Mechanisms of Mitotic Inhibition in Corneal Endothelium: Contact Inhibition and TGF-β2

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Purpose. Contact inhibition has been implicated as an important antiproliferative mechanism in developing and mature corneal endothelium. Although exogenous TGF-β2 and TGF-β2 in aqueous humor suppress S-phase entry in cultured rat corneal endothelial cells, it is not known whether TGF-β2 contributes to the mitotic inhibition that occurs during in vivo endothelial development. TGF-β receptors I, II, and III must be coexpressed for a TGF-β2–induced intracellular signal to be transmitted. The current study was conducted to determine whether TGF-β2 contributes to mitotic inhibition during endothelial development, by investigating when these receptors become coexpressed in the endothelium of neonatal rats. Cyclin-dependent kinase inhibitors (CKIs), such as p27kip1 and p15INK4b, help mediate mitotic inhibition in other cell types. The role of CKIs in inhibiting proliferation in corneal endothelium was examined by first determining the kinetics of p27kip1 expression in neonatal rat corneal endothelium. Studies were then extended to cultured cells to more directly compare the effects of TGF-β2 and cell–cell contact on the relative protein and mRNA expression of the CKIs, p27kip1, and p15INK4b.

Methods. Immunocytochemistry (ICC) detected TGF-β receptors I, II, and III (RI, RII, RIII, respectively) in the endothelium of rat corneas on postnatal days 1, 10, and 21, and in adult (3-month-old) rats. ICC for p27kip1 was conducted on postnatal days 1, 7, 14, and 21. Samples were taken for p27kip1 RT-PCR on postnatal days 7, 14, and 21 and from adult rats. The effect of TGF-β2 on p27kip1 and p15INK4b expression was determined in G_0-phase synchronized subconfluent rat corneal endothelial cells incubated for 24 hours in 10% serum ± 5 ng/mL TGF-β2. CKI expression was also examined in fully confluent cultures. RT-PCR and Western blot analysis detected p27kip1 and p15INK4b mRNA and protein expression, respectively. The effect of releasing cells from cell–cell contact on proliferation and p27kip1 protein expression was studied in confluent cultures treated for 1 hour with and without 2.0 mg/mL di-sodium EDTA and then maintained for 24 hours in 10% serum. Cultures were then either fixed for ICC of Ki67, a marker of actively cycling cells, or extracted for Western blot determination of p27kip1 protein.

Results. Positive staining for TGF-β RIII was detected on postnatal day 10, and staining for RI and RII was detected on postnatal day 21. The endothelium stained positively for p27kip1 on postnatal day 1 and thereafter, and p27kip1 PCR product was detectable at the earliest time point tested (postnatal day 7). In cultured cells, TGF-β2 and cell–cell contact had relatively little effect on expression of p27kip1 or p15INK4b mRNA. TGF-β2 lowered the levels of both proteins, but p27kip1 remained at a higher level than p15INK4b. In confluent cultures, p15INK4b protein was reduced; however, p27kip1 protein levels increased 20-fold. Positive staining for Ki67 was detected, and p27kip1 protein levels substantially decreased in EDTA-treated confluent cells compared with the untreated control.

Conclusions. Previous studies from this laboratory showed that corneal endothelial cell proliferation ceases in neonatal rat by postnatal day 13. This timing correlated with the formation of stable cell–cell contacts, implicating contact inhibition as an important mechanism of growth arrest during endothelial development. The current studies showed that coexpression of TGF-β RI, RII, and RIII occurred too late for TGF-β2 to have a significant role in inhibiting proliferation during endothelial development. Studies in cultured cells suggest that p27kip1 mediates inhibition of proliferation induced by TGF-β2, although the response to this cytokine was relatively weak. ICC and RT-PCR of p27kip1 in neonatal endothelium and RT-PCR and Western blot studies in cultured cells indicate that contact inhibition is mediated, in large part, through the activity of p27kip1. These results, together with previous data from this laboratory, strongly suggest that contact inhibition is an important mechanism responsible for inducing cell cycle arrest during corneal endothelial development and for maintaining the mature monolayer in a nonproliferative state. In both cases, contact-induced inhibition is mediated, at least in part, by p27kip1. TGF-β2 appears not to induce mitotic arrest in the developing endothelium, but may function to maintain the mature endothelium in a nonreplicative state should cell–cell contact be lost in the monolayer. (*Invest Ophthalmol Vis Sci.* 2002;43:2152–2159)

Corneal endothelial cells in vivo are inhibited in the G_1-phase of the cell cycle,[1,2] but retain the ability to proliferate.[3–5] That these cells retain proliferative capacity but do not divide leads to the hypothesis that the endothelium must be actively maintained in a nonproliferative state. This laboratory has been conducting studies to identify mechanisms responsible for inducing or maintaining mitotic quiescence in these cells. Among the factors that appear to contribute to the nonreplicative status of the endothelium are the apparent lack of autocrine or paracrine stimulation by positive growth factors, age, transforming growth factor (TGF)-β, and contact inhibition. Aqueous humor, which bathes the posterior of the endothelium, does not have significant concentrations of positive growth factors.[6] In addition, although endothelial cells express mRNA and protein for a number of growth factors and their receptors,[7,8] there is little apparent autocrine or paracrine mitogenic stimulation, even after cell loss.[9] Age is a factor, in that cells from older individuals enter the cell cycle more slowly than those from younger individuals and require stronger mitogenic stimulation than their younger counterparts.[5] Specific age-related changes leading to this differential response are currently unknown. Telomere length does not seem to be a limiting factor, because telomere lengths in human corneal endothelial cells appear adequate to permit

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additional rounds of division before replicative senescence.[4] TGF-β may play a role in inhibiting proliferation in the endothelium. Human corneal endothelial cells in vivo express TGF-β receptor types I, II, and III[10] (RI, RII, and RIII, respectively), which are required for TGF-β-induced signal transduction.[11–13] Cultured human corneal endothelial cells synthesize mRNA[7] and protein for TGF-β[8] and cells in vivo are exposed to latent TGF-β2 in aqueous humor.[14,15] In addition, studies using cultured rat[16] and rabbit corneal endothelial cells[17] indicate that exogenous TGF-β1[17] and -β2[16,17] and TGF-β2 in aqueous humor[16] suppress entry into the S-phase. As in many cell types, contact inhibition plays an important role in inhibiting proliferation in corneal endothelium.[18] This appears to be true, even in the endothelium from older human donors. Studies from this laboratory reveal that, in ex vivo human corneas from donors 50 years of age or older, exposure of the intact endothelial monolayer to strong mitogenic stimulation alone does not promote proliferation. However, cells proliferate in response to this stimulation, if cell–cell contacts are broken by mechanical wounding[5] or treatment of the monolayer with EDTA.[19]

Cell cycle studies indicate that exposure of cells to mitogens leads to increased synthesis of cyclin D1, a regulatory factor that binds to and activates cyclin-dependent kinase-4 (CDK4).[20] Cyclin D1-CDK4 complexes mediate phosphorylation of the retinoblastoma gene product pRb.[21] pRb actively binds and inactivates E2F, a transcription factor that must be activated for S-phase entry.[22] Hyperphosphorylation of pRb by the cyclin D1-CDK4 complex promotes E2F activation and the subsequent transcription of S-phase genes.[23] Cyclin E is a regulatory protein synthesized later in G_1-phase than cyclin D. Association of cyclin E with CDK2 activates its kinase activity, which is also important for S-phase entry.[20] Maintenance of cells in G_1-phase arrest requires a complex regulatory mechanism, mediated at least in part by members of the Cip/Kip and INK families of cyclin-dependent kinase inhibitors (CKIs). Members of the Cip/Kip protein family bind to cyclin-CDK complexes, preventing kinase activation.[24–26] In many cell types, p27kip1, a member of the Cip/Kip family, helps mediate cell cycle arrest induced by cell–cell contact and TGF-β.[27–29] p27Kip1 binds and inhibits only G1-phase cyclin-CDK complexes. Overexpression of p27kip1 inhibits entry into the S-phase in normal and malignant cells,[30,31] whereas, downregulation of p27kip1 in (−/−) knockout mice results in unregulated cell growth and tumor formation.[32] The INK family of CKIs bind directly to CDK4 and prevent its binding to and activation by cyclin D.[33–37] INK proteins also compete effectively with cyclin D for binding to CDK4, thus inactivating existing cyclin D-CDK4 complexes.[33,37] The INK family member, p15INK4b, has been reported to induce release of p27kip1 from cyclin D-CDK4, resulting in increased binding of p27kip1 to cyclin E-CDK2 complexes and subsequent inactivation of CDK2 kinase activity.[27] p15INK4b mediates the antiproliferative effect of TGF-β in some cells,[34] whereas the INK family member p16INK4a has been implicated in mediating contact-dependent inhibition of FH109 human diploid lung fibroblasts.[38] Human corneal endothelial cells in vivo express p27kip1 and p16INK4a.[1,2] Adult rat corneal endothelial cells in vivo and in culture express p27kip1, but express p15INK4b instead of p16INK4a (Joyce NC, unpublished observation, 1999).

Evidence that cell–cell contact and TGF-β2 have an antiproliferative effect on corneal endothelium has come mainly from tissue culture or ex vivo studies; however, there has been little information about their relative contribution to inhibition of proliferation in vivo. Neonatal rat cornea makes an excellent model for these studies. We showed previously[18] that, at birth, rat corneal endothelium is immature, and cells continue to proliferate until approximately postnatal day 13. This 2-week window permits the study of mechanisms that contribute to maturation of the endothelium in vivo. Relative expression of membrane and matrix markers demonstrated a temporal correlation between the gradual cessation of proliferation and the formation of stable cell–cell and cell–substrate contacts that are characteristic of mature endothelium. This correlation suggests that, during formation of the endothelial monolayer, proliferation of endothelial cells is arrested by a contact inhibition-like mechanism. On postnatal day 1, cells stained positively for p27kip1, suggesting that this CKI could be responsible, at least in part, for the gradual inhibition of proliferation that occurs during maturation of the monolayer. Strong positive staining for p27kip1 was also evident in adult endothelium.

The current studies used the neonatal rat model to explore the relative contribution of cell–cell contact and TGF-β to inhibition of corneal endothelial proliferation in vivo and to examine the role of p27kip1 in mediating this inhibition. The kinetics of the expression of TGF-β RI, RII, and RIII were examined to determine when, during the process of endothelial monolayer maturation, a TGF-β2-induced intracellular signal could be transmitted. The kinetics of p27kip1 protein and expression of mRNA were studied to determine whether this CKI could help mediate the inhibition of proliferation that occurs during maturation of the monolayer. Studies were then extended to cultured rat corneal endothelial cells to examine more directly the effect of cell–cell contact and TGF-β2 on the relative protein and mRNA expression of the CKIs p27kip1 and p15INK4b.

Materials and Methods

Immunolocalization in Neonatal Rat Corneas

For immunolocalization of TGF-β receptors, eyes were dissected from male Sprague–Dawley rats on postnatal days 1, 10, and 21, and corneas were obtained from adult (3-month-old) rats. All rats were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For immunolocalization of p27kip1, eyes were obtained on postnatal days 1, 7, 14, and 21. Fresh-frozen 6-μm transverse sections were cut, fixed, and incubated for immunocytochemistry, as previously described.[18] Rabbit anti-TGF-β RI was used at a concentration of 0.1 μg/mL, rabbit anti-RII at 0.25 μg/mL, goat anti-RIII at 10 μg/mL, and rabbit anti-p27kip1 at a dilution of 1:200. FITC-conjugated anti-rabbit IgG diluted at 1:200 or FITC-conjugated anti-goat IgG diluted at 1:500 were used as secondary antibodies. Negative control sections were incubated in secondary antibody alone. Studies were repeated at least three times per time point. Positive staining of TGF-β receptors was visualized on a fluorescence microscope (Eclipse E800; Nikon, Melville, NY) equipped with a digital camera (SPOT; Micro Video Instruments, Avon, MA). Staining for p27kip1 was visualized on a confocal fluorescence microscope (TCS 4D; Leica, Deerfield, IL) equipped with a laser (DMRBE; Leitz, Wetzlar, Germany) and software (SCANware, ver. 4.2; Leica Lasertechnick, Heidelberg, Germany). Images presented in a single figure were processed using the same brightness and contrast levels to permit comparison.

Isolation of RNA from Neonatal and Adult Rat Corneal Endothelium

Corneas were obtained from male Sprague–Dawley rats on postnatal days 7, 14, and 28 and from adult (3-month-old) rats. A filter strip method was used to obtain RNA for RT-PCR studies. Corneas were placed endothelium-side up on a clean glass surface. Nitrocellulose filter strips (Millipore, Bedford, MA), cut 3 to 5 mm wide, were held at one end with blunt forceps. The other end of the strip was directly applied to the endothelial surface. Pressure was applied to the strip using a glass rod until liquid saturated the membrane. The strip was cut

at the point where the liquid mark ended and the moistened portion was placed into 500 μL RNA extraction reagent (TRIzol; Invitrogen Corp., Carlsbad, CA) and homogenized with a tissue grinder (or pellet pestle mixer), and an additional 500 μL of extraction reagent was added. Isolation of total RNA was performed on the nitrocellulose strips as directed by the manufacturer (Invitrogen). Multiple neonatal rats were used to obtain a single RNA sample. Nine rats were used for the postnatal day 7 RNA sample, 10 rats for day 14, and 10 to 12 rats for the day-28 and adult samples. In preliminary studies, RNA obtained by the filter strip method was tested by RT-PCR using primer sets for marker mRNAs that would distinguish endothelial cells from corneal keratocytes or epithelial cells. This method has been used to identify endothelial cells in explant cultures of rat cornea.[16] RNAs tested in this manner showed a profile expected for corneal endothelial cells and indicated that the samples were not contaminated by epithelial cells or keratocytes (data not shown).

Culture of Rat Corneal Endothelial Cells

Corneas were obtained from adult male Sprague–Dawley rats and endothelial cells were grown in explant culture as previously described.[16] Primary cultures were then trypsinized, resuspended in medium 199 supplemented with 50 μg/mL gentamicin (Invitrogen Corp.) and 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT), and seeded into T75 flasks (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 2.5×10^5 cells per flask. For studies requiring a fully confluent culture, cells were grown to confluence and maintained for at least one additional week in medium 199, supplemented with 50 μg/mL gentamicin and 10% FBS. For studies requiring subconfluent cultures, cells were seeded at the same density and allowed to attach to the tissue culture flask for 24 hours in medium containing 10% FBS. Cells were then synchronized in G_0-phase of the cell cycle (quiescence) by incubation for 48 hours in medium containing 0.5% FBS. After synchronization, cells were incubated for 24 hours in medium containing 10% FBS alone or supplemented with 5 ng/mL TGF-β2 (R&D Systems, Minneapolis, MN). Confluent or subconfluent cells, treated as indicated, were then extracted to obtain total RNA or protein for RT-PCR or Western blot studies, respectively. Total RNA was extracted from cultured cells, as described for neonatal endothelium. Protein was extracted and Western blot analysis prepared as described later.

Reverse Transcription–Polymerase Chain Reaction

cDNA was synthesized by reverse transcription from 1 μg of total RNA in a final reaction volume of 20 μL using reagents from a commercially available kit (Promega, Pittsburgh, PA). Primers specific for p27kip1 and p15INK4b were designed in accordance with previously described criteria[39] using primer-analysis software (Oligo; National Biosciences, Inc., Plymouth, MN) and based on mouse p27kip1 mRNA sequence (GenBank accession number U09968)[25] or mouse p15INK4b mRNA sequence (GenBank accession number AF059567; GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at http://www.ncbi.nlm.nih.gov/Genbank).[40] Primers for p27kip1 were upstream sequence, 5′-TGGAGGGCAGATACGAATGG-3′ downstream sequence, 5′-GGGGAACCGTCTGAAACATT-3′ and should yield a 327-bp product. Primers for p15INK4 were upstream sequence, 5′-GGAATTCCTGGAAGCCGGCGCAGATC-3′ downstream sequence, 5′-GCTCTAGAGCGTGTCCAGGAAGCCTTCC-3′ and should yield a 190-bp product.

PCR was performed in a total reaction volume of 50 μL containing 0.5 μg cDNA, 2.5 μM of both the upstream and downstream primers, and reagents from a commercially available kit (Qiagen, Valencia, CA). A hot-start method was used to enhance the yield and specificity of the reaction.[41] p15INK4b primers were run for 30 cycles. Cycling conditions for these primers included denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 2 minutes. A final extension step at 72°C for 7 minutes was added at the end of the 30 cycles. p27Kip1 primers were run for the same number of cycles, and cycling conditions were the same with the exception of the annealing temperature (55°C). PCR products and a 100-bp DNA ladder were separated on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide. RT-PCR was repeated at least twice for each experimental condition. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH; 452 bp; Clontech, Palo Alto, CA) was used as a positive control. For a negative control, PCR was run using PCR reaction mixture with primers but no nucleic acid. Genomic DNA contamination was checked by performing PCR using the reaction mixture with G3PDH primers, but replacing cDNA with total RNA. None of the samples used in these experiments yielded detectable levels of PCR product for G3PDH when RNA was used as a template. Therefore, all the PCR products obtained resulted from amplification of mRNA and not genomic DNA contamination.

Western Blot Analysis of G_1-Phase Inhibitor Protein Expression

Cultured cells were trypsinized and then suspended and homogenized in 1% Triton X-100 (Sigma, St. Louis, MO), 250 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (all from Sigma). Equal protein was loaded on 10% polyacrylamide gels for SDS-PAGE. Peptides were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), and nonspecific binding was blocked by incubation for 1 hour at room temperature in 5% milk diluted in PBS. Rabbit anti-p27kip1 and goat anti-p15INK4b were diluted 1:200 in blocking solution. Membranes were incubated with primary antibody for 1 hour, washed, reblocked, and then exposed for 1 hour to peroxidase-conjugated donkey anti-rabbit IgG or donkey anti-goat IgG, diluted 1:200 in blocking solution. The same blots were probed with rabbit anti-nonmuscle myosin (Biomedical Technologies, Inc., Stoughton, MA) to control for protein load. After a thorough wash, peptides were detected using chemiluminescent substrate (SuperSignal West Pico; Pierce, Rockford, IL).

Densitometric Analysis

Images of PCR gels and Western blots were obtained using an image-analysis system (BDS Image, ver.1.3; Biological Detection Systems Inc., Pittsburgh, PA). Semiquantitative analysis of the images was made using NIH-Image version 1.61 (NIH Image; W. Rasband, National Institutes of Health, Bethesda, MD; available by FTP from zippy.nimh.nih.gov or on floppy disc from NTIS, Springfield, VA). G3PDH was used to normalize the densitometry results for the PCR experiments. Nonmuscle myosin was used to normalize results for the Western blot analysis. All results represent the average density of positive bands obtained from two to three separate experiments.

EDTA Treatment of Confluent Cultures

Primary cultures of corneal endothelial cells were seeded into two-well chamber slides (Nalge Nunc International, Naperville, IL) or into T75 flasks. Cells were grown to confluence and then maintained in culture medium containing 10% FBS for another week to ensure formation of a fully confluent monolayer. Confluent cultures were then washed, incubated for 1 hour in the presence or absence of 2.0 mg/mL disodium EDTA.$2H_2O$ (Life Technologies, Grand Island, NY) diluted in Hanks' buffered salt solution (Invitrogen Corp.), rewashed, and incubated for 24 hours in medium containing 10% FBS. To test the effect of EDTA on proliferation, cells in two-well chamber slides were fixed in methanol and prepared as described earlier for immunolocalization of Ki67, a marker of actively cycling cells.[42] Mouse anti-Ki67 was diluted 1:50, whereas FITC-conjugated anti-mouse IgG was diluted at 1:200. Western blot analysis, prepared as described herein, tested the effect of EDTA treatment on p27kip1 protein levels.

Antibodies

Rabbit anti-TGF-β RI, anti-TGF-β RII, anti-p27kip1, and goat anti-p15INK4b were obtained from Santa Cruz Biotechnology, Inc. (Santa

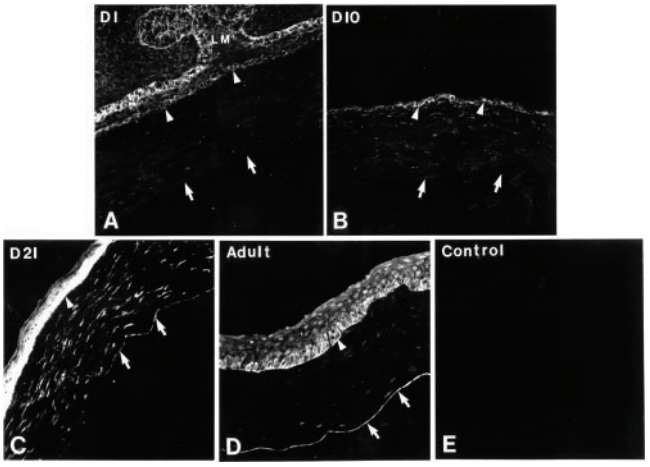

FIGURE 1. Immunolocalization of TGF−β RI in rat corneas on postnatal days 1 (**A**, D1), 10 (**B**, D10), and 21 (**C**, D21) and in adult cornea from a 3-month old rat (**D**). *Arrowheads*: corneal epithelial cells; *arrows*: endothelial cells. Eyelids were closed on postnatal day 1, and the lid margin is visible (**A**, LM). By postnatal day 10, eyelid opening had occurred, and the lids are no longer visible in the tissue section. Control sections (**E**) were incubated in secondary antibody alone. Original magnification, ×40.

Cruz, CA) goat anti-TGF-β RIII from R&D Systems; mouse monoclonal anti-Ki67 from Novocastra (Newcastle, UK); and FITC-conjugated anti-rabbit IgG, anti-goat IgG, anti-mouse IgG, and peroxidase-conjugated anti-rabbit and anti-goat IgG from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

RESULTS

Detection of TGF-β Receptors in Neonatal Rat Cornea

Studies were first conducted to determine whether TGF-β might play a role in inhibiting proliferation during corneal endothelial development in vivo. The rationale for detecting TGF-β receptors to answer this question is based on the fact that, for a TGF-β-induced signal to be transmitted, cells must coexpress TGF-β receptor types I and II (RI, RII).[11,12] In addition, for optimal binding of TGF-β2, which is the predominant TGF-β isoform in aqueous humor, cells must also express receptor type III (RIII: β-glycan), a highly glycosylated molecule that binds the β2 isoform with high affinity.[13] TGF-β RI, RII, and RIII were immunolocalized in neonatal rat corneas to determine when, during postnatal development, endothelial cells coexpress these receptors. Corneas from adult (3-month-old) rats were used as a positive control in these studies. Figure 1 shows that, on postnatal day 1, positive staining for TGF-β RI was visible in conjunctival epithelial cells, in epithelial cells of the lid margin, and in the single layer of corneal epithelial cells. The circumferential pattern in these cells was consistent with a plasma membrane localization. No positive staining was visible in the endothelium at this time. On days 10 and 21, positive staining for R1 was observed in corneal epithelium and was most intense in the basal cell layers. A low level of positive staining was present in stromal keratocytes by postnatal day 10. In contrast, positive staining for RI was not visible in the endothelium until postnatal day 21. In adult cornea, intense staining was clearly visible in the basal cells of the epithelium and in the endothelium. The level of positive staining in keratocytes appeared lower in adult cornea than that on postnatal day 21. Intense staining for TGF-β RII (Fig. 2) was visible in conjunctival epithelial cells and cells of the lid margin on

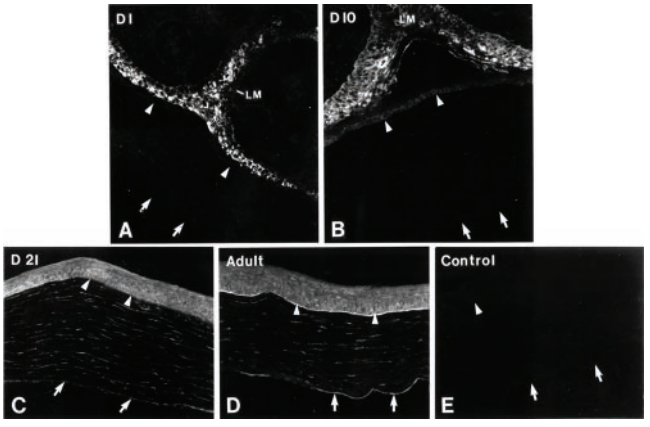

FIGURE 2. Immunolocalization of TGF-β RII in rat corneas on postnatal days 1 (**A**, D1), 10 (**B**, D10), and 21 (**C**, D21), and in adult cornea from a 3-month old rat (**D**). *Arrowheads*: corneal epithelial cells; *arrows*: endothelial cells. Eyelids were closed on postnatal day 1, and the lid margin is visible (**A**, LM). Note that, on postnatal day 10 (**B**), eyelid opening was in progress. Control sections (**E**) were incubated in secondary antibody alone. Original magnification, ×40.

postnatal day 1, but the corneal epithelium was not positively stained until day 10. Staining for RII was not visible in the endothelium or keratocytes until day 21. At no time was staining in corneal epithelium or endothelium as intense as in conjunctival epithelial cells. Positive staining for TGF-β RIII (Fig. 3) was visible in conjunctival epithelial cells, epithelial cells of the lid margin, and corneal epithelium on postnatal day 1. Posterior keratocytes were stained at low levels at this time, but no staining was evident in the endothelium. On postnatal day 10 and thereafter, the endothelium was intensely stained for RIII. In adult cornea, endothelial cells and keratocytes were intensely stained for RIII, whereas, only light staining was observed in the epithelium.

Kinetics of p27Kip1 Expression in Neonatal Corneal Endothelium

Because p27kip1 is an important G_1-phase regulator, it was important to determine whether this protein could mediate

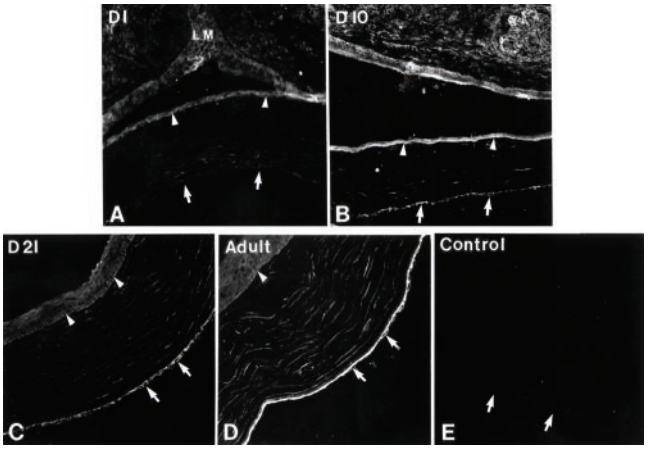

FIGURE 3. Immunolocalization of TGF-β RIII (β-glycan) in rat corneas on postnatal days 1 (**A**, D1), 10 (**B**, D10), and 21 (**C**, D21), and in adult cornea from a 3-month old rat (**D**). *Arrowheads*: corneal epithelial cells; *arrows*: endothelial cells. Note that eyelid opening was in progress on days 1 (**A**) and 10 (**B**), and the lid margin is visible (**A**, LM). Control sections (**E**) were incubated in secondary antibody alone. Original magnification, ×40.

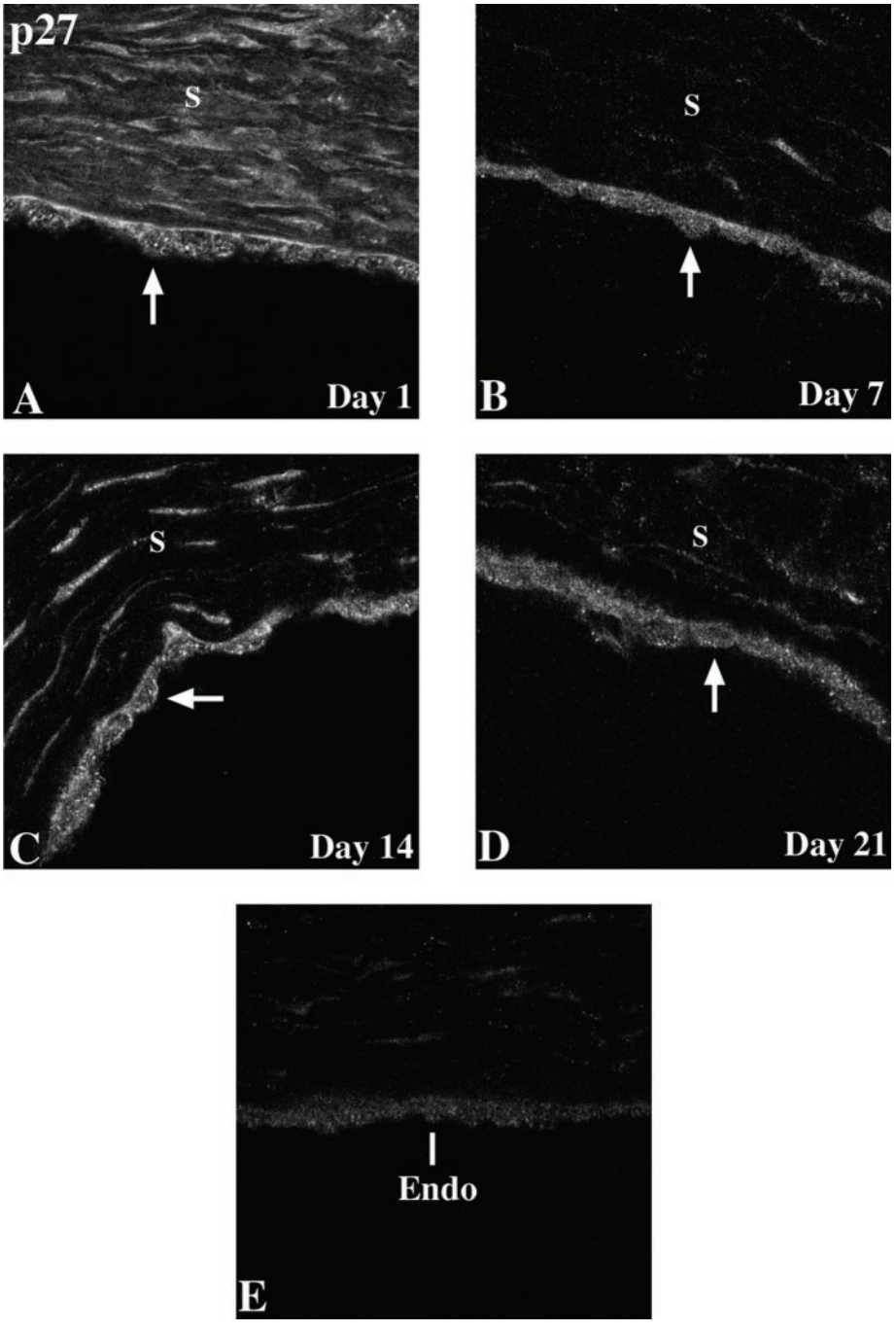

FIGURE 4. Immunolocalization of p27kip1 in neonatal rat corneal endothelium on postnatal days 1 (**A**), 7 (**B**), 14 (**C**), and 21 (**D**). Negative control (**E**), corneal sections were incubated with secondary antibody. *Arrows*: single endothelial cells. S, stroma; Endo, endothelium. Original magnification, ×40.

mitotic inhibition during corneal endothelial development. To answer this question, the relative kinetics of p27kip1 expression were determined in the endothelium of neonatal rat cornea by immunolocalization and RT-PCR. As shown in Figure 4, immunostaining for p27kip1 was visible in the endothelium beginning on postnatal day 1 and thereafter. Punctate nuclear staining, as well as a more diffuse cytoplasmic staining pattern, was visible at all time points tested. Figure 5A shows representative results of the RT-PCR study. PCR reaction product of the size expected for p27kip1 was obtained from samples on postnatal days 7, 14, and 28, as well as from adult endothelium. Semiquantitative densitometric analysis of the PCR results indicated that the amount of p27kip1 PCR product relative to the G3PDH control was similar on days 7 (0.40) and 14 (0.41), but increased approximately twofold by day 28 (0.79) and remained at a similar level in adult endothelium (0.71).

Effect of TGF-β2 and Cell–Cell Contact on CKI Expression in Cultured Cells

Studies were then expanded to a tissue culture model to determine more directly the effect of TGF-β2 and cell–cell contact on the relative mRNA and protein expression of p27kip1 and p15INK4b. Fully confluent, nonproliferating cultures maintained in 10% FBS were prepared for analysis. In addition, subconfluent cultures were synchronized in the G_0-phase of the cell cycle before incubation for 24 hours in 10% FBS in the presence or absence of TGF-β2, as indicated in the Materials and Methods section. Timing for this analysis was

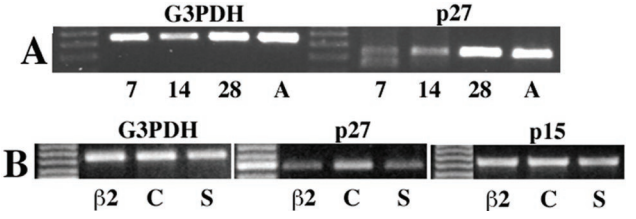

FIGURE 5. RT-PCR study of CKI protein expression in neonatal rat corneal endothelium (**A**) and in cultured rat corneal endothelial cells prepared under different culture conditions (**B**). In (**A**), PCR reaction products of the expected molecular weight were obtained for G3PDH (452 bp) and p27kip1 (327 bp). Note that two PCR products were obtained for p27kip1 on postnatal day 7. Sequencing identified the *top* band (at the molecular weight expected for p27kip1) as the authentic product, whereas the *bottom* band was determined to be nonspecific. For the blot in (**B**), total RNA was extracted from synchronized subconfluent rat corneal endothelial cells incubated for 24 hours in 10% FBS plus 5 ng/mL TGF-β2 (β2), from fully confluent cultures (C), and synchronized subconfluent cells incubated for 24 hours in 10% FBS alone (S). PCR reaction products of the expected molecular weight were obtained for G3PDH, p27kip1, and p15INK4b (190 bp). A portion of the 100-bp DNA ladder is shown to the *left* of each set of samples.

based on the results of previous [^{3}H]-thymidine incorporation studies.[16] Figure 5B shows the RT-PCR results and Table 1 presents the semiquantitative densitometric analysis of these results relative to G3PDH. In this analysis, the density of PCR products in TGF-β2-treated and confluent cells was compared with that of subconfluent cells maintained in 10% FBS alone. Analysis of the data indicates that TGF-β treatment had little effect on p27kip1 mRNA levels compared with that of untreated subconfluent cells, but increased p15INK4b mRNA levels approximately threefold. In confluent cells, p27kip1 mRNA increased approximately 1.75-fold and p15INK4b increased almost 3-fold. Parallel experiments measured protein levels by Western blot analysis. Table 2 shows the semiquantitative densitometric results relative to nonmuscle myosin. As in Table 1, the density of the protein bands in TGF-β2-treated and confluent cells was compared with that of subconfluent cells maintained in 10% FBS alone. In TGF-β2-treated cells, p15INK4b levels decreased by approximately 25%, but TGF-β2 had little effect on p27kip1 protein levels. In confluent cells, p15INK4b protein levels also decreased by approximately 25%. In contrast, p27kip1 protein levels increased almost 20-fold over that of subconfluent cells.

EDTA Decreases p27kip1 Expression in Confluent Cells

Previous studies in this laboratory showed that EDTA treatment of human corneal endothelium in situ releases cells from cell–cell contact and promotes proliferation in the presence of appropriate mitogens.[19] To investigate the role of p27kip1 in this relationship, confluent cultures of rat corneal endothelial cells were treated with EDTA, followed by incubation for 24 hours in medium containing 10% FBS, as indicated in the Materials and Methods section. Control confluent cultures were maintained in 10% FBS throughout the experiment. The effect of EDTA treatment on proliferation was determined by staining for Ki67, a marker of actively cycling cells.[42] The micrographs in Figure 6 demonstrate the absence of Ki67 staining in confluent cultures maintained in 10% FBS, but significant Ki67-positive staining in the nuclei of confluent cultures pretreated with EDTA. The effect of EDTA-induced release of cell–cell contacts on p27kip1 levels is demonstrated by the Western blot in Figure 6. Confluent cells maintained in 10% FBS alone expressed high levels of p27kip1 protein; however, when cultures were pretreated with EDTA and then 10% FBS, they expressed much lower levels of this protein. Together, these results demonstrate a correlation between the nonproliferation of contact-inhibited cells and high levels of p27kip1. p27kip1 levels can be reduced by EDTA-induced release of cell–cell contacts.

TABLE 1. CKI mRNA Expression in TGF-β2–Treated Subconfluent Cells and in Confluent Cells Compared with That in Subconfluent Cells in 10% FBS

CKI	Subconfluent Cells (+)TGF-β2*	Difference†	Confluent Cells*	Difference†
p27kip1	0.57	1.10×	0.91	1.75×
p15INK4b	1.14	3.08×	1.08	2.92×

Results are the average of three separate experiments.
* Average density compared with G3PDH control.
† Difference compared with subconfluent cells incubated in 10% FBS alone.

TABLE 2. CKI Protein Expression in TGF-β2–Treated Subconfluent Cells and in Confluent Cells Compared with That in Subconfluent Cells in 10% FBS

CKI	Subconfluent Cells (+)TGF-β2*	Difference†	Confluent Cells*	Difference†
p27kip1	0.27	0.93×	5.74	19.8×
p15INK4b	0.22	0.76×	0.55	0.74×

Results are the average of three separate experiments.
* Average density compared with nonmuscle myosin control.
† Difference compared with subconfluent cells incubated in 10% FBS alone.

DISCUSSION

Previous studies[18] quantifying bromodeoxyuridine (BrdU)-positive endothelial cells in neonatal rat cornea indicate that al-

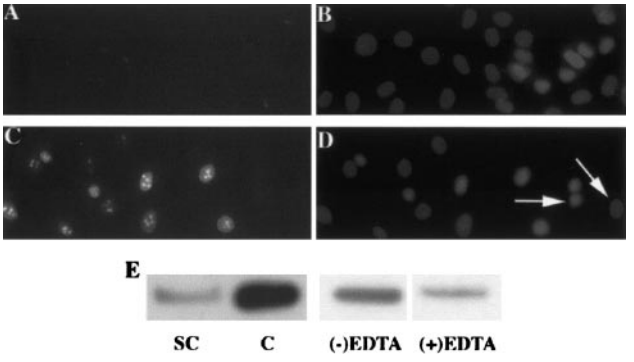

FIGURE 6. Effects of EDTA treatment on proliferation (**A–D**) and p27kip1 expression (**E**) in cultured rat corneal endothelial cells. Confluent cells maintained in 10% FBS did not stain positively for Ki67, indicating nonproliferation under this condition (**A**). (**B**) Same area of the culture as in (**A**) stained with DAPI to indicate the presence of nuclei. Punctate Ki67 staining is visible in the nuclei of confluent cells pretreated with EDTA and maintained for 24 hours in 10% FBS (**C**). (**D**) Corresponding area of the culture stained with DAPI. *Arrows*: cells not positively stained for Ki67 in (**C**). (**E**) Western blot comparing p27kip1 protein expression in subconfluent (SC) and confluent (C) cells maintained in 10% FBS and in confluent cultures treated (+) or not (−) with EDTA. Original magnification, ×40

most 12% of the cells are proliferating on postnatal day 1. This percentage decreases linearly with time, so that, by postnatal day 13 and thereafter, no positive cells are detected. The kinetics of this decrease in proliferation correlates well with the kinetics of increasing stability of cell–cell contacts, as indicated by the gradual development of plasma membrane polarity. Together, these results suggest that contact inhibition plays a role in inducing mitotic arrest in developing corneal endothelium. In the current studies, we explored whether TGF-β2 might also contribute to mitotic arrest during endothelial development. Results indicate that coexpression of the TGF-β receptors RI, RII, and RIII did not correlate with the proliferation kinetics. Although RIII was expressed by postnatal day 10, coexpression of RI and RII was not observed until postnatal day 21, approximately 1 week after proliferation ceases. Because coexpression of RI and RII is essential for intracellular signal transduction, it is reasonable to conclude that TGF-β2 signals cannot be transduced until 2 to 3 weeks after birth. Comparison of these results with those for contact inhibition strongly suggests that contact inhibition is responsible for mitotic inhibition during corneal endothelial development.

Age is clearly an important factor in the ability of human corneal endothelial cells to respond to a mitogenic stimulus.[5] Contact inhibition also appears to be important in maintaining mitotic quiescence, because endothelium from older individuals enters the cell cycle on strong mitogenic stimulation only after cell–cell contacts are broken.[5,19] It is possible that contact inhibition would make the endothelium sufficiently refractory to positive growth factors that cells would be prevented from replicating in vivo, even though these factors might be present in the microenvironment.[7,8] The findings that TGF-β receptors I, II, and III are expressed in adult human corneal endothelial cells[10] and that exogenous TGF-β2[16,17] and TGF-β2 in aqueous humor[16] suppress S-phase entry in cultured rat and rabbit endothelial cells suggest that TGF-β2 could inhibit proliferation in the mature endothelial monolayer. Together, our studies lead to the hypothesis that, in the mature endothelium in vivo, TGF-β2 exerts its antiproliferative effect by acting as a fail-safe mechanism to inhibit proliferation should cell–cell contact be compromised.

It is expected that, by postnatal day 1, proliferation of endothelial cells would already be decreasing because of the gradual formation of stable cell–cell and cell–substrate contacts. As such, mediators of contact-induced mitotic inhibition should also be expressed early in the postnatal period. The kinetics of p27kip1 protein and mRNA expression suggest that its is an important mediator of contact-induced inhibition of proliferation during development of the endothelial monolayer.

Studies of the effect of TGF-β treatment on expression of p27kip1 and p15INK4b in cultured corneal endothelial cells showed that this cytokine did not substantially alter p27kip1 protein levels, but decreased p15INK4b by 25%, thus effectively increasing p27kip1 levels relative to p15INK4b. Under similar experimental conditions, TGF-β2 suppressed, but did not completely inhibit, S-phase entry in synchronized rat corneal endothelial cells.[16] The observed effect of TGF-β2 on CKI expression may help explain, in part, why this cytokine is capable of suppressing, rather than totally inhibiting cell cycle progression in these cells. In contrast, the RT-PCR, Western blot, and EDTA studies reported herein demonstrated a strong correlation between high levels of p27kip1 protein and inhibition of proliferation in contact inhibited cells, providing evidence that p27kip1 is an important mediator of contact inhibition in corneal endothelium, as has been shown in other cell types.[25,28,29] Of interest is the finding that p15INK4b protein levels were lower in confluent cultures than in subconfluent cells. The specific reason for this reciprocal expression is currently unknown and a subject for further study.

Evaluation of the effect of TGF-β2 and contact inhibition on relative CKI expression in cultured rat corneal endothelial cells revealed a disparity in relative levels of p27kip1 protein and mRNA. This disparity has been observed by others[43] and reflects the fact that p27kip1 protein concentrations are regulated posttranslationally. Multiple mechanisms may help maintain high p27kip1 protein levels relative to mRNA, including p27kip1 sequestration by cyclin D-CDK complexes,[44] enhanced ribosomal association of p27kip1 mRNA[44] yielding large amounts of protein relative to mRNA, stabilization of p27kip1 protein by phosphorylation on Ser-10,[45] and decreased degradation by the ubiquitin-proteasome pathway[46,47] or by proteolytic processing of the cyclin-binding domain.[47] Recent studies by Kim et al.[48,49] have reported the effects of cell density and TGF-β2 on p27kip1 protein expression in cultured rabbit corneal endothelial cells. In their studies, p27kip1 expression was density dependent and TGF-β2, when added to actively cycling cells, inhibited phosphorylation of p27kip1 on Thr-187, which is required for its nuclear export and subsequent degradation.[50]

An important question not addressed in the current studies is the relative activity of cyclin-CDK complexes in TGF-β2-treated and confluent cells. The binding of CKIs to specific cyclin-CDK complexes and the subsequent inactivation of the kinase activity of those complexes is central to the effective regulation of S-phase entry. Future studies will evaluate the effect of TGF-β2 and cell–cell contact on CKI binding to cyclin D-CDK4 and cyclin E-CDK2 complexes and on the relative kinase activity of these complexes to determine more specifically how proliferation is regulated by these mechanisms in corneal endothelial cells.

In summary, contact inhibition appears to play an important role in inhibiting proliferation of corneal endothelium, both during corneal development and in the mature monolayer. This inhibition appears to be mediated, at least in part, by the activity of p27kip1. TGF-β does not appear to play a significant inhibitory role during endothelial development or in the mature endothelium. However, this cytokine may be of importance in maintaining the endothelium in a nonreplicative state should cell–cell contact be lost in the monolayer. Overall, TGF$-\beta$ appears to alter the relative protein levels of CKIs so that p27kip1 is the most abundant, suggesting that p27kip1 may also mediate the suppressive effect of this cytokine on S-phase entry.

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