



Altered KSPG expression by keratocytes following corneal injury

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Purpose: Keratocytes synthesize keratan-sulfate proteoglycans (KSPG), lumican and keratocan, to develop and maintain proper collagen interfibrillar spacing and fibril diameter characteristics of the transparent cornea. The purposes of this study are to compare the expression patterns of KSPGs and keratin 12 (K12) respectively by corneal keratocytes and epithelial cells after three different types of injuries; partial and total epithelial debridement and alkali burn.

Methods: Corneas of 8-12 week old C57Bl/6J or FVBN mice were wounded by partial epithelial (2 mm in diameter) and total epithelial debridement, and alkali burn (0.1 M NaOH, 30 s) and were allowed to heal for various periods of time, from 1 to 84 days. The corneas were then subjected to light microscopy, in situ and Northern hybridization and RT-PCR for examining the expression of K12 and KSPG in the corneal epithelium and stroma, respectively. Immunohistochemistry with anti- α -smooth muscle actin (α -SMA) was used to identify myofibroblasts in the stroma of injured cornea.

Results: In 2-3 days, partial epithelial denuded corneas were resurfaced by corneal epithelium positive for K12, and stromal edema caused by debridement disappeared. Total epithelial debridement wounded corneas were resurfaced by conjunctival epithelial cells in 2 weeks. Stromal edema in the total epithelial debridement corneas began to subside after 6 weeks. Corneal epithelial cells resurfaced alkali burned corneas within 3-5 days. In situ and Northern hybridization showed a decrease in keratocan and lumican expression at 6 weeks and increased at 12 weeks post-injury in all wound types. α -SMA positive myofibroblasts in the cornea were detected via immunostaining at the time point when KSPG expression was lowest, 6 weeks post-injury.

Conclusions: The results suggest keratocan and lumican are down-regulated during wound healing at 6 weeks and returned to higher levels at 12 weeks post-injury; implicating that the cells repopulating the injured corneal stroma regained the characteristic function of keratocytes independent of the wound types. However, complete epithelial removal results in irreversible loss of K12 expression.

The corneal surface consists of a multi-layered (5-7 cell layers) non-keratinized, stratified squamous epithelium. In part the strength of the corneal epithelium is maintained by its tissue-specific expression of intermediate filaments consisting of paired K3/K12 keratins, which is a characteristic of corneal-type epithelial differentiation [1]. The next layer is the stroma that comprises 90% of the corneal thickness in humans and accounts for the major contribution towards corneal curvature and clarity, and refractive power. Collagen I, V, VI, and XII make up the majority of the stroma [2-5] along with the dermatan sulfate proteoglycan decorin [6], and the keratan sulfate proteoglycans (KSPG), e.g., lumican [7], keratocan [8], and osteoglycin/mimecan [9].

Large epithelial wounds and alkali burns often lead to poor visual outcome, including conjunctivalization, stromal scarring, recurrent epithelium erosions, vascularization, and limbal deficiency [10-17]. The resultant corneal stroma after

wound healing is one of disorganized collagen fibrils and opacity. This outcome is significantly different from the uniform collagen fibril diameter and interfibrillar spacing allowing virtually unobscured light resonance resulting from corneal development [18]. The divergence of these two mechanisms suggests the involvement of other factors and/or a difference in gene expression during wound healing. Keratocytes are the major cell-type in the corneal stroma expressing the KSPGs and collagen [19-21] and MMPs for tissue remodeling during wound healing. One early event well documented is a population of keratocytes undergo apoptosis following a corneal wound [22-28]. It remains elusive whether the stromal cells repopulate the injured corneas maintain keratocyte phenotype.

Previous reports demonstrate differences (inflammation, necrosis, ulceration, and scarring) in the wound healing process of various wound types, specifically noting a unique healing process for the alkali burn wound [29-33]. One possible explanation is the variation in cytokines and growth factors expressed after a specific wound type [31,34]. Potential source for the initial factors are the tear, the remaining keratocytes and epithelial cells [31-37]. These signaling molecules have the potential to recruit inflammatory cells, promote apoptosis, and contribute to changes of gene expression patterns accounting for opaque scar tissue formation [38-45].

The purpose of this study was to examine the expression of corneal KSPGs as an indication of keratocyte phenotype

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during the wound healing process of three different wound types. We hypothesize the wound healing process alters KSPG expression in the entire corneal stroma. To examine this hypothesis, we observed the expression patterns of lumican and keratocan in response to alkali and epithelial debridement

wounds at 6 weeks and 12 week post-injury. Furthermore, we examined the ability of the cells resurfacing the cornea to express K12 after complete epithelial debridement. Here we report the irreversible loss of K12 expression following total debridement of the corneal epithelium. We also report a down-regulation of KSPG expression at 6 weeks post-injury as compared to uninjured corneas, followed by an increase at 12 weeks as compared to 6 weeks in all three wound types. Our observations are consistent with the notion that cells repopulating the injured stroma can regain the characteristics of keratocytes.

METHODS

Wound generation: C57Bl/6J or FVBN albino mice (8-12 weeks of age) were utilized in this study after pre-operative examination for exclusion criteria such as ocular disease, wound, or infection. Animal care and use conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. All animal protocols were approved by IACUC at the University of Cincinnati. Mice were anesthetized by intraperitoneal injections of ketamine (2.0 mg/gm body weight) and xylazine (0.4 mg/gm body weight). One drop of 0.5% proparacaine hydrochloride ophthalmic solution was applied to each eye prior to surgery as a topical anesthetic. The mice were subject to surgery and observed during a recovery period until awakening in a heated chamber. Using a dissecting microscope and light source, a centrally located circular 2 mm corneal epithelial wound was generated using an Algerbrush II™ corneal rust ring remover with a 0.5 mm burr (Alger Equipment Co., Inc., Lago Vista, TX) to create the partial epithelial debridement wound. The eye was then topically stained with fluorescein, analyzed with light microscopy, and photographed to determine the rate of epithelial wound healing. The mice were then carefully observed during a recovery period in a heated chamber. Total epithelial debridement wounds were generated in a similar fashion, except the entire corneal epithelium, including the limbal region was removed with the corneal rust ring remover. Alkali burn wounds were generated in the following manner: A 2 mm diameter circular piece of filter paper soaked in 0.1 M NaOH was centrally placed on the eye for 45 s. The filter paper was removed and the eye then flushed with PBS to remove any remaining alkali agent. The epithelium lesion was evaluated with topical staining of fluorescein and photography.

RT-PCR: RT-PCR was performed on 10 µg of RNA extracted from wounded or unwounded mouse eyes for K12 as previously described [46]. cDNA was synthesized using 40 µl of 5X reverse transcription buffer, 20 µl of 0.1 M dithiothreitol, 8 µl of 25 mM dNTPs, 10 µl of RNasin (40 units/µl, Promega, Madison, WI), 10 µl of 50 mM random hexamers (Pharmacia, Piscataway, NJ), 10 µl of avian myeloblastosis virus reverse transcriptase (9.5 units/ml, Promega), and 1 µg of heat-denatured corneal RNA extracts from either wounded or non-wounded mouse corneal tissue. Diethylpyrocarbonate-treated water was added to bring the final reaction volume to 200 µl. The reaction was then incubated at 22 °C for 10 min, 42 °C for 90 min, and 100 °C for 2 min and immediately placed on

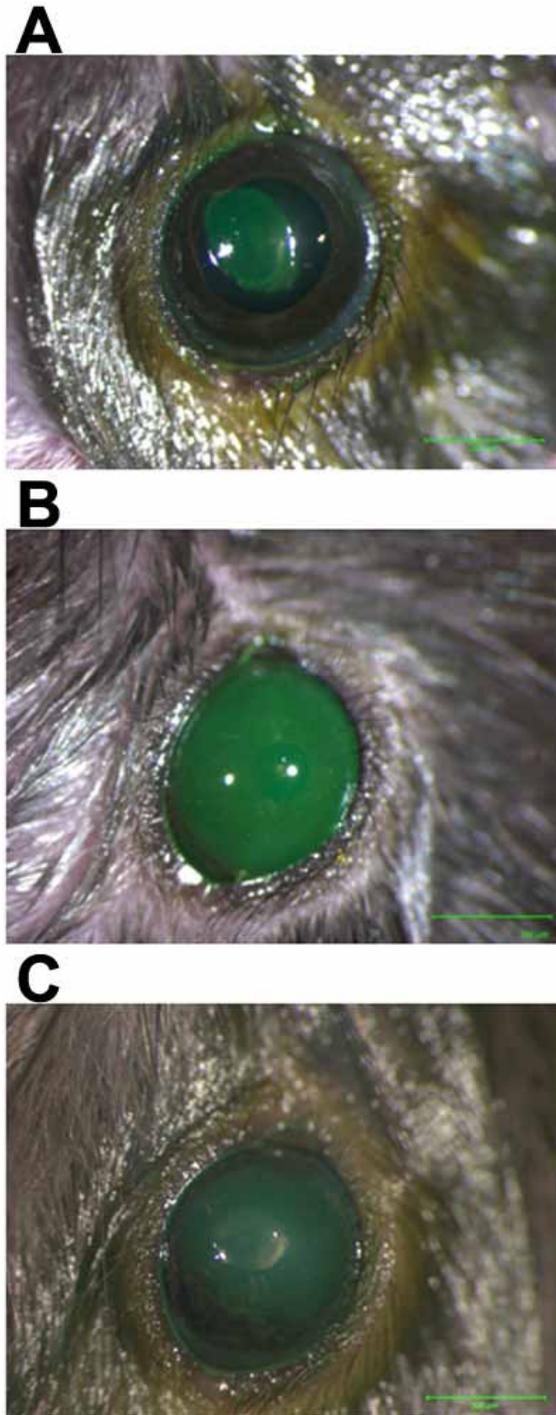


Figure 1. Fluorescein staining of the three wound types. Topical fluorescein staining of mouse corneas immediately after partial epithelial debridement (A), total epithelial debridement (B), and alkali burn (C). The positive green staining denotes an area where epithelial cells have been removed.

ice. A 20 μ l aliquot of the described RT reactions was added to 80 μ l of a PCR mixture containing 8 μ l of 10X PCR buffer (no $MgCl_2$), 8 μ l of 25 mM $MgCl_2$, 10 μ l of 20 ng/ μ l primers, 0.5 μ l of Taq polymerase (5 units/ μ l, Promega, Madison, WI), and 45.5 μ l of ddH₂O. The PCR reaction was performed for 35 cycles at 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min for 35 cycles, followed by a 5-min extension time at 72 °C at the end of 35 cycles. The primers used for generation of the 437 bp PCR product for K12 were I₁D 5'-CGA GAG TGG TAT GAA ACA-3' and I₁R 5'-GAG TGA TGG TGT CTG CGG-3' as previously described [46].

In situ hybridization of keratocan mRNA: Antisense and sense digoxigenin-labeled riboprobes (Boehringer Mannheim, Indianapolis, IN) of keratocan mRNA were synthesized as previously reported [47]. To identify the cell types that express keratocan, mouse eyes were enucleated and fixed with 4% paraformaldehyde at 4 °C and embedded in paraffin as described previously. Paraffin sections (5 μ m thick) mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) were deparaffinized and processed for in situ hybridization with sense (control) and antisense riboprobes of mouse lumican and keratocan. To remove nonspecifically bound probes, slides were subjected to a stringent wash with 0.5X SSC at 65 °C and treated with 20 μ g/ml RNase (Sigma, St. Louis, MO) at

room temperature for 1 h, followed by washing with 0.2X SSC at 65 °C as described previously [48]. The hybridization signals were visualized with anti-digoxigenin antibody-alkaline phosphatase conjugates using procedures recommended by Boehringer Mannheim. Finally, the sections were counterstained with 0.5% neutral red and mounted.

Northern hybridization: Total RNA was extracted from mouse tissues using TRI-reagent™ (Molecular Research Center, Cincinnati, OH) according to the manufacturer's recommendations. Total RNA (10 μ g) was separated by electrophoresis in 1.3% agarose containing 2 M formaldehyde buffered with TBE (Tris borate/EDTA). The RNAs were then transferred to Magna-Charge™ membranes (MSI, Westborough, MA) and hybridized with ³²P-labeled mouse lumican and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA in a hybridization solution containing 50% formamide at 41 °C overnight as described previously [48]. The excess ³²P-probes were removed by stringent washing three times with 0.1X SSC and 1% SDS at 65 °C for 30 min each. Hybridization signals were detected with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Hybridization signals were then standardized using the GAPDH loading control and calculated after subtraction of background noise as relative expression using unwounded cornea signal densities as 100.

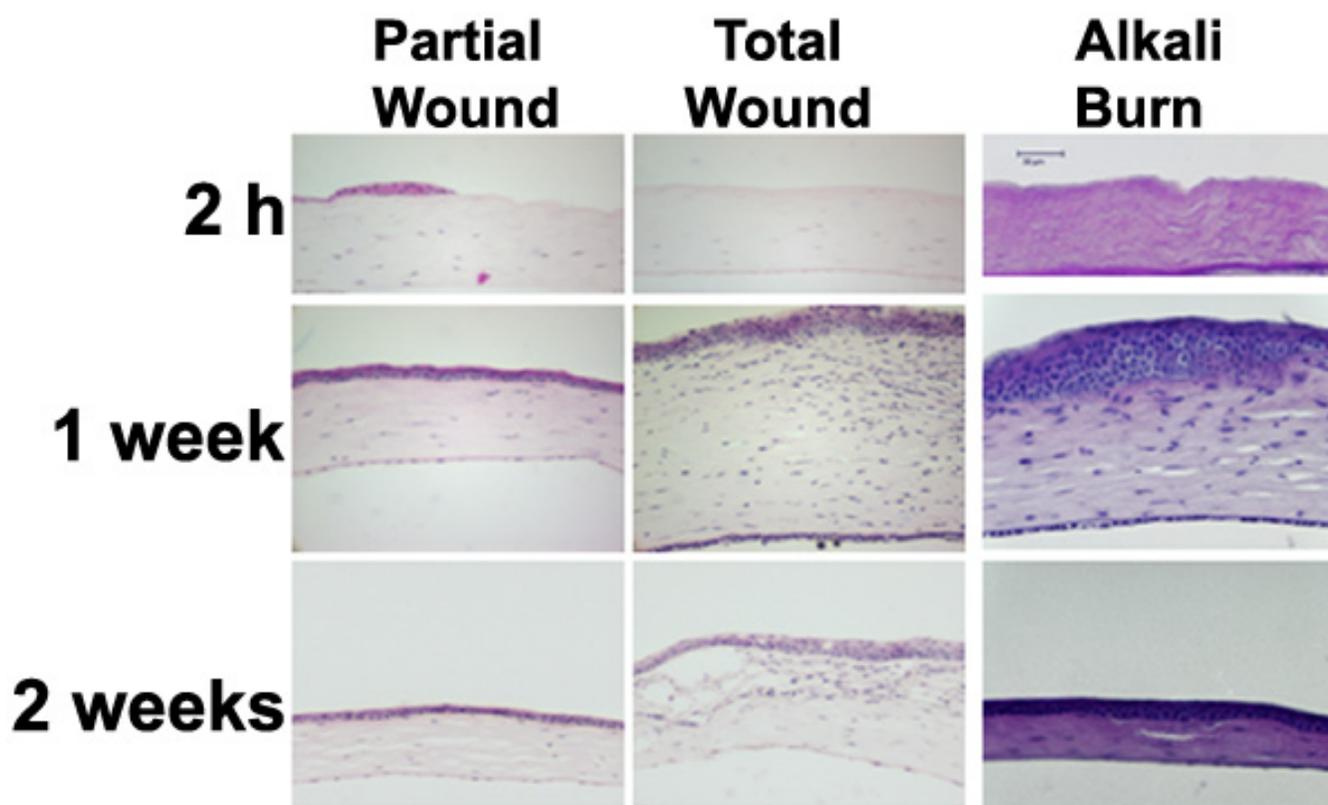


Figure 2. Hematoxylin and eosin staining of mouse cornea sections. Paraffin sections of mouse eyes at 2 h, 1 week, and 2 weeks post-wound stained with hematoxylin and eosin followed by light microscopy. Epithelial removal can be seen at 2 h post-injury in all wound types. Edema is most noticeable at 1 week post-injury especially in the total epithelial debridement and alkali burn. Partial debridement corneas return to a normal morphology 2 weeks post-wound; whereas total epithelial debridement corneas still possess a large amount of edema and conjunctival infiltration.

Immunohistochemistry: To determine the presence of myofibroblasts in the corneal stroma, paraffin sections from normal and injured corneas that had healed for various periods of times were subjected to immunohistochemistry with anti- α -SMA antibody directly conjugated to Alkaline Phosphatase (Sigma) and nonimmune rabbit IgG (control). Immunohistochemistry was performed as described previously [47]. The immune reactivity was detected with fast red staining for AP using light microscopy.

RESULTS

Three types of wounds, partial epithelial debridement, total epithelial debridement, and alkali burn, analyzed in this study were generated on wild-type mouse corneas and immediately stained topically with fluorescein to identify and confirm the removal of epithelial cells. Figure 1 shows *in vivo* photographs (15x magnification) using a stereomicroscope of C57Bl/6J mice eyes topically stained with fluorescein immediately after the creation of a partial epithelial debridement wound (Figure 1A), total epithelial debridement wound (Figure 1B), and alkali burn (Figure 1C). To understand the gross impact of the

wounds at the epithelial and stroma level, 5 μ m corneal sections were stained with hematoxylin and eosin (H& E). Figure 2 shows H& E staining of 5 μ m paraffin cross-sections of mouse eye of partial epithelial debridement, total epithelial debridement, and alkali burn at 2 h, 1 week, and 2 weeks after injury (400x magnification). Stromal edema is present in all wound types at 1 week post-wound, but was significantly higher in the total epithelial debridement and alkali burn. The epithelium of each wound type has resurfaced at this time point as well. The presence of edema varied in each wound type with the most notable difference between the partial and total epithelial debridements. Epithelium hyperplasia occurs most notably at 1 week post-injury in the alkali burn region. The resurfacing of the total epithelial debridement by stratified epithelial cells from the conjunctiva, as shown by the presence of goblet cells, prompted further analysis as to whether environmental cues would prompt these cells to transdifferentiate and express K12. To confirm conjunctivalization of the corneal epithelial layer with no transdifferentiation in the absence of limbal stem cells, K12 *in situ* hybridization was performed for the partial and total

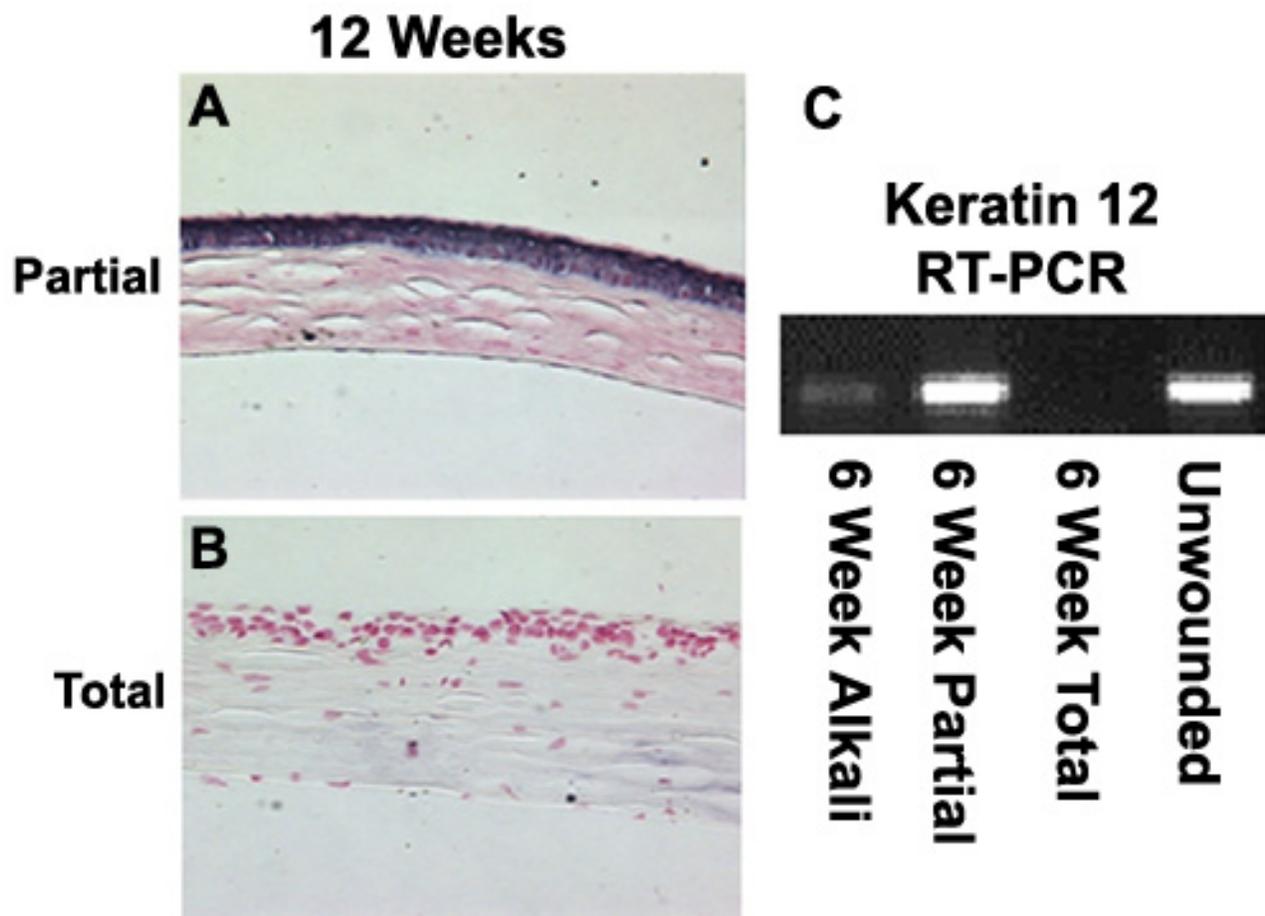


Figure 3. Keratin 12 *in situ* hybridization and RT-PCR. K12 *in situ* hybridization with a digoxigenin riboprobe was performed on the epithelial debridement. K12 mRNA is present in the partial debridement (A), but not the total debridement (B) 12 weeks post-injury suggesting a permanent loss of K12 upon limbal stem cell removal. K12 RT-PCR (C) was also performed on all 3 wound types and an unwounded cornea. K12 expression is detected to a lesser degree in the alkali burn, but no reaction is detected in the total debridement.

epithelial debridements (Figure 3). Positive hybridization signals for K12 can be seen in the partial wound epithelium (Figure 3A), but not in the total wound (Figure 3B), further confirming the irreversible loss of K12 expression upon creation of a limbal deficiency. This was further confirmed by the failure of detecting a 437 bp product in the total debridement for K12 mRNA using RT-PCR at 6 weeks post-injury (Figure 3C).

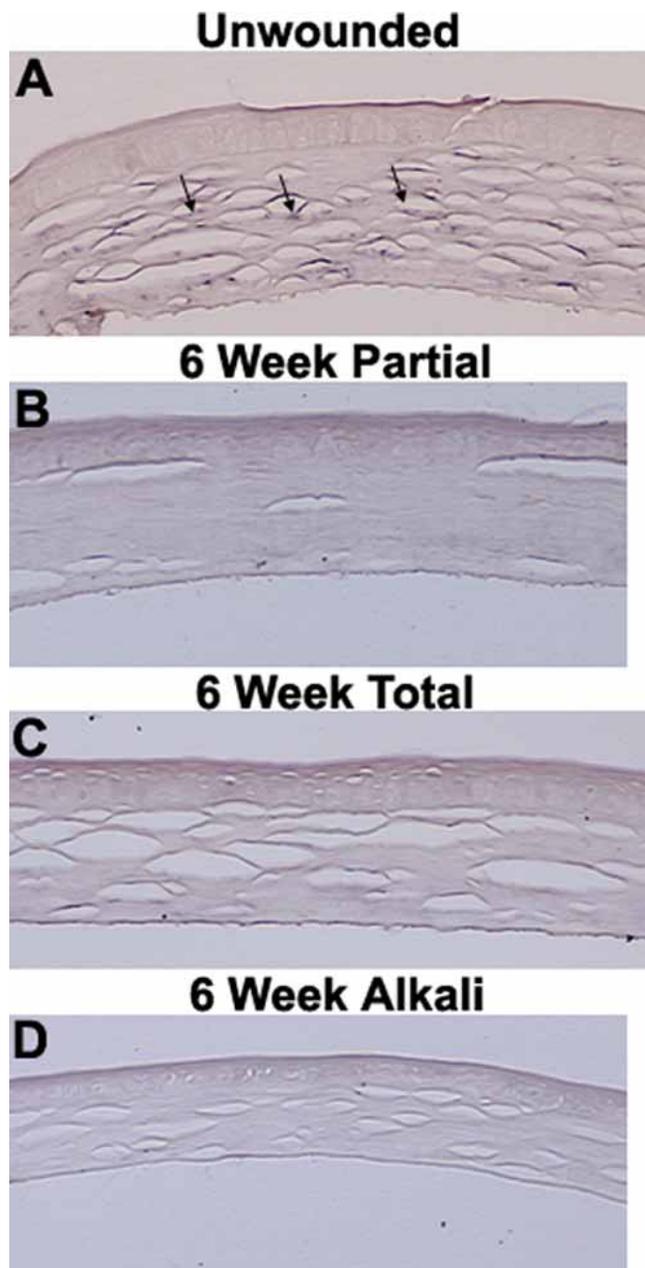


Figure 4. Six weeks post-wound keratocan in situ hybridization. In situ hybridization for the keratan-sulfate proteoglycan keratocan was performed in mouse eye sections at 6 weeks post-wound. Positive keratocan mRNA signal is denoted by the presence of purple (arrowheads). Unwounded mouse corneas are positive for keratocan (arrowheads), but wounded corneas have little or no keratocan expression.

To investigate the impact of the wound types on the keratocytes in the corneal stroma, the expression of two corneal KSPGs, lumican and keratocan, were analyzed during the wound healing process. Keratocytes typically populating the corneal stroma are quiescent cells and secrete collagen and KSPGs [19-21]. Keratocan is a corneal stroma specific protein whose expression was used to analyze the keratocyte response to injury. Figure 4 shows in situ hybridization of paraffin-embedded sections with a digoxigenin-labeled keratocan riboprobe at 6 and 12 weeks post-wound. The presence of a purple color denotes a positive signal for keratocan mRNA expression. Keratocan mRNA can only be detected in the unwounded control using in situ hybridization at 6 weeks post-injury. Results from 6 weeks post-wound sections indicate that a decrease in keratocan expression occurred for all three wound types when compared to a non-wounded control. To identify

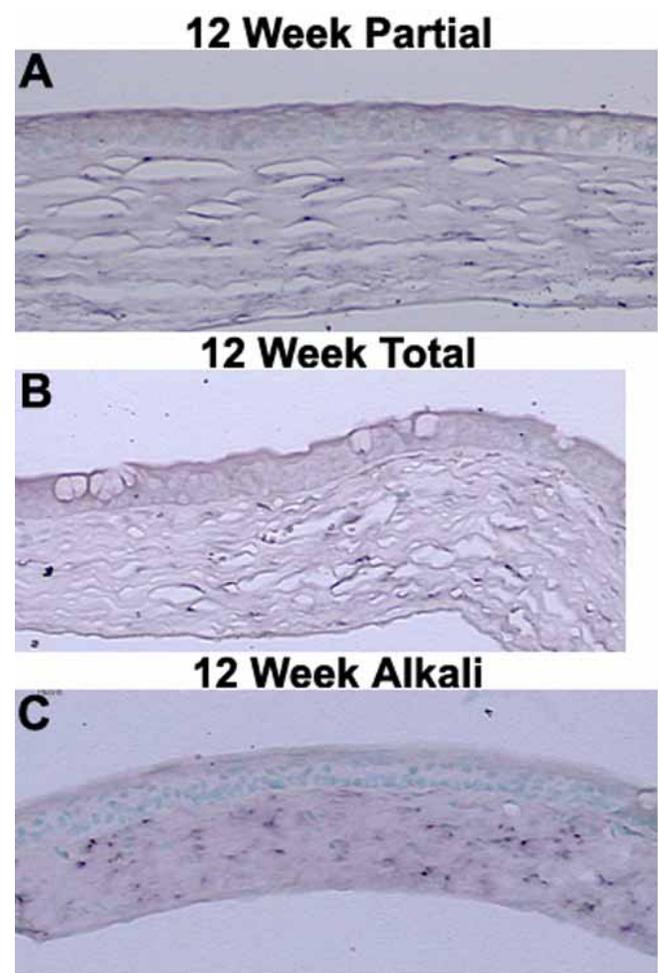


Figure 5. Twelve weeks post-wound keratocan in situ hybridization. In situ hybridization for the keratan-sulfate proteoglycan keratocan was performed in mouse eye sections at 12 weeks post-wound. Unlike 6 week keratocan expression, wounded mouse corneas are positive for keratocan implying a decrease in keratocan up to 6 weeks post-injury, and an upregulation prior to 12 weeks post-injury. A decreased amount of keratocan expression is still apparent as compared to a non-wounded control, most apparent in the alkali burn.

if the wound healing process resulted in a permanent decrease or loss in keratocan expression, further analysis of keratocan expression was performed at 12 weeks after injury using in situ hybridization. All of the wound types show the presence of a positive keratocan mRNA signal in the corneal stroma. The data shown in Figure 5 from 12 weeks post-wounded corneas does demonstrate the return of keratocan expression, but not to the level of uninjured cornea. Taking the fluctuation of keratocan data into account, another corneal KSPG was analyzed to determine whether this fluctuation was protein specific or pertained to all KSPGs in the stroma. Lumican and keratocan mRNA expression were analyzed using Northern blot analysis at 6 weeks and 12 weeks post-injury with GAPDH

serving as a loading control (Figure 6). Northern blot signals were calculated electronically and standardized using the corresponding GAPDH control. All samples were then quantitated on a relative scale using the unwounded cornea expression levels as 1 (100%) and standardization using GAPDH and graphed for lumican (Figure 6C) and keratocan (Figure 6D). A parallel expression pattern to that of keratocan in situ is also seen with lumican and keratocan Northern data, suggesting the loss of typical keratocyte gene expression during the wound healing process. The corneal KSPGs were expressed

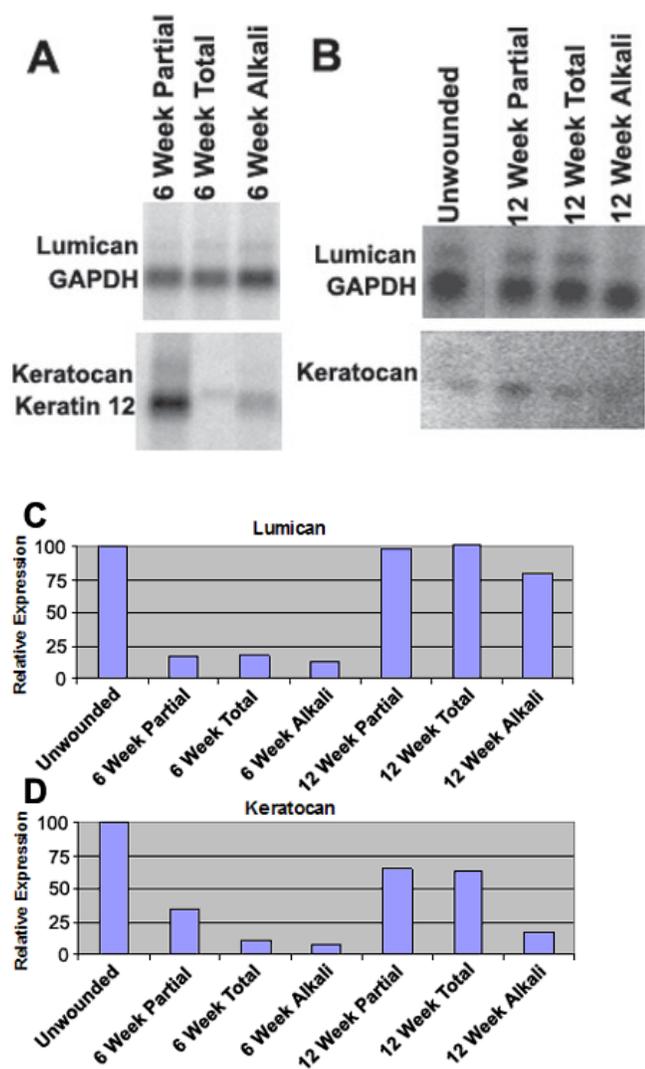


Figure 6. Lumican and keratocan northern hybridization. Lumican and keratocan Northern hybridization at 6 weeks (A) and 12 weeks (B) post-injury for the 3 wound types and an unwounded cornea with a GAPDH loading control. Hybridization signals were quantitated and graphed as relative expression as compared to the unwounded control for lumican (C) and keratocan (D). A decrease in lumican and keratocan mRNA is detected 6 weeks post-injury in all 3 wound types. An increase in expression is detected 12 weeks post-injury.

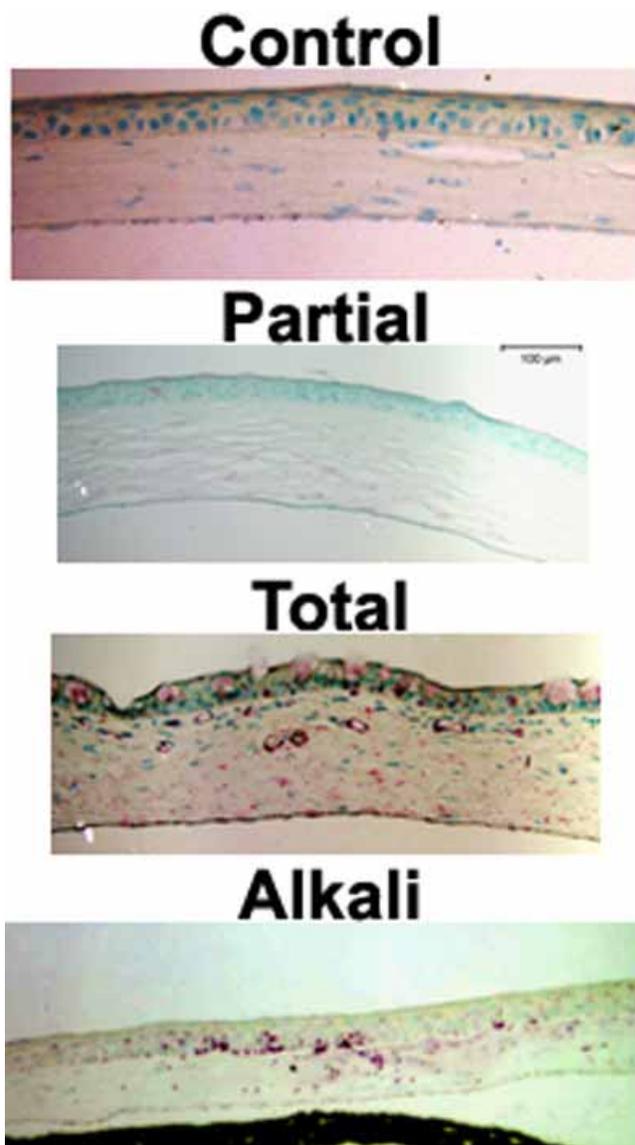


Figure 7. α -Smooth muscle actin immunostaining of corneas 6 weeks post-injury. α -SMA immunostaining performed on partial debridement, total debridement, and alkali burn corneas 6 wks post-injury and a non-wounded control. The presence of the red color (AP reaction) in the corneal stroma denotes a positive reaction for α -SMA. Partial epithelial debridement corneas have low levels of α -SMA expression; whereas, total and alkali burn corneas have high levels of α -SMA expression due to the large presence of blood vessels and myofibroblasts.

at the lowest level at 6 weeks post-injury suggesting a loss of keratocytes or a change in gene expression. At 12 weeks post-injury lumican mRNA levels in all the partial and total wounds returned normal levels while the alkali burn was approximately 75% of unwounded control. Keratocan levels only returned to approximately 65% in the partial and total wound and only 17% in the alkali burn suggesting extensive damage to the keratocytes and stroma.

Typical wound healing mechanisms involve the infiltration of myofibroblasts into the wounded tissue [49,50]. It is possible the keratocyte population was replaced by myofibroblasts to aid in wound healing. To determine the presence of myofibroblasts in the stroma of injured corneas at the point where KSPG expression was lowest, α -SMA immunostaining was performed on samples 6 weeks post-injury. Figure 7 shows myofibroblasts detection in all wound types, but a higher expression of α -SMA is seen in the alkali burn and total wound samples relative to the partial wound samples. The presence of myofibroblasts correlates with the previous findings with respect to KSPG expression.

DISCUSSION

In the present study corneal response to three different wound types was analyzed at the epithelial and stromal level with respect to K12 and KSPG expression. Much debate still exists as to the ability of cells migrating into the corneal epithelial region to express K12. Here we report, removal of the entire epithelium results in permanent K12 expression loss. At the stromal level, we report KSPGs have a decrease in expression at 6 weeks, but expression increases 12 weeks post-injury. This type of lumican and keratocan expression comparison in the adult animal using different wound types has not been previously reported.

Here we report 2 genes typically expressed by keratocytes are down-regulated during the wound healing process. Under normal conditions, keratocytes are the major cell population of the stroma thought to be responsible for expressing components necessary for maintaining the orthogonal matrix of collagen and proteoglycans necessary for corneal transparency [19,51-53]. Previous studies have shown the removal of corneal epithelial cells regardless of method e.g., mechanical or chemical, results in apoptosis of the keratocytes directly under the debridement region [54-56]. The mechanism to repair the corneal stroma and the role of the remaining keratocytes during wound healing is not clearly defined. Also elusive is the actual source of the cells responsible for stromal repair and the subsequent keratocytes which repopulate the stroma.

Although the function and expression pattern characterization of these cells has been attempted in vitro by several groups [19,52,57-59], no unequivocal evidence has been provided in an in vivo model. To date the actual cellular events in the corneal stroma remain elusive for various reasons. Primarily, a single molecular marker for keratocytes has yet to be identified. Traditionally, keratocan has been used to identify a keratocyte, but cultured corneal stroma cells lose the expression of keratocan as reported by Gendron et. al. [59] and here we report the decrease in keratocan expression following a

wound. In essence, the actual gene expression pattern of the keratocyte differs in an in vitro and in vivo model and varies with environmental and growth factors as well [57,58,60-63]. It is for these reasons keratocyte cell biology remains elusive.

Even though a large population of α -SMA positive cells exists in the corneal stroma at 6 weeks post-injury, this does not provide insight as to whether these cells migrated into the stroma from surrounding tissues or were transformants of the previously quiescent keratocytes. Furthermore once the wound is healed, it is unknown as to the source of the keratocyte population restored in the corneal stroma. The real answers to these problems lie at identifying the source of origin for the corneal keratocyte, which still remains unknown [10,11,16]. Our results indicate that the cells repopulating the injured corneal stroma regain the characteristics of keratocytes and express keratocan in all three wound types. Nevertheless, the source of keratocytes following injury remains unknown.

It is also not known if mesenchymal stem cells exist in ocular tissues and/or other tissues. Environmental cues e.g., growth factors and cytokines, may modulate various gene expression patterns in the keratocyte dependent upon the state of the stroma. This would suggest the cell population of the corneal stroma does not change during the wound healing process, but solely the gene expressing patterns changed during the process. Another contributing factor to this may be the cytokines released by corneal epithelial cells, which is apparently lost upon complete epithelial removal. Again, without a definitive cellular marker constitutively expressed by the keratocyte, the answer to the cellular population of the corneal stroma is clouded. The keratocyte generates the necessary components to maintain a highly organized orthogonal matrix in the stroma, but the ensuing corneal opacity and scarring following a wound is the result of fibroblasts and/or myofibroblasts. Further analysis of these cells under varying conditions and a detailed analysis to their source will provide many insights into corneal cell biology and biochemistry.

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