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Cellular Changes of Phenotype and Collagenase-1 Expression in Healing Corneal Stromal cells

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Matrix metalloproteinases (MMPs) have been implicated in corneal diseases of the eye. Among the MMPs, collagenase-1 (MMP-1) participates in remodeling the extracellular matrix of corneal stroma during wound healing. Here the temporal expression patterns of both IL-1α and collagenase-1 were examined during regenerative corneal wound healing. IL-1α was highly expressed in the healing epithelium at 3 d after epithelial abrasion wound, thereafter its expression returned to normal level. Primary cultured quiescent stromal cells (keratocytes) from uninjured normal cornea were round-shaped and did not expresse collagenase-1. However, freshly-cultured stromal cells isolated 3 d after epithelial abrasion wound (abrasion wound) underwent marked morphological changes, and a certain percentage of cell populations was transformed into fibroblasts. By immunofluorescence, collagenase-1 expression was detected only in spindle-shaped fibroblasts. Interestingly, most freshly-isolated cells from remodeling corneal repair tissue 10 d after abrasion injury were found to have fibroblastic morphology, and also constitutively expressed a high level of collagenase-1 *in vitro* primary culture. However, its expression was not found in the cells isolated 14 d after wound and cells seemed to have returned to normal quiescent stromal cells. Taken together, these results suggest that differentially expressed IL-1α in the epithelium might play important roles in wound healing, and important signalling pathways are altered as cells take on the fibroblastic morphology.

The cornea contains three layers, the outer squamous epithelium, inner endothelium, and central stroma that contains quiescent stromal cells (keratocytes) embedded within a thick collagenous matrix. Epithelial injury is an important factor modulating keratocytes apoptosis. Upon epithelial abrasion, the epithelia at the margin of undamaged areas begin to migrate and resurface the damaged area, and regenerate completely within several days of post injury depending on the wound size. At the same time, the keratocytes underlying the epithelial wound regions are programmed to die within several hours after injury (Wilson et al., 2001). In contrast, keratocytes located at the wound edge of stroma lose their quiescence and begin to undergo mitosis, transform into activated fibroblast by chemotactic cytokines released from regenerating epithelia, and then migrate into the damaged acellular area (Weimer, 1960; Fini, 1999). These activated fibroblasts synthesize new extracellular matrix (ECM) molecules and cell-ECM adhesion molecules as well as induce collagenase-1, which is not synthesized by stromal cells in uninjured normal cornea (Fini, 1999; Girard et al., 1993).

There are at least 25 members of the matrix metalloproteinases (MMPs) family and collectively, these proteases can degrade all components of the ECM (Yong et al., 2001). Collagenase (MMP-1) is capable of cleaving native interstitial collagens at neutral pH of the extracellular space, and then the denatured collagen triple helix is further degraded by other MMPs (Matsubara et al., 1991). As a rule, resident cells synthesize collagenase only upon demand for remodeling such as early development, metamorphogenesis, and wound healing, and the presence of collagenase in a tissue indicates that remodeling is occurring (Fini et al., 1998). Abnormal MMP function can have profound effects on the composition and organization of the ECM due to imbalance between the synthesis and degradation of ECM and lead to pathological conditions, particularly tumor invasion and metastasis, ulceration, fibrosis, and arthritis (Fini, 1999).

During skin wound healing, induced collagenase expression occurs in the migrating epidermis of skin and proliferating fibroblasts within the repairing dermis (Okada

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et al., 1997; Vaalamo et al., 1997). Like skin, collagenase-1 expression occurs in epithelial cells migrating to resurface corneal wounds of human (Fini et al., 1996). Many diffusible cytokines and growth factors including IL-1α and transforming growth factor-β have been identified to act as paracrine or autocrine mediators of collagenase expression depending on tissues and cell types (Kanamori and Brown, 1993; Lyons et al., 1993; Reitamo et al., 1994; Sciavolino et al., 1994; Tamai et al., 1995; Tan et al., 1995).

The typical repair of incision injury in the adult mammals resulted in deposition of fibrotic repair tissue (Girard et al., 1993). However, short-term tissue remodeling in epithelial abrasion wound eventually restores the cornea to normal transparency at the site of injury. Therefore, it is this unique regenerative aspect that makes the cornea particularly interesting for the study of controlling mechanisms for remodeling (Girard et al., 1993). Up to now, almost all studies were carried out to understand mechanisms of transcriptional and translational regulation of MMP by treatment with various cytokines and reagents using passaged cell cultures isolated from the normal corneal stroma or cell lines that might not accurately reflect the healing tissue pattern of collagenase-1 regulation *in vivo*. It has been demonstrated that the primary cultured keratocytes isolated from normal uninjured cornea are not competent for collagenase synthesis until cell shape becomes like a fibroblast (Fini, 1999). The tight association of cell phenotypic changes and collagenase expression during wound healing *in vivo* has never been directly revealed.

To begin defining the timing of the fibroblasts returning to the normal quiescent stromal keratocytes upon abrasion injury, both cell morphology and translation levels of collagenase-1 expression were examined from primary cultured cells isolated from differential time points after wound. Understanding of this process will provide the information on the appropriate time points to study the control of expression of collagenase-1 that occurs during corneal repair *in vivo*.

Materials and Methods

Abrasion wounds

New Zealand white rabbits (3 kg) were anesthetized with intraperitoneal injection of Avertin. Animals were then given the topical anesthetic Aclaine (Alcon, Ft Worth, Texas) and pupils were dilated with drops of 0.5% cyclogyl (Alcon). Briefly, the central corneal epithelium was debrided using an alger brush within a 7.2 mm region, leaving the basement membrane intact. Corneal wounds were allowed to heal for 3 d, 10 d, 14 d, and 3 wks.

Corneal stromal cell culture

According to the standard protocol, the corneas dissected at 3, 10, 14, and 20 d after abrasion wound as well as normal uninjured corneas were treated with trypsin at 4°C overnight. The next day both epithelial and endothelial layer were gently scraped with a scalpel and stromas were incubated with collagenase (5 mg/ml) for 3 h to release stromal cells. Equal numbers of freshly-isolated cells were cultured in MEM in the presence of 10% fetal calf serum (FCS) and left to attach and spread on the dish overnight. The next day, the phenotypic differences of the cells were examined by phase contrast microscopy and the cells were coltured for 24 h in serum free MEM medium. Competence for collagenase-1 expression in response to trypsin or cytochalasin B (CB) (remodelling competence) was assayed by immunolocalization and western blotting.

Immunofluorescence

Freshly-isolated stromal cells were seeded at 1×10^4 cells per eight-chamber slide (Tissue-Tek; VWR Scientific) and allowed to attach and spread overnight to increase sensitivity of detection in MEM-10% FCS. For visualizing collagenase-1, the medium was then removed, the cell layer washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 5 min. Cells were then permeabilized in 0.2% Triton X-100 for 2 min, incubated with a polyclonal anti-collagenase-1 antibody (DSHB., U. Iowa), followed by rhodamine conjugated goat anti-rabbit IgG (Chemicon). Images of staining were captured on a Nikon fluorescence microscope.

Immunohistochemistry

In brief, the portion of ulcerated human cornea were fixed with formalin and embedded in OCT. For immunostaining, cryostat sections (7 μm) were dipped into methanol : 3% hydrogen peroxide (4 : 1) for 15 min to remove endogeneous peroxidase activity, rinsed with PBS, and blocked with normal horse serum for 1 h. Sections were incubated with anti collagenase-1 (1 : 30 dilution; R&D System) in a humidified chamber at room temperature for 30 min. After washing, sections were incubated with diluted biotinylated secondary antibody for 30 min. The tissues were then washed twice in PBS for 5 min, and incubated sections with Vectastain ABC reagent (Vector) for 30 min. After washing with PBS for 15 min, the sections were incubated in peroxidase substrate solution until desired stain intensity developed. The tissues were then washed in PBS, mounted with crystal mounting media, and observed under Nikon microscope equipped with digital camera.

Western blotting

For analyzing amounts of secreted collagenase-1, freshly-isolated cells were plated in 24-well culture plates at

2×10^5 cells per well. The next day, the medium was changed to serum-free medium and allowed to condition their media for 24 h. Media were then collected and secreted proteins were precipitated with 10% ice-cold trichloroacetic acid (TCA), the precipitates were washed twice with 100% acetone to remove remaining TCA, and precipitates were dissolved in RIPA buffer (1% NP-40, 0.5% deoxycholate, and 0.1% SDS). Total secreted protein from equivalent cell number was then run on 10% SDS-PAGE under non-reducing conditions and transferred to PVDF membrane. Blots were incubated with polyclonal collagenase-1 antibody and with horseradish peroxidase-conjugated secondary antibody. The signal was detected by using the ECL detection system (Amersham Pharmacia Biotech).

Northern blot analysis

Total RNA was extracted using RNAzol B (CNNA; Biotech) according to manufacturer's instructions directly from corneal epithelium after appropriate time period. Twenty μg of total RNA were electrophoresed on a 1.2% agarose gel, transferred onto nylon membranes, and ultraviolet wave crosslinked. Integrity and equal loading of samples were assessed by methylene blue staining of the transferred RNA. The membranes were hybridized with rabbit IL-1α cDNA inserts (^{32}P-deoxycytidine 5'-triphosphate; NEN) labeled by the random priming. After overnight hybridization at 65°C, the blots were washed for 20 min in 2×SSC with 0.1% SDS at 65°C, followed by a 10 min wash in 0.1×SSC with 0.1% SDS at 65°C. The membranes were autoradiographed with intensifying screens at -70°C for up to 2 d.

Results

Corneal ulcer formation is associated with collagenase-1

To examine the expression and localization of collagenase-1 in patients who developed corneal melts, immunohistochemistry was performed. As shown in Fig. 1, high levels of collagenase-1 were expressed in the ulcerated cornea. This result indicates that uncontrolled collagenase-1 expression should play an important role in the regulation of severe ECM degradation associated with ulceration.

Transformation of cells to fibroblasts in the process of wound healing

Previous studies have shown that primary cultured stromal cells from uninjured normal cornea initially showed round-shaped morphology due to unorganized cortical actin cytoskeleton (Jester et al., 1994; Fini, 1999). However, after a 3 d culture in serum conditions, cells develop a spindle shape and an organized actin

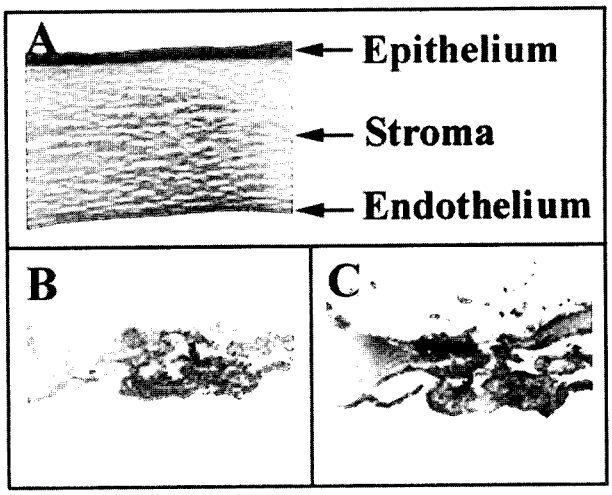

Fig. 1. Collagenase-1 was highly expressed in ulcerated human cornea. A, Normal human cornea was mainly composed of intact epithelium, stromal layer, and endothelium. B, Ulcerated cornea was severely deteriorated. C, High levels of collagenase-1 were detected by immunohistochemistry in the ulcerated cornea. A and B, Haematoxylin and Eosin stain.

cytoskeleton that is more characteristic of wound healing cells. In an attempt to examine whether changes in phenotypic difference occurr during remodeling of corneal repair tissue, isolated stromal cells from control and abrasion wound corneas were primary cultured. As shown in Fig. 2A, cells from quadruplicate cultures for 18 h were fixed and their morphology was examined. Compared with control cultures, a percentage of cultured stromal cells from the 3 d post-abrasion wound adopted the fibroblast phenotype. Furthermore, all cells isolated 10 d after wound exhibited fibroblast phenotype. However, cultured cells isolated 14 d after wounds showed keratocytic morphology, indicating that they have completely returned to normal quiescent stromal keratocytes in the wound by morphological criteria. Taken together, these results suggest that important signalling pathways are altered as cells take on the fibroblast phenotype.

Temporal expression of collagenase in activated stromal cell

To examine whether cell shape change was required for expression of collagenase during wound healing, immunofluorescence staining was performed (Fig. 2B). Immunoreactive intracellular collagenase-1 was not detectable in stromal cells from control cornea. In contrast, its expression was detected only in fibroblast phenotype of primary cultured cells isolated 3 d post-wound, but its expression was not detectable in round-shaped cells. Surprisingly, all the fibroblastic morphology of cells isolated from the repairing stroma at day 10 were intracellular collagenase-1 positive, suggesting that

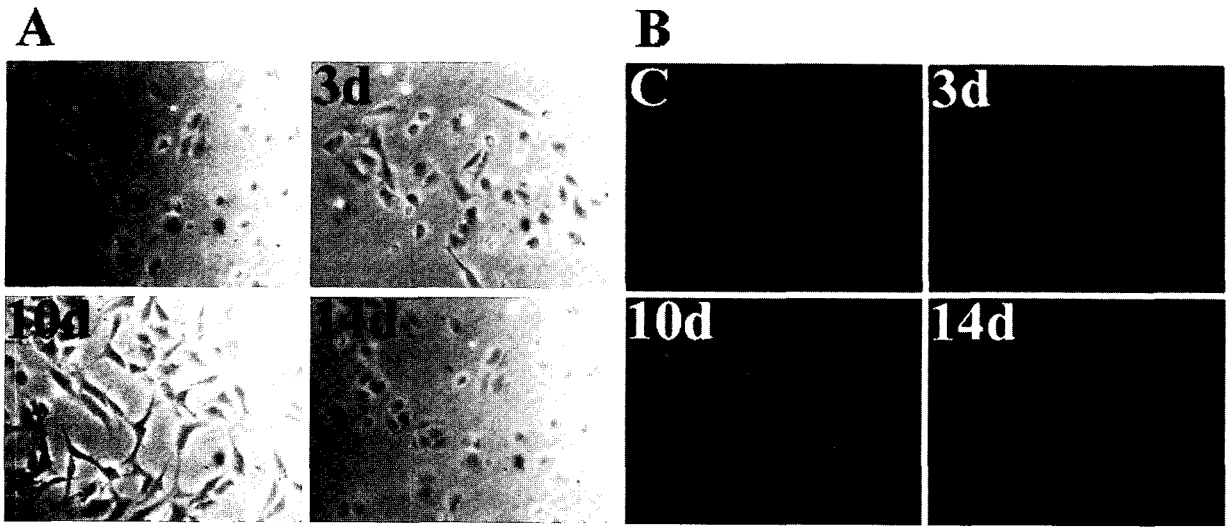

Fig. 2. Collagenase-1 expression is detected only in activated fibroblasts isolated from abrasion wounds. Freshly-isolated stromal cells were plated in the presence of 10% serum, and changes of both phenotype and collagenase-1 expression were examined the next day (A and B). A, Under phase contrast microscope, the cells isolated from both uninjured normal and 14 d wounds looked like keratocytes. However, most cells isolated from 10 d wounds have fibroblastic morphology. Interestingly, cells at day 3 presented a heterogeneous morphology and a certain percentage of cells showed fibroblastic morphology. B, The cells isolated from both uninjured normal and 14 d wounds showed no expression of collagenase-1. However, most of the activated fibroblasts isolated from 10 d wounds expressed collagenase-1. In cells from 3 d wounds, only cells of fibroblast morphology expressed collagenase-1.

most of cells were fully activated. However, the staining was not detected in the round-shaped cells isolated from tissue 14 d after wound, indicating that most of cells have returned to normal quiescent stromal cells.

To confirm that collagenase was secreted only in cells from repairing stroma, western blot analyses were performed on equal volumes of conditioned medium samples. As expected, collagenase-1 was not detected in the samples of both normal uninjured stromal cells and 14 d wounds, while it was easily detectable as early as 3 d after injury. Moreover, its production was increased over time, reaching its peak at day 10 of healing stroma (Fig. 3).

To test whether the integrity of the cytoskeleton plays a role in collagenase-1 expression in primary cultured cells, the effects of cytochalasin B (CB) was examined.

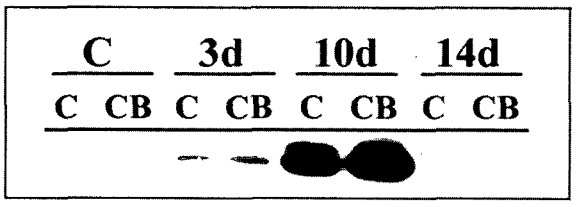

Fig. 3. Collagenase-1 synthesis was stimulated only in activated fibroblasts by CB treatment. Conditioned media from primary cultured cells as mentioned materials and methods was used for western blot analysis. The cells isolated from both uninjured normal and 14 day wounds showed no expression of collagenase-1 in response to CB treatment which is potent collagenase-1 stimulator in serum subcultured fibroblasts. It is notable that CB has a slight effect for collagenase-1 induction in activated fibroblasts isolated from 10 days wounds. Abbreviations: C: control; CB: cytochalasin B.

It is well known that CB disrupts microfilament function and architecture, and it caused significant changes in cell morphology with cells adopting a round shape compared to the classical elongated spindle shape of untreated fibroblast (data not shown). After cells were treated with 10 μg/ml of CB for 18 h, the conditioned media were collected and then analyzed by western blot. CB had no effect on collagenase-1 induction in primary cultured stromal cells from both uninjured normal and 14 d wounds (Fig. 3). On the other hand, CB had stimulatory effects on the production of collagenase-1 in cultured cells at day 10, suggesting that collagenase expression was associated with the cytoskeleton.

Expression of IL-1α from wounded cornea

To examine the role of IL-1α during wound healing, both immunohistochemistry and Northern blot analysis were performed. By immunohistochemistry, expression of IL-1α was detected at a low level in normal epithelium (Fig. 4Aa), but its expression was increased in the regenerating epithelium 18 h after wound (Fig. 4Ab). Moreover, northern analysis revealed that expression of two alternative spliced forms of IL-1α transcripts were detected at low levels in uninjured normal epithelium (Fig. 4B), but their expression levels were increased up to 3 d after wound. Thereafter, their expression levels were decreased as similar as expression pattern of normal cornea. Taken together, these results indicate that temporal up-regulation of IL-1α expression is required for regenerative wound healing.

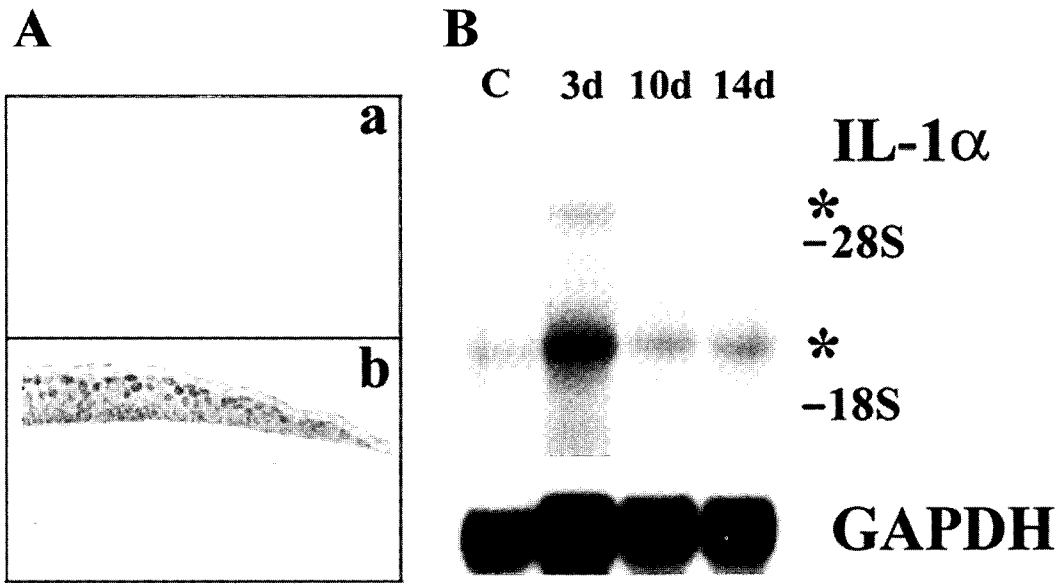

Fig. 4. IL-1α was differentially expressed in the epithelium of healing cornea. A, Immunohistochemistry showed that low levels of IL-1α were expressed in normal epithelium (a), but the expression was up-regulated in regenerating epithelium for 18 h (b). B, IL-1α mRNA was highly expressed 3 d after wound epithelium and its expression returned to normal level 10 d after wound. The asterisk (*) indicates the IL-1α transcript.

Discussion

The data presented show that both temporal cell shape changes and collagenase-1 expression occur only by activated fibroblasts during wound healing. Previously, it has been shown that primary stromal cells isolated from uninjured cornea exhibit many hallmarks of quiescent stromal cells *in vivo* (Fini, 1999). As the cells are allowed to proliferate in culture for several days they begin to undergo a transition to an active form which presumably mimicks their *in vivo* wound response. However, they are not competent to express collagenase-1 until IL-1α, PMA, and CB treatment. Until now, direct evidence correlating cell shape change and collagenase expression has never been reported *in vivo*. It has been hypothesized that both temporally and spatially expressed cytokines and growth factors across the sites of tissue remodeling might differentially modulate collagenase-1 as well as ECM expression. The present data provide the first direct evidence that both temporal cell shape changes and collagenase-1 expression occurring only in activated fibroblasts are closely correlated with wound healing process.

It was found that initial cultures of uninjured corneal stromal cells are deficient in activating collagenase-1 in response to PMA and CB due to incompetency for IL-1α autocrine loop activation (Fini, 1999), and deficiency of primary cells in organization of the actin cytoskeleton as well as lack expression of the fibronectin receptor (Fini, 1999). However, only after 3 d in culture stromal cells begin to gain competence for induction of collagenase-1 expression in response to CB that alter cell morphology due to increased ability of cells to produce IL-1α (Fini, 1999), which allows for self-activation (autocrine) of collagenase-1 expression. Alteration of the actin cytoskeleton occurs during many remodeling situations *in vivo* such as wound contraction and cell migration (Fini et al., 1998).

Interestingly, there were mixed cell populations including round-shaped stromal cells and fibroblasts in primary cultured cells isolated 3 d after wound (Fig. 2A). This result suggests that high level of IL-1α expressed from healing epithelium (Fig. 4A and B) diffused into the stroma and stromal cells begin to acquire IL-1α autocrine loop and fibronectin receptors as well as organization of cytoskeleton, and transform into active fibroblasts responsible for collagenase-1 expression. However, not all cells in the damaged area in the 3 d wound will respond in an identical manner to the diffused IL-1α from epithelium. Based on this result, collagenase-1 levels in tissue extracts do not necessarily reflect the response of whole cell populations, but rather of the individual cells. Even at low levels of IL-1α mRNA expression from epithelium at day 10 (Fig. 4), all the stromal cells isolated 10 days after wound showed fibroblastic phenotypes expressing collagenase-1 that was up-regulated by CB treatment (Figs. 2 and 3).

It has been shown that collagenase gene expression is induced by UV light exposure and "aging" of cell cultures via multiple passaging through autocrine IL-1α loop (Kumar et al., 1992; Petersen et al., 1992). The autocrine cytokine, IL-1α, is absolutely required as an

intermediate for induction of collagenase synthesis in response to CB in rabbit fibroblast culture (Fini et al., 1994; West-Mays et al., 1995; 1997). Thus it seems likely that all cells at day 10, regardless of low levels of IL-1α signals from epithelium, are already acquiring IL-1α autocrine loop controlling collagenase-1 expression *in vivo*. There exists a possibility that fibroblast-matrix interactions *in vivo* can send signals for collagenase expression through cytoskeletal remodeling and ligation of cell surface integrin receptors. However, at 14 d after wound, all stromal cells have returned to normal keratocytes based on their round-shaped morphology and the absence of collagenase-1 expression, possibly due to lack of IL-1α autocrine loop. Considering the potentially destructive effects of uncontrolled tissue degradation by collagenase-1 (Fig. 1), the balance between matrix synthesis and degradation should be precisely regulated to maintain the structural integrity and function of tissues. Therefore, it can be speculated that control of its constitutive expression may decrease the deteriorative roles of collagenase in many pathological states.

In summary, the present data show that stromal fibroblasts synthesize and secrete collagenase-1 in the remodeling corneal stroma. To my knowledge, this is the first study which establishes that temporal cell-shape changes and collagenase-1 synthesis by resident is a marker for short-term remodeling. The final goal of this study is to determine the important signaling mediators and elucidate the specific signaling pathways involved in collagenase-1 production during wound healing *in vivo*. This information is key to understanding and neutralizing the stromal reaction to ultimately prevent corneal ulcer or fibrosis, and possibly induction of cancer. However, further works are required to fully understand the molecular nature of this phenomenon.

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