Thymosin-β4 Modulates Corneal Matrix Metalloproteinase Levels and Polymorphonuclear Cell Infiltration after Alkali Injury

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PURPOSE. Corneal alkali injury is highly caustic, and present clinical therapies are limited. The purpose of this study was to investigate the ability of thymosin-β4 (Tβ4) to promote healing in an alkali injury model and the mechanisms involved in that process.

METHODS. Corneas of BALB/c mice were injured with NaOH, irrigated copiously with PBS, and treated topically with either Tβ4 or PBS twice daily. At various time points after injury (PI), corneas from the Tβ4-treated group were examined for polymorphonuclear leukocyte (PMN) infiltration, chemokine, and matrix metalloproteinase (MMP)/tissue inhibitor of metalloproteinase (TIMP) expression.

RESULTS. Tβ4-treated corneas demonstrated improved corneal clarity at day 7 PI. Whereas Tβ4 decreased corneal MMP-2 and -9 and MT6-MMP levels after alkali injury, no change in TIMP-1 and -2 expression was detected. Tβ4 treatment also decreased corneal KC (CXCL1) and macrophage inflammatory protein (MIP)-2 chemokine expression and PMN infiltration. Immunohistochemistry studies demonstrated MMP-9 expression at the leading edge of the epithelial wound, in the the limbus (containing stem cells), and in stromal PMNs.

CONCLUSIONS. Tβ4 treatment decreases corneal inflammation and modulates the MMP/TIMP balance and thereby promotes corneal wound repair and clarity after alkali injury. These results suggest that Tβ4 may be useful clinically to treat severe inflammation-mediated corneal injuries. (Invest Ophthalmol Vis Sci. 2005;46:2388–2395) DOI:10.1167/iovs.04-1368

Alkali injuries of the eye often cause extensive damage to the cornea and anterior segment, resulting in permanent visual impairment. Multiple facets of ocular alkali injury interfere with the proper healing process and result in formation of scar tissue, recurrent corneal erosions, and nonhealing defects.1 A major component that influences visual outcome after chemical insult is the severity of the host inflammatory response. Although most of the ensuing ocular complications stem from the massive infiltration of polymorphonuclear leucocytes (PMNs) into the stroma, the precise mechanisms of corneal damage and wound healing in this injury are not completely understood. The acute inflammation is characterized by a rapid infiltration of PMNs into the cornea that is followed by a chronic inflammatory phase. The extended inflammatory cell migration into the cornea results in the release of proteolytic enzymes into the extracellular matrix (ECM), thereby damaging the normal cornea cytoarchitecture.3

Matrix metalloproteinases (MMPs) are a family of zinc and calcium-dependent enzymes that are capable of degrading components of the ECM.3,5 Tissue remodeling and the extent of proteolysis are dependent on the activation state of the MMPs and the balance between active MMPs and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).4 After wounding, failure of the cornea to re-epithelialize correlates with increased levels of MMPs.7 In addition, there are several reports showing that treatment of alkali-injured corneas with synthetic MMP inhibitors significantly improves basement membrane integrity.8–10 These data support the notion that expression of corneal MMPs impedes wound repair after corneal injury. Therapeutic strategies that modulate corneal MMP expression may prevent and/or arrest stromal ulceration that often follows inflammation-mediated damage.

An MMP that has been shown to play a role in inflammation is MMP-9 (gelatinase B). It is synthesized by the resident corneal cells, and reduction of its synthesis correlates with inhibition of corneal basement membrane dissolution.1 In human corneas with non-alkali-burn repair defects, MMP-9 is synthesized by cells in the basal layer of the epithelium directly adjacent to the basement membrane, suggesting that it may participate in dissolution of this structure.7 Zhang et al.11 suggested a correlation between MMP-9 expression after corneal alkali injury with the wound-healing response. However, little is known about the regulation of MMP-9 expression after alkali injury.

In addition to MMP-9, we also examined expression of MMP-2 and leukolysin because of their presumptive roles in inflammation and wound healing. Expression of MMP-2 after wounding is associated with extracellular matrix remodeling and epithelial cell migration.12 Leukolysin/MT6-MMP (MMP-25) is a GPI-anchored matrix MMP primarily expressed by neutrophils. The proteolytic targets for leukolysin at the inflammatory sites and its role in wound healing remain unknown, although PMNs may mediate tissue destruction by deploying leukolysin at inflammatory sites.13

Thymosin-β4 (Tβ4) is a small 43-amino-acid, 4.9-kDa protein originally isolated from bovine thymus that until recently was thought to function primarily as a G-actin sequestering protein.14–17 Tβ4 is a ubiquitous polypeptide, highly conserved across species, and is found in serum and a variety of tissues and cell types; yet, no receptors for the protein have been identified.18–20 Tβ4 levels are highest in platelets and PMNs, which are among the first formed elements and cells, respectively, to enter a wound and release their factors, some of which recruit additional cells to the wound site.21 Although

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the mechanism(s) of action of exogenous Tβ4 on wound repair remain unclear, high levels of Tβ4 present in human wound fluid (15 μg/mL) suggest its importance in wound healing.22 Previously, we reported that Tβ4 promotes corneal wound healing and decreases inflammation after alkali injury.23 Here, we extended these findings to the BALB/c inbred strain of mouse and focused on the effects of Tβ4 treatment on PMN infiltration and MMP expression after corneal alkali injury.

MATERIALS AND METHODS

Alkali Injury and Ocular Response after Wounding

Six- to 8-week-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were humanely treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were anesthetized with ether (Fisher Scientific, Fairlawn, NJ) and a 2-mm disc of filter paper (size 50: Whatman, Clifton, NJ) soaked in 1 N NaOH was applied for 30 seconds to the central cornea. The eyes were irrigated copiously with PBS and then treated topically with either Tβ4 (5 μg/5 μL phosphate-buffered saline [PBS]; RegeneRx, Inc., Bethesda, MD) or a similar volume of PBS twice daily for up to 7 days. At day 7 after injury (PI), animals (n = 10/group) treated with PBS or Tβ4 were killed, and the ocular anterior segments were evaluated and photographed at the slit lamp level to illustrate the disease response at this time point.

Histopathology

At days 1, 3, and 7 PI, mice (n = 4 per group per time point) that were treated with PBS or Tβ4 were killed, and their eyes were enucleated. Eyes were fixed and prepared for histopathology, as described previously.24 Briefly, the eyes were fixed in a solution of 1% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M Sorensen’s phosphate buffer (pH 7.4; 1:1:1) at 4°C for a total of 1.5 hours. Eyes were then transferred into fresh fixative for an additional 1.5 hours, dehydrated in graded ethanol, and embedded in Epon-araldite. Richardson-stained sections25 from PBS- and Tβ4-treated eyes were examined, and representative 10-μm sections from the central (through the visual axis) and peripheral cornea were photographed with a microscope (Axioskop; Carl Zeiss Meditec, Inc., Thornwood, NY).

Semiquantitative RTPCR

Individual corneas (excluding the limbus) from PBS- and Tβ4-treated mice (n = 5/group/time point) were stored in RNAlater (Ambion, Inc., Austin, TX) at −20°C until processing. Corneas from unwounded animals were collected and processed for baseline reference. Each cornea was then ground manually with a pestle in 1 mL extraction reagent (TRizol; Invitrogen Corp., Carlsbad, CA), and total RNA was isolated according to the manufacturer’s protocol. All reverse transcription (RT) reagents were purchased from Invitrogen. Briefly, RT was performed in 0.5 mL sterile tubes using equal volumes of RNA and reverse transcriptase (SuperScript III; Invitrogen-Gibco) of choice in a buffer containing 20 μg/mL hexadecyltrimethylammonium bromide (HTAB; Sigma-Aldrich, St. Louis, MO). Samples were freeze-thawed three times and after centrifugation, a 0.1-mL aliquot of the supernatant was added to 2.9 mL of 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HTAB; Sigma-Aldrich, St. Louis, MO). Samples were then incubated at 20°C for 1 hour. Supernatants were aliquoted into sterile tubes and frozen at −80°C. Zymograms were run as previously described.26 Purified mouse pro-MMP-9 was used as the control. Triplicate ELISA analyses for murine pro-MMP-9 and the murine IL-8-like chemokine, KC (R&D Systems, Inc., Minneapolis, MN) were performed with fresh aliquots of the individual corneal lysates, according to the manufacturer’s protocol. The average concentrations of each concentration point were calculated. Statistical analysis was performed with the unpaired Student’s t-test with significance set at P < 0.05.

Myeloperoxidase Assays

A myeloperoxidase (MPO) assay was used to quantitate the number of PMNs in the PBS- and Tβ4-treated corneas, as previously described, where MPO activity is related to neutrophil (PMN) concentration.27,28 Briefly, corneas were excised (n = 3 per group per time point) at days 1, 3, and 7 PI and homogenized with glass tissue grinders in 1.0 mL of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB; Sigma-Aldrich, St. Louis, MO). Samples were freeze-thawed three times and after centrifugation, a 0.1-mL aliquot of the supernatant was added to 2.9 mL of 50 mM phosphate buffer containing o-dianisidine dihydrochloride (16.7 mg/100 mL) and hydrogen peroxide (0.0005%; Sigma-Aldrich). The absorbance at 450 nm was monitored for 5 minutes with a spectrophotometer (Genesys 2; Spectronics, Rochester, NY). The slope of the line was determined for each sample and used to calculate units of MPO/cornea. One unit of MPO activity is equivalent to 2 × 10⁵ PMN/mL.29

RESULTS

Tβ4 and Corneal Healing

Eyes from BALB/c mice were evaluated by slit lamp on day 7 PI with 1 N NaOH and treatment with either PBS or Tβ4 (n = 10 per group/time point). Of the 10 mice treated with PBS, 7 had readily apparent hyphema and total corneal opacification. In contrast, 2 of 10 Tβ4-treated eyes had hyphema and possessed the same degree of corneal opacity as the PBS-treated eyes. Figure 1 is a representative slit lamp photograph at day 7 PI demonstrating dense corneal scarring, causing the absence of the red reflex that is readily apparent in the Tβ4-treated eye. These slit lamp observations suggest that Tβ4 treatment improves corneal healing and clarity in this alkali injury model.

Histopathological Analysis of Corneas after Alkali Injury

At days 1, 3, and 7 PI, eyes treated with either PBS or Tβ4 (n = 5 per group/time point) were examined histologically to evaluate corneal integrity and inflammation (Fig. 2). Sectioning was performed by a masked observer (RPB). Figures 2A and 2B illustrate the corneal epithelial defects present in both the PBS- and Tβ4-treated eyes and the presence of early inflammatory infiltrate. By day 3 (Figs. 2C, 2D), some re-epithelialization was then 35 cycles of 94°C for 30 seconds, annealing temperature for 15 seconds, 65°C for 30 seconds, and a final extension at 65°C for 10 minutes. All PCRs were optimized and subsequently amplified on a gradient cycler/DNA engine (PTC-200; MJResearch, Inc., Incline Village, NV). PCR products were visualized by electrophoresis on 2% agarose-1000 gels (Invitrogen), stained with 0.5 μg/mL ethidium bromide, and photographed under UV light. Integrated density values (IDVs) of the β-actin and/or GAPDH standards were used to calculate the corrected IDVs of the genes analyzed.
present in both groups of eyes, and a more marked keratitis was evidenced by the presence of PMNs in each cornea. By day 7 (Figs. 2E, 2F), the inflammatory infiltrate of PMNs was significantly reduced in the Tβ4-treated eyes. The markedly improved corneal appearance in the Tβ4-treated eyes is clearly evident (Fig. 2F), suggesting that Tβ4 may be affecting corneal PMN infiltration in the alkali burn model, thereby promoting superior healing and corneal clarity.

**Corneal PMN Infiltration after Tβ4 Treatment**

Based on the histologic appearance of the corneas, we next quantitatively assessed the effects of Tβ4 on corneal PMN infiltration after alkali injury by using an established MPO assay28 on the excised corneas from PBS- and Tβ4-treated eyes at days 1, 3, and 7 PI (n = 3 per group per time point). Although no significant quantitative differences in PMNs were detected at days 1 and 3 PI (data not shown), at day 7 PI, there was a threefold higher level of MPO activity (P = 0.0002) detected in corneas from PBS- versus Tβ4-treated mice (Fig. 3). The higher PMN MPO activity in the PBS-treated eyes was consistent with the histopathological sections and the slit lamp findings that Tβ4 treatment decreases anterior chamber and corneal PMN infiltration after alkali injury.

**Corneal Chemokine Levels after Tβ4 Treatment**

We next examined the levels of corneal gene transcript and protein levels for two key murine PMN chemokines: MIP-2 and KC. Figures 4A and 4B depict graphically and by RT-PCR, respectively, the almost 10-fold increase in MIP-2 gene transcript levels at day 1 after injury in PBS- versus Tβ4-treated corneas (n = 5). By day 3 PI, corneal MIP-2 gene expression had decreased in both the PBS- and Tβ4-treated corneas and did not differ between the two groups at both days 3 and 7 PI. MIP-2 mRNA was undetectable in the unwounded corneas.

KC expression levels were similar between the PBS- and Tβ4-treated corneas at day 1 PI. By day 3 PI, and similarly at day 7, Tβ4-treated corneas (n = 5 per group per time point) demonstrated approximately a twofold decrease in KC gene transcript levels (P = 0.0162 and P = 0.0083, respectively; Fig. 5A) which approached the levels detected in the unwounded corneas. Similarly, KC protein levels (Fig. 5B) were markedly reduced in the Tβ4-treated corneas (n = 5 per group per time point) at days 3 (P < 0.0001) and 7 (P = 0.0002) PI. These results strongly suggest that topical Tβ4 treatment after alkali injury downregulates the expression of the potent PMN chemotactants MIP-2 and KC in the cornea. In turn, the decreased corneal chemokine expression may be responsible for the observed decreased PMN infiltration.
Corneal MMP Levels after Tβ4 Treatment

Alterations in MMP-9 expression have been linked to improper corneal wound healing and the formation of persistent epithelial defects and chronic corneal ulcers. Therefore, we hypothesized that Tβ4’s ability to promote corneal wound healing and to modulate inflammation after alkali injury may also involve MMP regulation. To test this hypothesis, we studied the expression of MMP-9, MMP-2, MT6-MMP (leukolysin), and their inhibitors TIMP-1 and TIMP-2 in PBS- and Tβ4-treated corneas after alkali injury (Figs. 6, 7).

Although at day 1 PI, there was no significant difference in MMP-9 gene transcription between the two groups, at days 3 and 7 PI, Tβ4 treatment decreased MMP-9 gene expression twofold (*P = 0.0125 and **P < 0.0001, respectively; Fig. 6A).

ELISA analysis of corneal pro-MMP-9 levels showed no statistically significant differences between PBS- and Tβ4-treated corneas at day 1 PI. However, at day 3 PI, Tβ4 treatment decreased pro-MMP-9 levels more than 10-fold (*P = 0.0028), in agreement with the RT-PCR data. At day 7 PI, Tβ4-treated corneal levels remained over five times lower than in PBS-treated eyes (*P = 0.0001; Fig. 6B).

Corneal MMP-9 levels were also assayed by gelatin zymography (Fig. 6C). In agreement with the ELISA results, MMP-9 levels were markedly lower in the Tβ4, than in the PBS-treated corneas at 3 and 7 days PI. Thus, topical Tβ4 treatment after corneal alkali injury markedly reduced corneal MMP-9 gene and protein expression.

In addition to MMP-9, at day 7 PI, RT-PCR analysis showed that Tβ4-treated corneas had decreased gene transcript levels of MMP-2 and MT6-MMP (Figs. 7A, 7B). ELISA results for MMP-2 in Tβ4-treated corneas showed a trend toward decreased protein levels, but the results were limited by the assay’s detection...
limits. Zymography analysis demonstrated extremely low levels of MMP-2 expression in the PBS-treated corneas at days 3 and 7 PI and no detectable expression in the T\(\beta_4\)-treated eyes at any of the time points assayed (Fig. 7C). TIMP levels did not change in both groups, as determined by RTPCR (data not shown). These results show that T\(\beta_4\) differentially regulates the expression of various MMPs and TIMPs in the cornea and suggest that by altering the MMP/TIMP balance, T\(\beta_4\) may promote corneal repair and matrix remodeling by processes linking inflammation and corneal MMP expression.

**DISCUSSION**

In the present study, topical T\(\beta_4\) treatment promoted corneal clarity in a BALB/c model of alkali injury. These novel findings extend those of our previous studies of topical T\(\beta_4\) treatment, to demonstrate quantitatively that the healing effect of T\(\beta_4\) on the cornea is associated with a decrease in PMN infiltration, downregulation of the chemokines MIP-2 and KC, and a decrease in the expression of three major MMPs: the gelatinases (MMP-2 and -9) and MT6-MMP. The relationship of corneal

**FIGURE 6.** T\(\beta_4\) effects on corneal MMP-9 expression after alkali injury. Gene (A) and biochemical analysis by ELISA (B) and zymography (C) of MMP-9 expression at days 1, 3, and 7 PI. RT-PCR showed that T\(\beta_4\) decreased corneal MMP-9 gene expression at days 3 and 7 PI. The graphic depiction in (A) represents the relative amounts of MMP-9 mRNA measured in alkali-injured corneas (\(n = 5\) per group per time point) treated topically twice daily with either PBS or T\(\beta_4\) (5 \(\mu\)g). Treated corneas express 2.2-fold lower levels of MMP-9 mRNA compared with untreated controls at day 3 PI (\(P = 0.0125\)) and day 7 PI (\(P < 0.0001\)). \(\beta\)-Actin was used as a standard to normalize values. Likewise, T\(\beta_4\)-treated corneas expressed >10-fold (\(P = 0.0028\)) lower levels of pro-MMP-9 protein at day 3 and >5-fold decreased amounts (\(P < 0.0001\)) at day 7 PI. (C) Zymography results. Similar to (B), T\(\beta_4\) treatment markedly decreased corneal MMP-9 enzymatic activity (\(n = 5\) per group per time point) at days 3 and 7 PI. All experiments were repeated at least three times.

**FIGURE 7.** Corneal MMP-2 and leukolysin (MT6 MMP) expression in PBS- and T\(\beta_4\)-treated corneas after alkali injury. Semiquantitative RT-PCR for MMP-2 (A) and leukolysin (B) demonstrated decreased gene transcript levels in T\(\beta_4\)-treated corneas (\(n = 5\) per group per time point) for MMP-2 (\(P < 0.0001\)) and leukolysin (\(P < 0.0001\)) at day 7 PI. (C) Zymography results from corneas indicated low levels of MMP-2 activity in both the PBS- and T\(\beta_4\)-treated corneas; yet, at days 3 and 7 PI, gelatinase activity was higher in the PBS- versus T\(\beta_4\)-treated corneas. Experiments were repeated at least three times.
inflammation (chemokine expression), MMP expression, and wound healing with \( \text{T}_{\beta 4} \) has not been studied previously. Because the mechanisms of action of \( \text{T}_{\beta 4} \) in wound healing are not well defined, our data shed new light on possible pathways that may be modulated during repair. A major component that influences visual outcome and ocular morbidity after chemical burn is the severity of the host inflammatory response. PMN infiltration during corneal ulceration and into injured corneal tissue during wound repair is a well-recognized phenomenon.30,51 Because most of the ensuing ocular complications stem from the massive PMN infiltration of the stroma,2 therapeutic strategies that limit PMN infiltration of the corneal stroma may prevent and ameliorate the ocular morbidity that follows inflammation-mediated damage. In this study, \( \text{T}_{\beta 4} \) treatment significantly inhibited the expression of two murine chemokines, KC and MIP-2, suggesting that \( \text{T}_{\beta 4} \) inhibits PMN infiltration by downregulating these proinflammatory mediators. Regarding the KC RT-PCR and ELISA data, whereas similar expression trends were noted at days 3 and 7 PI, there appeared to be a slight increase in the KC protein levels on day 7 in the \( \text{T}_{\beta 4} \)-treated corneas. Although the graphic representation of the mRNA (Fig. 5A) shows a slight decrease between days 3 and 7 PI, the difference is not statistically significant. It is possible that in the individual corneas assayed, posttranscriptional and posttranslational modifications of proteins may occur, and that may explain the discrepancy in the protein levels for these days (Fig. 5B). After corneal alkali burn, our results show that the expression of KC and MIP-2 may be responsible for triggering PMN influx into the cornea to initiate an intense inflammatory cascade. These molecules are functionally homologous to human IL-8 and exhibit potent PMN chemotactic activity when mediating neutrophil recruitment in response to tissue injury and infection.42–45 We monitored the kinetics of PMN recruitment to the cornea after alkali injury by measuring MPO activity levels, with and without \( \text{T}_{\beta 4} \) treatment. Our data show that \( \text{T}_{\beta 4} \) treatment reduced corneal MPO levels, indicating that \( \text{T}_{\beta 4} \) is a potent inhibitor of PMN infiltration after corneal injury, consistent with reports of \( \text{T}_{\beta 4} \) as an anti-inflammatory agent and previous studies showing that \( \text{T}_{\beta 4} \) inhibits PMN migration in vivo.25,36 However, in vitro studies showed that \( \text{T}_{\beta 4} \) had no effect on PMN migration37 suggesting that the anti-PMN activity of \( \text{T}_{\beta 4} \) in vivo may involve indirect effects on PMNs, possibly by downregulation of chemokine production as shown here. Whether \( \text{T}_{\beta 4} \) exerts its effects on PMNs directly or indirectly is an intriguing question. As the anti-inflammatory properties of \( \text{T}_{\beta 4} \) are beginning to be elucidated, other recent studies have shown the activation-responsive expression of the lymph-specific form of \( \text{T}_{\beta 4} \) may be one mechanism by which dendritic epidermal T cells and possibly other intraepithelial lymphocytes downregulate local inflammation.46 In a separate study, \( \text{T}_{\beta 4} \) lowered circulating levels of inflammatory cytokines and intermediates after lipopolysaccharide (LPS) administration in vivo. In addition, \( \text{T}_{\beta 4} \) levels rapidly disappeared in the blood after LPS administration or during septic shock, suggesting that \( \text{T}_{\beta 4} \) may be involved in early events leading to activation of the inflammatory cascade and ultimately the clinical sequelae of sepsis.47 Thus, the anti-inflammatory effects found with topical \( \text{T}_{\beta 4} \) treatment in the current study may have clinical relevance as a novel therapeutic agent for treating corneal inflammation.

The regulation of chemokine gene expression in vivo may be important, as their expression must be closely controlled to minimize excessive and unnecessary inflammation and tissue damage. Prolonged and dysregulated inflammation has been attributed to chronic wound conditions, such as those in diabetes and to leg ulcers, skin wounds, and ocular injuries.40–45 Although the infiltration of PMNs into injured tissue is known to protect wounds from infection, excessive or dysregulated PMN infiltration has been reported to inhibit the wound-repair process in diabetes44 and to slow corneal epithelial wound healing in vitro.45 In addition to PMN recruitment, chemokines also contribute to the regulation of epithelialization and tissue remodeling and are important modulators of skin wound healing.46 MIP-2 and KC appear to play a role in skin wound healing, as evidenced by CXCR2 (the keratinocyte receptor for IL-8) knockout (−/−) mice, which demonstrate severely retarded re-epithelialization.47 Further, distinct temporal patterns of MIP-2 and KC chemokine gene expression have been observed after surgical and dermal burn injuries.48,49 Indeed, elevated levels of MIP-2 production have been associated with PMN persistence and corneal perforation in bacterial keratitis.50,51 Thus, we propose that \( \text{T}_{\beta 4} \) administration may decrease the corneal inflammatory response in dysregulated inflammatory conditions.

In the burned cornea, PMN infiltration is particularly important, because their influx is an early event and if uncontrolled, their release of various proteases can have a deleterious effect on stromal repair.52 The activities of proteases, including MMP-1, -2, and 9 and MT1-MMPs, and serine proteases play pivotal roles in corneal ulceration induced by alkali burns and are known to be produced by PMNs, injured epithelial cells, and stromal keratocytes.11,53–59 Yet, little is known about the precise role of MPPs during corneal wound repair after alkali injury. The data presented herein indicate that \( \text{T}_{\beta 4} \) treatment after alkali burn regulates corneal expression of MMP-2 and -9 and leukolysin. The downregulation of leukolysin, a specific PMN MMP is consistent with the anti-inflammatory effects of \( \text{T}_{\beta 4} \).60 In contrast, \( \text{T}_{\beta 4} \) had no effect on the levels of TIMP-1 or -2 expression, suggesting that \( \text{T}_{\beta 4} \) alters the balance of proteases and their inhibitors in vivo in favor of matrix repair. The effect of \( \text{T}_{\beta 4} \) on corneal MMP-9 is significant, because this protease is the primary MMP that is synthesized and secreted by basal corneal epithelial cells at the leading edge of the epithelium migrating to heal a wound. Excessive synthesis of MMP-9 has been shown to contribute to epithelial repair defects and corneal melting.51–53 The pattern of corneal MMP-9 synthesis is consistent with the timing of basement membrane degradation—rapid increase in expression within a day of wounding—and provides a mechanism for control of basement membrane remodeling.29,64–65 The decreased inflammatory response seen in this study after alkali insult with \( \text{T}_{\beta 4} \) treatment may affect epithelial-stromal interactions through cytokine and MMP expression and prevent poorly controlled matrix remodeling that can lead to stromal thinning and epithelial hyperplasia, which are changes in cornea structure associated with chronic epithelial injury. In turn, the migration of activated keratocytes into the wound and their production of new extracellular matrix and remodeling may be regulated to achieve superior healing and visual clarity.

In addition to its role in ECM remodeling, it is well known that MMP-9 can modulate the inflammatory response by cleaving IL-8, a key PMN chemoattractant, thereby increasing its potency 10-fold.66–68 In the current results, after alkali injury MMP-9 was highly expressed at the leading edge of the corneal epithelial wound, in the stroma, in infiltrating PMNs, and in the corneal limbus. Indeed, our findings showing the presence of MMP-9 in the limbal region suggest that it is involved in corneal repair and regeneration after alkali injury. Corneal stem cells residing at the limbus serve as a proliferative reserve, and limbal cell transplantation is an effective procedure for restoring the corneal surface integrity after chemical injury.69 Yet, there is little known about the role of limbal stem cell expression of MMP-9 in the pathogenesis of corneal ulceration and/or repair after alkali injury. It is plausible to speculate that \( \text{T}_{\beta 4} \) treatment modulates limbal cell MMP-9 expression and their migration into the wounded cornea, thereby promoting repair.
In conclusion, the ability of Th4 treatment to promote corneal clarity and decrease corneal chemokine and MMP expression after alkali injury suggests that Th4 may act as a key regulatory wound-healing agent. It remains uncertain whether Th4 promotes corneal healing by acting directly on PMNs and thereby decreasing MMP levels. Recent studies have reported that Th4 is specifically translocated into the cell nucleus by an active transport mechanism, requiring an unidentified soluble cytoplasmic factor. Thus, it is plausible to hypothesize that Th4 may be acting as a transcription factor mediator in addition to its other known cellular functions. Ongoing studies are focusing on the molecular mechanisms by which Th4 exerts its anti-inflammatory effects and are aimed at elucidating further the effects of Th4 on downstream inflammatory signaling pathways involved in wound-healing process. Further understanding of Th4's mechanism of action in promoting corneal wound repair may help in the development of Th4 as a potential therapeutic agent for corneal wound healing and inflammatory disorders.

References
40. Hofer HP, Kukovetz E, Egger G, Wildburger R, Quehenberger F, Schar RJ. Polymorphonuclear leucocyte migration response in uneventful wound healing following trauma surgery: a contribu-


