Cornea | June 2001

TGF-β Receptor Types I and II Are Differentially Expressed during Corneal Epithelial Wound Repair

James D. Zieske; Audrey E. K. Hutcheon; Xiaoqing Guo; Eui-Hong Chung; Nancy C. Joyce

+ Author Affiliations

Investigative Ophthalmology & Visual Science June 2001, Vol.42, 1465-1471. doi:

Abstract

PURPOSE. It has been demonstrated that cells migrating to cover an epithelial débridement wound exit the cell cycle and that the cell-cycle inhibitor $p15^{INK4b}$ is upregulated in these cells. TGF-β signaling has been implicated in both of these processes, and this study was conducted to determine whether the expression and localization of TGF-β receptor (TβR)-I and -II are altered during corneal epithelial wound repair.

METHODS. Three-millimeter superficial keratectomy wounds and 3-mm débridement wounds were made in central rat cornea and allowed to heal in vivo for 1 to 48 hours. Immunofluorescence microscopy and Western blot analysis were used to determine the localization and expression of TβR-I and -II. Unwounded rat corneas served as control samples. To determine the effect of epidermal growth factor (EGF) and TGF-β1 on $p15^{INK4b}$ and TβR-I and -II expression, human corneal epithelial cells were grown in culture to 50% to 60% confluence, and EGF (5 ng/ml) and/or TGF-β1 (2 ng/ml) were added for 6 hours. Cells were harvested and $p15^{INK4b}$ and TBR-I and -II levels were assayed by using Western blot analysis.

RESULTS. In unwounded corneas, TβR-I and TβR-II were present at low levels across the cornea, with higher levels in limbal epithelium. Both TβR-I and -II were

upregulated after wounding. However, levels of TβR-II appeared to increase in the epithelial cells that had migrated to cover the wound area, whereas TβR-I was upregulated in the entire corneal epithelium. Western blot analysis indicated that both TβR-I and -II were upregulated threefold after wounding. In cultured cells, EGF and TGF-β1 stimulated TβR-II; however, neither one stimulated TβR-I expression. TGF-β1 stimulated $p15^{INK4b}$ protein levels threefold.

CONCLUSIONS. After wounding, TβR-I and TβR-II were both expressed at high levels in cells migrating to cover a corneal wound, suggesting that TGF-β signaling is involved in blocking migrating cells from progressing through the cell cycle. This blockage, at least in part, involves the inhibitor $p15^{INK4b}$. In addition, although both TβR-I and TβR-II are upregulated during wound repair, they appear to be differentially regulated.

Corneal epithelial wound repair is an ordered process that is regulated at least in part by soluble growth factors.[1,2] A variety of growth factors have been postulated to be involved in the healing process, including members of the epidermal growth factor (EGF) family, the transforming growth factor (TGF)-β family, hepatocyte growth factor (HGF), the fibroblast growth factor (FGF) family, and platelet-derived growth factor (PDGF). Several cytokine have also been implicated to play a role in wound repair (see Refs. [2,3,4,5,6,7] for review). All these growth factors and cytokines function by binding membrane-spanning receptors that, when activated, trigger a signaling cascade. The EGF receptor, which is one of these membrane-spanning receptors, has been demonstrated by several groups to be present in corneal epithelial cells.[3,8,9,10,11,12]

We have recently shown that EGF receptor is present across the entire cornea and is activated within 15 minutes after corneal epithelial débridement and that corneal epithelial cells extending from the leading edge to the limbus can bind EGF-fluorescein isothiocyanate (FITC) after wounding.[13] In addition, we have found that inhibition of the EGF receptor kinase activity slowed wound repair by almost 50%. These findings indicate that activation of the EGF receptor is involved in corneal wound healing. One puzzling aspect of these results, however, is the finding that although all corneal basal cells appear

to be activated by EGF,[13] cells distal to the wound are stimulated to proliferate, whereas cells migrating to cover the wound are not.[14][15][16][17][18][19][20] One possible explanation for these findings is that cells migrating to cover the wound area are inhibited from progressing through the cell cycle. This type of cell cycle inhibition is frequently associated with the action of TGF-β.[21][22][23] One of the target genes of TGF-β signaling is the cell-cycle–dependent kinase inhibitor $p15^{INK4b}$.[24][25][26] This protein was found to be upregulated during treatment of epidermal keratinocytes with TGF-β,[27] and $p15^{INK4b}$ was subsequently found to inhibit the cell cycle by binding cyclin-dependent kinase-4 and preventing its interaction with cyclin D. Our previous finding[28] that $p15^{INK4b}$ is upregulated in migrating epithelium suggests that TGF-β is preferentially signaling in these cells.

There are three isoforms of TGF-β (termed TGF-β1, -2, and -3) in mammalian cells. These isoforms are part of the TGF-β superfamily that consists of a large number of structurally related proteins, including activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs). These factors regulate cell proliferation, differentiation, motility, adhesion, and death. In epithelial cells, TGF-β inhibits cell proliferation and stimulates the synthesis of extracellular matrix (see Refs.[21][22][23][24][25][26] for review.)[21][22][23][24][25][26] The TGF-β superfamily signals through a family of cell surface serine-threonine kinase receptors. These receptors are divided into two subfamilies: type I and type II receptors. Type I receptors have several names, one of which is activin receptor-like kinase (ALK). There are at least six ALKs, and ALK5, also known as TGF-β receptor (TβR)-I, is the receptor most commonly associated with TGF-β. ALK1 also binds TGF-β but does so less strongly than TβR-I and is not known to mediate a TGF-β response.[22] In vertebrates, the type II receptor that selectively binds TGF-β is known as TβR-II. In the currently accepted method of TGF-β signaling, the ligand binds to TβR-II, leading to the formation of a receptor complex with TβR-I and the phosphorylation and activation of TβR-I. Activated TβR-I then phosphorylates signaling proteins termed SMAD2 and SMAD3. This phosphorylation results in the dissociation of SMAD2 and/or SMAD3 from the receptor and allows them to complex with SMAD4 and move into the nucleus. The SMAD–SMAD4 complex, along with other factors, then binds to DNA and activates transcription. TβR-I and TβR-II are both required for signaling.

In addition to TβR-I and TβR-II, a third family of receptors has been identified, termed TβR-

III. Members of this family appear to be accessory receptors, in that they do not have an intrinsic signaling function. TβR-III appears to function by binding members of the TGF-β family and then passing the ligands along to TβR-II. This function appears to be of primary importance for TGF-β2, which on its own does not have a high affinity for TβR-II. TβR-III may also serve to concentrate ligand(s) at the cell surface. Both TβR-I and TβR-II have been reported to be present in human [29] and rat [30] corneal epithelium. In addition, the localization of the receptors appears to be altered during wound repair in rat corneas. [31]

In the current investigation, we examined whether signaling through the TGF-β receptor family may be involved in the inhibition of proliferation in the migrating cells and whether the spatial and temporal expressions of TβR-I and TβR-II are altered during wound repair, leading to the inhibition of cell proliferation in migrating corneal epithelial cells.

Materials and Methods

Animal Model

Adult Sprague-Dawley rats of either sex were used in most experiments. Rats were anesthetized with an intramuscular injection of rodent anesthesia cocktail containing ketamine (21.5 mg/kg body weight), xylazine (4.3 mg/kg body weight), and acepromazine (0.7 mg/kg body weight) followed by topical application of 0.5% proparacaine. Either a 3-mm débridement [32] or keratectomy [33] wound was made. The corneas were allowed to heal from 1 to 48 hours. Rats were killed with an intraperitoneal injection of sodium pentobarbital. All protocols in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunofluorescence Microscopy

Immunofluorescence, using 6-μm cryostat sections, was performed as previously published. [9] The polyclonal antibody against TβR-I (R-20), TβR-II (C-16; Santa Cruz Biotechnology, Santa Cruz, CA), or laminin (Chemicon, Temecula, CA) was placed on the sections and incubated for 1 hour at room temperature, followed by a 1-hour incubation of secondary antibody, FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and coverslips were mounted (Vectashield; Vector Laboratories, Inc., Burlingame, CA). Negative controls consisted of secondary antibody alone, irrelevant

antibodies, or primary antibody preadsorbed with its respective antigen. The sections were viewed and photographed under a microscope (Eclipse E800; Nikon, Melville, NY) equipped with a digital SPOT camera (Micro Video Instruments, Avon, MA).

Electrophoresis and Immunoblotting

Western blot analysis, as previously described, [19] was used to quantify TβR-I and -II after débridement. A 3-mm trephine was used to demarcate the corneal epithelium, and the epithelium inside the demarcation was scraped and collected as a control. The corneas were allowed to heal, and the rats were killed from 1 to 48 hours after wounding. The epithelium within a 4-mm trephine area was collected and the protein extracted. Epithelium from 12 wounded corneas was pooled for each time point. Equal amounts of total protein were loaded for each time point, electrophoresed on an 8% tris-glycine gel (Novex, San Diego, CA), and electrophoretically transferred to a transfer membrane (Immobilon-P; Owl Separation Systems, Woburn, MA). Relative amounts of protein were confirmed by Coomassie blue staining. The membrane was incubated for 1 hour at room temperature in blocking reagent (Blotto; Santa Cruz; 5% for TβR-I; or 10% for TβR-II). The membrane was then incubated with either anti-TβR-I (H-100) or anti-TβR-II (L-21; Santa Cruz) in the blocking reagent for 1 hour. After a washing, the membrane was incubated for 1 hour with peroxidase-conjugated goat anti-rabbit IgG (New England BioLabs, Inc., Beverly, MA) diluted 1:2000 in the blocking reagent. The membrane was soaked in chemiluminescent substrate (SuperSignal; Pierce, Rockford, IL) for 5 minutes, exposed to film (Hyperfilm ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK), and developed using an x-ray film processor (X-Omat; Eastman Kodak, Rochester, NY). Band intensities were quantified by computer (NIH Image 1.61/68K; National Institutes of Health, Bethesda, MD; available in the public domain at http//:www.nih.gov/od/oba). Western blot analyses were repeated at least three times. Statistical analyses were performed using a paired *t*-test. $P < 0.05$ was considered significant.

Cell Culture

Human corneas were obtained from National Disease Research Interchange (Philadelphia, PA). A 9-mm trephine was used to remove the central cornea. The limbal ring was rinsed with Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} (Gibco, Baltimore,

MD), containing gentamicin (Gibco) at a concentration of 20 μg/ml, for 2 to 3 minutes. The ring was then cut into six to seven pieces of equal size, placed into a dispase solution (25 caseinolytic units/ml), containing gentamicin (5 μg/ml) in Hanks' balanced salt solution (Gibco), and incubated for 18 to 24 hours at 2°C to 8°C. After incubation, the epithelial layer was separated and placed in a tissue culture dish containing trypsin-EDTA solution (Gibco). The epithelium was incubated at 37°C for 5 to 6 minutes, during which time it was aspirated with a small pipette every 2 to 3 minutes to dissociate the cells. The trypsin action was then stopped by the addition of 10% fetal bovine serum (FBS; Gibco) in Dulbecco's PBS, and the cells were centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in keratinocyte/serum-free medium (SFM; Gibco) with 0.09 mM $CaCl_2$ and seeded onto coated T25 tissue culture flasks (FNC Coating mix; Biological Research, Jamesville, MD). When the cells reached 80% confluence, they were split and seeded onto coated T75 tissue culture flasks. When 50% to 60% confluent, the cells were treated with normal medium (keratinocyte-SFM), medium plus EGF (5 ng/ml), medium plus TGF-β1 (2 ng/ml; R&D Systems, Inc., Minneapolis, MN), or medium plus both EGF and TGF-β1. Cells were cultured for 6 hours. They were then harvested and protein was isolated. Equal amounts of protein were loaded onto a tris-glycine gradient gel of 10% to 20% polyacrylamide. The gel was electrophoresed, the protein was transferred to membranes (Immobilon-P; Owl Separation Systems), and the membranes were probed with anti-$p15^{INK4b}$ (Upstate Biotechnology), anti-TβR-I, or anti-TβR-II. Protein levels were quantitated as previously described.

Results

We have previously reported [29] that TβR-I and TβR-II are present in human corneal epithelium and that both are preferentially localized in the limbus. Similar localization was demonstrated in the rat corneal epithelium (Fig. 1) . Both TβR-I and TβR-II were present at low levels in the central cornea (Figs. 1B 1D) and at much higher apparent levels in the limbus (Figs. 1A 1C) . Both TβR-I and TβR-II were preferentially localized in the limbal basal cells and had a membranous localization consistent with a cell surface receptor. When the antibodies were preadsorbed with their corresponding blocking peptides, the intensity of binding decreased to background levels (Fig. 1A1 C1).

To determine whether the localization of TβR-I or TβR-II was altered during wound repair, 3-mm débridement wounds were made and allowed to heal in vivo for various periods. As seen in Figure 2 , both TβR-I and TβR-II appeared to be upregulated after wounding. TβR-I levels were elevated across the entire cornea (Figs. 2C 2D) . At the leading edge of migrating epithelium, TβR-I was localized in multiple cell layers (Figs. 2A 2D) ; however, it was preferentially localized in the basal cell layer distal to the wound edge (Fig. 2C) . In contrast, TβR-II upregulation was far more confined. As early as 4 hours after wounding, elevated levels of TβR-II were observed in cells at the leading edge (Fig. 2B) . No apparent upregulation of TβR-II in cells distal to the original wound was seen at any time point (Fig. 2E) . The contrast between TβR-I and TβR-II localizations can be clearly seen 8 hours after wounding (Figs. 2C 2D 2E 2F) , when TβR-I levels were elevated across the entire cornea, and TβR-II levels appeared to be elevated only in the cells migrating to cover the débrided area. This pattern was maintained until 48 hours after wounding (Figs. 2G 2H) . No discernible change in either TβR-I or TβR-II localization was seen at any time point in the limbus (data not shown).

During the course of the examination of alterations in TβR-II localization, it appeared that the elevation of TβR-II was confined to the cells migrating to cover the original 3-mm débridement zone. To confirm this possibility, we examined TβR-I and TβR-II localization in superficial keratectomy wounds. In this wound model, which removes the epithelium, the basement membrane, and a portion of the anterior stroma, the edge of the original wound area can be precisely localized by reacting the tissue with antibodies against basement membrane components such as laminin. As seen in Figure 3 , TβR-II expression was preferentially upregulated in the epithelial cells that had migrated across the original wound area. This pattern was observed at all time points examined. One difference that was observed in the localization of TβR-II in débridement versus keratectomy wounds was that the cells at the very tip of the leading edge expressed very low levels of TβR-II in the keratectomy wounds (Fig. 3D) . Localization of TβR-I in the keratectomy wounds was similar to that in débridement wounds (data not shown).

To confirm that TβR-I and TβR-II expression was elevated during epithelial wound healing, we assayed protein levels of the receptors using Western blot analysis. In these experiments, 3-mm wounds were created and allowed to heal 1 to 48 hours, after which a

4-mm area of epithelium was harvested. We harvested a 4-mm rather than a 3-mm area, because the amount of tissue within the 3-mm area at early time points was insufficient to perform Western blot analysis. As seen in Figure 4 , protein levels for both TβR-I and TβR-II were enhanced after wounding, in agreement with the immunofluorescence data. Similar, although not identical, kinetics were seen for both proteins with peak levels of TβR-I seen 16 hours after wounding, and peak levels of TβR-II seen at 24 hours. The most apparent difference in kinetics was that TβR-II levels actually appeared to decrease immediately after wounding and then to steadily increase until 24 hours, whereas TβR-I did not exhibit an initial decrease.

In previous experiments, we had observed that the cell-cycle inhibitor $p15^{INK4b}$ was expressed at high levels in cells migrating to cover a débridement wound. [28] The inhibitor $p15^{INK4b}$ has been shown in epidermal cells to be stimulated by TGF-β. [27] To confirm that $p15^{INK4b}$ was stimulated by TGF-β in corneal epithelial cells, human corneal epithelial cells were cultured with or without TGF-β1 for 6 hours, and $p15^{INK4b}$ protein levels were assayed. As seen in Figure 5 , TGF-β stimulated a 3.1 ± 0.18-fold increase in $p15^{INK4b}$ levels. In an interesting observation, EGF blunted this response (Fig. 5) .

Finally, because TβR-I and TβR-II were differentially expressed after wounding, this suggested that the receptors were under different regulational control. To support this possibility, we examined the effect of EGF and TGF-β on the cultured cells to determine whether these growth factors might have differential effects on expression. As seen in Figure 6 , neither EGF nor TGF-β1 significantly affected TβR-I protein levels. However, TGF-β1 stimulated a 1.7-fold enhancement of TβR-II protein levels. In addition, the combination of EGF and TGF-β stimulated a 2.3-fold increase.

Discussion

One of the intriguing aspects of corneal epithelial wound repair is that wounding appears to stimulate cell proliferation in cells distal to the wound; however, cells migrating to cover the original wound appear to be inhibited from proliferating. [14] [15] [16] [17] [18] [19] [20] We have previously reported that the cell-cycle–dependent kinase inhibitor $p15^{INK4b}$ appears to be preferentially upregulated in cells that are migrating to cover the wound area. [28] We

postulated that the upregulation of $p15^{INK4b}$ might generate migratory and proliferative phenotypes. Because TGF-β is known to upregulate $p15^{INK4b}$ in other cell types, we postulated that TGF-β signaling might play a role in creating these phenotypes. [28] In the current investigation, we examined the localization and expression of TβR-I and TβR-II to determine whether these parameters were consistent with a role for TGF-β in inhibiting cell proliferation in migrating corneal epithelium. Our findings appear to agree with this hypothesis. First, we found that TGF-β1 stimulated the synthesis of $p15^{INK4b}$ in corneal epithelial cells (Fig. 5) . Second, we observed that both TβR-I and TβR-II were upregulated after wounding (Figs. 1 2 3 4) . This enhancement in receptor levels gives the cells the potential for enhanced levels of TGF-β signaling. Perhaps our most interesting finding is that TβR-II appeared to be preferentially upregulated in the epithelial cells migrating to cover the wound area (Figs. 2 3) . Because both TβR-I and TβR-II are required for TGF-β signaling, our findings suggest that TGF-β maximally affects migrating cells. This is consistent with our previous finding that $p15^{INK4b}$ is preferentially upregulated in these cells. Potential sources of TGF-β include tears, keratocytes, and the epithelium itself (see Refs. [1] [2] [5] [6] [7] and [11] for review).

Only limited reports have been made of the localization and expression of TβR-I and TβR-II in corneal epithelium. The localization of TβR-I and TβR-II in the current investigation is in agreement with published reports in unwounded human corneal epithelium. where both TβR-I and TβR-II were present at low levels in central cornea and at much higher levels in limbal epithelium. [29] Our results are also in general agreement with Obata et al., [30] who found TβR-I and TβR-II in unwounded rat corneal epithelium. They did not compare limbal versus central corneal expression. Mita et al. [31] also reported that both TβR-I and TβR-II are expressed in the corneal epithelium after excimer laser keratectomy. They did not quantify alterations in protein levels. Of note, they observed that TβR-II was not localized at the very tip of the leading edge 24 hours after wounding. This is in agreement with our observations after superficial keratectomy. The significance of this is not clear, but a possible explanation is that the receptors at the leading edge have bound ligand and become internalized. [34] [35] To our knowledge, there have been no published reports indicating that TβR-I and TβR-II are differentially regulated in corneal epithelial wound healing; however, our findings are in agreement with observations made in skin wound models. [36] [37] Frank et al. [36] found that both TβR-I and TβR-II was upregulated in the

epidermis after wounding and that the kinetics of their expression is different. Coupled with the finding that TβR-I and TβR-II are differentially affected by glucocorticoids led to the their conclusion that the two receptors are under different regulational control.

Because both TβR-I and TβR-II are required for TGF-β signal transduction, it was somewhat surprising that the two receptors appeared to be differentially localized and regulated during wound repair. A possible explanation is that TβR-I and TβR-II can also heterodimerize with other members of the TGF-β superfamily of receptors. Thus, it may be advantageous to have the receptors under different controls. Nevertheless, it appears that TβR-II regulation is the key regarding our original question of whether TGF-β signaling is involved in the absence of cell proliferation in migrating cells. Whereas TβR-I is present across the cornea, TβR-II appears to be preferentially upregulated in cells that are migrating over the wound area. This expression pattern is consistent only with cells' being subject to high levels of TGF-β signaling in the wound area.

However, these findings raise the additional question of what is the signal for TβR-II upregulation. Because TβR-II is upregulated both in débridement wounds (where an intact basement membrane is present) and keratectomy wounds (where the basement membrane is removed), it does not seem likely that the signal is a molecule normally present in the basement membrane. A possible explanation is that the signal is deposited onto the wound area. The signal could be an extracellular matrix component such as fibronectin, [33] the unprocessed form of laminin 5, [38] amyloid precursor-like protein (APLP)2, [39] or other unknown proteins that are deposited or secreted on the matrix during wound repair. Alternatively, the signal could be a matrix-binding growth factor (such as heparin-binding EGF) that may preferentially coat the wound matrix. These growth factors may be present in the tear film or released by the wounded epithelium. Little is known about the regulation of TβR-II expression in vivo. However, expression appears to be regulated by the ets-related transcription factor (also known as ESX and ESE-1). [40] EGF in turn stimulates this family of transcription factors. [41] How TβR-II is stimulated is currently under investigation.

Based on our investigations and the findings of others, [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [18] [19] [20] we propose the following model of corneal epithelial wound repair. In unwounded

tissue, members of the EGF family that are present in the tear film cannot access the EGF receptors that are present primarily in the basal cell layer.[13] After wounding, these growth factors, along with growth factors released by the epithelium and underlying keratocytes rapidly stimulate EGF receptors across the entire cornea.[13] Activation of the EGF receptor stimulates both cell proliferation and migration. The activation, in turn, stimulates an autocrine loop of EGF receptor ligand synthesis.[13] In an unknown mechanism, both TβR-I and TβR-II are upregulated after wounding, with TβR-II being preferentially localized to cells migrating over the original wound. The enhanced level of TβR-I and TβR-II allows these cells to be preferentially stimulated by TGF-β, in turn stimulating the upregulation of $p15^{INK4b}$. This inhibitor (most likely in concert with other inhibitors of the cell cycle) blocks cell proliferation in the cells migrating across the wound, effectively spatially separating the proliferative and migratory responses to wounding. The separation of the two responses could give rise to more efficient healing. Indeed, the overexpression of another cell-cycle–dependent kinase inhibitor, $p27^{Kip1}$, has been shown in another system to stimulate migration rates.[42]

Obviously, many important questions remain to be resolved in this proposal. What regulates TβR-II? What is the role of other growth factors besides TGF-β? Are ligands for TβR-I and TβR-II present? These questions will be the subject of our future investigations.

Supported by National Eye Institute Grants R01 EY05665 (JDZ) and R01 EY05767 (NCJ).

Submitted for publication January 3, 2001; accepted February 28, 2001.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: James D. Zieske, Cornea Unit, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114-2500. zieske@vision.eri.harvard.edu

FIGURE 1.

View Original Download Slide

Immunolocalization of TβR-I (**A, B**) and TβR-II (**C, D**) in unwounded limbal (**A, C**) and central corneal (**B, D**) epithelium of the adult rat. TβR-I and TβR-II were preferentially localized in the basal cells of the limbal epithelium. Binding of anti–TβR-I (**A1**) and anti–TβR-II (**C1**) was blocked by preadsorption with the appropriate peptides. Scale bars, 50 μm.

FIGURE 2.

View Original Download Slide

Micrographs demonstrating immunolocalization of TβR-I and TβR-II after wounding. (**A**) TβR-I at leading edge, 4 hours after débridement. (**B**) TβR-II at leading edge, 4 hours after débridement. Intense localization of TβR-II are present only at the very tip of the leading edge. (**C**) TβR-I distal to the original wound, 8 hours after débridement. Intense binding of anti-TβR-I was observed throughout the basal epithelial layer. (**D**) TβR-I at the leading edge, 8 hours after débridement. (**E**) TβR-II distal to original wound, 8 hours after débridement. There was a relative absence of TβR-II in comparison with TβR-I. (**F**) TβR-II at the leading edge, 8 hours after wounding. TβR-II appeared to be maximally upregulated in the cells that had migrated over the original wound. The localization of TβR-I (**C, D**) and TβR-II (**E, F**) are on adjacent sections. (**G**) TβR-II at the edge of the original wound, 48 hours after wounding. Only low levels of TβR-II were present toward the limbus, whereas much higher levels were observed toward the central wound area (*bracket*). (**H**) TβR-II in the débridement area, 48 hours after wounding. TβR-II levels still appeared to be elevated compared with those in unwounded corneas. Scale bars, 50 μm.

FIGURE 3.

View Original Download Slide

Immunolocalization of TβR-II (**A, C, D**) and laminin (**B, E, F**) 4 hours (**A, B**) and 16 hours (**C–F**) after superficial keratectomy. Localization of TβR-II was closely correlated with the original wound area, as indicated by the absence of laminin. (**A**, **B**) are adjacent sections; (**C**, **D**) are a montage of a single section; (**E**, **F**) are a montage of an adjacent section. *Arrow*: tip of the leading edge. Scale bars, 50 μm.

FIGURE 4.

View Original Download Slide

(**A**) Representative Western blot analyses of unwounded (UnW) and wounded corneal epithelium harvested at 1 to 48 hours after a central 3-mm débridement. Equal amounts of protein were loaded on each lane. Blots were reacted with anti-TβR-I or anti-TβR-II. (**B**) Quantitation of the relative levels of TβR-I (*hatched bars*) and TβR-II (*filled bars*) protein from three separate experiments. Data are expressed as the mean ± SEM. *Significant difference in the level of TβR-I and TβR-II protein from unwounded control at all points from 16 to 48 hours ($P < 0.05$).

FIGURE 5.

View Original Download Slide

(**A**) Representative Western blot analysis of primary human corneal epithelial cells in culture for 6 hours with no added growth factors (negative control), 5 ng/ml EGF, 2 ng/ml TGF-β1, or both EGF and TGF-β1. Equal amounts of protein were loaded on each lane. Blots were reacted with anti-$p15^{INK4b}$. (**B**) Quantitation of the relative level of $p15^{INK4b}$

protein from three separate experiments. Data are expressed as the mean ± SEM. *Significant increase in the level of $p15^{INK4b}$ protein in the TGF-β1-treated cells compared with the untreated cells ($P < 0.05$).

FIGURE 6.

View Original Download Slide

(**A**) Representative Western blot analysis of primary human corneal epithelial cells in culture for 6 hours with no added growth factors (negative control), 5 ng/ml EGF, 2 ng/ml TGF-β1, or both EGF and TGF-β1. Equal amounts of protein were loaded on each lane. Blots were reacted with anti-TβR-I or anti-TβR-II. (**B**) Quantitation of the relative levels of TβR-I (*hatched bars*) and TβR-II (*filled bars*) protein from three separate experiments. Values are expressed as the mean ± SEM.* Significant difference in the level of TβR-II protein in the TGF-β1 plus EGF-treated cells compared with the untreated cells ($P < 0.05$).

1 Zieske J, Gipson I. Agents that affect corneal wound healing: modulation of structure and function. Albert D Jakobiec F eds. *Principles and Practice of Ophthalmology.* 2000;364–372. WB Saunders Philadelphia.

2 Imanishi J, Kamiyama K, Iguchi I, Kita M, Sotozono C, Kinoshita S. Growth factors: importance in wound and maintenance of transparency of the cornea. *Prog Retinal Eye Res.* 2000;19:113–129. [CrossRef]

3 Wilson SE, Chen L, Mohan RR, Liang Q, Liu J. Expression of HGF, KGF, EGF and receptor messenger RNAs following corneal epithelial wounding. *Exp Eye Res.* 1999;68:377–397. [CrossRef] [PubMed]

4 Obata H, Kaji Y, Yamada H, Kato M, Tsuru T, Yamashita H. Expression of transforming growth factor-beta superfamily receptors in rat eyes. *Acta Ophthalmol Scand.* 1999;77:151–156. [CrossRef] [PubMed]

5 Nishida T, Tanaka T. Extracellular matrix and growth factors in corneal wound healing. *Curr Opin Ophthalmol.* 1996;7:2–11.

6 Gipson IK, Inatomi T. Extracellular matrix and growth factors in corneal wound healing. *Curr Sci.*

1995;6:3–10.

7 Schultz G, Khaw PT, Oxford K, MaCauley S, Van Setten G, Chegini N. Growth factors and ocular wound healing. *Eye.* 1994;8:184–187. [CrossRef] [PubMed]

8 Frati L, Daniele S, Delogu A, Covelli I. Selective binding of the epidermal growth factor and its specific effects on the epithelial cells. *Exp Eye Res.* 1972;14:135–141. [CrossRef] [PubMed]

9 Zieske JD, Wasson M. Regional variation in distribution of EGF receptor in developing and adult corneal epithelium. *J Cell Sci.* 1993;106:145–152. [PubMed]

10 Murata T, Ishibashi T, Inomata H. Localizations of epidermal growth factor receptor and proliferating cell nuclear antigen during corneal wound healing. *Graefes Arch Clin Exp Ophthalmol.* 1993;231:104–108. [CrossRef] [PubMed]

11 Wilson SE, He Y-G, Lloyd SA. EGF, EGF receptor, basic FGF, TGF beta-1, and IL-1 alpha messenger RNA production in human corneal epithelial cells and stromal fibroblasts. *Invest Ophthalmol Vis Sci.* 1992;33:1756–1765. [PubMed]

12 Hongo M, Itoi M, Yamaguchi N, Imanishi J. Distribution of epidermal growth factor (EGF) receptors in rabbit corneal epithelial cells, keratocytes and endothelial cells, and the changes induced by transforming growth factor-beta 1. *Exp Eye Res.* 1992;54:9–16. [CrossRef] [PubMed]

13 Zieske J, Takahashi H, Hutcheon A, Dalbone A. Activation of epidermal growth factor receptor during corneal epithelial migration. *Invest Ophthalmol Vis Sci.* 2000;41:1346–1355. [PubMed]

14 Hanna C. Proliferation and migration of epithelial cells during corneal wound repair in the rabbit and the rat. *Am J Ophthalmol.* 1966;61:55–63. [CrossRef] [PubMed]

15 Arey LB, Covode WM. The method of repair in epithelial wounds of the cornea. *Anat Rec.* 1943;86:75–86. [CrossRef]

16 Hara J. An autoradiographic study of regeneration of the corneal epithelium. *Folia Ophthalmol.* 1962;13:443–466.

17 Haaskjold E, Sandvig KU, Bjerknes R, Kravik K. The early cell kinetic response during healing of corneal epithelial wounds. *Ophthalmic Surg.* 1992;23:680–684. [PubMed]

18 Sandvig KU, Haaskjold E, Bjerknes R, Refsum SB, Kravik K. Cell kinetics of conjunctival and corneal epithelium during regeneration of different-sized corneal epithelial defects. *Acta Ophthalmol.* 1994;72:43–48.

19 Chung E-H, Hutcheon AEK, Joyce NC, Zieske JD. Synchronization of the G1/S transition in response

to corneal débridement. *Invest Ophthalmol Vis Sci.* 1999;40:1952–1958. [PubMed]

20 Zagon IS, Sassani JW, Ruth TB, McLaughlin PJ. Cellular dynamics of corneal wound re-epithelialization in the rat. III: mitotic activity. *Brain Res.* 2000;882:169–179. [CrossRef] [PubMed]

21 Massagué J, Polyak K. Mammalian antiproliferative signals and their targets. *Curr Opin Genetics Dev.* 1995;5:91–96. [CrossRef]

22 Massagué J. TGF-β signal transduction. *Annu Rev Biochem.* 1998;67:753–791. [CrossRef] [PubMed]

23 Reed SI, Bailly E, Dulic V, Hengst L, Resnitzky D, Slingerland J. G_1 control in mammalian cells. *J Cell Sci.* 1994;18(suppl)69–73.

24 Carnero A, Hannon GJ. The INK4 family of CDK inhibitors. *Curr Top Microbiol Immunol.* 1998;227:43–55. [PubMed]

25 Pavletich NP. Mechanisms of cyclin-dependent kinase regulation: structures of Cdks, their cyclin activators, and Cip and INK4 inhibitors. *J Mol Biol.* 1999;287:821–828. [CrossRef] [PubMed]

26 Roussel MF. The INK4 family of cell cycle inhibitors in cancer. *Oncogene.* 1999;18:5311–5317. [CrossRef] [PubMed]

27 Hannon GJ, Beach D. $p15^{INK4b}$ is a potential effector of TGF-β-induced cell cycle arrest. *Nature.* 1994;371:257–261. [CrossRef] [PubMed]

28 Zieske J. Expression of cyclin-dependent kinase inhibitors during corneal wound repair. *Prog Retinal Eye Res.* 2000;19:257–270. [CrossRef]

29 Joyce NC, Zieske JD. Transforming growth factor-β receptor expression in human cornea. *Invest Ophthalmol Vis Sci.* 1997;38:1922–1928. [PubMed]

30 Obata H, Kaburaki T, Kato M, Yamashita H. Expression of TGF-beta type I and type II receptors in rat eyes. *Curr Eye Res.* 1996;15:335–340. [CrossRef] [PubMed]

31 Mita T, Yamashita H, Kaji Y, et al. Effects of transforming growth factor beta on corneal epithelial and stromal cell function in a rat wound healing model after excimer laser keratectomy. *Graefes Arch Clin Exp Ophthalmol.* 1998;236:834–843. [CrossRef] [PubMed]

32 Zieske JD, Gipson IK. Protein synthesis during corneal epithelial wound healing. *Invest Ophthalmol Vis Sci.* 1986;27:1–7.

33 Zieske JD, Higashijima SC, Spurr-Michaud SJ, Gipson IK. Biosynthetic responses of the rabbit cornea to a keratectomy wound. *Invest Ophthalmol Vis Sci.* 1987;28:1668–1677. [PubMed]

34 Wakefield LM, Smith DM, Masui T, Harris CC, Sporn MB. Distribution and modulation of the cellular receptor for transforming growth factor-beta. *J Cell Biol.* 1987;105:965–975. [CrossRef] [PubMed]

35 Zwaagstra JC, Kassam Z, O'Connor-McCourt MD. Down-regulation of transforming growth factor-β receptors: cooperativity between the types I, II, and III receptors and modulation at the cell surface. *Exp Cell Res.* 1999;252:352–362. [CrossRef] [PubMed]

36 Frank S, Madlener M, Werner S. Transforming growth factors β1, β2, and β3 and their receptors are differentially regulated during normal and impaired wound healing. *J Biol Chem.* 1996;271:10188–10193. [CrossRef] [PubMed]

37 Gold LI, Sung JJ, Siebert JW, Longaker MT. Type I (RI) and type II (RII) receptors for transforming growth factor-β isoforms are expressed subsequent to transforming growth factor-β ligands during excisional wound repair. *Am J Pathol.* 1997;150:209–222. [PubMed]

38 Nguyen BP, Ryan MC, Gil SG, Carter WG. Deposition of laminin 5 in epidermal wounds regulates integrin signaling and adhesion. *Curr Opin Cell Biol.* 2000;12:554–562. [CrossRef] [PubMed]

39 Guo J, Thinakaran G, Guo Y, Sisodia SS, Yu FX. A role for amyloid precursor-like protein 2 in corneal epithelial wound healing. *Invest Ophthalmol Vis Sci.* 1998;39:292–300. [PubMed]

40 Choi SG, Yi Y, Kim YS, et al. A novel ets-related transcription factor, ERT/ESX/ESE-1, regulates expression of the transforming growth factor-beta type II receptor. *J Biol Chem.* 1998;273:110–117. [CrossRef] [PubMed]

41 Watabe T, Yoshida K, Shindoh M, et al. The Ets-1 and Ets-2 transcription factors activate the promoters for invasion-associated urokinase and collagenase genes in response to epidermal growth factor. *Int J Cancer.* 1998;77:128–137. [CrossRef] [PubMed]

42 Nagahara H, Vocero-Akbani A, Snyder E, et al. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nat Med.* 1998;4:1449–1452. [CrossRef] [PubMed]