells (the source of RANKL) and antigen-presenting dendritic cells (the target for RANKL) are implicated in vascular inflammatory

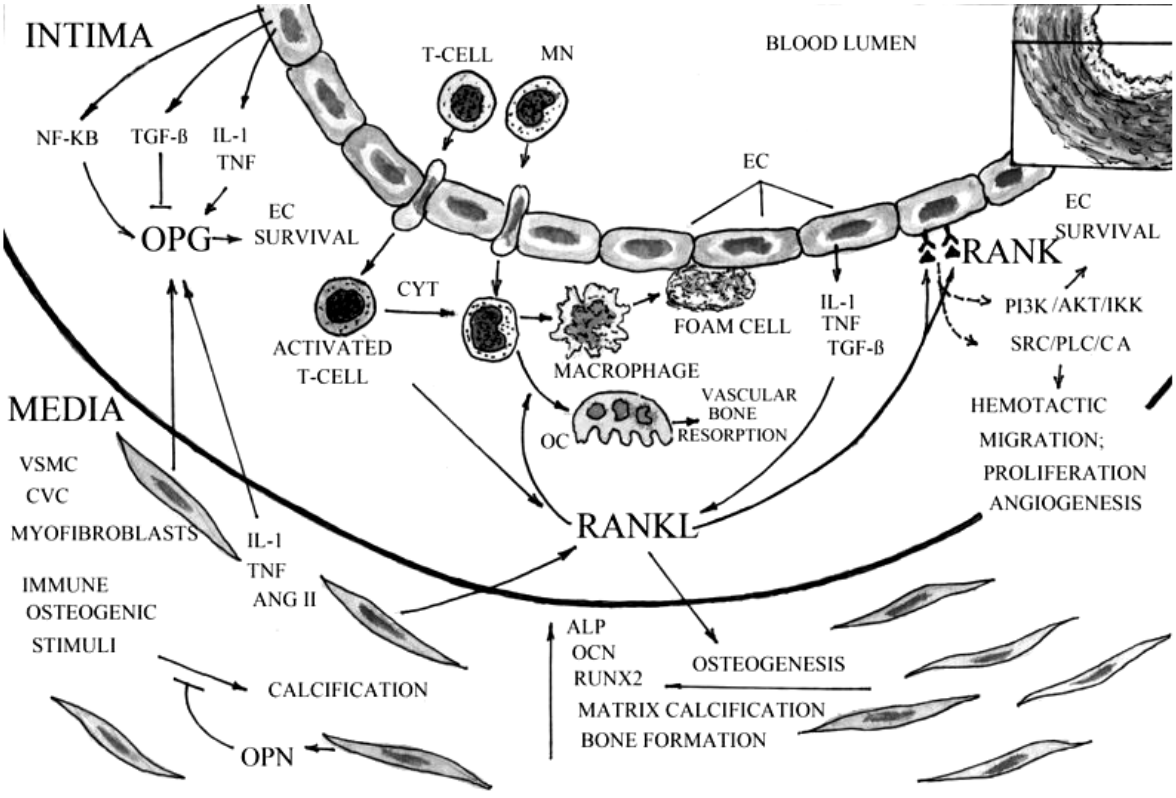

Fig. 3. Schematic diagram of potential expression, regulation, and function of RANKL, RANK, and OPG in atherosclerotic vascular calcification. Soluble or membrane-associated RANKL may be produced by inflammatory cytokine-stimulated endothelial cells (ECs), activated T cells recruited into the tissue, vascular smooth muscle cells (VSMCs) undergoing osteogenic differentiation, or ECs in contact with CD44-expressing VSMCs. On interacting with the RANK receptor (increased on ECs by VEGF), RANKL might contribute to the atherosclerotic process through: (1) triggering EC survival, proliferation, chemotactic migration, or angiogenesis; (2) stimulating monocyte MMP-9 activity, transmigration through the EC barrier, and development into osteoclasts (OCs) that resorb bone in advanced lesions; and (3) promoting an osteogenic differentiation program in VSMCs that leads to the synthesis of bone proteins and matrix calcification within the arterial vessel. OPG, a soluble decoy receptor that antagonizes RANKL actions in bone tissue, is constitutively produced by VSMCs and may be induced by inflammatory modulators or down regulated by anti-inflammatory stimuli in VSMCs and ECs. OPG is also generated by ECs following their $\alpha_v\beta_3$ engagement of OPN (both of which are increased by VEGF) and may serve as an EC survival signal to oppose the pro-apoptotic actions of TRAIL highly produced by atherosclerotic VSMCs. Elevated OPG levels may help protect against arterial damage and vascular calcification, reflect a general state of EC dysfunction, rise in the serum of patients with diseases associated with vascular calcification, and/or possibly represent counter-regulatory attempts to offset the effects of a RANKL increase. Further details are provided in the text; MN – monocyte; NF-κB – nuclear factor kappa B; TGF-β – transforming growth factor beta; IL-1 – interleukin-1; TNF – tumor necrosis factor-α; OPG – osteoprotegerin; RANK – receptor activator nuclear factor-κB; RANKL – receptor activator nuclear factor-κB ligand; CYT – cytokine; PI3K/AKT/IKK – phosphatidylinositol 3-kinase / serine / threonine protein kinase B / inhibitor kappa B kinase; SRC/PLC/CA – tyrosin protein kinase / phospholypase C / calcium; CVC – calcification of vascular cells; ANG II – angiotensin II; OPN – osteopontin; ALP – alkaline phosphatase; OCN – osteocalcin; RUNX2 – runt-related transcription factor 2

diseases. It has been speculated that RANKL and its antagonist OPG represent an important cytokine system in vascular biology [129]. Protection against mineralization in the vessel wall is thought to be achieved by a modulation of OPG and RANKL expression, inhibiting osteoclast maturation and thus preventing the subsequent release of calcium and mineral from bone. Although expression levels of RANKL can often remain unchanged, the anti-osteoclastogenic cytokine OPG is more often reduced in osteolysis patients or after dexamethasone treatment, thus elevating RANKL:OPG ratios [136]. These findings would suggest that the OPG-RANKL-RANK autocrine/paracrine axis is dysregulated under certain pathological situations, with a parallel osteoporotic bone loss in conjunction with bone deposition in the vasculature [123]. Until now, the evidence linking RANKL with vascular calcification has been circumstantial and indirect. S. Panizo et al. [134] report direct links between RANKL/RANK signaling, elevated levels of BMP4, and increased vascular calcification both *in vitro* and *in vivo*. They demonstrate an involvement of the IKK-α pathway, the alternative pathway of activation of NF-κB signaling,

and eliminate involvement of an apoptotic process in RANKL-induced mineralization. First, using an *in vitro* model of calcification, they show that addition of RANKL to VSMCs in culture accelerates mineralization, as assessed by an increase in alkaline phosphatase activity, calcium incorporation, and von Kossa staining, effects that are all inhibited by addition of its soluble decoy receptor, OPG. The authors also used small interfering (si)RNA to knock down RANK, abolishing the effect of RANKL induction of calcification, indicating a direct role of RANKL binding to RANK causing osteogenic differentiation of the VSMCs. To assess the mechanism underpinning this effect, S. Panizo et al. [134] investigated the downstream signaling pathways activated by RANKL, namely the NF-κB transcription factor cascade, involving the 2 kinases IKK-β and IKK-α, which are associated with the classical and alternative pathways of NF-κB activation respectively. Of note, S. Panizo et al. [134] demonstrated that inhibition of IKK-β, using siRNA knockdown, had no effect on calcification, and whereas blocking IKK-α obliterated RANKL-induced VSMC mineralization. In addition, they showed that after incubation of the cells with RANKL, nuclear levels of RelB were elevated, adding further confirmation of the alternative pathway of NF-κB activation. BMPs are members of the transforming growth factor-β family, initially shown to be involved in bone formation but in vascular calcification [137]. A striking finding of S. Panizo et al. [134] concerns the elevated BMP4 mRNA abundance and protein secretion resulting from RANK activation, which can be attenuated when they inhibit IKK-α. In addition, they show that addition of Noggin, an inhibitor of BMP4, blunted the increase in calcification induced by RANKL, providing evidence for the direct link between RANKL and BMP4-mediated calcification. Finally, the authors confirm RANKL-induced biomineralization *in vivo*, using a rat model of calcification. The use of calcitriol, a synthetic vitamin D analog, increases the absorption of calcium from renal tubular cells and also stimulates osteoclastic calcium resorption from bone. Animals with ⅝ nephrectomy show an increase in calcification, an effect enhanced by the addition of calcitriol. The interesting finding from the study by S. Panizo et al. [134] is that the local vascular expression of RANKL and BMP4 increased within the vicinity of calcification in the rat arteries, whereas systemic RANKL and OPG levels were unchanged and elevated, respectively, concluding that paracrine and autocrine regulatory pathways may differ and a disruption in the RANKL:OPG ratio has a more direct effect on the deposition of a mineralized matrix in the vessel wall than levels of RANKL *per se*. In another study, RANKL indirectly promoted smooth muscle cell calcification by enhancing macrophage paracrine pro-calcific activity through release of Il-6 and TNF-α [138]. These studies suggest that RANK/RANKL may be important in promoting vascular calcification, while OPG inhibits vascular calcification.

Role OPG in vascular calcification and atherosclerosis

Osteoprotegerin (OPG), a member of the tumor necrosis factor receptor superfamily, is a soluble decoy receptor for the osteoclast differentiation factor receptor-activator of nuclear factor κB ligand (RANKL) that inhibits interaction between RANKL and its membrane-bound receptor RANK (Fig. 2, A–B). The RANKL-OPG-RANK axis has been shown to regulate bone remodeling and was more recently found to be involved in carcinogenesis as well as central thermoregulation. This system has also been linked to the development of atherosclerosis and plaque destabilization [127, 139]. RANKL exhibits several properties with relevance to atherogenesis, such as promotion of inflammatory responses in T cells and dendritic cells, induction of chemotactic properties in monocytes, induction of MMP activity in vascular smooth muscle cells (SMC), and RANKL has also been found to have prothrombotic properties [74, 125, 129]. In observational studies, elevated circulating OPG levels have been associated with prevalence and severity of coronary artery disease, cerebrovascular disease, and peripheral vascular disease [140]. Circulating OPG levels are increased in patients with acute coronary syndrome, and its enhanced expression has been found within symptomatic carotid plaques. Elevated OPG levels have also been associated with the degree of coronary calcification in the general population as a marker of coronary atherosclerosis [141]. OPG has been reported to predict survival in patients with heart failure after acute myocardial infarction, to predict heart failure hospitalization and mortality in patients with acute coronary syndrome, and to be associated with long-term mortality in patients with ischemic stroke. There are also a few studies that show a relationship between OPG and CVD and related mortality in the general population. In this issue, W. Lieb et al. [142] describe the clinical correlates, from subclinical disease to mortality, of serum OPG in 3250 Framingham study participants. They found that OPG was related to risk factors, such as age, smoking, diabetes, systolic blood pressure, and prevalent CVD, as well as CVD-related mortality. There was also a (non-significant) relation between OPG and coronary calcification, and the Dallas heart study demonstrated a relation of OPG to coronary as well

as aortic calcifications. In addition, A. Vik et al. [143] have recently shown that OPG was an independent predictor of plaque growth in the general population in women but not in men, indicating gender-specific actions of OPG in plaque progression. Taken together, all these studies suggest that OPG may be a new promising marker for risk prediction in CVD. On the other hand, although the pathogenic effects of the RANKL/OPG/RANK axis in atherogenesis has been related to RANKL activities (Fig. 3), W. Lieb et al. [142] did not find any relation between serum levels of RANKL and CVD, which is in line with the EPIC-Norfolk and the Tromsø studies. Moreover, genetic OPG inactivation accelerates advanced atherosclerotic lesion progression in older apolipoprotein $E^{-/-}$ mice, and OPG treatment promotes SMC accumulation, collagen fiber formation, and development of fibrous caps in apolipoprotein E deficient mice, suggesting a protective rather than harmful effect of OPG in atherogenesis [144]. The reasons for these apparently discrepant findings are at present not clear, but several potential explanations may exist. A reliable biomarker in CVD is not necessarily an important mediator in atherosclerosis but rather a stable marker of up-stream pathways that are involved in the pathogenesis of CVD. In fact, the leading role of C-reactive protein as an inflammatory biomarker in CVD is not primarily based on its pathogenic role in these disorders but rather on its ability to reflect upstream inflammatory activity. OPG has been shown to be modulated by various up-stream inflammatory mediators, such as IL-1 and tumor necrosis factor α [72, 145]. Moreover, because OPG circulates at much higher levels than RANKL, it may be a more stable overall measure of RANKL-RANK activity than soluble RANKL. Therefore, the role of OPG as a marker in CVD may not be related to its role as a mediator but reflect its role as a stable marker of activity in the RANKL/OPG/RANK axis, as well as the activities in inflammatory pathways that are involved in atherogenesis. Thus, mirroring several interaction pathways with relevance to atherosclerosis, such as inflammation, matrix degradation, and vascular calcification. Moreover, S.M. Venuraju et al. [135] reported OPG to be expressed at higher levels in symptomatic carotid plaques than in asymptomatic carotid plaques, and it is possible that the relation between circulating OPG levels and CVD may, at least in part, reflect shedding from atherosclerotic lesions. However, several questions remain unresolved. First, although OPG is a known inhibitor of RANKL, its biological effect may depend on the molar ratio between RANKL and OPG. Thus, although OPG under low RANKL/OPG ratios attenuates RANKL-mediated effects, it has during high RANKL/OPG ratios been found to enhance the RANKL-mediated effects on MMP levels in vascular SMC. Also, OPG has been shown to exert chemotactic properties, and SMC incubated with OPG develop impaired cell proliferation, increased apoptosis, increased MMP-2 and MMP-9 levels, and enhanced IL-6 production [141]. Furthermore, in addition to its ability to bind RANKL, OPG seems to also bind tumor necrosis factor-related apoptosis inducing ligand but the role of this interaction, with potential antiapoptotic net effects, in atherogenesis has not been studied. Second, in line with some other studies, W. Lieb et al. [142] found an inverse correlation between circulating RANKL and OPG levels. It is important to clarify if OPG and RANKL are differently regulated or if these findings merely reflect enhanced binding of circulating RANKL to OPG in excess of OPG, leading to decreased levels of free RANKL. Moreover, although several studies have suggested a role for RANKL-OPG-RANK axis in atherogenesis, OPG was recently found to inhibit vascular calcification without affecting atherosclerosis in low-density lipoprotein receptor-deficient mice [72, 130, 146]. In the Tromsø study, OPG was associated with plaque progression but not with novel plaque formation, suggesting a restricted role in atherogenesis [143]. Furthermore, drugs targeting the RANKL-OPG-RANK axis have not been related to CVD events in randomized trials, but importantly, these studies were not designed to evaluate their effect on CVD. Although several studies suggest the involvement of RANKL-OPG-RANK axis in coronary artery disease and related atherosclerotic disorders [123, 135, 147] more evidence is needed to evaluate the predictive and diagnostic value of serum OPG levels for clinical use as well as the pathogenic importance of these mediators in the process of atherosclerosis and plaque rupture.

OPG and vascular disease

Since its initially discovered in 1997 as a key regulator in bone turnover, OPG has become a subject of intense research in its role as a common mediator of bone metabolism and vascular calcification and vascular diseases [148, 149, 150]. Extracellular matrix calcification is a normal physiological process, necessary for proper development of tissues like bone, teeth and cartilage. However, when it occurs in tissues that normally are not mineralized, calcification can lead to serious consequences. Researches *in vitro*, and in animal models show that OPG inhibits vascular calcification [72, 130, 146]. Paradoxically, clinical researches have shown that OPG serum levels is increased in patients with progressive cardiovascular disease [151], correlates

with presence and severity of coronary artery disease, is associated with left ventricular hypertrophy and C-reactive protein, is increased in chronic haemodialysis patients [147, 152], in type 2 diabetic patients with microvascular complications, and in heart failure after acute myocardial infarction. This has led to an interesting debate about potential role of OPG as a vascular disease biomarker. The exact mechanism by which OPG affects cardiovascular pathology is still unclear. The need for complete picture is a subject to valuable research, that shows that OPG is not only the marker but the mediator in vascular pathology that modulates osteogenic, inflammatory and apoptotic response [72, 125, 132].

Role of cathepsin K in development vascular calcification (atherosclerosis)

Cathepsin K, a potent extracellular matrix degrading cysteine protease, has been linked to the pathogenesis of osteoporosis and cardiovascular diseases [19, 22, 24]. Cathepsin cysteine proteases have been described to play a role in several cardiovascular diseases, including restenosis and neointima formation, aneurysm formation, and atherosclerosis. Cathepsin K expression in normal arteries is low. Early human atherosclerotic lesions showed cathepsin K expression in the intima and medial SMCs. In advanced atherosclerotic plaques, cathepsin K was localized mainly in macrophages and SMCs of the fibrous cap. A.O. Samokhin et al. [153] report the effects of cathepsin K deficiency ($ctsK^{-/-}$) on atherosclerotic plaque formation in brachiocephalic arteries in an aggressive atherosclerosis model using apoE-deficient mice on cholate-containing high fat diet (HFD). On this diet, $apoE^{-/-}$ mice displayed severe lesions with buried fibrous caps after 8 weeks, whereas the $apoE^{-/-}$ $ctsK^{-/-}$ mice revealed a significantly decreased number of buried fibrous caps accompanied by increased collagen content in plaque areas and fibrous cap thickness. After 16 weeks of HFD, $ctsK^{-/-}$ mice had smaller plaque areas and maintained the structure of the tunica media in terms of their smooth muscle cell content and elastic lamina integrity. Overall macrophage content in the tunica media was lower in $ctsK^{-/-}$ mice, but higher in the plaque area after 8 weeks of HFD. Decreased apoptosis rates in atherosclerotic plaques in brachiocephalic arteries of cathepsin K-deficient indicated a lower level of inflammation. Therefore, cathepsin K deficiency appears to increase lesion stability in brachiocephalic arteries by maintaining the integrity of the tunica media and by decreasing plaque vulnerability to rupture. Cathepsin K protein levels were increased in atherosclerotic lesions when compared with normal arteries, whereas cathepsin K mRNA levels were similar in both atherosclerotic and normal tissues [154]. Cathepsin K deficiency also affects the macrophages and lipid metabolism in atherosclerotic plaques. Along with the reduction of plaque collagen content, J. Guo et al. [155] demonstrated a marked increase in the numbers of macrophages in plaques of leucocyte cathepsin K-deficient mice. S.P. Lutgens et al. [19] have shown that plaque-resident macrophages are also larger due to their transformation into foam cells in whole-body cathepsin K-deficient mice. Therefore, cathepsin K deficiency enhances the formation of foam cells, which destabilize atherosclerotic plaques, but the reason for this is unclear. Furthermore, the uptake of modified lipoproteins is enhanced in cathepsin K-deficient macrophages and involves caveolin-1 and the scavenger receptor CD-36 pathway and the efflux of cholesterol is depressed in cathepsin K-deficient macrophages [156]. Modified lipoproteins likewise act to destabilize atherosclerotic plaques. These findings indicate that lowered levels of cathepsin K generally favour both foam cell formation and retention of lipoproteins in the plaque and hence cause plaque destabilization rather than stabilization. If reduction of cathepsin K does influence atherosclerotic plaque stability, can manipulation of cathepsin K be therapeutically useful? Plaque area in whole-body cathepsin K-deficient mice was reduced by almost half, suggesting that lowering cathepsin K levels, perhaps by using specific inhibitors of cathepsin K, might prove to be useful in the treatment of atherogenesis. Because of the known negative effect of cathepsin K on bone formation, several cathepsin K inhibitors, odanacatib, for example, have been tested for treating osteoporosis [21], but there are no data for their use in countering atherosclerosis in animals or humans. *In vivo* knockout studies revealed that cathepsin K is important in ECM degradation [19], since deficiency of these cathepsin reduces plaque size [18, 157], atherosclerotic plaque progression, and the number of elastin breaks. Deficiency of cystatin C exerts opposite effects on atherosclerotic plaque size and elastin breaks. Cathepsin K also plays an essential role in lipid metabolism [157]. Especially cathepsin F, but also cathepsins B, K, and S, are important in the degradation of lipid. The role of cathepsins in cholesterol efflux seems to depend on the localization of cathepsins. Extracellular cathepsins degrade cholesterol acceptors, thereby reducing cholesterol efflux and increasing foam cell formation. Intracellular deficiency of cathepsin K also reduces cholesterol efflux independent of cholesterol acceptor degradation [154]. This latter observation suggests a protective role for cathepsin K in foam cell formation, which is further substantiated by the increase in lipid uptake and

storage by macrophages when cathepsin K is absent [156]. Although to date no *in vivo* studies are available on the role of cathepsin K in neointima and aneurysm formation, changes in the expression levels of cathepsins K, L, S, and the natural inhibitor of cathepsins, cystatin C, in diseased arteries strongly suggest a role for these proteins in neointima and aneurysm formation [158]. Degradation of the ECM by cathepsin cysteine proteases may facilitate the migration and invasion of SMCs and macrophages, thereby contributing to neointima formation. The inflammatory status as seen in aneurysms may contribute to cathepsin activity, thereby increasing ECM degradation and possibly contributing to the formation of aneurysms. Furthermore, cathepsins K and S have been described to play a role in neovascularization by degradation of the ECM. However, *in vivo* data exploring the role of cathepsins in relation to neovascularization in cardiovascular disease are still lacking. Recent findings also indicate a possible role for cathepsins as a diagnostic tool both as imaging device and as biomarker. The current quest for cathepsins as diagnostic tool therefore seems a reasonable goal in atherosclerosis research. Furthermore, inhibitors of cathepsin K showed effectiveness in clinical evaluation for the treatment of osteoporosis, suggesting that cathepsin inhibitors may also have therapeutic effects for the treatment of atherosclerosis [20].

New perspective in dual therapy atherosclerosis and osteoporosis: the RANKL-RANK-OPG system and cathepsin K as a potential therapeutic target

Vascular calcification, a key manifestation of atherosclerosis, is a tightly regulated process with similarities to bone remodeling [10, 15, 159]. Several proteins implicated in normal bone metabolism (e.g., osteocalcin, osteonectin, osteoprotegrin, bone morphogenic protein) have also been shown to localize to the arterial wall and the artherosclerotic plaque [140, 160]. Mice deficient in osteoprotegrin, an indirect inhibitor of osteoclastogenesis, have both severe osteoporosis and extensive arterial calcification [70, 161, 162]. Interestingly, calcification of the aorta has been shown to correlate with incidence of vertebral fractures and low BMD in human subjects, and is considered to be an independent predictor of cardiovascular mortality [10]. One example of the potential association between osteoporosis and atherosclerosis are anti-resorptive treatments like estrogen replacement therapies and bisphosphonates, which appear to have cardio-protective effects as exhibited by a positive impact on lipid metabolism and reduced atherosclerotic plaque formation [163, 164]. New experimental therapeutic horizons have been opened up, promoting the generation of dual-purpose treatment, which will retard the progression of atherosclerotic plaques and enhance bone density. Briefly, they involve: (1) inhibition of RANKL activity; (2) inhibition function of cathepsin K.

Inhibitors RANKL function (denosumab, omentin-1)

Less than a decade ago substantial changes in our understanding of bone metabolism and vascular calcification emerged by the discovery of the outstanding importance of the osteoprotegerin (OPG) – receptor activator of nuclear factor-κB (RANK) – RANK ligand (RANKL) system for bone and vascular cells regulatory processes. Recent discoveries in bone biology have demonstrated that RANKL, a cytokine member of the tumor necrosis factor superfamily, is an essential mediator of osteoclast formation, function and survival (Fig. 2). Denosumab, the first in class RANKL-inhibitor, is a recombinant human IgG2 antibody with affinity and specificity for RANKL. By binding to RANKL, denosumab prevents the RANKL/RANK interaction on the osteoblast which leads to the inhibition of osteoclast formation, function, and survival, thereby decreasing bone resorption and increasing bone mass and strength in both cortical and trabecular bone [165]. Administered as a subcutaneous injection every six months, denosumab has been shown to decrease bone turnover and to increase bone mineral density in postmenopausal women with low bone mass and osteoporosis. In these patients denosumab significantly reduced the risk of vertebral fractures, hip fractures and nonvertebral fractures. In all clinical trials published to date [166, 167, 168, 169], denosumab was well tolerated with an incidence of adverse events, including infections and malignancy, generally similar to subjects receiving placebo or alendronate. The denosumab therapeutic regimen consisting in a subcutaneous injection every 6 months may increase patient compliance and persistence with a further benefit from treatment. By providing a new molecular target for osteoporosis treatment, denosumab is a promising drug for the treatment of postmenopausal osteoporosis and the prevention of fragility fractures. Denosumab was approved by the Food and Drug Administration in June 2010 as a new treatment for postmenopausal osteoporosis in women who are at high risk for fracture. As described above, RANKL also has direct effects to promote vascular smooth muscle cell calcification [170] and osteoclast like cell formation [171]. Interestingly, that the results of research on influence of denosumab on vascular calcification are controversial. Thus, E.J. Samelson et al. [172] evaluated the effects of denosumab, an inhibitor of RANKL, on the progression of vascular calcification in a large, randomized clinical

trial. Authors found no evidence that treatment with this drug contributed to the progression of abdominal aortic calcification or to increased risk of cardiovascular adverse events in 60–90 year old women with osteoporosis and high cardiovascular risk. On the other hand, S. Helas et al. [173] demonstrated a reduction of vascular calcium deposition in glucocorticoid-induced osteoporotic mice by administering denosumab. In confirmation of these results in a recent study, D.A. Lerman et al. [174] demonstrated that denosumab inhibited induced calcium deposition to basal levels in porcine valvular interstitial cells model. Denosumab holds promise as a novel treatment for vascular calcification (atherosclerosis) and osteoporosis and is currently being investigated in an ongoing randomized controlled trial (SALTIERE II and RANKL Inhibition in Aortic Stenosis) [175, 176]. The purpose of this study is to present the effects of 4 years of continued denosumab treatment of vascular calcification from a phase II study.

Omentin-1 attenuates arterial calcification and bone loss

Omentin-1 (also intelectin-1) is a novel visceral adipose tissue-derived cytokine. The level of circulating Omentin-1 is high in plasma and is decreased in patients with obesity and increased after weight loss. It correlates positively with adiponectin and negatively with body mass index and the leptin level [177]. Omentin-1 has been reported to inhibit osteoblast differentiation *in vitro* [178, 179]. In co-culture systems of osteoblasts and osteoclast precursors, omentin-1 reduced osteoclast formation by stimulating OPG and inhibiting the RANKL production in osteoblasts [180]. This suggested that omentin-1 may play a pivotal role in the dynamic balance of bone formation and bone resorption [181]. Arterial calcification is positively associated with visceral adiposity, but the mechanisms remain unclear. Several experimental studies demonstrated the protective role of omentin-1 in the regulation of atherogenesis [182]. This study demonstrated that recombinant human omentin-1 could induce OPG and inhibit RANKL production in primary mouse osteoblasts and calcifying vascular smooth muscle cells (CVSMs) *in vitro*, and adenovirus-mediated over-expression of human omentin-1 in $OPG^{-/-}$ mice could ameliorate bone loss and arterial calcification *in vivo*. All these actions were dependent on the PI3K/Akt signaling pathway [180, 183]. This study investigated the effects of omentin on the osteoblastic differentiation of CVSMCs, a subpopulation of aortic smooth muscle cells putatively involved in vascular calcification. Omentin inhibited mRNA expression of alkaline phosphatase (ALP) and osteocalcin; omentin also suppressed ALP activity, osteocalcin protein production, and the matrix mineralization. Furthermore, omentin selectively activated phosphatidylinositol 3-kinase (PI3K) downstream effector Akt. Moreover, inhibition of PI3K or Akt activation reversed the effects of omentin on ALP activity and the matrix mineralization. These results demonstrate for the first time that omentin can inhibit osteoblastic differentiation of CVSMCs via PI3K/Akt signaling pathway [179], suggesting that lower omentin levels in obese (especially visceral obese) subjects contribute to the development of arterial calcification, and omentin plays a protective role against arterial calcification. Therefore, therapeutic approaches aimed at increasing circulating omentin levels could be valuable for the prevention or treatment of atherosclerosis and osteoporosis.

Cathepsin K inhibitor odanacatib

Odanacatib (ODN, MK-0822; Merck & Co., Inc., Whitehouse Station, NJ, USA) is a potent (*in vitro* IC50 = 0.20 nm), orally active and selective CatK inhibitor (≥ 300-fold selectivity against all other known human cathepsins) [184]. Cathepsin K was identified as a therapeutic target for the treatment of osteoporosis because of its key role in the resorption of the organic matrix of bone. Targeting one particular function of the osteoclast rather than its production or survival has conferred some theoretical advantages over the anti-resorptive agents currently in use. Evidence to date has shown that selective cathepsin K inhibitors substantially reduced bone resorption by preventing cathepsin K degradation of type I collagen in several animal models and in clinical trials. At 36 months, increases in BMD similar to those of zolendronate and denosumab were observed, although these changes are not from head-to-head comparisons [185, 186]. However, cathepsin K inhibition has shown a quality that is absent in other classes of anti-resorptive agents: it has resulted in greater suppression of bone resorption than bone formation, suggesting a dissociation between bone resorption and bone formation. Even after 5 years of treatment with odanacatib in humans, while lumbar spine and hip BMD increases correlated with sustained suppression of bone resorption, there was little suppression of bone formation markers, in comparison with the known reduction of these markers by bisphosphonates [186]. In OVX monkeys, odanacatib not only suppressed bone resorption but also showed a compartment-specific action on bone formation with increased periosteal bone formation and cortical thickness in the femur. These results of the effects of cathepsin K inhibitors are consistent with the dissociation of bone resorption and formation, suggesting an additional influence on bone modeling.

Whether a similar compartment-specific action of odanacatib on bone resorption and formation can be demonstrated in humans remains to be determined. The increases in spine and hip BMD observed with odanacatib were comparable to those observed with the bisphosphonate alendronate and the RANKL-inhibitor denosumab [185, 186]. Interestingly, while there was a smaller reduction in markers of bone resorption in comparison with other powerful anti-resorptive agents, the reduction in levels of formation markers was much smaller. Furthermore, histomorphometry of bone biopsies performed in a subset of 32 patients included in the phase II trial showed that the modest reduction in bone formation markers was not accompanied by a suppression of the bone formation rate. These findings suggest a decoupling between bone formation and resorption. It was hypothesized that as the inhibition of cathepsin K suppresses osteoclast function but does not impair osteoclast viability, it may preserve the osteoclast-osteoblast crosstalk and maintains bone formation. In addition, unlike conventional anti-resorptives, odanacatib displayed site specific effects on trabecular versus cortical bone formation with marked increases in periosteal bone formation and cortical thickness in ovariectomized monkeys. Although their clinical relevance remains to be confirmed, these findings would represent a major advance in the field of bone research. A randomized, placebo-controlled phase III fracture endpoint trial in more than 16 000 postmenopausal women with low bone mass is currently ongoing with expected results in 2016 (NCT00529373). Once available, the results of this study will unveil a comprehensive efficacy and safety profile of odanacatib for the treatment of postmenopausal osteoporosis. Over the past decade, several pharmaceutical companies have become interested in cysteine protease inhibitor development. Individual cathepsins involve physiological and pathophysiological processes, although redundancies may exist, favoring the application of pharmacological inactivation of each individual cathepsin using its selective inhibitors. Development of selective cathepsin inhibitors has been mainly focused on cathepsins S and K because of their involvement in osteoporosis. To our knowledge, compound 6, a nitrile-based specific cathepsin S inhibitor was applied for the first time to evaluate cathepsin inhibitor-mediated vasculoprotective effects on atherosclerosis in an experimental animal model. Cathepsin S inhibitor treatment mice display fewer elastic lamina breaks, infiltrated macrophages, and buried fibrous caps and lessen atherosclerotic plaque size in ApoE$^{-/-}$ mice. Recently, it was demonstrated that *in vivo* administration of a broad-spectrum synthetic cathepsin inhibitor E64*d* lessened hypertension-induced cardiac and renal fibrosis and dysfunction in a Dahl salt-sensitive rat model [187]. Furthermore, cathepsin K inhibitor odanacatib showed effectiveness in clinical evaluation for the treatment of osteoporosis, suggesting that cathepsin inhibitors may also have therapeutic effects for the treatment of atherosclerosis [18, 188, 189]. Until now, however, no data were available on the effect of odanacatib or other inhibitors cathepsin K (relacatib; SB-462795) in animal or human atherosclerosis.

Conclusion

Among the degenerative conditions associated with aging, atherosclerosis and osteoporosis are the critical healthy problems. The role these chronic diseases play in the decline in quality of life and as a major cause of morbidity and mortality cannot be overlooked. Although cardiovascular diseases and osteoporosis have been considered independent processes, increasing evidence suggests the existence of a biological linkage between the bone and vascular system. The association between bone mass loss and carotid atherosclerosis, coronary artery disease, arterial disease of lower limbs, and aortic calcification has been demonstrated in several studies. Some of them show that the progression of the arterial plaque parallels the bone loss; however the nature of the possible link remains uncertain. Several hypotheses have been suggested to explain this association, which include age-related mechanisms, diabetes mellitus, estrogen deficiency, hypovitaminosis D and K, cigarette smoking, and renal failure. Inflammatory cytokines and oxidized LDL have been suggested as crucial determinants of both calcification in the vascular intima and reduction in osteoblast activity. Discovery of the OPG-RANK-RANKL system and cathepsin K was a major breakthrough in understanding the regulation of vascular calcification and bone remodeling. Another achievement was the finding of their involvement in the control of immune and vascular systems. Animal studies' results are clear and dissolving doubts about the role of this axis in the pathogenesis of vascular calcifications and osteoporosis. Therefore, atherosclerosis and osteoporosis are linked by biological association. This encourages the search for therapeutic strategies having both cardiovascular and skeletal beneficial effects. The drugs that may concordantly enhance bone density and reduce the progression of atherosclerosis include anti-RANKL antibodies denosumab and odanacatib, an inhibitor of the cathepsin K. Available evidence comes from experimental animals and human studies. All these treatments however lack controlled clinical studies designed to demonstrate dual effects of these inhibitors in animal or human atherosclerosis and osteoporosis.

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Authors' contribution

All authors have read and approved the final manuscript.

Competing interest

The authors declare no conflict of interest.

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Кальцификация сосудов, атеросклероз и потеря костной массы (остеопороз): новые патофизиологические механизмы и перспективы развития медикаментозной терапии

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Кальцификация, или эктопическая минерализация, кровеносных сосудов – активный процесс, регулируемый клетками, который получает все большее признание как общий сердечно-сосудистый фактор риска. Эктопическая минерализация артерий часто сопровождается уменьшением плотности костной ткани или нарушением костного обмена с развитием остеопороза. Последние данные подтверждают связь остеопороза с атеросклерозом, что свидетельствует о параллельном прогрессировании дегенеративных процессов в этих двух тканях, увеличивающем частоту летальных и нелетальных сердечно-сосудистых событий и повышающем риск переломов. У пациентов с остеопорозом имеется более высокий риск сердечно-сосудистых заболеваний, чем у лиц со здоровой костной тканью. В артериальной стенке найдено много белков, участвующих в процессах костеобразования и костной резорбции. Кальцификация сосудов подразумевает в большей степени остеогенную и в меньшей – хондрогенную дифференцировку остеобластов и остеокластоподобных клеток. Показано, что в атеросклеротической бляшке также экспрессируются многие регуляторы костеобразования и костной резорбции, некоторые структурные белки кости, такие как остеопротегерин (OPG) и лиганд-рецептор активатора ядерного фактора кВ (RANKL). После связывания RANKL с RANK происходит активация остеокластов, усиливается костная резорбция и процессы кальцификации сосудов. OPG, белок, гомологичный рецептору активатора ядерного фактора кВ (RANK), может связываться с RANKL, блокируя связывание последнего с RANK, что ведет к угнетению дифференцировки преостеокластов в зрелые остеокласты, снижению способности остеокластов резорбировать минеральный матрикс кости и кальцификации сосудов. Самые последние данные подтверждают, что катепсин К (цистеинпротеаза) может активно разрушать коллаген I и II типов – основной компонент матрикса кости и атеросклеротической бляшки. Эти данные еще больше подчеркивают перспективность использования катепсина К как мишени действия новых молекул для лечения остеопороза и атеросклероза. Таким образом, открытие системы цитокинов RANKL-RANK-OPG и важнейшей роли катепсина К в ремоделировании костной ткани, сосудистой кальцификации и атеросклероза – шаг вперед в понимании механизмов развития заболеваний и, возможно, в разработке новых лекарств двойного действия. Новые препараты для лечения остеопороза и атеросклероза, способствующие усовершенствованию и повышению эффективности существующих методов лечения, – это недавно зарегистрированный антагонист лиганда рецептора активатора ядерного фактора кВ моноклональное антитело деносумаб и ингибитор катепсина К оданакатиб, который в настоящее время находится в третьей фазе клинических испытаний.

Ключевые слова: атеросклероз, остеопороз, общие механизмы, система RANKL-RANK-OPG, катепсин К, деносумаб, оданакатиб

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