Enhanced Expression of Osteopontin in Human Diabetic Artery and Analysis of Its Functional Role in Accelerated Atherogenesis

Minoru Takemoto, Koutaro Yokote, Motonobu Nishimura, Takashi Shigematsu, Toshio Hasegawa, Shigeyuki Kon, Toshimitsu Uede, Taro Matsumoto, Yasushi Saito, Seijiro Mori

Abstract—We have previously reported that high glucose stimulates osteopontin (OPN) expression through protein kinase C–dependent pathways as well as hexosamine pathways in cultured rat aortic smooth muscle cells. The finding prompted us to study in vivo expression of OPN in diabetes mellitus. In the present study, we found by immunohistochemistry that medial layers of the carotid arteries of streptozotocin-induced diabetic rats and the forearm arteries of diabetic patients stained positively for OPN antibodies, whereas the staining from arteries of control rats and nondiabetic patients was negative. We also found that OPN stimulated the migration and enhanced platelet-derived growth factor (PDGF)-mediated DNA synthesis of cultured rat aortic smooth muscle cells. OPN and PDGF synergistically activated focal adhesion kinase as well as extracellular signal–regulated kinase; this finding seems to explain the OPN-induced enhancement of PDGF-mediated DNA synthesis. Taken together, our present results raise a possibility that OPN plays a role in the development of diabetic vascular complications. (Arterioscler Thromb Vasc Biol. 2000;20:624-628.)

Key Words: diabetic macroangiopathy ■ osteopontin ■ platelet-derived growth factor ■ vascular smooth muscle cells

It is generally accepted that diabetic patients often suffer from atherosclerotic vascular diseases, such as ischemic heart disease and arteriosclerosis obliterans.1,2 It is also known that diabetic vascular lesions tend to undergo restenosis after angioplasty and that diffuse calcification of the affected arteries is a characteristic feature of diabetic vascular diseases.3–5 However, the reason for the accelerated atherosclerosis in diabetes mellitus has not been fully elucidated.

Osteopontin (OPN) is a multifunctional phosphoprotein secreted by many cell types, such as osteoclasts, lymphocytes, macrophages, epithelial cells, and vascular smooth muscle cells (SMCs).6 Overexpression of OPN has been found in several physiological as well as pathological conditions, including immunologic disorders,7 neoplastic transformation,8 progression of metastases,8 formation of urinary stones,9 and wound healing.10

It has been reported that OPN protein and mRNA are expressed in the neointima as well as in calcified atheromatous plaque.11 A neutralizing antibody against OPN has been found to inhibit rat carotid neointimal formation after endothelial denudation.12 It has also been reported that OPN inhibits the calcification of vascular SMCs in culture.13 These reports have suggested that OPN contributes not only to the tissue calcification process but also to the development of atherosclerosis, especially in the process of intimal thickening.

We have recently reported that high glucose levels stimulate OPN expression through protein kinase C–dependent pathways as well as hexosamine pathways in cultured rat aortic SMCs.14 The present study was undertaken to gain more insight into the mechanism of diabetic vascular complications. We first demonstrate that OPN protein is highly expressed in the medial layers of the arteries of diabetic rats and patients. Furthermore, OPN stimulates migration and enhances platelet-derived growth factor (PDGF)-mediated DNA synthesis of SMCs, possibly by promoting the activation of focal adhesion kinase (FAK) as well as extracellular signal–regulated kinase (ERK). The present data suggest that OPN plays a role in the accelerated atherogenesis in diabetes mellitus.

Methods

Reagents

Recombinant OPN was affinity-purified by using an antibody column filled with a monoclonal anti-human OPN antibody (10A16, IBL) from conditioned medium of full-length human OPN cDNA-transfected CHO-K1 cells.15 Recombinant human PDGF-BB was purchased from R&D Systems. A monoclonal anti-rat OPN antibody (MPIIIB10) was from American Research Products. A monoclonal
anti-α-smooth muscle actin antibody (1A4) was from Sigma Chemical Co. [Methyl-\textsuperscript{3}H]thymidine was from Amersham Pharmacia Biotech.

**Tissue Preparation**
Male Wistar rats weighing ~200 g were administered either 50 mg/kg streptozotocin (STZ, Sigma) or normal saline into tail veins as described previously.\textsuperscript{16} Four weeks later, the carotid arteries were thoroughly perfused with 4% paraformaldehyde in PBS, excised, fixed with 4% paraformaldehyde in PBS for 4 hours at 4°C, rinsed with PBS, and incubated with 30% sucrose in PBS for 3 hours at 4°C. Then the fixed arteries were mounted in Tissue-Tek embedding OCT compound (Miles Inc), snap-frozen, and subsequently stored at −70°C before sectioning.\textsuperscript{17} A piece of vascular wall was excised from the forearm arteries of 6 patients with end-stage renal disease during a shunt operation after obtaining written informed consent for their excised arteries to be used in the present experiment. Of the 6 patients, 5 were men; the ages of the 6 patients ranged between 40 and 80 years, with a mean age of 62 years. Three of the patients were diabetics, 2 patients had been treated by diet restriction only, and the other patient had been treated by diet restriction and subcutaneous insulin infusion. The samples were fixed in 4% paraformaldehyde at 4°C for 24 hours, mounted in Tissue-Tek embedding OCT compound, snap-frozen, and subsequently stored at −70°C before sectioning.\textsuperscript{17}

**Immunohistochemistry**
Immunohistochemistry was performed essentially as described.\textsuperscript{18} For the identification of OPN, we used the anti-human OPN antibody (10A16, 5 μg/mL) as well as the anti-rat OPN antibody (MPIIIB10, 1:250 dilution), which also cross-reacts with human OPN, according to the manufacturer’s instructions. For the identification of SMCs, we used the anti-α-smooth muscle actin antibody (1A4, 1:1000 dilution). Calcium deposition was assessed by von Kossa staining.\textsuperscript{19}

**Cell Culture**
Rat aortic SMCs were isolated by the explant method from adult male Wistar rats, and the primary culture and subculture were carried out as described.\textsuperscript{20} Cells at the 4th to 9th passages were used for the present study.

**Migration Assay**
A chemotactic response was assayed essentially as described\textsuperscript{21} with use of a 96-well microchemotaxis chamber (Neuro Probe). Cells that had migrated to the lower surface of the membrane were fixed and stained, and the number of the cells was estimated by using a microplate reader (TOSHO) at 600 nm. Experiments were performed in quadruplicate and were repeated at least 3 times.

\[[^{3}H] \text{Thymidine Incorporation Assay}\]
DNA synthesis was estimated by measuring [\textsuperscript{3}H]thymidine incorporation into trichloroacetic acid–precipitable materials of cultured cells as described.\textsuperscript{22} Experiments were performed in quadruplicate and were repeated at least 3 times.

**Assays for FAK and ERK**
Confluent SMCs in 6-cm dishes were serum-starved for 18 hours. Then the cells were treated with different concentrations of PDGF-BB and OPN at 37°C for 10 minutes. After incubation, activities of FAK\textsuperscript{23} and ERK\textsuperscript{24} were assayed in the cells exactly as described. Densitometric analyses of the detected bands on gels were performed by use of an imaging scanner (EPSON ES 8000) with NIH Image 1.44 software.

**Statistical Analysis**
Data are expressed as mean±SEM. The significance of differences was evaluated by Student t test.

**Results**

**OPN Protein Is Highly Expressed in Medial Layers of Carotid Arteries in STZ-Induced Diabetic Rats and Forearm Arteries in Diabetic Patients**
We have previously found that high glucose levels potently enhance OPN expression in cultured rat aortic SMCs.\textsuperscript{14} The finding prompted us to study the in vivo expression of OPN in diabetes mellitus. We examined OPN expression in STZ-induced diabetic rats by immunohistochemistry with use of a monoclonal anti-rat OPN antibody (MPIIIB10). Two diabetic rats and 2 control rats were examined. The mean blood glucose level of the diabetic rats (29.6 mmol/L) was much higher than that of the control rats (9.3 mmol/L). As shown in Figure 1, the staining of the medial layers of the carotid arteries derived from both of the diabetic rats was positive for the OPN antibody (panel B), whereas the staining of the arteries from the control rats was negative (panel A). These results led us to further examine OPN expression in human samples. A piece of the forearm arteries was collected during shunt operations from age-matched diabetic (n=3) and non-diabetic (n=3) patients. As shown in Figure 1, medial layers of the samples derived from all the diabetic patients stained positively for a monoclonal anti-human OPN antibody (10A16, panel D), whereas the staining of samples from the nondiabetic patients was negative (panel C). The results were confirmed by using another monoclonal anti-OPN antibody (MPIIIB10). The OPN-stained areas also stained positively with an anti-α-smooth muscle actin antibody (1A4; data not shown), suggesting that the OPN was produced by SMCs. No obvious relation was found between OPN expression and calcium deposition (data not shown).

**OPN Not Only Stimulates Migration but Also Enhances PDGF-BB–Mediated DNA Synthesis of SMCs**
To evaluate a possible role of OPN in atherogenesis, biological effects of recombinant human OPN on cultured rat aortic SMCs were analyzed. We first examined the effect of OPN on the migration of SMCs by modified Boyden chamber assay. As shown in Figure 2, OPN dose-dependently enhanced SMC migration. The migratory response was confirmed to be chemotactic, as assessed by checkerboard analyses (data not shown).

We next examined the effect of OPN on DNA synthesis of SMCs by a [\textsuperscript{3}H]thymidine incorporation assay. It was found that OPN by itself did not stimulate DNA synthesis of SMCs (data not shown). However, as shown in Figure 3, OPN enhanced DNA synthesis of SMCs, which were simultaneously stimulated with low-dose (1 ng/mL) PDGF-BB.

**PDGF-BB–Stimulated DNA Synthesis Is Mediated Possibly by Promoting Activation of FAK and ERK**
OPN is known to be a ligand for some integrins.\textsuperscript{25} Thus, we examined the effect of OPN on the activation of FAK, a downstream mediator of the integrin signaling pathway. After incubation of SMCs with PDGF-BB and/or OPN, the cell lysates were immunoprecipitated with the anti-FAK antibody,
and the samples were subjected to Western blotting. The blot was first probed with an anti-phosphotyrosine antibody. As shown in Figure 4, treatment with 1 ng/mL PDGF-BB did not appreciably enhance phosphorylation of FAK (top panel, lane 2). Likewise, treatment of OPN alone did not appreciably enhance FAK phosphorylation (data not shown). However, in the presence of 1 ng/mL PDGF-BB, OPN potently enhanced FAK phosphorylation (top panel, lanes 3 and 4). The same blot was then stripped and reprobed with the anti-FAK antibody. The intensity of the 125-kDa FAK band was not different among the lanes (middle panel).

The results led us to further examine the effect of OPN on activation of ERK, a putative downstream mediator of FAK.

Figure 1. OPN protein is highly expressed in medial layers of the carotid arteries in STZ-induced diabetic rats and the forearm arteries in patients with diabetes mellitus. The rat carotid arteries were removed and subjected to immunostaining with the anti-OPN antibody (MPIIIB10). A piece of the human forearm arteries was removed during shunt operation and subjected to immunostaining with the anti-OPN antibody (10A16). Immunodetection was performed by the ABC peroxidase method. A, Sample from 1 of 2 control rats (magnification ×400). B, Sample from 1 of 2 diabetic rats (magnification ×400). C, Sample from 1 of 3 nondiabetic patients (magnification ×400). D, Sample from 1 of 3 diabetic patients (magnification ×400).

Figure 2. OPN stimulates migration of cultured rat aortic SMCs. Cells (5×10⁵) were placed on the upper chamber, and OPN was added to the lower chamber and then incubated for 4 hours at 37°C. After incubation, cells that had migrated to the lower surface of the membrane were fixed and stained. The measurement of migrant cells was performed by a microplate reader. Data are mean±SEM of quadruplicate determinations. *P<0.05 vs control; **P<0.005 vs control.

Figure 3. OPN enhances PDGF-BB–mediated DNA synthesis in cultured rat aortic SMCs. Cells were serum-starved for 48 hours and then incubated with the indicated concentrations of PDGF-BB and OPN at 37°C for 18 hours. Then [³H]thymidine (1 μCi/mL) was added, and the cells were maintained in culture for an additional 8 hours. After removal of the culture medium, trichloroacetic acid–precipitable radioactivity was measured with a scintillation counter. Data are mean±SEM of quadruplicate determinations. *P<0.01 vs control.
SMCs were serum-starved and stimulated with PDGF-BB and/or OPN. After stimulation, ERK was collected by immunoprecipitation and subjected to in vitro kinase assay in the presence of Elk-1. ERK activity was estimated by the degree of Elk-1 phosphorylation, which was detected by immuno blotting with the specific antibody against phosphorylated Elk-1. As shown in Figure 5, treatment with 1 ng/mL PDGF-BB activated ERK to some degree (lane 3). Treatment with OPN alone also slightly activated ERK (lane 4), but in the presence of 1 ng/mL PDGF-BB, OPN potently activated ERK (lanes 5 and 6).

Discussion

In the present study, we show that OPN is highly expressed in medial layers of the carotid arteries in STZ-induced diabetic rats as well as in the forearm arteries of patients with diabetes mellitus (Figure 1). Our present study also shows that OPN stimulates migration and enhances PDGF-BB–mediated DNA synthesis of cultured rat aortic SMCs (Figures 2 and 3). These results, together with our previous finding that high glucose levels stimulate OPN expression in cultured rat aortic SMCs, imply that OPN plays a role in the development of diabetic vascular complications.

In vivo expression of OPN has previously been analyzed in several diabetic animal models. Towler et al demonstrated the upregulation of OPN expression in the aortas of high-fat diet–induced diabetic mice. Fischer et al reported that the upregulation of OPN expression in the renal cortex of STZ-induced diabetic rats was mediated by bradykinin. We also found the enhanced expression of OPN mRNA in the aortas of STZ-induced diabetic rats (data not shown). However, data concerning OPN expression in human subjects with diabetes mellitus have not yet been reported. In the present study, we have demonstrated for the first time that OPN expression is also enhanced in human diabetic arteries, confirming that the diabetes-induced upregulation of OPN expression is a general phenomenon observed across species.

OPN was shown to promote chemotaxis of different cell types, including vascular SMCs, macrophages, and osteoclasts. OPN was also shown to promote proliferation of cultured human coronary artery SMCs and primary prostate epithelial cells. It has been reported that the OPN-stimulated migration of vascular SMCs is mediated by interaction of OPN with β3 integrin and, likewise, that the growth-promoting effect of OPN is mediated by αvβ3 integrin. In the present study, we have also found that OPN stimulates migration and enhances PDGF-BB–mediated DNA synthesis of cultured rat aortic SMCs; thus, we examined the effect of OPN on downstream events of the integrin signaling pathway. As shown in Figures 4 and 5, our present data clearly indicate that OPN and PDGF-BB synergistically activate FAK and ERK; this finding seems to explain the OPN-induced enhancement of PDGF-BB–mediated DNA synthesis observed in the present study.

We have previously reported that overexpression of the PDGF β-receptor in SMCs is a causative element in the accelerated growth of diabetic SMCs. The report, together with our present findings, raises a possibility that diabetes-induced upregulation of the PDGF β-receptor expression and OPN production in vascular SMCs synergistically augment their migration and proliferation and, thereby, facilitate the development of atherosclerosis in diabetes mellitus. Further study is necessary to examine this possibility.

Acknowledgments

This study was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan (Nos. 11144206 and 11838002) and from the Sumitomo Foundation for Medicine and Welfare.

References


Figure 5. PDGF-BB and OPN synergistically activate ERK in cultured rat aortic SMCs. Cells were seeded onto 6-cm dishes and serum-starved for 18 hours. Then the cells were incubated with the indicated concentrations of PDGF-BB and OPN at 37°C for 10 minutes. After incubation, the cells were lysed, and the lysates were immunoprecipitated with the anti-FAK antibody and separated by SDS-PAGE, followed by immunoblotting with the anti-phosphotyrosine antibody (top). The same blot was then stripped and reprobed with the anti-FAK antibody (middle). Degree of FAK phosphorylation was estimated by the ratio of phosphoprotein to total protein of FAK measured with the image analyzer. The data represent the average values of 2 independent experiments (bottom).


Enhanced Expression of Osteopontin in Human Diabetic Artery and Analysis of Its Functional Role in Accelerated Atherogenesis

Minoru Takemoto, Koutaro Yokote, Motonobu Nishimura, Takashi Shigematsu, Toshio Hasegawa, Shigeyuki Kon, Toshimitsu Uede, Taro Matsumoto, Yasushi Saito and Seijiro Mori

Arterioscler Thromb Vasc Biol. 2000;20:624-628
doi: 10.1161/01.ATV.20.3.624
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/20/3/624

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/