Osteopontin Deficiency Attenuates Atherosclerosis in Female Apolipoprotein E–Deficient Mice

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Objective—Osteopontin (OPN), a noncollagenous adhesive protein, is implicated in atherosclerosis, in which macrophages within atherosclerotic plaques express OPN. However, it is not known whether the elevated OPN expression is a cause or result of atherosclerosis.

Methods and Results—We generated mice that lacked OPN and crossed them with apolipoprotein (apo) E–deficient mice and analyzed these mice with a mixed C57BL/6×129 background after 36 weeks on a normal chow diet. In female mice, OP<sup>+/−</sup>E<sup>−/−</sup> and OP<sup>−/−</sup>E<sup>−/−</sup> mice had significantly smaller atherosclerotic and inflammatory lesions compared with OP<sup>+/+</sup>E<sup>−/−</sup> mice, and that was reflected by smaller area of MOMA-2–positive staining. In male mice, however, there was no significant difference in the atherosclerosis lesion areas among 3 genotypes. In both OP<sup>−/−</sup>E<sup>−/−</sup> and OP<sup>+/+</sup>E<sup>−/−</sup> mice, typical atherosclerotic lesions were detected, which include necrotic core, foamy cell collections, and cholesterol clefts. However, we found that vascular mineral-deposited areas in 60-week-old male OP<sup>−/−</sup>E<sup>−/−</sup> mice were significantly increased compared with those in OP<sup>+/+</sup>E<sup>−/−</sup> male mice.

Conclusions—These results suggest that OPN plays a promoting effect in atherosclerosis and inhibitory effect in vascular calcification. The suppression of OPN expression in females should be considered a therapeutic possibility in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2003;23:1029-1034.)

Key Words: osteopontin ■ atherosclerosis ■ macrophage ■ calcification ■ lipid metabolism

Atherosclerosis is characterized as a chronic inflammatory process of the vessel wall. Atherosclerosis is initiated by the infiltration of monocytes and T-lymphocytes into activated endothelium, followed by their migration into the intima and subsequent lipid accumulation within macrophages. In late stages of atherosclerosis, calcification is a common advanced complication. Osteopontin (OPN), a non-collagenous adhesive protein, was first found at sites of dystrophic calcification and is synthesized at high levels by collagenous adhesive protein, was first found at sites of

plaque. The expression of OPN protein was detected in not only macrophages but also vascular smooth muscle cells within atherosclerotic lesion. In addition, it was shown that vascular smooth muscle cells during the proliferative and migratory phase, but not the quiescent and contractile phase, expressed OPN in a model of balloon catheter injury of rat carotid artery. More importantly, Liaw et al reported that neutralizing antibodies directed against OPN inhibited rat carotid neointimal thickening after endothelial denudation. Taken together, these results suggested that OPN can play a pivotal role in the early stage of atherosclerosis, including proliferation and migration of smooth muscle cells as well as at the late stage of atherosclerosis, characterized by calcified atheromatous plaque formation.

To more directly address the question of whether OPN initiates the development of atherosclerotic lesion and plays a role in calcification, we took advantage of OPN-deficient mice we had recently generated. We crossed them with apolipoprotein (apo) E–deficient mice and made OPN and apoE double-deficient mice. In mice deficient for OPN gene expression, OPN mRNA was not detected in any organs. In this study, we determine whether the absence of OPN influences atherogenesis and calcification in vivo in hyperlipidemic apoE-deficient mice.

Methods

Mice

The creation of the OPN null mouse used in this study has been described previously. C57BL/6 apoE null male mice (E<sup>−/−</sup>) (Jackson Laboratory, Bar Harbor, Me) were bred to OPN null (OP<sup>−/−</sup>) female mice on a C57BL/6×129 background (~1:1). Heterozygous F1 progeny were interbred, yielding 9 possible F2 genotypes. OPN
wild-type (WT), OPN heterozygous, and OPN null mice among apoE null mice were designated as OP+/E−, OP+/-E−, and OP−/-E−, respectively, and were intercrossed to yield F3 progeny, which served as subjects in this experiment on a C57BL/6×129 background (n=3:1). Screening for apoE was done by phenotypic assays. Blood was obtained, and apoE deficiency in these mice was detected by elevation of serum cholesterol as described previously.8

OPN genotyping was performed by the polymerase chain reaction analysis of tail DNA as described previously.7

**Diet and Experimental Design**

The pups were weaned at 3 weeks of age and then maintained on normal chow diet. At 36 weeks of age, blood was collected by means of left ventricular puncture. The heart and aorta from the aortic root to the iliac branch were removed, fixed in 10% phosphate-buffered formalin for histopathology, frozen in OCT embedding medium using liquid nitrogen-cooled isopentane for immunohistochemistry, or processed for oil red staining. Additional animals were killed at 26 or 60 weeks, and atherosclerosis was determined by an en face method.

**Analysis of Atherosclerotic Lesions**

The degree of atherosclerosis was determined by quantifying oil red O-stained en face lesions in pinned out aortas.9 Briefly, the mice were perfused, first with PBS and then with 4% paraformaldehyde. The aorta was opened longitudinally from the aortic root to the iliac branch, and the aorta from the iliac bifurcation to a point equidistant between the aortic valve and the brachiocephalic artery was removed, pinned out flat on a black wax surface, and stained with oil red O. The aortas were then photographed, and the total surface and the entire lesion areas were measured by planimetry.

**Histopathology**

Serial 5-μm sections were taken from the aortic valve area. Sections were stained with H&E, Masson’s trichrome stain for fibrosis, and von Kossa stain for mineral salts and calcification.

**Immunohistochemistry**

A monoclonal antibody, OPN 2.2 reacting against mouse OPN, was used in this study.10 A monoclonal antibody, reacting against α-smooth muscle actin, clone 1A4, was purchased from Nichirei, Tokyo, Japan. A monoclonal antibody, reacting against macrophage, MOMA-2, was purchased from Serotec, Tokyo, Japan. The sections were stained with OPN2.2, MOMA-2, or 1A4 followed by biotin-conjugated goat anti-rat IgG (for OPN2.2 and MOMA-2) or rabbit anti-mouse IgG (for α-smooth muscle actin, 1A4) followed by Streptavidin-biotin peroxidase complex (Histofine kit; Nichirei) and counterstained with hematoxylin.

**Titration of Plasma OPN and Lipid Measurements**

The concentration of plasma OPN was determined by ELISA (Immuno-Biological Laboratories Co). Plasma levels of total cholesterol (Determiner TC555, KYOWA MEDEX), triglyceride (Determiner TG, KYOWA MEDEX), and HDL cholesterol (Determiner HDL, KYOWA MEDEX) were measured.

**Statistical Analysis**

Results were expressed as mean±SEM. The statistical significance of the difference between groups was estimated using Student’s t test; P<0.05 was regarded as statistically significant.

**Results**

**Absence of Osteopontin Attenuates Atherosclerosis in Female ApoE Null Mice**

Male and female mice of 3 genotypes were analyzed at 36 weeks of age. The atherosclerotic lesion area in the entire aorta was carefully determined on 93 mice, and individual data points are plotted by genotype and sex in Figure 1. There were 2 clear results. In female mice, OP+/E− and OP−/-E− mice had significantly smaller atherosclerotic lesions compared with OP+/E− mice, showing an OPN gene dosage effect on atherosclerotic lesion areas in female mice (Figure 1A). In male mice, however, there was no significant difference in the atherosclerosis lesion areas among 3 genotypes (Figure 1B). In both sexes combined (Figure 1C), there was still a significant difference between OP+/E− and OP−/-E− mice. The reduction rates of lesion areas were approximately 34.0% in OP+/E− and 42.5% in OP−/-E− compared with OP+/E− mice. Figure 1D shows a summary of these results. To confirm that atherosclerotic lesion area in male mice was not different among 3 genotypes, we examined the time course of the progression of atherosclerotic lesion areas in male mice. We found that there was no significant difference in atherosclerotic lesion areas between the OP+/E− and OP−/-E− male mice at 26 weeks (OP+/E− [n=7] 12.0±2.6% versus OP−/-E− [n=7] 14.6±5.0%, P=0.65) and 60 weeks (OP+/E− [n=3] 57.0±8.9% versus OP−/-E− [n=3] 55.4±4.2%, P=0.88), respectively. Representative macroscopic findings in female mice are demonstrated in Figure 2, showing the reduction in lesions (red-stained areas) in female OP+/-E− mice (Figure 2B) compared with OP+/-E− (Figure 2A).
Absence of Osteopontin Attenuates Infiltration of Inflammatory Cells in Female ApoE Null Mice

After 36 weeks, aortic walls in the OP\(^{-/}\)E\(^{-/-}\) female mice were severely infiltrated by various inflammatory cells (Figure 3A). The major cell type among those inflammatory cells was macrophages, as depicted by the positive staining with MOMA-2 antibody (Figure 3C). In contrast, inflammation in the aortic walls of OP\(^{+/+}\)E\(^{-/-}\) mice was less severe (Figure 3B), as reflected by reduced areas of MOMA-2-positive staining (Figure 3D). We confirmed histologically that the atherosclerotic lesions in OP\(^{+/+}\)E\(^{-/-}\) mice expressed OPN (Figure 3E), whereas those in OP\(^{-/-}\)E\(^{-/-}\) mice were completely OPN negative (Figure 3F). Next we examined the nature of the atherosclerotic lesions. In both OP\(^{+/+}\)E\(^{-/-}\) and OP\(^{-/-}\)E\(^{-/-}\) mice, typical atherosclerotic lesions detected included a necrotic core, foamy cell collections, and cholesterol clefts (Figures 4A and 4B). Fibrous caps were outlined by the positive staining of α-smooth muscle actin similarly in both OP\(^{+/+}\)E\(^{-/-}\) and OP\(^{-/-}\)E\(^{-/-}\) mice (Figures 4C and 4D). We then examined the degree of fibrosis in the aortic walls by Masson’s trichrome staining. Although the vascular fibrotic areas in the OP\(^{+/+}\)E\(^{-/-}\) female mice had a tendency to be smaller than those in OP\(^{+/+}\)E\(^{-/-}\) mice (Figures 4E and 4F), quantitative analysis showed that vascular fibrotic area was not significantly different between mice of the 2 genotypes. Because previous reports showed that OPN expression in OPN transgenic (TG) mice is associated with a significant increase in medial thickening with aging in vivo,\(^1\) we went on to examine the extent of staining for α-actin, indicative of smooth muscle cells, in the vascular wall. We found that the absence of OPN does not influence medial thickening (data not shown).

Absence of Osteopontin Augments Calcification Within Atheromatous Plaque in Male ApoE Null Mice

We were able to examine the late effects of OPN deficiency on vascular walls in only male mice. Von-Kossa-stained

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**Figure 2.** Representative macroscopic findings of oil red O-stained en face aortal preparations of 36-week-old female mice fed a normal chow diet, showing red lipid-rich atherosclerotic lesions. A, OP\(^{+/+}\)E\(^{-/-}\); B, OP\(^{-/-}\)E\(^{-/-}\).

**Figure 3.** Representative aortic root sections of 36-week-old female mice fed a normal chow diet. The aortas were obtained from OP\(^{+/+}\)E\(^{-/-}\) (A, C, and E) and OP\(^{-/-}\)E\(^{-/-}\) (B, D, and F) mice and were stained with H&E (A and B), MOMA-2 (C and D), or OPN2.2 (E and F). The MOMA-2-positive staining areas were analyzed quantitatively using 7 OP\(^{+/+}\)E\(^{-/-}\) and 8 OP\(^{-/-}\)E\(^{-/-}\) mice. Data represent mean positive areas ±SE. *P<0.05 vs OP\(^{+/+}\)E\(^{-/-}\) group. (A and B, original magnification ×85; C and D, original magnification ×85; E and F, original magnification ×340).

**Figure 4.** Representative aortic root sections of 36-week-old female mice fed a normal chow diet. H&E-stained section of the aortic sinus of the OP\(^{+/+}\)E\(^{-/-}\) (A) and OP\(^{-/-}\)E\(^{-/-}\) (B) mice. Immunohistochemical staining for α-smooth muscle actin in OP\(^{+/+}\)E\(^{-/-}\) (C) and OP\(^{-/-}\)E\(^{-/-}\) (D) mice. Masson’s trichrome staining of OP\(^{+/+}\)E\(^{-/-}\) (E) and OP\(^{-/-}\)E\(^{-/-}\) (F) mice. Quantitative analysis showed that the vascular fibrotic areas in OP\(^{+/+}\)E\(^{-/-}\) mice (n=7; black bar) tended to be smaller than those in OP\(^{-/-}\)E\(^{-/-}\) (n=8; white bar), although not significantly. Data are reported as mean±SE. (A and B, original magnification ×85; C and D, original magnification ×340; E and F, original magnification ×85).
sections showed calcification of the advanced vascular lesion in OP\textsuperscript{+/−}/E\textsuperscript{−/−} male and OP\textsuperscript{−/−}/E\textsuperscript{−/−} mice at 60 weeks (Figures 5A and 5B), and these areas were stained strongly for OPN in OP\textsuperscript{+/−}/E\textsuperscript{−/−} (C) but not in OP\textsuperscript{−/−}/E\textsuperscript{−/−} (D) mice (Figure 5D). The calcified lesion areas seemed bigger in OP\textsuperscript{−/−}/E\textsuperscript{−/−} mice. To confirm that observation, quantitative evaluation of Von-Kossa positive vascular calcification areas was carried out. We found that vascular mineral-deposited areas in 60-week-old male OP\textsuperscript{−/−}/E\textsuperscript{−/−} mice were significantly increased compared with those in OP\textsuperscript{+/−}/E\textsuperscript{−/−} male mice (Figure 5E), suggesting that the absence of OPN augments vascular calcification in vivo.

Characteristics of Lipid Metabolism in ApoE Null Mice With 3 Genotypes of OPN

Because lipid metabolism critically influences the complex processes of atherosclerosis, total cholesterol, triglyceride, and non–HDL cholesterol levels were determined at 36 weeks to see whether any of these factors were altered by the OPN genotypes (see http://atvb.ahajournals.org/). As expected, OPN was not detectable in OP\textsuperscript{−/−}/E\textsuperscript{−/−} mice, and low levels of plasma OPN were detected in OP\textsuperscript{+/−}/E\textsuperscript{−/−} mice. In female mice, there was no significant effect of OPN genotype on total cholesterol, triglyceride, and non–HDL cholesterol levels. These data indicate that the reduction in atherosclerotic lesions observed in OP\textsuperscript{−/−}/E\textsuperscript{−/−} mice is not attributable to alterations in plasma lipid metabolism. In male mice, on the other hand, there was a significant effect of OPN genotype on plasma total cholesterol, triglyceride, and non–HDL cholesterol. Most striking, triglyceride levels in OP\textsuperscript{−/−}/E\textsuperscript{−/−} mice were approximately 4-fold greater than that in OP\textsuperscript{+/−}/E\textsuperscript{−/−} mice, and total cholesterol level in OP\textsuperscript{−/−}/E\textsuperscript{−/−} mice was about one and a half times as much as that in OP\textsuperscript{+/−}/E\textsuperscript{−/−} mice. Using an ANCOVA model, a significant negative correlation was found between the plasma OPN level and plasma non-HDL cholesterol level ($r^2=0.209$, $P=0.0021$, $n=43$, data not shown).

Discussion

OPN is known to induce chemotactic movement of vascular smooth muscle cells, macrophages, and fibroblasts$^{5,12-17}$ and this activity might be critically involved in the formation of atherosclerotic lesions. We had previously reported that the lesion size of atherosclerotic plaques in OPN TG mice fed a high-fat diet was significantly increased compared with control littermates.$^{18}$ To more directly assess the cause/result relationship between OPN and atherosclerosis or promoting/inhibitory effect of OPN on atherosclerosis, we generated mice that lacked OPN and crossed them with apoE null mice that develop severe atherosclerosis. In this study, we determined whether OPN influences atherogenesis in vivo in hyperlipidemic apoE null mice.

Dual Role of OPN in Complex Atherosclerotic Processes

The development of atherosclerosis involves multiple processes. In early stages of atherosclerosis, monocyte transendothelial migration and differentiation into macrophages and macrophage uptake of modified lipoprotein are key events. OPN was previously shown to have a critical role in macrophage infiltration in response to pathological stimuli in vivo.$^{15}$ In female mice, OP\textsuperscript{−/−}/E\textsuperscript{−/−} mice had significantly smaller atherosclerotic as well as macrophage-positive areas compared with OP\textsuperscript{+/−}/E\textsuperscript{−/−} mice. We also found that the infiltration of inflammatory cells in and around atherosclerotic lesions in OP\textsuperscript{+/−}/E\textsuperscript{−/−} mice was significantly more extensive compared with OP\textsuperscript{−/−}/E\textsuperscript{−/−} mice. These results are consistent with previous findings that OPN is chemotactic for not only macrophage but also for lymphocytes.$^{19}$ These lymphocytes might modulate the complex processes of atherogenesis.$^{20}$ The inhibitory effect of OPN deficiency in atherosclerotic processes is thus 30% to 40% in this study. It has been shown that other macrophage chemotactic factors, including monocyte chemoattractant protein-1, are also involved in atherosclerotic processes.$^{21}$ It is possible that blockade of both OPN and monocyte chemoattractant protein-1 can inhibit atherosclerotic processes in synergistic fashion.

The effects of OPN deficiency on inhibiting atherosclerosis and inflammatory response were much reduced in male...
compared with female mice. However, OPN-deficient male mice exhibited significantly high triglyceride and total cholesterol levels compared with control OP+/−/−mice. Because high triglyceride and total cholesterol levels are risk factors for atherosclerosis,22 this observation suggests a protective role of OPN in male mice.

In late stages of atherosclerosis, lesions are often associated with dystrophic calcification, which leads to serious complications, including plaque rupture and thrombosis. Previous studies showed that OPN was abundant at the site of calcification in atherosclerosis plaques23 and in calcified aortic valves,24 suggesting that OPN had a critical role in regulating calcification formation. It has been demonstrated that exogenously added OPN is a potent inhibitor of vascular calcification in vitro by a mechanism that likely involves direct inhibition of apatite growth by binding to crystal surfaces.25,26 Furthermore, recent reports showed that mice deficient in both matrix GlA protein and OPN had increased calcification in their arteries compared with mice that were wild-type for OPN and homozygous for matrix GlA protein deficiency, suggesting an inhibitory effect of OPN in vascular calcification.27 Those reports were consistent with our findings that calcified areas were increased in OP+/−/−mice compared with OP+/+/−mice. Although we were only able to study the inhibitory effect of OPN on vascular wall calcification in male mice, it is possible that this effect can be found in female mice as well. However, additional study is needed to confirm this hypothesis. These results collectively suggest that OPN might be one of the candidates to modulate the vascular calcification processes.

**OPN and Cholesterol Metabolism**

Previous work demonstrated that a significant negative correlation was found between the plasma OPN level and serum total cholesterol levels in humans.28 Because most subjects in that study were male, these results were consistent with our results that significant negative correlation was found between the plasma OPN level and plasma non–HDL cholesterol concentration in male mice, suggesting either the involvement of OPN in cholesterol metabolism or the involvement of cholesterol in OPN expression.

A linkage among cytokines, cholesterol metabolism, and atherosclerosis has been reported. It was shown that OPN is a critical inducer of the Th1 cytokine interleukin (IL)-12.19 Both IL-12–treated apoE-deficient mice and OPN TG mice fed high-fat diet exhibited accelerated atherosclerosis with lower plasma total cholesterol level compared with control mice.18,20 Taken together, Th-1 type cytokines, such as interferon-γ, IL-12, and OPN, seemed to promote atherosclerosis and have a tendency to decrease the plasma total cholesterol levels. These observations suggest that OPN promotes and modifies atherosclerosis as a immunomodulatory factor. However, additional studies are needed to determine the molecular and cellular mechanisms involved.

**Study Limitations**

In our study, the OPN-deficient mice were twice backcrossed with C57BL/6 mice, resulting in animals that have approximately 75% C57BL/6 and 25% 129/Sv. The possibility that a 129/Sv gene contributed to the differences in atherosclerosis and lipid metabolism cannot be completely excluded, particularly if this gene was linked to OPN. For example, previous reports showed that atherosclerotic lesions were not affected by PAI-1 deficiency in congenic C57BL/6 mice,30 whereas they were increased in PAI-1−/− mice with a mixed C57BL/6×129Sv/J genetic background31; the differences between these findings are likely attributable to differences in genetic background. Every gene, including disease candidate genes such as PAI-1 and OPN, is potentially influenced by modifier genes. In a congenic background, a particular set of modifier genes is present. Depending on which modifier genes are present and how strongly the gene candidate is influenced by these modifier genes, the phenotype of a mutant mouse can significantly vary (and even exhibit opposite features) in different congenic backgrounds. Conversely, in a mixed genetic background, modifier genes of either background will codetermine the phenotype of a gene-inactivated mouse. Therefore, if the genetic backgrounds varied, the role of OPN on atherosclerosis might be influenced by other modifier genes in this genetic background. Because the OPN gene disruption was not available on the C57BL/6 background at the start of this study, parental OPN-deficient mice on a mixed C57BL/6×129 background (1:1) were used, and F3 progeny on a mixed C57BL/6×129 background (≈3:1) served as subjects in this study. Because F1 and F2 mice were intercrossed to generate mice homozygous for OPN and apoE, the offspring had a random mix of 129 and C57BL/6 chromosomal DNA throughout the genome. The differences of background could influence the development of atherosclerosis and lipid metabolism. In this regard, we carefully examined a large number of F3 progeny mice in this study to minimize the genetic variability and ensure the data. However, additional studies are warranted using C57BL/6 backcrossed mice to determine the OPN effects on atherosclerosis excluding the influences of differences of genetic background. However, it should be kept in mind that humans have mixed genetic backgrounds, so the data generated in the mixed mouse background may be most relevant to human disease.

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