IL-8 Gene Expression in Cultures of Human Corneal Epithelial Cells and Keratocytes

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Purpose. To determine if human corneal keratocytes and epithelial cells synthesize and release IL-8 after stimulation with selected proinflammatory cytokines.

Methods. Human corneal keratocytes and epithelial cells were isolated from human corneal buttons and grown independently in vitro. Epithelial cell cultures stained positive in immunofluorescent tests with antibody specific for keratin (AE1/AE3), whereas keratocyte cultures were unreactive. Both cell types reacted with anti-vimentin antibody. Cultures of the two cell types were treated with various concentrations of human recombinant interleukin-1 alpha or TNF-alpha. Culture supernatants were then assayed at timed intervals by enzyme-linked immunosorbent assay for IL-8 content. Cytokine mRNA levels in cell lysates were monitored by Northern blot analysis.

Results. Exposure of corneal keratocytes and epithelial cells to either interleukin-1 alpha or TNF-alpha stimulated IL-8 mRNA synthesis and IL-8 production in a dose-response fashion. It was also found that TNF-alpha stimulated the synthesis of comparable amounts of IL-8 in both cell types. However, when IL-8 synthesis between the two cell types was compared after interleukin-1 alpha stimulation it was found that keratocytes synthesized 33 times more IL-8 than did epithelial cells.

Conclusions. The results establish that pro-inflammatory cytokines can induce IL-8 synthesis in both human corneal epithelial cells and human corneal keratocytes. They also suggest that interleukin-1 alpha may play a more active role in amplifying inflammatory responses in the stroma than in the epithelial layer of the cornea. Investig Ophthalmol Vis Sci. 1993;34:3199-3206.

The cornea of the eye represents a specialized epidermal tissue. It plays an important role in vision by transmitting optical images to the retina and by protecting intraocular contents from infectious agents. Like the skin, the cornea is highly susceptible to the deleterious effects of inflammatory cells. However, in this tissue the vascularization, fibrosis, and scarring that can follow inflammation may lead to blindness. IL-8 is a proinflammatory cytokine that can be released by both epithelial cells and fibroblasts at a number of body surfaces. This cytokine plays an important role in inflammation through its capacity to recruit T-cells and nonspecific inflammatory cells into sites of inflammation and by its capacity to activate neutrophils. Because IL-8 has the capacity to amplify inflammatory processes at body surfaces, one would anticipate that its synthesis would be highly regulated in the cornea because it would help to minimize tissue damage and vision loss.

It has long been possible to grow large numbers of human corneal keratocytes in tissue culture. Here we report a technique to grow large numbers of human corneal epithelial cells in vitro. The ability to grow epithelial cells as well as keratocytes in pure culture provided a unique opportunity to compare the capacity of the two corneal cell types to synthesize IL-8. Interleukin-1 alpha (IL-1 alpha) and tumor necrosis factor-alpha (TNF-alpha) are two primary cytokines known to stimulate synthesis of IL-8 in a number of nonocular cell types. Accordingly, we investigated how readily these two cytokines would induce IL-8 production in human corneal epithelial cells and in human corneal keratocytes. We report here that both cultured cell types could synthesize IL-8. However, we found that IL-1 alpha was a more potent inducer of IL-8 in keratocytes than in epithelial cells.
MATERIALS AND METHODS

Establishment of Human Corneal Epithelial and Keratocyte Cell Cultures

Corneas were obtained from the National Disease Research Interchange (Philadelphia, PA), or the Alabama Eye and Tissue Bank (Mobile, AL), and placed into culture within 4 days of enucleation. After trimming off the corneoscleral rims and peeling off the endothelial cell layer, the corneas were washed repeatedly in RPMI-1640 medium (Gibco, Grand Island, NY). To dissociate the epithelial layer from the stroma, the concave side of the cornea was placed in a 60-mm petri dish on a drop of Dispase, grade II (Boehringer Mannheim, Indianapolis, IN) containing 25 caseinolytic units/ml Dispase and 20 μg/ml gentamicin in Hanks balanced salt solution. Care was taken to prevent direct contact of epithelial layer with Dispase. After incubation with Dispase at 4°C for 24 hours in a humidified environment, the epithelial cell layer was lifted from the stroma with a pair of jeweler’s forceps.

A single cell suspension was prepared by trypsinizing the epithelial cell layer in 1.5 ml of 0.05% trypsin in 0.53 mmol/l ethylenediaminetetraacetic acid for 10 to 15 minutes at 37°C, and then passing the preparation through a 20 g hypodermic needle 4 to 5 times with moderate pressure. Trypsinization was halted by addition of 10 mg/ml soybean trypsin inhibitor (Gibco, Grand Island, NY) in phosphate-buffered saline without Ca++ and Mg++. The cell suspension was centrifuged at 1000g for 10 minutes and resuspended in 5 μg/ml gentamicin. The cells from each cornea were seeded into two Falcon Primaria positively charged 25 cm² tissue culture flasks (Becton Dickinson, Lincoln Park, NJ) with 5 ml medium per flask, and incubated at 37°C in a 5% CO2 atmosphere. After 24 hours, unattached cells and medium were discarded and 5 ml fresh medium was added. The epithelial cells were subcultured when they were approximately 90% confluent. The media was aspirated and replaced with 2 ml media containing the desired concentration of either recombinant human TNF-α (rTNF-α) (Genzyme, Cambridge, MA) or recombinant human IL-1α (rIL-1α) (R&D Systems, Minneapolis, MN). At selected times after induction, media was removed and frozen at −20°C for subsequent cytokine analysis. The cells were then removed from the flasks and counted in a hemocytometer.

Keratocyte cultures were grown in Dulbecco’s modified Eagle’s medium containing 10% FCS until they were approximately 90% confluent. The medium was then replaced with Opti-MEM (Gibco) without FCS. To eliminate possible stimulation of cytokine production by FCS, Dulbecco’s modified Eagle’s medium was replaced with Opti-MEM 3 days before induction experiments and changed daily. The medium was then aspirated and replaced with Opti-MEM containing either human rTNF-α or human rIL-1α. The supernatants were removed from flasks of treated cells at varying times and frozen at −20°C for subsequent cytokine analysis. All reagents and media were found to be negative for lipopolysaccharide by the Limulus amebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, MA).

Cytokine Assays

Immunoreactive IL-8 was quantified using enzyme-linked immunosorbent assay kits obtained from R&D.
IL-8 Gene Expression in Human Corneas

RNA Isolation and Northern Blot Analysis

Epithelial and keratocyte cultures in 25 cm² flasks were established from individual corneal donors. After each culture was treated with the desired cytokine, total cellular RNA was isolated from cell cultures by either the rapid total RNA isolation method described by Maniatis et al [16] or isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method. [17] Fifteen micrograms of the RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde, [16] and then stained with 1 μg/ml ethidium bromide to confirm that all of the lanes contained equal amounts of RNA. Gels were then blotted onto Magnagraph nylon filters (Micron Separations Inc., Westborough, MA), and baked 1 hour at 80°C under vacuum. The RNA blots generated from the two cell types were placed together in a heat-sealable bag and hybridized overnight at 42°C with a 32P-labeled cDNA probe for IL-8. The blots were then removed from the bag and washed at high stringency as described by the membrane manufacturer’s instructions. The blots were then stripped and reprobed with a 32P-labeled cDNA probe for glyceraldehyde-3-phosphate dehydrogenase. The cell numbers were estimated by counting cells in identical flasks that were not lysed by extraction buffer.

Preparation of Radiolabeled Probes

The IL-8 cDNA clone was donated by Dr. J. Oppenheim (Laboratory of Molecular Immunoregulation, NCI-FCRDC, Frederick, MD). The probe for glyceraldehyde-3-phosphate dehydrogenase was purchased from ATCC (Rockville, MD). Probes were labeled by random primer extension using the DECAprime DNA labeling kit (Ambion, Inc., Austin, TX) and [α-32P]CTP (Amersham Corp., Arlington Heights, IL) to a specific activity of ≥ 4 x 10⁶ cpm/μg.

RESULTS

Generation of Epithelial and Keratocyte Cell Cultures from Human Corneas

Figure 1 shows the morphology of epithelial cells and keratocytes 2 weeks after being placed in culture. The corneal epithelium layer gave rise to cultures composed almost entirely of polygonal-shaped cells characteristic of stratified squamous epithelium. Monolayers of cells established from the stromal layer of corneal buttons were devoid of polygonal-shaped cells. These cultures were composed entirely of flat spindle-shaped cells possessing the characteristic shape of fibroblasts.

To confirm that different corneal cell types had been established in culture, we performed immunofluorescent studies using anti-keratin monoclonal antibody (Figure 2). Keratin is known to be present in the cytoplasm of epithelial cells, but absent in keratocytes. [18-20] It was found that corneal epithelial monolayers reacted strongly with anti-keratin whereas corneal keratocyte monolayers failed to exhibit fluorescence. Both cell types reacted with anti-vimentin whereas neither cell line reacted with anti-HSV glycoprotein D monoclonal antibody (data not shown). Thus, the fluorescent antibody studies confirmed that two distinct types of corneal cells had been established in tissue culture.

IL-8 Gene Expression in Corneal Epithelial Cells and Keratocytes Exposed to IL-1α and TNF-α

IL-8 gene expression in human corneal epithelial cells exposed to IL-1α or TNF-α was initially evaluated by
FIGURE 2. Indirect immunofluorescent staining of secondary cultures of human corneal epithelial cells and keratocytes. Human corneal epithelial cells reacted with anti-vimentin (panel A) and anti-keratin AE1/AE3 (panel C). Human corneal keratocytes reacted with anti-vimentin (panel B) but did not react with anti-keratin AE1/AE3 (panel D). Neither cell type showed immunofluorescence after reaction with an antibody to an irrelevant antigen (data not shown). (160X).

Northern blotting techniques. The results in Figure 3 are representative of three independent experiments involving three different corneal donors. In unstimulated cells, IL-8–specific mRNA was not detected after hybridization to the 32P-labeled IL-8 cDNA probe (Figure 3A). In contrast, IL-8–specific mRNA was readily detectable by 3 hours postexposure in TNF-α– and IL-1α–treated cells. Maximal levels of IL-8 mRNA were detected at 3 hours postexposure. The levels of IL-8–specific mRNA then declined during the next 15 hours of incubation. Medium overlaying the cultures was assayed for IL-8 by enzyme-linked immunosorbent assay (Figure 3B). It was found that both IL-1α– and TNF-α–stimulated epithelial cells synthesized more than 30 ng/million cells of IL-8.

Keratocyte cell cultures established from the identical corneal donors as epithelial cell cultures were also evaluated for their response to IL-1α and TNF-α by Northern blots and enzyme-linked immunosorbent assays. A representative experiment is shown in Figure 4. In unstimulated keratocytes, IL-8–specific mRNA was undetectable (Figure 4A). After addition of TNF-α, IL-8 mRNA appeared with kinetics similar to and at levels comparable with that seen in TNF-α–stimulated epithelial cells. IL-8 mRNA was also detected in keratocytes after exposure to IL-1α. However, in these cells, maximal levels of IL-8 mRNA was reached at 6 hours postexposure rather than at 3 hours postexposure as noted in epithelial cultures.

Medium collected from individual flasks of keratoctyes was analyzed for IL-8 by enzyme-linked immunosorbent assay. Exposure of keratocytes to TNF-α resulted in the synthesis of 22 ng/million cells of IL-8. This was very similar to the amounts of IL-8 synthesized by epithelial cells exposed to TNF-α. In contrast, keratocytes exposed to IL-1α synthesized more than 650 ng/million cells of IL-8 during the 18 hours of incubation. This was more than 24 times the amount...
IL-8 Gene Expression in Corneal Epithelial Cells and Keratocytes Established from Additional Corneal Donors

To determine if the pattern of response of human corneal epithelial cells and keratocytes to IL-1α and TNF-α could be extrapolated to additional corneal donors, we investigated IL-8 synthesis in cell cultures established from four additional pairs of corneas. TNF-α significantly enhanced IL-8 synthesis in both epithelial cells and keratocytes generated from these corneas in a dose-dependent fashion (Figure 5A and B). Furthermore, the amounts of IL-8 synthesized by epithelial cells was not significantly different from the amounts synthesized by keratocytes (P > 0.05). Exposure of epithelial cells and keratocytes cell cultures to IL-1α also significantly enhanced IL-8 synthesis in a dose-dependent fashion (Figure 5, C and D). However, keratocytes on average synthesized 39 times more IL-8 than did epithelial cells (note differences in ordinate scale in Figure 5, C and D). This difference was highly significant (P ≤ 0.01).

DISCUSSION

IL-8 has been demonstrated within keratocytes in the stroma layer of whole human corneal buttons by histochemistry and in situ hybridization techniques. However, it was not known if the epithelial layer of the eye could also synthesize this proinflammatory cytokine. For this study, we developed a procedure to separate and culture epithelial cells from human corneas so IL-8 gene expression could be studied in these cells after exposure to IL-1α and TNF-α. It was found that exposure of epithelial cells to either of these two cytokines resulted in an increase in levels of IL-8-specific mRNA and IL-8 synthesis. These results therefore establish that corneal epithelial cells like corneal keratocytes can synthesize IL-8.

The development of techniques to grow independent cultures of corneal epithelial cells and keratocytes from individual corneal donors made it possible to compare IL-8 gene expression in the two cell types. We found that the expression of the IL-8 gene in epithelial cells and keratocytes after exposure to TNF-α was nearly identical in terms of the protein produced. In contrast, keratocytes exposed to IL-1α produced more than 33 times more IL-8 than that observed in IL-1α stimulated epithelial cells. These results therefore suggest that during inflammatory events involving release of TNF-α the epithelial and stromal layers of the cornea have the capacity to synthesize and release comparable quantities of IL-8. However, many times more IL-8 may be synthesized by the stroma as compared to the epithelial layer of the cornea during inflammatory events involving release of IL-1α.

It is possible that keratocyte cultures exposed to IL-1α produced more IL-8 than did epithelial cell cultures because they were more metabolically active and therefore able to support greater gene activity. However, two lines of evidence argue against this interpretation. First, all of our experiments were performed with subconfluent monolayers of epithelial cells. These cultures contained rapidly dividing cells at the
FIGURE 5. IL-8 synthesis in human corneal epithelial cells and keratocytes after treatment with IL-1α or TNF-α. Epithelial cell and keratocyte cultures established from four corneal donors were exposed to increasing concentrations of either IL-1α or TNF-α. Unstimulated cultures received media without IL-1α and TNF-α. At selected times after exposure, culture medium was removed and assayed for IL-8 by enzyme-linked immunosorbent assay. Values shown are the mean ± SEM of experiments performed on individual epithelial cell and keratocyte cultures established from the four donors (N = 4 at each dose and time point).

time of exposure to IL-1α or TNF-α, and had not lost their characteristic epithelial cell morphology. Second, if the epithelial cell cultures used in this study were quiescent in vitro one would anticipate that they would respond to other inducing cytokines with less vigor than keratocyte cultures. However, it was found that both cell types produced nearly identical levels of IL-8 after exposure to TNF-α. It was only after the two cell types were exposed to IL-1α that they exhibited dramatic differences in the quantities of IL-8 produced. Collectively, our results support the conclusion that differences in IL-8 synthesis reflect the fact that IL-8 gene expression in corneal keratocytes is more responsive to IL-1α than is IL-8 gene expression in corneal epithelial cells. At least two mechanisms could have accounted for the differences seen in IL-8 gene expression. First, it is known that epithelial cells as well as fibroblasts possess receptors for both IL-1α and TNF-α.22-28 Thus, it is possible that corneal keratocytes responded more vigorously to IL-1α than did epithelial cells because corneal keratocytes expressed more IL-1α receptors. Second, the differential expression of IL-8 could have been due to differences in the mechanisms by which IL-1α enhanced IL-8 mRNA levels. It is known that intracellular levels of proinflammatory cytokine mRNA can be regulated at the transcriptional level as well as at the level of mRNA stability.29-35 Thus, IL-1α may have enhanced the synthesis and/or stability of IL-8 mRNA molecules in keratocytes more so than in epithelial cells.
Both epithelial cells and keratocytes are possible sources of IL-1α and TNF-α within the eye. In addition, mononuclear inflammatory cells that readily infiltrate the eye after tissue damage can synthesize and release IL-1α and TNF-α. The results of this study suggest that corneal epithelial cells and keratocytes can both respond to these two cytokines by secreting IL-8 and thereby contribute to the amplification of inflammation on the eye surface. Our study suggests that corneal keratocytes have the capacity to synthesize many times more IL-8 than do corneal epithelial cells, so they may play a more important role than epithelial cells in amplifying inflammatory events that lead to vision loss. More studies are needed to determine if the results observed in vitro can be extrapolated to inflamed corneal tissue within the human eye.

Key Words
epithelial, keratocytes, TNF-α, IL-1α, IL-8

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