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Osteopontin Transgenic Mice Fed a High-Cholesterol Diet Develop Early Fatty-Streak Lesions

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Background—Osteopontin (OPN) is a noncollagenous adhesion protein found at the site of atherosclerotic lesions. However, it has not yet been clarified whether or not OPN can promote atherosclerotic lesions.

Methods and Results—We investigated the contribution of OPN to atherosclerosis by evaluating aortic sinus lesions of both OPN transgenic (Tg) and non-Tg mice fed an atherogenic diet (1.25% cholesterol) for 16 weeks. The atherosclerotic lesions were found to be significantly larger in OPN-Tg compared with those in non-Tg ($17\,859 \pm 2010$ versus $6469 \pm 485 \mu\text{m}^2$, $P < 0.01$). The lesions in both mice were fatty-streak lesions with an accumulation of mononuclear cells and lipids. We next investigated the production of interleukin (IL)-10 by macrophages from both mice. Compared with the non-Tg mice, a 42% ($P < 0.01$) and 73% ($P < 0.001$) decrease in the IL-10 production was identified in the OPN-Tg mice either without or with lipopolysaccharide.

Conclusions—The expression of OPN induces fatty-streak lesion formation in mice fed an atherogenic diet and inhibits IL-10 production by macrophages, thus suggesting that OPN plays an important role in the development of fatty-streak lesions in vivo. (*Circulation*. 2003;107:679-681.)

Key Words: atherosclerosis ■ genes ■ interleukins

Atherosclerosis is initiated by the infiltration of monocytes and T lymphocytes into an activated endothelium, and the expression of osteopontin (OPN) protein was detected in both macrophages and vascular smooth muscle cells (SMCs) within atherosclerotic lesions.¹⁻⁴ We recently reported that OPN transgenic (Tg) mice showed an increase in the medial thickness of artery without injury and an increase in the neointimal formation after injury.⁵ Taken together, these results suggest that OPN may play an important role in the initial step of atherosclerosis, including the proliferation and migration of smooth muscle cells. However, whether or not OPN can promote atherosclerotic lesions remains to be elucidated. The purpose of the present study was to determine whether OPN has a promoting or inhibitory effect on atherosclerosis in OPN-Tg mice.

Methods

Animals and Diet

Previously characterized OPN-Tg mice⁵ bred in our laboratory were used under protocols approved by the National Defense Medical College Board for Studies in Experimental Animals. In the present study, we compared heterozygous OPN-Tg to non-Tg littermates (non-Tg). Beginning at 8 weeks after birth and continuing for 16 weeks, both OPN-Tg and non-Tg mice were fed a high-fat/cholesterol diet containing 15% fat, 1.25% cholesterol, and 0.5% sodium cholate, hereafter referred to as the atherogenic diet. These mice

were killed at 24 weeks of age to undergo a plasma lipoprotein analysis and lesion assay.

Plasma Lipid Measurements

On the day of analysis, food was removed from the cages in the morning, and the mice were fasted for 7 hours. The plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride levels were then measured by enzymatic assays as previously described.⁶

Quantification of Aortic Sinus Lesions

The sections containing aortic sinus were prepared as previously reported,⁷ with modifications. The area of the lesion was measured with the National Institutes of Health Image 1.55 (public domain software). The values reported represented the mean lesion area from 5 sections for each animal. The extent of atherosclerosis in mouse aorta was also determined with an "en face" method.⁸

Immunohistochemical Studies

Frozen sections were incubated with either a primary rat monoclonal antibody against mouse macrophage, clone MOMA-2 (BioSource International), or a primary goat polyclonal antibody against mouse Interleukin (IL)-10 (PharMingen). Immunostaining was visualized as previously described.⁵

Analysis of the Ability of IL-10 Production by Macrophages

Resident peritoneal macrophages from both non-Tg and OPN-Tg mice obtained by peritoneal lavage with phosphate-buffered saline

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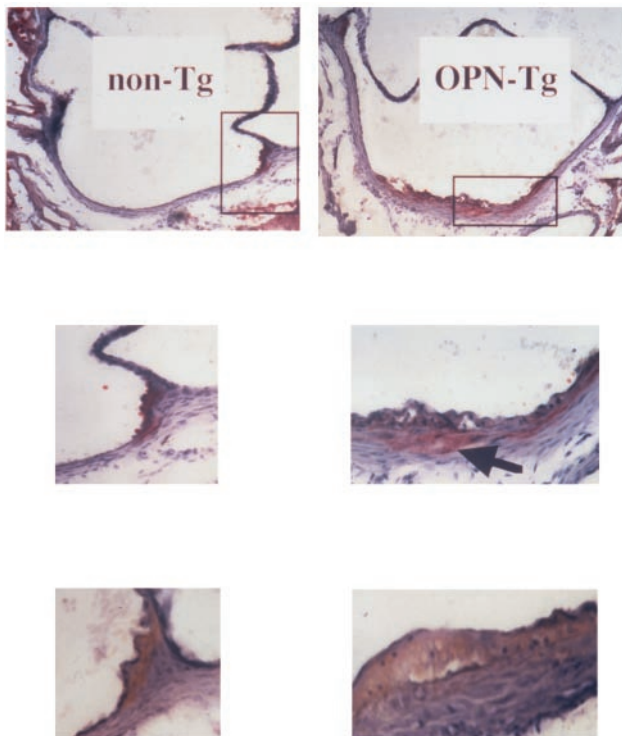


Figure 1. Representative light photomicrographs of aortic atherosclerotic lesions of both non-Tg (left) and OPN-Tg (right). Sections were stained with oil red O and hematoxylin (upper and middle panels). Boxed areas are shown in middle of panels. Arrow shows lipids were contained in the medial layer. The bottom panels show MOMA-2 staining of both mice. Original magnification $\times 25$ (top panels), $\times 50$ (bottom panels), and $\times 100$ (bottom panels).

were treated with red cell lysis buffer and were incubated (10^5 macrophages per $100 \mu\text{L}$) for 2 hours. The adherent fraction was incubated with lipopolysaccharide (LPS) (30 ng/mL) for 48 hours. Supernatant IL-10 was assayed with commercial ELISA kits (R&D Systems).

Statistical Analysis

The results are shown as the mean \pm SE. Two groups were compared with either Student's *t* test or Student-Newman Keuls's test with a one-way analysis of variance. $P < 0.05$ was regarded as a significant difference.

Results

Development of Fatty-Streak Lesions

Our previous study demonstrated an increase in the medial thickness of the artery, but no evidence of the formation of early form cells in the OPN-Tg mice fed normal chow.⁵ To investigate the impact of OPN on the development of atherosclerotic lesions in vivo, studies were conducted with murine models on an atherogenic diet. After 16 weeks on the atherogenic diet, the total cholesterol (non-Tg: 361.2 ± 15.1 versus OPN-Tg: $325.7 \pm 11.3 \text{ mg/dL}$), triglyceride (21.3 ± 2.7 versus $29.2 \pm 2.9 \text{ mg/dL}$), and HDL cholesterol levels (58.0 ± 3.8 versus $44.1 \pm 3.2 \text{ mg/dL}$), were comparable between the non-Tg ($n = 12$) and OPN-Tg mice ($n = 12$). Figure 1 shows the aortas of both a non-Tg and an OPN-Tg mouse on the atherogenic diet for 16 weeks. There was a marked difference in the lesion size between the OPN-Tg and non-Tg

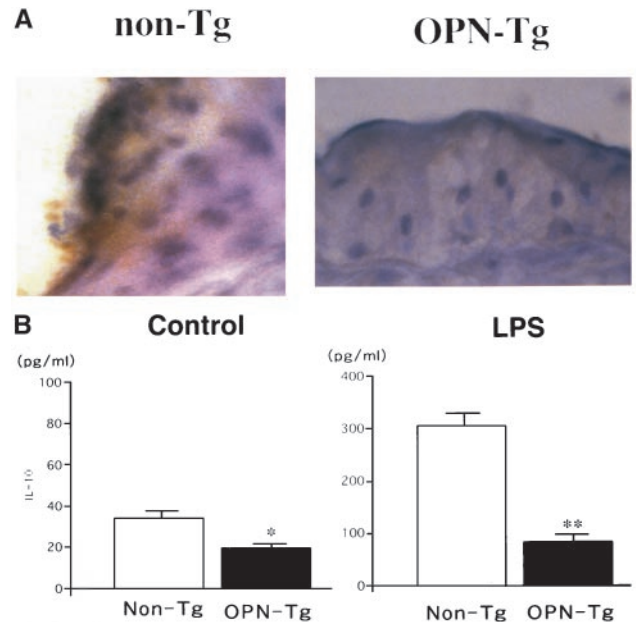


Figure 2. A, Representative photomicrographs showing the immunohistochemical staining for IL-10 in atherosclerotic lesions of non-Tg (left) and OPN-Tg (right). IL-10 expression was detected in the lesion of non-Tg (brown staining). Original magnification $\times 150$. B, Production of IL-10 by peritoneal macrophages from non-Tg and OPN-Tg mice. The left panel shows the production of IL-10 without LPS and the right panel shows production of IL-10 by macrophages stimulated with LPS. Values represent the mean \pm SE. * $P < 0.01$; ** $P < 0.001$.

mouse, with the OPN-Tg mouse displaying a significantly larger fatty-streak lesion formation than the non-Tg mouse (Figure 1, upper panels). Furthermore, the thickness of the medial layer of the aorta was greater in the OPN-Tg mouse (Figure 1, middle panels). MOMA-2 staining revealed that the lesions in both mice consisted mainly of foam cells of macrophage (Figure 1, bottom panels), but more lipids were contained in the medial layer, which was not stained by MOMA-2 in the OPN-Tg mouse (Figure 1, arrow, right middle panel). After the mice were fed an atherogenic diet for 16 weeks, the aortic sinus lesions were increased in the OPN-Tg mice to a mean of $17\,859 \pm 2010 \mu\text{m}^2$ ($n = 12$) versus the non-Tg to a mean of $6469 \pm 485 \mu\text{m}^2$ ($n = 12$, $P < 0.001$). Analysis of the extent of atherosclerosis in the aortas en face yielded similar results with a 187% increase in the lesion aorta of OPN-Tg mice compared with non-Tg mice ($0.89 \pm 0.23\%$ and $0.31 \pm 0.01\%$, $P < 0.001$). These results suggest that an overexpression of OPN may promote early fatty-streak lesions formation in OPN-Tg mice on an atherogenic diet.

OPN Modulation of Macrophage IL-10 Production

The recent mice studies showed that mice deficient in OPN gene expression demonstrated an increase in production of IL-10,⁹ and showed that IL-10 overexpression can inhibit fatty-streak formation in the that were mice fed an atherogenic diet.^{10,11} Based on the findings of these reports, we performed immunohistochemical analysis. As shown in Figure 2A, non-Tg mice expressed detectable levels of IL-10 compared with OPN-Tg mice. To clarify the contribution of

IL-10, we next examined the production of IL-10 in mice. Figure 2B shows the production of IL-10 by peritoneal macrophages from OPN-Tg and non-Tg mice. Figure 2B (left) shows the production of IL-10 without stimulation, in which both peritoneal macrophage groups released little IL-10. However, the IL-10 levels were less in the OPN-Tg mice than in the non-Tg mice (19.8 ± 1.7 versus 34.0 ± 2.9 pg/mL, $P < 0.01$). Next, the induction of IL-10 production by LPS was performed. These results are shown in Figure 2B (right). The LPS-dependent IL-10 production of macrophages was significantly less in the OPN-Tg mice than in the non-Tg mice (81.8 ± 7.4 versus 304.8 ± 15.0 pg/mL, $P < 0.0001$). These results show that OPN-Tg may decrease in the production of IL-10, especially under the inflammatory conditions, and a decrease of IL-10 may contribute to the susceptibility to atherosclerosis in OPN-Tg mice.

Discussion

We recently reported that there was no neointimal formation in our OPN-Tg mice fed normal chow, although OPN-Tg mice showed increased medial thickening dependent on aging. However, in the response to arterial injury, more neointima formed in OPN-Tg than in non-Tg mice.⁵ These results suggest that the migration of SMCs and neointima formation by OPN are induced when the intima is impaired. Endothelial dysfunction has been reported to be induced by hypercholesterolemia.¹² As a result, we think hypercholesterolemia induced more atherosclerotic lesions in OPN-Tg than in non-Tg.

A previous study showed the expression of OPN in peritoneal cells of OPN-Tg to significantly increase,¹³ and the present study demonstrated the production of IL-10 by macrophages from OPN-Tg to decrease. These results are compatible with the results of the previous study, in which OPN suppressed the LPS-dependent IL-10 response of peritoneal macrophages.⁹ We think the decrease in IL-10 must contribute to the development of fatty-streak lesions. Indeed, previous studies showed that IL-10 overexpression can inhibit fatty-streak formation in mice fed an atherogenic diet,^{10,11} and that IL-10 can induce endothelial cells to inhibit oxidized phospholipids-induced monocyte-endothelial interactions *in vitro*.¹⁰ These reports suggested that IL-10 might inhibit atherosclerosis. Although our findings suggest that the inhibition of IL-10 in OPN-Tg may play one of the most important roles in the development of atherosclerosis, we also believe that other factors most likely play a role in this phenomenon. For example, OPN has recently been classified as a T-helper 1 cytokine because of its ability to enhance interferon- γ and IL-12 production and to diminish IL-10.⁹ The strong influence toward a T-helper 1 phenotype has previously been demonstrated in an atherosclerotic model,^{14,15} and therefore, we think that these factors may cause an increase of fatty-streak lesions in OPN-Tg mice. Further research is needed to clarify the mechanisms why OPN promotes atherogenesis.

Although the HDL levels decreased in the OPN-Tg, no significant difference was seen between OPN-Tg and non-Tg, and the total cholesterol levels tended to decrease in non-Tg. We thus believe that the HDL levels only slightly contributed to the increased fatty-streak lesions in OPN-Tg. The present study shows that early fatty-streak formation was promoted by OPN but the findings didn't show whether or not OPN has any effect on more mature and advanced lesions. We thus believe that the OPN overexpression must be bred onto the apoE-deficient background to determine whether or not OPN has any effect on advanced lesions. However, it remains important to determine whether OPN can be modulated by treatment, and whether or not such an approach can be used to modulate the immune responses and reduce atherosclerosis.

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References

- Hirota S, Imakita M, Kohri K, et al. Expression of osteopontin messenger RNA by macrophages in atherosclerotic plaques: a possible association with calcification. *Am J Pathol.* 1993;143:1003–1008.
- Giachelli CM, Bae N, Almeida M, et al. Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. *J Clin Invest.* 1993;92:1686–1696.
- Ikedo T, Shirasawa T, Esaki Y, et al. Osteopontin mRNA is expressed by smooth muscle-derived foam cells in human atherosclerotic lesions of the aorta. *J Clin Invest.* 1993;92:2814–2820.
- Shanahan CM, Cary NR, Metcalfe JC, et al. High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. *J Clin Invest.* 1994;93:2393–2402.
- Isoda K, Nishikawa K, Kamezawa Y, et al. Osteopontin plays an important role in the development of medial thickening and neointimal formation. *Circ Res.* 2002;91:77–82.
- Hedrick CC, Castellani LW, Warden CH, et al. Influence of mouse apolipoprotein A-II on plasma lipoproteins in transgenic mice. *J Biol Chem.* 1993;268:20676–20682.
- Johnson RC, Chapman SM, Dong ZM, et al. Absence of P-selectin delays fatty streak formation in mice. *J Clin Invest.* 1997;99:1037–1043.
- Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J Lipid Res.* 1995;36:2320–2328.
- Ashkar S, Weber GF, Panoutsakopoulou V, et al. Eta-1 (Osteopontin): an early component of type-1 (cell-mediated) immunity. *Science.* 2000;287:860–864.
- Mallat Z, Besnard S, Duriez M, et al. Protective role of interleukin-10 in atherosclerosis. *Circ Res.* 1999;85:e17–e24.
- Pinderski Oslund LJ, Hedrick CC, Olvera T, et al. Interleukin-10 blocks atherosclerotic events *in vitro* and *in vivo*. *Arterioscler Thromb Vasc Biol.* 1999;19:2847–2853.
- Busse R, Fleming I. Endothelial dysfunction in atherosclerosis. *J Vasc Res.* 1996;33:181–194.
- Iizuka J, Katagiri Y, Tada N, et al. Introduction of an osteopontin gene confers the increase in B1 cell population and the production of anti-DNA autoantibodies. *Lab Invest.* 1998;78:1523–1533.
- Zhou X, Paulsson G, Stemme S, et al. Hypercholesterolemia is associated with a T helper 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J Clin Invest.* 1998;101:1717–1725.
- Frostegard J, Ulfgren AK, Nyberg P, et al. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis.* 1999;145:33–43.