Increased Expression of Thrombospondin-1 in Vessel Wall of Diabetic Zucker Rat

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Background—Thrombospondin-1 (TSP-1) expression in the vascular wall has been related to the development of atherosclerotic lesions and restenosis. TSP-1 promotes the development of neointima and has recently been associated with atherogenesis at a genetic level. Because TSP-1 expression is responsive to glucose stimulation in mesangial cells, we hypothesized that glucose may stimulate its production by vascular cells. Thus, TSP-1 expression in the blood vessel wall may increase, providing a molecular link between diabetes and accelerated vascular lesion development.

Methods and Results—To determine whether the expression level of TSP-1 in vessel wall is increased in diabetes, aorta and carotid arteries of Zucker rats were used for immunostaining, Western blotting, and in situ RNA hybridization. A significant increase in TSP-1 expression was found in the adventitia of blood vessels from diabetic rats. Consistent with the well-known antiangiogenic effect of TSP-1, the number of vasa vasorum was reduced in aortas from diabetic rats. In cultured endothelial cells, vascular smooth muscle cells, and fibroblasts, TSP-1 expression increased in response to glucose stimulation (>30-fold). After balloon catheter injury to carotid arteries, expression of TSP-1 protein and mRNA was higher at all time points in the vessels of diabetic rats.

Conclusions—Increased expression of TSP-1 in blood vessels in diabetes may represent a new link between diabetes, atherogenesis, and accelerated restenosis. This increase in TSP-1 production may be a direct response of vascular cells to glucose. (Circulation. 2003;107:3209-3215.)

Key Words: diabetes mellitus • aorta • cardiovascular diseases • glucose

Despite the advances made in therapeutic approaches to control blood glucose and insulin levels in diabetic patients, the precise and constant regulation of these levels remains problematic and leads to pathogenic consequences. Atherosclerosis is the most prevalent of these complications. Accelerated restenosis of blood vessels after surgical interventions represents yet another dangerous complication of diabetes. Nevertheless, the molecular basis for the atherosclerotic and restenotic complications of diabetes is not well understood.

Analysis of literature suggests that thrombospondin-1 (TSP-1), a multifunctional protein that interacts with a number of matrix proteins and cell surface receptors, may represent an important link between diabetes, high glucose levels, and the accelerated development of atherosclerotic lesions and restenosis in diabetes. Among the evidence for this linkage are the following: (1) TSP-1 levels are elevated in the plasmas and certain tissues of diabetic patients and diabetic animal models.1–6 (2) TSP-1 expression is regulated by glucose in mesangial cells in vitro.6,7 (3) TSP-1 induces vascular smooth muscle cell (SMC) proliferation and prevents endothelial cell (EC) growth, both of which effects would have proatherogenic consequences.8–11 (4) TSP-1 influences neointima formation after balloon injury of arteries.12,13 and its expression is increased in stent-induced neointima.14 (5) The results of a recent study have linked the TSP family, including TSP-1, with development of atherosclerosis at a genetic level.15–17

Given the emerging evidence for involvement of TSP-1 in atherosclerosis, changes in TSP-1 levels in the vascular wall exposed to high glucose may contribute to accelerated atherogenesis and restenosis in diabetes. To test the hypothesis that vascular cells respond to high glucose levels by producing more TSP-1, we examined the expression of TSP-1 protein and mRNA in vessel wall cells in vitro and in the vessels of lean and diabetic Zucker rats under basal conditions and in response to vascular injury.

Methods

Animal Procedures

Zucker rats18 9 to 12 weeks of age from Genetic Models, Inc (Indianapolis, Ind), were used in the described experiments, with at least 4 animals used at each time point.

Arterial Injury and Tissue Preparation

Injury to the carotid artery was performed by balloon deendothelialization as described previously.19,20 Animals were killed at 6 hours,
Detection of TSP-1 Expression in Vessel Wall of Zucker Rats

**TSP-1 Immunostaining**
Tissues were embedded into paraffin, and sections were stained with monoclonal anti-TSP-1 antibody (NeoMarkers, clone A6.1). TSP-1 expression was visualized with diaminobenzidine (DAB) chromogenic substrate. For every section, a negative control without anti-TSP-1 antibody was processed simultaneously. Staining of matching sets of tissues from lean and diabetic rats was done simultaneously. Sections were counterstained with hematoxylin and eosin (except von Willebrand factor [vWF] staining). vWF was visualized with anti-vWF antibody (AccurateChem).

**RNA Isolation and In Situ RNA Hybridization**
RNA isolation was performed with Trizol reagent (Invitrogen). Biotinylated RNA probe was synthesized in vitro with the Riboprobe system T7/SP6 (Promega). The 5′ terminal fragment of TSP-1 (600 base pairs of clone TS-33 from ATCC) was subcloned into pGEM-T vector (Promega) and used to synthesize antisense and sense riboprobes. One hundred fifty nanograms of mRNA per slide was used for hybridization. Sections were fixed in 2% 3-aminopropylthiethoxysilane in acetone, and the endogenous peroxidase activity was blocked. Then the sections were deproteinized with 40 μg/mL proteinase K, fixed in 4% paraformaldehyde, and dehydrated. Hybridization was performed for 3 hours at 55°C; slides were washed in 0.2× SSC overnight, blocked with BSA, and incubated with streptavidin-peroxidase (Sigma). Staining was developed with DAB (Sigma), and slides were mounted with Vectashield (Vector).

**Morphometry**
Morphometric analysis of the arterial segments was performed with computerized digital microscopic planimetry software (Image-Pro Plus 4.5.1, Media Cybernetics).

**Extraction of TSP-1 From Vessel Tissue**
Tissues collected from lean and diabetic rats were homogenized in buffer containing 0.6% SDS, 5% Triton X-100, and protease inhibitors and incubated for 2 hours at 4°C. After 15 minutes of centrifugation at 15 000g, the supernatants were used for Western blotting.

**Stimulation of Cultured Cells With Glucose**
Confluent human umbilical vein ECs, human aortic SMCs, and human foreskin fibroblasts were preincubated in 5 mmol/L glucose for 24 hours, then stimulated with 30 mmol/L cell culture–tested endotoxin-free (D-+)-glucose for 0.5 to 48 hours. After incubation, cells were lysed in hypotonic buffer with 0.66% NP-40 and centrifuged at 12 000g for 15 minutes. The supernatants were used for Western blotting (50 μg of protein per sample). Anti-TSP-1 antibody (NeoMarkers, clone A6.1) was used for TSP-1 detection.

**Statistical Analysis**
Values are mean±SEM. Probability values, derived with a t test, of ≤0.05 were considered significant.

**Results**

**TSP-1 Expression in Aorta and Carotid Artery of Diabetic Zucker Rats**
The expression of TSP-1 in blood vessel wall of diabetic Zucker rats was compared with that of lean Zucker rats. Uninjured aorta and carotid arteries were collected from 9- to 12-week-old animals, and TSP-1 distribution was detected by immunohistochemistry. Counterstaining with hematoxylin and eosin allowed identification of the intimal, medial, and adventitial layers of the vessel wall. TSP-1 expression was elevated in vessel wall of diabetic compared with lean rats (Figure 1), and the increase was primarily localized to the extracellular matrix of adventitia, where the TSP-1 was evenly distributed along the vessel. Luminal ECs and cells of adventitia were stained positive for TSP-1. Western blotting of protein extracts from vessel tissues confirmed a marked difference in the amounts of TSP-1 in vessel wall of lean and diabetic Zucker rats: TSP-1 was barely detectable in tissues of lean animals and was abundant in aorta and carotid arteries of diabetic rats (Figure 1E). The quantification of Western blots by densitometry confirmed the significant increase in TSP-1 levels in the diabetic vessels (Figure 1F). The additional lower band recognized by the antibody apparently represents proteolytically processed TSP-1. This band reacted...
TSP-1 Expression in Vascular Cells Stimulated by Glucose

We used cultured vascular cells to consider whether glucose might directly stimulate TSP-1 expression. Three major cell types present in the vessel wall (ECs, SMCs, and fibroblasts) were stimulated with 30 mmol/L glucose for different times. All 3 cell types responded to glucose by increasing their expression of TSP-1 protein (Figure 3). The stimulation of TSP-1 production by glucose was consistent and significant; at 24 hours, in ECs, TSP-1 levels increased 12.9±7.2-fold \((P=0.05)\), and in SMCs, they increased 7.2±3.8-fold \((P=0.001)\) as quantified by densitometric scans of Western blots (Figure 3B). The most dramatic effect was seen with the fibroblasts; the 30.5±13.6-fold \((P=0.017)\) increase is consistent with the presence of TSP-1 in the adventitia of diabetic vessels, where these cells predominate. Glucose produced a more pronounced effect than sorbitol, which indicates that the increase was not simply due to a change in osmolarity.

mRNA was isolated from cells stimulated with glucose and used for Northern blotting. mRNA levels increased after 6 hours in all 3 cell types in response to glucose stimulation, reaching a maximum by 24 hours (Figure 4A).

Even though glucose levels in the blood of diabetic patients may be regulated, they still fluctuate, as indicated by the presence of glycation products in vascular wall in diabetics.\(^2\) As shown in Figure 4B, even short-term (30 minutes to 3 hours) exposure of fibroblasts to high glucose is sufficient to stimulate TSP-1 mRNA levels 24 to 48 hours later.

Vasa Vasorum in the Aorta of Diabetic Zucker Rats

A well-established function of TSP-1 is its antiangiogenic activity (reviewed elsewhere\(^2\)–\(^4\)), and its increased expression in the adventitia of diabetic rats may result in inhibited development of the vasa vasorum, capillaries within the walls of large blood vessel. A decreased blood supply could create ischemic conditions in the inner layers of large blood vessels that contribute to SMC proliferation and atherosclerotic lesion progression. To determine whether there is an inverse correlation between the number of capillaries in aortas of diabetic rats and the level of TSP-1 in adventitia, we visualized ECs with anti-vWF immunostaining. A marked difference in immunostaining for vWF in aortas from lean and diabetic rats was noted (Figure 5A) and was confirmed quantitatively (Figure 5B). The area of immunostaining in aortas (excluding luminal endothelium and intercostal arteries) was 3.1-fold less in diabetic than lean rats \((P=0.028)\). The mean intensity of immunostaining, which reflects the amount of vWF produced per EC, was the same (169±20.7 in aortas from lean rats and 168±22.4 in aortas from diabetic rats), which suggests that vWF expression per cell did not change in diabetic animals.

TSP-1 mRNA and Protein Expression in Carotid Arteries of Lean and Diabetic Zucker Rats After Balloon Catheter Injury

To examine TSP-1 expression in diabetic vessels after injury, balloon catheterization was performed on lean and diabetic rats. Right (injured) and left (uninjured) carotid arteries were

with 3 other antibodies recognizing different epitopes in the N- and C-terminal parts of TSP-1 (clones D4.6, C6.7, and MBC200.1 from NeoMarkers) but not with an antibody to the extreme N-terminal TSP-1 peptide (sc12312 from Santa Cruz). This reactivity pattern suggests that the N-terminus of the protein in the diabetic vessel wall had been cleaved. Consistent with this interpretation, the sc12312 antibody recognized an \(\sim40\)-kDa fragment in the gel, which could account for size difference between the 2 TSP-1 bands (data not shown).

TSP-1 mRNA Expression in Aorta and Carotid Artery of Zucker Rats

TSP-1 within the vessel wall might be synthesized by vascular cells, in which case TSP-1 mRNA would be present in the vessel wall. Alternatively, TSP-1 in the vessel wall might be derived from platelets, which are highly activated in diabetes and are a rich source of TSP-1. We sought to detect TSP-1 mRNA by in situ hybridization in uninjured aortas and carotid arteries (Figure 2). There was no hybridization with sense TSP-1 RNA used as a negative specificity control (Figure 2C). Little staining was detected with anti-sense TSP-1 RNA in vessels of lean rats; however, in diabetic rats, mRNA expression was increased dramatically in the cells of both the adventitia and media (Figure 2B), and this was confirmed by quantification of the staining intensity (Figure 2D).

Figure 2. Increased TSP-1 mRNA in vessel wall of diabetic Zucker rats. In situ hybridization was performed to detect TSP-1 mRNA in uninjured aortas of 12-week-old lean (A) and diabetic (B) Zucker rats (magnification \(\times10\)). C, Negative control: hybridization with sense TSP-1 RNA. Increase in mRNA in carotid arteries (Figure 2). There was no hybridization with 3 other antibodies recognizing different epitopes in the N- and C-terminal parts of TSP-1 (clones D4.6, C6.7, and MBC200.1 from NeoMarkers) but not with an antibody to the extreme N-terminal TSP-1 peptide (sc12312 from Santa Cruz). This reactivity pattern suggests that the N-terminus of the protein in the diabetic vessel wall had been cleaved. Consistent with this interpretation, the sc12312 antibody recognized an \(\sim40\)-kDa fragment in the gel, which could account for size difference between the 2 TSP-1 bands (data not shown).
collected at various times, and in situ hybridization was performed to visualize TSP-1 mRNA (Figure 6). Injury of the carotid artery of lean rat resulted in an increase in TSP-1 mRNA levels 24 hours after injury, which was sustained for up to 10 days. In diabetic vessels, in which mRNA levels were increased before injury, a rapid and marked downregulation of mRNA occurred within 6 hours after injury (Figure 6D). However, mRNA levels then increased by 24 hours after injury and remained elevated compared with nondiabetic vessels thereafter (2.5-fold at 24 hours \(P=0.034\), 3-fold at 48 hours \(P=0.0005\), 2.2-fold at 10 days \(P=0.01\), and 4-fold at 21 days \(P=0.001\); Figure 7).

Extracts from injured arteries were subjected to Western blotting to detect TSP-1 (Figure 8). The extracts from

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**Figure 3.** Glucose stimulation of TSP-1 expression in cultured vascular cells. ECs, SMCs, and fibroblasts (F) were stimulated with 30 mmol/L glucose for different times, and TSP-1 in cell lysates was detected by Western blotting. Equal loading was controlled by staining of membrane with Coomassie (A). Evaluation of relative TSP-1 amounts was done by densitometry of at least 4 blots for each cell type with NIH Image program (B). F indicates fibroblasts.

**Figure 4.** Increased mRNA expression in cultured vascular cells after glucose stimulation. Cells were stimulated with glucose as described in Methods and Figure 3, and isolated RNA (15 μg/sample) was used for Northern blotting. Ethidium bromide staining of ribosomal RNA was used to control RNA loading in samples (A). After 3-hour stimulation with glucose, cells were washed 2 times, and new growth medium was added for 48 hours. mRNA was then isolated and used for Northern blotting (B). HFF indicates human foreskin fibroblasts; HASMC, human aortic SMCs; and HUVEC, human umbilical vein ECs.
noninjured arteries of lean rats did not contain detectable TSP-1. At 48 hours after injury, TSP-1 of the expected size was detected, and trace amounts remained for up to 21 days after injury. In diabetic rats, TSP-1 was present in noninjured arteries; its levels were higher than in vessels from lean rats at all time points after injury, and it was still clearly detectable 21 days after injury (Figure 8).

**Discussion**

There are several lines of published evidence that suggest a relationship between TSP-1, diabetes, and atherosclerosis. Surprisingly, there is no direct information on TSP-1 expression and distribution in the vessel wall in diabetes, and the goal of the present study was to provide these missing data. We used Zucker rat, a model of type II diabetes that is known to respond to mechanical injury with accelerated restenosis and to proatherogenic stimuli with changes characteristic of early atherogenic response. The detection of TSP-1 in the vessel wall by immunohistochemistry and Western blotting demonstrated that its level was increased markedly in diabetic compared with nondiabetic vessels, and the TSP-1 protein accumulated primarily in the adventitia.

The increased levels of TSP-1 mRNA in diabetic vessels suggest that TSP-1 is synthesized by vascular cells rather than being derived from activated platelets. The capacity of vascular cells to respond to glucose by increasing TSP-1 production was demonstrated with cultured ECs, SMCs, and fibroblasts. With each cell type, the expression of TSP-1 increased dramatically on glucose stimulation. These results are not only compatible with the in vivo observations but also suggest that glucose itself may be the stimulus of enhanced TSP-1 production. TSP-1 mRNA may be regulated by glucose at the transcriptional or mRNA stability levels; both types of regulation have been implicated in the regulation of gene expression by glucose. Remarkably, short-term stimulation with glucose resulted in prolonged production of TSP-1.
TSP-1 was detected by Western blotting in extracts of carotid arteries. Carotid arteries were collected from lean and diabetic Zucker rats after balloon catheterization. Increased levels of TSP-1 mRNA in extracts of carotid arteries. Carotid arteries were collected from lean and diabetic Zucker rats after balloon catheterization.

mRNA. This observation may be particularly pertinent to diabetic patients, who experience fluctuations in glucose levels.

The present data describing the increased expression of TSP-1 in large vessels in response to high glucose complement the information on the role of TSP-1 in diabetic complications. TSP-1 was suggested to influence diabetic nephropathy and diabetic retinal vasculopathy. Thus, TSP-1 may be centrally involved in regulating the development of vascular complications in diabetes. However, the relationship between high glucose and TSP-1 expression is complex; unlike in mesangial cells and large vessels, TSP-1 is downregulated in the diabetic eye and in microvascular ECs.

We examined levels of TSP-1 mRNA in the carotid artery before and after balloon injury in diabetic and control Zucker rats. TSP-1 mRNA expression in diabetic arteries was consistently more robust than in nondiabetic vessels. Multiple reports indicate that TSP-1 expression in vascular wall is significantly increased in human restenotic arteries and after experimental mechanical injury to vessels in animal models. TSP-1 stimulates SMC proliferation after injury, and the blockade of TSP-1 with a specific antibody reduces intimal hyperplasia. The combined effects of injury, hyperglycemia, and the pro-proliferative consequences of the increased TSP-1 expression may contribute to the more aggressive development of intimal hyperplasia in diabetics. This prediction is consistent with the genetic link between TSP-1 and atherosclerosis.

The dysfunction of ECs in diabetes is well established, and TSP-1 certainly could contribute to this dysfunction because of its antiproliferative and apoptotic effects on ECs. Although TSP-1 in diabetic vessels may affect the metabolism of luminal EC monolayer and SMCs, the large amounts present in adventitia must result ultimately in compromised growth and remodeling of vasa vasorum. Indeed, the present data showed that the number of vasa vasorum decreased in diabetic aorta, inversely correlating with the expression of TSP-1, a potent antiangiogenic agent. In large blood vessels, this may lead to ischemic conditions in the inner layers of the vessel wall. Such oxygen stress could stimulate SMC proliferation and initiation of atherosclerotic lesions. Thus, the increased expression of TSP-1, which directly influences key events in the development of atherosclerotic lesions, provides a molecular connection between diabetes, hyperglycemia, and accelerated atherosclerosis and restenosis.

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