Osteoprotegerin Inactivation Accelerates Advanced Atherosclerotic Lesion Progression and Calcification in Older ApoE<i>k</i> Mice

Brian J. Bennett, Marta Scatena, Elizabeth A. Kirk, Marcello Rattazzi, Rebecca M. Varon, Michelle Averill, Stephen M. Schwartz, Cecilia M. Giachelli, Michael E. Rosenfeld

Objective—Osteoprotegerin (OPG), a member of the tumor necrosis factor (TNF) superfamily of proteins, plays an important role in bone remodeling and is expressed in both mouse and human atherosclerotic lesions. The current study was designed to assess whether OPG plays a role in the progression and calcification of advanced atherosclerotic lesions in apoE<i>k</i> mice.

Methods and Results—Atherosclerotic lesion area and composition and aortic calcium content were examined in mice deficient in both OPG and apolipoprotein E (OPG<i>−/−</i>.apoE<i>−/−</i> mice) at 20, 40, and 60 weeks of age. Littermate OPG<i>+/+</i>.apoE<i>−/−</i> mice were used as controls. The average cross-sectional area of lesions in the innominate arteries was increased in OPG<i>−/−</i>.apoE<i>−/−</i> mice at 40 and 60 weeks of age. The increase in lesion area was coupled with a reduced cellularity and an increase in connective tissue including laminated layers of elastin. Sixty-week-old OPG<i>−/−</i>.apoE<i>−/−</i> mice also had an increase in the area of calcification of the lesions. There were no differences in markers of plaque stability. In vitro, OPG induced matrix metalloproteinase-9 (MMP-9) activity in macrophages and smooth muscle cells and acted as a survival factor for serum-deprived smooth muscle cells.

Conclusion—OPG inhibits advanced plaque progression by preventing an increase in lesion size and lesion calcification. OPG may act as a survival factor and may modulate MMP9 production in vascular cells. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: apolipoprotein E-deficient mice ■ atherosclerosis ■ osteoprotegerin ■ vascular calcification

Calcification of advanced atherosclerotic lesions has been well-documented. There is accumulating evidence that atherosclerosis-associated calcification is an active, cell-regulated process. The presence of ossified bone within plaques and the expression of osteogenic cell markers have been previously reported. Most of these proteins are known to be important in bone remodeling and are expressed in both mouse and human atherosclerotic lesions. These proteins such as osteoprotegerin (OPG), matrix-gla protein, osteopontin, and pyrophosphatase have been identified as inhibitors of vascular calcification.

OPG is a critical modulator of bone resorption through its action as a decoy receptor for the receptor activator of NFκB ligand (RANKL). OPG is able to block interaction of RANKL with its receptor RANK, thereby inhibiting osteoclast differentiation. Furthermore, OPG has been shown to have a role in modulating adaptive immunity. OPG-deficient mice develop severe osteoporosis. In one report in which the OPG-deficient mice were of a mixed genetic background, the mice also developed severe medial calcification of the blood vessels. Because of these immunologic and bone regulatory properties, OPG has been considered as a potential regulator of atherosclerotic lesion development and calcification. To determine whether OPG plays a role in the calcification and chondrocyte metaplasia that has been reported in advanced atherosclerotic lesions in mice, we generated mice deficient in both OPG and apoE (OPG<i>−/−</i>.apoE<i>−/−</i>) and have compared them to wild-type controls (OPG<i>+/+</i>.apoE<i>−/−</i>). We reasoned that when crossed with apoE<i>−/−</i> mice that develop highly calcified lesions in the innominate arteries, calcification of the intima would be more rapid and extensive. The data support this hypothesis as we report here that the loss of OPG leads to larger atherosclerotic lesions in the innominate arteries at 40 and 60 weeks of age coupled with more rapid and extensive calcification of both the media and intima.
Methods

Animals
Double-knockout OPG−/−.apoE−/− mice were generated by mating OPG−/− mice (a generous gift of Edward Clark, University of Washington, Seattle, Wash), with apoE−/− mice. Both strains were on a C57Bl/6 genetic background. Genotypes were confirmed by polymerase chain reaction (PCR), and all experiments were performed using male and female mice from generations F4 to F7, with littermate OPG+/−.apoE−/− mice serving as controls. Mice were maintained in a temperature controlled room on a 12-hour light cycle. All animals were fed a standard rodent diet containing 4% fat for the duration of the study. At 20, 40, and 60 weeks of age, blood was collected via the retro-orbital sinus into tubes containing EDTA, and the animals were euthanized by pharmacological overdose with ketamine/xylazine. The animals were then perfusion-fixed with 10% buffered formalin via the left ventricle for 4 minutes. The entire innominate.brachiocephalic artery from each animal was dissected out, embedded in paraffin, and serially sectioned (5 μm). All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Morphometry
Every fifth section was stained with a modified Movat’s penta-chrome stain. To identify vascular calcification, adjacent sections were stained with the von Kossa stain for calcium phosphate mineral which in the artery wall is known to form hydroxyapatite.19 The lesion area, total cell count per lesion, total number of chondrocyte-like cells, and the area of calcium deposition were determined in each stained section using computer assisted image analysis (Image Pro; Media Cybernetics, Silver Spring, Md). Plaque composition and stability were evaluated by assessing the frequency of the following features: thickness of the fibrous cap (thin fibrous cap was defined as occupying ≤3 cell layers), size of the necrotic core (a large necrotic core was defined as occupying >50% of the volume of the plaque), intraplaque hemorrhage (defined as the presence of red blood cells), medial erosion (defined as the replacement of the normal media by foam cells), presence of laminated elastic fibers, presence of chondrocyte-like cells, and lateral xanthomas (defined as the presence of aggregates of macrophage-derived foam cells situated on the lateral margins of the plaques). These were recorded as binary outcomes, and the frequency for each animal was determined.

Aortic Calcium Content
Aortic calcium content was assessed as previously described.10 Aortas were lyophilized and decalcified with 0.6 mmol/L HCl at 37°C for 48 hours. Calcium released from the lyophilized tissues was determined colorimetrically by the o-cresolphthalein complex one method (calci-diagnostics kit; Tecno Diagnostic, Analheim, Calif).

Plasma OPG and RANKL
Plasma OPG and RANKL levels were evaluated using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, Minn).

Plasma Cholesterol
Total plasma and high-density lipoprotein (HDL) cholesterol concentrations were measured using a commercially available cholesterol oxidase enzymatic kit. (Diagnostic Chemicals Limited, Oxford, Conn). HDL cholesterol concentration was determined after precipitation of very-low-density lipoprotein/low-density lipoprotein (LDL) by 20% polyethylene glycol.

Smooth Muscle Cell Isolation and Survival Assay
Smooth muscle cells (SMCs) were isolated as previously described by Speer et al. Briefly, aortas were harvested from 4-week old OPG+/−.apoE−/− mice, dissected free of adventitia, and de-endothelialized mechanically with a Teflon cell scraper. The aortic medias from 4 to 8 mice were pooled, digested with elastase/collagenase, and plated in serum-containing media. The yield was between 50 000 to 100 000 cells per aorta. Cells between passages 3 to 8 were used for these studies. SMCs were plated on tissue culture plates. A day later, cells were treated with 4 nM of OPG (R&D Systems) or vehicle in serum-free medium. The percentage of apoptotic cells was determined by nuclear fragmentation 24, 48, and 72 hours later as described by Pritzker et al.21 Briefly, cells were stained with 4 μg/mL of the nuclear stain Hoechst (Roche) and the number of apoptotic nuclei in 4 fields per triplicate cultures was determined and averaged.

MMP-9 Expression and Zymography
Bone marrow macrophages were prepared as follows. Bone marrow was harvested from femurs and cell suspensions were plated and cultured for 7 days in the presence of macrophage colony-stimulating factor. The resulting adherent cells were used in subsequent experiments. The bone marrow derived macrophages, RAW 264.1 cells, and SMCs were plated on tissue culture polystyrene dishes. A day later, cells were treated with 4 nM of OPG (R&D Systems) or vehicle in serum-free medium for 24 hours.

Western Blot
Equal volumes of cell supernatant were separated on 10% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (NEN Life Science Products, Boston, Mass) and probed with an anti-mouse MMP-9 antibody (R&D Systems). After washing, the blots were probed with an HRP-conjugated secondary antibody and detected by enhanced chemiluminescence (Analytical Sciences, Boston, Mass).

Zymography
SDS-PAGE containing 1 mg/mL of gelatin was used to assess levels and activity of MMP-2 and MMP-9. Cell supernatant was mixed with 4× loading buffer before loading onto the gel.

Statistical Analysis
In vivo comparisons between OPG−/−.apoE−/− and OPG+/−.apoE−/− mice and in vitro comparisons from cell culture studies were performed by 2-tailed unpaired Student t test. Frequency of medial conversion at 20 weeks was assessed by χ² analysis. For frequency measures and non-normally distributed data, analysis was performed using the Mann-Whitney test. Significance is denoted by P<0.05.

Results

Lesion Area: Innominate Artery
We examined the lesion area in the innominate artery of 20-, 40-, and 60-week-old female and male OPG−/−.apoE−/− and OPG+/−.apoE−/− mice. Data from both sexes showed similar trends: there was no difference between groups at 20 weeks of age in the average area of lesion in the innominate arteries (average of the area from all of the step sections). However, there was a significant increase in average lesion area at 40 and 60 weeks for both males and females (Table 1).

Plaque Composition: Innominate Artery
Next, we determined the frequency of lesions composition between groups. There were no differences in the frequency of markers of plaque stability at 20, 40, and 60 weeks of age (thin fibrous cap, large central necrotic core, intra-plaque hemorrhage; data not shown). However, there was an increased frequency of lesions containing laminated elastic fibers and an increased frequency of lesions with larger areas of collagen and proteoglycan deposition within the plaques of the OPG−/−.apoE−/− mice (Table 2 and Figure 3).
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Area of Plaque Calcification

In one report of OPG−/− mice on a mixed genetic background, medial calcification occurred in the aorta and renal artery of one-third of the mice. In this study, 38% of 20-week-old OPG−/− apoE−/− mice exhibited calcification in the medial layer of the innominate artery as determined by Von Kossa staining, whereas control mice were totally devoid of medial calcification (Figure 1; $\chi^2$ analysis, 0.039). There was no intimal calcification at 20 weeks of age in either genotype. At 40 weeks of age there was a nonsignificant increase in average calcified area in the intimal layer of OPG−/− apoE−/− compared with OPG−/− apoE−/− mice (15 559 μm² in the OPG−/− apoE−/− mice versus 8 693 μm² for controls; $P=0.26$). At 60 weeks of age the increase in intimal calcified area was significantly greater in OPG−/− apoE−/− compared with OPG−/− apoE−/− mice (68 243 μm² for OPG−/− apoE−/− versus 33 161 μm² for controls; $P<0.04$; Figure 3). When expressed as percent of total lesion area, the calcified lesion area was significantly greater in OPG−/− apoE−/− mice at 60 weeks of age (24±4% for OPG−/− apoE−/− versus 14±2% for controls; $P<0.05$).

Aortic Calcium Content

The amount of extractable calcium that was deposited in the whole aorta was significantly greater in OPG−/− apoE−/− mice at 40 and 60 weeks of age compared with OPG+/+apoE−/− mice (Table 1). The calcium content of the aorta of 20-week-old male OPG−/− apoE E−/− mice was 4-fold greater than the aortic content of 20-week-old OPG+/+ apoE−/− mice.

Plaque Cellularity

We have previously reported that lesion cellularity declines concomitantly with increased deposition of hydroxyapatite in very advanced lesions in the innominate arteries of the apoE−/− mice. In the present study, we observed 40% additional reduction in total cellularity of the lesions of the OPG−/− apoE−/− mice as compared with the OPG+/+ apoE−/− mice at both 40 and 60 weeks of age ($P<0.02$ at 40 weeks,

### TABLE 1. Aortic Calcium Content and Average Lesion Area in the Innominate Arteries

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>20</th>
<th>40</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic calcium content mmol/mg dry weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG−/− apoE−/−</td>
<td>0.28±0.17 (3)</td>
<td>1.42±2.13 (6)</td>
<td>6.24±2.17* (5)</td>
</tr>
<tr>
<td>OPG+/− apoE−/−</td>
<td>0.20±0.08 (4)</td>
<td>0.39±0.27 (8)</td>
<td>0.55±0.016 (3)</td>
</tr>
<tr>
<td>OPG−/− apo E−/−</td>
<td>0.80±0.35* (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average lesion area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG−/− apoE−/−</td>
<td>21 224±14 863 (4)</td>
<td>120 029±57 631* (7)</td>
<td>253 431±30 050* (5)</td>
</tr>
<tr>
<td>OPG+/− apoE−/−</td>
<td>37 294±35 300 (4)</td>
<td>71 418±31 867 (9)</td>
<td>189 660±47 557 (4)</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic calcium content mmol/mg dry weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG−/− apoE−/−</td>
<td>0.49±0.18 (8)</td>
<td>1.04±0.46* (7)</td>
<td>4.36±1.86* (5)</td>
</tr>
<tr>
<td>OPG+/− apoE−/−</td>
<td>0.18±0.11 (7)</td>
<td>0.52±0.31 (9)</td>
<td>2.13±1.71 (9)</td>
</tr>
<tr>
<td>Average lesion area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG−/− apoE−/−</td>
<td>51 275±38 002 (9)</td>
<td>154 502±33 099* (10)</td>
<td>255 591±47 705* (5)</td>
</tr>
<tr>
<td>OPG+/− apoE−/−</td>
<td>33 635±30 723 (8)</td>
<td>123 570±21 723 (9)</td>
<td>210 798±28 051 (11)</td>
</tr>
</tbody>
</table>

All values are mean±SD.

* $P<0.05$, † $P=0.06$, Mann-Whitney test.

Numbers in parenthesis indicate number of mice in each group.

### TABLE 2. Frequency of Matrix-Rich Lesions in the Innominate Arteries

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>20</th>
<th>40</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of collagen-rich lesion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG+/− apoE−/−</td>
<td>33%±27% (13)</td>
<td>57%±32% (17)</td>
<td>80%±12% (10)</td>
</tr>
<tr>
<td>OPG−/− apoE−/−</td>
<td>40%±31% (12)</td>
<td>71%±27%† (18)</td>
<td>84%±9% (15)</td>
</tr>
<tr>
<td>Frequency of proteoglycan-rich lesion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG+/− apoE−/−</td>
<td>36%±27% (13)</td>
<td>57%±31% (17)</td>
<td>80%±12% (10)</td>
</tr>
<tr>
<td>OPG−/− apoE−/−</td>
<td>43%±30% (12)</td>
<td>76%±27%* (18)</td>
<td>84%±9% (15)</td>
</tr>
<tr>
<td>Frequency of laminated elastin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG+/− apoE−/−</td>
<td>16%±31% (13)</td>
<td>29%±38% (17)</td>
<td>40%±43% (10)</td>
</tr>
<tr>
<td>OPG−/− apoE−/−</td>
<td>14%±30% (12)</td>
<td>49%±1043% (18)</td>
<td>84%±29%* (15)</td>
</tr>
</tbody>
</table>

All values mean±SD.

* $P<0.05$.

† $P=0.06$, Mann-Whitney test.

Numbers in parenthesis indicate number of mice in each group.
The total number of chondrocyte-like cells also declined with age; however, the number of chondrocyte-like cells per mm lesion area did not differ between strains at any time point (data not shown).

**OPG as Survival Factor for SMCs**

We have recently determined that in human endothelial cells, OPG prevents cell death in part by inhibiting TRAIL activity. OPG is also a survival factor for arterial SMCs. As shown in Figure 3, OPG treatment inhibited cell death induced by serum-deprivation in aortic SMC from apoE

**OPG Induces MMP-9 Activity**

ApoE

mice deficient in MMP-9 have a marked decrease in aneurysm formation, suggesting that MMP-9 can play a role in regulating the connective tissue content of advanced lesions in the apoE

mice. Because there is a greater frequency of advanced lesions with an increased content of collagen, proteoglycan and laminated elastin in the OPG

mice (Figure 4), it is conceivable that the lack of OPG results in down-regulation of MMP-9 or other matrix degrading enzymes. As shown in Figure 4, OPG treatment potently induced increased levels of MMP-9 in RAW264.1 macrophage-like cells as well as in aortic SMCs from apoE

mice. Furthermore, OPG was a potent inducer of MMP-9 activity in bone marrow-derived macrophages (Figure 4).

**Plasma Cholesterol**

Total plasma cholesterol and HDL cholesterol levels did not differ significantly between OPG

apoE

apoE

mice at any time point (Table 3).

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**Figure 1.** Comparative morphology of advanced lesions in the innominate artery of OPG

apoE

apoE

mice. Light micrographs of representative lesions stained with the Movat's stain from 20-week-old mice (A and D), 40-week-old mice (B and E), and 60-week-old mice (C and F). OPG

apoE

apoE

mice (A-C) and OPG

apoE

apoE

mice (D-F). These micrographs show examples of the increases in plaque size and alterations in cellularity and calcification. Arrows denote areas highlighted in insets. Insets show the von Kossa staining for hydroxyapatite of the same areas in adjacent slides. Magnification 100x for the Movat's stained sections and 400x for the von Kossa stained sections.

**Figure 2.** Average area of calcification of advanced atherosclerotic lesions in the innominate artery of OPG

apoE

apoE

and OPG

apoE

mice. Calcified plaque area was measured on von Kossa-stained sections. Data are presented as the mean±SEM. P<0.05 at 60 weeks of age.
Plasma OPG and RANKL

Plasma levels of OPG and RANKL were measured in a subset of OPG+/−.apoE+/− and OPG+/+.apoE+/− mice. Measurement of plasma OPG levels confirmed the absence of OPG in the OPG+/−.apoE+/− mice. Interestingly, plasma levels of RANKL were 4-fold higher in OPG+/+.apoE+/− mice as compared with controls (data not shown).

Discussion

There is increasing interest in the role that calcification plays in the atherogenic process and in understanding the mechanisms by which calcification is induced within lesions.23 Current hypotheses suggest that vascular calcification may recapitulate endochondral ossification of bone,24 and involves chondrocytes, osteoblasts, and osteoclast-like activity.25 OPG plays an integral role in modulating bone resorption through its action as a decoy receptor for RANKL.12 OPG-deficient mice develop severe osteoporosis,8,13,14 and in some cases medial calcification8 and administration of OPG rescues the vascular calcification phenotype.26 Furthermore, OPG has an additional role in adaptive immunity as inactivation of OPG leads to increased immature B-cells in the spleen and a reduced antibody response.13

There is currently little evidence of a direct role for OPG in the atherogenic process. However, a potential role for OPG/RANKL in atherosclerosis has recently been outlined in several reviews.15,16,27 This is in light of data showing a correlation between plasma OPG levels and the severity and extent of coronary heart disease28 and heart failure29 in humans. Furthermore, plasma OPG appears to be predictive of poor prognosis in at least one cohort of patients30 and polymorphisms in the human OPG gene are associated with increased carotid intimal thickness.31 OPG is expressed in human atherosclerotic lesions,6,32 and we have recently reported that chondrocyte-like cells within advanced atherosclerotic lesions in the innominate arteries of older apoE−/− mice express OPG.17

In the current study, we show that the absence of OPG leads to increased lesion area and calcification at 40 and 60 weeks, suggesting that OPG does play a role in the progression of advanced atherosclerotic lesions in mice. The increase in lesion area in the innominate arteries is caused by both an...
increase in the area of calcification as well as an increase in extracellular matrix components such as collagen, proteoglycans, and laminated elastin (Table 2 and Figure 4). The increase in lesion area is not caused by increases in the cellular content, because there was a significant decrease in the total cellularity of the lesions in the OPG-deficient mice (Figure 3). The increased deposition of connective tissue in the OPG-deficient mice likely occurs during the formation of the fibro-fatty nodules in animals between 35 and 50 weeks of age, in which the lesions are still highly cellular but before large-scale calcification of the nodules that we previously showed occurs normally in apoE−/− mice by 75 weeks of age.17 It is unclear what the source of the laminated elastin is and why the elastin is deposited in these unusual layers. It is also unclear whether this phenomenon is associated with the process of calcification. However, previous studies have suggested that elastin can be a scaffold for deposition of hydroxyapatite35 and with formation of the multiple layers of elastin, there may be an increased surface area to support calcification.

Remodeling of advanced atherosclerotic lesions and changes in plaque morphology are multifactorial processes. One class of proteins thought to have effects on the composition of atherosclerotic lesions are MMPs. Our in vitro data indicates that OPG induces MMP-9 in bone marrow-derived macrophages, RAW264.7 cells, and aortic SMCs from apoE−/− mice. It is therefore possible that reduced MMP-9 activity is partially responsible for the increased connective tissue content of the lesions in OPG−/−apoE−/− mice. In keeping with our current observations, it has recently been demonstrated that OPG treatment induces MMP-9 activity34 and increases total MMP activity35 in cultured human SMCs.

In our previous studies of the time course of plaque calcification,17 we found that chondrocytes are temporally and spatially associated with the deposition of hydroxyapatite. Our de novo hypothesis was that OPG inactivation would increase the chondroplastic conversion leading to increased calcification. Contrary to this hypothesis, there was a decrease in the total cellularity of the lesions in the OPG−/−apoE−/− mice, including the number of chondrocytes by 40 weeks of age. Thus, the absence of OPG does not appear to stimulate chondrocyte metaplasia. Alternatively, the death of intraplaque cells including the chondrocytes may increase calcification through the release of apoptotic bodies. Both apoptotic vesicles36 and matrix vesicles37 from chondrocytes have been implicated in endochondral ossification in bone and in calcification of atherosclerotic lesions.38,39 We and others have observed that OPG functions as a survival factor for endothelial cells in vitro in part by blocking trail-induced apoptosis.31,40 Our current data showing increased survival of serum deprived mouse aortic smooth muscle cells after treatment with OPG (Figure 3) are consistent with these previous observations.

In the inominate artery and aorta of older apoE−/− mice, both the intima and media normally become calcified,17 and both intimal and medial calcification are more pronounced in the absence of OPG. For example, at 20 weeks of age, 5 of 13 OPG−/−apoE−/− mice exhibited medial calcification in the inominate artery, whereas none of the age-matched apoE−/− mice had medial calcification (P=0.039). Furthermore, at 20, 40, and 60 weeks of age there were highly significant increases in the total calcium content of the aorta (intima and media combined). We also observed a very small increase in the aortic calcium content of 20-week-old, male OPG−/−apoE−/− mice (Table 1). This is not entirely consistent with the previously reported large increase in medial calcification of the aorta in OPG−/− mice in the absence of hyperlipidemia and atherosclerosis.8 This difference may reflect differences in the background strain of the mice as others have reported no medial calcification in OPG−/− mice on a pure C57BL/6 background.14

SMCs can be converted to osteogenic cells in vitro suggesting that they may play a role in vascular calcification in vivo.41,42 In the current study we found that the increase in medial calcification preceded the increase in lesion area and lesion calcification. Thus, it is conceivable that the absence of OPG leads to an increase in smooth muscle cell derived osteogenic cells in the media that are then recruited into the developing lesion. However, as noted, OPG is a potent survival factor for SMCs (Figure 3) and the death of SMCs has been shown to increase calcification in vitro.38 Therefore, OPG may function indirectly to prevent calcification by protecting arterial smooth muscle cells from apoptosis.

The increase in lesion area and calcification may also be due to increased secretion of pro-inflammatory factors from cells resident within both the media and intima. This is supported by recent work demonstrating that calcium crystals can be internalized by macrophages and in turn stimulates secretion of pro-inflammatory cytokines.43 Furthermore, pro-inflammatory cytokines such as tumor necrosis factor-α increase the conversion of SMCs to an osteogenic phenotype in vitro.42,44 Thus there is the possibility that calcification itself increases plaque burden by further activating pro-
inflammatory signals and the conversion of SMCs to osteogenic cells.

The increased vascular calcification of OPG−/−apoE−/− mice may ultimately be caused by altered mineral metabolism in the bone or kidney. The OPG−/−apoE−/− mice are highly osteoporotic and similar to previous reports, we have observed a significant increase in circulating alkaline phosphatase in these mice (data not shown). Recently, osteoporosis has been identified as an independent risk factor for mortality in renal patients who are also at increased risk for cardiovascular disease. In a subset of mice we did not observe differences in serum phosphate or calcium (data not shown).

Differences in circulating lipoproteins could explain both the increase in lesion size and calcification as previous reports have demonstrated increased plaque burden and coronary artery calcification with increased circulating LDL and decreased HDL. In the current study there was no difference in HDL cholesterol between OPG−/−apoE−/− and OPG+/−apoE−/− mice.

The increase in lesion size in the OPG−/−apoE−/− mice may also be caused by the role of OPG in adaptive immunity. Data from Major et al indicate that B-cell deficiency in LDL-receptor−/− mice increases atherosclerosis. B-cells from LDL-receptor−/− and apoE−/− mice produce antibodies that recognize oxidized LDL and immunization of atherosclerotic mice with modified forms of LDL has been shown to reduce lesion development. As noted, OPG−/− mice have immature B-cell development and impaired antibody production and administration of recombinant OPG stimulates production of specific antibodies.

Interestingly, circulating levels of unbound RANKL are 4-fold higher in the OPG−/−apoE−/− mice (data not shown). Although the assay used to measure RANKL cannot differentiate between free RANKL and RANKL bound to OPG, these results suggest that increased RANKL levels may be associated with atherosclerosis progression. Like OPG, RANKL plays a role in lymphocyte development and has been shown to activate the inflammatory response in macrophages. RANKL is expressed in human plaques and we recently reported expression of RANKL by chondrocyte-like cells in advanced lesions in the innominate artery of apoE−/− mice.

In conclusion, the results of these studies have for the first time shown that OPG has a protective role in the progression and calcification of advanced atherosclerotic lesions in the innominate arteries of apoE−/− mice. The mechanism(s) by which OPG mediates these effects are currently unknown but may involve regulation of the inflammatory response and cell survival.

Acknowledgments

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Disclosures

None.

References


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