Objective: To determine the levels of 2 endogenous inhibitors of angiogenesis, thrombospondin 1 (TSP-1) and pigment epithelium–derived factor (PEDF), in the vitreous fluid from patients with and without diabetes.

Methods: The levels of TSP-1 and PEDF in vitreous samples from diabetic and age-matched nondiabetic patients were determined by Western blot analysis.

Results: We observed significant amounts of TSP-1 and PEDF in the vitreous samples of control eyes. The TSP-1 levels varied in samples from patients with diabetes. In contrast, PEDF levels showed little or no change in vitreous samples from patients with or without diabetes. However, the PEDF protein exhibited variation in its molecular weight among the samples. We consistently observed lower levels of TSP-1 in diabetic patients who expressed the higher-molecular-weight PEDF isoform.

Conclusions: In diabetes, changes in the TSP-1 level may play a role in shifting the angiogenic balance and contributing to the pathogenesis of diabetic retinopathy. Although the PEDF level did not change, the diabetic samples with the higher-molecular-weight PEDF isoform consistently showed lower levels of TSP-1.

Clinical Relevance: The presence of the higher-molecular-weight PEDF isoform may be associated with greater risk of severe diabetic retinopathy.
TSP-1 is present at high levels in vitreous samples prepared from control rats, whereas it is absent in vitreous samples prepared from diabetic rats. This was associated with significant early vasculopathies observed in diabetic animals. We also showed that exposure of microvascular endothelial cells (ECs), including retinal ECs, to high glucose levels results in decreased production of TSP-1 and enhanced migration of retinal ECs. Furthermore, retinal ECs prepared from TSP-1−/− deficient mice maintain a proangiogenic phenotype in culture. Together, these studies indicate that TSP-1 plays a critical role in retinal vascular homeostasis as decreased production during diabetes may contribute to the pathogenesis of diabetic retinopathy. However, to our knowledge, the level of TSP-1 in the eyes of patients with diabetes has not been previously evaluated. Its contribution to the development and progression of diabetic retinopathy requires investigation.

Pigment epithelium–derived factor, a 50-kDa neurotrophic glycoprotein, is also an endogenous inhibitor of angiogenesis and may play a role in retinal vascular homeostasis. We recently showed that the development of retinal vasculature proceeds at a faster rate in PEDF-deficient mice and that the retinal vasculature is more sensitive to hyperoxia-mediated vessel obliteration during oxygen-induced ischemic retinopathy. This is in contrast to what we observed in the TSP-1−/− deficient mice, which exhibited protection from vessel obliteration in response to hyperoxia. Therefore, the roles these molecules play in retinal vascular homeostasis are distinct and need further evaluation.

Like TSP-1, PEDF inhibits angiogenesis in a number of models and inhibits EC proliferation and migration in culture. It was previously reported that the vitreous level of PEDF is lower in patients with proliferative diabetic retinopathy compared with nondiabetic control subjects. In contrast, others have reported that the vitreous levels of PEDF are higher in diabetic patients with active proliferative retinopathy than in those with no neovascularization. Therefore, for improved management of diabetic retinopathy, a better understanding of the pathogenesis of this disease is crucial. The aim of this study was to examine whether alterations in TSP-1 and PEDF levels occur during diabetes.

**PATIENTS**

Patients were recruited from the Department of Ophthalmology and Visual Sciences, University of Wisconsin School of Medicine and Public Health. Patients with diabetic retinopathy and age-matched control subjects were recruited from November 1, 2001, to July 31, 2003. The diagnosis of diabetes was made by the criteria of the American Diabetes Association reported in 1997.24 Patient history and duration of diabetes were gathered from medical records. All of the patients signed informed consent for their participation in the study. The study design and protocol were approved by the institutional review board of the University of Wisconsin School of Medicine and Public Health.

**METHODS**

**VITREOUS SAMPLE COLLECTION**

At the beginning of vitrectomy surgery, 0.1 to 0.5 mL of undiluted vitreous was removed via a vitrectomy cutting instrument attached to a sterile 1-mL syringe before turning on the infusion. Each syringe was capped and immediately placed on ice. Vitreous samples were immediately centrifuged at 16 000g for 10 minutes at 4°C to remove cellular debris, transferred to a clean tube, and stored at −80°C until being analyzed.

**WESTERN BLOTTING**

Western blot analysis of vitreous samples was performed to determine the levels of TSP-1 and PEDF. Vitreous samples (50 μL) were combined with 10 μL of 6× sodium dodecyl sulfate loading buffer, boiled, electrophoresed in 4% to 20% polyacrylamide gel (Invitrogen, Carlsbad, California) under nonreducing conditions, and transferred to a nitrocellulose membrane. The membrane was blocked for 1 hour in blocking solution (3% bovine serum albumin and 3% nondairy creamer), prepared in TRIS-buffered saline (20mM TRIS, pH 7.6, 150mM sodium chloride) containing 0.1% Tween 20, and then incubated with a mouse antihuman TSP-1 antibody (A61; Neo Marker, Fremont, California) for 1 hour at room temperature. We have shown that this antibody reacts with TSP-1 under nonreducing conditions in conditioned medium prepared from wild-type but not TSP-1−/− retinal ECs. The immune complexes were detected with a goat-antimouse horseradish peroxidase–conjugated secondary antibody (1:1 000; Pierce, Rockford, Illinois) followed by enhanced chemiluminescence detection (Amersham Biosciences, Piscataway, New Jersey). The same blot was reprobed with an antihuman PEDF antibody (1:1000; Millipore, Temecula, California) as described earlier. For reprobing, blots were washed once in TRIS-buffered saline with 0.1% Tween 20 and stripped by 2 washes with 25 mL of prewarmed stripping solution (62.5mM TRIS hydrochloride, pH 6.8, 2% sodium dodecyl sulfate, 100mM β-mercaptoethanol) at 65°C. Blots were then washed twice with TRIS-buffered saline with 0.1% Tween 20 (5 minutes for each wash) at room temperature before proceeding with blotting.

**DATA ANALYSIS**

The Western blots were evaluated in a masked fashion and the bands were evaluated by their intensities using ImageJ software (National Institutes of Health, Bethesda, Maryland). For quantitative assessment of the data, the band intensities of TSP-1 were determined for each sample; the level of TSP-1 is reported relative to the level of PEDF for each sample because the PEDF level was minimally affected among the samples. The data were scored based on the TSP-1 level relative to the PEDF level: ++++ for TSP-1 values of 2.0 to 2.5; +++ for TSP-1 values of 1.0 to 1.9; ++ for TSP-1 values of 0.5 to 0.9; and + for TSP-1 values less than 0.5. The PEDF level was minimally changed among the samples and is shown as ++++ to indicate the high level and minimal change among the samples.

**BASELINE CHARACTERISTICS**

This study involved a total of 21 control subjects without diabetes who had a mean (SD) age of 69.7 (7.73) years and an idiopathic macular hole or epiretinal membrane (Table 1) as well as 35 diabetic patients with diabetic
retinopathy who had a mean (SD) age of 62.9 (7.57) years (Table 2). The mean (SD) duration of diabetes for diabetic patients was 22.2 (10.0) years. All of the diabetic patients had proliferative retinopathy. Sex and age were comparable between the diabetic and control groups (Table 1 and Table 2).

ALTERATIONS IN TSP-1 LEVEL IN VITREOUS SAMPLES FROM DIABETIC PATIENTS

We previously showed that TSP-1 is present at high levels in vitreous samples from normal rats, whereas it is significantly diminished with diabetes. We examined the level of TSP-1 in vitreous samples from all of the patients by Western blotting. The Figure shows a representative TSP-1 Western blot of vitreous samples from control subjects and diabetic patients as well as the quantitative assessment of the data. The data for the remaining samples are summarized in Table 1 and Table 2. Our results showed that a significant amount of TSP-1 is present in vitreous samples from control eyes. However, the level of TSP-1 in vitreous samples varied among patients with diabetes. To our knowledge, this is the first report of the detection of TSP-1 and its potential changes in vitreous samples from patients with diabetes.

LACK OF ALTERATIONS IN PEDF LEVEL IN VITREOUS SAMPLES FROM DIABETIC PATIENTS

We next determined whether the PEDF level changed in vitreous samples of diabetic patients compared with control subjects. The Figure shows a representative PEDF Western blot of vitreous samples from control subjects and diabetic patients. The results from the remaining samples are summarized in Table 1 and Table 2. We observed little or no differences in the level of PEDF detected in vitreous samples from diabetic patients compared with control subjects. However, PEDF exhibited a variation in its molecular weight among the samples (Figure, Table 1, and Table 2). Thus, our data suggest that in vitreous samples from control subjects and diabetic patients, there are 2 isoforms of PEDF that vary in their molecular weight. This may be owing in part to various polymorphisms previously reported in the human PEDF gene or its glycosylation. We consistently observed lower levels of TSP-1 in vitreous samples of diabetic patients with the higher-molecular-weight isoform of PEDF.

COMMENT

In this study, we analyzed the levels of 2 major endogenous inhibitors of angiogenesis, TSP-1 and PEDF, in the vitreous samples from patients with diabetes compared with those from control subjects. Our hypothesis is that decreased production of TSP-1 occurs during diabetes and contributes to the development and progression of diabetic retinopathy. We observed variation in the levels of TSP-1 in vitreous samples prepared from diabetic patients compared with control subjects. This ranged from minimal changes in TSP-1 levels to little or no detectable TSP-1 in vitreous samples from diabetic patients. In contrast, we detected significant levels of PEDF in all of
the vitreous samples with minimal effects of diabetes on the PEDF level. However, we observed 2 major isoforms of PEDF, which migrated differently on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, in human vitreous samples. We observed that the TSP-1 level was consistently lower in vitreous samples prepared from diabetic patients with the higher-molecular-weight isoform of PEDF. Thus, the neuroprotective and/or antiangiogenic activity of PEDF rather than its level may contribute to the development and progression of diabetic retinopathy. The exact identity and the neuroprotective and/or antiangiogenic activity of these isoforms are subjects of future investigation.

The tight regulation of angiogenesis is achieved by a balanced production of positive and negative factors. Alterations in this balance under various pathological conditions such as diabetes result in angiogenesis. Vascular endothelial growth factor acts as an EC mitogen in vitro and promotes vascular permeability and angiogenesis in vivo.\textsuperscript{30,31} Intraocular VEGF concentrations increase during the periods of active intraocular neovascularization in patients with proliferative diabetic retinopathy.\textsuperscript{2} However, little is known about the potential changes in the expression and/or activity of endogenous inhibitors of angiogenesis during diabetes.

Many endogenous inhibitors of angiogenesis, including TSP-1 and its closely related family member TSP-2, angiostatin, endostatin, PEDF, interferon \(\alpha/\beta\), and platelet factor 4, have been reported.\textsuperscript{33,34} Thrombospondin 1 was the first identified endogenous inhibitor of angiogenesis whose decreased expression during progression of many solid tumors is associated with activation of the angiogenic switch.\textsuperscript{7,35} We consider TSP-1 to be an angiogenesis inhibitor associated with the development and progression of diabetic retinopathy.\textsuperscript{8} Thrombospondin 1 is present in vitreous and aqueous humor at high levels and is produced by almost all known cell types in the eye, including retinal ECs, astrocytes, and pericytes, and elsewhere, including retinal pigment epithelial cells, corneal epithelial cells and ECs, and trabecular meshwork.

### Table 2. Characterization of Diabetic Patients

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<tr>
<th>Patient No./ Sex/Age, y</th>
<th>Diagnosis</th>
<th>Duration of Diabetes, y</th>
<th>Previous PRP</th>
<th>TSP-1 Score(^a)</th>
<th>PEDF Score (PEDF Isoform)(^a)</th>
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</table>

Abbreviations: H, higher molecular weight; L, lower molecular weight; PDR, proliferative diabetic retinopathy; PEDF, pigment epithelium–derived factor; PRP, panretinal photocoagulation; TSP-1, thrombospondin 1.

\(^a\) The TSP-1 levels are scored relative to the PEDF levels: ++++ for TSP-1 values of 2.0 to 2.5; ++++ for TSP-1 values of 1.0 to 1.9; +++ for TSP-1 values of 0.5 to 0.9; and + or +/− for TSP-1 values less than 0.5. The PEDF levels were minimally changed among the samples and are shown as ++++ to indicate the high level and minimal change among the samples.
It specifically inhibits EC proliferation and migration and blocks angiogenesis and tumor growth.\textsuperscript{13,14} Thrombospondin 1 and its antiangiogenic peptides effectively inhibit new blood vessel growth during oxygen-induced ischemic retinopathy.\textsuperscript{9,15} However, the possibility that administration of TSP-1 and/or its antiangiogenic peptides may inhibit the development and progression of proliferative diabetic retinopathy needs evaluation. Production of TSP-1 and TSP-2 by astrocytes has recently been shown to be essential for appropriate synaptogenesis and retinal neuronal functions.\textsuperscript{42} Thus, alterations in TSP-1 levels may also affect retinal neuronal functions, contributing to visual dysfunctions associated with diabetes.\textsuperscript{43,44} Furthermore, decreased production of TSP-1, PEDF, and endostatin observed in eyes with age-related macular degeneration\textsuperscript{39,45} suggests an important role for these angiogenesis inhibitors in modulation of choroidal vascular homeostasis.

In diabetic retinopathy, the total protein concentration of the vitreous is generally increased perhaps owing to the loss of retinal EC barrier function, an early dysfunction associated with diabetes. Because change in the total vitreous protein content is a likely concern and an important early characteristic of diabetic retinopathy, we used volumes of vitreous rather than total protein concentration in our analysis to normalize across all of the samples. In addition, to circumvent the possibility of vitreous hemorrhage affecting our results, we removed cell debris and platelets, major sources of TSP-1, before further analysis of the samples. However, this does not rule out the potential release of TSP-1 from activated platelets, resulting in increased levels of TSP-1. Although obtaining vitreous samples from patients in early stages of diabetic retinopathy may be challenging, it will help to address these concerns. Alternatively, one can evaluate TSP-1 levels following laser treatment and quiescence of retinal vasculature. However, the limited changes observed in vitreous levels of PEDF suggest a minimal contribution from contaminating serum.

The TSP-1 concentration was unaffected in some diabetic patients and was dramatically down-regulated in others. Because we did not further classify the patients with diabetes into active (highly active neovascularization with rapidly proliferative membrane and fresh vitreous hemorrhage or retinal detachment) or quiescent (chronic neovascularization with extensive panretinal laser photocoagulation and minimal background retinopathy) groups, there are 3 possible explanations for the results seen here. First, diabetic retinopathy was active in patients with low TSP-1 levels in the vitreous fluid. Second, diabetic retinopathy may be quiescent in patients with a high level of TSP-1 in the vitreous fluid. Third, retinopathy may be active or inactive when the TSP-1 level is high or low, respectively, depending on the concentration of stimulators such as VEGF. Thus, the activity of diabetic retinopathy rather than its severity more accurately reflects the effects of angiogenic stimulators and inhibitors. Unfortunately, the lack of sufficient patient information was prohibitory in delineating these possibilities. Further investigation of VEGF levels and better classification of retinopathies are needed for more accurate assessment of the TSP-1 change during diabetes and its effect on the development and progression of diabetic retinopathy.

Pigment epithelium–derived factor is also a potent inhibitor of ocular angiogenesis.\textsuperscript{13} Alterations in PEDF levels in patients with diabetes compared with nondiabetic patients have been controversial and not clearly resolved. It was reported that the level of PEDF in vitreous was lower in patients with diabetes.\textsuperscript{20,21} There are also reports that the PEDF concentration was higher in the vitreous from patients with diabetes.\textsuperscript{22,23} Our study showed that PEDF was at consistently high levels in the eyes of diabetic patients and control subjects. However, we detected 2 different isoforms of PEDF in human vitreous samples with different migration or molecular weight (Figure). One isoform migrated more quickly than the other on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. We consistently observed lower levels of TSP-1 in patients with the higher-molecular-weight PEDF isoform.

Multiple polymorphisms in the human PEDF gene\textsuperscript{28} and an association between some of these polymorphisms (the promoter and exon 3 coding region) and diabetic retinopathy and age-related macular degenera-
tion have been reported. However, it is not known whether these polymorphisms result in production of proteins with different molecular weights or expression levels. The identity and the level of transcripts generated by these polymorphisms as well as the identity and activity of their potential products require further investigation. A potential polymorphism reported in intron 5 (just upstream from the splice acceptor site) may be a potential candidate for generation of an alternatively spliced transcript producing a protein of a different size. Thus, the identity of the PEDF polymorphisms and their protein products as well as their association with the severity of diabetic retinopathy will be very informative. Furthermore, this may allow for the development of a screening method for identification of diabetic patients who are at greater risk for the development of proliferative diabetic retinopathy.

As endogenous inhibitors of angiogenesis, TSP-1 and PEDF exhibit different expression patterns and functions in retinal vascular development and angiogenesis. Although the level of PEDF was not dramatically changed in the eyes of patients with diabetes, the involvement of different PEDF isoforms in the pathogenesis of diabetic retinopathy needs further investigation, especially in regard to their effect on TSP-1 expression. It is possible that different isoforms of PEDF have different antiangiogenic potential during development and progression of diabetic retinopathy, perhaps through modulation of the TSP-1 level. Although vitamin A has been shown to increase expression of TSP-1 and PEDF in retinal pigment epithelial cells, nothing is known about the regulation of TSP-1 by PEDF. Thus, further investigations are needed to address the relationship of proangiogenic factors such as VEGF, isoforms of PEDF, and TSP-1 expression in the pathogenesis of diabetic retinopathy.

In summary, we have demonstrated that the vitreous TSP-1 levels varied among diabetic samples and were consistently lower in the diabetic patients who expressed the higher-molecular-weight isoform of PEDF. The vitreous levels of PEDF, however, did not vary significantly among diabetic patients and control subjects. Thus, decreased production of TSP-1 along with expression of the high-molecular-weight isoform of PEDF in patients with diabetes may indicate a greater risk of developing severe retinopathies.

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