Regulation of Vascular Calcification by Osteoclast Regulatory Factors RANKL and Osteoprotegerin

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Abstract—Vascular calcification often occurs with advancing age, atherosclerosis, various metabolic disorders such as diabetes mellitus and end-stage renal disease, or in rare genetic diseases, leading to serious clinical consequences. Such mineralization can occur at various sites (cardiac valves, arterial intima or media, capillaries), involve localized or diffuse widespread calcification, and result from numerous causes that provoke active inflammatory and osteogenic processes or disordered mineral homeostasis. 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 calcific vasculopathies. One emerging area in vascular biology involves the RANKL/RANK/OPG system, molecules of the tumor necrosis factor-related family recently discovered to be critical regulators of immune and skeletal biology. Evidence is accumulating that such signals may be expressed, regulated, and function in vascular physiology and pathology in unique ways to promote endothelial cell survival, angiogenesis, monocyte or endothelial cell recruitment, and smooth muscle cell osteogenesis and calcification. Concerted research efforts are greatly needed to understand these potential roles, clarify whether RANKL (receptor activator of nuclear factor κB ligand) promotes and osteoprotegerin (OPG) protects against vascular calcification, define how OPG genetic polymorphisms relate to cardiovascular disease, and learn whether elevated serum OPG levels reflect endothelial dysfunction in patients. Overall, the RANKL/RANK/OPG system may mediate important and complex links between the vascular, skeletal, and immune systems. Thus, these molecules may play a central role in regulating the development of vascular calcification coincident with declines in skeletal mineralization with age, osteoporosis, or disease. (Circ Res. 2004;95:1046-1057.)

Key Words: RANKL ■ OPG ■ calcification ■ atherosclerosis ■ blood vessels
Cardiac valves may also calcify after mechanical injury, infection, or inflammation.\textsuperscript{3-4,8} The precise mechanisms driving vascular calcification and its clinical consequences reflect the type and extent of mineralization provoked. Although intensely studied for many years, our understanding of regulatory factors and molecular pathways that control such processes is still incomplete. New insights are emerging in several areas, including recent research on a newly discovered group of molecules belonging to the tumor necrosis factor (TNF)-related family, which suggests key unexpected roles for them in normal vascular biology and pathophysiology. The purpose of this review is to highlight how these regulatory factors and molecular pathways that control such processes governing vascular calcification, and to suggest potential directions for future research.

### Types of Vascular Calcification

From a histoanatomical perspective, vascular calcification may be classified into four general types based on its location, association with plaque, and mode of formation.\textsuperscript{1-4} Whereas a minor form of widespread nonspecific organ and soft tissue calcification derives from abnormal calcium/phosphate products, three more common types of vascular calcification may occur by actively regulated processes in the absence of raised calcium/phosphate levels. Morphologically, these latter types of calcification are distinguishable by whether calcification is confined to intimal layers, or extends into subintimal or medial layers in association with macrophages and lipid and vascular smooth muscle cells (VSMCs) as in atherosclerosis, or in arterial medial layers because of elastin fiber mineralization as in end-stage renal disease or diabetes mellitus. Like most biological processes, vascular calcification by active mechanisms is subject to complex regulatory 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.\textsuperscript{1-6} Mounting evidence suggests that RANK, RANKL, and OPG may participate in multiple aspects of these processes governing vascular calcification.

### RANK/RANKL/OPG Links Between the Skeletal, Immune, and Vascular Systems

Since discovery of the tripartite RANK, RANKL, and OPG system, much has been learned about their developmental, homeostatic, and pathological roles in controlling cell recruitment, differentiation, function, and survival in skeletal and immune biology.\textsuperscript{9-13} However, such questions are only beginning to be explored in the context of vascular biology and pathophysiology. While deepening our understanding and providing further support for skeletal-like bone formation and remodeling during vascular calcification, this work is also revealing surprising functions for these TNF family molecules as potential mediators of vascular biology.

### RANK, RANKL, and OPG Essential Roles in the Skeletal and Immune Systems

RANKL, a 316-amino acid transmembrane protein, is highly expressed by T cells in lymphoid tissue and osteoblast (OB)/stromal cells in trabecular bone, especially areas undergoing active bone remodeling or inflammatory osteolysis.\textsuperscript{9-13} RANKL also exists in two biologically active soluble forms, either secreted (from T cells) or proteolytically cleaved from cell surfaces.\textsuperscript{9,10,13} RANKL binds as a homotrimer to RANK, a 616-amino acid transmembrane receptor (also a trimer), on dendritic cells (DCs), osteoclasts (OCs), and osteoprogenitor (OPG) may be involved in regulating vascular calcification, and to suggest potential directions for future research.
the resorptive activity and survival of mature OCs. Because OPG directly counters all of these RANKL-mediated actions through RANK, the RANKL-to-OPG balance critically determines bone remodeling and net bone mass. Imbalances in the RANKL/OPG ratio or RANK signaling underlie the pathology of many skeletal disorders exhibiting excessive bone loss, excessive bone formation, or disordered bone remodeling (eg, rheumatoid arthritis, periarticular osteolysis, periodontal disease, tumor-associated osteolysis, various osteopetroses, or Paget disease). Mechanistically, most osteocytic signals exert their resorptive or bone-sparing effects through altering the expression of RANKL and/or OPG and, thus, RANKL is the final common mediator of many of their actions in bone. Specifically, RANKL is upregulated in OB/stromal cells by a wide array of signals, such as 1α,25-dihydroxyvitamin D3 (VD3), parathyroid hormone (PTH), glucocorticoids, prostaglandin E2, interleukin (IL)-1α, TNF-α, IL-6, IL-11, IL-17, calcium, or immunosuppressants (cyclosporin A), and it is downregulated by transforming growth factor (TGF)-β. OPG is increased by some of these same stimuli (VD3, IL-1α, TNF-α, IL-6, IL-11, IL-17, calcium) and by estrogen, TGF-β, or bone morphogenetic protein (BMP)-2, and decreased by PTH (administered continuously, not intermittently), glucocorticoids, prostaglandin E2, insulin-like growth factor 1, or immunosuppressants. In vivo, RANKL administered to normal adult mice increases OC size and resorptive activity while inducing systemic hypercalcemia, whereas targeted disruption of RANKL inhibits OC formation and function and results in a severely osteopetrotic phenotype. A similar phenotype is observed in mice lacking RANK. Conversely, transgenic OPG expression in mice causes osteopetrosis, whereas OPG knockout mice are severely osteoporotic and exhibit excessive bone turnover in both cancellous and cortical bone because of unopposed actions of RANKL to stimulate OC formation, activity, and survival. OPG administration to animals prevents bone loss attributable to estrogen deficiency or tumor-induced osteolysis, and OPG has shown some initial promise in early human clinical trials.

Besides regulating bone mass, RANKL and OPG are essential for DC functions (survival, immunostimulatory activity), lymph node organogenesis, and lymphocyte development. Binding of RANKL on T cells (CD4+ or CD8+ T cells) to RANK on the DC surface enhances DC survival (via Bcl-xL and Src/phosphatidylinositol 3-kinase [PI3K]/Akt) and the ability of DCs to produce inflammatory cytokines (IL-1β, IL-6, IL-12, IL-15) and stimulate naïve T-cell proliferation and survival. RANKL is therefore implicated in DC antigen surveillance, T-cell memory formation, induction of immunological tolerance, and autoimmunity disease prevention. Increased RANKL production by activated T cells is linked to bone and joint destruction in a rat model of adjuvant arthritis and to osteolysis in periodontitis. OPG-deficient mice exhibit altered B-cell maturation and antibody responses, whereas mice lacking RANKL or RANK have defective T-cell and B-cell maturation and peripheral lymph nodes do not develop.

Overall, the RANKL/RANK/OPG axis is clearly of central significance in controlling the immune and skeletal systems, and these discoveries have forged fundamental links to spawn the new field of osteoimmunology. Research is now revealing that the vascular system also integrates into this RANKL/RANK/OPG multifaceted network in direct and indirect fashions. Vascular endothelial cells (ECs) are primary coordinators of the inflammatory response, and immune-mediated mechanisms are intimately involved in numerous vascular diseases, including vascular calcification. Moreover, vascular calcification can involve differentiation of osteogenic cells from VSMC or calcifying vascular cells (CVCs), expression of multiple bone-related molecules, formation of mineralized bone-like structures, and the participation of T cells, macrophages, and ECs that may serve as sources or targets of RANKL/RANKL/OPG actions. Thus, many of the same signals that modulate RANKL and OPG in bone or immune cells may also regulate their expression in vascular cells. From an indirect perspective, it is likely that the RANKL/RANKL/OPG axis exerts important effects on the vascular system through both immunomodulatory and osteogenesis-related mechanisms. In addition, RANKL and OPG might also directly target vascular cells to modify their differentiation, morphology, function, or survival as discussed below.

**Emerging Roles for RANKL, RANK, and OPG in the Vascular System**

Vascular calcification is associated with osteoporotic bone loss, but the reasons for this are unclear. As discussed below, the discovery that mice lacking OPG had severe osteoporosis and arterial calcification provided the first clue that OPG might be a key molecule linking these vascular and skeletal phenotypes. Recent work suggests that OPG might be an important autocrine/paracrine regulator of vascular calcification and perhaps an indicator of vascular disease in which serum levels are elevated in patients with diseases that involve vascular calcification. However, exactly what role OPG (or RANKL/RANK) might play in vessel calcification is still not understood.

**Relationship Between Vascular Calcification and Osteoporosis**

Vascular calcification and osteoporosis frequently occur together and share many of the same risk factors (eg, aging, inflammatory disease, glucocorticoid use, chronic renal failure, or estrogen deficiency). Osteoporotic patients have a higher incidence of arterial calcification, and their lateral lumbar spinal radiographs often reveal dense calcium deposits of the aorta situated directly adjacent to osteopenic vertebrae. Conversely, epidemiological studies indicate that the incidence of osteoporosis is elevated in people with atherosclerosis, cardiovascular disease, or aortic calcifications. In fact, the progression of calcification in atherosclerosis closely parallels the loss of bone in postmenopausal women, leading to the concurrent formation of bone in vessels while it is lost in the skeleton. Agents known to reduce OC bone resorption and diminish bone loss (eg, bisphosphonates, OPG, or OC vacuolar H+ ATPase inhibitors) may also prevent arterial calcification in animals. Thus, it has been proposed that an imbalance in calcium allocation allows its movement from bone to the vascular wall via...
mechanisms that involve OPG as a crucial tie between the bone and vascular systems.9,21

**OPG Knockout Mice and Vascular Calcification**

In addition to early onset osteoporosis, two-thirds of OPG knockout mice unexpectedly have late medial calcification of renal and aortic arteries, sites of abundant endogenous OPG expression in normal animals.18,22 This suggested that OPG might normally play an important vascular protective role in vivo and, further, that OPG may represent a factor linking osteoporosis and vascular calcification.9,18,21 Histologically, calcified arteries in the OPG−/− mice were detectable by 2 weeks and marked by 2 months of age, with aortic calcification primarily in the media (with mild to moderate intimal and medial proliferation) and calcified lesions in the renal arteries not associated with mineralization of any smaller vessels.18 Interestingly, calcified arteries of OPG−/− mice express RANKL and RANK, proteins undetectable in normal murine arteries, and large multinucleated RANK+ OC-like cells formed in close proximity to cells (possibly CVCs) expressing RANKL.32 These features resemble aspects of human atherosclerotic lesions, wherein RANKL and RANK often may be evident in calcified (but not normal) vessels and OC-like cells appear adjacent to RANKL-expressing cells.27,28 However, OPG−/− mice do not have atherosclerotic plaques in either their aortic or their renal arteries.18 Furthermore, the vascular phenotype of OPG−/− mice contrasts with human OPG deficiency (attributable to a homozygous gene deletion) because the latter does not appear to involve vascular abnormalities (whereas it causes juvenile Paget disease).29

Rescue/reversal studies showed that systemic (transgenic) OPG delivery in OPG−/− mice from midgestation through adulthood prevented vascular calcification, whereas transient OPG administration (postnatal injection) could not reverse arterial mineralization once it had occurred.32 In contrast, both treatments effectively reversed osteoporotic bone loss in OPG−/− mice. OPG prevented vascular calcification by blocking a bone remodeling-like process resembling osteoclastogenesis, consistent with increases in RANK (in OC-like cells) and RANKL (in adjacent cells) seen in the bone-like calcified arterial walls of OPG−/− mice.22 Such tissue-related differences support the idea that endogenous OPG may prevent mineralization in vascular tissues but promote mineralization in skeletal tissues; thus, OPG replacement would be expected to readily halt further OC-mediated bone loss but be unable to correct the overmineralization of arteries caused by an earlier lack of OPG.

**OPG and Other Animal Models**

A vascular-protective role for OPG is also indicated by rat studies in which OPG administration (parenteral) prevented calcification induced by warfarin (a vitamin K antagonist) or high vitamin D doses.26 Thus, OPG inhibited medial calcification in the arterial elastic lamellae of the aorta and other arteries of these rats without lowering serum calcium or phosphorus levels. Vascular calcification also occurs in other mouse models, including those lacking another physiological inhibitor of mineralization, matrix GLA protein (MGP), a γ-carboxylated mineral-binding protein produced by VSMC and chondrocytes that is found in the extracellular matrix of blood vessels and cartilage.1-6,30 MGP may suppress mineralization through binding BMP-2, a potent osteogenic signal upregulated in calcified lesions of human atherosclerosis that promotes mesenchymal cell differentiation, and undercarboxylation of MGP (and other proteins) by warfarin treatment is associated with extensive vascular calcification in animals.1,2,4-6,27 Compared with the mild arterial calcification in OPG−/− mice, MGP−/− animals exhibit widespread severe calcification of all arteries (and cartilage).1-6,30 Whether MGP and OPG interface in coregulating vessel mineralization is unknown. Various other mutant mice in which calcifications of small or large vessels develop have been described in detail elsewhere, including mice deficient in SMAD 6 (a key inhibitor of BMP signaling).1,3,5 Also of note are mice harboring an insertional mutation in the β-glucosidase–related gene, klotho, a humoral factor that can break-down glycolipids, because such animals display a complex premature aging phenotype with development of arterial medial calcification, low-turnover osteopenia, and increased serum OPG and Pi levels.31-34 Endogenous klotho protein provides cardiovascular protection through nitric oxide elicited by humoral factors and thereby guards against endothelial dysfunction in vivo.33 Klotho protein is downregulated in rat kidneys (its primary site of expression) under conditions simulating chronic hypertension, diabetes mellitus, or chronic

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Information was summarized from published reports cited. myoFb indicates myofibroblast; PLC, phospholipase C; ALP, alkaline phosphatase; OCN, osteocalcin; ?, no effects have yet been reported.
renal failure, diseases associated with humans with vascular calcification and elevated serum OPG levels, and klotho may negatively regulate OPG expression. Thus, klotho−/− mice mimic features of human senile osteoporosis, and their phenotype underscores the intriguing association between vascular calcification, bone resorption, and raised serum OPG levels (discussed in detail later).  

RANKL, RANK, and OPG Expression in Normal and Pathological Vasculature  

OPG is produced by a wide range of tissues, including the cardiovascular system in which the heart, arteries, and veins all express OPG. Both ECs and VSMCs constitutively express OPG, and such levels are particularly high in aortic and renal arteries. By comparison, RANKL and RANK are frequently undetectable in normal vessels and noncalcified arteries or valves. Although weak RANKL expression has been reported, for example, in human aortic lesions, small capillaries and venules of murine skin, or murine bone metaphyseal vessels. Although OPG may be unchanged or decline, RANKL or RANK may become upregulated and expressed together with OPG in atherosclerotic lesions, calcified vessels, or valves. An increased RANKL/OPG ratio seems consistent with the inflammatory nature of atherosclerosis because OPG often decreases while RANKL rises in ECs or other cells in periodontal, articular, or other inflamed tissues. In certain respects, atherosclerotic calcification resembles embryonic bone formation because arterial calcifications can develop into lamellar bone, with trabeculae, lacunae, islands of marrow, and cells that possess key features of bone-forming OBs and bone-resorbing OCs. Moreover, initial mineralized matrix may be subsequently replaced by osteoid, invaded by angiogenic vessels, and remodeled into mature bone as in endochondral ossification. RANKL and OPG have been immunodetected in both early and advanced human atherosclerotic lesions, with OPG located at the margins of mineralized lamellar bone-like structures, RANKL associated with the adjoining matrix, and OC-like RANK− cells situated nearby RANKL-expressing cells. Perhaps RANKL (and OPG) production by OB-like cells regulates OC formation and activity in atherogenic lesions. Furthermore, because RANKL can stimulate chemokine release, matrix metalloproteinase (MMP)-9 activity, and monocyte/macrophage matrix migration, it might contribute to these crucial processes during cell recruitment and infiltration into atherogenic lesions. Like atherosclerosis, calcific aorta valve stenosis involves pronounced inflammation with activated macrophages and T cells, initiation of an active bone developmental program, and increased RANKL staining along with fewer OPG-expressing cells in focal calcification areas. However, in arterial medial calcification, OPG was expressed in areas surrounding calcified regions, whereas little RANKL was detected. Overall, the occurrences of vascular calcification with little or no change in OPG and increases in RANKL (in human atherosclerotic or valvular lesions and OPG−/− mice) have led to the proposal that elevated RANKL levels might somehow favor vessel calcification. However, it has also been convincingly demonstrated that serum OPG levels often rise in vascular calcification diseases and, thus, is not clear if circulating levels of OPG may be involved in directly promoting vascular calcification, reflect biological attempts to correct an overmineralization process and exert vasculo-protective effects, provide an indicator of vascular pathology, or have other significance.  

Relationships Between Serum OPG and RANKL Levels and Vascular Calcification  

Because of the enormous surface area of the endothelium throughout the body as well as the relatively substantial levels of constitutive and regulated OPG produced by ECs and VSMCs, vascular cells may significantly contribute to circulating OPG levels measured in human serum. Because inflammation is a primary causative factor for atherosclerosis and a major contributing factor to medial calcification, altered serum OPG levels may reflect the development or status of vascular disease. Recent reports suggest that increased circulating OPG levels often occur in vascular calcification or cardiovascular disease. Thus, circulating OPG levels were greater in postmenopausal women with a high rate of bone turnover (especially those with severe osteoporosis). Serum OPG levels have correlated with bone resorption markers, serum calcium and PTH levels, and decreased bone mineral density, whereas they were inversely related to serum osteocalcin levels (a marker of bone formation) and unrelated to VD3 levels. However, negative or no associations between serum OPG levels and these parameters have also been reported. Elevated serum OPG levels have correlated with increasing age, diabetes, hypertension, increased cardiovascular mortality, the presence and severity of coronary artery disease, and chronic renal failure. Conversely, serum OPG levels declined in patients receiving glucocorticoid or immunosuppressive therapies. Serum OPG levels may also be related to sex hormone levels because they were higher in women than men, diminished in postmenopausal women, increased by estrogen replacement therapy or oral contraceptives, and positively associated with bioavailable testosterone levels or, in some cases, estrogen levels. These findings agree with studies in which estrogen increased, and PTH reduced, OPG production in OB/stromal cells and with in vivo studies wherein systemic PTH reduced local OPG production in rat bone and circulating OPG levels in postmenopausal women. However, serum OPG levels have also been positively associated or unrelated to PTH levels. Generally, serum RANKL levels appear unaltered and unrelated to serum OPG levels, although they have sometimes declined as serum OPG levels increased. Overall, these studies strongly indicate that serum OPG levels frequently rise in clinical conditions that favor atherosclerosis, vessel calcification, or vascular dysfunction, and they suggest OPG may have an important role in the cause or progression of vascular calcification. Interestingly, a nucleotide polymorphism (T950C) in the OPG promoter correlates with increased intimal thickening in the common carotid artery (an indicator of early atherosclerosis) and increased forearm blood flow (a measure of vasodilatory capacity) in healthy individuals.
morphism therefore is associated with structural and functional changes of the vasculature that may predispose to cardiovascular disease. In addition, linkage of two OPG genetic polymorphisms was associated with an increased risk of coronary artery disease in white men, and serum OPG levels correlated with one of these polymorphisms.64

Despite the initial discovery that mice lacking OPG develop arterial calcification, increased serum OPG levels occur along with vascular calcification in normal male mice with advancing age or in kl/kl mutant mice that exhibit rapid aging and osteoporosis.18,31,65 Thus, there may be certain similarities between animals and humans in the role of circulatory OPG relative to the vasculature and, more broadly, in pathological mechanisms governing vascular calcification. Moreover, whereas serum OPG levels increase with age in mice, OPG levels within bone decrease in parallel with reduced OB and increased OC functions,68 again highlighting the potential for OPG to serve as a link between skeletal bone loss and vascular calcification. The possibility is also raised that embryonic arterial calcification attributable to a developmental lack of OPG may proceed via disparate mechanisms from those controlling vascular calcification in adult pathologies that are associated with rises in OPG. In this regard, it is noteworthy that the important mineralization inhibitor MGP, which prevents arterial calcification during embryonic and postnatal development and is expressed in atherosclerotic lesions (but whose serum levels inversely correlate with vascular calcification, unlike OPG), was unexpectedly shown recently to interface with other mineralization signals (BMP-2) to directly stimulate CVC osteogenic differentiation and mineralization under certain conditions.45,66

RANKL/RANK/OPG Regulation and Direct Actions in Vascular Cells

OPG is produced by both ECs and VSMCs, regulated in these cells by multiple stimuli, and implicated in EC physiology in vitro and in vivo. Furthermore, RANKL is upregulated in stimulated ECs, may increase in VSMCs during vascular calcification, and may exert complex autocrine/paracrine effects on ECs and VSMCs that contribute to atherosclerosis or vascular calcification processes as discussed below. Such regulation is summarized in Tables 1 and 2.

OPG and Vascular Cells

In keeping with the inflammatory nature of atherosclerosis and the ability of proinflammatory cytokines to upregulate OPG expression in human and murine OB/stromal cells,9–13 OPG expression and release are stimulated by IL-1 and/or TNF-α in ECs or VSMCs.38,67,68 In vitro, potential inhibitory effects of raised OPG levels in ECs on OC development appear to be overridden by concurrent RANKL increases induced by these cytokines.10,38 In vivo, OPG might be released from ECs in a polarized fashion and preferentially rise either in the circulation or in tissue. These questions require further study. Unlike stromal cells, ECs have not responded to VD3 or PTH with increases or decreases, respectively, in OPG expression.38 Because VD3 is implicated in medial calcification, OPG inhibits VD3-induced ectopic calcification in blood vessels and other sites, and PTH inhibits osteogenic vascular calcification and raises circulating levels of osteopontin (OPN), which acts as a mineralization inhibitor and trigger for OPG production in ECs.3,26,69 It will be interesting to investigate in future studies whether either of these hormonal stimuli regulate OPG in VSMCs and consequently impact on OC or EC pathophysiology. Similarly, the potential for estrogen (which can promote CVC osteogenic differentiation and calcification), BMP-2 (a promoter of osteogenesis and calcification in CVCs, VSMCs, and aortic myofibroblasts), or calcium to stimulate OPG expression in VSMCs as they do in bone marrow stromal cells, or for lipid-lowering statins to alter vascular OPG production, are currently unknown and are important areas of inquiry to pursue.10,66,69,70

Beyond IL-1 and TNF-α, OPG is modestly elevated in VSMCs by basic fibroblast growth factor (bFGF) or angiotensin II (Ang II), and is strongly upregulated by platelet-derived growth factor (PDGF), three important regulators of vascular pathogenesis.68 In particular, PDGF promotes atherosclerosis through stimulating VSMC proliferation and migration (partly through upregulating integrins αβ1 and α5β1), VSMC binding and retention of monocytes in the subendothelium, EC angiogenesis and MMP-3 production, disruption of the elastic layer, and VSMC expression of peroxisome proliferator-activated receptor (PPAR)-γ (which increases in intimal VSMCs in atherosclerosis).7,68,71–73 Conversely, OPG production is inhibited by TGF-β in ECs, or by PPAR-γ ligands or immunosuppressants in VSMCs, agents associated with anti-inflammatory, anti-atherogenic, and/or pro-apoptotic actions in vitro or in vivo.55,74,75 As in other tissues, TGF-β actions are complex and context-dependent and, although TGF-β generally behaves as an anti-inflammatory and crucial plaque-stabilizing factor, it can also stimulate vascular cell osteogenesis and calcification, cell proliferation and migration, and MMPs and matrix remodeling.8,27,44,46,76 Both TGF-β and PPAR-γ ligands have major roles in vascular disease and participate in a reciprocal regulatory feedback loop in VSMCs.77 PPARs are present in all vascular-associated cells (ECs, VSMCs, and monocytes/macrophages), critically regulate atherosclerotic processes and exert beneficial effects on the cardiovascular system, and functionally link obesity, hypertension, and diabetes, all of which are associated with vascular disease and calcification.74,77,78 Many of the same signals that induce OPG in human VSMCs (eg, PDGF, bFGF, angiotensin II, TNF-α, and IL-1β) also stimulate their PPAR-γ expression, possibly providing a negative feedback route in the presence of PPAR-γ ligands for restraining the levels of OPG generated.79 This mechanism is consistent with reports that OPG is reduced (along with MGP) in osteogenic VSMCs of calcified arteries.79 It is intriguing to consider that PPAR-γ might collaborate with OPG in differentially regulating calcification mechanisms between the vascular and skeletal systems. Thus, in bone, PPAR-γ is expressed by monocyte/macrophage cells but not skeletal OBs, and PPAR-γ-activating ligands do not affect OB OPG production, although they inhibit differentiation of bone-resorbing OCs from monocyte/macrophage precursors.74,80 These actions would favor high OPG levels and greater mineralized bone mass. However, in the vascular...
system, PPAR-γ ligands downregulate OPG in VSMC, suppress VSMC growth and survival, and inhibit vascular mineralization.24 Consequently, further research is needed to learn if and how OPG might interface with PPAR-γ ligands in regulating mineralization within the skeletal and vascular systems.

Functionally, OPG was recently and unexpectedly discovered to mediate the integrin-dependent survival of serum-deprived ECs, an effect that is directly opposite to its apoptosis-inducing actions in OC precursor cells.81–83 Thus, OPG engagement of αvβ3 on the EC surface triggered a nuclear factor κB (NF-κB)–dependent generation of OPG that was essential for conveying the anti-apoptotic actions of OPN-induced NF-κB activation in EC.81 Interestingly, neither RANKL nor RANK were involved in this pathway; instead, OPG-induced EC survival appears attributable to its ability to directly bind and prevent TRAIL interaction with death-inducing TRAIL receptors on ECs.85 Because all four of these molecules—OPN, αvβ3, OPG, and TRAIL—are expressed in atherosclerotic or calcified vessels, this OPG-mediated anti-apoptotic mechanism might be operational and relevant under such conditions. OPN, a well-established inhibitor of hydroxypatite formation (in vitro) and vascular calcification (in vivo), is highly expressed by VSMCs and intimal macrophages in human arteries near sites of vessel mineralization and upregulated by inflammatory and osteogenic stimuli.2,3,5,30 EC surface expression of integrin αvβ3, important for angiogenesis elicited by bFGF or other stimuli, is also stimulated by inflammatory cytokines abundant in atherosclerotic sites.81,82 Therefore, inflammatory-activated ECs might generate elevated OPG levels through both direct (inflammatory cytokine-induced) and indirect (OPN/αvβ3–mediated) mechanisms, thereby significantly raising serum OPG levels detected in vascular calcification diseases. Possibly, EC-derived OPG may serve as an important autocrine/paracrine factor to protect against arterial calcification and the vascular-damaging effects of inflammatory cytokines through enhancing EC survival, via conveying integrin/NF-κB–induced survival signals and/or by directly binding and neutralizing the pro-apoptotic actions of TRAIL released from VSMCs.86 This agrees with the demonstrated anti-atherogenic and vasculoprotective actions of OPG in developing mice or in suppressing warfarin or VD3–induced calcification in rats, and with genetic evidence correlating human OPG promoter polymorphisms with susceptibility for coronary artery disease or atherosclerotic-like vascular changes.18,22,26,63,64 A role for OPG in EC survival is also indirectly suggested by microarray studies wherein OPG, vascular endothelial growth factor (VEGF), and VEGF receptors were among those genes more highly expressed by ECs less prone to apoptosis.84 Interestingly, VEGF induces both αvβ3 and OPN in ECs,85 and VEGF receptor 2 (which conveys VEGF pro-angiogenic signals) can directly associate with αvβ3.86 In contrast to ECs, whether OPG influences VSMC physiology is unknown at present but is worth probing, especially because PDGF promotes angiogenesis, atherogenesis, and both OPG and αvβ3 expression in VSMCs (as well as PPAR-γ) and PDGF receptors directly interact in a selective manner with β3 (but not β1 or β5) integrin.68,72,86

**RANKL/RANK and Vascular Cells**

Like OPG, RANKL mRNA and protein is expressed in ECs and upregulated by inflammatory cytokines, but not by PTH or VD₃ (which elevate RANKL in bone OB/stromal cells).38 Thus, IL-1α and TNF-α co-induce RANKL and OPG in ECs (as they do in OB/stromal cells), although maximal RANKL expression may occur later and persist longer than peak elevations in OPG expression.38 RANKL induced on the surface of ECs is functional and capable of causing in vitro formation of bone-resorptive OCs in cocultures with human monocyte38 or murine87 precursors. Because RANKL also stimulates the recruitment, survival, and bone-resorptive activity of OCs,9–13,15,47,49,50 inflammatory-activated ECs expressing RANKL are poised to contribute to the in vivo appearance, function, and longevity of OC-like cells, which arise in advanced calcified atherosclerotic lesions and participate in remodeling bone formed in the vessel wall.22,27,39,46,88 EC-associated RANKL might also enhance the recruitment and infiltration of monocyte/macrophages47–50 that stimulate VSMC mineralization in atherosclerosis1,20,44,52,88 and trigger monocyte production of MMP-947 that supports cell infiltration and atherosclerotic plaque growth.

Beyond inflammatory cytokines, RANKL can also be upregulated in ECs by at least two other mechanisms: via direct EC contact with CD44 receptors on metastatic tumor cells or via EC exposure to TGF-β (which is abundantly produced by many cells, matrix-incorporated and liberated, and involved in vascular angiogenesis and calcification). In the first case, RANKL mRNA and protein expression were rapidly induced in bone EC (without any OPG changes) during their direct cell contact with metastatic myeloma cells via a CD44–mediated mechanism, leading to the formation of OCs from murine precursors in vitro or pathological osteolytic bone lesions in mice in vivo.87 Because CD44 can be expressed by aortic medial CVCs,89 induced by IL-1β in VSMCs,90 and increased in atherosclerosis wherein it promotes inflammatory cell recruitment, vascular cell activation, and VSMC differentiation,81 it will be interesting to investigate whether CD44 might upregulate RANKL in ECs of calcifying vessels. In the second case, TGF-β rapidly increased RANKL while decreasing OPG in human umbilical vein endothelial cells or bone ECs, enabling increased OC formation in vitro.75 TGF-β appears to oppositely regulate RANKL and OPG in the vascular and skeletal systems, raising RANKL and lowering OPG in ECs but elevating OPG and suppressing RANKL in OB/stromal cells.9,10,75 Ultimately, TGF-β is positively associated with enhanced mineralization in both tissue types. Thus, whereas TGF-β–mediated reduction of the RANKL/OPG ratio in bone would favor skeletal mineralization through inhibiting OC resorption of bone, TGF-β elevation of the RANKL/OPG ratio in the vasculature may be an important facet of the mechanism by which TGF-β stimulates vascular cell osteogenesis and calcification, cell proliferation and migration, and matrix remodeling.27,92 This is bolstered by recent evidence that RANKL might directly promote osteogenic and angiogenic processes in vascular cells.36,93,94 In contrast to ECs, no reports to my knowledge have yet described RANKL regulation in VSMCs, despite the fact that RANKL mRNA and
protein are detected in them, VSMCs acquire an osteogenic phenotype during vascular calcification (which therefore may be associated with changes in RANKL and OPG expression), and RANKL expression may increase in atherosclerotic or valvular tissues, whereas OPG declines in differentiating VSMC during vascular calcification.22,28,39,67,74 However, others have reported weak RANKL and strong OPG expression in the neointima of atherosclerotic lesions and medial arterial layer of Monckeberg sclerosis, indicating the need for further work to clarify these relationships in human vascular disease.37

Functionally, RANKL was recently shown to affect EC and VSMC physiology, inducing angiogenesis and survival in ECs and osteogenic differentiation and calcification in VSMCs. RANKL enhancement of EC survival under serum deprivation conditions might seem incongruous with the strong previous evidence that OPG can mediate EC survival.81,83 However, in nonproliferating human umbilical vein endothelial cells subjected to serum deprivation or treatment with TNF-α or lipopolysaccharide, RANKL partially prevented their apoptosis via stimulating an essential PI3K/Akt pathway.67 Because potential survival effects of OPG were not assessed here,67 whereas RANKL actions were not investigated in the αβ3/NF-κB-related studies,81,83 and because different experimental designs (EC types, culture conditions, assays) were used, further research is required to understand when and how EC survival might be enhanced either by OPG or by RANKL. RANKL was also unexpect-
edily found to stimulate EC proliferation, chemotactic migration, and capillary tube formation in vitro, and to elicit neoangiogenesis (comparable to bFGF) in vivo in two different animal models.93 These actions involved RANKL binding to RANK on the EC surface, thereby initiating Src-phospholipase C–Ca<sup>2+</sup> signals that directly promoted EC angiogenesis (without elaboration of an intermediary proangiogenic signal like VEGF).93 Interestingly, VEGF induced RANK mRNA and protein expression in EC (through Flk-1), enabling greater angiogenic capillary tube formation by ECs exposed to RANKL.94 These findings are notable because Flk-1 (VEGFR-2) and VEGF expression are increased in arterial walls or vascular cells in atherosclerosis and apoE<sup>−/−</sup> mice exhibiting neointima thickening and plaque formation.95 VEGF is upregulated in macrophages by oxidized lipids that elicit foam cell formation,96 antiangiogenic therapy limits plaque growth and vascular neovascularization,97 and both RANK and RANKL may increase (whereas OPG decreases) in calcifying atheromatous lesions.36,39 Together, these observations suggest that RANKL production (by activated T cells, ECs, or calcifying VSMCs or CVCs) may help to initiate and perpetuate angiogenic processes that exacerbate vascular inflammation and calcification. These novel findings also provide further insights into how the VEGF-Flk-1 pathway, a major driving force for angiogenesis, may be important in the development of atherosclerosis through stimulating neovascularization that supports cell recruitment, intimal-media cell contact, plaque progression and rupture, and a persistent inflammatory state.45,97 RANKL may also directly promote an osteogenic phenotype in medial adventitial cells.36 Specifically, isolated vascular aortic myofibroblasts cultured under mineralizing conditions have responded to exogenous RANKL by increasing their bone-specific alkaline phosphatase activity, osteocalcin expression, cbfa-1/DNA binding, matrix calcification, and nodule formation.36 Consistent with this, RANKL immunostaining increased, whereas OPG decreased, in calcified versus normal human aortic valves.36 Thus, RANKL might be involved in promoting vascular calcification, whereas OPG serves a protective role in the vascular system.18,22,36,93,94 If so, RANKL might collaborate with other potent signals to trigger both angiogenic and osteogenic programs that underlie the fundamental disease nature of calcific vasculopathies. Whether OPG antagonizes any of these RANKL-mediated actions in ECs or VSMCs is currently unknown but, from a broader perspective, it appears that elevated circulating OPG levels that occur in vascular diseases, although potentially protective, may be insufficient to fully counteract such effects. To date, there are no reports regarding potential biological actions of OPG on VSMCs or related cells, and such insights therefore await further research.

Conclusions and Future Directions
Highly complex mechanisms are involved in the initiation and progression of vascular calcification in various human pathologies. Although such diseases, particularly atherosclerosis, have been intensively studied for years, surprising new insights continue to arise that provide us with a deeper understanding and suggest new avenues for further research or potential strategies for novel clinical interventions. The RANKL/RANK/OPG system represents one newly emerging area of inquiry in vascular biology, which builds on a strong foundation of exciting findings recently codiscovered in the immunology and osteobiology fields. Tentative roles for these molecules and their interactions with other key signals in atherosclerosis are depicted in the Figure. Understandably, more questions than answers currently exist relative to these new potential roles of RANKL, RANK, and OPG in normal or pathological vascular biology. Numerous unresolved issues in need of further exploration include questions such as, why do serum OPG levels often rise in vascular calcification? Are they a cause or effect of such processes? Do vascular tissue levels of OPG increase, decrease, or not relate to calcification? What happens to RANKL expression in calcifying vascular tissue? Are RANK and RANKL important in vascular calcification through effects on immune cells, ECs, VSMCs, and monocytes or their derived macrophages or OCs? Which immune or vascular cells produce OPG or RANKL and when? Does RANKL stimulate EC survival, angiogenesis, monocyte recruitment, MMP activity, or VSMC osteogenesis in vivo during vascular calcification? Do RANKL or OPG exert other unique or heretofore unknown actions on vascular cells? Are the roles of RANKL or OPG different during embryonic vascular development compared with their later functions in adult vascular pathology or even during various stages of vascular dysfunction? How do important systemic or local modulators, drugs, and specific clinical conditions influence the RANKL/RANK/OPG axis to possibly impact on vascular cells and affect vascular ossification? How might such knowledge be exploited for diagnostic, therapeutic, or preventative purposes? Answers to these and further intriguing questions are eagerly awaited and will no doubt be forthcoming in the near future.

Acknowledgments
The author was supported by NIH grant DK46547. The author greatly appreciates the valuable contribution of Dr Philip Osdoby in the figure preparation.

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doi: 10.1161/01.RES.0000149165.99974.12

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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