Interleukin-1 and Transforming Growth Factor Beta: Commonly Opposing, but Sometimes Supporting, Master Regulators of the Corneal Wound Healing Response to Injury

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Citation: Wilson SE. Interleukin (IL)-1α/IL-1β and transforming growth factor (TGF)β1/TGFβ2 have both been promoted as “master regulators” of the corneal wound healing response due to the large number of processes each regulates after injury or infection. The purpose of this review is to highlight the interactions between these systems in regulating corneal wound healing.

METHODS. We conducted a systematic review of the literature.

RESULTS. Both regulator pairs bind to receptors expressed on keratocytes, corneal fibroblasts, and myofibroblasts, as well as bone marrow-derived cells that include fibrocytes. IL-1α and IL-1β modulate healing functions, such as keratocyte apoptosis, chemokine production by corneal fibroblasts, hepatocyte growth factor (HGF), and keratinocyte growth factor (KGF) production by keratocytes and corneal fibroblasts, expression of metalloproteinases and collagenases by corneal fibroblasts, and myofibroblast apoptosis. TGFβ1 and TGFβ2 stimulate the development of myofibroblasts from keratocyte and fibrocyte progenitor cells, and adequate stromal levels are requisite for the persistence of myofibroblasts. Conversely, TGFβ3, although it functions via the same TGF beta I and II receptors, may, at least in some circumstances, play a more antifibrotic role—although it also upregulates the expression of many profibrotic genes.

CONCLUSIONS. The overall effects of these two growth factor-cytokine-receptor systems in controlling the corneal wound healing response must be coordinated during the wound healing response to injury or infection. The activities of both systems must be downregulated in coordinated fashion to terminate the response to injury and eliminate fibrosis.

TRANSLATIONAL RELEVANCE. A better standing of the IL-1 and TGFβ systems will likely lead to better approaches to control the excessive healing response to infections and injuries leading to scarring corneal fibrosis.

Keywords: cornea, growth factors, growth factor receptors, transforming growth factor (TGF) beta-1, TGF beta-2, interleukin-1 (IL) alpha, interleukin-1 beta, stromal-epithelial interactions, fibrosis, corneal scar

The Corneal Wound Healing Response to Injury

Injuries to the cornea most commonly occur via the corneal epithelium in the form of traumatic, infectious, toxic, or surgical injuries to the epithelium alone or to the epithelium and underlying stroma.1 Another route of injury, however, typically mediated by some type of immune response, can enter the peripheral stroma directly via the limbal blood vessels.2 Finally, the corneal endothelium can be injured either via a perforating injury through the epithelium and deep stroma, or directly through processes such as herpes simplex endothelitis.3 This paper, for the most part, will focus on anterior injury models, with reference to the other modes of injury when applicable. This topic has recently been reviewed,1 but a brief overview will be helpful.

The first notable change in the stroma after epithelial or epithelial-stromal injury is apoptosis of the underlying keratocytes (Figs. 1A, 1B).5 The site and extent of this apoptosis, and whether there is also or in some cases exclusively necrosis (for example in severe alkali burns6) of keratocytes, is dependent primarily on the type and extent of the injury.4 This stromal apoptosis response occurs, at least to a limited extent, even if the injury is confined to the epithelium, for example, in a simple corneal abrasion or herpes simplex epithelial keratitis. Importantly, a posterior corneal keratocyte apoptosis response and similar ensuing posterior corneal wound response can occur with injuries to the corneal endothelium.7,8

Depending on the extent of injury, and likely genetic influences and possibly other unknown factors, the ensuing wound healing response typically goes one of two
FIGURE 1. Nonfibrotic and fibrotic healing responses to injury in the cornea. (A) In the uninjured cornea the stroma is populated with quiescent keratocytes. Inactive TGFβ1 is produced in small amounts by corneal epithelial and endothelial cells, while small amounts of inactive TGFβ1 and TGFβ2 are also present in the tears—restricted from passage through the epithelium by an epithelial barrier function. The EBM and Descemet's membrane prevent passage of TGFβ1 or TGFβ2 into the stroma, although small amounts of TGFβ1 or TGFβ2 below the level of IHC detection may be sequestered in the stromal matrix (not shown). IL-1α76–78 (and also inactive IL-1β81) are within corneal epithelial cells. (B) Within minutes to hours of epithelial-stromal injury, including the EBM, inactive TGFβ1 production is upregulated in the
epithelium, and TGFβ1 and TGFβ2 are present at increased levels in the tears (from the lacrimal gland and possibly conjunctiva, goblets cells and other cells), and enter the stroma in high levels in the absence of EBM.9 TGFβ1 and TGFβ2 are activated by collagenases and metalloproteases (and thrombospondin-1) in the stroma, and integrins in the epithelium (as well as possibly other activators).58–60 IL-1α (and pro-IL-1β) are released from injured corneal epithelial cells (IL-1β is activated by neutrophil serine proteases and other enzymes).34,35 and high concentrations of IL-1α and IL-1β trigger apoptosis of subepithelial keratocytes via upregulation of Fas ligand by keratocytes (that constitutively express Fas).35,171 Surrounding keratocytes that escape the wave of apoptosis transition to corneal fibroblasts and, driven by TGFβ1 and TGFβ2, begin development into myofibroblasts.9 IL-1α and IL-1β also upregulate surviving corneal fibroblast production of HGF and KGF that modulate corneal epithelial cell migration, proliferation and differentiation to heal the epithelial defect.81 Fibrocytes (not shown) attracted from limbal blood vessels by chemokines produced by corneal fibroblasts also begin TGFβ1- and TGFβ2-driven development into myofibroblasts.12,116 Limited amounts of TGFβ1 and TGFβ2 are also produced by both vimentin-positive and vimentin-negative stromal cells9 (not shown). C) In corneas that heal without scarring fibrosis, the EBM is regenerated by the coordinated action of the healed epithelium and cooperating keratocytes/corneal fibroblasts (that produce EBM components such as perlecan and nidogens), and epithelial barrier function is re-established.9 Deprived of sufficient epithelial and tear TGFβ1 and TGFβ2 by the regenerated EBM, myofibroblast precursors and corneal fibroblasts either undergo apoptosis or revert to keratocytes.9 Little disordered extracellular matrix is produced and stromal opacity is limited.1 D) If the EBM is not regenerated in a timely manner (typically a few weeks), then myofibroblast precursors, driven by epithelial, tear, and, possibly, stromal cell-derived TGFβ1 and TGFβ2, complete development into mature myofibroblasts, that are themselves opaque due to decreased production of corneal crystallins,23 and produce large amounts of stromal disordered extracellular matrix associated with scarring fibrosis.3 This scarring fibrosis persists for months or years, or even indefinitely, until such time as the EBM is once again regenerated through the coordinated action of corneal fibroblasts/keratocytes and corneal epithelial cells (not shown).9,129 Thereby deprived of requisite TGFβ1 and/or TGFβ2, the myofibroblasts undergo late apoptosis9,7,29 or revert to precursor cells. Keratocytes repopulate the subepithelial stroma, and reorganize the disordered extracellular matrix and re-establish transparency (not shown). A similar posterior stromal keratocyte apoptosis and scarring fibrosis response can occur after injury to the endothelium and Descemet’s membrane (not shown). Severe corneal injuries involving both the EBM and Descemet’s membrane can produce fibrosis of the full-thickness stroma (not shown).14 Illustration by David Schumick, BS, CMI. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2021. All Rights Reserved.

Regeneration of the epithelial basement membrane (EBM) determines whether these myofibroblast precursors complete their development and secrete large quantities of disordered collagen type 1, collagen type 2, and other disordered extracellular matrix materials that are found in fibrosis.17,18 The EBM normally regenerates within 1 to 2 weeks via the cooperative interactions of the corneal epithelial cells and corneal fibroblasts and/or keratocytes (see Fig. 1C).9,19 The first step in regeneration of the EBM is closure of the epithelium because if an epithelial defect develops and persists for more than 1 to 2 weeks, myofibroblasts and scarring fibrosis invariably develop.20 Even if the epithelial defect does close, the EBM may not be regenerated by the coordinated production and localization of EBM components by epithelial cells, subepithelial corneal fibroblasts, and/or keratocytes, depending on the extent of injury (and the level of initial keratocyte loss via apoptosis/necrosis), stromal surface irregularity, and likely other factors (see Fig. 1D).18,19,21 Two recent studies have pointed to perlecan production and incorporation by corneal fibroblasts and/or keratocytes into the nascent EBM as a major factor in normal versus defective regeneration of the mature, fully functional EBM.3,22 The reason that mature EBM regeneration is the key to nonfibrotic versus fibrotic corneal stromal healing is because the fully regenerated EBM is the paramount regulator of ongoing entry of TGFβ1 and TGFβ2, as well as PDGF, from the overlying epithelium and tears.9,18,21 When the EBM normally regenerates, TGF beta-1 and TGF beta-2 from the tears and epithelium are prevented from entering the underlying stroma. However, in the absence of fully mature EBM with normal perlecan, and possibly other EBM components derived from corneal fibroblasts and keratocytes, TGFβ1 and TGFβ2 gain ongoing entry into the corneal stroma in sufficient amounts to drive the development of mature myofibroblasts, and maintain their viability (see Fig. 1D). If at any time point mature EBM is regenerated, then myofibroblast precursors and any mature myofibroblasts that have developed undergo apoptosis. Corneal fibroblasts and keratocytes then repopulate that area of stroma and restore the
normal collagen and extracellular matrix (ECM) structure of the stroma associated with transparency. If mature EBM is not fully regenerated, then myofibroblast precursors and myofibroblasts receive ongoing TGFβ1 and TGFβ2 from the epithelium and tears, and to a lesser extent corneal stromal cells, and persistent scarring fibrosis is established in the stroma. Importantly, a lower level of stromal opacity, that is clinically referred to as “haze,” can be generated by corneal fibroblasts that do not develop into mature myofibroblasts.8,24

A similar fibrosis process can occur in the posterior cornea following injury to the corneal endothelial cells and associated Descemet’s basement membrane.8,24,25 The source of TGFβ1 and TGFβ2 after these posterior injuries are the aqueous humor and residual peripheral endothelial cells.8,24,25 In this case, however, there is much less tendency for Descemet’s membrane to be regenerated by coordinated action of endothelial cells and overlying keratocytes or corneal fibroblasts, and the posterior corneal fibrosis tends to persist indefinitely.14,24,25 Full-thickness corneal fibrosis can occur after extensive injuries damaging both the EBM and Descemet’s basement membrane, although there is more tendency for the EBM to eventually regenerate and the anterior stromal fibrosis to be resolved.14

Even in corneas that develop extensive myofibroblasts and fibrosis, the fibrosis can eventually decrease, or even resolve, if the EBM is repaired.18,21,24,26 This process likely occurs by eventual penetration of keratocytes and/or corneal fibroblasts through the fibrotic tissue to cooperate with the overlying basal epithelial cells in regeneration of the normal mature EBM, with the ultrastructural regeneration of lamina lucida and lamina densa signaling this process.18,21,24,25 When this occurs, persistent myofibroblasts, or even recently developed myofibroblasts, because myofibroblast and fibrosis turnover is dynamic over time,9 are deprived of mandatory supplies of TGFβ1 and TGFβ2, and subsequently undergo apoptosis.4,47

Peripheral corneal wound healing and scarring that occurs with disorders, such as peripheral ulcerative keratitis and Mooren’s ulcers,26,29 likely share some characteristics with anterior and posterior corneal injuries, but likely have more involvement of bone marrow-derived cells, such as monocytes, macrophages, and lymphocytes. The wound healing responses in these peripheral corneal disorders have not been well-characterized.

**General Overview of the Interleukin-1 Cytokines, Antagonists, and Receptors**

The interleukin (IL)-1 family of cytokines comprises 11 members that includes 7 pro-inflammatory agonists (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ) and 4 antagonists (IL-1 receptor antagonist [IL-1Ra], IL-36 receptor antagonist, IL-37, and IL-38).30,31 The IL-1 receptor family includes 10 members that includes cytokine-specific receptors, coreceptors, and inhibitory receptors.30,31

IL-1 cytokines do not possess a secretory sequence, with the exception of IL-1Ra, and are secreted via injury or death of cells.30,31 Pro-IL-1β and pro-IL-18 are produced as biologically inert pro-peptides that require cleavage by caspase-1 upon inflammasome activation to generate the active cytokines. The inflammasome is a cytosolic multiprotein signaling complex that is part of the innate immune system and has a critical role in activating inflammatory responses in the cornea and other organs.32,33 In addition, IL-36 also requires N-terminal processing for activation.30,31 However, pro-IL-1β can be cleaved and activated by neutrophil serine proteases, such as proteinase 3, also known as PRTN3, and elastase, as well as by mast cell-derived serine proteases, including chymase.34,35

IL-1α and IL-33 are typically released by cell damage or cell death, and, therefore, have been classified as “alarmins.” Even though the full-length pro-IL-1α and pro-IL-33 can bind their receptors and trigger intracellular signaling, the activities of both of these cytokines are markedly enhanced by protease cleavage.30,37

Previously published work has revealed important insights into the functions of IL-1β, IL-33, IL-36α, IL-36β, and IL-36γ and their antagonists and receptors. The recent publication of new biologic agents for the treatment of alkali burns in rats30,31 and to increase corneal transplant survival in mice.30,31 Although it is well-tolerated in humans and animals, it has not been used widely, in part possibly to the expense of treatment and clinical trials to expand its use.

IL-1 receptor, type 2 (IL-1R2) is a decoy receptor expressed by many cells, including keratinocytes, endometrial epithelial cells, T and B cells, monocytes, and
polymorphonuclear cells.\(^{30}\) IL-1R2 binds IL-1\(\alpha\) and IL-1\(\beta\) with high affinity, but this small protein has a short cytoplasmic terminus that is incapable of signal transduction.\(^{30,32}\) As a decoy, IL-1R2 also modulates the effects of IL-1\(\alpha\) and IL-1\(\beta\) on cells that express it.

Both IL-1\(\alpha\) and IL-1\(\beta\) bind to and produce their effects via IL1R1.\(^{30,31,32}\) Binding of IL-1\(\alpha\) or IL-1\(\beta\) to IL1R1 leads to the activation of a series of protein kinases that subsequently trigger an increase in the expression of numerous pro-inflammatory genes,\(^{30,33}\) and in some cells triggers apoptosis,\(^{34,35}\) likely depending on the local concentration of IL-1\(\alpha\) and IL-1\(\beta\).

**GENERAL OVERVIEW OF THE TGF\(\beta\) FAMILY OF GROWTH FACTORS AND RECEPTORS**

TGF\(\beta\)1 was the first member identified in the TGF family.\(^{36}\) However, this large family of modulators now includes 33 related proteins, including the TGF betas, bone morphogenic proteins (BMPs), activins, growth and differentiation factors (GDFs), nodal, Mullerian inhibiting substance, and a large number of related receptors. This review focuses on the family members TGF\(\beta\)1, TGF\(\beta\)2, and TGF\(\beta\)3, and their receptors.

TGF\(\beta\)1, TGF\(\beta\)2, and TGF\(\beta\)3 are produced as a homodimeric pro-proteins that undergo proteolytic cleavage in the trans-golgi network by furin-like enzymes. This gives rise to a C-terminal mature TGF\(\beta\) dimer and N-terminal pro-peptide known as latency-associated peptide (LAP).\(^{37}\) LAP remains noncovalently associated with mature TGF\(\beta\), which keeps the TGF\(\beta\) inactive in a “small latent complex” (SLC). This SLC forms a complex with another protein called latent TGF\(\beta\) binding protein (LTBP) through intermolecular disulfide bonds, and constitutes the “large latent complex” (LLC).\(^{37}\) This is the most abundant secreted form of each TGF\(\beta\) isoform in most tissues.\(^{31}\) Each latent TGF\(\beta\) isoform is activated in vivo by several molecules in the tissues where they function—such as thrombospondin 1 (TSP-1), integrins, including integrin \(\alpha V\beta 6\), matrix metalloproteinase (MMP) 2, and MMP9.\(^{38-40}\)

All three isoforms of TGF-beta signal via three TGF\(\beta\) receptors—TGFBR1, TGFBR2, and TGFBR3.\(^{36,37}\) “Canonical” TGF-beta signaling occurs after one of the TGF\(\beta\) isoforms with high affinity, but does not have kinase activity. It facilitates binding of TGF\(\beta\)2 to TGFBR2,\(^{71}\) and, therefore, could be an important regulator of TGF\(\beta\)2 responses over TGF\(\beta\)1 and TGF\(\beta\)3 effects in some cells. TGFBR3 has also been shown to trigger noncanonical pathways, suggesting it is not merely a coreceptor.\(^{72}\)

Thus, TGF\(\beta\)1, TGF\(\beta\)2, and TGF\(\beta\)3 compete for the same receptors and the stoichiometry of the available, activated isoforms is likely very important in the TGF\(\beta\) signaling effect on a particular cell,\(^{73,74}\) and the context of the surrounding cellular milieu, including other cytokines and growth factors that are binding and activating their receptors.

Several agonists or antagonists have been shown to facilitate or inhibit binding of activated TGF\(\beta\)1, TGF\(\beta\)2, and TGF\(\beta\)3 to their receptor complexes.\(^{75}\) For example, KCP/CRIM2, CHRDL1, and BMPER/CV-2 act as both agonists and antagonists, depending on the particular growth factor and whether or not other factors are present in the environment.\(^{76}\) Other modulators, such as follistatin (FST), FSTL1, BMPER/CV-2, and Lefty, can bind to TGFBR1 or TGFBR2 to form an inactive, nonsignaling complex, and thereby down-regulate the cells’ response to TGF\(\beta\)1, TGF\(\beta\)2, and TGF\(\beta\)3.\(^{75}\)

**EXPRESSION OF IL-1\(\alpha\), IL-1\(\beta\), IL-1RA, AND IL-1 RECEPTOR IN THE CORNEA**

IL-1\(\alpha\) is constitutively produced by the corneal epithelial (Fig. 2) and corneal endothelial cells,\(^{76-78}\) as it is in most epithelial, endothelial, and stromal cells studied to date.\(^{31,79}\) Although IL-1\(\alpha\) protein expression is typically minimal in the stroma of unwounded corneas (see Fig. 2),\(^{76-78}\) IL-1\(\alpha\) protein, however, has been detected in stromal cells in...
wounded corneas and it has been shown to be active in an autocrine loop in corneal fibroblasts in culture. IL-1β expression has been reported to be restricted primarily to immune cells, although IL-1β has been detected in epithelium and endothelium in unwounded human corneas in one study. In another study, IL-1α and IL-1β proteins were found in both SMA-positive cells (myofibroblasts) and SMA-negative cells (keratocytes, corneal fibroblasts, and immune cells) after photorefractive keratectomy injury in rabbits.

Each of the forms of IL-1Ra is produced by corneal epithelial cells, including ex vivo human corneal epithelium, and stromal cells, but not by corneal endothelial cells. Corneal epithelium expresses high levels of IL-1Ra, as it does IL-1α, suggesting the IL-1Ra modulates IL-1 function when both are released by corneal epithelial injury. Expression of IL-1ra isoforms has apparently not been studied after corneal injury in situ.

IL-1 type I receptors are expressed by all corneal cells, including myofibroblasts, although the IL-1 receptor family has become more complex and the expression of many of the other IL-1 receptor family members has not been studied in corneal cells.

IL-1α and IL-1β are released and activated by injury or death of the corneal epithelial or endothelial cells after injury and modulate numerous functions of stromal cells during the wound healing process. The specific effect released IL-1α or IL-1β has on a particular stromal cell likely depends on factors, such as the specific stromal cell phenotype, the localized concentration of the cytokine, the context of other growth factors and cytokines simultaneously activating receptors on that cell, and other yet to be discovered dynamics.

**Expression of TGFβs and TGFβ Receptors in the Cornea**

TGFβ1 and TGFβ2 are present in normal tears and TGFβ1, TGFβ2, and TGFβ3 are present in the aqueous humor. One source of the TGFβ in tears is the corneal epithelium because normal ocular surface epithelium produces TGFβ1 (Fig. 3), and TGFβ3. These growth factor isotypes are likely released with normal turnover of epithelial cells. TGFβ2 is not expressed in the unwounded rabbit corneal epithelium (see Fig. 3). Normal unwounded corneal endothelial cells also produce TGFβ1 (see Fig. 3C), but not TGFβ2 (see Fig. 3D).

In unwounded rabbit corneas (Figs. 3A, 3B), stromal cell production of TGFβ1 or TGFβ2 protein is relatively low. TGFβ2 is not detectable in unwounded rabbit corneal epithelial cells, but is present on the epithelial surface of unwounded corneas (see Fig. 3B) and is detectable on the stromal surface immediately after photorefractive keratectomy (PRK) in rabbits (see Fig. 3D)—likely deposited there from the tears. However, after PRK injury to the cornea, TGFβ1 (see Fig. 3E) and TGFβ2 (see Fig. 3F) proteins are detectable in many stromal cells that include keratocytes, corneal fibroblasts, myofibroblasts, and some bone marrow-derived cells. TGFβ1 and TGFβ2 mRNAs are also upregulated in corneal epithelial and endothelial cells proximate to the injury. TGFβ-2 LAP has also been detected in human corneal epithelium and stroma, and TGFβ3 LAP is present in the subepithelial stroma.

TGF beta receptors 1 and 2 have been detected by immunohistochemistry in the epithelium and stroma of unwounded and wounded rat corneas. TGF beta receptor 1, 2, and 3 are expressed by human corneal stromal fibroblasts in vitro.

**Corneal Cellular Functions With Known Interactive Regulation by IL-1 and TGFβ**

**IL-1-Mediated Myofibroblast Apoptosis Antagonistic to profibrotic TGFβ**

IL-1α or IL-1β triggers myofibroblast apoptosis in vitro if the available concentration of apoptosis-suppressive TGFβ is low. TGFβ1 from tears and corneal epithelium (and TGFβ2 from tears) penetrates into the stroma and drives development of mature myofibroblasts from keratocyte and fibrocyte precursor cells. This process continues until such time that the damaged basement membranes are regenerated or replaced, which leads to a drop in stromal TGFβ1 and TGFβ2 coming from the epithelium, tear film, and/or aqueous humor. SMA-negative cells, such as corneal fibroblasts, keratocytes, and/or inflammatory cells that enter the stroma, produce IL-1α and IL-1β that act in paracrine fashion to trigger myofibroblast apoptosis. This occurs especially in the region where there is fibrosis in the cornea after injury and SMA-positive myofibroblasts are present at high density. That study also showed that a small percentage of SMA-positive myofibroblasts present in an area of fibrosis in the stroma after injury produce IL-1α and/or IL-1β, suggesting that myofibroblast apoptosis is also regulated via autocrine mechanisms in corneas with fibrosis.

These in vitro interactions were confirmed in a subsequent study using B6; 129S1-Il1r1tm1Roml/J homozygous IL-1RI knockout mice compared to control B6129SF2/J mice that had myofibroblast-generating irregular phototherapeutic keratotomy (PTK) performed with an excimer laser in one eye. SMA-positive myofibroblast density was significantly higher in the IL-1RI knockout group than in the control group at 1 month, 3 months, and 6 months after the irregular PTK. In addition, at 6 months after the irregular PTK, the mean TUNEL+ stromal cells in the subepithelial 50 μm of stroma was significantly lower in the IL-1RI knockout group compared to the control group.

These in vitro and in situ studies showed that IL-1α, likely by TGFβ-unopposed autocrine or paracrine mechanisms, is an important modulator of TGFβ-promoted myofibroblast viability during corneal wound healing. The working hypothesis is that as the EBM is fully regenerated after fibrosis-generating epithelial-stromal injury, TGFβ1 and TGFβ2 levels at TGF beta-dependent myofibroblasts drop to the point that IL-1α, produced by the myofibroblasts themselves and/or surrounding corneal fibroblasts, triggers myofibroblast apoptosis.

**Opposing IL-1- and TGFβ-modulation of Expression of EBM Components**

IL-1α and TGFβ also have opposing effects on the expression of some basement membrane components by stromal cells that participate in regeneration of the EBM.
**FIGURE 3. Immunohistochemistry for TGFβ1 and TGFβ2 in rabbit corneas.**

(A) TGFβ1 is present in the unwounded corneal epithelium (e), but few cells in the stroma (s) express the growth factor. (B) In the unwounded cornea, TGFβ2 is not detected in epithelial cells, but there are deposits of TGFβ2 adherent to the epithelial surface (arrowheads). A rare cell in the stroma (s) expressed TGFβ2 (arrows). (C) The normal unwounded corneal endothelium (arrowheads) expresses TGFβ1, as do a few posterior stromal cells (arrows). (D) Immediately following -9 D PRK in rabbits, TGFβ2 is detected on the stromal surface (arrows), likely deposited from the tears. Note no TGFβ2 is detected in corneal endothelial cells (arrowheads). (E) At 2 days after -9 D PRK, TGFβ1 protein is detected in the healing epithelium (e) and in cells (arrows) in the stroma (s) that could include keratocytes, corneal fibroblasts, and bone marrow-derived immune cells. The TGFβ1 associated with the monolayer of healing corneal epithelial cells is likely derived from the epithelial cells themselves and the tears. (F) At 2 days after -9 D PRK, TGFβ2 is detected in the healing epithelium (e) and in cells (arrows) in the stroma (s) that could include keratocytes, corneal fibroblasts, and bone marrow-derived immune cells. (G) At 3 weeks after -4.5 D PRK in the rabbit cornea, epithelial TGFβ1 is present at higher levels than in the unwounded cornea, but is confined to the superficial epithelium (arrowheads). A line of vimentin+ cells (arrows) is present in the subepithelial stroma, but few of these cells have developed into SMA+ myofibroblasts. (H) Conversely, at 3 weeks after higher injury -9 D PRK in the rabbit cornea, epithelial TGFβ1 is present throughout the hyperplastic corneal epithelium, albeit at higher concentration in the superficial epithelium. A cluster of SMA+ vimentin+ myofibroblasts (arrows) is present in the subepithelial stroma. Some of these myofibroblasts or nearby cells are TGFβ1+. The asterisk (*) indicates artifactual separation of the epithelium from the stroma that occurs during tissue sectioning due to a defective EBM at this point after -9 D PRK. Magnification 200 times, except D is 100 times. Blue is DAPI in all panels. See reference #9 for other views and time points.

IL-1β upregulate perlecan mRNA expression in keratocytes, but not in corneal fibroblasts or myofibroblasts. Perlecan is a major component in the EBM that modulates TGFβ1, TGFβ2, PDGF AA, and PDGF BB penetration into the stroma and defective assembly of perlecan into the regenerating EBM has a role in the development of myofibroblasts associated with stromal fibrosis. Perlecan protein expression was found to be increased in anterior stromal cells at one and two days after -9 diopters (D) PRK, but that subepithelial localization of perlecan was disrupted at 7 days and later time points after injury when myofibroblasts populated the anterior stroma in corneas that developed fibrosis.

TGFβ1 (or TGFβ3) also markedly downregulates nidogen-1 or nidogen-2 mRNA expression in keratocytes. Interestingly, in a PRK corneal fibrosis model in rabbits, perlecan protein expression was found to be increased in anterior stromal cells at one and two days after -9 diopters (D) PRK, but that subepithelial localization of perlecan was disrupted at 7 days and later time points after injury when myofibroblasts populated the anterior stroma in corneas that developed fibrosis.
Thus, IL-1 likely promotes regeneration of the EBM after injury by upregulation of critical EBM components in keratocytes that survive the initial wave of apoptosis after corneal epithelial injury, or corneal fibroblasts that repopulate the subepithelial stroma. Conversely, TGF-β1 (or TGF-β3) may tend to impede EBM repair by downregulating production of components needed for EBM repair that are produced by keratocytes and corneal fibroblasts.

### IL-1α and TGFβ Modulation of the Chemotaxis and Development of Fibrocytes After Epithelial-Stromal Injury

#### IL-1 Modulation of Cytokine Networking by Keratocytes/Corneal Fibroblasts After Injury.

Within an hour after injury to the corneal epithelium or endothelium, large numbers of immune cells migrate into the corneal stroma from the limbal blood vessels. These cells are attracted into the stroma by IL-1 and tumor necrosis factor alpha (TNF-α) released by the injured epithelium and/or endothelium via both direct chemotaxis and a cascade effect known as cytokine networking. Thus, IL-1α, IL-1β, and TNFα bind to receptors on keratocytes that survive the wave of apoptosis in the anterior stroma—likely due to lower concentrations of IL-1 and TNFα that penetrated into the deeper stroma—and stimulates these keratocytes to produce chemokines such as CCL2 (monocyte chemoattractant protein-1 [MCP-1]; Fig. 4), granulocyte colony-stimulating factor (G-CSF), CXCL5 (also called neutrophil-activating peptide or ENA-78), and monocyte-derived neutrophil chemotactic factor (MDNCF). These chemokines, and other cytokines like IL-7, IL-9, and IL-17 upregulated in keratocytes and corneal fibroblasts, amplify the chemotactic effects on bone marrow-derived cells and draw lymphocytes, neutrophils, macrophages, fibrocytes, and other blood-derived cells into the corneal stroma to deal with the injury or infection in the cornea via this cytokine networking process.

Fibrocytes are especially important bone marrow-derived cells in the fibrotic responses to severe epithelial-stromal injuries in the cornea due to their being one of the precursors to myofibroblasts. Studies in other organs have shown that when CCL2 binds functional CCR2 receptors on mouse or human fibrocytes, there is not only chemotaxis triggered by a gradient of CCL2 in the tissue, but also induction of fibrocyte proliferation and differentiation towards myofibroblasts. Conversely, fibroblasts do not express CCR2 receptors and do not respond to CCL2.

#### TGFβ modulation of Corneal Fibroblast and Fibrocyte Development Into Myofibroblasts.

Once the fibrocytes arrive in the healing tissue, they are dependent on one or more TGFβ isoforms for survival and development into mature SMA-positive myofibroblasts, similar to corneal fibroblasts. Thus, TGFβ1 and TGFβ2, in conjunction with PDGF, drive the development of myofibroblasts from keratocytes, and their progeny corneal fibroblasts, both in vitro and in situ. Similarly, TGFβ1 and TGFβ2 drive the development of fibrocytes into mature SMA-positive myofibroblasts. Recent studies, however, have shown that corneal fibroblast-derived and fibrocyte-derived myofibroblasts are not equivalent cells, but likely cooperate in the generation of tissue fibrosis.

Myofibroblasts are rarely seen in unwounded corneas. Interestingly, if TGFβ is overexpressed at high levels in the lens in vivo, myofibroblasts and fibrosis are generated in the overlying cornea. TGFβ1 is expressed in the normal unwounded corneal epithelium (see Fig. 3A) and endothelium (see Fig. 3C), and is present in the tears and aqueous humor. TGFβ2 is also present in tears and at least at low levels in aqueous humor. Necessarily, there are systems in place to regulate TGFβ1 and TGFβ2 entry into the stroma in uninjured corneas to preclude needless keratocyte development into fibrosis-producing myofibroblasts. Studies demonstrated an apical epithelial barrier and epithelial basement membrane regulate TGFβ1 and TGFβ2, and likely PDGF, entry into the stroma at sufficiently high levels to...
drive myofibroblast development from both keratocyte and fibrocyte precursors.

From immunocytochemistry studies of localization of TGFβ1 and TGFβ2 proteins after PRK injury in rabbits, it's apparent an apical epithelial barrier modulates penetration of tear TGFβ1 and TGFβ2 into the full-thickness epithelium. This occurs possibly at the level of epithelial tight junctions. Thus, at 3 weeks after lower injury -4.5 D PRK in rabbits (see Fig. 3G), TGFβ1 localization is re-established in the superficial epithelium and few myofibroblasts typically develop. Conversely, at 3 weeks after high injury -9 D PRK (see Fig. 3H), there is full-thickness epithelial penetration of large amounts of TGFβ1, and large numbers of SMA-positive myofibroblasts develop in the anterior stroma. The specific components that contribute to the apical epithelial barrier that modulates TGFβ1 and TGFβ2 epithelial penetration need further investigation.

Some stromal cells—both SMA-positive myofibroblasts and SMA-negative cells—produced TGFβ1 and TGFβ2, but the amount of this stromal production is highly variable between different corneas in rabbits. The stromal SMA-negative cells producing TGFβ1 and TGFβ2 likely include keratocyte-derived corneal fibroblasts and bone marrow-derived cells, such as fibrocytes, but further work is needed to characterize these cells.

More is known about the EBM and Descemet's basement membrane components that bind or block trans-EBM movement of TGFβ1 and TGFβ2. These include collagen type IV and perlecan. Perlecan is a major component in both of these corneal basement membranes and it produces a high negative charge due to three heparan sulfate side chains. Thus, perlecan also provides a nonspecific barrier to TGFβ penetration through either EBM or Descemet's basement membrane. Therefore, for sufficiently high and prolonged levels of TGFβ1 and TGFβ2 needed to drive myofibroblast development from precursor cells to be available in the stroma, corneal injuries must include the EBM and/or Descemet's basement membrane.

TGFβ expression and localization patterns in corneal cells are very different in unwounded compared to wounded corneas (see Fig. 3), and also at different time points after wounding—depending on the status of EBM and Descemet's membrane. Injuries or infections. As an example of this regulation, IL-1α and IL-1β are major up-regulators of HGF and KGF mRNA and protein production by keratocytes and corneal fibroblasts. Thus, injury to the epithelium triggers the release of IL-1, that then up-regulates HGF and KGF production, that function to promote the healing of the injured epithelium.

However, HGF has also been shown to have an anti-fibrotic role, possibly in promoting myofibroblast apoptosis when TGFβ levels in the surrounding tissue drop to levels incompatible with survival. Thus, whereas IL-1 can directly promote myofibroblast apoptosis via autocrine or paracrine effects, it may also have an indirect effect in promoting HGF production by surrounding corneal fibroblasts and keratocytes that then drives myofibroblast apoptosis.

Opposing Effects of IL-1 and TGFβ on Expression of Metalloproteinases and Collagenases by Corneal Fibroblasts

Metalloproteinases and collagenases have a critical role in the stromal wound healing response because they are involved in the degradation of normal matrix during the early response to injury or infection, as well as maintenance and removal of the disordered extracellular matrix that is deposited in the stroma after injury, infections, or surgeries. IL-1 (or tumor necrosis factor alpha) upregulates the expression of metalloproteinases and collagenases by corneal stromal cells, and also regulates them in corneal epithelial cells. This upregulation of metalloproteinases and collagenases must be tightly regulated or severe damage to the corneal stroma could be produced by even trivial injuries or infections. As an example of this regulation, IL-1 receptor antagonist produced by corneal epithelial cells downregulates MMP-2 produced by corneal fibroblasts. A decrease in IL-1α and IL-1β released by the regenerated epithelium is likely another major regulator of the release of collagenases and matrix metalloproteinases by stromal cells, such as corneal fibroblasts and myofibroblasts.
However, TGFβ may have opposing effects on the modulation of collagenases or metalloproteinases by corneal fibroblasts. West-Mays and coworkers\textsuperscript{145} showed that TGFβ1 inhibited collagenase production by rabbit corneal fibroblasts. TGFβ1 was also found to downregulate MMP-3 (stromelysin) expression in rat fibroblasts.\textsuperscript{146} In another in vitro study,\textsuperscript{147} this group found that TGFβ2 inhibited collagenase synthesis by rabbit corneal stromal cells. Finally, in yet another study by this group,\textsuperscript{148} the effects of IL-1α, IL-1β, or TGFβ1 on collagenase, stromelysin, and gelatinase were investigated in cultures of rabbit corneal fibroblasts. They found that recombinant human IL-1α or IL-1β increased collagenase, stromelysin, and gelatinase (both 92-kD and 72-kD). Conversely, they found that expression of collagenase and stromelysin were repressed, whereas expression of 72-kD gelatinase was increased, by treatment of corneal fibroblasts with recombinant human TGFβ1.

These studies suggest that in vivo IL-1 and TGFβ are likely to have opposing effects on the expression of key collagens and metalloproteinases that are active in matrix degradation and regeneration after both nonfibrotic and fibrotic corneal injuries where both corneal fibroblasts and myofibroblasts produce disordered ECM.

**Cross-Regulation Between the IL-1 and TGF Beta Systems**

Many studies have shown that TGFβ1, TGFβ2, and TGFβ3 are the primary modulators of corneal scarring fibrosis in vitro and in situ in every organ studied.\textsuperscript{149–153} This is the case for the cornea, too. For example, Gupta and coworkers\textsuperscript{154} showed that targeted delivery of Smad7, the major intracellular negative regulator of TGFβ signaling, to the corneal stroma decreased stromal scarring after fibrosis-inducing injury. These investigators also showed that delivery of soluble TGFβ receptor II could attenuate TGFβ1-induced MFB development from corneal fibroblasts in vitro\textsuperscript{155} and that gene transfer of decorin, a natural proteoglycan inhibitor of TGFβ, inhibited the development of human corneal fibroblasts into myofibroblasts in vitro.\textsuperscript{156} Yang et al.\textsuperscript{157} showed that TGFβ-induced myofibroblast development is highly dependent on a positive feedback loop in which p-SMAD2-induced reactive oxygen species (ROS) activates transient receptor potential vanilloid 1 (TRPV1) channel, TRPV1 causes activation of p38, the latter in turn further enhances the activation of SMAD2 to establish a recurrent loop that greatly extends the residency of the activated state of SMAD2 that drives myofibroblast development. Studies have also shown there are concentration-dependent effects of TGFβ1 on corneal wound healing.\textsuperscript{158} In addition, blockade of TGFβ receptor II markedly reduces the fibrotic response to corneal injury in mice in situ\textsuperscript{155} and TGFβ1 directly modulates the development of corneal fibroblasts into myofibroblasts in vitro.\textsuperscript{154}

As was detailed earlier, TGFβ1, TGFβ2, and TGFβ3 signal through the same type I and type II receptors, and the same downstream signaling pathways, and yet the knockout phenotype of each isoform is different.\textsuperscript{159} The genes expressing TGFβ1, -β2, and -β3, and have differing promoters that differentially regulate the expression of these genes in tissues during development, homeostasis, and the response to injury.\textsuperscript{159,161} TGFβ1 and TGFβ2 have differing expression patterns and likely different but overlapping roles during wound healing in the cornea compared to TGFβ3.\textsuperscript{156,162,163} TGFβ1 and TGFβ2 have profibrotic effects that include promotion of myofibroblast development from keratocyte and fibrocyte precursor cells and, conversely, in at least in some systems, TGFβ3 tends to have antifibrotic effects in adult animals.\textsuperscript{162–164}

Supporting this role for TGFβ3, Karamichos and coworkers\textsuperscript{158} showed that the addition of TGFβ1 or TGFβ2 to human corneal fibroblasts cultured in a 3-dimensional construct stimulated the formation of a fibrotic matrix compared to control cultures, whereas the addition of TGFβ3 resulted in the production of a nonfibrotic matrix. This group also showed that PDGF receptor a was a key modulator of the differential effect of TGFβ1 (increases alpha-smooth muscle actin expression) compared to TGFβ3 (decreases alpha-smooth muscle actin expression) in human corneal fibroblasts—effects that would promote versus inhibit myofibroblast generation, respectively.\textsuperscript{158} They also showed that fibriellar collagen secreted by human corneal fibroblasts in the absence of TGFβ3 showed uniform parallel alignment in cultures.\textsuperscript{166} However, in the presence of TGFβ3, the collagen bundles made by the corneal fibroblasts had orthogonal layers indicative of the formation of lamellae in corneas. Finally, in cross-section projections, without TGFβ3, the corneal fibroblasts were flattened and largely localized on the Transwell membrane at the bottom of each well. Conversely, with TGFβ3, corneal fibroblasts were multilayered—as they are in corneas in situ. Construct thickness and collagen organization was also enhanced by TGFβ3.

In other very informative experiments,\textsuperscript{167} Zieske and colleagues also showed that TGFβ1 and TGFβ3 had similar early effects on the expression of fibrosis-related genes in human corneal fibroblasts. With longer exposure of 3 day’s duration to each TGFβ isoform, however, there was differential expression of fibrosis-related genes in the human corneal fibroblasts—especially for genes that were involved in the modulation of ECM. For example, Smad7 (antagonist of signaling by TGF-type 1 receptor superfamilies) member protein expression was significantly decreased by TGFβ1 but TGFβ3 had no significant effect on Smad7 protein expression. Thrombospondin-1 protein production in human corneal fibroblasts was significantly increased by TGFβ3 (2.5-fold higher than controls), whereas TGFβ1 had no significant effect on thrombospondin-1 protein expression in corneal fibroblasts. Collagen type I protein production was significantly increased and Smad3 (a TGF-beta receptor cytoplasmic protein that is responsible for downstream cellular signaling of the TGF-beta receptors) was dramatically decreased by both TGFβ1 and TGFβ3. Of the 84 fibrosis-related genes analyzed in corneal fibroblasts in this study, however, after 3 days of exposure to TGFβ1 or TGFβ3, only 5 of the 84 genes were upregulated by TGFβ3 compared to TGFβ1—MMP1, plasminogen activator urokinase, integrin alpha-1, thrombospondin-1, and IL-1α (which was 2.7 times the fold upregulated by TGFβ3 compared to TGFβ1). Both TGFβ1 and TGFβ3 upregulated IL-1α after 4 hours of exposure compared to controls (2.6 times and 2.0 times, respectively), but were not significantly different from each other. No effect of TGFβ1 or TGFβ3 on IL-1α expression was seen at 3 days. These results show, however, that TGFβ1 and TGFβ3 do modulate the expression of IL-1α and IL-1β. What is perhaps most surprising about this study is how few differences in the expression of fibrotic genes in corneal fibroblasts were found between TGFβ1 and TGFβ3—only 1 difference in the 84 genes and 5 differences
in the 84 genes evaluated at 4 hours and 3 days, respectively. Thus, 83 of 84 genes and 79 of 84 fibrotic genes were similarly regulated by TGFβ1 and TGFβ3 in corneal fibroblasts after 4 hours or 3 days, respectively. In the author's thinking, the great similarity in modulation by TGFβ1 and TGFβ3 calls into question the rather simple labels of "profibrotic" versus antifibrotic" for these two TGFβ isoforms. This is especially the case if one looks at specific fibrotic genes that were similarly regulated by the two isoforms in this study. For example, the most profoundly downregulated gene in corneal fibroblasts by both TGFβ1 and TGFβ3 after 3 days was HGF (-73.5 times and -56 times downregulated, respectively). If TGFβ3 were truly antifibrotic, perhaps upregulation of HGF by this isoform would have been expected given recent studies showing HGF has an anti-fibrotic effect on myofibroblast viability.\textsuperscript{141–145} Perhaps this greater similarity than difference in the modulation of fibrotic genes by TGFβ1 and TGFβ3 provides one explanation for why TGFβ3 failed to modulate scarring in skin in phase III clinical trials that included 350 adult patients, if skin fibroblasts have similar responses to the TGFβ isoforms as corneal fibroblasts.\textsuperscript{168} Thus, TGFβ3 appears to have fibro-modulatory differences from TGFβ1, but to not be truly "antifibrotic," which was always difficult to explain based on the isoforms signaling via the same receptors and sharing so many similarities in the signal transduction pathways that are activated.\textsuperscript{56–75}

In other organs, IL-1 isoforms have been shown to differentially modulate the expression of TGFβ isoforms. Thus, in human articular chondrocytes \textit{in vitro}, IL-1β downregulated TGFβ1 mRNA expression but upregulated TGFβ3 isoform mRNA expression.\textsuperscript{169} Similarly, IL-1β selectively induced TGFβ3 protein synthesis but reduced synthesis of the TGFβ1 and TGFβ2 proteins in articular chondrocytes. Li and Tseng\textsuperscript{170} found IL-1β did not affect TGFβ1 expression in human corneal or limbal fibroblasts. Otherwise, there has been little investigation of potential effects of the IL-1 isoforms on the expression of TGFβ isoforms, or their receptors, in corneal stromal cells, but such studies could be revealing based on the cross-talk between these two systems in other organs.

**Summation**

Thus, in some respects, IL-1α and IL-1β have complementary roles to TGFβ1 and TGFβ2 in promoting the development of myofibroblasts after epithelial-stromal injuries that produce fibrosis. However, other functions of IL-1α and IL-1β seemingly oppose TGFβ1 and TGFβ2 effects in promoting fibrosis.

**Corneal Cellular Functions Regulated by IL-1 Without Known TGFβ Involvement**

**The Keratocyte Apoptosis Response to Epithelial or Endothelial Corneal Injury Via Modulation of the Fas-Fas Ligand System**

Immediately after injury to the epithelium\textsuperscript{5} or endothelium\textsuperscript{7} underlying or overlying, respectively, keratocytes undergo apoptosis (Fig. 5). Studies have shown that this programmed cell death is likely mediated via activation of the Fas-Fas ligand system—with high concentrations of epithelial and/or endothelial IL-1 penetrating into the adjacent stroma, binding IL-1 receptors on keratocytes, and stimulating production of autocrine Fas ligand.\textsuperscript{55,171} Because keratocytes constitutively produce the receptor Fas,\textsuperscript{35,171} this rapid increase in Fas ligand stimulates the cells to undergo "autocrine suicide" apoptosis. This process initiates the stromal wound healing response after either epithelial and/or endothelial injury. Presumably, this keratocyte apoptosis is IL-1 concentration dependent, since it extends only 30 to 50 μm depth into the stroma from either the epithelium and/or endothelium that is injured (see Fig. 5).

As of yet, there have been no reports of TGFβ influencing the keratocyte apoptosis response to epithelial injury.

**Conclusions**

The specific effects of the IL-1 and TGFβ isoforms on corneal stromal cells—including keratocytes, corneal fibroblasts, fibrocytes, and myofibroblasts—likely depends on the cells’ overall milieu of growth factors, cytokines (including IL-1α and IL-1β), integrins, and other modulators, as well as the status of the associated ECM, including adjacent corneal basement membranes, and the expression of the IL-1 and TGFβ receptor family members in the cells.

The IL-1 cytokine-receptor system modulates both early and late events in the corneal responses to injuries, including the early keratocyte apoptosis response and late myofibroblast apoptosis. IL-1 also regulates the expression of HGF and KGF by keratocytes and corneal fibroblasts that control corneal epithelial healing, as well as the expression of collagenases and metalloproteinases needed for breakdown and remodeling of stromal matrix. IL-1, also a controller of cytokine networking whereby corneal...
fibroblasts produce chemokines that modulate the influx of bone marrow-derived cells, including fibrocytes, fights infectious agents and contributes to the development of myofibroblasts.

The TGFβ growth factor-receptor system is equally important in modulating the development of keratocyte-derived corneal fibroblasts and bone marrow-derived fibrocytes into myofibroblasts. TGFβ/s also maintain mature myofibroblasts once they develop, and removal of a requisite source of the TGFβ/s leads to myofibroblast apoptosis.

In some functions, such as myofibroblast viability, basement membrane component production, and collagenase/metalloproteinase expression, the two systems oppose each other to finely tune the overall corneal healing response. In other functions, such as promoting the early development of myofibroblasts after epithelial-stromal injuries, the two systems work hand in hand to promote the development of corneal fibrosis. Thus, the two systems function in coordination as “co-master regulators” of the overall wound healing response to a particular corneal injury or infection. There remains a great deal of work to be done to better understand the cross-talk that likely occurs between the IL-1 and TGFβ systems in corneal homeostasis, wound healing, and fibrosis.

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TGFβ and IL-1 Systems in the Cornea

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TGFβ and IL-1 Systems in the Cornea

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