Role of Osteoprotegerin and Its Ligands and Competing Receptors in Atherosclerotic Calcification

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Vascular calcification significantly impairs cardiovascular physiology, and its mechanism is under investigation. Many of the same factors that modulate bone osteogenesis, including cytokines, hormones, and lipids, also modulate vascular calcification, acting through many of the same transcription factors. In some cases, such as for lipids and cytokines, the net effect on calcification is positive in the artery wall and negative in bone. The mechanism for this reciprocal relation is not established. A recent series of reports points to the possibility that two bone regulatory factors, receptor activator of NF-κB ligand (RANKL) and its soluble decoy receptor, osteoprotegerin (OPG), govern vascular calcification and may explain the phenomenon. Both RANKL and OPG are widely accepted as the final common pathway for most factors and processes affecting bone resorption. Binding of RANKL to its cognate receptor RANK induces NF-κB signaling, which stimulates osteoclastic differentiation in preosteoclasts and induces bone morphogenetic protein (BMP-2) expression in chondrocytes. A role for RANKL and its receptors in vascular calcification is spported by several findings: a vascular calcification phenotype in mice genetically deficient in OPG; an increase in expression of RANKL, and a decrease in expression of OPG, in calcified arteries; clinical associations between coronary disease and serum OPG and RANKL levels; and RANKL induction of calcification and osteoblastic differentiation in valvular myofibroblasts.

Key words: vascular calcification, osteoprotegerin, biomineralization, atherosclerosis

Cardiovascular disease, most often due to atherosclerosis, is the leading cause of mortality and morbidity in the United States,[1] and osteoporosis is the most common, disease of bone.[2] Atherosclerosis, vascular calcification, and osteoporosis correlate in an age-independent manner with one another and with hyperlipidemia and osteoprotegerin levels.[3–11] The mechanisms for these links are not understood.

Vascular calcification is present in half of middle-aged individuals and in 90% of those over age 70 years.[12] It particularly affects patients with atherosclerosis, diabetes, and end-stage renal disease.[13] It contributes substantially to cardiovascular morbidity and mortality by destroying the artery's normal elastance, leading to systolic hypertension, left ventricular hypertrophy, diastolic hypotension, coronary insufficiency, congestive heart failure, aortic stenosis, and, potentially, plaque rupture.[12,14–21]

Intimal and Medial Calcification

Intimal (atherosclerotic) calcification colocalizes with atherosclerosis and correlates with plaque burden and cardiovascular events.[22–24] Medial calcification occurs independently of atherosclerosis, primarily in patients with end-stage renal disease and diabetes. Intimal calcification appears to be driven by inflammatory factors such as cytokines and atherogenic lipids.[25,26] In contrast, medial calcification appears to be promoted by a variety of factors, including high leptin levels,[27] hyperphosphatemia,[28–30] uremia,[31] hypercalcemia,[32] hyperparathyroidism,[33] warfarin,[34] 1-alpha,25-dihydroxyvitamin D_3,[21,34–36] and pyrophosphate deficiency.[37] In a given individual, both intimal and medial vascular calcification may occur, and more than one mechanism may be operative. The final common pathway, if any, is not known.

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Similarity to Osteogenesis

Atherosclerotic calcification shares many features with embryonic osteogenesis. It involves the same histologic transitional stages, including amorphous mineral remodeling, invasion of preosteoblasts, and, finally, formation of full bone tissue with trabecular architecture.[38–41] The full complement of osteogenic factors and bone proteins is present in atherosclerotic calcification, including bone morphogenetic protein 2 (BMP-2), which governs mesenchymal lineage acquisition via Smad-dependent signals, Msx-2, $Cbfa_1$, and downstream effector molecules such as osteopontin, matrix γ-carboxyglutamic acid protein (MGP), and bone sialoprotein.[42–49] Thus, calcified atherosclerotic lesions resemble bone in many ways.

In Vitro Vascular Osteogenesis

Vascular smooth muscle cells (SMCs) and closely related cells, such as microvascular pericytes, calcifying vascular cells, adventitial myofibroblasts, and cardiac valvular myofibroblasts, express osteoblastic differentiation markers and produce the bone mineral hydroxyapatite, with a characteristic time course[44,46,48,50–55] nearly identical to that in skeletal bone cell osteogenesis.[56] The process is governed by BMP-2 and master osteogenic transcriptional regulators, such as $Cbfa_1$ and Msx-2.[57] It is modulated by atherogenic lipids and lipoproteins,[25] lipoprotein receptor–related protein 5 and Wnt pathway members,[49] transforming growth factor (TGF)-β,[58] cyclic adenosine monophosphate agonists,[48] 1-alpha,25-dihydroxyvitamin D_3,[59] warfarin,[34] tumor necrosis factor (TNF)-α,[60,61] and monocyte coculture.[62] It is inhibited by high-density lipoprotein,[63] collagen IV and laminin,[64] osteopontin,[65] extracellular phosphate,[51] parathyroid hormone (PTH)-related peptide,[59] and PTH itself, which, in turn, down-regulates Msx-2.[66]

Bone-Vascular Connection

In vivo, bone and vascular mineralization may be linked through endocrinologic mechanisms or they may be independent, depending on the circumstances. In metastatic vascular calcification, as occurs in patients with osteolytic cancer or vitamin D overdose, vascular calcification results from excess serum calcium. Extremely rapid bone resorption, beyond that of ordinary osteoporosis, can overcome the normally tight endocrine regulation of serum calcium. As serum calcium increases, the serum calcium-phosphate product eventually exceeds the threshold required for spontaneous crystallization in many tissues, including arteries.[67] High-dose vitamin D has been used to produce experimental vascular calcification in rodents, and factors that arrest the bone resorption consequently inhibit the vascular calcification.[68,69] Because of this potential effect of excess bone resorption on vascular calcification, genetic interventions to assess the direct effects of genes on vascular calcification may need to be vascular specific.

In many mouse models of vascular calcification, including mice deficient in fibrillin, MGP, and carbonic anhydrase, the skeleton is either normal or hypermineralized, indicating that vascular calcification does not require high bone-resorptive activity.[70–72] Even some mouse models with both vascular calcification and osteoporosis have normal serum calcium, suggesting that the bone phenotype is not sufficiently severe to induce metastatic calcification.

In atherosclerotic calcification, serum calcium and phosphate are not elevated, and the association with osteoporosis may be attributable to a common cause such as hyperlipidemia and/or inflammation, which have reciprocal effects on osteogenic differentiation in bone versus vascular tissues.[25,73,74] Although the reciprocal response to inflammation recapitulates the reciprocal response to chronic infection (calcification in soft tissue versus osteolysis in bone), the signaling mechanism is not known. One possibility is that the reciprocal response is mediated via RANKL activity, which has been shown to promote osteoclastogenesis in bone and osteogenesis in SMCs.

RANKL and OPG in Bone

Regulation of receptor activator of nuclear factor-κB (RANK) is now considered the final common pathway for most mediators of bone catabolism.[75] Binding of RANK to its ligand, RANKL, is essential for the development and activation of osteoclasts.[76] In bone, RANKL is expressed as a transmembrane protein on osteoblasts and marrow stromal cells, and when it binds to RANK on the surface of preosteoclasts, it induces osteoclastic differentiation and maturation.[77] OPG blocks interaction of RANKL with RANK, thus preventing osteoclast differentiation and bone resorption.[77–79] High RANKL states cause osteoporosis owing to excess resorption by osteoclasts.

RANKL expression in osteoblast lineage cells is low at baseline,[80] but it is up-regulated by osteolytic factors such as inflammatory cytokines, 1,25-dihydroxyvitamin D_3, and dexamethasone in regions of bone that are undergoing rapid turnover or osteolysis. These osteolytic factors also inhibit OPG expression.[81–87] RANKL can be cleaved by the metalloproteinase TNF-α converting

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enzyme to release a soluble form (sRANKL). Like other TNF receptor family members, RANK acts primarily via nuclear factor (NF)-κB and Jun-N-terminal kinase activation in preosteoclasts.[84] It also activates p44 and p38 mitogen-activated protein kinases, as well as the phosphatidyl inositol-3 kinase pathway.[88]

As an aside, although RANKL, RANK, and OPG recently were assigned new nomenclature, "tumor necrosis factor superfamily member 11" and "TNF receptor superfamily members 11a and 11b," respectively, the simpler nomenclature used in this review is generally recommended for the bone literature.[89]

Mouse Models

In mouse models lacking RANKL/RANK signaling, such as RANKL-deficient,[76] RANK-deficient,[90] and OPG-overexpressing[78] mice, the lack of osteoclastic resorption results in rigid and overmineralized "osteopetrotic" bone with limited marrow cavities. No vascular phenotype has been reported, and no vascular findings are expected at baseline if RANKL is the operative factor.

In contrast, mouse models with unopposed RANKL activity develop osteoporosis. Soluble RANKL treatment induces overactive osteoclastic resorption in mice.[77] Mice lacking OPG develop osteoporosis.[91] Mice with ubiquitous RANKL overexpression die at the late fetal stage.[92] A transgenic mouse expressing soluble RANKL in the liver (driven by a serum amyloid P promoter, SAP-*rankl*^{tg}) and released in the circulation has osteoporosis, hypercalcemia, and abnormalities of lymphocytes, monocytes, and dendritic cells.[92] In these mice, the indirect effects of the bone phenotype would also confound interpretation of a vascular phenotype.

RANKL and OPG in the Artery Wall

All three members of the RANKL/OPG/RANK system are expressed in vascular cells:

1. RANK is expressed in cultured umbilical and microvascular endothelial cells (EC).[88,93] Although not found in normal arteries, it is expressed in the calcified arteries of *opg*$^{(-/-)}$ mice.[94]
2. RANKL is not expressed at baseline in cultured endothelial cells (ECs) or SMCs,[88,95] but it is induced in ECs by inflammatory cytokines and factors from actively remodeling bone such as TGF-β.[11,96] RANKL immunoreactivity is not found in normal mouse arteries,[94] but it is present in calcified vascular tissue in humans[47,55,97] and in *opg*$^{(-/-)}$ mice.[94]
3. OPG is expressed by cultured arterial ECs[11,93,98] and SMCs.[11,95,99] OPG immunoreactivity is found in calcified human atherosclerosis,[97] but, in contrast with RANKL, it is expressed at higher levels in the normal artery wall.[94,100]

RANKL and OPG in Vascular Calcification

The mechanism of vascular calcification in *opg*$^{(-/-)}$ mice is not known. Given that these mice have normal serum calcium and the vascular calcification is not reversed by correction of the osteoporosis,[91,94] it is most likely not attributable (directly) to the osteoporosis. Interestingly, in vitro evidence has shown that RANKL induces osteoblastic differentiation and mineralization of cultured cardiac valvular (myofibroblastic) cells.[55] The molecular regulatory mechanism for this phenomenon is unknown. In osteoclasts, NF-κB is a major signaling mechanism for RANKL activation of RANK. Interestingly, NF-κB response elements have been identified in the 5′ regulatory region of BMP-2, and NF-κB induces BMP-2 in chondrocytes.[101,102] Chondrocytes from p50/p52 double mutants showed decreased BMP-2.[101] One possibility is that RANKL induces vascular osteogenesis by activating NF-κB signaling and up-regulating BMP-2 in SMCs.

Whereas OPG deficiency leads to mineralization in the artery wall, it leads to demineralization in bone. A possible explanation for this reciprocal effect is the difference in the availability of osteoclast progenitor cells. Normal bone has a rich supply of preosteoclasts from the marrow, whereas normal artery wall has few, if any. Thus, in bone, but not artery wall, unopposed RANKL activity stimulates osteoclastic differentiation and resorption. Given that the net result is an osteoporotic phenotype, the osteoclastogenic effect of RANKL apparently overrides any possible promineralization effect of RANKL in bone, where preosteoclasts are abundant. But, in the artery wall, a promineralization effect of RANKL may be unmasked by the lack of preosteoclasts.

TRAIL in Vascular Calcification

OPG also serves as a decoy receptor for another ligand, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), which is expressed in the artery wall, together with its receptor (TRAILR), which is encoded by the gene *dr5*.[103] Previous studies have not linked TRAIL to vascular calcification. However, TRAIL is known to induce apoptosis in certain cells, including ECs.[104] It is possible that TRAIL promotes vascular calcification by inducing apoptosis. Apoptotic bodies may serve as nucleation sites for crystal formation similar to matrix vesicles, and Proudfoot

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and colleagues showed evidence of apoptosis in vascular cell calcification.[105] Another mechanism by which TRAIL could contribute to vascular calcification is by occupying OPG sites, which would indirectly increase the effective availability of RANKL.

Clinical Cardiovascular Disease

Recent animal and clinical studies suggest that serum OPG and RANKL levels are associated with cardiovascular disease. Serum OPG correlates positively with cardiovascular disease and mortality,[106–109] whereas serum RANKL levels correlate inversely with cardiovascular disease.[109] Although this may appear to be paradoxical, it may reflect an incomplete compensatory response. It is not unusual for serum levels of protective factors to be elevated in compensatory response to the diseases they prevent. For example, matrix Gla protein, which is recognized as an inhibitory factor in vascular calcification, is increased in calcified lesions, and serum OPG is elevated in a variety of conditions involving persistent immune activation as a partial compensatory response to proinflammatory activity of other members of the TNF family.[110] Similarly, natriuretic factors are elevated in heart failure, yet they are successfully used as treatment for heart failure. As another consideration, OPG could be selectively increased in the circulation and not in the artery wall by factors that selectively induce OPG expression in the endothelium. This is because ECs segregate and store OPG in their Weibel-Palade bodies, which are selectively exocytosed into the circulation. Thus, the serum levels of OPG may be increased independently of levels in the medial layer of the artery wall.

Summary

The RANKL/OPG/RANK system governs biomineralization of the skeleton, and growing evidence suggests that it has a role, possibly reciprocal, in biomineralization of the artery wall. Overall, the evidence clearly and consistently indicates that RANKL stimulates differentiation and maturation of osteoclast progenitor cells and that the net effect of high RANKL activity on bone in vivo is excess resorption and osteoporosis. Consequently, deficiency of its decoy receptor, OPG, would have the same effects, and the net effect of reduced OPG activity on bone in vivo is osteoporosis. In contrast, recent evidence now suggests that RANKL also stimulates osteoblastic differentiation and mineralization in vascular cells. Atherosclerotic calcification may result in part from the unopposed activity of RANKL. Given that normal arteries lack osteoclast progenitor cells, high RANKL activity would be expected to stimulate calcification in the vasculature. This would explain the finding of aortic calcification in the OPG-deficient mice. Given that inflammatory factors, as are present in atherosclerotic lesions, promote RANKL and inhibit OPG, it is possible that the reciprocal effects of hyperlipidemia on bone and artery are mediated through disturbance of the RANKL/OPG balance toward a RANKL-dominant state.

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