Transforming Growth Factor-β Receptor Expression in Human Cornea

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**Purpose.** Limbal basal cells and corneal endothelial cells appear to be inhibited in the G1 phase of the cell cycle. As a preliminary to determining whether transforming growth factor-beta (TGF-β) might mediate this inhibition, investigation was made to determine whether human corneal and limbal cells express TGF-β receptor types I (RI), II (RII), and III (RIII).

**Methods.** Corneas from eight human donors, aged stillborn to 85 years, were fresh frozen, cryostat sectioned, and prepared for indirect immunofluorescence localization of RI, RII, and RIII, using an established protocol. Corneas from donors 50 years of age or older were used to prepare RNA from the epithelium and endothelium. Reverse transcription-polymerase chain reaction was conducted using primers specific for each TGF-β receptor type.

**Results.** Immunolocalization patterns for RI, RII, and RIII were similar, regardless of donor age. Binding of RI and RII antibodies was barely detectable in central corneal epithelium; however, most limbal basal cells stained positively for RI and RII. All layers of central corneal epithelium and the suprabasal layers of the limbus stained positively for RIII, whereas staining for this receptor was markedly decreased in limbal basal cells. Corneal endothelium bound the antibody for all three TGF-β receptor types. In the same tissue sections, antibody staining for the RII protein was more intense in corneal endothelial cells than in limbal basal cells. Polymerase chain reaction product for RI, RII, and RIII was detected in the epithelium and in the endothelium.

**Conclusions.** Limbal basal cells and corneal endothelial cells expressed mRNA and protein for TGF-β receptor types I, II, and III, suggesting that both cell types can transmit a TGF-β-induced signal. These two cell types may differ in their relative response to those TGF-β isoforms that require binding to RII for signal transduction, in that staining intensity for RII was relatively low in limbal basal cells compared with that in the endothelium. That limbal basal and corneal endothelial cells express receptors for TGF-β suggests that this cytokine could mediate G1 phase arrest in these two cell types. Invest Ophthalmol Vis Sci. 1997;38:1922–1928.

The human cornea and limbus contain populations of cells in different nonproliferative states. Suprabasal cells of the cornea are terminally differentiated and cannot be stimulated to enter the cell cycle. Corneal epithelial basal cells are generally in a quiescent, G0-like state, but enter the cell cycle and divide to replace cells lost from the epithelial surface. Limbal basal cells proliferate only infrequently, while retaining the capacity to divide multiple times. The corneal endothelium is considered a nonrenewing cell population, because cell proliferation does not keep pace with the rate of cell loss. The relative expression of cell-cycle-associated proteins in corneal cells in situ has been determined using immunolocalization techniques.

Corneal epithelial suprabasal cells and the majority of basal cells do not stain positively for cyclins, the regulatory proteins required for cell cycle progression, or for Ki67, a marker of actively cycling cells. Limbal basal cells and corneal endothelial cells stain relatively intensely for the G1 cyclins D and E. In these cells, cyclin A, a regulatory protein active during the S and early G2 phases, is barely detectable above control levels and Ki67 is undetectable. These results suggest that the majority of limbal basal cells and corneal endothelio-
lial cells have entered the cell cycle but are arrested in the G1 phase.

G<sub>1</sub>-phase arrest can be considered an active process by which cells that have entered the division cycle are prevented from proceeding through the G1→S phase transition and from committing the cell to complete mitosis. Transforming growth factor-beta (TGF-β) inhibits proliferation of many cell types by arresting cells at the G1→S-phase transition and may be responsible, at least in part, for inducing G1-phase arrest in ocular cells. Kurosaka and Nagamoto showed that proliferation of cultured bovine lens epithelial cells is inhibited by TGF-β<sub>2</sub> and human aqueous humor, which contains relatively high concentrations of the TGF-β<sub>2</sub> isoform. In these experiments, inhibition of proliferation induced by aqueous humor was reversed by the addition of TGF-β<sub>2</sub>-neutralizing antibody to the culture medium.

For TGF-β to affect cell function, it must be accessible to the cells, and the cells must express the receptors needed to bind TGF-β efficiently and to transduce the signal. Three isoforms of TGF-β (β<sub>1</sub>, β<sub>2</sub>, and β<sub>3</sub>) have been identified in mammalian cells. In the eye, TGF-β is found in a number of tissues, including those of the trabecular meshwork, ciliary epithelium, retina, vitreous, and choroid. Within the cornea, the epithelium appears to synthesize TGF-β; however, a consensus has not been reached on the specific isoforms synthesized. Corneal endothelial cells synthesize mRNA and protein for TGF-β<sub>1</sub>,<sub>12,16</sub> and are also exposed to the TGF-β<sub>2</sub> isoform present in aqueous humor. That TGF-β may be available to limbal basal and corneal endothelial cells presents the possibility that this cytokine could be involved in inducing G<sub>1</sub>-phase arrest in these cells through an autocrine or a paracrine mechanism.

For TGF-β to induce an intracellular signal, TGF-β receptor types I (RI) and II (RII) must be expressed on the cell surface. Receptor type II binds TGF-β<sub>1</sub> and -β<sub>3</sub> isoforms with high affinity, but by itself cannot induce signal transduction. Binding of ligands to RII results in recruitment of RI and formation of a ligand–RI–RII complex. Recruitment of RI into the complex results in phosphorylation of RI by RII and in the subsequent induction of an intracellular signal. The TGF-β<sub>2</sub> isoform of TGF-β does not bind to RII with high affinity but binds avidly to TGF-β receptor type III (RIII), also known as β-glycan. Receptor type III does not directly participate in signal transduction but cooperates with RI and RII to produce a transmembrane signal by binding TGF-β<sub>2</sub> and presenting it to RII. Relatively little is known about the distribution or function of TGF-β receptors in the eye. Results of binding studies suggest that porcine trabecular meshwork cells express RII and RIII but do not express RI. Receptor types I and II have been localized by immunohistochemistry in rat eyes; however, no comprehensive study has been conducted to determine the mRNA and protein expression for TGF-β receptors in human corneal and limbal cells.

The goal of the current study was to determine whether human corneal and limbal cells express mRNA and protein for TGF-β receptor types I, II, and III. Results of indirect immunofluorescence and reverse transcription-polymerase chain reaction (RT-PCR) studies indicate that limbal basal and corneal endothelial cells express protein and mRNA for all three receptor types, suggesting that they would be sensitive to a TGF-β-induced signal and that TGF-β might play a role in the G<sub>1</sub>-phase arrest of these cells.

**METHODS**

**Human Corneal Tissue**

Donor human corneas were obtained through National Disease Research Interchange (Philadelphia, PA). All corneas were preserved in Optisol (Chiron, Irvine, CA) and received on wet ice in the laboratory within 24 to 36 hours after death. Criteria for exclusion of corneas from these studies included history of endothelial dystrophy, presence of central gutta, low endothelial cell count, ocular inflammation or disease and diabetes.

**Immunocytochemical Localization**

Corneas were obtained from donors whose ages were stillborn, 3 months, and 5, 21, 37, 67, 77, and 85 years. When received in the laboratory, corneas were sectioned into quadrants, fresh frozen in Tissue-Tek OCT Compound (Miles, Elkhart, IN), and stored at −70°C until ready for cryostat sectioning. The indirect immunocytochemical localization protocol was essentially that of Spurr and Gipson. Briefly, 6-μm transverse corneal sections were applied to formalin-fixed, gelatin-coated slides and air-dried. All steps were carried out at room temperature. Slides were rinsed in phosphate-buffered saline (PBS) and incubated for 15 minutes with 2% bovine serum albumin in PBS to block nonspecific binding. Primary and secondary antibodies were prepared in the same blocking buffer. Primary antibody was applied to the tissue sections for 2 hours in a moist chamber. Anti-TGF-β RI was used at a concentration of 0.1 μg/ml, anti-RII at 0.25 μg/ml, and anti-RIII at 10 μg/ml. After primary antibody incubation, sections were washed with PBS and incubated for 15 minutes with blocking buffer. Fluorescein-conjugated antirabbit or antigoat IgG was used as the secondary antibody, depending on the primary antibody source. Secondary antibody, diluted to 1:200 to 1:500, was applied to the sections for 1 hour in a...
FIGURE 1. Transforming growth factor-β type I receptor localization in (A) central and in (B) peripheral corneal epithelium, and in (C) limbal epithelium. The micrograph in (D) shows lack of staining in sections of epithelium incubated with a secondary antibody alone. A similar control in (E) shows autofluorescence of Descemet’s membrane and unstained corneal endothelial cells (arrows). Corneal endothelial cells (F) stain intensely for RI. Note the circumferential pattern of type I receptor binding in (B) and (C). DM = Descemet’s membrane; RI = receptor type I. Magnification, ×400.

moist chamber. Sections were washed, as described earlier, and mounted in paraphenylene diamine–glycerol for visualization and photography using a Nikon UFX II photomicroscope (Donsanto, Natick, MA). Negative controls consisted of tissue sections incubated with secondary antibody alone or with primary antibody previously incubated with an excess of the corresponding peptide antigen, followed by staining with the secondary antibody. For each experiment, film negatives were printed at the same exposure to permit comparison of relative staining intensity. Sodium dodecyl sulfate extracts were prepared from SV40 transformed human corneal endothelial cells, an abundant source of human corneal endothelial cell protein (a gift of Dr. Steven Wilson, Cleveland Clinic Foundation, Cleveland, OH). Extracts were electrophoresed, transferred to nitrocellulose, and tested for antibody specificity by Western blot analysis, as previously described.²³ All antibodies reacted with a band of the correct molecular weight.

Antibodies

Affinity-purified rabbit antihuman TGF-β RI and anti-human TGF-β RII were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); goat antihuman TGF-β RIII detection antibody from R & D Systems, (Minneapolis, MN); and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies from Jackson Laboratories (West Grove, PA).

Reverse Transcription–Polymerase Chain Reaction

One to three corneal pairs from donors 50 years of age or older were used for RNA isolation. RNA isolation and RT–PCR were performed essentially as described previously.²³ Briefly, corneas were dissected immediately after receipt. Descemet’s membrane with associated endothelial cells was dissected from the cornea and the epithelium, including limbal epithelium, was removed by EDTA treatment.²⁴ Tissues of like type were pooled and placed directly into a denaturation solution, containing 4 M guanidine isothiocyanate, 0.02 M sodium citrate, and 0.5% sodium lauryl sarcosinate, provided in a micro-RNA isolation kit (Stratagene, LaJolla, CA). Total RNA was isolated according to the methods described in the kit. RNA from human epithelium was electrophoresed and stained with acridine orange to document RNA integrity. Complementary DNA (cDNA) was prepared from 1 µg of total RNA by reverse transcription in a volume of 20 µl, using reagents from a commercially available kit (Promega, Pittsburgh, PA).

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933196/)
FIGURE 3. Transforming growth factor-β type II receptor localization in (A) central and (B) peripheral corneal epithelium, and in (C) limbal epithelium. The micrograph in (D) shows lack of staining in sections of epithelium incubated with a secondary antibody alone. (E) A similar control shows autofluorescence of Descemet’s membrane (DM) and unstained corneal endothelial cells (arrows). Corneal endothelial cells (F) stain intensely for RII. Note the circumferential staining pattern for receptor type II, which is particularly obvious in (C). RII = receptor type II. Magnification, ×400.

Primers were designed according to established criteria: TGF-β RI upstream sequence, 5’-CGTTACAGTCGTTTCTGCCACCT-3’, and downstream sequence, 5’-AGACGAGCACAACACTGGCCAGC-3’; RII upstream sequence, 5’-TGTGAGAAGCAGGCAGGAAGTC-3’, and downstream sequence, 5’-GGACATCTTCTGCTAGATCA-3’; and RIII upstream sequence, 5’-CCGGGTGTGACTGTCAACCAAATCA-3’, and downstream sequence, 5’-CTGTGCTGACTTCCATCAG-3’. Polymerase chain reaction was performed in a reaction mixture containing 1 μg cDNA and 2.5 μM each of the upstream and downstream primers, plus reagents from a commercially available kit (GibcoBRL Life Technologies, Gaithersburg, MD). Specificity and yield of the PCR products were enhanced using the “hot start” approach. For all three experiments, PCR was performed for 40 cycles in a thermal cycler. Polymerase chain reaction cycle conditions included denaturation at 95°C for 1 minute, annealing at the required temperature for 1 minute, and extension at 72°C for 2 minutes. A 5-minute extension was added at the end of 40 cycles of PCR. Annealing temperatures were as follows: RI = 56°C; RII = 50°C; RIII = 52°C. Polymerase chain reaction products and 100-bp DNA-ladder molecular weight markers were electrophoresed in 1.5% agarose gels containing 0.5 μg/ml ethidium bromide and were photographed.

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH; Clontech, Palo Alto, CA) acted as a positive control for the PCR. Negative controls consisted of the PCR reaction mixture, including primers, but without cDNA. To ensure that the total RNA samples were not contaminated with genomic DNA, a negative control using 1 μg total RNA was substituted for cDNA in our PCR reaction mixture, along with 2.5 μM each of upstream and downstream G3PDH primers. According to the supplier, these primers are capable of amplifying genomic DNA and of generating the same size PCR product as that obtained using cDNA. No total RNA samples used in these studies yielded a G3PDH PCR product under these conditions, indicating that the PCR products were obtained from mRNA and not from contaminating genomic DNA (data not shown). To confirm the specificity of the PCR results, products from each PCR reaction were digested with restriction enzymes that were chosen on the basis of the expected nucleotide sequence of each product. All three PCR reactions for TGF-β receptors yielded cleavage products of the size expected from the sequence data (data not shown).

RESULTS

Transforming Growth Factor-β Receptor Type I

A gradient of TGF-β RI protein expression was exhibited within the corneal and limbal epithelium, as indicated by the micrographs in Figure 1. In the central cornea (Fig. 1A), RI antibody staining was detectable above background level (Fig. 1D); however, with proximity to the limbus, an increasing number of single cells or groups of cells within the basal layer stained intensely for RI (Fig. 1B). In some cases, positively stained cells just above the basal layer exhibited stalk-like structures, suggesting that the cell bodies were displaced from the basal layer, but that the cells remained attached to the epithelial basement membrane. Most, but not all, limbal basal cells stained in-
FIGURE 4. Reverse transcription-polymerase chain reaction for transforming growth factor-β type II receptor (lanes 2 and 4) and G3PDH (lanes 3 and 5) from human donor corneal epithelium (lanes 2 and 3) and endothelium (lanes 4 and 5). A single polymerase chain reaction product for receptor type II (650 bp) was obtained from both corneal samples, although the product obtained from the epithelial sample was relatively faint. The G3PDH (452 bp) acted as a positive control for the polymerase chain reaction, whereas the negative control (lane 1) consisted of all reagents in the reaction mixture except complementary DNA. Lane 6 = 100 bp DNA ladder; G3PDH = glyceraldehyde-3-phosphate dehydrogenase.

tensely with the RI antibody (Fig. 1C). The circumferential pattern of antibody-binding reflects the expected localization of TGF-β RI to the plasma membrane. Corneal endothelium bound the RI antibody intensely in comparison with background (cf. Figs. 1E and 1F). Polymerase chain reaction product for RI (314 bp) was detected in samples prepared from donor epithelium and endothelium (Fig. 2).

Transforming Growth Factor-β Receptor Type II
As was observed with RI, a gradient of TGF-β RII protein expression was detected in the corneal and the limbal epithelium. Receptor type II antibody-binding within the central epithelium (Fig. 3A) was detectable above background level (Fig. 3D). The number of intensely stained basal cells increased with proximity to the limbus (Fig. 3B). Most, but not all, limbal basal cells exhibited intense circumferential staining for RII, whereas suprabasal cells were less intensely stained (Fig. 3C). Corneal endothelium stained intensely for TGF-β RII compared with background (cf. Figs. 3E and 3F). Polymerase chain reaction product for RII (650 bp) was detected in samples prepared from donor epithelium and endothelium (Fig. 4). In all samples tested, RII PCR product obtained from donor epithelial RNA was visualized as a faint band compared with that seen in the the G3PDH positive control. Efforts to increase the product yield by varying the annealing temperature or altering the magnesium concentration were unsuccessful.

Transforming Growth Factor-β Receptor Type III
Antibodies that specifically recognized RIII bound in a characteristic pattern, but the relative intensity of staining varied in a non-age-dependent manner among the eight corneas studied. Figure 5 illustrates the results in a cornea that stained intensely with anti-TGF-β RIII. Antibodies bound in a circumferential pattern to virtually all basal and suprabasal cells within the central epithelium of the cornea (Fig. 5A). In comparison, individual basal cells and groups of basal cells in the peripheral epithelium stained less intensely with the RIII antibody (Fig. 5B). In the limbus (Fig. 5C), suprabasal cells stained intensely for RIII, whereas staining was barely detectable in the majority of limbal basal cells. Corneal endothelial cells consistently stained intensely with the RIII antibody (Fig. 5D). In addition to producing a circumferential pattern indicative of plasma membrane binding, this antibody also appeared to stain nuclei, particularly in epithelial cells. Polymerase chain reaction product of the expected molecular weight (342 bp) for RIII was detected in donor epithelium and endothelium (Fig. 6).

DISCUSSION
Limbal basal cells and corneal endothelial cells express RI and RII, as is indicated by the immunocyto-
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FIGURE 6. Reverse transcription-polymerase chain reaction for TGF-β type III receptor (lanes 2 and 4) and G3PDH (lanes 3 and 5) from donor epithelium (lanes 2 and 3) and endothelium (lanes 4 and 5). A single polymerase chain reaction product for receptor type III (342 bp) was obtained from both corneal samples. The G3PDH (432 bp) acted as a positive control for the reaction, whereas the negative control (lane 1) consisted of all reagents in the reaction mixture except complementary DNA. Lane 6 = 100 bp DNA ladder; G3PDH = glyceraldehyde-3-phosphate dehydrogenase.

chemical and RT-PCR data. Reverse transcription-polymerase chain reaction using RNA from donor corneal epithelium consistently yielded a faint band with the expected molecular weight for RII. The "epithelial" tissue samples used for RNA extraction consisted of corneal epithelium (which stained for RII just above background level) and of limbal epithelium (of which only the basal cells stained intensely for RII). The faint band of PCR product may have resulted from the relatively low amount of mRNA for RII present in this sample. It may also reflect the relative synthesis or turnover rate in this mRNA species. Receptor types I and II must be coexpressed in cells for transduction of a TGF-β-induced signal to occur. The expression of mRNA and protein for RI and RII in limbal basal and corneal endothelial cells indicates that a TGF-β-induced signal could be transmitted in these cells. TGF-β1, β2, and β3 isoforms bind with high affinity to RII; however, optimal binding of the TGF-β2 and -3 isoforms requires additional interaction with the type III (β-glycan) receptor. Immunocytochemical localization showed that staining for RII in limbal basal cells was less intense than that in suprabasal cells of the limbal epithelium or in corneal epithelial cells, suggesting that the β2 isoform may not be involved significantly in signal transduction in limbal basal cells. The intense staining for RII in corneal and limbal suprabasal cells suggests that these cells might bind TGF-β isoforms produced within the epithelium or supplied through the tears, thereby protecting limbal basal cells from inappropriate growth factor stimulation. The expression of TGF-β RII in corneal endothelium suggests that these cells could efficiently bind the TGF-β2 that is present in aqueous humor.

It is intriguing that limbal basal cells and corneal endothelial cells, which exhibit characteristics of G1-phase-arrested cells, also express relatively high levels of RI and RII protein. The immunocytochemical and RT-PCR data do not provide evidence that these receptors are active; and there is, as yet, no direct evidence that TGF-β induces G1-phase arrest in these cells. Nevertheless, the fact that G1-phase-arrested limbal basal and corneal endothelial cells express the TGF-β receptor types required for TGF-β binding and signal transduction strongly suggests that TGF-β could mediate G1-phase arrest in these two important cell types.

Key Words
cell proliferation, corneal endothelium, corneal epithelium, transforming growth factor-β receptors

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References
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