**Repair Phenotype in Corneal Fibroblasts Is Controlled by an Interleukin-1α Autocrine Feedback Loop**

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**Purpose.** To explore the role of autocrine interleukin-1α (IL-1α) as a central regulator of the repair phenotype in corneal fibroblasts.

**Methods.** Disruption of the actin cytoskeleton with cytochalasin B (CB), which mimics changes in shape that occur in repair tissues, was used to stimulate repair gene expression in early-passage fibroblasts. Changes in expression of IL-1α, IL-8, collagenase, and ENA-78 were determined by Northern blot analysis, radioimmunoassay, and an enzyme-amplified sensitivity immunoassay (EASIA). Expression of repair genes was also examined in repair fibroblasts, isolated from healing, penetrating keratectomy wounds in rabbits.

**Results.** Blocking IL-1α activity prevented both constitutive and stimulated increases in synthesis of IL-8 and collagenase in early-passage cultures of corneal fibroblasts, demonstrating the role of IL-1α as a necessary intermediate for expression of these genes. Evidence is also presented that the IL-1α autocrine controls expression of an IL-8 related factor, ENA-78. Unlike early-passage fibroblasts, fibroblasts freshly isolated from the uninjured cornea did not express IL-1α. However, fibroblasts freshly isolated from remodeling corneal repair tissue 3 weeks after injury were found to express substantial levels of IL-1α, regulated through an autocrine feedback loop. Neutralization experiments demonstrated that the IL-1α autocrine is largely responsible for controlling both collagenase and IL-8 synthesis in repair fibroblasts, as it is in early-passage fibroblasts.

**Conclusions.** These findings provide evidence that activation of an autocrine IL-1α feedback loop is an important mechanism by which fibroblasts adopt a repair phenotype during remodeling of the cornea. Invest Ophthalmol Vis Sci. 1997;38:1367-1379.

The structure of tissues is maintained through a dynamic process of synthesis, degradation, and reorganization of extracellular matrix components. This mechanism, referred to as remodeling, contributes to morphogenesis of organs during development and participates in the reshaping of adult structures after injury. We have used the rabbit cornea as a model for studying the mechanism of tissue remodeling. The cornea consists of three layers of tissue, the epithelium, stroma and endothelium, each of which are homogenous in cell type and composition of the extracellular matrix. One of the major components of the extracellular matrix of the corneal stroma is the collagen fiber, which provides tensile strength to the tissue. Interstitial collagens have the unique ability to catalyze cleavage of the native collagen types I, II, and III at the neutral pH of the extracellular space. These enzymes are therefore considered key players in normal tissue remodeling events as well as important mediators of a number of connective tissue disorders, including arthritis and corneal ulcers.

To characterize the molecular and biochemical events responsible for activating fibroblasts to express collagenase at the onset of tissue remodeling, we have used an in vitro culture model of diploid fibroblasts.
derived from the rabbit corneal stroma. Because the uninjured corneal stroma is relatively homogeneous in cell type, composed of collagen fibers and fibrocytes, we are able to isolate a pure population of fibroblasts for primary culture. Primary fibroblasts, directly isolated from the uninjured cornea, do not express collagenase. In addition, these cells are not competent to make collagenase in response to such stimulators as phorbol 12 myristate 13-acetate (PMA), CB, or trypsin, which have in common the capacity to alter cell shape through a reorganization of the actin cytoskeleton. However, after only a single passage, the fibroblasts begin to synthesize collagenase constitutively and are competent to respond to shape-altering agents. This reorganization is relevant in biologic remodeling because similar cytoskeletal alterations occur in vivo as cells release from and reorganize the extracellular matrix. The stimulated increase in collagenase expression observed after treatment of early-passage fibroblasts with either PMA or CB is preceded by a lag time of 6 to 20 hours. Furthermore, if stimulated cells are cotreated with an inhibitor of protein synthesis, induction of collagenase mRNA is blocked. These data suggested that an intermediate protein must be synthesized for subsequent stimulation of collagenase. We have recently identified Interleukin-1α (IL-1α) as the necessary intermediate. In these studies we demonstrated that neutralization of IL-1α activity prevented stimulation of collagenase expression by PMA or CB in early-passage fibroblast cultures. We further showed that the levels of endogenous IL-1α are maintained through an autocrine feedback loop that is activated by these agents. Most important, our findings revealed that primary corneal fibroblasts, which do not express collagenase, similarly are unable to synthesize the IL-1α autocrine. These cells do, however, make collagenase at normal levels when IL-1α is supplied exogenously. Thus, competence in expressing collagenase is dependent on activation of the IL-1α autocrine loop, which tissue fibroblasts adopt with increased time in culture.

Does this culture model have an in vivo correlate? The simple nature of the cornea, compared with that of the skin, for example, has enabled investigators to carefully document the cellular events that occur during corneal repair. Initial events occurring within hours after injury include a resurfacing of the wound with a migrating sheet of epithelial cells. Concomitant with this event is the transformation of quiescent cells in the stroma at the edge of the damaged area to repair fibroblasts. These fibroblasts migrate into the damaged region and begin to deposit early repair matrix. Results from our laboratory demonstrated that within 24 hours after injury these fibroblasts begin to synthesize collagenase (MMP-1) and the related matrix metalloproteinase, stromelysin (MMP-3). Although synthesis of enzymes peaked at 5 weeks after injury, it was still detectable as long as 9 months into the repair process. These findings suggest that collagenase and stromelysin participate in the long-term remodeling of corneal repair tissue, a mechanism that restores tensile strength and clarity to the tissue. However, the mechanism by which MMPs are activated in vivo remains unknown. Our in vitro findings suggest that IL-1α is a candidate for the role of intermediate in activation of new collagenase synthesis in repair fibroblasts in vivo.

The transition from quiescent stromal cell to repair fibroblast after injury involves the activation of a number of other genes in addition to collagenase. These include such contractile cytoskeletal proteins as α-smooth muscle-specific actin, the extracellular matrix receptor α5β1 integrin, and fibronectin. The repair phenotype is also characterized by a number of new cell behaviors, including entry into the cell cycle and an increase in migratory activity. Expression of many of these genes and cell behaviors is characteristic of early-passage fibroblasts in culture and exogenously added cytokines similar to those found in repair tissue (IL-8 or TGFβ) can stimulate these activities. Similar to IL-1α, it seems likely that these cytokines can also act in an autocrine manner. Although IL-1α is an important mediator of CB-stimulated collagenase gene expression, its capacity to induce collagenase synthesis in corneal fibroblasts can also be inhibited with cycloheximide. This suggests the requirement of an additional protein or proteins in the cascade leading to new collagenase synthesis. Could this also be a cytokine? We have found that early-passage corneal fibroblast cultures synthesize a number of cytokines in addition to IL-1α, including TGFβ and IL-8, IL-6, and platelet-derived growth factor (PDGF) (KJ Strissel, JA West-Mays, ME Fini, unpublished data), several of which have been shown to stimulate or inhibit collagenase expression when added exogenously to cell cultures. Each of these autocrine cytokines may act independently in regulating the repair fibroblast phenotype. However, in that cytokines are known to regulate expression of other cytokines, it seems more likely that they act together in an autocrine network.

In this study, we test the hypothesis that IL-1α is a central signaling intermediate for controlling the repair phenotype of corneal fibroblasts. We concentrate our attention on mechanisms regulating IL-8 family members and collagenase. We show that expression of IL-8, like collagenase, is dependent on synthesis of IL-1α in early-passage fibroblasts. However, IL-8 does not appear to be a part of the autocrine cascade leading to new collagenase expression. We then demonstrate that primary fibroblasts, directly isolated from corneal repair tissue are equivalent to early-passage...
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cells in their competency for expressing IL-1α. Our findings further show that similar to early-passage fibroblasts, repair fibroblasts require IL-1α for new IL-8 and collagenase synthesis.

METHODS

Fibroblast Culture

Animal procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. New Zealand White rabbits (3 kg) were killed by intravenous injection of sodium pentobarbital. Corneal fibroblasts were isolated and cultured from healthy corneas as previously described. Briefly, corneas were dissected from the rabbits and 9-mm central discs were removed by trephination. The corneal stromas were separated from the overlying epithelium and underlying endothelium by overnight treatment with trypsin at 4°C. The fibroblasts were freed from the corneal stroma by digestion in bacterial collagenase (5 mg/ml). For studies on primary fibroblasts, cells were plated for an experiment immediately after isolation. To prepare passaged cultures, the cells released from six corneas were plated into a 100-mm cell-culture dish. When these cultures had multiplied to confluence, they were removed from the dish with trypsin and split into three new culture dishes (first passage).Passaging was continued in this way. The cells were used for an experiment before the fourth or fifth passage.

Cell Culture Treatment Protocol

Equal densities (1.5 × 10⁵ to 2 × 10⁵ cells/well) of primary fibroblasts, or passaged fibroblasts freshly trypsinized from their culture dish, were plated in 24-well cluster dishes with medium containing 10% supplemented calf serum (Hyclone, Logan, UT). The corneal stromas were separated from the corneal epithelium and underlying endothelium by overnight treatment with trypsin at 4°C. The fibroblasts were freed from the corneal stroma by digestion in bacterial collagenase (5 mg/ml). For studies on primary fibroblasts, cells were plated for an experiment immediately after isolation. To prepare passaged cultures, the cells released from six corneas were plated into a 100-mm cell-culture dish. When these cultures had multiplied to confluence, they were removed from the dish with trypsin and split into three new culture dishes (first passage). Passaging was continued in this way. The cells were used for an experiment before the fourth or fifth passage.

Analysis of Collagenase Protein Synthesis

During treatment of the cells, ¹⁴C]-Methionine (New England Nuclear, Boston, MA) was added to culture medium at 110 μCi/ml for biosynthetic labeling of proteins. After treatment, media containing secreted cell proteins was collected, and equal-sized samples were run on 8% sodium dodecyl sulfate–polyacrylamide gels. Gels were dried and autoradiographed to exhibit synthesized and secreted proteins. The total ¹⁴C]-labeled, secreted protein in each gel lane was quantified by volume densitometry (Molecular Dynamics, Sunnyvale, CA) and compared to ensure that treatments did not affect overall secreted protein synthesis. The relative amount of collagenase was determined by immunoprecipitation from pooled treatment replicates with 10 μl of sheep antiserum or 25 μl of an "oligoclonal" mixture of five monoclonal antibodies reactive against rabbit collagenase-1. In previous experiments, media were also analyzed with nonimmune sheep serum as a control for antibody specificity. The total amount of collagenase synthesized and secreted was determined by volume densitometry.

Assays for Quantifying Interleukin-1α and Interleukin-8 Protein

Cells were plated and treated as outlined earlier. After treatment, culture medium and cells were collected from each well and assayed for total IL-1α content by radioimmunoassay using a kit specific for the rabbit species (Cytokine Sciences). To determine the corresponding levels of IL-8 protein, the culture medium and cell lysates were analyzed using an enzyme-amplified sensitivity immunoassay (EASIA), which was specific for human IL-8 (Incstar, Stillwater, MN), but which was reactive against rabbit IL-8 in initial tests. Each sample was assayed in duplicate or triplicate and determinations were averaged. Statistically significant differences were determined by Student’s t-test. A value of \( P < 0.05 \) was considered significant.

Molecular Cloning of Interleukin-8-Related, Human Neutrophil-Activating Protein ENA-78 cDNA

To isolate cDNAs for cytokines expressed by rabbit corneal fibroblasts, we cloned one of the IL-8 family members, termed ENA-78. This rabbit cDNA fragment was cloned using standard methodology for reverse transcription–polymerase chain reaction: Total RNA, free of genomic DNA from fibroblast cultures taken from rabbit corneal stroma, was isolated by gua-
The cDNA was reverse-transcribed (Superscript, BRL, Bethesda, MD) in a 20-μl reaction containing 2 to 5 μg of total RNA and then primed with an antisense oligonucleotide primer. Five μl (25%) of the reverse-transcribed product was amplified with 1 U of Taq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT), using the same antisense and an appropriate sense primer. Amplification was performed in a 50-μl reaction in a Perkin-Elmer-Cetus thermocycler at 93°C for 1 minute, 58°C for 1 minute, and 70°C for 30 cycles. Amplified products were cloned into the vector pCR II, using the TA cloning kit (Invitrogen, San Diego, CA) and sequenced using the Sequenase kit (USB, Cleveland, OH). Sequence data were entered into the Genbank DNA sequence data-base. The cloned rabbit fragment of 380 base pairs showed 85% identity and 88% similarity at the conceptual translation level with human ENA-78.

RNA Analysis
Confluent plates of passaged cultures were split 1:3 into 100-mm dishes and allowed to multiply until they reached approximately 90% confluence. Before starting an experiment, the culture medium was changed, and treatment reagents were added. In all experiments, the cells were treated for 24 hours. Total RNA was isolated and analyzed by Northern blot analysis. Rabbit cDNA probes for collagenase, IL-1α, and IL-8 were labeled with 32P by random priming. Loading equivalence between gel lanes was ascertained by probing for glyceraldehyde-3-phosphate dehydrogenase message using a human cDNA probe.

Corneal Surgery
The following animal procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional animal welfare committee. Penetrating keratectomy was performed to remove a full-thickness section of the cornea of one eye of eight New Zealand White rabbits using a 2-mm trephine, as previously described. The contralateral eye was not injured and served as a control. Eyes were monitored to ensure that none became inflamed. After 3 weeks of healing, the animals were euthanized by lethal injection of sodium pentobarbital, and the corneas were removed. Using the 2-mm trephine, the repair tissue that had come to occupy the original excisional wound was isolated. Primary cultures of repair fibroblasts from the corneal buttons were isolated using the procedure identical to that described for healthy corneas. Identical sections were removed from the uninjured cornea of each rabbit, and these fibroblasts served as controls. The primary fibroblasts were plated for experiments immediately after isolation.

RESULTS
Endogenous Interleukin-1α Regulates Expression of Interleukin-8 in Early-Passage Corneal Fibroblasts
In initial experiments, we attempted to learn whether disruption of the actin cytoskeleton induces IL-8 expression as it induces IL-1α. In a representative experiment (Fig. 1), early-passage corneal fibroblasts were plated at equal density into 24-well culture dishes and either left untrtreated or treated with cytochalasin B or interleukin-1 for 24 hours. Total interleukin-8 protein (in picograms) was determined by enzyme-amplified sensitivity immunoassay for each sample. Error bars represent standard deviation from the mean. Con = untreated control cultures.

FIGURE 1. Effects of cytochalasin B and human recombinant interleukin-1 on levels of interleukin-8 protein in early-passage corneal fibroblasts. Culture medium was collected from triplicate wells containing equal numbers of cells left untreated or were treated with cytochalasin B or interleukin-1 for 24 hours. Total interleukin-8 protein (in picograms) was determined by enzyme-amplified sensitivity immunoassay for each sample. Error bars represent standard deviation from the mean. Con = untreated control cultures.

In early-passage corneal fibroblasts, IL-1α stimulated IL-8 production significantly more than CB, which induced IL-8 production similarly to CB. IL-8 production was also induced by EASIA in early-passage corneal fibroblasts treated with IL-1α, which was similar to findings for CB.
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FIGURE 2. Effects of neutralization by interleukin-1α on cytochalasin B-induced levels of interleukin-8 in early-passage corneal fibroblasts. Cell lysate was collected from triplicate wells of equal numbers of cells left untreated, treated with cytochalasin B, or with cytochalasin B and increasing doses of interleukin-1 receptor antagonist for 24 hours (10, 100, and 1000 ng/ml). Total picograms of interleukin-8 protein were determined by enzyme-amplified sensitivity immunoassay for each sample. Error bars represent standard deviation from the mean.

We next determined whether endogenous IL-1α, stimulated in response to disruption of the actin cytoskeleton, regulates levels of IL-8 by treating early-passage cells with CB alone, or simultaneously with IL-1 receptor antagonist (IL-1ra) to block IL-1α activity for 24 hours. After treatment, the levels of IL-8 protein were measured by EASIA. If inhibiting IL-1α activity reduces the levels of IL-8 stimulated by CB, we can conclude that IL-1α is a necessary mediator of CB-induced IL-8 expression. In results of a representative experiment (Fig. 2), a significant increase (1.2-fold; P = 0.006) in the amount of IL-8 protein was found in the cell lysates (cell-associated) of cultures treated with CB (390 ± 39.9 pg) versus that found in untreated control medium (20 pg).

We have similarly found that the constitutive levels of IL-8 and IL-1α mRNAs precede that of collagenase mRNA. Expression levels of IL-8 mRNA were found in treated cells. Importantly, treatment with CB also produced a significant upregulation of ENA-78 message levels. Northern blot analysis was employed to determine the relative changes in ENA-78 message levels in untreated cultures. Therefore, the elevated constitutive levels of IL-8 in these cultures are probably caused by increased levels of IL-1α.

Together these data demonstrate that IL-1α is a required mediator of IL-8 synthesis induced in response to disruption of the actin cytoskeleton in early-passage corneal fibroblasts.

Induction of mRNA Levels of an Interleukin-8-Related Factor

Earlier work has shown that IL-1 can stimulate secretion of IL-8-related factors, including human neutrophil-activating protein, also known as ENA-78, which, like IL-8, belongs to the CXC family of peptides. Actions of ENA-78 are similar to those of other members of the IL-8 family, including activation of neutrophils and induction of chemotaxis. Thus, we have cloned the rabbit cDNA for ENA-78 we tested whether this cytokine, similar to IL-8, could be induced by disruption of the actin cytoskeleton. In a representative experiment (Fig. 3A), early-passage corneal fibroblasts were either left untreated or were treated with CB. After 24 hours of treatment, cells were collected for Northern blot analysis to determine the relative changes in message levels for ENA-78 that may accompany those changes we have observed for collagenase and IL-1α. Substantial increases in IL-1α and collagenase mRNAs were observed in treated cells. Importantly, treatment with CB also produced a significant upregulation in ENA-78 message levels. Northern blot analysis was further used to compare the timing of IL-1α and ENA-78 inductions in response to CB. Representative treatment times with CB of 8, 15, and 20 hours are shown (Fig. 3B). A small increase in IL-1α message is first observed after 8 hours of treatment with CB. Induction of ENA-78 mRNA is then observed at the next measured time point of 10 hours. The increases in ENA-78 and IL-1α mRNAs precede that of collagenase, which is observed at 15 hours. Thus, like IL-8, ENA-78 is induced by disruption of the actin cytoskeleton. Furthermore, the time of induction of ENA-78 relative to that of IL-1α, is consistent with the hypothe-
Time (hours)

FIGURE 3. Effects of cytochalasin B on mRNA levels in interleukin-1α, collagenase, and ENA-78 (an interleukin-8-related chemokine) in early-passage corneal fibroblasts. (A) Cells were treated with cytochalasin B or were left untreated, and RNA was collected 24 hours later. This Northern blot was first probed for interleukin-1α and then was stripped and reprobed for collagenase and ENA-78. The blot also was probed with GAPD (not shown) to ensure that the lanes were equally loaded. (B) Cells were treated with cytochalasin B or were left untreated and were collected for isolation of RNA, during a 20-hour period. Northern blotted RNA was probed as in A, and reprobed with GAPD. Relative mRNA levels were quantitated by densitometry. The fold stimulation, calculated by subtracting the unstimulated level of mRNA for that time point from the stimulated level, is graphed. CL = collagenase; con = untreated control cultures; GAPD = glyceraldehyde-3-phosphate dehydrogenase.

Effect of Interleukin-8 on New Collagenase Synthesis

The autocrine IL-1α is one step closer to being the direct stimulator of collagenase synthesis than are agents that disrupt the actin cytoskeleton. However, protein synthesis inhibitors block stimulation of collagenase synthesis by exogenous IL-1, suggesting a need for an additional protein to be synthesized. In the following experiment we tested whether IL-8 may play a role in the cascade of events leading to expression of new collagenase genes. Early-passage fibroblasts were either left untreated or were treated with exogenous IL-8 (10 ng/ml) for 24 hours. As a positive control, cells were also treated with exogenous IL-1α (10 ng/ml). Newly synthesized proteins labeled with [35S]methionine were displayed on 8% polyacrylamide gels (Fig. 4A). Total protein synthesis appeared relatively equal in all cultures. However, synthesis of a 53-kDa protein was selectively stimulated in the fibroblasts treated with exogenous IL-1α. In contrast, cells treated with exogenous IL-8 did not exhibit a change in synthesis of the 53-kDa protein. To confirm the identity of the 53-kDa protein as collagenase, the duplicate samples were pooled and incubated with a specific antiserum against rabbit collagenase to immunoprecipitate and quantitate the protein (Fig. 4B). These data confirmed that treatment with IL-8 did not cause a change in synthesis of the collagenase protein. Repeated experiments had been performed, using different dosages of exogenous IL-8 (1, 10, and 100 ng/ml) only to confirm the findings above. To further test whether IL-8 is a mediator in the expression of collagenase genes, we treated cells with CB, a stimula-

FIGURE 4. Effects of treatment with exogenous interleukin-8 on synthesis of collagenase. (A) Culture medium was collected from duplicate wells of cells left untreated (−) or treated with interleukin-8 for 24 hours. For comparison, duplicate wells were also treated with interleukin-N. The autoradiograph displays the [35S]methionine-labeled secreted protein profile. Arrows point to the 53- and 51-kDa proteins corresponding to collagenase and the related metalloproteinase, stromelysin, respectively. (B) Replicate samples from each culture well analyzed in (A) were pooled and subjected to immunoprecipitation analysis with collagenase antiserum, or nonimmune serum (not shown). Arrows indicate the 53-kDa collagenase proenzyme and its 57-kDa glycosylated form.
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Autocrine Interleukin-1α Synthesis in Repair Fibroblasts

To learn whether the transition in the cell culture to competency for expressing IL-1α might have a correlate in vivo during tissue repair, we used a corneal keratectomy model, which allows the isolation of pure repair tissues. We determined whether IL-1α is synthesized constitutively in fibroblasts freshly isolated from this 3-week-old repair tissue, and whether this is sustained through an autocrine feedback loop. The levels of IL-1α protein were measured by radioimmunoassay analysis from duplicates of primary cultures of wound fibroblasts that were either left untreated or treated overnight with inhibitors of IL-1α activity (IL-1α neutralizing antibody or IL-1ra). Undetectable levels of IL-1α protein were measured in the cell lysates (cell-associated IL-1α) of primary cultures of fibroblasts from uninjured corneas. In contrast, cell lysates from the wound fibroblasts in the primary cultures exhibited substantial levels of IL-1α protein (128 ± 27.5 pg). Interestingly, primary cultures of wound fibroblasts treated with IL-1ra have significantly reduced (42.8 ± 17.3 pg; 3-fold; P < 0.001) levels of cell-associated IL-1α protein in comparison with that found in the wound fibroblasts in the untreated cultures. Those fibroblasts supplied with IL-1α antibody had an even greater reduction (4 ± 0 pg; 32-fold; P < 0.001) in cell-associated IL-1α protein than that seen in the untreated cultures. Small but detectable amounts of IL-1α protein (5.5 pg) were released into the cell-conditioned medium from the cultured wound fibroblasts compared with undetectable levels in control cultures (not shown); and likewise, a corresponding reduction to undetectable levels of IL-1α protein in the supernatants from primary cultures of wound fibroblasts treated with IL-1α antibody was also found (not shown).

Endogenous Interleukin-1α Regulates Collagenase and Interleukin-8 Synthesis in Repair Fibroblasts

Considering that the IL-1α loop was not activated in fibroblasts freshly isolated from the uninjured cornea, we predicted that these cells would not express IL-8 constitutively, whereas wound fibroblasts would express IL-8 under control of the IL-1α autocrine. To test this hypothesis, we first determined the levels of IL-8 protein in cultures of fibroblasts isolated from uninjured tissue using EASIA assay (Fig. 6). As predicted, we found that untreated primary cultures, which do not synthesize detectable levels of IL-1α, do not express detectable levels of IL-8 protein. However, treatment of cells with exogenous IL-1 produced a substantial increase in the amount of IL-8 protein secreted into the cell culture medium (437 ± 98.6 pg; P < 0.001). In fact these levels were similar to those measured in early-passage fibroblasts treated with CB. A small, but significant increase (30 ± 10.6 pg; P < 0.001) was also found in the cell lysates (not shown). These experiments demonstrate that expression of IL-8 is responsive to exogenous IL-1, suggesting that the lack of constitutive expression in primary cells is caused by a lack of IL-1α loop activation.

In a representative experiment (Fig. 7), levels of IL-8 in cultured wound fibroblasts were determined by EASIA assay. We found that, unlike primary control fibroblasts, wound fibroblasts synthesize substantial levels of IL-8 (549 pg ± 22.7 pg; P < 0.001) protein.
We further examined whether IL-1α is regulating IL-8 synthesis in the repair fibroblasts. To accomplish this, wound fibroblasts were treated with IL-1ra (1000 ng/ml) to block IL-1α activity. Results show (Fig. 7) that although the IL-1ra did not block all of the IL-8 protein synthesized, its levels were significantly reduced (1.4-fold; \( P < 0.001 \)). These findings show that IL-8 synthesis in repair fibroblasts is at least partially dependent on activation of the IL-1α intermediate.

To test whether the IL-1α autocrine loop regulates collagenase expression in repair fibroblasts, primary fibroblasts from the wounded region were isolated from the stromas and cultured in the presence of \(^{35}\)S-methionine for 24 hours to label newly synthesized proteins. Primary fibroblasts freshly harvested from the central region of the uninjured, contralateral corneas served as controls. Newly synthesized proteins secreted into the culture media from duplicate cultures were collected and displayed on 8% polyacrylamide gels. Synthesis of a 53-kDa protein, which corresponds to the molecular weight of collagenase, was selectively stimulated in the wound fibroblasts (not shown). To confirm the identity of the 53-kDa protein as collagenase and to quantitate its levels, the duplicate samples were pooled and incubated with a specific antiserum against rabbit collagenase to immunoprecipitate and isolate the protein. A representative experiment is shown in Figure 8. The results show that fibroblasts from the healing, penetrating keratectomy wounds synthesize a substantial amount of collagenase versus the undetectable levels found in control primary fibroblasts. These findings corroborate results of our earlier studies, which demonstrate that primary fibroblasts extracted from healing corneas synthesize significant levels of collagenase. However, unique to this study is our investigation as to whether the IL-1α intermediate contributes to the synthesis of new collagenase in the repair fibroblasts. To determine
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In this study, we supplied the duplicate cultures of wound-repair fibroblasts (Fig. 8) with either IL-1α or IL-1α neutralizing antibody to interfere with IL-1α activity. Addition of the IL-1α antibody to wound fibroblasts caused a substantial reduction of immunoprecipitated collagenase (3.2-fold reduction) in comparison with wound fibroblasts without antibody. Addition of the IL-1α antibody also reduced the amount of immunoprecipitated collagenase—only slightly, however (1.2-fold reduction), in comparison with that measured in the untreated wound fibroblasts. Thus, by blocking IL-1α activity, collagenase synthesis in repair fibroblasts was significantly suppressed.

Together these findings show that, unlike fibroblasts from the uninjured cornea, repair fibroblasts are competent to express the IL-1α autocrine, and that synthesis of IL-8 and collagenase is at least partially dependent on activation of this intermediate.

DISCUSSION

In a recent study, we demonstrated that the ability of early-passage corneal fibroblasts to synthesize collagenase in response to agents that alter the actin cytoskeleton is dependent on activation of an IL-1α autocrine feedback loop. In the current investigation, we have expanded on these findings and have shown that IL-1α is also a necessary mediator for synthesis of additional genes stimulated by a shape-altering agent, including IL-8 and ENA-78. Our findings further suggest a similar role for IL-1α in vivo: In this case, repair fibroblasts freshly isolated from healing corneal wounds in rabbits synthesize the cytokine IL-1α when placed in culture. Neutralization of IL-1α activity in the repair fibroblasts resulted in a reduction in the amount of IL-1α protein synthesized, demonstrating that levels of endogenous IL-1α in these cells are also maintained through an autocrine feedback loop. The IL-1α autocrine appears to be largely responsible for controlling expression of the repair genes, IL-8 and collagenase. This is unlike fibroblasts freshly isolated from the uninjured rabbit cornea, which do not express IL-1α in culture and therefore are not competent to express IL-8 or collagenase. Together these findings show that resident tissue fibroblasts become competent to activate the IL-1α feedback loop after wounding and that this loop plays a central role in regulating expression of genes characteristic of the repair–remodeling phenotype.

Classically, IL-1 (both in the IL-1α and IL-1β forms) is considered to be a proinflammatory cytokine that contributes to tissue destruction and inflammation, particularly in such disease circumstances as those in rheumatoid arthritis. However, recent work has revealed that IL-1 also acts as a regulator of fibroblast behavior associated with normal repair. For example, IL-1 can modulate focal adhesions of fibroblasts, and can regulate their migration and proliferation. Recently, we have shown that fibroblasts from the uninjured rabbit cornea acquire competence for IL-1α expression after passage in culture and that expression of this cytokine is a required intermediate for collagenase synthesis stimulated by agents that alter the actin cytoskeleton like CB, PMA, and trypsin. A similar role for IL-1α has also been demonstrated in human fibroblasts stimulated with ultraviolet irradiation. In this case, ultraviolet-A irradiation of dermal fibroblasts induces expression of a cytokine network consisting of IL-1α, IL-1β, and IL-6, which, through interrelated autocrine loops, stimulates collagenase synthesis. Similarly, serotonin-induced collagenase expression in smooth muscle cells cultured from the rat uterus has been shown to be mediated by an IL-1 feedback loop. Finally, the IL-1α autocrine has been shown to mediate synthesis of a related matrix metalloproteinase,stromelysin (MMP-3), in cultured rabbit articular chondrocytes in response to an extracellular matrix peptide. Findings in these cell culture studies collectively implicate autocrine IL-1α as a common mediator for tissue remodeling by virtue of its role as a regulator of expression of the enzymes required for this process.

The results reported in this study contain the first demonstration of IL-1α loop activation in a normal remodeling situation. Although not a direct demonstration of a regulatory connection, IL-1α and collagen gene have, however, been colocalized to mesenchymal cells during the acute remodeling phase of heart morphogenesis in the rat. Evidence in the literature suggests that activation of an IL-1α feedback loop may also occur in some abnormal remodeling situations. For example, cultured dermal fibroblasts from patients with systemic sclerosis constitutively, unlike healthy dermal fibroblasts, express IL-1α mRNA and protein. Expression of IL-1α has also been reported in metastatic cancer cells of fibroblast origin. These results suggest that activation of the IL-1α loop may be a transition of general importance that is occurring in a number of different cell types and that it is not limited to normal tissue remodeling of the cornea.

We emphasize that our experiments did not determine whether corneal repair fibroblasts were synthesizing IL-1α in situ. In fact, it seems likely that much of the IL-1α synthesis we observed in cell culture was stimulated by the treatments used to remove the cells from the cornea, in that these treatments can cause actin cytoskeletal rearrangements. What we have determined is cell competency for IL-1α synthesis. Fibroblasts, freshly isolated from the healing cornea, are phenotypically different from those of the nonremodeling cornea, in that they have acquired competence to synthesize the IL-1α autocrine. Like fibroblasts that...
have been passaged in vitro in culture, the repair fibroblasts must have undergone a transition in vivo to become capable of making the IL-1α autocrine. Thus, we have shown that transition to the “IL-1α-producing” phenotype is not simply a cell-culture phenomenon but is a molecular event that occurs in vivo as cells become activated during tissue repair. We hypothesize that in vivo, these competent cells would then be able to respond, by synthesizing IL-1α, to the changes in cell shape that occur as cells release from or exert tension on the extracellular matrix during repair tissue remodeling.

Our experiments in early-passage cultures of corneal fibroblasts revealed that IL-1α is a necessary intermediate for constitutive synthesis of IL-8, and for stimulated synthesis in response to shape change. In addition, Northern blot data suggest that IL-1α may also regulate other members of the CXC family of peptides (ENA-78) to which IL-8 belongs. The regulation of IL-8 by IL-1 is not unique to corneal fibroblasts. For example, a recent investigation showed that endogenous IL-8 synthesis in cultures of renal fibroblasts is controlled by an intrinsic IL-1α feedback loop. It has become well known that exogenous IL-1α has been shown to stimulate IL-8 production in a number of different cells, including macrophages, epithelial cells, endothelial cells, and fibroblasts. A unique contribution of this study, however, is the observation that IL-1α-producing phenotype controls subsequent expression of IL-8.

Interleukin-8 has been shown to play an important role in inflammation because of its capacity to recruit T cells and nonspecific inflammatory cells into sites of inflammation, and also because of its effects on neutrophils, including degranulation and the upregulation of integrin receptors. It is also known to induce an angiogenic response in a number of tissues, including the cornea. However, we have found that fibroblasts directly cultured from healing corneas, when inflammation is not apparent, are competent in their ability of repair fibroblasts to express IL-8 is dependent on activation of the IL-1α loop. Thus, transition to the IL-1α-producing phenotype controls subsequent expression of IL-8.

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cultural evidence has implicated the repair epithelium as a source of substances required for activation of the repair fibroblast. Messenger RNA for a number of cytokines have been localized to the corneal epithelium, including IL-1β, IL-6, and IL-10. Corneal epithelial cells in culture synthesize two major cytokines that control collagenase expression: TGF-β2 and IL-1α. These cytokines might also be released into the extracellular space after wounding. Although short exposure to IL-1 does not activate the IL-1α loop, it is possible that longer exposure to this cytokine may enable the cell to reach a particular threshold for competence. Although activation in culture may be mediated by a different external stimulator, it seems likely that activation in vitro or in vivo may share common intracellular pathways. Using our cultured corneal cell transition model, we are investigating competency in expressing the IL-1α autocrine.

The findings of this study demonstrate that competency in the expression of IL-1α feedback loop is a key event in the transition to the repair phenotype in corneal fibroblasts. This cytokine acts as a mediator for such effector genes as collagenase but also for additional cytokines, IL-8, for example. Data from other investigations have demonstrated that IL-1α also controls expression of additional cytokines including, PDGF, IL-6, IL-8, IL-10, and IL-12. All of these cytokines are expressed by corneal fibroblasts as described in this article. Thus, we surmise that IL-1α may act as a focal point for an interconnecting cascade of autocrine cytokines that regulate cellular behaviors associated with corneal remodeling and the expression of repair genes. This cytokine may therefore be an important therapeutic target in fibrotic diseases that involve inappropriate or excessive expression of repair genes.

Key Words
- collagenase, cornea, interleukin-1, interleukin-8, wound healing

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