Expression of Collagen I, Smooth Muscle \( \alpha \)-Actin, and Vimentin During the Healing of Alkali-Burned and Lacerated Corneas

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**Purposes.** Alkali-burned corneas can seldom heal properly to restore corneal transparency. To provide a better understanding of this devastating corneal injury, we compared the expression of collagen I, smooth muscle \( \alpha \)-actin (\( \alpha \)-SMA), and vimentin in lacerated and alkali-burned rabbit corneas.

**Methods.** A radiolabeled cDNA probe of \( \alpha_1(I) \) chain was used in slot-blot hybridization to determine the levels of \( \alpha_1(I) \) mRNA in alkali-burned corneas. In situ hybridization was used to identify the cell types that express the \( \alpha_1(I) \) chain. Antibodies against collagen I, \( \alpha \)-SMA, and vimentin were used in immunohistochemical studies to determine the tissue distribution of collagen I and to identify cells expressing \( \alpha \)-SMA and vimentin.

**Results.** The levels of \( \alpha_1(I) \) mRNA in alkali-burned corneas increased steadily after the alkali burn and reached a plateau within 2 weeks. One day after alkali burn, specific in situ hybridization signals were detected in stromal cells immediately surrounding the edge of the corneal injury. As the healing proceeded, the fibroblastic cells migrated into the injured stroma, and they showed positive reactions by in situ hybridization and by immunostaining with anti-collagen I probes. In alkali-burned corneas, retrocorneal membranes were formed 1 week after injury. This fibrillar membrane was stained by anti-collagen I antibody, and the fibroblastic cells in the membrane were hybridized by the \( ^3 \)H-labeled \( \alpha_1(I) \) cDNA probe. No retrocorneal membrane was formed in the lacerated corneas, even after the injured corneas were allowed to heal for 3 weeks. The epithelial cells in the epithelial plug of lacerated corneas were positive by in situ hybridization, whereas the epithelial cells in the regenerated epithelium of alkali-burned cornea was not. Antibodies against \( \alpha \)-SMA reacted with the migrating fibroblastic cells but did not react with epithelial cells or endothelial cells in the injured corneas. Anti-vimentin antibody reacted with fibroblastic cells, endothelial cells, and keratocytes in normal and injured corneas, and with the basal epithelial cells of injured corneas.

**Conclusions.** During wound healing, the keratocytes that migrate to injured stroma transform into myofibroblasts. These myofibroblasts express high levels of \( \alpha_1(I) \) mRNA, \( \alpha \)-SMA, and vimentin. The healing of alkali-burned corneas differ from that of lacerated corneas in that the retrocorneal membranes are formed in the former but not in the latter. In addition, the epithelial cells of alkali-burned corneas lack \( \alpha_1(I) \) mRNA, whereas it is found in the epithelium of lacerated corneas. These differences may result from the persistence of inflammatory cells in the alkali-burned corneas. Invest Ophthalmol Vis Sci. 1993;34:3320–3328.

Alkali burn causes one of the most severe corneal injuries. The most serious alkali burns often involve limbal epithelium and other surrounding ocular tissues (conjunctiva, sclera, and so on) and lead to the loss of vision from corneal ulceration and perforation within a short time (1 to 3 weeks).

1-5 In contrast, less severe alkali burns can heal, but the healing is characterized by the persistence of inflammatory cells in the injured corneas, recurrent epithelial defects, and neovascularization. These characteristics interfere with the proper healing process and result in the formation of scar tissue, recurrent corneal erosions, or both.1,2,5-8

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The cause or causes of this devastating process is not well understood, and the treatment of alkali-burned corneas is usually not satisfactory. Seldom do alkali-burned corneas heal properly to restore vision.

Collagen constitutes about 80% of the organic constituents of the corneal stroma. Collagen I is the major collagenous component found in stroma, with the well-organized collagen I fibrils contributing to corneal transparency. However, after an alkali burn, polymorphonuclear leukocytes infiltrate the injured corneas, and the proteolytic enzymes, oxidative derivatives, or both, released by the inflammatory cells can cause severe loss of the extracellular matrix. Meanwhile, the stromal cells (keratocytes) that survive the alkali burn may proliferate and synthesize components of extracellular matrix for the repair of the injured corneas. Stromal ulceration takes place when the rate of degradation of extracellular matrix components (e.g., collagen, proteoglycans) exceeds the rate of synthesis. Many studies have focused on the degradation of the extracellular matrix after corneal alkali burns. However, there is little information available regarding the synthesis of the extracellular matrix during the healing of the alkali-burned corneas. In contrast, many investigators have examined the metabolism of fibrillar collagens during the healing of lacerated corneas. For example, increases in the synthesis of collagen I, III, and V were reported by Cintron, et al. We previously reported that stromal cells play a major role in the healing of lacerated corneas. The stromal cells not only synthesize collagen I to repair the injured tissues but actively participate in the process of remodeling the extracellular matrix by phagocytizing collagen fibrils during the healing of lacerated corneas.

It has been suggested that myofibroblasts participate in the healing of mechanical wounds. Myofibroblasts that synthesize and secrete collagen I during wound healing are characterized by the expression of smooth muscle (α-SMA). It has been suggested that myofibroblasts contribute to wound contraction of mechanical injuries. Thus, it is of interest to examine whether myofibroblasts may play a similar role in the healing of alkali burns.

In the present study, we compared the expression of collagen I by alkali-burned corneas and lacerated corneas after allowing them to heal for various periods of time. Slot-blot hybridization and in situ hybridization with radiolabeled cDNA of collagen I mRNA was used to analyze the expression of collagen I. Antibodies against collagen I, smooth muscle α-actin (α-SMA), and vimentin were used in immunohistochemical studies of the injured corneas. Our results indicate that fibroblastic cells are the major cell type that synthesizes collagen I in the stroma of alkali-burned and lacerated corneas. The fibroblastic cells have the characteristics of myofibroblasts because they express α-SMA in addition to vimentin. Alkali-burned corneas formed a retrocorneal membrane consisting of collagen I, whereas the lacerated corneas did not form such a membrane.

**MATERIALS AND METHODS**

32P-Labeled dNTP, γ-ATP, and [3H]dCTP were purchased from DuPont-New England Nuclear (Boston, MA). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA). BAS membranes were purchased from Schleicher and Schuell, Inc. (Keene, NH). Polyclonal anti-collagen I antibody was purchased from Southern Biotechnology Associates (Birmingham, AL). Monoclonal antibodies against α-SMA, vimentin, and desmin were obtained from Dakopatts, (Copenhagen, Denmark). Biotinylated second antibodies and ABC (avidin biotin peroxidase complex) reagents were purchased from Vector (Burlingame, CA). All other chemicals and reagents were obtained from either Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise specified.

**Animal Experiments**

Adult albino rabbits of either sex weighing 3 to 4 kg were purchased from Clerco Research Farm (Cincinnati, OH). Animal experiments were performed in compliance with the ARVO Resolution on the Use of Animals in Research. Rabbits were anesthetized with a combined intramuscular administration of ketamine (30 mg/kg) and rompun (5 mg/kg). A drop of proparacaine-HCl (0.5%) was applied directly to the eye. Only one eye of each experimental rabbit was injured. The contralateral eyes were discarded because occasionally pathologic changes were detected in the uninjured eyes (our unpublished observation). For control experiments, corneas from untreated animals were used. To create the alkali burn, a filter paper 8 mm in diameter soaked in 1 M NaOH was applied to the center of the cornea for 1 minute and followed by a rinse with 20 ml of phosphate-buffered saline (PBS) containing 0.15 M NaCl and 0.01 M sodium phosphate, pH 7.5. To create a laceration wound, an 8 mm penetrating incision was made in the center of the cornea with a Micro-Sharp blade (Becton-Dickinson, Franklin Lakes, NJ). Buprenorphine (0.3 mg/kg) was administered subcutaneously immediately after injury and 3 times daily for 3 days. The injured corneas were allowed to heal for various periods of time from 1 to 21 days. The rabbits were killed with an overdose of sodium pentobarbital intravenously (65 mg/kg). Corneal buttons (10 mm in diameter) were excised so that each excised cornea contained a portion of uninjured tissue. The excised corneas were immediately frozen in liquid nitrogen.

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and stored at −70°C until use or were subjected to in situ hybridization as described below.

Extraction of Total RNA

All solutions were autoclaved in the presence of 0.1% diethylpyrocarbonate (DEPC), and all glassware and appliances were either baked overnight or soaked in 1 M NaOH for 2 hours. Total RNA was isolated from alkali-burned rabbit corneas and normal rabbit corneas with RNAzol (Molecular Research Center, Inc., Cincinnati, OH), as previously described. The RNAs were resuspended in a solution containing 0.5% SDS and 0.1 U/ml RNasin (Promega, Madison, WI) and stored at −70°C until use.

Slot-Blot Hybridization

About 20 µg of total RNA from each sample were denatured in 50% formamide at 65°C for 5 minutes and were twofold serially diluted. The RNA was then transblotted to BAS membranes with a slot-blower (Schleicher & Schuell) and hybridized with the 32P-labeled cDNA of a1(I) mRNA, as described previously.

In Situ Hybridization

The normal, alkali-burned, and lacerated rabbit corneas were fixed in 4% methanol-free EM-grade formaldehyde (Polysciences, Inc., Warrington, PA) in 0.05 M sodium PBS, pH 7.4, at 4°C for 1 hour, and then immersed in 30% sucrose in DEPC-treated water for 2 hours at 4°C. The specimens were embedded in OCT compound (Miles, Elkhart, IN), quick frozen in ethanol/dry ice, and stored at −70°C. Frozen sections of 5 µm thickness were cut by a cryostat and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA). The sections were then subjected to hybridization with 3H-labeled cDNA probes of rabbit a1(I) mRNA, as previously described.

A 3H-labeled DNA probe was prepared by [3H]-dCTP incorporation using random primers as previously described. The specificity of the probe was 2 × 107 cpm/µg. The hybridization reaction was carried out at 45°C overnight. The sections were washed under stringent conditions to reduce the background, as previously described. The slides were then dehydrated in graded ethanol, air dried, and dipped in 50% Kodak NT-2 Nuclear emulsion (Eastman-Kodak, Manchester, NY); after exposure for 2 to 4 weeks, the slides were developed for 3 minutes in a Kodak D-19 developer at 15°C, counterstained with hematoxylin and eosin, and mounted.

Immunohistochemistry

For detection of collagen I, frozen sections of 5 µm thickness were prepared from the OCT-embedded, unfixed rabbit corneas by a cryostat and mounted on Superfrost/Plus microscope slides (Fisher Scientific). Cryosections were subjected to immunostaining using the ABC method (avidin-biotin-peroxidase complex), described by Chida et al. For detection of α-SMA, vimentin, and desmin, sections (3 µm) of paraffin-embedded corneas were mounted on precleaned glass slides without adhesive. The sections were deparaffinized and rehydrated with PBS before incubation with antibodies.

Briefly, the tissue sections were incubated with 0.3% H2O2 in methanol at room temperature for 30 minutes to eliminate endogenous peroxidase activity in tissue. The sections were rinsed with PBS and incubated at room temperature for 120 minutes with preimmune serum of the source of biotinylated second antibody (10X diluted) to block nonspecific absorption of second antibodies to the tissue sections. The sections were incubated with goat polyclonal anti-collagen I antibodies (1 µg/ml) or appropriately diluted monoclonal antibodies (0.1 to 1 µg/ml) at room temperature for 60 minutes or 4°C overnight. The sections were washed with PBS and further incubated with biotinylated secondary antibody for 60 minutes at room temperature, followed by incubation with streptavidin-biotin-peroxidase complex at room temperature for 60 minutes. The sections were soaked in a solution containing 0.2 mg/ml of 3-3'-diaminobenzidine hydrochloride, 0.005% of H2O2, and 50 mM Tris-HCl buffer, pH 7.6, for 3 to 5 minutes and counterstained with Mayer's hematoxylin. Photomicrograms were prepared with a Nikon Diaphot microscope (Nikon, Garden City, NY).

RESULTS

Slot-Blot Hybridization With 32P-Labeled cDNA of α(I) mRNA

Total RNA was extracted from alkali-burned rabbit corneas that had healed for 1, 2, 3, 5, 7, 14, and 21 days with RNAzol, and it was subjected to slot-blot hybridization with 32P-labeled cDNA of α(I) mRNA as previously described. Figure 1 demonstrates that levels of α(I) mRNA steadily increase and reach a plateau as the injured corneas are allowed to heal for more than 14 days. These results indicate that the alkali-burned corneas actively synthesize collagen I in attempts to repair the destroyed extracellular matrix induced by the alkali treatment.

In Situ Hybridization With 3H-Labeled cDNA Probe of α(I) mRNA

To identify the cells synthesizing collagen I in alkali-burned corneas, the injured corneas that had healed for 1 day and for 1, 2, and 3 weeks were subjected to in
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In an attempt to examine whether the lacerated cornea may heal differently, a series of similar experiments of in situ hybridization was performed with lacerated corneas healed for 1, 2, and 3 weeks. Fibroblastic cells migrate into the wound of the lacerated cornea and express high levels of α1(I) mRNA. Figure 2e shows a typical dark-field micrograph from a lacerated cornea healed for 2 weeks. Similar results were obtained with lacerated corneas that had healed for 1 and 3 weeks (data not shown). It is of interest to note that lacerated corneas do not form the retrocorneal membrane (Fig. 2e). The endothelial cells in the uninjured portion of the cornea either do not express or express a very low level of α1(I) mRNA (Fig. 3d), whereas the epithelial cells in the regenerated epithelial plug have a low level but specific expression of α1(I) mRNA (Figs. 2c, 3a).

Immunostaining With Antibody Against Collagen I

To verify whether collagen I exists in the retrocorneal membrane, goat antibody against collagen I was used in immunohistochemical studies with alkali-burned and lacerated corneas. Figure 4a shows the reaction of anti-collagen I antibody with the denatured collagenous components in the alkali-injured stroma healed for 1 day. Figure 4b demonstrates the presence of collagen I in the granulomatous tissue between the regenerated epithelium and stroma of alkali-burned corneas healed for 5 weeks. Figure 4c shows that the antibody reacts with the retrocorneal membrane but does not react with the Descemet's membrane. Similar immunohistochemical studies were performed with lacerated corneas healed for 1, 2, and 3 weeks. The results indicate that the lacerated corneas did not form retrocorneal membrane, and no fibrous structure can be detected underneath the endothelium of the uninjured portion of the lacerated corneas (data not shown).

Immunostaining of Alkali-Burned Rabbit Corneas With Antibodies Against Smooth Muscle α-Actin, Vimentin, and Desmin

To characterize the fibroblastic cells in the alkali-burned corneas, antibodies against α-SMA, vimentin, and desmin were used in immunohistochemical studies with alkali-injured cornea healed for 3 weeks. Figure 5a shows that anti-α-SMA does not react with either keratocytes or epithelial cells in the normal corneas. In alkali-burned cornea healed for 2 and 3 weeks, the antibody reacts with the fibroblastic cells in stroma and retrocorneal membrane (Figs. 5b, 7a, 7c). The anti-α-SMA antibody does not react with the epithelial cells in alkali-burned and lacerated corneas (Figs. 5b, 7a, 7c). The endothelial cells in both normal
FIGURE 2 In situ hybridization of injured corneas with 3H-labeled cDNA of α1(I) mRNA. The alkali-injured corneas were allowed to heal for 1 day and for 1, 2, and 3 weeks. The corneas were then subjected to in situ hybridization with 3H-labeled ARC55 cDNA probes encoding the 3'-end of the α1(I) mRNA. Dark-field micrograms: Panel a, alkali-burned corneas healed for 24 hours; panel b, alkali-burned corneas 1 week after injury; panel c, alkali-burned corneas 2 weeks after injury; panel d, alkali-burned corneas 3 weeks after injury; panel e, lacerated corneas healed for 2 weeks; panel f, bright-field microgram of panel b. Large arrows indicate the sites of injury. Small arrows and stars indicate cells hybridized by the 3H-labeled probe. D, Descemet’s membrane; RCM, retrocorneal membrane.

(data not shown) and lacerated corneas (Fig. 7g) are not stained by the anti-α-SMA antibody.

In another experiment, antibody against vimentin was used to stain the normal, alkali-injured, and lacerated corneas. The anti-vimentin antibody reacts with keratocytes in normal corneas and fibroblastic cells in alkali-injured stroma (Figs. 6, 7b), fibroblastic cells in retrocorneal membranes (Fig. 7d), endothelial cells of normal corneas (data not shown), and in lacerated corneas healed for 2 weeks (Fig. 7h). The epithelial cells in
FIGURE 3. In situ hybridization of injured corneas with $^3$H-labeled cDNA of $\alpha$I(I) mRNA. Panels a and d, lacerated cornea healed for 2 weeks; panels b and e, alkali-burned cornea healed for 1 day; panels c and f, alkali-burned cornea healed for 3 weeks. epi, epithelium; D, Descemet's membrane. Magnification, bar = 0.2 mm.

FIGURE 4. Immunostaining of alkali-burned corneas with antibodies against collagen I. Injured corneas healed for 1 day and 3 weeks were subjected to immunostaining with specific anti-collagen I antibodies. Panel a, cornea 24 hours after injury. Degenerated stroma still reacts to the anti-collagen I antibodies (X270). Panels b and c, 3 weeks after injury; panel b, the newly formed granulation tissue between epithelium and stroma (*) (X270); panel c, retrocorneal fibrilar membrane. Magnification (X350), bar = 0.1 mm.

FIGURE 5. Immunostaining of normal and alkali-burned corneas with antibody against smooth muscle $\alpha$-actin. 0.1 $\mu$g/ml of anti-$\alpha$-actin monoclonal antibody was used in the immunostaining. Panel a, normal cornea; panel b, alkali-burned corneas healed for 3 weeks. Magnification, bar = 0.1 mm.

FIGURE 6. Immunostaining of normal and alkali-burned corneas with antibody against vimentin. 0.88 $\mu$g/ml of anti-vimentin monoclonal antibody was used. Panel a, normal cornea; panel b, alkali-burned cornea healed for 3 weeks. Magnification, bar = 0.1 mm.
normal corneas are not stained by anti-vimentin antibody (Fig. 6a), but the basal epithelial cells in alkali-burned and lacerated corneas are stained by the antibody (Figs. 7b, 7f). The anti-desmin antibody does not react with any cell type in normal and injured corneas, but it does react with ocular muscle cells (data not shown).

DISCUSSION
In the present study, we measured the amounts of \( \alpha 1(1) \) mRNA in alkali-injured rabbit corneas. Our results indicate that the amount of \( \alpha 1(1) \) mRNA steadily increases as the alkali-burned corneas heal, and it reaches a plateau after 14 days of injury (Fig. 1). We previously reported that the increase of the amount of \( \alpha 1(1) \) mRNA in lacerated rabbit corneas followed a biphasic kinetics. The difference in the levels of \( \alpha 1(1) \) mRNA can be explained by the fact that alkali burn causes severe cell death, whereas laceration does not. The increase of \( \alpha 1(1) \) mRNA reflects the proliferation of fibroblastic cells and increased cellular activities when the alkali-burned corneas are allowed to heal (Fig. 2).

The cell types that express \( \alpha 1(1) \) mRNA are not exactly identical in alkali-burned and lacerated corneas, as judged by in situ hybridization (Figs. 2, 3). The alkali-burned corneas often form a retrocorneal membrane within a week after injury, and the fibroblastic cells in this membrane express high levels of \( \alpha 1(1) \) mRNA.
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mRNA. However, the lacerated corneas do not form such retrocorneal membrane. Another interesting observation is that although the epithelial cells of lacerated corneas have a relatively low but specific expression of α1(I) mRNA, the epithelial cells of alkali-burned corneas do not have detectable activity in expressing α1(I) mRNA. The reasons for the differences in the expression of α1(I) mRNA by the epithelial cells in alkali-burned and lacerated corneas remain unknown. It is possible, however, that the persistence of PMN or other inflammatory cells1–3 in alkali-burned corneas may be responsible for the variation in the expression of α1(I) mRNA by the epithelial cells. It is known that chemotactants derived from the proteolytic reaction and oxidative burst of PMN, as well as cytokines released by other inflammatory cells, can modulate the functions of cells residing in the injured tissues.34 Kay et al have demonstrated that PMN secrete factors that stimulate production of basic fibroblast growth factor by cultured corneal endothelial cells.35,36 Basic fibroblast growth factor alters the synthesis of collagen I by the endothelial cells. The scheme is consistent with our observations of the persistence of inflammatory cells in alkali-burned corneas and the synthesis of collagen I by the fibroblastic cells in the retrocorneal membrane. Many investigators have also demonstrated that cytokines (interleukin 2 and γ-interferon) modulate the expression of collagen genes in vivo and in vitro.37–41

Our immunohistochemical studies indicate that the fibroblastic cells in the injured stromas and in the retrocorneal membrane have characteristics of myofibroblasts, in that these fibroblastic cells are stained by the antibodies against vimentin and α-SMA (Figs. 5, 6, 7) but not by anti-desmin antibody. Vimentin has been shown to be expressed by most mesenchymal cells.18 However, the expression of α-SMA is restricted to the smooth muscle cells and some fibroblastic cells—the so-designated myofibroblasts.19–20 It should be mentioned that stromal cells (keratocytes) in the normal cornea do not react with the anti-α-actin antibody (Fig. 5), indicating that there are phenotypic changes of stromal fibroblastic cells in the injured stroma and in the newly formed retrocorneal membrane. Recently, it has been suggested that myofibroblasts play a role in the contraction of incision cornea wounds.20 It is likely that these fibroblastic cells contribute to wound contraction in our experimental model of alkali burns. However, further studies are needed to elucidate this hypothesis.

The fibroblastic cells found in the injured stroma are most likely derived from the keratocytes in the injured tissue. However, the source of the fibroblastic cells in the retrocorneal membrane is not known. It is possible that the rabbit corneal endothelial cells may proliferate and transform to the fibroblastic cell type. This hypothesis is especially intriguing when one considers that corneal endothelial cells are derived from mesenchymal neurocrest during embryonic development, just as are the stromal keratocytes.11 The results of immunohistochemical studies of anti-α-actin and anti-vimentin are consistent with the notion that the fibroblastic cells in retrocorneal membrane are of endothelial cell origin.18 It has been demonstrated recently that the endothelial cells can enter the cell cycle, possibly because of the presence of cytokines, growth factors, or both.32,43 It is plausible to speculate that cytokines secreted by PMN or other inflammatory cells persisting in the alkali-burned corneas may induce the proliferation and transformation of endothelial cells to myofibroblasts, which form the retrocorneal membranes. Although the alkali burns (8 mm in diameter) in our studies are relatively mild and do not usually involve other ocular tissues (e.g., trabecular meshwork or iris ciliary body), it is possible that the fibroblastic cells in the retrocorneal membrane may derive from these surrounding ocular tissues.

The anti-vimentin antibody does not react with the epithelial cells of normal corneas. Sundar-Raj et al recently demonstrated that vimentin is transiently expressed by epithelial cells in nonpenetrating lacerated corneas that healed for fewer than 5 days.44 However, in the present study we found that the basal epithelial cells still express vimentin 2 weeks after injury (Fig. 7b, 7f). The reason for the differences between our observations and that of Sundar-Raj et al is not known. However, experimental designs (alkali burn, penetrating incision versus nonpenetrating incision) may account for our differing results.

Key Words
alkali-burned corneas, lacerated corneas, wound-healing, collagen I, myofibroblast

References