Osteopontin Plays an Important Role in the Development of Medial Thickening and Neointimal Formation

Kikuo Isoda, Kenichirou Nishikawa, Yashuhiro Kamezawa, Mikoto Yoshida, Masatoshi Kusuhara, Masao Moroi, Norihiro Tada, Fumitaka Ohsuza

Abstract—Osteopontin (OPN) is a soluble secreted phosphoprotein that binds with high affinity to several integrins and it has been found at the site of atherosclerotic lesions. However, the role of OPN expression in vivo is still poorly understood. To investigate the physiological role of OPN in detail, we generated transgenic mice (Tg) overexpressing the OPN gene under control of the cytomegalovirus enhancer/chicken β-actin promoter. We detected OPN mRNAs in almost all tissues of 3 lines of Tg mice by Northern blotting. The serum levels of OPN were significantly higher in Tg than in non-Tg mice (782 ± 107 versus 182 ± 44 ng/mL; P < 0.001). Compared with non-Tg mice, a 73% (88 ± 6 versus 51 ± 7 μm; P < 0.001) and 94% (126 ± 15 versus 73 ± 11 μm; P < 0.0001) increase in the medial thickness of the aorta was determined in Tg mice at 16 and 32 weeks after birth. However, we found no evidence of inflammatory cells adhering to endothelial cells, intimal hyperplasia, or calcification in any region of Tg mice without artery injury. We then investigated the effect of cuff-induced injury to the femoral artery. The intimal thickening in Tg mice increased 2.9-fold more than that in non-Tg mice (4.9 ± 1.9 versus 1.7 ± 0.4 μm; P = 0.022). The expression of OPN induces both medial thickening without injury and neointimal formation after injury, thus suggesting that OPN plays a role in the development of atherosclerosis, vascular remodeling, and restenosis after angioplasty in vivo. (Circ Res. 2002;91:

Key Words: aorta • atherosclerosis • genes • inflammation • smooth muscle

Osteopontin (OPN) is a phosphoprotein that was originally isolated from the bone. It possesses the tripeptide sequence Arg-Gly-Asp like other extracellular matrix proteins and serum proteins such as fibronectin, vitronectin, and collagen.1–2 OPN functions as a cell adhesion and migration molecule that can bind to several ligands including αβ, integrin, CD44, and fibronectin.3–5 A wide range of cell types including some epithelia, macrophages, T cells, and vascular smooth muscle cells (SMCs) express OPN in a constitutive or inducible fashion.6–9 OPN has recently emerged as a key factor in both vascular remodeling and in the development of atherosclerosis.10–12 In vitro studies have shown that OPN also promotes the proliferation of cultured rat vascular SMCs13 and human coronary artery SMCs.14 Furthermore, a previous study has shown OPN to stimulate Arg-Gly-Asp-dependent endothelial migration in vitro.15 However, the function of OPN in the vessel walls is still not yet well understood. The present study uses OPN transgenic mice (Tg) to determine the effect of OPN on SMCs in vivo and to investigate the effect of cuff-induced injury to the femoral artery in vivo. Our results show that OPN overexpression is associated with a significant increase in medial thickening with aging in vivo and in intimal thickening after arterial injury.

Materials and Methods

Construction of Transgene

We prepared a transgene construct containing the cytomegalovirus enhancer/chicken β-actin (CAG) promoter with cDNA to murine OPN. The CAG-OPN transgene was constructed by inserting the 1.35-kb EcoRI fragment containing mouse OPN cDNA sequence into the EcoRI site of the third exon of the rabbit β-globin gene in pBsCAG-2.16,17 OPN cDNA contains 34 bp of the 5’ noncoding region, 885 bp of the coding region, and 429 bp of the 3’ untranslated region. Sequencing revealed that the OPN cDNA was identical to the described mouse OPN cDNA.18 The resulting plasmid was termed pCAG/OPN-9. The CAG-OPN transgene was isolated from the pCAG/OPN-9 backbone by Sall and BamHI digestion.

Generation and Identification of Transgenic Mice

Transgenic animals were generated by microinjecting purified CAG-OPN construct into the pronuclei of hybrid eggs from B6C3F1 × C57BL/6 strain parents. The tails of the offspring were biopsied at 3 weeks of age. Genomic DNA was isolated using proteinase K. Transgenic mice were identified by Southern blotting. Genomic DNA (10 μg) was digested with EcoRI, which released the 1.35-kb internal fragment of the CAG-OPN construct. All blotted
membranes were probed with 1.35-kb mouse OPN cDNA labeled with \(^{32}\)P dCTP using a random prime labeling kit (Boehringer Mannheim). The National Defense Medical College Board for Studies in Experimental Animals approved the studies.

**Northern Blot Analysis**
Fifteen micrograms of RNAs were subjected to electrophoresis and then were transferred to a nylon membrane. The RNAs were fixed on this membrane. Hybridization was performed as described previously.\(^{19}\)

**Femoral Artery Injury**
The animals were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). The left femoral artery was exposed under sterile conditions, and a nonocclusive, flexible polyethylene cuff (length 2 mm; internal diameter 0.56 mm; Becton Dickinson) was placed around the femoral artery to induce intimal thickening, as described.\(^{20}\) The right femoral artery was dissected from the surrounding tissues (sham-operated), but no cuff was put in place. The mice were killed 14 days after cuff placement for a morphometric analysis.

**Tissue Preparation and Histology**
After tail-cuff, systolic blood pressure was measured in the mice, then the animals were euthanized with pentobarbital and perfused with 0.9% NaCl followed by 10% formalin through a 22-gauge angiocatheter placed in the left ventricle of the heart. The aorta and femoral artery were fixed in 10% formalin for 48 hours and embedded in paraffin. Serial 10-μm sections cut from the aorta or femoral artery were examined by staining with hematoxylin–eosin and Masson’s trichrome, as well as by immunohistochemistry.

**Morphometry**
We examined the hematoxylin–eosin–stained tissue specimens by morphometry. The thickness of the intima and media were measured in each aorta from uninjured mice. Regarding the morphometric analyses of the femoral artery, 10 cross-sections from the cuffed left femoral artery and the control right femoral artery were photographed for each animal. For each artery section, the thickness of the intima and media were measured. Regarding the area/volume calculations, 4 measurements were made using an image analysis computer program (NIH Image; National Institutes of Health): luminal circumference, luminal area, area inside the inner elastic lamina, and area inside the outer elastic lamina. The mean vascular diameter was calculated as luminal circumference/π. The intima was defined as the area between the lumen and the internal elastic lamina. The media was defined as the area between the internal and external elastic laminas. The volumes of the intima and media were calculated by integrating the areas over the length of the cuffed region.\(^{20}\)

The observers of the sections were blinded to the genotype of the mice. The intraassay variability, using independent observers or analyzing adjacent sections from the same vessel, was <3.

**Immunohistochemistry and Enzyme Immunoassay (EIA)**
Immunohistochemistry was proceeded on paraffin-embedded sections. The following primary antibodies were used: a rat anti-mouse IgG antibody raised against OPN (Immuno-Biological Laboratory), a primary rat monoclonal antibody against mouse macrophage, clone MOMA-2 (BioSource International), and polyclonal rabbit anti-mouse proliferation cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology). The sections were visualized using a Vectastain ABC kit (Vector Laboratories, Inc) with diaminobenzidine as the substrate. The plasma OPN concentration was measured using an EIA kit (Immuno-Biological Laboratory).

**Substrate Gel Zymography**
The protein extracts of aortic tissue were prepared as previously reported,\(^{21}\) and samples (10 μg) were resolved by nonreducing 10% SDS-PAGE through gels containing 1 mg/mL gelatin. The gels were washed with 2.5% Triton X-100 to remove the SDS, and then were incubated overnight at 37°C in 50 mmol/L Tris-HCl, pH 8.5, 5 mmol/L CaCl₂, and 0.5 mmol/L ZnCl₂. The zones of lysis were visualized after staining the gels with 0.5% Coomassie blue R-250. A densitometric analysis was performed using the NIH Image.

**Aortic SMC Explant Migration**
The descending aorta was thoroughly dissected free from connective tissue, cut open longitudinally, and the intima and a thin portion of the subjacent media were removed. The descending aorta was weighed and trimmed to normalize the weight to 10 mg. The aorta was cut into 4 pieces and each piece was placed into a separate well of a 6-well plate containing DMEM+20% FBS.

**Statistical Analysis**
The results are shown as the mean±standard deviation. Two groups were compared using Student’s t test or Student-Newman Keuls’s test with the 1-way analysis of variance. A value of \(P<0.05\) was regarded as a significant difference.

**Results**
**Generation of Transgenic Mice**
We obtained 7 lines of Tg, and 3 lines of them showed high expression of OPN. We examined the expression of OPN mRNA in 6-week-old homozygous Tg mice and in non-Tg mice. These 3 lines expressed high levels of OPN mRNA in almost all tissue specimens, and the kidney and aorta expressed OPN mRNA in both Tg and non-Tg mice (Figures 1A and 1B). The serum levels of OPN determined by EIA were significantly higher in OPN-Tg than in non-Tg mice (Tg, 782±107 ng/mL [n=6]; non-Tg, 182±44 ng/mL [n=6], 6 weeks old; \(P<0.0001\)). Homozygous Tg mice showed poor growth, osteopenia, and a higher death rate than the non-Tg mice. Despite an absence of death in non-Tg mice at 12 months, the cumulative mortality rate of Tg mice at the same time was 43% (35 of 81 Tg mice were found to have died spontaneously). The dead Tg mice showed a high rate of malignant tumors in liver and/or intestine and/or lung (data not shown). Because homozygous Tg mice were infertile, we crossed heterozygous male and heterozygous female mice to obtain homozygous offspring. Regarding blood pressure, systolic blood pressure of OPN-Tg mice was as low as that of non-Tg mice (OPN-Tg [n=10, 32 weeks old], 108±12 mm Hg versus non-Tg [n=10, 32-week-old], 101±12 mm Hg; \(P=NS\)). Male and female mice did not differ markedly.

**Histology of Aorta**
We characterized the changes mainly in the vessel. Figure 2 shows the ascending aorta of OPN-Tg and non-Tg mice 32 weeks after birth that were fed with normal chow. Hematoxylin and eosin staining (Figures 2A and 2B) revealed an increase in the medial thickness and the number of nuclei of SMCs, but no evidence of inflammatory cells adhering to endothelial cells, intimal hyperplasia, or calcification in any region in the OPN-Tg mice. Furthermore, no macrophages (staining for MOMA-2) were detected in either OPN-Tg or non-Tg mice (data not shown). Masson’s trichrome-stained sections (Figures 2C and 2D) showed the destruction of the elastic lamina in OPN-Tg. Figures 2E and 2F showed that the medial layers of the aorta derived from OPN-Tg mice were...
positive for the anti-mouse OPN antibody, whereas the aorta from the non-Tg mice was negative despite little expression of OPN mRNA. Moreover, OPN was essentially detected in the SMCs of the media of OPN-Tg mice. We next performed PCNA staining to find any evidence of cell proliferation in the SMCs of the media of OPN-Tg mice. The nuclei of SMCs of OPN-Tg mice stained positively for PCNA (Figure 3A). In contrast, the nucleus of non-Tg mice did not stain for PCNA (Figure 3B). These results indicated that the increased medial thickness in OPN-Tg is actually due to an increased proliferation of SMCs.

Figure 1. OPN mRNA expression in various tissues from OPN-Tg (A) and non-Tg (B) mice. Northern blots of 15 μg total RNA from each tissue were hybridized with a 32P-labeled 1.35-kb mouse OPN probe. GAPDH served as an inner marker. Signal intensity of each gene was normalized for GAPDH.

Figure 2. Histology and immunochemical staining of OPN-Tg (left) and non-Tg (right) mouse aortas harvested 32 weeks after birth. Sections were adjacent sections processed for hematoxylin and eosin staining (A and B), elastin staining (C and D), and immunohistochemical staining for OPN (E and F). Bar=50 μm.
We next investigated the change of medial thickness dependent on aging (Figure 4). At 6 weeks old, the medial thickness of the aorta in the OPN-Tg tended to be larger (but not significant) than that of non-Tg mice (OPN-Tg \( n=10 \), 41±4 \( \mu \)m; non-Tg \( n=10 \), 36±4 \( \mu \)m, NS). At 16 weeks old, the medial thickness of the aorta in the OPN-Tg mice increased by 73% (88±6 \( \mu \)m, \( n=10 \); \( P<0.001 \)). At 32 weeks old, the medial thickness in OPN-Tg mice increased by 94% (126±15 \( \mu \)m, \( n=10 \)) compared with non-Tg mice (73±11 \( \mu \)m, \( n=10 \); \( P<0.0001 \)). These findings indicated the period between adolescence and young adulthood (between 6 weeks and 16 weeks old) to be the time when the biggest increase in the medial thickness occurs (from 14% to 73%) and that this increase in the medial thickness in OPN-Tg may be related to body development.

**Intimal Thickening After Injury**

We investigated the effect of cuff-induced injury to the femoral arteries of OPN-Tg mice at 16 weeks old. Figure 5 shows representative cross sections of femoral arteries harvested 14 days after the induction of injury. The intima appears within the internal elastic lamina. More neointima forms in response to cuff injury in OPN-Tg than in non-Tg mice. Although inflammatory cells are located within the adventitia of both non-Tg and OPN-Tg mice, very little neointima forms in non-Tg. The morphometric measurements of the intimal and medial thickness 14 days after injury in both groups of mice are shown in the Table. No control intima was present in the femoral arteries of either OPN-Tg or non-Tg mice, and the control medial thickness in OPN-Tg mice was larger than that in non-Tg mice like aorta. The extent of the cuffed intimal thickening in OPN-Tg mice increased 2.9-fold more than that in non-Tg mice (4.9±1.9 versus 1.7±0.4 \( \mu \)m; \( P=0.022 \)). The overexpression of OPN was also associated with a 1.3-fold increase in cuffed medial thickening in comparison to the medial thickening observed in non-Tg mice (16.1±2.7 versus 12.7±1.8 \( \mu \)m; \( P=0.046 \)). Because the cuffed medial thickness increased less than the cuffed intimal thickness, the intima/media ratio was much higher in OPN-Tg (26.1±9.6%), than in non-Tg (12.9±3.5%; \( P=0.034 \)) mice.

![Figure 3](image1.png)

**Figure 3.** Immunochemical staining for PCNA of OPN-Tg (A) and non-Tg (B) mouse aorta harvested 6 weeks after birth. Mayer's hematoxylin counterstaining is shown. Arrowheads indicate positive nuclei. Bar=20 \( \mu \)m.

![Figure 4](image2.png)

**Figure 4.** Comparison of the medial thickness of the ascending aorta between 6- (left), 16- (middle), and 32- (right) week-old OPN-Tg and non-Tg mice. Values represent the mean±SD. NS indicates not significant. \( *P<0.001 \); \( **P<0.0001 \).

![Figure 5](image3.png)

**Figure 5.** Sections of the femoral arteries harvested 14 days after the induction of injury stained with hematoxylin and eosin. Cross section of the femoral arteries from OPN-Tg (A) and non-Tg (B) mice. Intima is shown within the internal elastic lamina. OPN-Tg mice show more neointimal formation in response to cuff injury than non-Tg mice. Bar=20 \( \mu \)m.
Zymography
We performed zymography to confirm whether MMP-2 and MMP-9 was expressed in the aorta of either OPN-Tg or non-Tg. The amount of active MMP-2 increased by 6.1 ± 1.9-fold (P < 0.01) in the aortas from OPN-Tg mice compared with those from non-Tg mice, and the pro-MMP-2 also differed (1.6 ± 0.4-fold; P < 0.05) (Figure 6). Pro-MMP-9 also increased in the aortas from OPN-Tg mice (3.3 ± 0.4-fold; P < 0.0001). These findings indicate that OPN might stimulate MMP-2 and MMP-9 production.

SMC Migration
To assess the ex vivo effects of OPN on SMC migration in the OPN-Tg, we examined the ability of SMCs to migrate out from aortic explants. Explant migration of aortic SMCs was performed with OPN-Tg or non-Tg. After 8 days from the aortic explant, a 16- to 27-fold promotion of migration compared with non-Tg was observed in the OPN-Tg (Figure 7). This result correlates with the findings obtained in the cuff model and suggest that OPN strongly contributes to the promotion of SMC migration.

Discussion
The present study shows that high levels of OPN are expressed in the medial layer of the aorta in OPN-Tg mice and that medial thickening in these animals increased from 73% to 94% more than that in non-Tg mice. This study also shows that more neointima formed after arterial injury in OPN-Tg than in non-Tg mice. These results suggest that OPN plays an important role in the pathological processes associated with arterial SMC proliferation, such as atherosclerosis and restenosis.

The present study demonstrates that the level of OPN expression significantly increased in the SMCs of OPN-Tg mice and that medial thickening increased significantly more in OPN-Tg than in non-Tg mice. Furthermore PCNA staining showed evidence of cell proliferation in the SMCs of the media of OPN-Tg mice. These findings suggest OPN expression to be associated with SMC proliferation. Recent reports show that OPN is upregulated in the aortas of mice with diabetes induced by a high-fat diet22 and in the forearm arteries of patients with diabetes mellitus.23 These reports suggest that OPN production in vascular SMCs synergically

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### Table 1: Response to Vessel Injury in OPN-Tg and Non-Tg Mice

<table>
<thead>
<tr>
<th></th>
<th>OPN-Tg</th>
<th>Non-Tg</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Control intima, μm</td>
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<td>5</td>
<td></td>
</tr>
<tr>
<td>Control media, μm</td>
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<td>NS</td>
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<tr>
<td>Cuffed intima, μm</td>
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<td>13.5±1.3</td>
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<tr>
<td>Cuffed media, μm</td>
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<td>12.7±1.8</td>
<td>0.046</td>
</tr>
<tr>
<td>Cuffed I/M ratio, %</td>
<td>26.1±9.6</td>
<td>12.9±3.5</td>
<td>0.034</td>
</tr>
</tbody>
</table>

I/M ratio indicates intima/media ratio (μm³/μm³×100); NS, not significant. Values represent the mean±SD.

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Figure 6. Representative gelatin zymographic analysis of protein extracts from the aortas of OPN-Tg and non-Tg. Active MMP-2 and pro-MMP-9 were more prevalent in OPN-Tg. Purified MMP-2 and MMP-9 protein were used standard (STD) in zymography (left lane).

Figure 7. Explants of the aortas. SMCs started to grow out from the explants after 5 to 10 days in culture. At 8 days after explant, the SMC number increased more in OPN-Tg (A) than in non-Tg (B). Bar=200 μm.
augment their migration and proliferation and, thereby, facilitate the development of atherosclerosis in diabetes mellitus. The suggestions of our and other’s studies are supported by the results of other in vitro studies. Giachelli et al reported the OPN mRNA level to be higher in cultured pup cells, which have a high proliferative capacity, than in adult SMCs. Gadeau et al analyzed the OPN expression in serum-stimulated quiescent SMCs and in asynchronously cycling SMCs in vitro and demonstrated that OPN overexpression is associated with SMC proliferation. These results suggest that an OPN expression is closely related to arterial SMC proliferation both in vitro and in vivo.

Regarding the factors of medial thickening, there are several ones, such as hemodynamic factors and inflammation. Blood pressure-induced stretch provokes remodeling in vascular SMCs and transmural pressure promotes SMC proliferation. However, no such hemodynamic effects significantly affected the medial layer thickening induced by OPN overexpression, because a pressure study revealed that the systolic blood pressure of OPN-Tg mice was as low as that of non-Tg mice. We therefore believe that the medial thickening in OPN-Tg mice was not due to hemodynamic factors. Although the adhesion of mononuclear cells to, and their infiltration into, the blood vessel wall is associated with the induction of medial thickening, we also believe that inflammation is not responsible for the medial thickening observed in our Tg mice. Because the analysis of hematoxylin and eosin–stained sections (Figures 2A and 2B) showed no evidence of inflammatory cells adhering to endothelial cells or their infiltration into the vessel wall in any region in the OPN-Tg mice.

Our analysis of zymography confirmed that MMP-2 or MMP-9 did increase in the OPN-Tg aorta, but they were not expressed to a significant degree in the normal mouse aorta. This result is partly supported by the results of a previous in vitro study in which the stimulation of SMCs with OPN was reported to increase matrix metalloproteinases. Furthermore, another in vitro study showed that OPN-stimulated MMP-2 activation occurred through NF-κB–mediated induction of membrane type 1 MMP, and thus, that report suggested that OPN-induced MMP-2 production could be regulated at the transcriptional level. The increase in the amount of MMP-2 and MMP-9 in the OPN-Tg may thus play an important role in the destruction of the elastic lamina and the promotion of the explant migration of aortic SMCs.

OPN promotes rat and bovine SMC migration. High levels of OPN mRNA and protein are detectable in the rat and human aorta and carotid arteries during neointima formation. Moreover, suggested that the migration of SMCs from their original location in the arterial media toward the intima may be closely related to high circulating levels of OPN in the blood. However, there was no neointimal formation in our OPN-Tg mice without cuff-induced injury, although the levels of both OPN mRNA and serum OPN protein in OPN-Tg mice were high and immunohistochemistry revealed that SMCs of the aorta derived from OPN-Tg mice were positive for the anti-mouse OPN antibody. Furthermore, the explant migration of aortic SMCs increased in OPN-Tg mice. These results suggest that SMC migration and neointima formation by OPN are induced when the intima is impaired. Indeed, most in vivo studies, which suggest that OPN may play a role in SMC migration and/or neointima formation, were performed under such conditions, namely atherosclerosis and after angioplasty.

The migration of SMCs to the site of injury caused by angioplasty and a subsequent proliferation suggest the mechanisms of restenosis. OPN mRNA is expressed in the both rat aorta and carotid arteries, and the expression levels are significantly elevated after balloon angioplasty, thus suggesting an important role for this matrix protein in vascular remodeling. Our results indicate that OPN overexpression increases the neointimal proliferative response to vessel injury from cuff placement. Taken together, these data imply that OPN plays a role in the development of restenosis after angioplasty and these findings also lead us to assume that the inhibition of OPN gene expression at sites of injury may thus be viewed as a new therapeutic approach for preventing restenosis. Indeed, previous studies have demonstrated that a blockade of αβ integrin, an OPN receptor and mediator of SMC migration after angioplasty, resulted in a reduction of neointimal formation. Furthermore, another recent study reported that neutralizing antibodies against OPN decreased the neointimal areas and cell numbers after endothelial denudation.

In conclusion, this study demonstrated that OPN plays an important role in the development of vascular medial thickening without injury and in neointima formation after arterial injury in vivo.

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References
smooth muscle cell phenotypes in vitro and is identical to osteopontin (secreted phosphoprotein I, 2aR). Biochem Biophys Res Commun. 1991; 177:867–873.


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