TGF and TGF-β3 Immunoreactivity Within the Ciliary Epithelium

Nancy S. Peress† and Edward Perillo†*

Purpose. To determine whether the ciliary epithelium exhibits immunoreactivity for antibodies to transforming growth factor beta (TGF-β) 2 and TGF-β3. The hypothesis was that because the aqueous humor contains mainly biologically active TGF-β2, with little TGF-β1, the epithelium largely responsible for its composition would also contain this isoform of TGF-β. The authors anticipated TGF-β3 immunoreactivity because TGF-β3 often co-localizes with TGF-β2.

Methods. The authors followed a standard immunohistochemical protocol using the avidin-biotin complex and newly available rabbit antibodies to synthetic peptide sequences of TGF-β2 and TGF-β3. Formalin-fixed, paraffin-embedded samples of freshly obtained rabbit and human autopsy eyes were studied. Specificity was supported by specific peptide absorption of antisera before tissue incubation.

Results. The pigmented and nonpigmented ciliary epithelia of rabbit and human eyes were stained by antibodies to both TGF-β2 and TGFβ-3, and the staining was inhibited by preabsorption of antibodies by peptides of TGF-β2 and TGF-β3.

Conclusions. The authors conclude that the ciliary epithelium exhibits TGF-β2- and TGF-β3-like immunoreactivity that, based upon complementary work from other laboratories, is probably synthesized by this epithelium and is not simply absorbed by it from the aqueous humor. Invest Ophthalmol Vis Sci. 1994;35:453–457.

Transforming growth factor-beta (TGF-β) proteins are a family of cytokines that influence inflammation and cell proliferation.[1] The aqueous humor, a fluid with known immunosuppressive properties, contains high concentrations of latent TGF-β, in particular TGF-β2, which accounts for most of the aqueous TGF-β biologic activity.[2–4]

We used commercially available, well-characterized rabbit antibodies to two mammalian forms of the TGF-β family, TGF-β2 and TGF-β3—which tend to co-localize—to study the expression of these proteins in the ciliary epithelium of human and rabbit eye. Our findings, of significant immunoreactivity within the cytoplasm of the pigmented and nonpigmented epithelial cells of the ciliary epithelium, complement a recent demonstration that cultured mammalian ciliary epithelial cells produce latent TGF-β mainly of the type 2 isoform.[5] Our results are in direct contrast to a recent immunohistochemical study of the ocular distribution of these cytokines that failed to demonstrate immunoreactivity within the ciliary epithelium.[6]

MATERIALS AND METHODS

To undertake this work, we first established optimal tissue conditions for positive epithelial cytoplasmic immunoreactivity. We used rabbit kidney, a known positive control tissue that has secretory epithelia with analogous physiological functions to the ciliary epithelium, to develop our protocol. Kidney samples were obtained immediately after sacrifice by lethal intravenous sodium pentathol injection. Tissue blocks were prepared for immunohistochemistry using four methods of fixation and processing that included: immediate quick freezing of tissue samples in liquid nitrogen chilled isopentane, followed by cryostat sectioning and brief (10 minutes) acetone fixation of glass affixed sec-

*From the *Department of Pathology, State University of New York, Stony Brook, and the †Department of Veterans Affairs Medical Center, Northport, New York.*
Supported by Veterans Administration Merit Grant: Intrinsic Immunologic Functions of Eye and Brain.
Submitted for publication June 4, 1993; revised July 28, 1993; accepted August 24, 1993.
Proprietary interest category: N.
Reprint requests: Nancy S. Peress, M.D., Department of Pathology, State University of New York at Stony Brook, BHS Tower 9, Stony Brook, NY 11794-8691.

tions; 24-hour fixation in freshly prepared 4% paraformaldehyde with subsequent cryoprotection by incubation in 20% sucrose, followed by quick freezing, as above, and subsequent thick-section frozen sectioning (16μ) to produce floating sections; 24-hour fixation in 10% neutral buffered formalin with subsequent dehydration in graded alcohols and standard embedment in paraffin; and 1-hour fixation in the B-5 mercuric chloride-formalin mixture followed by fixation in 10% neutral buffered formalin with processing as above for the formalin-fixed samples. We then compared results in air-dried glass-affixed and floating sections of kidney using dilutions of primary antibody (stock concentration 100 μg/ml) from 1/10 to 1/10,000 in a standard histochemical protocol consisting of preincubation with 0.1% H_2O_2 for 30 minutes, followed by 24-hour incubation at 4°C with the primary antibody in with 10% normal horse serum and 0.3% Triton X-100. Sections were then washed with phosphate buffered saline and incubated for 2 hours in 1:250 biotinylated goat anti-rabbit IgG, followed by 1 hour incubation with the Vectastain ABC reagent, avidin, and biotinylated horseradish peroxidase (Vector Labs, Burlingame, CA). The horseradish peroxidase substrate was visualized with a 50 mg/100 ml solution of 3,3' diaminobenzidine chromogen (Sigma, St. Louis, MO) in the presence of 0.03% H_2O_2. The controls included sections subjected to incubation in the secondary serum (goat) in lieu of primary antibody.

Once optimal conditions were established, controls also included sections incubated with primary antibody that had been preabsorbed with appropriate purified peptide, in a 10:1 ratio of peptide protein to antibody protein. Antibodies to TGF-β2 and TGF-β3 and their respective purified peptides were purchased from Santa Cruz Biotech, Inc.

The TGF-β2 antibody is an affinity-purified, polyclonal rabbit antibody raised against a peptide that corresponds to amino acid residues 352 to 377 mapping within the carboxy terminal region of human TGF-β2. It reacts with human TGF-β2 by Western immunoblotting, immunoprecipitation, and neutralization of biologic activity. It lacks detectable cross-reactivity with either TGF-β1 or TGFβ3. The TGF-β3 antibody is an affinity-purified polyclonal rabbit antibody raised against a peptide corresponding to amino acid residues 350 to 375 mapping within the carboxy terminal region of human TGF-β3. Antibody TGF-β3 reacts with human TGF-β3. By Western immunoblotting, antibody TGF-β3 is 400-fold more reactive with TGF-β3 than with TGF-β1, and it lacks detectable cross-reactivity with TGF-β2. In contrast, by immunoprecipitation and neutralization, antibody TGF-β3 specifically reacts only with TGF-β3 (Santa Cruz Biotech, Inc., Santa Cruz, CA).

With the establishment of optimal conditions, we studied both Dutch Black Belted and New Zealand White normal rabbit eyes, obtained by rapid enucleation after lethal sodium pentobarbital injection, and three adult autopsy eyes, obtained within 10 hours of death. The autopsy eyes were from a 54-year-old woman who died from disseminated T-cell lymphoma, a 32-year-old man who died from pneumonia, and a 22-year-old man with neuronal ceroid lipofuscinosis and terminal sepsis. The human eyes were free of disease in the uveal tract. There was retinal atrophy in the eye from the patient with lipofuscinosis.

This investigation adhered to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and the Declaration of Helsinki. Informed autopsy consent was obtained for the human tissue.

RESULTS

We found that the optimal tissue conditions for renal epithelial cytoplasmic immunoreactivity, our positive control tissue, and tissue preservation were 1/10 to 1/100 dilutions of the primary antibodies and tissues fixed in 10% neutral buffered formalin followed by standard paraffin embedment. Under these conditions, we saw strongly positive staining of renal tubular epithelium, which was markedly reduced by preabsorption of the primary antibody with immunogen peptide (peptide inhibitive), and we retained excellent histologic detail. The B-5, formalin-fixed tissue was also positive. This fixative combination was not preferable to formalin alone. The renal tubular epithelium did not exhibit distinctive immunostaining under conditions of paraformaldehyde fixation, cryoprotection, quick freezing, and subsequent frozen sectioning.

In the formalin-fixed, paraffin-embedded eyes with antibodies to both TGF-β2 and TGF-β3, we observed positive cytoplasmic staining of both pigmented and nonpigmented cells of the ciliary epithelium. The pigmented epithelial staining was best appreciated in the albino rabbit eyes. Absorption with appropriate peptide strongly inhibited subsequent epithelial immunoreactivity. In the no primary controls, which were incubated with normal goat serum in lieu of the primary antibody, there was no epithelial staining. The comparable findings in human and rabbit eyes are depicted in Figures 1 and 2, respectively. As can be seen in these photomicrographs, with both antibodies there was also light diffuse staining of the connective tissue and vascular region of the ciliary processes.

DISCUSSION

This study describes immunoreactive TGF-β2 and TGF-β3 within the pigmented and nonpigmented cili-

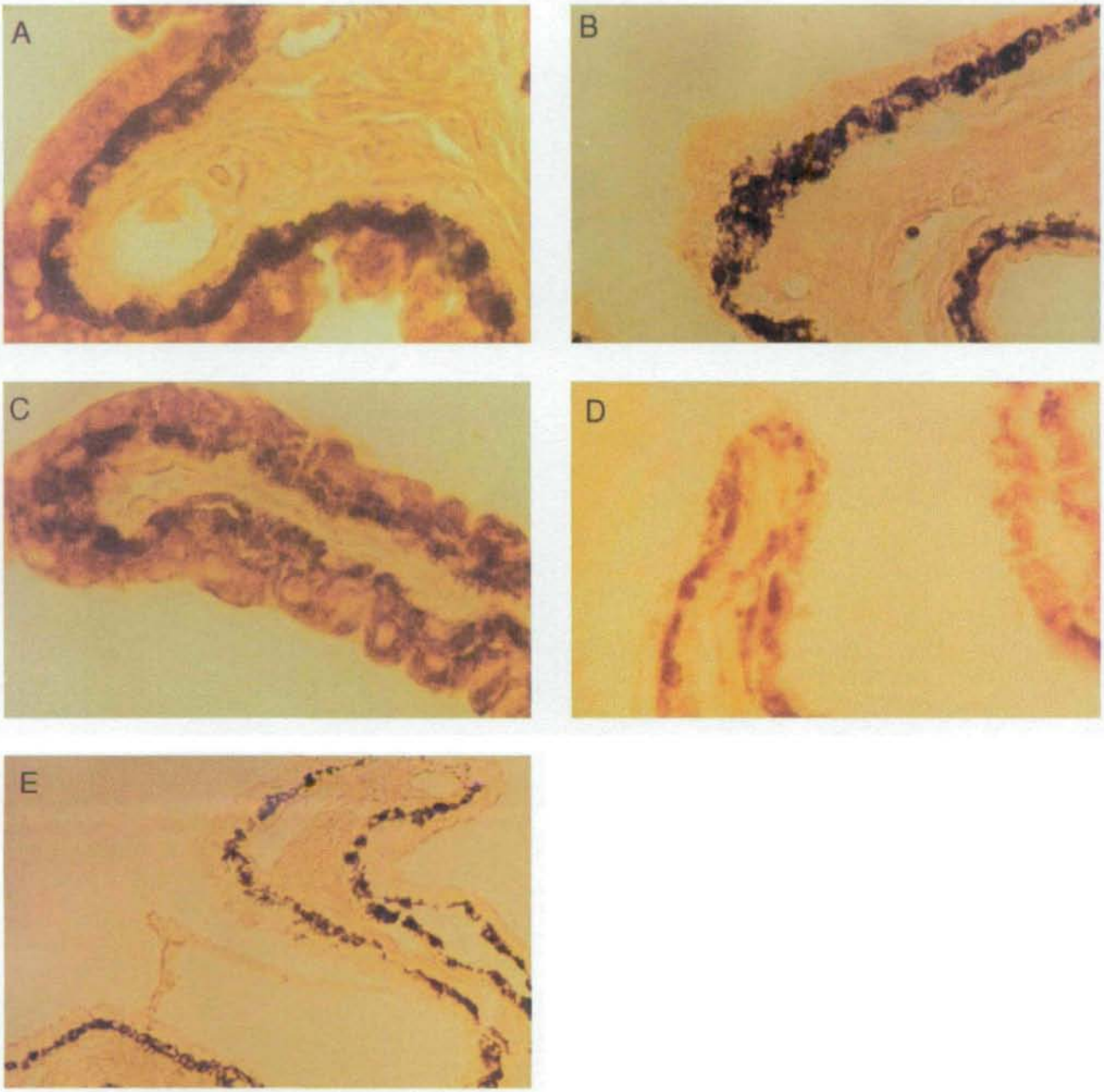

FIGURE 1. The photomicrographs in this figure are of human ciliary processes stained with anti-TGF-β2 (**a**), peptide preabsorbed anti-TGF-β2 (**b**), anti-TGF-β3 (**c**), peptide preabsorbed anti-TGF-β3 (**d**), and normal goat serum in lieu of a primary antibody, the no primary control (**e**). Magnifications are 800×, 800×, 800×, 600×, 400×, respectively. As can be seen in Figures 1a and 1c, the ciliary epithelium exhibits positive staining (brown reaction product) when immunoreacted with antisera to both TGF-β2 and TGF-β3. To some extent, the black pigment of the pigmented epithelium obscures staining of the inner layer of this double-layered epithelial structure. Epithelial positivity is markedly reduced by specific peptide preabsorption of the primary antisera (Figs. 1**b**, 1**d**), suggesting that the epithelial antibody staining observed in the nonabsorbed sections (**a** and **c**) is specific. The no primary control section (**e**) is free of significant epithelial staining, proof that the secondary antibody did not react directly with the epithelium.

ary epithelium of the ciliary body. It complements recent studies, employing bioassay for TGF-β1 and TGF-β2 protein activity, which found predominantly TGF-β2 within the aqueous humor and in supernatants of pigmented and nonpigmented ciliary epithelial monolayers.[3,4] We included antibodies to TGF-β3 in this study because it tends to co-localize with TGF-β2 and, hence, might also be present in ocular TGF-β2-positive cell populations.[1,7] To our knowledge, there are no reports of TGF-β3 in aqueous humor. Because

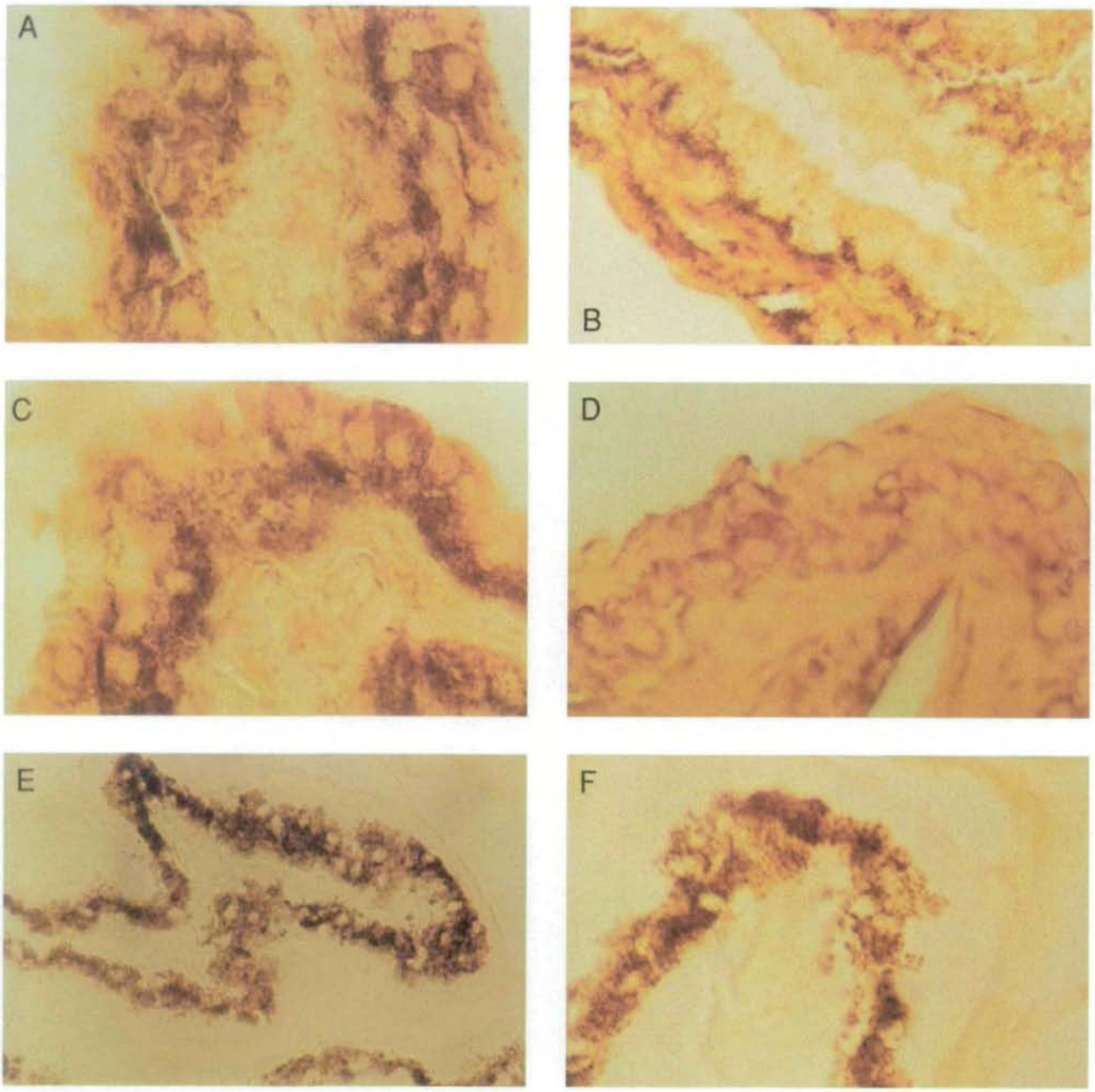

FIGURE 2. The photomicrographs in this figure are of rabbit ciliary processes stained with anti TGF-β2 (**a**), peptide preabsorbed anti-TGF-β2 (**b**), anti TGF-β3 (**c** and **d**), peptide preabsorbed anti-TGF-β3 (**e**) and normal goat serum in lieu of a primary antibody, the no primary control (**f**). Magnifications are 800×, 800×, 800×, 800×, 400× and 800×, respectively. In Figures 2a, 2c, and 2d, positive reaction product is seen within the cytoplasm of the ciliary epithelium of pigmented (**a** and **c**) and albino (**d**) rabbit eyes. In the albino rabbit eye (**d**), one can more easily appreciate staining of the inner epithelial layer. Peptide preabsorption (**b** and **e**) of these antibodies significantly reduced subsequent epithelial staining, suggesting that the antibody staining observed in the nonabsorbed sections (**a**, **c**, and **d**) is specific. The no primary control (**f**) is free of significant epithelial staining. This negative preparation proves that the secondary antibody, goat anti-rabbit IgG, did not react directly with the rabbit epithelium.

TGF-β2 messenger RNA has been detected in RNA extracted from samples of ciliary body and iris,[4] the constellation of these results suggests that this epithelium, which can both secrete and absorb, makes these proteins and releases them to the aqueous humor rather than simply absorbing them from the aqueous.

Our positive immunohistochemical findings in the ciliary epithelium for TGF-β2 and TGF-β3 are in sharp

contrast to the negative immunohistochemical results for these epithelial cells and proteins recently reported by Pasquale et al.[6] We think these different results can be explained by methodologic differences in the two studies. Pasquale et al[6] used rabbit antibodies that had been made and used successfully by another group, Flanders et al,[7] to study the embryonic nervous system. The Flanders study used formalin/Bouin's-fixed, paraffin-embedded brain tissue as we have in this study, whereas the ocular study by Pasquale used eyes that were fixed in paraformaldehyde and subsequently cryoprotected, quick frozen, and frozen sectioned. In our preliminary study to determine optimal conditions for demonstrating these epitopes within epithelial cells, as described above, we found that quick frozen sections of paraformaldehyde-fixed, cryoprotected samples did not yield cytoplasmic epithelial immunostaining. Thus, we did not use this method of tissue handling, a method we and others have found to be optimal for certain antigens. In our hands, for example, it has been especially useful for membrane bound, surface epitopes.[8] We think that this is the important methodologic difference between our study and the study by Pasquale et al.[6] Other potentially important differences include the use of rabbit antibodies from different sources made to different epitopes of the proteins of interest and our inclusion of Triton-X, a permeabilizing agent. The Flanders group[7] used hyaluronidase though this does not seem to have been used in the Pasquale study.[9]

In this study, we used kidney, a known positive epithelial tissue, to develop our protocol. The specificity of the immunoreactivity of the primary antibodies and the ciliary epithelium was supported by our inclusion of peptide preabsorbed primary antibodies. The absence of epithelial staining observed in our no primary controls established that the secondary antibodies did not react directly with the epithelium or with IgG, which may have been present in the tissue. The inclusion of human eyes in this study, which relied upon rabbit primary antibodies, further reduced the likelihood that the ciliary epithelial staining observed in both human and rabbit eyes was due to the presence of rabbit IgG.

In view of the unique immune environment of the eye, an immunologically privileged site, it is of interest that TGF-β2 and TGF-β3 are made by the ciliary epithelium and potentially secreted into the aqueous humor. These cytokines are made by immunologically activated systemic cells, such as activated macrophages, platelets, tumor cells in culture, and certain parenchymal cells (i.e., kidney).[1] They are immunomodulators with functions that include inhibition of T-cell activation and proliferation, inhibition of lymphokine production, inhibition of class II antigen expression, and inhibition of the generation of cytotoxic T-cells.[1] Their presence in the aqueous humor suggests that they contribute to the immunosuppressive properties of the aqueous and that they play a role in the intraocular expression of immune-mediated disorders.[2,3,4]

Key Words

TGF-beta, ciliary epithelium, cytokines, ocular

References

1. Roberts AB, Sporn MB. The transforming growth factor-βs. In: Sporn MB, Roberts AB, eds. *Growth Factors and their Receptors: Handbook of Experimental Pharmacology*. Heidelberg: Springer-Verlag; 1990:419–472.
2. Streilein JW, Wilbanks GA, Cousins SW. Immunoregulatory mechanisms of the eye. *J Immunol.* 1992;39: 185–200.
3. Cousins SW, McCabe MM, Danielpour D, Streilein JW. Identification of transforming growth factor-beta as an immunosuppressive factor in aqueous humor. *Invest Ophthalmol Vis Sci.* 1991;32:2201–2211.
4. Knisely TL, Bleicher PA, Vibbard CA, Granstein RD. Production of latent transforming growth factor-beta and other inhibitory factors by cultured murine iris and ciliary body cells. *Curr Eye Res.* 1991;10:761–771.
5. Helbig H, Kittredge KL, Coca-Prados M, Davis J, Palestine AF, Nussenblatt RB. Mammalian ciliary-body epithelial cells in culture produce transforming growth factor-beta. *Graefe's Arch Clin Exp Ophthalmol.* 1991;229:84–87.
6. Pasquale LR, Dorman-Pease ME, Lutty GA, Quigley HA, Jampel HD. Immunolocalization of TGF-β1, TGF-β2, and TGF-β3 in the anterior segment of the human eye. *Invest Ophthalmol Vis Sci.* 1993;34:23–30.
7. Flanders KC, Ludecke G, Engels S, et al. Localization and actions of transforming growth factor-βs in the embryonic nervous system. *Development.* 1991;113: 183–191.
8. Peress NS, Fleit HB, Perillo E, Kuljis R, Pezzullo C. Identification of Fc RI, II and III on normal human brain ramified microglia and on microglia in senile plaques in Alzheimer's Disease. *J Neuroimmunol.* 1993; 48:71–80.
9. Lutty GA, Merges C, Threlkeld AB, Crone S, McLeod DS. Heterogeneity in localization of isoforms of TGF-β in human retina, vitreous, and choroid. *Invest Ophthalmol Vis Sci.* 1993;34:477–487.