Ocular Gene Transfer of Active TGF-β Induces Changes in Anterior Segment Morphology and Elevated IOP in Rats

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PURPOSE. Transforming growth factor beta (TGF-β) is known to play a crucial role in wound healing and fibrotic tissue remodelling. A large body of evidence suggests a role for this cytokine in the pathogenesis of glaucoma; however, the mechanisms by which it affects anterior segment morphology are not well understood. Therefore, the purpose of this study was to examine the effects of TGF-β overexpression on anterior segment morphology and subsequent effects on intraocular pressure.

METHODS. Adenoviral gene transfer was used to deliver active TGF-β1 to the rat eye. Measurements of intraocular pressure were taken with a tonometer on days 0, 14, 21, and 29. Histologic analysis was undertaken to examine anterior segment morphology, and markers of matrix deposition and fibrosis were used.

RESULTS. Gene transfer of TGF-β in the anterior segment resulted in the formation of peripheral anterior synechiae (PAS), which consisted of a fibroproliferative region of corneal endothelial cells, matrix accumulation, and decrease in trabecular meshwork expression of α-smooth muscle actin. These features were accompanied by ocular hypertension.

Conclusions. Gene transfer of TGF-β into the anterior segment induces aberrant PAS associated with the transition of corneal endothelial cells and subsequent matrix deposition. These features are highly reminiscent of human iridocorneal endothelial (ICE) syndrome. Gene transfer of TGF-β can, therefore, be used to induce anatomic changes in the anterior segment in a rodent model that result in ocular hypertension. (Invest Ophthalmol Vis Sci. 2010;51:308–318) DOI:10.1167/iovs.09-3380

Transforming growth factor beta (TGF-β) is a pleiotropic cytokine involved in various aspects of tissue development, maintenance, and pathology. Through activation of receptor serine/threonine kinases, TGF-β initiates signaling cascades involving intracellular mediators including Smads and MAP kinases.7 Gene expression induced by these pathways typically include, but are not limited to, those involved in wound repair and matrix turnover, and its direct application to cells8 or in vivo organ systems via gene transfer7,8 induces a robust fibrotic response.

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TGF-β-induced fibrotic responses in the eye can involve not only the aberrant deposition of extracellular matrix but also significant alterations of the normal histologic architecture of anterior segment structures. Transgenic overexpression of TGF-β in the murine eye results in anterior segment dysgenesis in addition to pathologic changes involving matrix deposition, such as anterior subcapsular cataracts reminiscent of those seen in humans.5,10 For example, Flügel-Koch et al.9 reported that mice overexpressing TGF-β1 under the chicken βB-crystallin promoter lacked stromal layers of the iris and ciliary body and a corneal endothelium, resulting in direct corneal-ventricular contact. Srinivasan et al.11 also reported that mice overexpressing TGF-β1 under the chicken αA-crystallin promoter exhibited underdeveloped ciliary bodies in addition to adhesions involving the posterior cornea and iris.10 Together, these data indicate that TGF-β can induce pathologic changes in multiple tissues in the anterior segment, tissues that affect the outflow facility of the aqueous humor and ultimately intraocular pressure (IOP).

IOP is maintained by a balance between aqueous humor production by the ciliary body and its outflow through the drainage angle via the trabecular meshwork and Schlemm’s canal. Increases in IOP, known as ocular hypertension, can lead to optic nerve damage and subsequent visual field loss. Visual field loss is directly correlated with the loss of retinal ganglion cells (RGCs) in the retina. Ocular hypertension is, therefore, considered a risk factor for glaucoma in humans.12 Indeed, glaucoma is structurally defined by the condition of the outflow pathway, whether it is open or closed. In the case of closed-angle glaucoma, the iris may be in apposition with the lens or posterior cornea. It is, therefore, plausible that fibrotic changes in the anterior segment, as revealed by transgenic overexpression of TGF-β1 in the lens, may lead to pathologic changes in the outflow pathway similar to those in closed-angle glaucoma.13–15 In humans, TGF-β is found in elevated levels in the aqueous humor of patients with glaucoma.16–20 In addition, treatment of perfused anterior segments of humans21 and pigs22 with TGF-β2 revealed a decrease in outflow, an increase in IOP, and an increase in extracellular matrix (ECM) gene expression. Studies that make use of human trabecular meshwork cells further show that these cells express functional TGF-β receptors and that stimulation with TGF-β1 or TGF-β2 induces the expression of a number of ECM-related genes and apoptosis of these cells.24,25

Taken together, the above data demonstrate that TGF-β can induce fibrotic changes in anterior segment tissues, including those that comprise the outflow pathway. Thus, there appears to be a strong association between TGF-β, anterior segment pathology, and IOP, but how TGF-β can induce this cascade of events in the anterior chamber of living animals is not well understood. Thus, in the present study, we investigated how TGF-β1 overexpression impacts anterior segment morphology and whether this is associated with changes in IOP. To accom-

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MATERIALS AND METHODS

Recombinant Adenoviruses

Full-length porcine TGF-β1 cDNA was mutated at cysteines 223 and 225 (TGF-β1223/225) to create a constitutively active protein product, as described previously.76-79 This cDNA construct was used to generate a recombinant adenovirus vector in which the E1 region was replaced by the human cytomegalovirus promoter, driving expression of TGF-β1223/225, followed by the SV40 polyadenylation signal80 using a two-vector system developed by Graham et al.81 The resultant replication-deficient virus (AdTGF-β) was amplified in 293 cells, purified by cesium chloride gradient centrifugation; buffer was exchanged using a chromatography column (Sephadex PD-10; Sigma-Aldrich, St. Louis, MO) and plaque-titered on 293 cells. Adenoviral gene transfer of active TGF-β1 using this vector has previously shown that increased levels of TGF-β1 protein are produced.82-85 Porcine TGF-β1 amino acid sequence shares 96.2% sequence similarity to the rat homolog (NCBI FASTA alignment). The control vector expressing enhanced green fluorescent protein (AdGFP) was a gift from Mary Hitt (Department of Oncology, University of Alberta, Canada) and Robert Marr (Department of Neuroscience, Rosiland Franklin University, Chicago, IL). Briefly, an expression cassette with the murine cytomegalovirus immediately early promoter86 was used to drive expression of enhanced green fluorescent protein (eGFP). The eGFP sequence is identical with that in pEGFP-N1 (Clontech, Mountain View, CA). Immediately after the eGFP coding sequence is a portion of the SV40 polyadenylation signal. This fragment is duplicated just downstream, contiguous with the remainder of the polA signal. To generate AdGFP (Ad5 vector with E1 and E3 deleted), the expression cassette was inserted in an orientation parallel to the E1 region, which it replaces. The virus was rescued by site-specific recombination in 293 cells transfected with the expression cassette and pHBlux-ΔE1,ΔE3Cre.85 Growth and titration of AdGFP was carried out as described.

Animal Treatment

All animals were treated in accordance with the guidelines of the Canadian Council on Animal Care and according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Wistar rats (n = 3 for each treatment at each time point), each weighing 225 to 250 g, were purchased from Charles River Laboratories (Montréal, PQ, Canada). All animals were housed under specific pathogen-free conditions, and rodent laboratory food and water were provided ad libitum. All animal procedures were performed under inhalation anesthesia with isoflurane (MTC Pharmaceuticals, Cambridge, ON, Canada). AdTGF-β1 or AdGFP (as control vector) at 5 × 10^5 pfu were administered in a volume of 5 μL in phosphate-buffered saline (PBS). Briefly, animals were anesthetized with isoflurane, 1 drop of 0.5% proparacaine was applied to each eye, and the animal was placed under a dissecting microscope so that general eye structures could be visualized. Each globe was secured using blunt dissecting forceps. The tips of the forceps were placed on either side of the globe and, with the use of gentle, downward pressure, the globe was partially protruded and held in place. The 33-gauge needle was placed in an area between the central cornea and limbal region to avoid damaging the iris. Slow, gentle pressure was applied to introduce the needle into the anterior chamber and was directly verified visually under the dissecting microscope. A volume of 5 μL virus solution was slowly injected into the anterior chamber using the 33-gauge needle affixed to a 10-μL Hamilton syringe. After the solution was deposited, the needle was slowly removed at the same angle it was inserted. For assessment of the effects of TGF-β on anterior segment morphology and IOP, one eye was injected with AdTGF-β and the contralateral eye was injected with AdGFP. Eyes were covered with ophthalmic lubricating ointment (Lacrilube; Allergan, Irvine, CA) after injection, and animals were allowed to recover before returning to their cages.

IOP Readings

IOP readings were taken under isoflurane inhalation after a drop of 0.5% proparacaine solution (Sigma, Burlington, ON, Canada) was applied to each eye on day 0 before injection and at days 14, 21, and 29. All readings were performed during mid-afternoon work hours and were acquired with an applanation tonometer (Tono-Pen XL; Reichert, Depew, NY). This instrument is a handheld device that uses a 1-mm transducer tip, and its use in rats has been previously described. Readings were taken in accordance with the manufacturer’s instructions. Briefly, after four valid readings, the instrument displays an averaged reading with the SD. Those averaged readings with an SD of more than 5%, as indicated by the instrument, were not used. This process was repeated for each eye until three averaged readings, with a SD of 5% or less, was achieved. A number of studies have examined the reliability of the applanation tonometer (Tono-Pen XL; Reichert) to give consistent readings across a range of experimentally altered IOPs,34-36 but it is acknowledged that isoflurane anesthesia lowers IOP.57-58 Because the contralateral eye was injected with control vector, the possible IOP-lowering effects of isoflurane would be attributed to both eyes. Animals were killed 14, 21, and 29 days after injection by cervical dislocation, and eyes were enucleated.

Histology

After fixation in 10% neutral buffered formalin for 48 hours, tissues (n = 3 for each treatment and time point) were embedded in paraffin by routine methods. Four-micrometer thick mid-sagittal sections were cut and stained with hematoxylin and eosin to visualize general tissue architecture. For retinal histology, care was taken to ensure that images from representative sections were derived from mid-renal areas that were of similar distance from the optic nerve.

Immunofluorescence and TUNEL Assay

Studies to fluorescently localize α-smooth muscle actin (αSMA) used a monoclonal antibody conjugated to FITC (clone 1A4; Sigma-Aldrich, Oakville, ON, Canada; 1:200). In addition, sections were stained with polyclonal antibodies to collagen IV (Cedarlane Laboratories, Hornby, ON; 1:200), TGF-β 1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200), N-cadherin (Santa Cruz Biotechnology; 1:200), and GFP (BioShop Canada, Burlington, ON, Canada; 1:200). Secondary antibodies included goat anti-rabbit rhodamine and goat anti-rabbit FITC (BioShop Canada; 1:500). With the exception of TGF-β immunolocalization, all immunofluorescence procedures were performed as follows: after dehydration in a graded series of xylene and ethanol, paraffin sections were washed three times for 5 minutes with PBS (pH 7.5), boiled for 20 minutes in 10 mM sodium citrate (pH 6.09) for antigen retrieval, then washed an additional three times in PBS. Sections were then blocked with solution of 5% normal goat serum in PBS for 1 hour at room temperature. After three 5-minute washes in PBS, the primary antibody was added to the sections in a volume of 100 μL and allowed to incubate overnight at 4°C. After incubation, sections were washed three times for 5 minutes each in PBS, and 100 μL secondary antibody solution was added. The sections were incubated for 1 hour at room temperature. After incubation, sections were washed three times for 5 minutes each in PBS and then coverslipped with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) with DAPI as a nuclear counterstain. Sections lacking primary antibody served as negative controls. Immunolocalization of TGF-β was carried out as described, but all washing steps consisted of three washes of 10 minutes each with PBS containing 0.1% Tween-20 (BioShop Canada) followed by a final 10-minute wash with PBS. Secondary antibody used was goat anti-rabbit Alexa-Fluor 594 (Highly Cross-Absorbed Whole Antibody Conjugate; Invitrogen, Burlington, ON, Canada). Wash containers were placed on an orbital rotator using a setting of 60 rpm. TUNEL staining was carried out using a fluorescein in situ apoptosis detection kit (ApopTag; Mil-
lipse, Temecula, CA) according to the manufacturer’s instructions. A sample size (n) of 3 was used for each treatment and time point.

Microscopy

Fluorescently labeled sections were visualized using a fluorescence microscope (DMRA2; Leica Microsystems Canada, Inc., Richmond Hill, ON, Canada) fitted with a specialized digital camera (Q Imaging Retiga 1300i FAST; Surrey, BC, Canada). Images were captured using modular imaging software (OpenLab; PerkinElmer LAS, Shelton, CT). Cropping, rotating, and text addition to images were done using a graphics editing program (Photoshop 8; Adobe Systems Canada, Ottawa, ON).

RESULTS

Transgene Expression

Previous studies have shown that adenoviral gene transfer results in expression of reporter transgene in anterior chamber tissues after intracameral injection as early as day 4 after injection. To determine the tissues expressing transgene, GFP fluorescence was immunolocalized on day 14. As shown in Figure 1A, GFP expression was readily apparent in the anterior segment. Higher power views in Figures 1B, C, and D illustrate specific expression in the corneal endothelium, iris, and lens epithelium, respectively. These features were absent in untreated eyes (data not shown). Moreover, high expression in Schlemm’s canal is consistent with the presence of adenovirus present in the aqueous humor, which is removed from the anterior chamber via this structure. Additionally, the presence of GFP expression in other listed structures is also consistent with the presence of adenovirus in the aqueous humor because all these structures would be exposed to adenovirus.

To determine whether recombinant adenovirus infection resulted in increased TGF-β expression, we immunolocalized TGF-β in the anterior segment of both AdGFP and AdTGF-β-treated eyes on day 14 (Fig. 2). Fourteen days after infection, AdGFP-treated eyes show expression of TGF-β in the corneal endothelium (Fig. 2C), with minimal expression in the iris (Fig. 2E) and trabecular meshwork (Fig. 2G). In contrast, AdTGF-β–treated eyes demonstrate abundant expression in the multilayered corneal endothelium, corneal stroma, iris, iridocorneal adhesion (yellow star), and trabecular meshwork (Figs. 2D, F, H).

FIGURE 1. Expression of GFP transgene 14 days after adenovirus injection detected by anti-GFP immunolocalization. (A) Low-power (5×; scale bar, 200 μm) view of the anterior segment demonstrates the overall distribution of transgene expression, including boxed areas corresponding to the high-power views (B–D: 40×; scale bar, 50 μm). (B) Transgene expression that can readily be seen in the corneal endothelium (cen) beneath Descemet’s membrane (dm, yellow dotted line). Transgene expression is absent in the corneal epithelium (cep) and corneal stroma (cs). (C) Transgene expression that appears to be highest in the outermost layers of the anterior iris (ai) and posterior iris (pi) layers with positive yet variable expression within the body of these two layers. The lens epithelium (le) also demonstrates strong transgene expression. Expression on the surface of the lens capsule (lc) and fiber cell mass (fcm) appear to be artifactual. (D) Transgene expression that can be found throughout the trabecular meshwork (tm), with highest expression lining Schlemm’s canal (Sc). Faint expression can also be seen in the ciliary body epithelium (cbe).

FIGURE 2. Expression of TGF-β1 14 days after adenovirus injection detected by anti–TGF-β1 immunolocalization. Low-power (5×; scale bar, 200 μm) view of TGF-β1 expression in the anterior segments of (A) AdGFP- and (B) AdTGF-β–treated rat eyes. Included are boxed areas corresponding to the high-power views (C–F: 40×; scale bar, 50 μm). (G, H: 20×; scale bar, 100 μm). (C) AdGFP–treated eyes demonstrate TGF-β1 expression in the monolayer of corneal endothelium (cen) beneath Descemet’s membrane (dm, dotted yellow line). Expression is absent in the corneal stroma (cs). (D) AdTGF-β–treated eyes also demonstrate TGF-β1 expression in the multilayered corneal endothelium beneath Descemet’s membrane and in the corneal stroma. (E) Absence of TGF-β1 expression in AdGFP–treated eyes. (F) Abundant TGF-β1 expression in the multilayered corneal endothelium beneath Descemet’s membrane and in both the anterior iris (ai) and posterior iris (pi) layers. Additionally, there appears to be high expression in the multicellular region between the corneal endothelium and the anterior iris epithelium (yellow star). (G) Minimal TGF-β1 expression in the trabecular meshwork (tm) of AdGFP–treated eyes. In contrast, (H) abundant TGF-β1 expression in the trabecular meshwork and minimal expression in the ciliary body epithelium (cbe) of AdTGF-β–treated eyes.
IOP Measurements

Previous studies have shown in vitro, that application of recombinant TGF-β to perfused human anterior segments resulted in decreased outflow facility and ocular hypertension. We, therefore, sought to examine the effects of overexpression of TGF-β in vivo. Before and after intracameral injection with either AdTGF-β or AdGFP, IOPs were recorded using the tonometer (Tono-Pen XL; Reichert). Consistent with other rat models of glaucoma, the mean pressures before treatment (day 0) were 12.9 mm Hg (± 4.1 mm Hg) and 12.2 mm Hg (± 3.6 mm Hg) for AdTGF-β (n = 9) and control groups (n = 9), respectively (Fig. 3). Ocular inflammation in the anterior segment can occur as a consequence of adenoviral injection. A review by Bennett describes the innate immune response after adenovirus injection, which results in cellular infiltration predominantly composed of macrophages and neutrophils. Given that neutrophil infiltration was not present on day 14 (data not shown), we elected to commence readings 14 days after injection. At this time, the AdTGF-β–treated eyes demonstrated an IOP that peaked at 21.8 mm Hg (± 3.73 mm Hg; n = 6) compared with control vector-treated eyes (11.5 mm Hg ± 0.63 mm Hg; n = 6; P < 0.05). The IOP of AdTGF-β–treated eyes remained significantly higher than in controls on both days 21 (20.0 mm Hg ± 2.27 mm Hg [n = 6] versus 13.6 mm Hg [n = 6]; 0.3 mm Hg; P < 0.01) and 29 (19.2 mm Hg ± 1.8 mm Hg [n = 3] versus 12.4 mm Hg [n = 3]; P < 0.05).

Anterior Segment Morphology

To further understand the nature of IOP elevation in response to AdTGF-β, we examined the anterior segment for morphologic defects in histologic cross-sections stained with hematoxylin and cosin on day 14. Formation of iridocorneal adhesions (Fig. 4B) resulting in complete angle closure (Fig. 4D) and a markedly thicker cornea were predominant in the AdTGF-β group. The iridocorneal adhesions (Fig. 4F) were associated with a fibroproliferative area of what appeared to be endothelial cells beneath Descemet’s membrane combined with a thickened anterior iris. These two areas appeared to be joined by fibrous tissue (star). These features were not seen in day 14 control (AdGFP-treated) eyes (Figs. 4C, E).

N-Cadherin Expression

To further understand the nature of the fibroproliferative features in the AdTGF-β–treated eyes, we immunolocalized N-cadherin protein, a marker of endothelial cells, in the anterior chamber of both AdTGF-β– and AdGFP-treated eyes. As shown in Figure 5B, day 14 AdTGF-β–treated eyes showed iridocorneal adhesions that exhibited N-cadherin immunoreactivity within the adhesion, in contrast to the complete absence of adhesions in the AdGFP-treated eyes (Fig. 5A). The endothelial monolayer of cells beneath Descemet’s membrane in the AdGFP-treated eyes also stained positively for N-cadherin (Fig. 5C). In comparison, the AdTGF-β–treated eyes showed an aberrant accumulation of cells beneath Descemet’s membrane that stained positively for N-cadherin. These cells did not resemble the endothelial monolayer found in AdGFP-treated eyes but instead exhibited a spindle-shaped morphology. In the iris, N-cadherin expression was found in both posterior and anterior epithelial layers of the iris in AdTGF-β– and AdGFP-treated eyes (Figs. 5F, E, respectively). In both cases, N-cadherin expression appeared to be higher in the posterior iris. Additionally, the fibrous tissue in AdTGF-β–treated eyes found between the corneal endothelium and the anterior iris epithelium (Fig. 5F, red star) appeared to exhibit high expression levels of N-cadherin. In the drainage angle, N-cadherin expression ap-
6B show low-power views of αSMA immunolocalization on day 14 in both AdGFP- and AdTGF-β–treated eyes, respectively. Higher power views of the corneal endothelium reveal the expression of αSMA in the endothelium beneath Descemet’s membrane in both AdGFP- and AdTGF-β–treated eyes (Figs. 6C, D, respectively). In the iris, αSMA expression occurs in the papillary dilator muscle in both AdGFP- and AdTGF-β–treated eyes (Figs. 6E, F, respectively). Additionally, the fibrous tissue in AdTGF-β–treated eyes found between the corneal endothelium and the anterior iris epithelium (Fig. 6; red star) appeared to express αSMA. Interestingly, though αSMA expression was high in the trabecular meshwork of AdGFP-treated eyes (Fig. 6G), minimal expression was seen in AdTGF-β–treated eyes (Fig. 6H).

Collagen IV Expression

Another hallmark of fibrosis is the deposition of extracellular matrix molecules. We elected to immunolocalize collagen IV, a constituent of basement membrane structures. Figures 7A and 7B demonstrate day 14 low-power views of eyes treated with AdGFP and AdTGF-β, respectively. At low power, collagen IV expression can readily be seen in the corneal endothelium, iris, and lens capsule. In the corneal endothelium, expression was within Descemet’s membrane in AdGFP-treated eyes (Fig. 7C). In contrast, expression was also found in the multilayered endothelium of AdTGF-β–treated eyes, indicating collagen IV deposition within this multilayered structure. In the iris, collagen IV expression strongly outlined blood vessels in the anterior iris in both AdGFP- and AdTGF-β–treated eyes (Figs. 7E, F, respectively). Additionally, the fibrous tissue in AdTGF-β–treated eyes (yellow star) expressed very high levels of collagen IV. In both groups, collagen IV expression was also localized faintly to the stroma of both anterior and posterior iris epithelial layers. In the drainage angle, the trabecular meshwork expressed collagen IV along the trabecular beams that were readily apparent in AdGFP-treated eyes (Fig. 7G). Expression of collagen IV was present in the trabecular meshwork of AdTGF-β–treated eyes; however, the expression did not appear to be organized in beams, as seen in the AdGFP eyes (Fig. 7H).

Retinal Morphology

Given that ocular hypertension is highly associated with RGC loss, we next examined whether any morphologic changes in the retina were associated with AdTGF-β treatment. At 14 days, the AdTGF-β group demonstrated notable cell loss in all layers that was readily apparent when comparing AdGFP and AdTGF-β images of similar magnification (Figs. 8A, B, respectively). To determine the nature of this cell loss, a TUNEL assay was performed in histologic sections at day 14. AdTGF-β–treated eyes demonstrated TUNEL-positive cells (Fig. 8D), whereas AdGFP-treated eyes demonstrate a lack of TUNEL reactivity in all layers of the retina (Fig. 8C).

To determine whether this retinal degeneration occurred as a consequence of TGF-β overexpression, we immunolocalized TGF-β1 in retinas of both groups on day 14. Small amounts of TGF-β1 expression can be seen in retinas of both AdGFP- and AdTGF-β–treated eyes; however, the levels of expression do not appear to be substantially different (Figs. 8E, F, respectively).

Because TGF-β is predominantly known as a profibrogenic molecule, we evaluated the extent of fibrosis in the retina as a result of AdTGF-β treatment. On day 29, AdTGF-β–treated eyes demonstrated αSMA expression in the RGC and fiber cell layers (Fig. 8H). In contrast, day 29 AdGFP-treated eyes (Fig. 8G), showed no αSMA expression except in the vasculature (not shown).

α-Smooth Muscle Actin Expression

Because the iridocorneal adhesions and accumulation of cells beneath Descemet’s membrane exhibited a spindle shape morphology, we immunolocalized αSMA, a mesenchymal cell marker often involved in fibrotic disorders. Figures 6A and 6B show low-power views of αSMA immunolocalization on day 14 in both AdGFP- and AdTGF-β–treated eyes, respectively. Higher power views of the corneal endothelium reveal the expression of αSMA in the endothelium beneath Descemet’s membrane in both AdGFP- and AdTGF-β–treated eyes (Figs. 6C, D, respectively). In the iris, αSMA expression occurs in the papillary dilator muscle in both AdGFP- and AdTGF-β–treated eyes (Figs. 6E, F, respectively). Additionally, the fibrous tissue in AdTGF-β–treated eyes found between the corneal endothelium and the anterior iris epithelium (Fig. 6; red star) appeared to express αSMA. Interestingly, though αSMA expression was high in the trabecular meshwork of AdGFP-treated eyes (Fig. 6G), minimal expression was seen in AdTGF-β–treated eyes (Fig. 6H).

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DISCUSSION

Gene transfer methods have been used extensively to study molecular mechanisms of fibrosis in the eye.\(^4\)–\(^{51–54}\) Gene transfer of TGF-\(\beta\) to the anterior chamber is known to induce fibrotic responses such as the development of anterior subcapsular cataracts in mice, with concomitant expression of markers of epithelial to mesenchymal transition (EMT) and matrix deposition.\(^1\) When various cell types that comprise the anterior segment, such as those of the corneal endothelium\(^55–58\) and trabecular meshwork,\(^22,25,59–64\) were exposed to TGF-\(\beta\) in vitro, they were shown to undergo a fibrotic response. The specific mechanisms that give rise to these changes in the anterior segment, which result in a glaucoma-like phenotype in

**Cataract Formation**

Consistent with our previously published data in mice,\(^4\) morphologic changes in the rat lenses were also associated with AdTGF-\(\beta\)-treated eyes. At day 14, multilayering can readily be seen in the lenses of AdTGF-\(\beta\)-treated eyes (Fig. 9B), in contrast to the single layer of cuboidal epithelium seen in the lenses of AdGFP-treated eyes (Fig. 9A). Moreover, multilayering seen in the lenses of AdTGF-\(\beta\)-treated eyes was associated with \(\alpha\)SMA expression (Fig. 9D) and aberrant collagen IV expression (Fig. 9F), which were not expressed in the lenses AdGFP-treated eyes (Figs. 9C, E), indicating EMT and matrix deposition, respectively. Expression of TGF-\(\beta\)1 was found to be faintly present in the lens epithelium of AdGFP-treated eyes (Fig. 9G) and abundantly present in the multilayered epithelial plaque in AdTGF-\(\beta\)-treated eyes (Fig. 9H).

**Figure 6.** Expression of \(\alpha\)SMA in the anterior segment 14 days after adenovirus injection detected by anti-\(\alpha\)SMA immunolocalization. Low-power view (5×; scale bar, 200 \(\mu\)m) of (A) AdGFP- and (B) AdTGF-\(\beta\)-treated rat eyes. Included are boxed areas corresponding to higher power views (C–H: 40×; scale bar, 50 \(\mu\)m). (C) Expression of \(\alpha\)SMA in the corneal endothelium (cen) beneath Decemet’s membrane (dm, dotted yellow line) in AdGFP-treated eyes. (D) \(\alpha\)SMA expression in multilayered corneal endothelium of AdTGF-\(\beta\)-treated eyes beneath Decemet’s membrane. (E) Strong \(\alpha\)SMA expression in the iris delineating the papillary muscle, which also extends to the root of the iris (G), where the iris epithelium meets the ciliary body epithelium (che). (F) \(\alpha\)SMA expression in the iris of AdTGF-\(\beta\)-treated eyes, which extends to the root of the iris (H) in addition to scattered expression in the superficial layer of the anterior iris (ai) and the multicellular region between the corneal endothelium and anterior iris epithelium (red star). (G) Strong expression of \(\alpha\)SMA in the trabecular meshwork (tm) of AdGFP-treated eyes. (H) Faint but present expression of \(\alpha\)SMA in the trabecular meshwork.

**Figure 7.** Expression of collagen IV in the anterior segment 14 days after adenovirus injection detected by anti-collagen IV immunolocalization. Low-power view (5×; scale bar, 200 \(\mu\)m) of (A) AdGFP- and (B) AdTGF-\(\beta\)-treated rat eyes. Included are boxed areas corresponding to the higher power views (C–H: 40×; scale bar, 50 \(\mu\)m). (C) Expression of collagen IV in Decemet’s membrane (dm, dotted yellow line) in AdGFP-treated eyes. In contrast, (D) expression in Decemet’s membrane in addition to high expression in the multilayered corneal endothelium (cen) of AdTGF-\(\beta\)-treated eyes. (E) Collagen IV expression in the anterior iris (ai) and posterior iris (pi) of AdGFP-treated eyes. (F) Collagen IV expression in both the anterior and posterior iris layers in addition to the multicellular region between the corneal endothelium and anterior iris epithelium (yellow star). Collagen IV expression in the trabecular meshwork (tm) and ciliary body epithelium (che) of both (G) AdGFP- and (H) AdTGF-\(\beta\)-treated eyes.
and migration of corneal endothelial cells and iridocorneal adhesions. Mice that overexpress TGF-α73 and TGF-β110 under the αA-crystallin promoter also develop iridocorneal adhesions similar to PAS seen in humans. In humans, PAS formation is common in patients with closed-angle glaucoma. Clinical studies examined from evolutionary,74 ethnic,75–83 drug-induced,84–90 hereditary/genetic,91–93 and secondary-to-surgical-procedure24,95 perspectives suggest a role for TGF-β in the development of closed-angle glaucoma.96 Evidence for its direct involvement in PAS formation, however, has yet to be revealed. Yet the development of PAS is highly associated with surgical procedures involving the anterior chamber,97–99 trauma,100 and inflammatory conditions.101,102 circumstances in which TGF-β is likely to be activated. Thus, overexpression of

vivo, has remained relatively unexplored. Numerous mutant mouse strains have been generated that develop elevated IOP95; however, detailed analyses that characterize the changes in gene expression that accompany ocular hypertension are lacking. In the present study we have used gene transfer technology to deliver TGF-β and to examine the fibrotic response in the anterior segment and determine whether this would also result in ocular hypertension. We observed morphologic changes in anterior segment structures that included abnormal focal adhesions between the corneal endothelium and the anterior iris epithelium, described as peripheral anterior synechiae (PAS). These changes were accompanied by changes in gene expression and significant increases in IOP.

Peripheral Anterior Synechiae

Rodent models demonstrating glaucomatous features have included evidence of PAS formation (Robertson J. IOVS 2008;49; ARVO E-Abstract 5108; Robertson J. IOVS 2007;48; ARVO E-Abstract 5904).66,67 These models include ocular hypertension induced by laser treatment,68 the DBA2 mouse,69 and various gene knockouts,70–72 and they describe aberrant proliferation

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TGF-β, as we have shown in this study, may be directly involved in PAS formation.

Endothelial-to-Mesenchymal Transition

The PAS observed in the AdTGF-β-treated eyes appeared to develop from a fibroproliferative response of the corneal endothelium. For example, the AdTGF-β-treated eyes exhibited an accumulation of cells between Descemet’s membrane and the anterior iris epithelium that were immunoreactive to N-cadherin, similar to cell accumulations in the corneal endothelium. However, unlike the normal monolayer of endothelial cells observed in control eyes, these cells exhibited a spindle-shaped morphology suggestive of transformation to a mesenchymal cell phenotype. Indeed, TGF-β has been shown to induce endothelial-to-mesenchymal transition in corneal endothelial cells in vitro and to induce endothelial proliferation. Interestingly, clinical case studies describe patients with proliferative endotheliopathy or iridocorneal endothelial (ICE) syndrome who exhibit corneal endothelial changes similar to those found in our rat model. ICE syndrome involves aberrant proliferation of corneal endothelial cells that spread over the surface of the trabecular meshwork and anterior surface of the iris. Additionally, ICE syndrome is described to involve abnormal deposition of basement membrane material. Moreover, ocular hypertension is associated with ICE syndrome in humans similar to what we found in AdTGF-β-treated rats; therefore, it is plausible that the PAS we saw in rats treated with AdTGF-β may resemble cellular events leading to ICE syndrome associated with the development of closed-angle glaucoma in these patients.

Reduced α-Smooth Muscle Actin Expression

An examination of the trabecular meshwork after AdTGF-β treatment revealed that although changes in ECM deposition and architecture were not found, a decrease in αSMA levels was observed. This is in contrast to a number of in vitro studies demonstrating that trabecular meshwork cells in culture, when exposed to TGF-β, express higher levels of αSMA and confer a myofibroblast phenotype. Nonetheless, ocular hypertension has been correlated with a loss of αSMA expression in the trabecular meshwork of dogs with glaucoma. For example, Hassell et al. described a loss of αSMA in 50% of canine eyes with closed-angle glaucoma and proposed that this loss was associated with age and was exacerbated by the presence of glaucoma. Contractile elements in the trabecular meshwork are thought to facilitate aqueous outflow; therefore, loss of contractility could affect outflow resistance. Thus, consistent with these studies, we saw a decrease in αSMA expression in the trabecular meshwork of rats treated with AdTGF-β that experienced ocular hypertension but not in those treated with AdGFP. The morphologic phenotype seen in AdTGF-β–treated eyes appeared to be of an angle closure type, a finding also consistent with the canine studies.

One possible explanation for the decrease in αSMA in the trabecular meshwork of AdTGF-β–treated eyes might be related to the ability of TGF-β family members to elicit a biphasic response in cells that depended on both dose and duration of TGF-β expression. In vitro data typically represents a time course characterized by the number of hours or days in culture, whereas our in vivo data are scaled at weekly time intervals beginning with a 2-week time point. We did show increased TGF-β protein expression in the trabecular meshwork of AdTGF-β–treated eyes suggesting that the anterior segment structures were exposed to TGF-β in a chronic manner. A direct measurement of TGF-β in the aqueous humor was not possible because of the close apposition of the iris to the cornea in AdTGF-β–treated rat eyes (Fig. 2B). This arrangement increases the likelihood of injuring the iris and releasing blood, a rich source of TGF-β, and would have yielded erroneous results.

The phenomenon of a biphasic response to TGF-β has been reported by Cordeiro et al. who show that the stimulation of human tendon fibroblast cells with TGF-β family members elicits varied responses at different concentrations. Maximal contraction of two- and three-dimensional collagen matrices occurred at 10−9 M and was reduced at both higher and lower concentrations. Similar response dynamics were seen in both fibroblast proliferation and migration. Studies in extraocular tissues and fibroblast cell lines have also reported biphasic responses to TGF-β family members. It is, therefore, reasonable to hypothesize that anterior segment tissues in vivo may show a different response associated with elevations of TGF-β compared with in vitro stimulation, possibly because of the amount and duration of TGF-β elevation and the environmental cues that are inherently lacking in in vitro systems.

Retinal Morphology

The death of RGCs is often correlated with ocular hypertension. In this study, we observed a substantial cell loss in the retinas of AdTGF-β– but not AdGFP–treated eyes. TUNEL analysis confirmed that this cell loss was due in part to apoptosis. However, the nature of this cell loss may be attributed to either the ocular hypertension or the direct effect of elevated levels of TGF-β. Our analysis of TGF-β expression in the retina at day 14 indicated that endogenous or transgene expression was not substantially different between AdTGF-β– and AdGFP–treated eyes, suggesting that increased levels of TGF-β in the retina may not be responsible for the observed cell death. Analysis of αSMA expression at day 29 revealed epiretinal immunolocalization in AdTGF-β– but not AdGFP–treated eyes. Induction in αSMA expression by TGF-β is well documented. Therefore, this epiretinal localization of αSMA may suggest a direct effect of TGF-β on the retina. However, other factors known to induce αSMA expression, such as nerve growth factor (NGF) and its receptors trkA and p75, may be stimulated by the ensuing ocular hypertension. Thus, further studies are required to separate the effects of ocular hypertension and the TGF-β transgene on retinal morphology in AdTGF-β–treated eyes.

In conclusion, we have provided evidence indicating that TGF-β overexpression results in PAS formation, consisting of an aberrant accumulation of cells that appear to be of corneal endothelial origin. Loss of αSMA expression in the trabecular meshwork was also associated with AdTGF-β treatment. Importantly, these changes were accompanied by ocular hypertension. This model may, therefore, provide a new avenue of research for examining the molecular mechanisms leading to in vivo PAS formation and changes in anterior segment morphology that result in increases in IOP. Moreover, given that adenovirus gene transfer to the anterior chamber has already been reported in mice, this technique can be used in knockout mouse models to dissect the genetic requirements for PAS formation and ocular hypertension.

References


