The Roles of Lipid Oxidation Products and Receptor Activator of Nuclear Factor-κB Signaling in Atherosclerotic Calcification

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Abstract: This review focuses on the roles of oxylipids and receptor activator of nuclear factor-κB ligand signaling in calcific cardiovascular disease. Both intimal and valvular calcifications are closely associated with atherosclerosis, leading investigators to study the role of atherogenic oxidatively modified lipids (oxylipids). Results have identified the molecular signaling through which oxylipids induce osteogenic differentiation and calcification in vascular cells. A surprising concomitant finding was that, in bona fide osteoblasts from skeletal bone, oxylipids have the opposite effect, ie, inhibiting osteoblastic maturation. This is the basis for the lipid hypothesis of osteoporosis. Oxylipids also induce resorptive osteoclastic cells within the bone environment, raising the question of whether resorptive osteoclasts can be harnessed in the vascular context for cell-based therapy to remove artery wall mineral deposits. The challenge is that vascular cells produce antiosteoclastogenic factors, including the soluble decoy receptor for receptor activator of nuclear factor-κB ligand, possibly accounting for the paucity of resorptive cells and the dominance of mineral in atherosclerotic plaque. These factors may have therapeutic use in osteoclastogenic removal of mineral deposits from arteries. (Circ Res. 2011;108:1482-1493.)

Key Words: atherosclerosis ▪ calcification ▪ oxidized lipids ▪ receptor activator of nuclear factor-κB ligand

Recent meta-analysis confirmed previous studies suggesting that calcium supplement use is associated with a 30% increase in risk of myocardial infarction. The mechanism for such an association is not yet known, but attention has been focused on a possible relationship with vascular calcification. This review highlights growing evidence concerning vascular calcification with respect to the role of oxidatively modified phospholipids and pathways governing activity of bone-resorbing osteoclastic cells in the artery wall.

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As noted by Dwight Towler and colleagues, in many adults the cardiovascular system is the second most mineralized structure in the human body. The mineral deposits extend with age and lead to loss of aortic elasticity, hypertension, left ventricular hypertrophy, heart failure, and life-threatening aortic valvular stenosis. Findings from the past two decades indicate that mineralization occurs when vascular and valvular cells undergo osteochondrogenic differentiation and produce osteoid, bone extracellular matrix, and nanovesicular and microvesicular particles that, together, nucleate hydroxyapatite crystals and organize into bone tissue. Most evidence indicates that the process recapitulates the molecular events that govern skeletal bone formation. The capacity of cardiovascular cells to differentiate into bone cells has been confirmed repeatedly. Matrix vesicles, which nucleate hydroxyapatite mineral crystals, are present in calcific atherosclerosis and may promote plaque rupture. Conventional distinctions among many cell lineages have been increasingly blurred as investigators have reported unexpected transitions: endothelial cells differentiate to smooth muscle cells; adult mesangioblasts differentiate into myocytes and cardiomyocytes; adipocytes differentiate into vascular cells; osteoclasts differentiate into dendritic cells; dendritic cells differentiate into osteoclasts; smooth muscle cells (SMC) differentiate into osteoblasts and chondrocytes; and microvascular pericytes differentiate into osteoblasts, chondrocytes, myocytes, and adipocytes.

Despite the large body of evidence that calcific vascularopathy is driven by a wide range of paracrine factors elaborated by vascular SMC, endothelial cells, and leukocytes, the literature contains occasional suggestions that it is a passive or degenerative process. This impression appears to derive from a philosophical viewpoint and seems to derive from two observations: the finding that mice with targeted deletion of inhibitors of vascular calcification, such as matrix γ-carboxyglutamic acid protein, osteoprotegerin (OPG), or fetuin A, have calcific vasculopathy develop without addition of activators, and the fact that mineral crystal formation occurs extracellularly by physicochemical reactions. It is our view that the development of a phenotype in a mouse lacking an inhibitory factor is evidence for endogenous activators that balance endogenous inhibitors, rather than passivity, and that hydroxyapatite crystal formation also occurs extracellularly by physico-chemical reactions in skeletal mineralization. The reason skeletal mineralization is not considered a passive process is that osteoblasts actively synthesize extracellular matrix components and generate microenvironments that permit crystal formation. The same applies to vascular calcification. In an extreme reductionist sense, essentially all “active” biological processes reduce to purely physicochemical reactions. For example, in the phenomenon of calcium influx into cells, considered an active cellular process that governs a vast array of important cell signaling events, calcium ions passively follow an electrochemical gradient. Neither calcium influx nor biomineralization is “passive” in any meaningful biological sense.

This review focuses on the roles of oxylipids and receptor activator of nuclear factor-κB ligand (RANKL) signaling in calcific cardiovascular disease, which is categorized by location as intimal, medial, valvular, or microvascular. In at least the first three, the mechanism appears to involve a change in lineage of vascular cells that undergo osteogenic differentiation in intimal and valvular calcification and chondrogenic differentiation in the medial calcification. Both intimal and valvular calcification are closely associated with atherosclerosis, and this led investigators, including our group, to study the contribution of atherogenic factors, oxidatively modified lipids, “oxylipids” for short, to the process. Results have elucidated the molecular signaling through which oxylipids induce osteogenic differentiation and calcification. A surprising concomitant finding was that, in bona fide osteoblasts from skeletal bone, oxylipids have the opposite effect, ie, inhibiting osteoblastic maturation. Research in this area led to a novel concept, the lipid hypothesis of osteoporosis. Studies of oxylipids in bone provided evidence that they also induce resorptive osteoclasts within the bone environment. This now raises the exciting question of whether resorptive osteoclasts can be harnessed in the vascular context for cell-based therapy to remove harmful mineral deposits. The challenge is that vascular cells produce antosteoclastogenic factors, which may account for the paucity of resorptive cells and the dominance of mineral in atherosclerotic plaque. Bone biologists recently discovered that the ligand for receptor activator of nuclear factor-κB (RANK) is the pivotal factor governing osteoclastogenesis (Figure 1). The possibility of using vascular-specific RANKL as a treatment to promote vascular osteoelastic removal of calcific vasculopathy is of growing interest.

**Oxylipids**

Oxylipids arise in nature in a variety of forms, including oxidized phospholipids, oxysterols, and iso prostanes. Lipoproteins, such as low-density lipoprotein (LDL), are biological nanoparticles composed of several types of lipids and proteins that are subject to oxidation, including sterols, phospholipids, and apoproteins. Ex vivo, experimentalists generate oxidized lipoproteins using iron or copper catalysis. In vivo, oxylipids form by both enzymatic and nonenzymatic processes. Enzymatic modification may occur via lipooxygenases, myeloperoxidase, nitric oxide synthase, and NADPH oxidases. Nonenzymatic oxidation occurs in vivo, at least in part, via oxygen radicals released from adjacent cells as byproducts of energetic reactions.
metabolism. They may also be produced in vivo by divalent iron cations or heme. Oxidation of LDL is a seminal event mediating atherogenesis. The degree of oxidation greatly influences biological activity, with highly oxidized lipids sometimes having different bioactivity than mildly oxidized lipids, with mildly oxidized lipids often inducing a stronger inflammatory response. Recent studies now implicate oxylipids in the initiation of vascular calcification independently of their effect on atherosclerosis.

**Oxylipids and Cardiovascular Osteogenesis**

The capacity to undergo osteoblastic differentiation and produce a mineralized matrix of hydroxyapatite is a robust property of vascular cells, having been widely reproduced; however, little remains known outside of this field. This phenomenon occurs spontaneously, though slowly, in ordinary culture, and it is enhanced by transforming growth factor-β. In vitro, it is dose-dependently induced, approximately three-fold, by treatment with oxidized LDL. A recent report indicates that one mechanism for oxylipid-induced vascular osteogenesis is through induction of decorin, which triggers transforming growth factor-β. These effects of oxylipids may explain the reduced calcification in ethanol-pretreated bioprosthetic cardiac valves, where the most common cause of failure is overwhelming calcium deposition.

In vivo, hyperlipidemic (LDL-receptor–deficient) mice have calcific vasculopathy develop. Recently, a new lipid-related factor was shown to promote vascular cell osteogenesis, lysophosphatidylcholine. These findings suggested the lipid hypothesis of cardiovascular calcification and implicated clinical hyperlipidemia as a causal factor. Clinical evidence has supported a role for hyperlipidemia and oxylipids. Serum levels of oxidized LDL are positively associated with radiographic peripheral vascular calcification in patients with chronic kidney disease. Serum levels of

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**Figure 1. RANKL/RANK/OPG interactions.** When RANKL from osteoblasts binds to its receptor, RANK, on the surface of preosteoclastic cells, it induces osteoclastic differentiation. OPG, also from osteoblasts, acts as a soluble decoy receptor for RANKL and, thus, blocks osteoclastic differentiation (Illustration credit: Cosmocyte/Ben Smith).

**Figure 2. Working model of the effects of oxidized lipids on vascular and bone calcification based on in vitro and in vivo studies.** High levels of serum and tissue oxidized lipids may occur in genetic hyperlipidemia or with an atherogenic diet (or both). In the artery wall milieu, these oxylipids induce vascular calcification by direct action on smooth muscle cells (SMC) or adventitial myofibroblasts or through indirect induction of cytokine release from macrophages (or both). Infliximab, an inhibitor of tumor necrosis factor (TNF)-α, blocks this process. In the bone milieu, oxylipids inhibit bone calcification by direct action on osteoblasts or through indirect induction of cytokine release from T-lymphocytes (or both) (Illustration credit: Cosmocyte/Ben Smith).
lipoprotein(a) is associated with coronary artery calcification in women with diabetes.30 Old-order Amish people with a R350Q mutation in their apolipoprotein B100 have high-serum LDL and increased coronary calcification.31 Cholesterol levels are also associated with coronary artery calcification in asymptomatic individuals.32

The role of oxylipids in cardiac valvular calcification and ossification are addressed in another review of this series. During the last decade, other laboratories have developed the methods and evidence reversing former dogma that valvular stenosis results from simple degenerative wear-and-tear. Interestingly, valves on the left side of the heart, the aortic and mitral valves, are much more vulnerable to calcification than those of the right side, pulmonic and tricuspid valves, which are rarely affected. Toll-like receptor-4, which serves as a receptor for oxidized phospholipids and oxidized cholesteryl esters, is expressed at higher levels in left-side valves. In cultured valvular cells, Toll-like receptor-4 is associated with osteogenic differentiation and expression of the potent osteogenic differentiation factor, bone morphogenetic protein-2.33 Porcine aortic valves have greater activity of the potent osteogenic morphogen, bone morphogenetic protein-2, and the osteogenic marker, tissue-nonspecific alkaline phosphatase, than pulmonic valve leaflets, whereas pulmonic valves have more activity of the calcification inhibitor, matrix γ-carboxyglutamic acid protein.34 Oxidation-specific epitopes on lipids share molecular identity or mimicry, or both, with “pathogen-associated molecular patterns” and trigger innate immunity. Oxidized phospholipids and oxidized cholesteryl esters are also ligands for Toll-like receptor-4.35

**Oxidant Stress**

A unifying feature underlying the oxylipid effects appears to be oxidant stress. In vitro, oxylipids generate oxidant stress, measured fluorochromatically, and pure chemical oxidant stress with hydrogen peroxide directly promotes vascular cell osteogenesis, whereas chemical antioxidants counteract it.36 In vivo, oxidant signals were found at increased levels around calcium deposits in human valve leaflets and in valve leaflets of rabbits treated with high dietary cholesterol and its derivative, vitamin D. The cells in those locations had osteoblastic and osteoclastic markers.37 Recent studies now suggest that oxidant stress induced the osteogenic differentiation by induction of runt-related transcription factor-2 (Runx2), also known as core binding factor α-1, a master regulatory osteogenic transcription factor. The importance of runtrelated transcription factor-2 is underscored by the dramatic phenotype of null mice, which have complete absence of bone.38–41 Oxidant stress-induced calcification is enhanced in Runx2 overexpressing vascular cells in vitro, and it fails to induce calcification in Runx2-silenced vascular cells.42 Similarly, the aortic valve calcification induced by high cholesterol and vitamin D in rabbits was abrogated by the antioxidant, lipoic acid.43

**Atheroprotective Interventions**

As evidence that atherogenic oxylipids promote vascular calcification, factors that neutralize atherogenic oxylipids have protective effects. When treated with high-density lipoprotein, the alkaline phosphatase activity of vascular cells is greatly reduced and, with prolonged high-density lipoprotein treatment, the extent of mineral deposition is significantly reduced.43 Interest-ingly, oxidized high-density lipoprotein particles, which are proatherogenic, are also procalcific in vascular cells.44 Similarly, omega-3 fatty acids (fish oils), which are known to be antiatherogenic from the basic science to the clinical level, also inhibit vascular cell calcification in vitro by acting through p38 mitogen-activated protein kinase and peroxisome-proliferator activated receptor-γ pathways,45 and they are also effective in vivo.46 As further evidence for a role of lipids in vascular cell calcification, the farnesyl X receptor, a nuclear hormone receptor activated by derivatives of the isoprenyl lipid, farnesol, is upregulated during osteogenic differentiation of vascular cells.46 A synthetic farnesyl X receptor activator, INT-747, prevents vascular calcification in uremic hyperlipidemic mice.46

The most definitive evidence that oxylipids regulate cardiovascular calcification comes from an elegant study of aortic valves in Reversa mice. Reversa mice (Ldlr<sup>−/−</sup>/ApoB100/100/Mtp<sup>−/−</sup>Mx1-Cre<sup>−/−</sup>) have a “genetic switch” that allows rapid lipid-lowering with a simple intraperitoneal injection protocol that induces Mx1-Cre in the liver and shuts down expression of microsomal triglyceride transfer protein, blocking LDL release. Before the switch, Reversa mice had narrowed cusp openings, high levels of calcium deposition, pro-osteogenic protein expression, and lipids in the aortic valve, as well as high serum cholesterol and high levels of superoxide.47 After reversal of the hyperlipidemia via the genetic switch, these features normalized, including valvular calcium deposition, pro-osteogenic signaling, superoxide levels, and myofibroblast activation.47 Although the lipid-lowering did not reverse the aortic valve cusp narrowing, it did prevent progression.47

Based on the evidence for a role of lipids and hyperlipidemia in vascular calcification, it is reasonable to expect that HMG-CoA reductase inhibitors (“statins”) would prevent or reverse (or both) vascular calcification by reducing serum lipoprotein levels, with concomitant reduction in oxylipids and inflammation. In addition, the antinflammatory side effects of statins should provide additional protection. Furthermore, statins are now believed to promote breakdown of adenosine triphosphate (ATP) to form extracellular adenosine, which is a novel putative inhibitor of vascular calcification based on decreased SMC alkaline phosphatase (ALP) activity with adenosine treatment.48 However, unexpectedly, clinical studies have shown little or no benefit of statins in aortic or coronary calcification.49 Results in aortic valvular calcification have been similarly disappointing. Large, randomized, controlled trials of statins in calcific valvulopathy (SEAS and ASTRONOMER) found no significant effect on progression of aortic stenosis.50 The prospective randomized trial (Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression [SALTIRE]) of atorvastatin vs placebo yielded a negative result.51 One consideration is that patients with hyperlipidemia had to be excluded in this trial because it would be unethical to treat them with placebo instead of a statin. A recent prospective study (Rosuvastatin Affecting Aortic Valve Endothelium [RAAVE]) treating hypercholesteremic patients with rosuvastatin found a significantly slower rate of progression in these patients compared with patients with normal cholesterol levels who were left untreated.52 Thus, advanced calcific disease may not respond to statins, and the response of early-stage disease to statins remains to be determined.
One possible explanation for the failure of statins to regress advanced cardiovascular calcific disease is that in the context of skeletal osteoblasts, statins are believed to promote osteoblastic differentiation and mineralization.\textsuperscript{53} By the time arterial and valvular calcium deposits have advanced to the stage of clinically significant stenosis, the cells in these tissues may have already differentiated into osteoblasts,\textsuperscript{15} which, in their new identity, may respond positively to statins. Thus, statin treatment of advanced calcific valvulopathy may actually exacerbate the mineralization by osteoblast-like cells and effects of statins on early stages may be necessary.

**Interaction With Metabolic Factors**

Oxylipids also interact with some factors known to influence vascular calcification, such as vitamin D, and hyperphosphatemia. The effects of oxylipids are accentuated by vitamin D. In rats, adding vitamin D supplements to a high-cholesterol diet significantly enhances vessel calcium deposition and osteogenic differentiation measured by alkaline phosphatase expression and activity. This interaction raises public health concerns, given the recent zealous vitamin D supplementation in the context of the high-cholesterol American diet.\textsuperscript{54} Oxylipids synergize with hyperphosphatemia as well. They significantly enhance β-glycerophosphate–induced osteoblast differentiation of vascular cells via extracellular signal-regulated kinase and osterix-dependent mechanisms.\textsuperscript{55} Conversely, intermittent injections of recombinant human parathyroid hormone (teriparatide) significantly inhibited vascular calcification in hyperlipidemic mice.\textsuperscript{56}

**Inflammation**

Inflammation may be the underlying factor mediating the effects of oxidant stress on cardiovascular and skeletal bone calcification. Vascular calcification colocalizes with monocyte–macrophage infiltration in vivo in mice. Using near-infrared fluorescence with molecular imaging agents that target macrophages and active bone formation, Aikawa et al\textsuperscript{57} found a distinct colocalization of macrophages with bone formation within the aortas of apolipoprotein E-deficient mice. Oxylipids induce vascular cell osteogenesis in part through induction of monocyte cytokine release.\textsuperscript{58} Monocyte coculture and monocyte-conditioned media each significantly enhance vascular cell alkaline phosphatase activity and matrix mineralization in proportion to the number of monocytes, and tumor necrosis factor-α (TNF-α) was identified as one of the paracrine factors.\textsuperscript{58} Direct treatment of vascular cells with TNF-α also induces both ALP and mineralization.\textsuperscript{59} Definitive in vivo evidence for the role of TNF in vascular calcification comes from studies from Towler’s laboratory\textsuperscript{60} showing that vascular-specific overexpression of TNF-α promoted tissue-nonspecific alkaline phosphatase and Msn2 and Wnt signaling. They further showed the effect of abolishment by loss of Wnt signaling using the Wnt antagonist, Dikkopf1.\textsuperscript{60}

Inflammation is also a driving force in bone loss. Inflammatory osteolysis is well-known in chronic inflammatory arthritis, and osteolysis is a classical outcome of chronic infection. The loss of bone density in hyperlipidemic mice correlates and colocalizes with inflammatory burden, as indicated by in vivo fluorescence imaging of macrophages.\textsuperscript{57,61}

**Paradoxical Association With Osteoporosis**

Epidemiologically, both vascular calcification and osteoporosis are associated with hyperlipidemia.\textsuperscript{62,63} Hyperlipidemic mice have decreased skeletal osteogenesis, as shown by in vivo fluorescence imaging in the femurs of apolipoprotein E\textsuperscript{−/−} mice.\textsuperscript{61} Most studies examining the relation between calcific vasculopathy and osteoporosis show a strong association, and many find that it is not explained by a shared association with age.\textsuperscript{64–67} Osteoporosis also associates with atherosclerosis.\textsuperscript{68} Bone formation rate is lower in kidney patients with coronary calcification.\textsuperscript{69} Thus, patients with osteoporosis deposit calcium mineral in their arteries, adding theoretical concerns to the epidemiological evidence that calcium supplements may promote cardiovascular disease.\textsuperscript{70}

One proposed explanation for the link between osteoporosis and vascular calcification is that increased bone resorption may directly release excess calcium into the circulation in the form of calcium-phosphate-fetuin-matrix-γ-carboxyglutamic acid protein complexes, a concept supported by evidence that antiosteoporotic agents block vitamin D–induced vascular calcification in mice at the same doses that prevent osteoporosis.\textsuperscript{71} However, hypercalcemia is not typically seen in vascular calcification and osteoporosis, and vascular calcification occurs in the absence of excess bone resorption in a number of models.\textsuperscript{72–74}

**Lipid Hypothesis of Osteoporosis**

Alternatively, hyperlipidemia, with resultant oxidant stress and inflammation, may account for the link. In contrast to vascular cells, skeletal bone-derived osteoblasts lose their osteogenic activity in response to products of lipid oxidation, such as mildly oxidized LDL, in vitro.\textsuperscript{23} Oxylipids also accumulate in bone tissue.\textsuperscript{75,76} Osteoblasts have been shown to generate oxidized LDL, probably because of nonenzymatic oxidation by radicals released as waste from energetic metabolism. This may increase the local concentration of oxylipids.\textsuperscript{77} They have been detected by mass spectrometry in the bone marrow of hyperlipidemic mice and lipids demonstrated by histochemical staining in the subendothelial space of bone from osteoporotic humans.\textsuperscript{78} This subendothelial space is the site of maturation of developing osteoblasts, which proliferate but fail to mature at very low concentrations of oxidized LDL and die at high concentrations.\textsuperscript{77}

Hyperlipidemia impairs skeletal osteogenic differentiation in mice. Bone marrow stromal cells (mesenchymal stem cells/preosteoblasts) harvested from mice fed a high-fat atherogenic diet favor adipogenic vs osteogenic differentiation, leading to a “fatty” marrow.\textsuperscript{78} Skeletal bone density is also reduced in mice with diet-induced hyperlipidemia.\textsuperscript{79,80} In apparent opposition to this hypothesis, apolipoprotein E-deficient mice that are not treated with an atherogenic diet were found to have greater bone mass than in wild-type mice despite hyperlipidemia,\textsuperscript{81} raising questions about the lipid hypothesis of osteoporosis. However, one consideration is that the high-fat diet may be necessary for chronic inflammation and oxidant stress. Another possibility is that the lack of apolipoprotein E may prevent delivery from LDL to osteoblasts the fat-soluble vitamin K, which is required for matrix γ-carboxyglutamic acid protein and osteocalcin down-regulation of mineralization. Thus, lack of apolipoprotein E for
A number of signaling mechanisms may mediate oxylipid inhibition of bone formation. Oxylipids in their role as ligands of peroxisome proliferator-activated receptor-γ reduce β-catenin levels, blocking Wnt3a signaling.82 This phenomenon was linked to aging in mice, with an increase in the lipid oxidation product, 4-hydroxynonenal, together with increased expression of lipoxygenase in the skeletal tissues as a function of age.83

Oxylipids and Osteoblasts in Bone
Oxylipids have reciprocal effects on vascular and skeletal cells in vitro. Mildly oxidized LDL, but not native LDL, caused a dose-dependent increase in alkaline phosphatase activity and induced extensive calcification in calcifying vascular cells (CVC). In contrast, mildly oxidized LDL and its biologically active components inhibited differentiation of cells from the skeletal-derived MC3T3-E1 bone cell line.23 In vivo, femoral mineral content in C57BL/6 atherosclerosis-susceptible mice fed the high-fat diet was significantly reduced, and mineral density was lower compared with mice fed the chow diet.80,84

Oxylipids and Osteoclasts in Bone
In vitro, the isoprostane, isoprostaglandin E2, enhanced osteoclastic differentiation of marrow-derived preosteoclasts, as evidenced by increased tartrate-resistant acid phosphatase activity.85 Ex vivo, functional osteoclastic activity, measured as the number of resorption pits produced on synthetic hydroxyapatite-coated plates, known as osteologic disks, was significantly greater in bone marrow cells harvested from older hyperlipidemic mice (12-month-old, Ldlr−/−).76 Oxylipids and hyperlipidemia also have indirect effects on osteoclast differentiation via lymphocytes.84,86

Of potential clinical importance, hyperlipidemia abrogated the efficacy of anabolic parathyroid hormone treatment through effects on osteoblastic activity in vitro and in Ldlr−/− mice.87,88 The apolipoprotein A-I mimetic peptide, D-4F, which has antiatherogenic effects in hyperlipidemic mice,89 reverses adverse effects of hyperlipidemia on parathyroid hormone osteoanabolism, acting primarily through reduction in serum markers of bone resorption rather than rescue of bone formation (Figure 3).90 Thus, osteoclastic activity turns out to be a surprisingly important factor in vascular calcification, making knowledge of its control mechanisms important in understanding calcific vascular pathology.

The RANKL/RANK/OPG Axis
Osteoclastic activity is primarily governed by a triad of TNF receptor (TNFR)-related factors: RANK (TNFRSF11a), a member of the TNFR superfamily that is expressed on preosteoclasts and dendritic cells; its ligand, RANKL (TNF superfamily member 11), which is required for development and maturation of osteoclasts; and OPG (TNFR superfamily member 11b), a secreted glycoprotein and a soluble decoy receptor for RANKL. The rate of osteoclast formation as well as the catabolic and anabolic effects of a wide variety of
upstream hormones and cytokines on bone are now known to be mediated through alterations in the ratio of OPG to RANKL.\textsuperscript{91} The unexpected discovery of vascular calcification in OPG-deficient mice brought this regulatory mechanism to the attention of vascular biologists.

RANKL is found on the surface of, or is secreted from, osteoblasts, SMC, T-lymphocytes, and marrow stromal cells. On binding to RANK on the surface of preosteoclastic monocytes, it induces osteoclastic differentiation, fusion, and maturation.\textsuperscript{92} OPG, the first member of the triad discovered in bone,\textsuperscript{92,93} blocks this interaction of RANKL with RANK, thus interfering with osteoclast differentiation (Figure 1), hence the name osteoprotegerin, for its ability to “protect/prote¿ger” bone from resorption.\textsuperscript{94} Before the discovery of RANKL, in vitro osteoclastogenesis required coculture with osteoblastic lineage cells, now known to have provided RANKL; recombinant RANKL treatment now replaces the coculture. RANKL is minimally expressed in quiescent osteoblasts,\textsuperscript{95} but it is induced in regions of bone that are undergoing rapid turnover or osteolysis. It is also readily induced by parathyroid hormone 1-alpha,25-dihydroxyvitamin D_{3} (vitamin D) and dexamethasone. Although RANKL is normally expressed as a transmembrane protein, it can be cleaved by matrix metalloproteinases to a soluble form, which is active but less efficient.\textsuperscript{96}

Like other TNFR family members, RANKL activation of RANK signals via TRAF-induced nuclear factor-κB and Jun-N-terminal kinase activation.\textsuperscript{97} It also activates p44 and p38 mitogen-activated protein kinases as well as the phosphatidylinositol-3 kinase pathway.\textsuperscript{98} In skeletal osteoblasts, RANKL expression is induced by parathyroid hormone, dexamethasone, 1alpha,25-dihydroxyvitamin D(3), prostaglandin E2, and interleukin-11.\textsuperscript{97,99–103}

On an historical note, RANKL was originally identified on T lymphocytes and named “osteoclast differentiation factor” and as “TNF-related activation-induced cytokine.”\textsuperscript{104} and it promotes dendritic cell survival by binding to RANK Interestingly, dendritic cells are associated with atherosclerosis.\textsuperscript{5} With respect to nomenclature, more than one preferred name exists for each of these molecules. Recently, members of the human genome family standardized the nomenclature for TNF and TNFR superfamilies and chose TNF superfamily member 11, TNFR superfamily member 11a, and TNFR superfamily member 11b for RANKL, RANK, and OPG, respectively. In this review, we use the latter terms to reflect the nomenclature recommended for the bone literature.

In vivo, the absence of RANKL/RANK interaction leads to severe osteopetrosis because of deficient osteoclastic resorption. In addition to severe osteopetrosis, RANKL-deficient mice have immunologic defects develop and lack peripheral lymph nodes.\textsuperscript{105} Mice lacking RANK\textsuperscript{106} and mice overexpressing the soluble decoy receptor, OPG, also have severe osteopetrosis develop.\textsuperscript{94}

In contrast, mouse models with unopposed RANKL activity all had osteopetrosis develop, and one had vascular calcification develop. Although mice with ubiquitous RANKL overexpression died at the late fetal stage,\textsuperscript{107} OPG-deficient mice and those with high circulating levels of RANKL attributable to liver overexpression have osteopetrosis develop.\textsuperscript{107} Direct treatment with soluble RANKL induces overactive osteoclastic resorption in vivo.\textsuperscript{92} Unexpectedly, one strain of opg\textsuperscript{−/−} mice has large-vessel vascular calcification develop.\textsuperscript{108} This phenotype was not rescued by postnatal OPG injections.\textsuperscript{109} In mice with targeted deletion of OPG that were back-crossed to a slightly different background strain, there was no spontaneous vascular calcification, but the phenotype could be elicited by treatment with vitamin D and a high-phosphate diet.\textsuperscript{110}

### Serum Levels of RANKL and OPG in Cardiovascular Disease

Unexpectedly, cardiovascular disease correlates positively with serum levels of OPG and negatively with serum levels of RANKL,\textsuperscript{111} the opposite of that expected from in vitro studies.\textsuperscript{112,113} High serum levels of OPG associate with aortic stiffness,\textsuperscript{114} and mice fed an atherogenic diet have greater serum OPG levels than chow-fed mice.\textsuperscript{56} Serum OPG level correlates with fatal stroke and vascular mortality in older women\textsuperscript{115} and with coronary atherosclerosis severity in males.\textsuperscript{116,117} A single nucleotide polymorphism in the OPG gene is associated with increased carotid atherosclerotic thickness and forearm blood flow in normal subjects.\textsuperscript{118} Furthermore, in a 10-year prospective study, serum OPG correlated independently with cardiovascular mortality.\textsuperscript{119} Conversely, cardiovascular disease correlates negatively with serum RANKL levels.\textsuperscript{120} Some evidence suggests that high-serum OPG levels occur in a variety of conditions involving persistent immune activation and thus may be a compensatory response to enhanced activity of other members of the TNF family.\textsuperscript{121} The association of higher OPG levels with clinical cardiovascular disease together with evidence for a protective role of OPG in the artery wall suggest that OPG induction may represent an insufficient mitigating response, presumably a response, rather than a cause, of cardiovascular disease.

### RANKL/OPG/RANK Axis and Vascular Calcification

Some evidence suggests that RANKL directly induces SMC osteogenesis. In early studies of valvular interstitial cells, RANKL induced osteogenic differentiation in vitro.\textsuperscript{122} Similarly, in recent studies using rat SMC cultures, RANKL induced osteogenic markers via bone morphogenetic protein-4.\textsuperscript{113} Somewhat different findings from Osako et al\textsuperscript{112} showed in human aortic SMC that RANKL promoted osteogenic differentiation and calcification indirectly via induction of bone morphogenetic protein in endothelial cells and inhibition of matrix γ-carboxyglutamic acid protein in the SMC. Interestingly, these phenomena were blocked by estrogen.\textsuperscript{112} Interestingly, in murine SMC, CAMP, which induced both calcification and RANKL, the calcification was not inhibited by OPG, suggesting that vascular cell osteogenesis induced by the protein kinase A pathway is not mediated through RANKL.\textsuperscript{123}

Four lines of evidence support the hypothesis that the RANKL/OPG/RANK axis regulates vascular calcification. First, the finding of vascular calcification in the opg\textsuperscript{−/−} mouse suggests that unopposed activity of RANKL, or another OPG ligand, promotes vascular calcification.\textsuperscript{108} Similarly, OPG inactivation further worsens vascular calcification in hyperlipidemic mice.\textsuperscript{124} One possible mechanism is that high resorptive activity in bone leads to hypercalcemia and nonspecific mineral deposition in soft tissue. However, vascular calcification persists in
OPG-treated opg−/− mice, despite reversal of the osteoporosis. This suggests that either the vascular calcification in this model occurs early and is irreversible or does not depend on bone resorptive activity.125 Second, RANKL treatment in vitro induces osteoblastic differentiation and mineralization, as mentioned. Third, all three members of the regulatory trio are expressed in vascular cells or tissue. Last, OPG treatment inhibits vascular calcification in rodents; it prevents warfarin-induced vascular calcification in rats126 and hyperlipemic-induced vascular calcification in mice.109

RANK has been identified in cultured umbilical and microvascular endothelial cells.98,127 Although not found in normal arteries, it is expressed in calcified arteries of opg−/− mice.125 RANKL, though not expressed at baseline in cultured endothelial cells or SMC,98,128 is induced in endothelial cells by inflammatory cytokines and factors from actively remodeling bone such as transforming growth-factor-β129,136 RANKL immunoreactivity also is not found in normal mouse arteries,125 but it is present in calcified vascular tissue in humans122,131,132 and in opg−/− mice.125 OPG is expressed by cultured arterial endothelial cells127,129,133 and SMC.128,129,134 OPG immunoreactivity is also found in the normal artery wall but, in contrast with RANKL, its expression is less pronounced in calcified human atherosclerosis.125,132,135

RANKL/OPG/RANK Axis and Inflammation

In mineralized tissues, a RANKL-dominant condition is produced in inflammatory conditions such as arthritis and periodontitis.136 The same may be true for the inflammatory state of atherosclerosis. Cytokines, such as TNF-α, induce functional RANKL expression in endothelial cells.125 In monocytes, RANKL induces release of cytokines, including TNF-α.137,138 Thus, sites of chronic inflammation such as atherosclerosis may have a high RANKL-to-OPG ratio. There are two potential opposite results of a RANKL-dominant state, depending on the balance of circumstances. Mineralization may be augmented directly, given the evidence that RANKL induces interstitial cell osteogenesis, or indirectly by inducing TNF-α release from monocytes, which promotes SMC osteogenesis.59 Alternatively, or even simultaneously, RANKL in atherosclerosis may initiate resorption of mineral by inducing osteoclastogenesis of the abundant monocytes and macrophage colony-stimulating factor found in atherosclerotic lesions. Another consideration favoring a net resorptive outcome is the expression of monocyte recruitment factors, such as monocyte chemoattractant protein-1, in atherosclerotic plaque. A resorptive effect is further supported by the finding that soft tissue calcification undergoes rapid resorption in opg−/− mice, which have unopposed RANKL activity.139

Osteoclasts in the Artery Wall

Cells resembling osteoclasts have been identified in calcified atherosclerotic plaque, primarily at the edges of mineral deposits.15,125,140 and regression has been demonstrated in an animal model in which osteoclast-like cells resorb mineralized vascular tissue in a carbonic-anhydrase–dependent manner.141 Osteoclasts originate from monocytes142,143 and macrophages exposed to particulate matter are triggered to undergo osteoclastic differentiation.144 Because atherosclerotic lesions are rich in monocyte/macrophages and calcium mineral,145 they have an abundant source of preosteoclasts. Maturation to osteoclasts requires two factors: macrophage colony-stimulating factor and RANKL.102 Both factors are present in atherosclerotic lesions.122,131,146,147 Functional osteoclasts are multinucleated and express tartrate-resistant acid phosphatase, cathepsin K, calci- tonin receptors, H+/ATPase, and carbonic anhydrase II. Their cardinal feature is the capacity to generate resorption pits on mineralized surfaces. Osteoclast-like cells in atherosclerotic lesions are multinucleated and positive for tartrate-resistant acid phosphatase, cathepsin K, and carbonic anhydrase.140,141 Osteoclasts adhere to vascular mineral deposits via osteopontin to create an acidic and proteolytic microenvironment.148 Mice deficient in both osteopontin and apolipoprotein E, which are expected to have atherosclerosis but poor osteoclast and monocry function, have increased vascular calcification despite a decrease in atherosclerosis,149 strongly suggesting that vascular calcification may be regulated independently of atherosclerosis, and that osteoclastic resorption may occur in a physiologically significant manner to the point of calcific lesion regression.150

Many effects of oxylipids occur with or through the RANKL system, but they depend on the degree of oxidation. In cultures of bone marrow preosteoclasts, mildly oxidized lipids enhance osteoclastogenesis induced by RANKL treatment.85 In vivo, apolipoprotein E–deficient hyperlipidemic mice have increased RANKL, RANK, and osteopontin in their atherosclerotic plaque.112 Highly oxidized lipids, in contrast, prevented RANKL-induced osteoclastogenesis by preventing phosphorylation of extracellular signal-regulated kinase, p38 kinase, and Jun-N-terminal kinase, and DNA-binding activities of nuclear factor-κB and nuclear factor of activated T-cells transcription factors in human monocyte.151 These results underscore the dependence of oxylipid activity on the degree of oxidation similarly to the opposite activity of highly oxidized lipids vs mildly oxidized lipids in atherogenic activity. Mildly oxidized lipids also induce RANKL in T lymphocytes.86 Correspondingly, in vivo, T-cell–enriched bone marrow cells from hyperlipidemic mice had higher levels of RANKL expression and induced greater osteoclastogenic activity in a monocyte cell line compared with the same fraction of cells from normolipemic mice.84

Regression by Vascular Osteoclasts

The potential for cell-based therapy using osteoclastic resorption to regress calcium deposits has a strong theoretical basis. Cells with all the features of osteoclasts are present in artery wall calcific plaque,15,140 and osteoclastic cells have the ex vivo capacity to demineralize calcified elastin from the aorta.152 However, in atherosclerotic plaque, osteoclastic cells appear to be less numerous or less active than in skeletal bone. One possible explanation is that vascular SMC impede osteoclastogenic differentiation and activity of peripheral blood monocytes, in part, via secretion of OPG and interleukin-18.153

Much translational potential remains to be explored for vascular tissue in regenerative engineering. By serendipity, Shanahan’s group found spontaneous expression of the osteochondrogenic transcription factors, runt-related transcription factor-2 and Osterix, in normal human vessels from children, which the authors speculated may reflect a developmental remnant in the still immature vascularium of children. This remarkable finding of embryonic bone and cartilage transcrip-
tion factors offers a clue to the profound regenerative potential of vascular stem cells, and opens the possibility of future banking of stem cells years after cord blood is no longer available. The capacity of SMC to generate de novo and vascularized bone and cartilage in vivo, even in elderly patients with osteoporosis, offers intriguing possibilities for tissue engineering and clinical regenerative medicine.

In summary, oxidatively modified lipids, partly through induction of inflammatory cytokines, induce osteogenic differentiation and calcification in vascular cells. They have the opposite effect in skeletal osteoblasts. Within the bone environment, oxylipids also induce differentiation and resorptive activity in skeletal osteoclasts, promoting bone loss as well as reduced formation. These actions may account for the relationship between hyperlipidemia and osteoporosis as well as the age-independent paradoxical relationship between vascular calcification and osteoporosis. Oxylipids also interact in a complex manner with the osteoclastogenic factor, RANKL, and the hormone controlling calcium metabolism, parathyroid hormone, to regulate development of vascular calcification. Further elucidation of these mechanisms may lead to cell-based therapies capable of prevention and regression of calcific vasculopathy.

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None.

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