Osteoprotegerin Inhibits Vascular Calcification Without Affecting Atherosclerosis in \textit{ldlr}^{−/−} Mice

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**Background**—The role of osteoprotegerin in vascular disease is unclear. Recent observational studies show that serum osteoprotegerin levels are associated with the severity and progression of coronary artery disease, atherosclerosis, and vascular calcification in patients. However, genetic and treatment studies in mice suggest that osteoprotegerin may protect against vascular calcification.

**Methods and Results**—To test whether osteoprotegerin induces or prevents vascular disease, we treated atherogenic diet–fed \textit{ldlr}^{−/−} mice with recombinant osteoprotegerin (Fc-OPG) or vehicle for 5 months. Vehicle-treated mice developed significant, progressive atherosclerosis with increased plasma osteoprotegerin levels, consistent with observational studies, and \(\approx 15\%\) of these atherosclerotic lesions developed calcified cartilage-like metaplasia. Treatment with Fc-OPG significantly reduced the calcified lesion area without affecting atherosclerotic lesion size or number, vascular cytokines, or plasma cholesterol levels. Treatment also significantly reduced tissue levels of aortic osteocalcin, a marker of mineralization.

**Conclusions**—These data support a role for osteoprotegerin in the vasculature as an inhibitor of calcification and a marker, rather than a mediator, of atherosclerosis. (Circulation. 2008;117:411-420.)

Key Words: atherosclerosis \(\Rightarrow\) calcification \(\Rightarrow\) osteoprotegerin \(\Rightarrow\) RANK ligand

Vascular calcification contributes to morbidity and mortality in patients with advanced atherosclerosis, diabetes mellitus, and renal disease and is associated with reduced elasticity, increased cardiac work, raised blood pressure, and increased cardiovascular events.\(^1\) In atherosclerosis, calcium hydroxyapatite deposits colocalize with intimal plaque, and in renal disease, these deposits are localized predominantly to the medial arterial layer.\(^2\) Specific forms of vascular calcification include atherosclerotic calcification and calcific vasculopathy and share many cellular, molecular, and structural features with bone and bone formation. Fully formed bone has been noted in \(\approx 15\%\) of calcified carotid artery lesions,\(^3\) \(15\%\) of calcific aortic valves,\(^4\) and \(60\%\) of restenotic aortic valves.\(^5\) Regardless of morphology and location, the pathogenesis of vascular calcification appears to be driven by factors that regulate bone metabolism, including transcription factors (Msx2, Runx2, Osterix),\(^6\) bone morphogenic proteins,\(^7\) osteopontin, matrix \(\gamma\)-carboxyglutamic acid protein,\(^8\) and osteoprotegerin (OPG).\(^9\)

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OPG critically regulates bone metabolism and, together with other tumor necrosis factor–related family members, receptor activator of nuclear factor-κB ligand (RANKL), and its receptor RANK, has been implicated in both normal homeostatic and pathological control of bone resorption.\(^10\) OPG is a decoy receptor for RANKL, and the latter has been shown to induce, through its interaction with RANK, differentiation and activation of osteoclasts.\(^11\) OPG specifically inhibits osteoclastic bone resorption by interfering with RANKL binding to RANK.\(^10\) The importance of OPG as a regulator of bone metabolism is underscored by the osteoporotic phenotype of the OPG-null mouse.

Recently, several lines of evidence have supported a role for OPG in the vasculature. Recent observational studies have shown a positive correlation between endogenous serum OPG levels and the presence and severity of clinical coronary artery disease,\(^12\) stroke,\(^13\) cardiovascular morbidity\(^14\) and mortality,\(^15\) and the progression of atherosclerosis.\(^16\) Further evidence suggests that circulating OPG levels also are associated with the extent of vascular calcification.\(^17\) Additionally, in an animal model of atherosclerosis, Shao and colleagues\(^18\) have shown increased OPG serum levels in \textit{ldlr}^{−/−} mice on a Western diet. Taken together, these studies raise 3 possibilities: that vascular disease and OPG are causally related, that OPG is a marker for atherosclerosis, or that they share a common etiologic factor.
In contrast, animal studies have suggested a beneficial effect of OPG on vascular calcification. OPG expression is reduced in calcified vascular lesions,19,20 and Bennett and colleagues21 recently showed that OPG inactivation accelerates vascular calcification in apolE−/− mice. Furthermore, the OPG-null mouse develops medial calcification of the aorta and renal arteries.9 OPG treatment also prevents the vascular calcification induced by 1,25-dihydroxyvitamin D and warfarin in rodents.22 However, it is not known whether OPG treatment affects atherosclerotic calcification.

We hypothesized that OPG inhibits atherosclerotic calcification and that serum OPG levels do not increase the risk of atherosclerosis. To test this hypothesis, ldlr−/− mice were fed an atherogenic diet and treated with recombinant OPG (Fc-OPG) or vehicle. Results showed that Fc-OPG reduced vascular calcification without affecting atherosclerotic lesion number or size, supporting the view of OPG as an inhibitor of vascular calcification and a compensatory response to atherosclerosis. These findings have important clinical implications with respect to whether high serum OPG levels represent a risk factor for atherosclerosis.

Methods

Animal and Experimental Procedures

Eighty male ldlr−/− mice (age, 8 weeks) were fed an atherogenic high-fat diet (15.8% fat wt/wt, 1.25% cholesterol, 0.5% sodium cholate; Harlan Teklad, Madison, Wis), and at the same time, half were started on treatment with human Fc-OPG (10 mg/kg, Amgen Inc, Thousand Oaks, Calif) or vehicle (PBS, Gibco/Invitrogen, Carlsbad, Calif) injected subcutaneously 3 times per week for 2 or 5 months. Blood plasma was collected at baseline and at 2-week, 1-month, 2-month, and 5-month time points for cholesterol and biomarker analysis. At 2 months, 20 mice (10 control, 10 treated with Fc-OPG) were necropsied with no loss. At 5 months, the remaining mice were fed an atherogenic diet and treated with recombinant OPG (Fc-OPG) or vehicle. Results showed that Fc-OPG reduced vascular calcification without affecting atherosclerotic lesion number or size, supporting the view of OPG as an inhibitor of vascular calcification and a compensatory response to atherosclerosis. These findings have important clinical implications with respect to whether high serum OPG levels represent a risk factor for atherosclerosis.

Vascular Mineralization and Atherosclerosis

Three longitudinal sections of the thoracic aorta, including the aortic arch and ascending and descending aortic regions (≈50 mm apart), were stained with hematoxylin and eosin to assess atherosclerosis. Adjacent sections were stained for mineral with the von Kossa silver nitrate method. Image analysis software (Osteomeasure, Osteometrics, Decatur, Ga) was used to determine atherosclerotic lesion number and area, each normalized to the length of the media, and to determine the total number and area of calcified cartilage-like lesions.

Quantitative Polymerase Chain Reaction

Total RNA was isolated from the abdominal aortas of vehicle-treated ldlr−/− mice (on atherogenic diet) using the Trizol chloroform method, and cDNA synthesis was performed on 2.5 μg DNasel-treated RNA in duplicate. Quantitative polymerase chain reaction (PCR) was performed with the LightCycler 2.0 system (Roche Applied Science, Indianapolis, Ind) using primers specific for OPG (3′, GGAGAGCTGAAATTCTGTTGGA; 5′, GAAGACCCATCTGGACATTTT) and RANKL (3′, ATITGCCACCTCACCACATCA; 5′, TGGTACCAAGGCAGCAAGTGA), in conjunction with Universal Probes 21 and 89, respectively, and Hybridization Probe Master Mix. OPG and RANKL expression was normalized to β-actin (3′, GACAGACCTGTGTTGCCCATAGA; 5′, CATCCTCTTCCTCTTGAG) expression determined with SYBR Green Master Mix. The Trizol reagent was purchased from Invitrogen (Carlsbad, Calif), Omniscript reverse-transcription reagents from Qiagen (Valencia, Calif), DNaseI from Universal Probes, and PCR master mixes from Roche Applied Science.

Immunohistochemistry

To determine the cellular localization of RANKL aortic expression in ldlr−/− mice on an atherogenic diet, serial sections of heart tissue, including the aortic root and tricuspid valve, were immunostained with antibodies specific for RANKL (R&D Systems, Minneapolis, Minn), F4/80 (macrophages; Serotec Inc, Raleigh, NC), and CD3 (T cells; Laboratory Vision, Fremont, Calif). Briefly, deparaffinized and hydrated 5-μm tissue sections were pretreated with Antigen Retrieval Citra for RANKL (BioGenex, San Ramon, Calif), with BORG for CD3 (Biocare, Concord, Calif), or with 0.1% trypsin for F4/80 (Sigma, St Louis, Mo) and then incubated with primary antibody. RANKL was detected by biotinylated donkey anti-goat (Jackson Laboratory, West Grove, Pa); CD3 was detected by MACH2 horseradish peroxidase (Biocare); and F4/80 was detected by biotinylated rabbit anti-rat antibody (Vector Laboratories, Burlingame, Calif). Slides were quenched with 3% hydrogen peroxide (quenching for CD3 was done before the detection), followed by avidin-biotin treatment (Vector Laboratories). Reaction sites were visualized with diaminobenzidine tetrachloride (Dako Corp, Glostrup, Denmark) and counterstained with hematoxylin.

Biomarker Analysis

Blood plasma (EDTA) was collected from control and Fc-OPG-treated ldlr−/− mice (on atherogenic diet) at baseline and 2-week, 1-month, 2-month, and 5-month time points for analysis of circulating total cholesterol, OPG, RANKL, and osteocalcin levels. Tissue cytokine and osteocalcin levels were determined in protein extracts (50 mmol/L Tris buffer, pH 7.4, containing 0.1 mol/L sodium chloride and 0.1% Triton X-100) from the distal half of the abdominal aorta collected at the 2- and 5-month time points and then normalized to total tissue protein levels (BCA [bicinchoninic acid] Protein Assay, Pierce Co, Rockford, Ill). Total cholesterol levels were determined with a Hitachi 717 autoanalyzer (Roche Diagnostics); OPG and RANKL levels were determined by ELISA with commercially available kits (R&D Systems); and cytokine (22-plex) and osteocalcin levels were determined with Luminex bead–based multiplex assays (Linco Research, St Charles, Mo).

Statistical Analysis

Data represent mean±SD. For the analysis of the plasma data, mixed-models ANOVA was used so that all available data were incorporated into each analysis. For measurements made only at a single time (eg, lesion measurements at 5 months), comparisons between the Fc-OPG and control groups were made with a 2-tailed Student t test. Associations were tested with the Spearman rank-correlation method. Unless noted otherwise, results of group comparisons were confirmed by commercial resampling simulation software (Resampling Stats [www.resample.com], Arlington, Va), which assesses the probability that the observed differences between group means occurred by chance.23 For comparing incidence of calcified lesions, we used the resampling approach to Fisher’s exact test. A value of P<0.05 was used to determine statistical significance between groups. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Plasma Levels of OPG and RANKL in ldlr−/− Mice on Atherogenic Diet

The effect of an atherogenic diet on endogenous OPG and RANKL was assessed with plasma samples from vehicle-treated
ldlr(−/−) mice. Within 2 weeks of placing the mice on an atherogenic diet, plasma OPG levels were significantly elevated (3.8±0.7 ng/mL) compared with baseline (1.9±0.5 ng/mL) and reached a maximum 2.4-fold elevation at 1 month (4.5±1.1 ng/mL). Over the next 4 months, OPG levels did not increase further; however, at all times points, OPG levels remained significantly elevated compared with baseline (all P<0.01; Figure 1A).

In contrast to increased plasma OPG levels, plasma RANKL levels decreased significantly in ldlr(−/−) mice on an atherogenic diet. Two weeks after the initiation of the diet, RANKL levels were reduced by 3-fold (0.07±0.05 ng/mL) compared with baseline (0.22±0.04 ng/mL) and at 1 month were maximally suppressed by ∼15-fold (0.01±0.01 ng/mL; both P<0.001). Thereafter, RANKL plasma levels increased progressively with time on the atherogenic diet and were significantly increased at 5 months compared with the 1-month level (P<0.001); however, the 5-month levels were less than baseline (P<0.01; Figure 1B).

Expression of mRNA for OPG and RANKL in Vascular Tissue in ldlr(−/−) Mice on Atherogenic Diet
To determine whether vascular expression of OPG and RANKL correspond with plasma levels, total RNA was isolated from the abdominal aorta of vehicle-treated ldlr(−/−) mice fed an atherogenic diet for 2 and 5 months and analyzed by quantitative real-time PCR. Results showed that from 2 months to the 5-month time point, no change occurred in OPG message levels, whereas RANKL expression increased (0.48±0.18 versus 0.65±0.15 relative units; P<0.05).

We quantitatively assessed the aortic atherosclerotic lesions to determine whether a correlation was present between OPG levels and atherosclerosis or between RANKL levels and atherosclerosis, with all mice from both time points included. OPG plasma levels and aortic expression were not significantly associated with atherosclerotic lesion number (P=0.64; Figure 2A; and P=0.12; Figure 2B), area (P=0.17

Figure 1. Plasma OPG and RANKL in ldlr(−/−) mice fed an atherogenic diet over 5 months. Endogenous OPG (A) and RANKL (B) were assessed by ELISA. OPG levels increased early and remained elevated, whereas RANKL levels dropped markedly and then increased with disease progression. Values are mean±SD, *P<0.05 vs values at study onset.
and $P=0.41$), or size ($P=0.08$ and $P=0.13$). Unexpectedly, RANKL plasma levels correlated with atherosclerotic lesion number; both increased with the progression of vascular disease ($P<0.03$; Figure 2C). However, these circulating levels did not correlate with either atherosclerotic lesion area or lesion size. Similarly, RANKL message levels in the aorta were positively associated with atherosclerotic lesion number ($P<0.04$; Figure 2D), lesion area ($P<0.004$), and lesion size ($P<0.007$).

**Localization of RANKL in the Aortic Root of ldlr$^{−/−}$ Mice on Atherogenic Diet**

Results showed RANKL immunoreactivity primarily in clusters of neointimal cells, the endothelium, and the adventitia (Figure 3D, 3G, and 3J, respectively). In the intimal plaque, RANKL-positive staining was present in clusters of hypertrophic chondrocyte-like cells (Figure 3D) that were F4/80 positive (Figure 3E) and juxtaposed to calcified cartilage-like metaplasia (Figure 3B and 3C). The endothelial RANKL-positive staining (Figure 3G) was negative for both F4/80 and CD3 (Figure 3H and 3I, respectively). In the adventitia, RANKL-positive staining (Figure 3J) was associated with CD3-positive cells (Figure 3L), which were F4/80 negative (Figure 3K).

**Effect of Fc-OPG on Vascular Calcification**

To determine the effect of Fc-OPG on the calcification in experimental atherosclerosis lesions, we analyzed aortic sections from ldlr$^{−/−}$ mice fed an atherogenic diet and treated with Fc-OPG or vehicle for 5 months. Aortic sections stained for mineral by the von Kossa method revealed 2 distinct patterns of calcification: calcified cartilage-like metaplasia, which was found in ~1 of 6 atherosclerotic lesions, and small amorphic punctuate lesions. As shown in Figure 4 and the Table, Fc-OPG treatment reduced the number and area of the cartilage-like lesions compared with vehicle-treated mice by 93% ($P=0.009$) and 92% ($P=0.036$), respectively. Additionally, the incidence of all calcified lesions (including amorphous lesions) was reduced by 56% with Fc-OPG treatment compared with vehicle treatment ($P=0.046$; the Table).

The ability of Fc-OPG to suppress bone turnover in this study was confirmed by its ability to significantly suppress plasma TRAP-5b (tartrate resistant acid phosphatase-5b), an osteoclast-specific marker of bone resorption, in atherogenic diet–fed ldlr$^{−/−}$ mice compared with vehicle-treated mice ($P<0.003$ at 5 months; Table). Because an increase in calcium-phosphate product is known to promote metastatic vascular calcification, we assessed whether reduced bone resorption by Fc-OPG affects plasma calcium and phosphate levels at 5 months. Results showed no significant difference with Fc-OPG treatment versus vehicle treatment for calcium or phosphate at 5 months.

To assess whether Fc-OPG affects osteogenic differentiation in vascular tissue, we determined aortic osteocalcin levels. In vehicle-treated ldlr$^{−/−}$ mice fed an atherogenic diet, aortic osteocalcin mRNA levels were unchanged (data

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**Figure 2.** Correlation of OPG and RANKL levels with atherosclerosis in ldlr$^{−/−}$ mice on an atherogenic diet. Atherosclerotic lesion number was not significantly associated with OPG plasma (A) or mRNA (C) levels. However, significant positive correlations were noted for both RANKL plasma (B) and mRNA (D) levels. Spearman rank-order correlation, $P<0.05$. 

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not shown); however, protein levels increased 22-fold from 2 to 5 months (1.3 ± 0.7 to 29.3 ± 24.9 ng/mL). At both the 2- and 5-month time points, Fc-OPG treatment reduced aortic osteocalcin levels by 38% and 75%, respectively, compared with vehicle-treated mice (P = 0.03 at 2 months and P = 0.025 at 5 months; Figure 5A and 5B and the Table).

**Effects of Fc-OPG Treatment on Atherosclerosis**

To determine whether the effect of Fc-OPG on vascular calcification was related to an effect on cholesterol metabolism or atherosclerosis, we examined the effects of Fc-OPG on plasma cholesterol, vascular inflammatory cytokines, and extent of atherosclerosis. Cholesterol levels were
markedly elevated after the initiation of the atherogenic diet, increasing \( \approx 10 \)-fold, but were not significantly altered by treatment with Fc-OPG (Figure 6A). As a measure of the vascular inflammation associated with atherosclerosis, we determined tissue cytokine levels from the aorta. Interleukin-1\( \beta \), tumor necrosis factor-\( \alpha \), keratinocyte-derived cytokine (KC), interleukin-6, and monocyte chemoattractant protein-1 protein levels were increased in aortic tissue after 5 months of atherogenic diet but were not altered by Fc-OPG treatment (Figure 6B).

We next quantified the number, area, and size of the atherosclerotic lesions in the descending thoracic aorta. As shown in Figure 7 and the Table, both lesion number and area increased from 2 to 5 months and were unaffected by Fc-OPG treatment compared with vehicle-treated mice at both time points, although nonsignificant reductions in both parameters were noted.

**Discussion**

Recently, it has been suggested that OPG plays a role in atherosclerosis and calcific vasculopathy. Although observational studies show a positive relationship between serum OPG levels and clinical cardiovascular disease, the relationship is unlikely to be causal given that animal studies support a protective role for OPG. Because the role of OPG in the vasculature is unclear, we conducted studies in atherogenic diet–fed \( \text{ldlr}^{-/-} \) mice to determine whether induction of atherosclerosis affects plasma OPG levels and whether treatment with Fc-OPG affects diet-induced atherosclerosis and vascular calcification. Results showed that plasma OPG levels increased with initiation of an atherogenic diet, yet exogenous Fc-OPG treatment reduced vascular calcification. Interestingly, Fc-OPG treatment did not alter the progression or severity of atherosclerosis in these mice. These findings suggest that endogenous OPG may be a marker, rather than a mediator, of atherosclerosis and that exogenous Fc-OPG treatment may limit vascular calcification. These findings also suggest that the processes of atherosclerosis and vascular calcification can be uncoupled.

Plasma OPG elevation appears to be a marker of atherosclerosis onset rather than its severity or progression. Plasma OPG levels increased in mice within 2 weeks of starting the atherogenic diet and remained at the same high level 1 and 5 months later, despite the continued progression of atherosclerosis during that time. These findings are consistent with previous observational studies showing higher OPG levels in patients with cardiovascular disease.\(^{12,16}\) However, in contrast to these studies, we did not find a significant correlation...
between OPG levels and atherosclerotic lesion number, size, or area.

Although plasma RANKL levels decreased initially with the atherogenic diet, they increased after 2 months and were significantly associated with the number and severity of atherosclerotic lesions. This relationship may reflect an increase in both variables with time. The initial increases in circulating OPG may be a compensatory response to the increased RANKL levels. However, this is not likely given that the increase in OPG levels precedes that of RANKL.

The tissue source of the plasma OPG and RANKL in the present study, as in humans, is unclear. OPG is highly expressed in both bone and vasculature; RANKL is expressed predominantly in bone and myelomonocytic cells but rarely detected in the normal vasculature. Surprisingly, we found that aortic RANKL mRNA, but not OPG mRNA, increased with the progression of atherosclerosis from 2 to 5 months and that this pattern of expression was consistent with the changes in OPG and RANKL protein levels found in the circulation. RANKL has been associated with calcified vascular lesions and may be upregulated in vascular cells under pathological conditions. RANKL also might derive from immune cells in the increasing atheromatous lesions. Both vascular and immune cells may contribute to the increase in plasma RANKL levels as atherosclerosis progresses. Our data suggest that RANKL expression in the artery wall derives from the endothelium and adventitia, as well as from calcified and noncalcified ectopic cartilage within atherosclerotic lesions. Chondrocytes and calcified cartilage-like metaplasia are known to be present in mouse atherosclerotic lesions, and hypertrophic chondrocytes have been shown to express RANKL mRNA and protein. The positive staining for F4/80 of these same cells raises the question of whether they might be chondroclasts because immature osteoclasts also appear to express RANKL in culture; however, the morphology is more consistent with that of hypertrophic chondrocytes. These results are consistent with prior findings of RANKL expression in endothelial cells and atherosclerotic lesions, especially near areas of calcification. Additionally, we found RANKL immunoreactivity associated with CD3-positive T cells in the perivascular medial-adventitial layer. The increase in both plasma RANKL and aortic mRNA with the progression of atherosclerosis in this study is consistent with an increase in the size of the atherosclerotic lesions, the expansion of the associated endothelial cells, and the development of calcified cartilage-like vascular lesions.

The tissue source of the plasma OPG in the present study also is unclear. The early elevation in plasma OPG levels and lack of a progressive increase with disease suggest that the increase in plasma OPG may be a response to the initial vessel insult, not to the progression of atherosclerosis. Indeed, recent evidence suggests that damaged endothelial cells may release OPG from Weibel-Palade bodies in response to inflammation, thus increasing circulating levels. Although vascular endothelial cells may be the source of the circulating OPG with the onset of atheroscle-

### Table. Effects of Fc-OPG on Calcification-Related Parameters After 5 Months of Atherogenic Diet and Concurrent Treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Fc-OPG</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcified lesions, mean lesion, no./mouse</td>
<td>1.06±1.34</td>
<td>0.07±0.27</td>
<td>0.009</td>
</tr>
<tr>
<td>Area calcified, µm²</td>
<td>14 796±24 486</td>
<td>1084±4055</td>
<td>0.036</td>
</tr>
<tr>
<td>Incidence of calcification</td>
<td>11/17</td>
<td>4/14</td>
<td>0.046</td>
</tr>
<tr>
<td>TRAP-5b, U/L</td>
<td>9.4±5.1</td>
<td>4.9±1.7</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Serum calcium, mg/dL</td>
<td>9.5±1.0</td>
<td>9.6±1.6</td>
<td>NS</td>
</tr>
<tr>
<td>Serum phosphate, mg/dL</td>
<td>6.4±1.0</td>
<td>6.9±2.4</td>
<td>NS</td>
</tr>
<tr>
<td>Aortic osteocalcin, ng/mg total protein</td>
<td>29.3±24.8</td>
<td>7.5±10.5</td>
<td>0.025</td>
</tr>
<tr>
<td>Atherosclerotic lesion area, mm²/mm media length</td>
<td>0.065±0.04</td>
<td>0.060±0.04</td>
<td>NS</td>
</tr>
<tr>
<td>Atherosclerotic lesions, n/mm media length</td>
<td>0.235±0.10</td>
<td>0.231±0.13</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS indicates not significant; TRAP-5b, tartrate resistant acid phosphatase-5b.

### Figure 5. Aortic tissue osteocalcin levels. Osteocalcin protein in the aortic tissue was assessed by Luminex bead-based multiplex assays. Osteocalcin protein increased in both vehicle and Fc-OPG treated mice from 2 (A) to 5 (B) months (†P<0.05). However, compared with vehicle, Fc-OPG treated mice had significantly lower vascular osteocalcin protein levels at both time points (*P<0.05). Values are mean±SD.
rosis, it is not clear whether increased expression is required for the changes in circulating levels.

Importantly, our results demonstrate that OPG treatment attenuated the calcification associated with advanced atherosclerotic lesions, suggesting that OPG might play a protective role in that process. In support of this finding, we noted that Fc-OPG treatment was associated with significant reductions in aortic tissue protein, although not mRNA, levels of osteocalcin, an established marker of osteogenic differentiation and osteoblastic activity. Because we were unable to demonstrate a change in mRNA expression, the osteocalcin protein levels in the vascular tissue may reflect an accumulation in the mineralized matrix components or deposition from the serum. Nevertheless, the reduction in osteocalcin within the diseased vessels of Fc-OPG–treated mice is consistent with the observed reduction in vascular calcification.

Calcification typically follows the development of atherosclerotic lesions, and treatments that reduce atherosclerosis also prevent lesion mineralization. We evaluated plasma cholesterol levels, aortic cytokines, and atherosclerotic lesion severity to determine whether recombinant Fc-OPG treatment affected atherosclerosis. We found that each of these indexes increased with disease progression in vehicle-treated \emph{ldlr} \(-/-\) mice but were unaffected by Fc-OPG treatment. We also measured human Fc-OPG concentrations in the treated animals of this study to determine whether more marked increases in serum OPG might be associated with greater disease progression. The increase in endogenous plasma OPG that was associated with disease progression in vehicle-treated animals was comparatively modest (135% over baseline); however, treatment with Fc-OPG further increased total circulating OPG levels \(-10^5\)-fold (endogenous levels of 3 to 4 ng/mL with atherogenic diet in vehicle-treated mice versus 300 to 400 \(\mu\)g/mL with Fc-OPG treatment; data not shown). This high level of exposure to human Fc-OPG had no effect on the number or area of atherosclerotic lesions, which is consistent with the notion that RANKL inhibition per se does not exacerbate atherosclerosis.

Because OPG is a known inhibitor of bone resorption, it is possible that altered mineral metabolism causes the observed decrease in atherosclerotic calcification. An increase in the circulating calcium-phosphate product has been associated with ectopic mineralization, as in metastatic calcification, and recombinant OPG treatment has been shown to block this process.\textsuperscript{22} However, even in the context of significantly reduced bone resorption in Fc-OPG–treated \emph{ldlr} \(-/-\) mice, calcium and phosphate levels were in the normal range.
Nevertheless, it is possible that minimal but prolonged elevations in serum calcium and/or phosphate may directly or indirectly affect vascular calcification, as suggested by Price et al.22,36 Alternatively, it is possible that ligands for OPG such as RANKL and tumor necrosis factor-α–related apoptosis-inducing ligand (TRAIL) may play a role in vascular calcification. RANKL, for example, has been shown by Kaden and colleagues19 to induce alkaline phosphatase activity and calcification in vascular cells, and our present report shows increased RANKL expression associated with atherosclerotic lesions and the progression of vascular disease. Additionally, the effects of OPG may be mediated through the inhibition of TRAIL, which is expressed in the artery wall along with its receptor.27,38 OPG may inhibit atherosclerotic calcification by blocking endothelial cell apoptosis induced by TRAIL, thus reducing the number of apoptotic bodies that may serve as nucleation sites for mineralization.19 However, the physiological or pathological role of TRAIL in vivo is unclear.

Some of the observed changes over the 5-month time frame of the study may be attributable to normal aging. However, the rapid changes in plasma OPG seen with the onset of the atherogenic diet in this study suggest that atherogenesis affects OPG levels, and our findings recapitulate many of the changes associated with vascular disease in humans, including an early increase in plasma OPG and the development of atherosclerotic lesions that calcify. The results further showed that systemic long-term RANKL inhibition via Fc-OPG treatment did not alter the histological or biochemical progression of atherosclerosis but reduced vascular calcification in this animal model. The ability of OPG to suppress vascular calcification in these studies is consistent with previous observations22,28 and supports a recent report by Bennett et al.21 showing that OPG inactivation resulted in increased vascular calcification in apoE−/− mice. OPG would not be the first identified factor that is both protective and upregulated with heart disease. For example, serum levels of atrial natriuretic peptide increase with congestive heart failure, but the recombinant peptide is useful as therapy for heart failure.40 Similarly, OPG may be upregulated as an incomplete compensatory response to vessel insult, possibly limiting vascular calcification. Taken together, these data suggest that OPG is induced by atherosclerosis. At the same time, the inhibition of vascular calcification by Fc-OPG suggests that OPG may directly block calcification of lesions, independently of atherosclerosis development or progression, and implicates distinct mechanisms leading to the calcification of these lesions.

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Disclosures

Dr Morony, Dr Cattley, G. Van, D. Dwyer, Dr Stolina, and Dr Kostenuik are employees and stockholders of Amgen Inc. The other authors report no conflicts.

References

Several observational studies show that serum osteoprotegerin levels correlate positively with the severity and progression of coronary artery disease, atherosclerosis, and vascular calcification in patients. However, animal studies suggest that osteoprotegerin may protect against vascular calcification (eg, mice deficient in osteoprotegerin develop aortic calcification). To address this paradox, atherosclerotic ldlc/−/− mice were fed an atherogenic diet and treated with osteoprotegerin or vehicle. Although serum osteoprotegerin levels increased with initiation of the atherogenic diet (before treatment), exogenous recombinant osteoprotegerin treatment significantly reduced the calcified lesion area and level of the osteogenic marker osteocalcin in the aorta without a significant change in atherosclerosis or cholesterol level. These data support a role for osteoprotegerin in the vasculature as an inhibitor of calcification and a marker, rather than a mediator, of atherosclerosis.
Osteoprotegerin Inhibits Vascular Calcification Without Affecting Atherosclerosis in ldlr(−/−) Mice

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