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

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Abstract

This review focuses on the roles of oxylipids and receptor activator of nuclear factor-kappa B ligand (RANKL) signaling in calcific cardiovascular disease. Both intimal and valvular calcification 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, i.e. 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 anti-osteoclastogenic factors, including the soluble decoy receptor for RANKL, 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.

Keywords
Calcification; atherosclerosis; RANKL; oxidized lipids

A recent meta-analysis confirmed previous studies suggesting that calcium supplement use is associated with a 30 percent increase in risk of myocardial infarction.¹ 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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Disclosure
None

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As noted by Dwight Towler, 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 elastance, hypertension, left ventricular hypertrophy, heart failure, and life-threatening aortic valvular stenosis. Findings from the past 2 decades indicate that mineralization occurs when vascular and valvular cells undergo osteochondrogenic differentiation and produce osteoid, bone extracellular matrix, as well as 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. Indeed, conventional distinctions among many cell lineages have been increasingly blurred as investigators have reported unexpected transitions: endothelial cells differentiate to smooth muscle cells; adult mesoangioblasts into myocytes and cardiomyocytes; adipocytes into vascular cells; osteoclasts into dendritic cells; osteoblasts, chondrocytes, myocytes and adipocytes. Despite the large body of evidence that calcific vasculopathy 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 MGP, OPG, or fetuin A, develop calcific vasculopathy without addition of activators, and the fact that mineral crystal formation occurs extracellularly by physico-chemical reactions. It is our view that (1) 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 (2) 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-kappa B 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, which 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, i.e. 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 anti-osteoclastogenic 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-kappa B (RANK) is the pivotal factor governing osteoclastogenesis. The possibility of using vascular-specific RANKL as a treatment to promote vascular osteoclastogenic removal of calcific vasculopathy is of growing interest.¹⁷

OXYLIPIDS

Oxylipids, arise in nature in a variety of forms, including oxidized phospholipids, oxysterols, and isoprostanes. 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 lipoxygenases, myeloperoxidase, nitric oxide synthase, and NADPH oxidases. Nonenzymatic oxidation occurs in vivo, at least in part, via oxygen radicals released from adjacent cells as by-products of energetic metabolism. They may also be produced in vivo by divalent iron cations or heme. Oxidation of LDL is a seminal event mediating atherogenesis.¹⁸ 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,²¹²² having been widely reproduced; but it remains little known outside of this field. This phenomenon occurs spontaneously, though slowly, in ordinary culture, and it is enhanced by transforming growth factor-beta.²² In vitro, it is dose-dependently induced, approximately 3-fold, by treatment with oxidized LDL.³²³¹ A recent report indicates that one mechanism for oxylipid-induced vascular osteogenesis is through induction of decorin, which triggers transforming growth factor-beta.²⁴ 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.²⁵

In vivo, hyperlipidemic (LDL-receptor deficient) mice develop calcific vasculopathy.²⁶ Recently, a new lipid-related factor was shown to promote vascular cell osteogenesis, lysophosphatidylcholine.²⁷ These findings suggested the lipid hypothesis of cardiovascular calcification²⁸ and implicated clinical hyperlipidemia as a causal factor. Indeed, 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.²⁹ Serum levels of lipoprotein (a) is associated with coronary artery calcification in women with diabetes.³⁰ Old order Amish with a R3500Q 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. Laboratories of Mohler, Rajamannan, Otto, O’Brien, Heistad, and Miller 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 (TLR4), which serves as a receptor for oxidized phospholipids and oxidized cholesteryl esters, is expressed at higher levels in left-sided valves. In cultured valvular cells, TLR4 is associated with osteogenic differentiation and expression of the potent osteogenic differentiation factor, bone morphogenetic protein-2 (BMP2). Porcine aortic valves have greater activity of the potent osteogenic morphogen, BMP2, and the osteogenic marker, tissue non-specific alkaline phosphatase (TNAP), than pulmonic valve leaflets; whereas pulmonic valves have more activity of the calcification inhibitor, matrix gamma-carboxyglutamic acid protein (MGP). Oxidation-specific epitopes on lipids share molecular identity and/or mimicry with “pathogen-associated molecular patterns” and trigger innate immunity. Oxidized phospholipids and oxidized cholesteryl esters are ligands for TLR4.

**Oxidant stress**

A unifying feature underlying the oxylipid effects appears to be oxidant stress. In vitro, oxylipids generate oxidant stress, measured fluorochemically, and pure chemical oxidant stress with hydrogen peroxide directly promotes vascular cell osteogenesis, whereas chemical antioxidants counteract it. 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. 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 alpha-1 (Cbfα-1), a master regulatory osteogenic transcription factor. The importance of Runx2 is underscored by the dramatic phenotype null mice, the complete absence of bone. Oxidant stress-induced calcification is enhanced in Runx2 overexpressing vascular cells in vitro, and it fails to induce calcification in Runx2-silenced vascular cells. Similarly, the aortic valve calcification induced by high cholesterol and vitamin D in rabbits was abrogated by the antioxidant, lipoic acid.

**Atheroprotective interventions**

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

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>/Apob 100/100/ Mtp<sup>fl/fl</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. Following reversal of the hyperlipidemia, via the genetic switch, these features normalized, including valvular calcium deposition, pro-osteogenic signaling, superoxide levels, and myofibroblast activation. Although the lipid-lowering did not reverse the aortic valve cusp narrowing, it did prevent progression.

Based on the above evidence for a role of lipids and hyperlipidemia in vascular calcification, it is reasonable to expect that HMG-CoA reductase inhibitors (“statins”) would prevent and/or reverse vascular calcification, by reducing serum lipoprotein levels, with concomitant reduction in oxylipids and inflammation. In addition, the anti-inflammatory side effects of statins should provide additional protection. Furthermore, statins are now believed to promote breakdown of ATP to form extracellular adenosine, which is a novel putative inhibitor of vascular calcification, based on decreased SMC ALP activity with adenosine treatment. However, unexpectedly, clinical studies have shown little or no benefit of statins in aortic or coronary calcification. 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. The prospective randomized trial (Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression [SALTIRE]; of atorvastatin vs. placebo) yielded a negative result. One consideration is that patients with hyperlipidemia had to be excluded in this trial since 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. 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. 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, which may, in their new identity, 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 with 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. Oxylipids synergize with hyperphosphatemia as well. They significantly enhance beta-glycerophosphate-induced osteoblast differentiation of vascular cells via extracellular signal-regulated kinase (ERK1/2) and osterix-dependent mechanisms. Conversely, intermittent injections of recombinant human parathyroid hormone (teriparatide) significantly inhibited valvular calcification in hyperlipidemic mice.

**Inflammation**

Inflammation may be the underlying factor mediating the effects of oxidant stress on cardiovascular and skeletal bone calcification. Vascular calcification co-localizes with

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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 found a distinct colocalization of macrophages with bone formation within the aortas of apolipoprotein E (Apoe)-deficient mice. Oxylipids induce vascular cell osteogenesis in part through induction of monocyte cytokine release. Monocyte co-culture 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-alpha (TNF-alpha) was identified as one of the paracrine factors. Direct treatment of vascular cells with TNF-alpha also induces both ALP and mineralization. Definitive in vivo evidence for the role of TNF in vascular calcification comes from studies from Towler’s laboratory showing that vascular-specific overexpression of TNF-alpha promoted TNAP and Msx2 and wingless-int (Wnt) signaling. They further showed the effect of abolished by loss of Wnt signaling using the Wnt antagonist, Dkk1.

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.

**PARADOXICAL ASSOCIATION WITH OSTEOPOROSIS**

Epidemiologically, both vascular calcification and osteoporosis are associated with hyperlipidemia. Hyperlipidemic mice have decreased skeletal osteogenesis, as shown by in vivo fluorescence imaging in the femurs of Apoe−/− mice. 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. Osteoporosis also associates with atherosclerosis. Indeed, bone formation rate is lower in kidney patients with coronary calcification. Thus, patients with osteoporosis deposit calcium mineral in their arteries, adding theoretical concerns to the epidemiological evidence that calcium supplements may promote cardiovascular disease.

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-MGP complexes, a concept supported by evidence that anti-osteoporotic agents block vitamin D-induced vascular calcification in mice and at the same doses that prevent osteoporosis. 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.

**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. Oxylipids also accumulate in bone tissue. Osteoblasts have been shown to generate oxidized LDL, probably due to nonenzymatic oxidation by radicals released as waste from energetic metabolism. This may increase the local concentration of oxylipids. 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. 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.
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.  

Skeletal bone density is also reduced in mice with diet-induced hyperlipidemia. In apparent opposition to this hypothesis, Apoe deficient mice that are not treated with an atherogenic diet were found to have greater bone mass than in wild-type mice despite hyperlipidemia, 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 apoE may prevent delivery from LDL to osteoblasts the fat-soluble vitamin K, which is required for MGP and osteocalcin downregulation of mineralization. Thus, lack of Apoe for vitamin K transfer to osteoblasts would yield a net increase in bone mass.

A number of signaling mechanisms may mediate oxylipid inhibition of bone formation. Oxylipids, in their role as ligands of peroxisome proliferator-activated receptor-gamma (PPAR-gamma) reduce beta-catenin levels, blocking Wnt3a signaling. 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.

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 CVC. In contrast, mildly oxidized LDL and its biologically active components inhibited differentiation of cells from the skeletal-derived MC3T3-E1 bone cell line. In vivo, femoral mineral content in C57BL/6 atherosclerosis-susceptible mice on the high-fat diet was significantly reduced, and mineral density was lower compared with mice on the chow diet.

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 (TRAP) activity. 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^{-/-}). Oxylipids and hyperlipidemia also have indirect effects on osteoclast differentiation via lymphocytes.

Of potential clinical importance, hyperlipidemia abrogated the efficacy of anabolic PTH treatment through effects on osteoblastic activity in vitro and in Ldlr^{-/-} mice. The apolipoprotein A-I mimetic peptide, D-4F, which has anti-atherogenic effects in hyperlipidemic mice, reverses adverse effects of hyperlipidemia on PTH osteoanabolism, acting primarily through reduction in serum markers of bone resorption rather than rescue of bone formation. 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-related factors: RANK (TNFRSF11a) a member of the TNF receptor superfamily that is expressed on preosteoclasts and dendritic cells; its ligand, RANKL (TNFSF11), which is required for development and maturation of osteoclasts; and osteoprotegerin (OPG; TNFRSF11b), a

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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.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 secreted from, osteoblasts, smooth muscle cells, T-lymphocytes and marrow stromal cells. On binding to RANK on the surface of preosteoclasts, RANKL induces osteoclast differentiation, fusion and maturation.92 OPG, the first member of the triad discovered in bone,92, 93 blocks this interaction of RANKL with RANK, thus interfering with osteoclast differentiation (Figure), hence the name, osteoprotegerin, for its ability to “protect/protéger” bone from resorption.94 Before the discovery of RANKL, in vitro osteoclastogenesis required co-culture with osteoblastic lineage cells, now known to have provided RANKL; recombinant RANKL treatment now replaces the coculture. RANKL is minimally expressed in quiescent osteoblasts,95 but 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.96

Like other TNFR family members, RANKL activation of RANK signals via TRAF-induced nuclear factor kappa-B (NF-kB) and Jun-N-terminal kinase (JNK) activation.97 It also activates p44 and p38 mitogen-activated protein kinases (MAPK) as well as the phosphatidyl inositol-3 kinase (PI3k/Akt) pathway.98 In skeletal osteoblasts, RANKL expression is induced by parathyroid hormone, dexamethasone, 1alpha,25-dihydroxyvitamin D(3), prostaglandin E2, and interleukin-11 (IL-11).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”104 which promotes dendritic cell survival by binding to RANK. Interestingly, dendritic cells are associated with atherosclerosis.5 With respect to nomenclature, more than one preferred name exists for each of these molecules. Recently, members of the human genome field standardized the nomenclature for TNF and TNF receptor superfamilies, and chose TNFSF11 (tumor necrosis factor superfamily member 11), TNFRSF11a (tumor necrosis factor receptor superfamily member 11a), and TNFRSF11b 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 due to deficient osteoclastic resorption. In addition to severe osteopetrosis, RANKL deficient mice develop immunological defects and lack peripheral lymph nodes.105 Mice lacking RANK,106 and mice overexpressing the soluble decoy receptor, OPG, also develop severe osteopetrosis.94

In contrast, mouse models with unopposed RANKL activity all develop osteoporosis, and one develops vascular calcification. Though mice with ubiquitous RANKL overexpression died at the late fetal stage,107 OPG-deficient mice and those with high circulating levels of RANKL due to liver overexpression, develop osteoporosis.107 Direct treatment with soluble RANKL induces overactive osteoclastic resorption in vivo.92 Unexpectedly, one strain of Opg$^{-/-}$ mice develops large-vessel vascular calcification.108 This phenotype was not rescued by post-natal OPG injections.109 In mice with targeted deletion of OPG that were backcrossed to a slightly different background strain, there was no spontaneous vascular
calcification, but the phenotype could be elicited by treating 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 on 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 male subjects.\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}\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 BMP4.\textsuperscript{113} Somewhat different findings from Osako and colleagues showed, in human aortic SMC, RANKL promoted osteogenic differentiation and calcification indirectly via induction of BMP in endothelial cells and inhibition of MGP in the SMC.\textsuperscript{112} Interestingly, these phenomena were blocked by estrogen.\textsuperscript{112} Interestingly, in murine SMC, cyclic adenosine monophosphate (cAMP), which induced both calcification and RANKL, the calcification was not inhibited by OPG, suggesting that vascular cell osteogenesis induced by the PKA 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 \textit{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 \textit{Opg}\textsuperscript{−/−} 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.\textsuperscript{125} Second, RANKL treatment in vitro induces osteoblastic differentiation and mineralization, as mentioned above. Third, all three members of the regulatory trio are expressed in vascular cells or tissue. Lastly, OPG treatment inhibits vascular calcification in rodents; it prevents warfarin-induced vascular calcification in rats\textsuperscript{126} and hyperlipidemic-induced vascular calcification in mice.\textsuperscript{109}

RANK has been identified in cultured umbilical and microvascular endothelial cells.\textsuperscript{98, 127} Though not found in normal arteries, it is expressed in calcified arteries of \textit{Opg}\textsuperscript{−/−} mice.\textsuperscript{125} RANKL, though not expressed at baseline in cultured EC or SMC,\textsuperscript{98, 128} is induced in EC by inflammatory cytokines and factors from actively remodeling bone such as transforming growth-factor-beta.\textsuperscript{129, 130} RANKL immunoreactivity is also not found in normal mouse
arteries, but it is present in calcified vascular tissue in humans and in Opg−/− mice. OPG is expressed by cultured arterial endothelial cells and SMC. OPG immunoreactivity is also found in the normal artery wall, but, in contrast with RANKL, its expression is less pronounced in calcified human atherosclerosis.

### RANKL/OPG/RANK axis and inflammation

In mineralized tissues, a RANKL dominant condition is produced in inflammatory conditions such as arthritis and periodontitis. The same may be true for the inflammatory state of atherosclerosis. Cytokines, such as TNF-alpha, induce functional RANKL expression in endothelial cells. In monocytes, RANKL induces release of cytokines, including TNF-alpha. Thus, sites of chronic inflammation such as atherosclerosis may have a high RANKL: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, and/or indirectly by inducing TNF-alpha release from monocytes, which promotes SMC osteogenesis. Alternatively, or even simultaneously, RANKL in atherosclerosis may initiate resorption of mineral by inducing osteoclastogenesis of the abundant monocytes and M-CSF found in atherosclerotic lesions. Another consideration favoring a net pro-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.

### Osteoclasts in the artery wall

Cells resembling osteoclasts have been identified in calcified atherosclerotic plaque, primarily at the edges of mineral deposits, and regression has been demonstrated in an animal model, in which osteoclast-like cells resorb mineralized vascular tissue in a carbonic-anhydrase-dependent manner. Osteoclasts originate from monocytes and macrophages exposed to particulate matter are triggered to undergo osteoclastic differentiation. Since atherosclerotic lesions are rich in monocyte/macrophages and calcium mineral, they have an abundant source of preosteoclasts. Maturation to osteoclasts requires two factors: macrophage colony-stimulating factor (M-CSF), and RANKL. Both factors are present in atherosclerotic lesions. Functional osteoclasts are multinucleated and express TRAP, cathepsin K, calcitonin 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 TRAP, cathepsin K and carbonic anhydrase. Osteoclasts adhere to vascular mineral deposits via osteopontin (OPN) to create an acidic and proteolytic microenvironment. Mice deficient in both OPN and ApoE, which are expected to have atherosclerosis but poor osteoclast and monocyte function, have increased vascular calcification despite a decrease in atherosclerosis, 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.

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. In vivo, apoE-deficient hyperlipidemic mice have increased RANKL, RANK and OPN in their atherosclerotic plaque.
Highly oxidized lipids, in contrast, prevented RANKL-induced osteoclastogenesis by preventing phosphorylation of ERK, p38 and JNK kinases and DNA-binding activities of NF-κB and nuclear factor of activated T cells (NFAT) transcription factors in human monocyte. 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. 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.

**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, and osteoclastic cells have the ex vivo capacity to demineralize calcified elastin from the aorta. 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.

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, Runx2 and Osterix, in normal human vessels from children, which the authors speculated may reflect a developmental remnant in the still immature vasculature of children. This remarkable finding of embryonic bone and cartilage transcription 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. Indeed, the capacity of smooth muscle cells to generate de novo, 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, in part 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, PTH, 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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**Non-standard Abbreviations**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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Fig. 1. The 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.
Fig. 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 and/or an atherogenic diet. In the artery wall milieu, these oxylipids induce vascular calcification by direct action on SMC or adventitial myofibroblasts and/or through indirect induction of cytokine release from macrophages. Infliximab, an inhibitor of TNF-alpha, blocks this process. In the bone milieu, oxylipids inhibit bone calcification by direct action on osteoblasts and/or through indirect induction of cytokine release from T-lymphocytes.
Fig. 3. Working model of the effects of intermittent PTH on vascular and bone calcification based on in vitro and in vivo studies

In the artery wall, intermittent PTH blocks effects of hyperlipidemia (HL) and/or atherogenic (Ath) diet, also a diabetogenic diet, on induction of vascular calcification. In the bone milieu, oxylipids blunts the anabolic effects of intermittent PTH (as used in therapy for postmenopausal osteoporosis). The apoA-I-mimetic peptide, D-4F, by blocking formation of oxylipids, rescues the effect of PTH on bone. Of note, continuous exposure to PTH, as in clinical hyperparathyroidism, has different, often opposite, effects as intermittent exposure.