Elevated Transforming Growth Factor β 1 in Plasma of Primary Open-Angle Glaucoma Patients

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PURPOSE. To test the hypothesis that primary open-angle glaucoma (POAG) patients have a systemic elevation of transforming growth factor β 1 (TGF β 1).

METHODS. Plasma was prepared from blood samples drawn from patients of the Vanderbilt Eye Institute during clinic visits. Concentrations of total TGF β 1 and thrombospondin-1 (TSP1) in plasma were determined by ELISA. Statistical significance of differences between POAG and control samples was evaluated by Mann-Whitney test. Regression analysis was used to evaluate correlations between plasma TGF β 1 and patient age and between plasma TGF β 1 and TSP1.

RESULTS. Plasma samples were obtained from 148 POAG patients and 150 controls. Concentration of total TGF β 1 in the plasma of POAG patients (median = 3.25 ng/mL) was significantly higher ($P < 0.0001$) than in controls (median = 2.46 ng/mL). Plasma TGF β 1 was not correlated with age of patient ($P = 0.17$). Thrombospondin-1 concentration was also significantly higher ($P < 0.0001$) in POAG patients (median = 0.774 μ g/mL) as compared to controls (median = 0.567 μ g/mL). Plasma total TGF β 1 and TSP1 concentrations were linearly correlated ($P < 0.0001$).

CONCLUSIONS. Plasma samples from POAG patients display elevated total TGF β 1 compared to controls, consistent with elevated systemic TGF β 1 in POAG patients.

Keywords: glaucoma, transforming growth factor beta, plasma, platelet, aqueous humor

Transforming growth factor β s (TGF β s) are multifunctional cytokines that play central roles in a wide variety of physiological responses such as regulation of immune responses, cell proliferation and differentiation, vascular homeostasis, wound healing, and fibrosis.^{1–4} Depending on the specific microenvironment in which they act, TGF β s can exert opposing effects,⁵ for example, promoting or inhibiting immune reactions or cell proliferation. Mammals express three isoforms of TGF β encoded by separate genes: TGF β 1, TGF β 2, and TGF β 3. Transforming growth factor β s are secreted in a latent form that consists of dimers of latent TGF β bound to latent TGF β binding protein (LTBP).⁶ Transforming growth factor β 2 is the predominant isoform in aqueous humor (AH),^{7,8} while TGF β 1 is the predominant isoform in plasma.^{9–11} In the AH, TGF β 2 plays a central role in anterior chamber-associated immune deviation.¹²

Elevation of TGF β 2 in the AH of patients with primary open-angle glaucoma (POAG) was first reported in 1994 by Tripathi et al.¹³ A series of published studies from several laboratories followed,^{14–20} reporting consistent findings as reviewed by Prendes et al.,²¹ leading to a widely held consensus that TGF β 2 tends to be elevated in the AH of glaucoma patients. Transforming growth factor β 1 has also been shown to be elevated in AH from POAG patients, though its concentration is $\sim$ 40-fold lower than that of TGF β 2 in normal AH.^{17,22,23}

As recently reviewed by Fuchshofer and Tamm,²⁴ a pathogenic role for elevated TGF β 2 in glaucoma is supported by multiple studies showing, for example, that perfusion of human anterior segments with TGF β 2 results in decreased

outflow facility,^{25,26} and overexpression of TGF β 2 in rodent eyes leads to glaucoma phenotypes.^{27–29} Transforming growth factor β 2 overexpression is hypothesized to promote glaucoma through effects on extracellular matrix turnover, affecting both AH outflow facility^{24–26} and susceptibility of the optic nerve to pressure-induced damage.³⁰

Microfibrils are a major reservoir of latent TGF β s. All three TGF β isoforms can associate with microfibrils through interactions between LTBP and fibrillin-1, the principal component of microfibrils.^{31–34} Microfibrils are located in the extracellular matrix of a variety of tissues where they play a central role in localization and activation of TGF β signaling.^{31–34} In the eye, microfibrils are found in the trabecular meshwork, inner wall of Schlemm's canal, iris, ciliary body, and optic nerve head.^{35–37} In addition to microfibrils, platelets are another major reservoir of latent TGF β 1 that is released upon platelet degranulation.³⁸

Elevated systemic TGF β 1 levels and chronic activation of TGF β signaling are associated with a number of diseases caused by mutations in the fibrillin-1 gene (*FBN1*) and in other microfibril-associated genes.^{39–49} Microfibril defects may also cause glaucoma,⁵⁰ as suggested by the association of the microfibril gene *LTBP2*^{51–53} with primary congenital glaucoma in humans. Recently, we identified a mutation in a microfibril-related gene, *ADAMTS10*, as causative for POAG in dogs.^{54,55} Association of microfibril-related genes and glaucoma has led us to form the hypothesis that microfibril defects cause glaucoma.^{50,55} Our microfibril hypothesis of glaucoma would predict that glaucoma patients may have systemic elevation of TGF β 1, similar to observations in other microfibril-related diseases. To

investigate this possibility, we have compared TGFβ1 concentrations in plasma samples from POAG patients and control subjects in the present study.

METHODS

Study Participants

This study was approved by the institutional review board of Vanderbilt University and followed the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects after explanation of the nature and possible consequences of the study. All participants were patients of the glaucoma or retina services of the Vanderbilt Eye Institute. Clinical diagnosis of POAG was made by a fellowship-trained glaucoma specialist (RWK) based on optic nerve appearance characteristic of glaucoma, such as progressive cupping, thinning of the neuroretinal rim, notch formation, and disc hemorrhage. All patients underwent ancillary tests indicated by their clinical findings, such as visual field test (Humphrey visual field analyzer; Carl Zeiss Meditec, Dublin, CA, USA), fundus photographs of optic nerve (Zeiss 450+ fundus camera; Carl Zeiss Meditec), and spectral-domain optical coherence tomography (Cirrus OCT; Carl Zeiss Meditec). Elevated intraocular pressure was not used as a diagnostic criterion. All patients had open iridocorneal angles as determined by gonioscopy examination. Control patients were >50 years of age and were examined by ophthalmologists and found to be not affected by glaucoma. Patients were excluded from the study if they had been diagnosed with any form of glaucoma other than POAG, optic nerve degeneration not due to glaucoma, age-related macular degeneration, diabetic retinopathy, retinal vascular diseases, or an inherited retinopathy. Additional exclusion criteria were history of ocular inflammation or infection, surgery within the past 2 months, cancer, or renal failure. Family history of glaucoma was determined by patient interview. Current medications were determined by examining patient medical records.

Blood Collection and Plasma Preparation

Approximately 5 mL blood was collected from patients by venipuncture using a 23-gauge needle connected via tubing to an evacuated tube coated with lithium heparin (BD Vacutainer, catalog no. 366667; BD, Franklin Lakes, NJ, USA). Prior to blood draw, a tourniquet was applied to facilitate locating the cubital vein. The tourniquet was removed upon initiation of blood draw. Blood samples were kept on ice prior to plasma preparation. To prepare plasma, blood was centrifuged at 4°C for 10 minutes at 1500g in a 45° fixed-angle rotor within 2 hours of collection. Since platelets are a major source of TGFβ1 in blood,^{9,38} initial studies were performed to evaluate the effectiveness of a single centrifugation step in fully removing platelets from the plasma. Addition of a second centrifugation did not reduce plasma TGFβ1 concentration ($P = 0.86$), indicating that a single centrifugation step was sufficient to remove platelets (data not shown). Therefore, a single centrifugation step as described above was used to prepare plasma for all samples in this study. Immediately after centrifugation, plasma samples were aliquoted and stored in a -80°C freezer located in the eye clinic. All blood collection and plasma preparation was carried out by two dedicated study coordinators (LGB and MBP) using consistent technique at a single location (Vanderbilt Eye Institute).

ELISA Assays for TGFβ and Thrombospondin-1

Plasma concentrations of thrombospondin-1 (TSP1) and total (active plus latent) TGFβ1 and total TGFβ2 were determined

TABLE. Patient Characteristics

	Control	POAG	P Value*
Number of patients	150	148	
Age, y, mean (SD)	66.3 (7.8)	66.2 (12.2)	0.94
Female, <i>n</i> (%)	82 (54.7)	89 (60.1)	0.35
Caucasian, <i>n</i> (%)	143 (95.3)	136 (91.9)	0.25
African American, <i>n</i> (%)	6 (4.0)	9 (6.1)	0.44
Asian, <i>n</i> (%)	1 (0.7)	3 (2.0)	0.37
Primary family history,† <i>n</i> (%)	12 (8.0)	41 (27.7)	8.00×10^{-6}
Any family history,‡ <i>n</i> (%)	19 (12.7)	74 (50.0)	5.04×10^{-6}

* *P* values calculated by Student's *t*-test for age and by Fisher's exact test for other categories.

† Patient-reported history of glaucoma in primary relative (parent or sibling).

‡ Patient-reported history of glaucoma in any relative (except his or her children).

using well-established^{56–58} commercially available sandwich design ELISA kits (DuoSet ELISA; R&D Systems, Minneapolis, MN, USA), following the manufacturer's protocols including suggested sample diluents. Transforming growth factor β was converted to its active form by addition of 6 μL 1 N HCl to 12 μL plasma followed by 10-minute incubation at room temperature, neutralization by addition of 6 μL 1.2 N NaOH/0.5 M HEPES, and addition of 216 μL sample diluent. For TSP1 ELISA, plasma samples were diluted 1:11 in sample diluent. Duplicate wells were included for all samples. Each ELISA plate included approximately equal numbers of samples from control and POAG patients. Serial dilutions of recombinant protein standards were included in each plate to generate calibration curves.

Optical density of plates at 450 nm (OD₄₅₀) and 540 nm (OD₅₄₀) were determined with a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Correction for optical imperfections in the plates was made by subtracting OD₅₄₀ from OD₄₅₀. A calibration curve of OD₄₅₀ - OD₅₄₀ readings versus known concentration of the standards was calculated using a four-parameter logistic fitting routine included in the microplate software (SoftMax Pro, v.5.3.1; Molecular Devices). The working detection range of the ELISAs was approximately 0.62 to 40 ng/mL for TGFβs and 0.18 to 2.2 μg/mL for TSP1, based on the concentration range of calibration samples and dilution of the plasma samples.

Platelet Activation

The potent platelet-activating peptide TRAP6, which corresponds to amino acid residues 42 to 47 of the thrombin receptor (SFLLRN),⁵⁹ was added to 1-mL aliquots of blood for final concentrations of 0, 0.1, 1, and 10 μM. Plasma was prepared as above after 5-minute incubation at room temperature.

Statistics

Differences in TGFβ1 or TSP1 concentrations between POAG and controls were evaluated by two-tailed Mann-Whitney tests using the online resource VassarStats (<http://vassarstats.net/> [in the public domain]), with *P* values < 0.025 considered significant, taking multiple testing into consideration. Significance of correlations was evaluated by correlation and regression analysis using VassarStats. Comparisons between slopes of best-fit lines were made by analysis of covariance (ANCOVA) using GraphPad Prism software, version 6.04 (GraphPad Software, La Jolla, CA, USA). Significance of differences between control and POAG patient characteristics shown in the Table was examined by Student's *t*-test for age

FIGURE 1. Total TGF β 1 concentration in the plasma of control and POAG patients. TGF β 1 is significantly higher in the POAG group ($P < 0.0001$). Box plots are shown superimposed on individual data points (blue circles) with the lower and upper horizontal lines of the boxes representing the first and third quartiles, respectively, and the extent of the vertical lines above and below the boxes indicating the 90th and 10th percentiles, respectively.

and by Fisher's exact test for all other patient characteristics using an online resource (<http://www.langsrud.com/stat/index.html> [in the public domain]).

RESULTS

Plasma samples were obtained from 148 POAG patients and 150 control patients. The two groups did not significantly differ in age, sex, or ancestry (Table). Primary open-angle glaucoma patients reported family history of glaucoma at a significantly higher rate than control patients ($P < 10^{-5}$, Table), consistent with previous studies.^{60,61}

FIGURE 2. Total TGF β 1 concentration in the plasma is not correlated with patient age for either controls ([A], $P = 0.17$) or POAG patients ([B], $P = 0.79$). Plasma total TGF β 1 concentration was plotted versus age at time of blood draw. Equation of the best-fit line and R^2 value are shown in the upper left corner of each plot.

In an initial experiment comparing plasma samples from 10 control and 10 POAG patients, total TGF β 1 was readily detectable while total TGF β 2 was below limits of detection for all samples (data not shown). This relative abundance of TGF β 1 compared to TGF β 2 was consistent with previous studies showing TGF β 1 highly expressed in plasma, with low levels or no expression of TGF β 2.^{9–11,62} Active TGF β 1, determined with plasma samples not activated by acid treatment, was also below detection limits. Based on these initial findings, the present study focused on the total amount of the TGF β 1 isoform.

Total TGF β 1 was elevated in plasma samples from POAG patients as compared to controls (Fig. 1). Median TGF β 1 concentration for POAG was 3.25 ng/mL, with an intraquartile range of 2.4 to 4.9 ng/mL, compared to a median of 2.46 ng/mL and intraquartile range of 1.8 to 3.6 ng/mL for control plasma samples. While there was considerable variability between individuals and overlap between groups, the difference was significant ($P < 0.0001$). Plasma TGF β 1 was not linearly correlated with age (Fig. 2) for either the control ($P = 0.17$) or POAG samples ($P = 0.79$), consistent with previous studies.^{9,39}

Since platelets release abundant amounts of both TGF β 1 and TSP1 upon activation, plasma TSP1 concentration was measured as a marker of platelet degranulation in the same set of samples as used for TGF β 1 determinations. Similar to TGF β 1, TSP1 was elevated in POAG plasma compared to controls ($P < 0.0001$), with similar variability and overlap in distributions (Fig. 3). Median TSP1 concentration for POAG was 0.774 μ g/mL with intraquartile range of 0.551 to 1.022 μ g/mL compared to median concentration of 0.567 μ g/mL with intraquartile range of 0.366 to 0.869 μ g/mL for control plasma.

Plotting TGF β 1 versus TSP1 concentrations of each plasma sample revealed that concentrations of the two cytokines were significantly correlated ($P < 0.0001$) for both control and POAG groups (Figs. 4A, 4B). The slopes of the best-fit lines were not different between POAG and control ($P = 0.33$), suggesting that the extent of platelet degranulation was not different between groups.

To investigate the extent to which TGF β 1 and TSP1 in the plasma samples originates from platelet degranulation, experiments were performed in which varying doses of the platelet activator peptide TRAP6 were added to whole blood samples from four control patients prior to plasma preparation. Stimulation with TRAP6 resulted in a dose-dependent increase in TGF β 1 and TSP1 (Figs. 5A, 5B). Plotting TGF β 1 versus TSP1 concentrations of the TRAP6-stimulated plasma samples (Fig. 5C) revealed significant correlation ($P < 0.0001$) but with a slope of the best-fit line significantly different from the slopes of the best-fit lines of the nonstimulated plasma data ($P < 0.001$, Figs. 4A, 4B), suggesting that TGF β 1 and TSP1 are not solely derived from platelet degranulation.

FIGURE 3. TSP1 concentrations in the plasma of control and POAG patients. TSP1 is significantly higher in the POAG group ($P < 0.0001$). Individual data points (blue symbols) and superimposed box plots are presented as in Figure 1.

DISCUSSION

In this study, we discovered a significant elevation of systemic TGF β 1 in POAG patients as compared to controls (Fig. 1). This finding potentially places POAG within a group of diseases associated with elevated systemic TGF β 1 or elevated TGF β signaling. A fairly recent body of literature has emerged connecting elevated TGF β signaling or elevated systemic TGF β 1 with microfibril defects in a number of diseases.^{39–48} Neptune and coworkers originally identified this correlation in a known microfibril deficiency, Marfan syndrome, in which hyperactivated TGF β signaling is found in affected tissues and systemic TGF β 1 is elevated.^{39,40,63} Microfibril defects are a possible mechanism of the increased systemic TGF β 1 in POAG patients found in this investigation, and could also explain

elevated TGF β 2 and TGF β 1 in the AH of POAG patients as shown in a number of studies.^{13–20}

Sequestration of latent TGF β s by microfibrils in the extracellular matrix is a central mechanism of regulation of TGF β signaling.^{32–34} Microfibril deficiencies are associated with increased TGF β signaling and higher systemic TGF β 1 concentrations,^{39–48} likely due to incomplete sequestration of TGF β 1.³⁹ Our previous identification of a mutation in a microfibril gene, *ADAMTS10*, as causative for POAG in dogs,^{54,55} along with other studies showing microfibril genes associated with glaucoma,^{51–53,64} led us to form our microfibril hypothesis of glaucoma stating that microfibril defects could cause glaucoma. The microfibril hypothesis of glaucoma predicts elevated systemic TGF β 1 in glaucoma patients, as found in other diseases caused by microfibril deficiencies.^{50,55} The finding of elevated systemic TGF β 1 in POAG patients in this study (Fig. 1) is consistent with this prediction, providing additional evidence supporting the microfibril hypothesis of glaucoma.

Another potential source of plasma TGF β 1 is platelets, which are numerous and which contain relatively high amounts of TGF β 1 in their α -granules.³⁸ Platelet degranulation during blood draw can significantly contribute to TGF β 1 measured in plasma samples^{9,65} and is practically impossible to completely eliminate during blood sampling in a clinical setting. In the present study we minimized in vitro platelet degranulation by avoiding trauma to the vein, removing tourniquets after blood collection was initiated, keeping blood samples on ice, and preparing plasma shortly after blood draw. To investigate possible contributions from platelets, we chose TSP1 as a marker of platelet degranulation because it is highly expressed in platelet granules⁶⁶ and has been used as a marker of platelet activation in other studies.^{67,68} Thrombospondin-1 was significantly elevated in POAG plasma (Fig. 3), with a linear correlation between TGF β 1 and TSP1 in both POAG and control samples (Figs. 4A, 4B), suggestive of platelets contributing to plasma TGF β 1.

To investigate the extent of possible contributions from platelets, whole blood from several control patients was stimulated with TRAP6 peptide, which is a potent platelet activator,⁵⁹ prior to plasma preparation; TRAP6 caused a dose-dependent increase in plasma TGF β 1 and TSP1 (Figs. 5A, 5B). A linear correlation was found between TGF β 1 and TSP1 in the plasma samples from TRAP6-treated blood, with approximately 75% of the variation of TGF β 1 accounted for by variation in TSP1 based on the square of the Pearson correlation coefficient (Fig. 5C). In patient plasma samples that were not stimulated (Figs. 4A, 4B), variation in TGF β 1 was less dependent on TSP1 levels (36% and 22% for controls and POAG, respectively) as compared to the TRAP6-stimulated samples (Fig. 5C). These results suggest that while platelet degranulation likely contributed to plasma TGF β 1 in the patient samples, additional nonplatelet sources contributed as well. Correlations between TGF β 1 and TSP1 were not significantly different between cases

FIGURE 4. Correlation between TGF β 1 and TSP1. TGF β 1 versus TSP1 plots with best-fit lines for the control (A) and POAG (B) plasma samples reveal significant linear correlation ($P < 0.0001$). Equation of the best-fit line and R^2 value are shown in the upper right corner of each plot.

FIGURE 5. Correlation between TGFβ1 and TSP1 in plasma from blood samples stimulated with platelet activator TRAP6. Dose-dependent increases in TGFβ1 (A) and TSP1 (B) were found in samples from control donors ($n = 4$, not included in Figs. 1–4) that were stimulated with 0, 0.1, 1, and 10 μM TRAP6. Each donor is represented by a different color symbol. Plotting TGFβ1 versus TSP1 for the TRAP6-stimulated samples (C) reveals linear correlation ($P < 0.0001$). Equation of the best-fit line and R^2 value are shown in the upper right corner. The best-fit line for control plasma samples shown in Figure 4A is indicated by a dashed line.

and controls ($P = 0.33$, Figs. 4A, 4B), suggesting that the elevated TGFβ1 in POAG plasma was not due to increased platelet degranulation.

Our finding of elevated TSP1 in POAG (Fig. 3) may also be relevant to pathogenesis. In addition to being abundant in platelet granules, TSP1 is expressed by other cell types and has a rather complicated relationship with TGFβ1 and glaucoma. It is an activator of TGFβ1,⁶⁹ raising the possibility that levels of activated TGFβ1 may be increased in POAG, though we found activated TGFβ1 in plasma to be below detection in our assay. Expression of TSP1 has been shown to be induced by TGFβ1,⁷⁰ including in the trabecular meshwork,^{71,72} suggesting a possible feedforward mechanism leading to elevated TSP1 and activated TGFβ1. In relation to glaucoma, TSP1 protein expression was reported to be higher in the trabecular meshwork of glaucoma patients,⁷¹ and TSP1 mRNA expression is induced by stretch activation of cultured lamina cribrosa cells.⁷³ Furthermore, TSP1-null mice have lowered intraocular pressure,⁷⁴ suggesting a role for TSP1 in regulating intraocular pressure. The elevated TSP1 in POAG patients found in this study suggests that TSP1 could potentially contribute to POAG by itself or in concert with TGFβ1.

In conclusion, we have found elevated levels of TGFβ1 in plasma from POAG patients, as predicted by our microfibril hypothesis of glaucoma. If microfibril defects cause POAG, therapeutic approaches developed for microfibril deficiencies with similar elevations of TGFβ1 may be applied to POAG. Further study is needed to establish the mechanisms of increased TGFβ1 in POAG and the relationship between POAG and microfibrils.

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