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Thrombospondin-1, a Natural Inhibitor of Angiogenesis, Is Present in Vitreous and Aqueous Humor and Is Modulated by Hyperglycemia

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Negative regulators of angiogenesis play a major role in maintaining vascular homeostasis. Thrombospondin-1 (TSP1) is a natural inhibitor of angiogenesis. This report examines the presence of TSP1 in ocular samples and determines whether its production is altered in diabetes. Western blot analysis detected a 140 kDa antiangiogenic fragment of TSP1(gp140) in vitreous samples prepared from normal human and rat eyes. Intact TSP1 was detected in aqueous humor samples prepared from normal rat and bovine eyes. In contrast, TSP1 was virtually absent in vitreous and aqueous humor samples prepared from diabetic rat eyes. Furthermore, production of TSP1 by microvascular endothelial cells in culture was sensitive to high concentrations of glucose. Retinal blood vessels appeared nonuniform and dilated in diabetic animals when compared to control animals. These results demonstrate that TSP1 and its antiangiogenic fragment are present in aqueous humor and vitreous of normal rat eyes and are dramatically reduced in diabetes. Thus, TSP1 may play a role in ocular vascular homeostasis and its absence may contribute to vascular dysfunctions associated with diabetes. © 2000 Academic Press

Angiogenesis is tightly regulated by a balanced production of stimulators and inhibitors. It is alterations in this balance that result in formation of new blood vessels under many pathological conditions including diabetic retinopathy (1). TSP1 is a matricellular protein produced by a variety of cell types of both epithelial and mesenchymal origin (2). TSP1 and its core proteolytic fragments including gp140 inhibit angiogenesis *in vivo* (3) and endothelial cell migration and proliferation *in vitro* (4). TSP1 expression is also down regulated with malignant transformation, thus favoring an angiogenic phenotype (2). We have recently shown that TSP1 is a major regulator of endothelial cell phenotype and its expression favors the differentiated state of endothelium (2, 5, 6). This is accomplished by concerted regulation of expression of a number of genes with roles in angiogenesis.

Ocular vascularization is highly restricted and many regions remain free of blood vessels (7). For example, the cornea, lens and vitreous normally remain avascular. The vascularization of the superficial and deep layers of the retina are also restricted. However, under many pathological conditions such as diabetic retinopathy, these barriers are broken and excessive vascularization results with severe consequences (7, 8). Diabetic retinopathy affects the retinal vasculature resulting in retinal ischemia and, later, proliferative vascularization of the retina with blood vessels extending into the vitreous. This can result in bleeding, retinal detachment, and loss of vision. Therefore, agents which can inhibit angiogenesis have potential in the treatment of ocular diseases with a neovascular component.

Extracts of cornea, vitreous, and lens have been demonstrated to inhibit neovascularization *in vivo* and endothelial cell proliferation *in vitro* (9–14). However, the identity of the factor(s) which mediate these inhibitory effects remain largely unknown (15, 16). Pigment epithelium-derived factor, present in cornea and vitreous, has recently been shown to inhibit angiogenesis (17). Although, TSP1 is present in the extracellular matrix of the epiretinal membrane (18, 19), little is known about its role in the normal vitreous and in disease states involving the retina and vitreous. Here, we report the presence of TSP1, a natural inhibitor of angiogenesis, in normal ocular samples prepared from human, bovine, and rat eyes. In contrast, TSP1 was absent in the ocular samples prepared from diabetic

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rats. Retinal blood vessels appeared non-uniform, and dilated in diabetic retina when compared to normal retina. These results indicate that decreased production of TSP1 may contribute to early vascular dysfunctions associated with diabetes.

MATERIALS AND METHODS

Streptozotocin-induced diabetes. The animal studies presented here were carried out in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague-Dawley rats (~225 grams) were purchased from Sasco (Indianapolis, IN), housed individually, fed standard rat chow (rodent chow 5001; Ralston Purina, Richmond, IN), and water ad libitum. The animals were on a 12 h light/dark cycle. Diabetes was induced by intravenous administration of 55 mg/kg body weight streptozotocin (Sigma, St. Louis, MO) dissolved in phosphate buffered saline (PBS) immediately before injection. Induction of a diabetic state was confirmed by documenting elevated nonfasting morning plasma glucose levels (>200 mg/dl), increased 24 h urine volumes (>10-fold vs controls), and hyperphagia. Ocular samples were prepared from rats with diabetes of ~3-week duration. A group of 12 rats (6 control and 6 diabetic) were used in each set of experiments. These experiments were repeated at least three times.

Preparation of ocular samples and Western blot analysis. After three weeks of diabetes, rats were given a lethal dose of sodium pentobarbital by intraperitoneal injection and eyes were removed. Aqueous humor and vitreous were collected from each pair of eyes, mixed with 2× SDS sample buffer without a reducing agent, and boiled for 5 min. Vitreous samples from postmortem human eyes (obtained from Mid-America Tissue and Eye bank) ranging in age from 21–67 years, were prepared by removing equal amounts of vitreous from each eye, centrifuging it at 14,000 ×g for 15 min to remove cellular debris, transferring the clear sample to clean tubes, and mixing with 2× SDS sample buffer without a reducing agent. Ocular samples were analyzed for the presence of TSP1 by SDS-PAGE and Western blotting as described previously (20).

Antibodies. A rabbit polyclonal and a mouse monoclonal antibody (Mab A6.1) against human TSP1, which cross reacts with intact rat and bovine TSP1 under nonreducing conditions, were utilized. The TSP1 polyclonal antibody does not react with gp140 but reacts with the full length TSP1. The Mab recognizes both TSP1 and gp140. Blots were probed with both antibodies. However, the results with the Mab which recognizes gp140 in vitreous samples and the results with the polyclonal antibody which gives stronger reactivity to full length TSP1 are shown. A polyclonal rabbit serum, raised against the peptide encoded by exon 14 of murine PECAM-1, was utilized to stain retinal blood vessels. This antibody cross reacts with rat PECAM-1 in ocular vasculature (20).

Preparation of conditioned medium. Human dermal microvascular endothelial cells were cultured as previously described (19). Equal numbers of cells (3×10^5/60 mm dish) were plated in regular growth medium (5.5 mM glucose). Two days latter (at 70–80% confluence), plates were rinsed with serum free medium containing all the growth components and fed with 2 ml of this medium containing 5.5 mM glucose (low) or 25 mM glucose (high). Cells were allowed to condition the medium and an aliquot of conditioned medium was removed at designated times for Western analysis as described above.

Immunohistochemistry. The retina were isolated from dissected eyes, rinsed in PBS, placed in OCT compound (VWR Scientific, St. Louis, MO), frozen on dry ice, and stored at −70°C. Sections of 7 μm each were placed on polylysine coated slides (Sigma, St. Louis, MO). The sections were fixed in cold acetone, washed in PBS, and incubated in PBS blocking buffer (PBS containing 1% BSA, 0.3% Triton X-100, and 0.2% skim milk powder) for 15 min. The sections were then incubated with rabbit polyclonal antibodies to human TSP1 or murine PECAM-1 overnight at 4°C. The sections were incubated with indocarbocyanine (CY3)-labeled secondary antibody (Jackson ImmunoResearch, West Grove, PA) (for TSP1) or Histostain-SP kit and AEC development (Zymed, South San Francisco, CA) (for PECAM-1) as recently described (19).

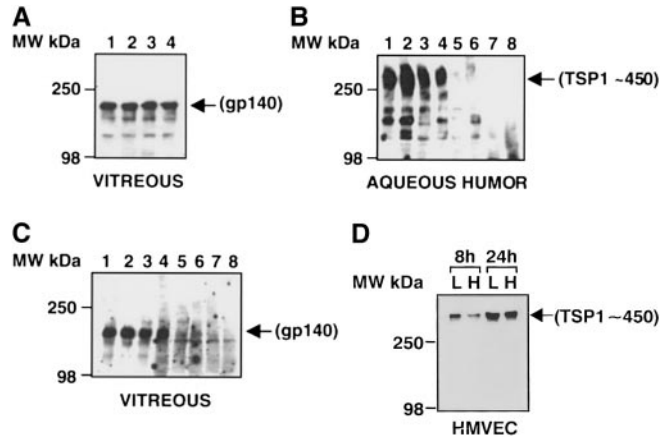

FIG. 1. Western blot analysis of TSP1 levels. Human vitreous from normal donors (A), rat aqueous humor (B), rat vitreous (C), and conditioned medium from HMVEC (D) were prepared and analyzed by SDS–PAGE under nonreducing conditions as described under Materials and Methods. Each lane contained equal volume (20 μl) of the sample. Proteins were transferred to nitrocellulose and blotted with an antibody to human TSP1 (Mab A6.1; A, C, and D or TSP1 polyclonal antibody; B). Results are representative of at least three independent experiments. Human ocular samples were from donor eyes ranging in age from 21 to 67 years. Rat ocular samples were control (1–4) or diabetic (5–8). HMVEC were incubated with serum-free medium containing 5.5 mM (low) or 25 mM (high) glucose. Conditioned medium was collected at indicated time points.

RESULTS

Presence of TSP1 at ocular avascular sites. The presence of TSP1 in vitreous and aqueous humor has not been previously demonstrated. Therefore, we examined the expression of TSP1 in ocular samples prepared from normal eyes. Vitreous samples prepared from normal cadaver donor eyes contained a 140 kDa TSP1 band (Fig. 1A) under non-reducing conditions. This fragment is very similar to gp140 (a fragment of TSP1 lacking the N-terminal heparin binding domain) identified in hamster cells as the anti-angiogenic factor whose expression is suppressed with malignant transformation (3). To our knowledge this is the first report of the presence of a gp140 fragment in any human tissue. A similar sized TSP1 band was also detected in vitreous samples prepared from normal rat eyes (Fig. 1C). We also detected full length TSP1 in the aqueous humor samples prepared from normal rat eyes (Fig. 1B) and bovine eyes (not shown).

Decreased production of TSP1 in diabetes. Diabetes specifically affects the retina resulting in vasculopa-

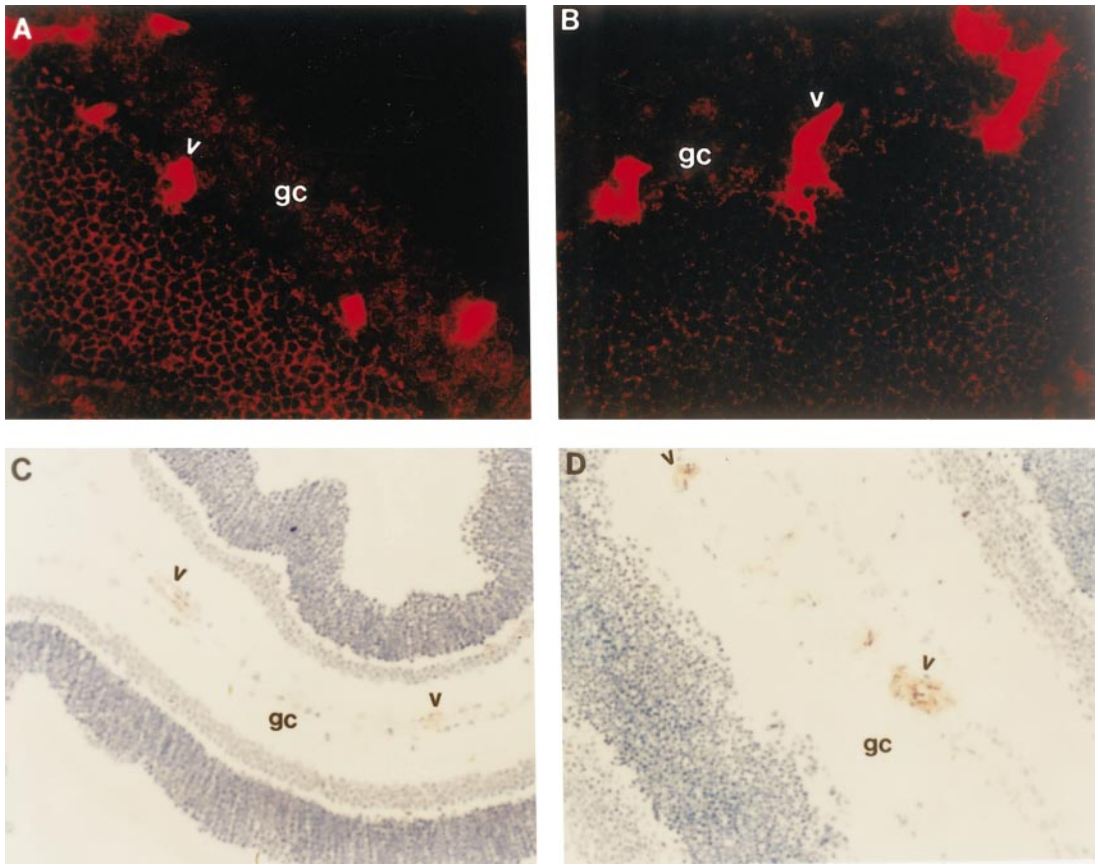

FIG. 2. Staining of sections prepared from normal (A and C) and diabetic (B and D) rat retina. Photomicrograph of sections originating from control and diabetic rat retina stained with a polyclonal anti-TSP1 (A and B, 40× objective) or a polyclonal anti-PECAM-1 antibody (C and D, 20× objective). Results are representative of more than five sets of retina. The arrows point to the blood vessels in the ganglion cell layer (gc). Please note staining of retinal layers with TSP1 and retinal blood vessels in the ganglion layer stained with TSP1 and PECAM-1. The retina sections in C and D are folded. The blood vessels appear uniform in normal retina while they are nonuniform, dilated, and appear tortuous in diabetic retina.

thies which ultimately lead to loss of vision. Our hypothesis is that changes in production of TSP1 may occur with the onset and progression of diabetes, thus contributing to vascular dysfunctions observed in this disease. We have utilized the streptozotocin-induced diabetic rat model to determine whether production of TSP1 is affected during diabetes. Streptozotocin-induced diabetes has dramatic effects on retinal vascular functions in rats despite the fact that these animals do not develop classic diabetic retinopathy (21–24). Alterations in protein kinase C activity and vascular dysfunctions have been observed within 1 to 2 weeks of diabetes (21, 22) while acellular capillaries were observed within 6 months of diabetes (24). Such early vascular dysfunctions may contribute to vasculopathies associated with long term diabetes. We examined expression of TSP1 in rats after three weeks of streptozotocin induced diabetes. Ocular samples were prepared from normal and diabetic rats and analyzed for the presence of TSP1 by Western blotting. Figures 1B and 1C demonstrate that levels of intact TSP1 and its antiangiogenic fragment (gp140) are decreased in aqueous humor and vitreous samples prepared from diabetic rats when compared to normal rats. Thus, TSP1 levels in both compartments decrease dramatically under hyperglycemic conditions, perhaps contributing to ocular vascular dysfunctions observed in early diabetes.

Effects of hyperglycemia on TSP1 production by endothelial cells. High serum glucose levels have been observed in diabetes and are considered to be responsible for many of the pathophysiologies associated with the disease. We next determined whether changes in the glucose levels could affect TSP1 production by endothelial cells *in vitro.* Figure 1D shows a Western blot of serum free conditioned medium prepared from cultures of human dermal microvascular endothelial cells which were exposed to physiological (low; 5.5 mM) or diabetic (high; 25 mM) levels of glucose for 8 or 24 h. At 8 h there was a dramatic decrease in the amount of TSP1 produced by cells exposed to high glucose levels.

Even after 24 h, we still observed significantly less TSP1 secreted by endothelial cells exposed to the higher level of glucose. These experiments were repeated three times with identical results. No differences were detected in the levels of laminin secreted under these conditions (not shown). Thus, high levels of glucose in diabetes may directly modulate the production of TSP1 by endothelial cells.

Alterations in the appearance of blood vessels in diabetes. We examined the appearance of blood vessels in ocular sections prepared from normal or diabetic animals after three weeks of diabetes. Immunohistochemical analysis of retinal sections prepared from normal or diabetic rats is shown in Fig. 2. Figures 2A and 2C show sections of normal rat retina stained with anti-TSP1 (Fig. 2A) or PECAM-1 (Fig. 2C). The TSP1 staining pattern observed is similar to that previously reported (25). We observed staining throughout the retinal layers with strong staining near the inner plexiform layer as well as at the retinal-vitreous boundary. Strong TSP1 immunoreactivity was also observed in the inner nuclear and outer plexiform layers. The retinal blood vessels also stained with TSP1 (in platelets), as seen for blood vessels in other tissues. PECAM-1 staining (Fig. 2C) demonstrates uniformly shaped retinal blood vessels in the ganglion cell layer. Figures 2B and 2D are sections of diabetic rat retina stained with TSP1 and PECAM-1 polyclonal antibodies, respectively. We observed an overall decrease in TSP1 levels throughout the diabetic retina when compared with similar sections from normal retina prepared under similar conditions. This decrease was strongest in the inner nuclear and outer plexiform layers. PECAM-1 staining, as well as TSP1 staining, demonstrate that the retinal blood vessels are variable in diameter and dilated in diabetic rat retina. The control IgG staining of similar sections was completely negative (not shown). In addition, the diabetic rat retina cell layers appeared thicker and disorganized when compared with control retina sections. Thus, the absence of TSP1 from normally avascular ocular compartments under hyperglycemic conditions may contribute to early vascular, and perhaps retinal, dysfunction associated with diabetes.

DISCUSSION

In this report, we demonstrate that (1) TSP1, a natural inhibitor of angiogenesis, is present in ocular samples prepared from normal eyes while it is dramatically decreased in diabetic rats; (2) expression of TSP1 is negatively regulated in microvascular endothelial cells under hyperglycemic conditions in culture; and (3) retinal blood vessels appear dilated and non-uniform in diabetic eyes. Together these data indicate that TSP1 may modulate normal ocular vascular homeostasis whose alterations under hyperglycemic conditions contribute to vascular dysfunctions associated with early diabetes.

Ocular vascularization is tightly regulated and exhibits a very restricted distribution. Thus, retinal capillaries normally do not invade into the vitreous or extend beyond the inner plexiform layer of the retina. In addition, the cornea and lens also normally remain avascular. Vascularization of these sites is associated with many ocular diseases (7, 8). It has been hypothesized that one or more inhibitors of angiogenesis maintain these sites in an avascular state. Antiangiogenic activity has been demonstrated in lens and corneal extracts as well as in normal vitreous samples (10–14). Our hypothesis is that TSP1 plays a role in retinal vascular homeostasis, and that suppression of TSP1 expression under hyperglycemic conditions may contribute to vascular dysfunctions associated with diabetes.

Here, we demonstrate that a naturally occurring antiangiogenic fragment of TSP1(gp140) is present in vitreous samples prepared from normal human, and rat eyes. To our knowledge this is the first report detecting this TSP1 fragment in a physiologically relevant site. We also detected intact TSP1 in aqueous humor samples prepared from normal rat and bovine eyes. Therefore, the presence of TSP1 and/or its antiangiogenic fragment at these sites may be involved in maintaining their avascularity. The virtual absence of intact TSP1 and gp140 in aqueous humor and vitreous samples prepared from diabetic rats strongly supports this notion. In addition, the strong staining of TSP1 at the retinal-vitreous border (not shown) and near the inner plexiform layer may contribute to restricted vascularization of the superficial and deep layer of the retina (Fig. 2; 25). The presence of heterogeneous and dilated blood vessels in the retina of diabetic eyes provide further support for a role of TSP1 in retinal vascular homeostasis. In addition, production of TSP1 by endothelial cells *in vitro* is inhibited by high levels of glucose. Therefore, abnormal serum glucose levels observed with diabetes may directly influence the production of TSP1 by endothelial cells, thus, contributing to vascular dysfunctions associated with diabetes.

The reason for detection of the 140 kDa fragment (gp140) of TSP1 in vitreous samples instead of full length TSP1 (as detected in aqueous humor) is not known. The presence of specific proteases in the vitreous and their production by retinal microvascular cells (26, 27) may be responsible. Indeed the 140 kDa fragment of TSP1 is the most stable fragment generated following initial digestion of TSP1 *in vitro* (4). Human eyes were obtained postmortem and gp140 could have been generated after death. However, the presence of gp140 in freshly prepared rat vitreous samples argues against this possibility.

The abnormal retinal blood vessels observed in diabetic retinopathy are morphologically similar to those

reported in PDGF-BB mutant mice. In these mice, smooth muscle/pericytes fail to cover the blood vessels resulting in dilated, tortuous, and leaky blood vessels with numerous microaneurysms (28). The disappearance of pericytes from the retinal blood vessels is an early hallmark of diabetic retinopathy (8). In addition, vascular endothelial growth factor (VEGF), a major angiogenic factor which contributes to neovascularization of retina in diabetes, can induce retinal vessel tortuosity (7, 29). Increased production of VEGF has been reported in streptozotocin induced diabetic rats with similar duration as that described here (29, 30). However, we presently do not know if the alterations we see in the appearance of diabetic retinal blood vessels are due to lack of pericytes and/or production of VEGF. The role of VEGF and its receptor in diabetes related ocular pathology has been demonstrated (24). Suzuma *et al.* (31) have recently reported a dynamic change in the expression of TSP1 and VEGF in ischemia-induced retinal neovascularization. The decreased expression of TSP1 with an early increase in expression of VEGF allows vascular endothelial cell migration and proliferation which is later inhibited with enhanced production of TSP1. This is consistent with our observation that expression of TSP1 in endothelial cells favors a differentiated, quiescent state of endothelium (2). TSP2, another member of the thrombospondin family with anti-angiogenic activity, is not expressed in the retina of avaian (32) or murine embryos (31) and thus its role in retinal vascularization is minimal. Therefore, TSP1 production may be an important negative feed back which prevents an excessive response to VEGF induced unchecked endothelial cell proliferation and vascular exudation in the retina.

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