Cytokine and Atherosclerosis: A Possible Role of Osteopontin in Development of Diabetic Macroangiopathy

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**I. Introduction**

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

Osteopontin (OPN) is a multifunctional phosphoprotein secreted by many cell types, such as osteoclasts, lymphocytes, macrophages, epithelial cells and vascular smooth muscle cells (SMC). It was reported that OPN protein and mRNA were expressed in neointima as well as calcified atheromatous plaque. A neutralizing antibody against OPN inhibited rat carotid neointimal formation after endothelial denudation. It was also reported that OPN inhibited calcification of vascular SMC in culture. These reports have suggested that OPN contributes not only to tissue calcification process but to the development of atherosclerosis especially in the process of intimal thickening.

We have recently reported that high glucose stimulates OPN expression through protein kinase C-dependent pathway as well as the hexosamine pathway in cultured rat aortic SMC. 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 medial layers of diabetic rats’ and patients’ arteries. Furthermore, OPN stimulates migration and enhances platelet-derived growth factor (PDGF)-mediated DNA synthesis of SMC 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.

**II. Methods and results**

OPN protein is highly expressed in medial layers of the carotid arteries in streptozotocin (STZ)-induced diabetic rats and the forearm arteries in diabetic patients.

We examined OPN expression in STZ-induced diabetic rats by immunohistochemistry using a monoclonal anti-rat OPN antibody (MP III B 10). As shown in Figure 1, medial layers of the carotid arteries derived from the diabetic rats stained positive with the OPN antibody (panel B), whereas the staining of 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 operation from age-matched patients of three diabetics and three non-diabetics. As shown in Figure 1, medial layers
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 (MP III B 10). A piece of the human forearm arteries was removed during shunt operation and subjected to immunostaining with the anti-OPN antibody (10 A 16). Immunodetection was performed by the ABC peroxidase method. A. A sample from one of two control rats (×400). B. A sample from one of two diabetic rats (×400). C. A sample from one of three non-diabetic patients (×400). D. A sample from one of three diabetic patients (×400).

of the samples derived from all the diabetic patients stained positive with a monoclonal anti-human OPN antibody (10 A 16) (panel D), whereas the staining of the non-diabetic patients was negative (panel C). The results were confirmed by using another monoclonal anti-OPN antibody (MP III B 10). The OPN-stained areas also stained positive with an anti-α-smooth muscle actin antibody (1 A 4) (data not shown), suggesting that the OPN was produced by SMC.

OPN not only stimulates migration but also enhances PDGF-BB-mediated DNA synthesis of SMC.

In order to evaluate a possible role of OPN in atherogenesis, biological effects of recombinant human OPN on cultured rat aortic SMC were analyzed. We first examined the effect of OPN on migration of SMC 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 SMC by [3H] thymidine incorporation assay. It was found that OPN by itself did not stimulate DNA synthesis of SMC (data not shown). However, as shown in Figure 3, OPN enhanced DNA synthesis of SMC which were simultaneously stimulated with low dose (1 ng/ml) of PDGF-BB.

OPN-induced enhancement of PDGF-BB-stimulated DNA synthesis is mediated possibly by promoting the activation of FAK as well as ERK.

OPN is known to be a ligand for some integrins.
Figure 2  OPN stimulates migration of cultured rat aortic SMC. Cells (5 × 10⁵) were placed on the upper chamber and OPN was added to the lower chamber, and then incubated for 4 h at 37°C. After incubation, migrated cells to the lower surface of the membrane were fixed and stained. The measurement of migrant cells was performed by a microplate reader. Data are means±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 SMC. Cells were serum-starved for 48 h and then incubated with the indicated concentrations of PDGF-BB and OPN at 37°C for 18 h. Then [³H] thymidine (1 μCi/ml) was added and the cells were maintained in culture for an additional 8 h. After removal of the culture medium, trichloroacetic acid-precipitable radioactivity was measured using a scintillation counter. Data are means±SEM of quadruplicate determinations. *p<0.01 vs. control.

We thus examined the effect of OPN on activation of FAK; a downstream mediator of the integrin signaling pathway. After treatment of SMC 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 the anti-phosphotyrosine antibody. As shown in Figure 4, treatment with 1 ng/ml PDGF-BB did not appreciably enhance phosphorylation of FAK (upper panel, lane 2). Likewise treatment of OPN alone did not appreciably enhance FAK phosphorylation (data not shown). But, in the presence of 1 ng/ml PDGF-BB, OPN potently enhanced FAK phosphor-
Figure 4  OPN potently enhances phosphorylation of FAK in the presence of PDGF-BB in cultured rat aortic SMC. Cells were seeded onto 6-cm dishes and serum-starved for 18 h. Then the cells were incubated with the indicated concentrations of PDGF-BB and OPN at 37°C for 10 min. 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-phosphorytrosine antibody (upper panel). The same blot was then stripped and reprobed with the anti-FAK antibody (lower panel).

Figure 5  PDGF-BB and OPN synergistically activate ERK in cultured rat aortic SMC. Cells were seeded onto 6-cm dishes and serum-starved for 18 h. Then the cells were incubated with the indicated concentrations of PDGF-BB and OPN at 37°C for 10 min. After incubation, the cells were lysed and the lysates were immunoprecipitated with the anti-phospho ERK antibody. The immunoprecipitated samples were subjected to in vitro kinase assay in the presence of Elk-1. Elk-1 phosphorylation was detected by immunoblotting with the anti-phospho Elk-1 antibody. Standard phospho Elk-1 run in parallel is also presented in lane 1.

As shown in Figure 5, treatment with 1 ng/ml PDGF-BB activated ERK in 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 5-6).

III. 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 the forearm arteries in the 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 SMC (Figures 2-3). These results, together with our previous finding that high glucose stimulates OPN expression in cultured rat aortic SMC, imply that OPN plays a role in the development of diabetic vascular complications.

We have previously reported that overexpression of the PDGF β-receptor in SMC is a causative element in the accelerated growth of diabetic SMC. 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 SMC synergistically augment their migration and proliferation and, thereby, facilitate the development of atherosclerosis in diabetes mellitus. Further study is necessary to prove this possibility.

References
