Differences in Interleukin-6 Gene Expression Between Cultured Human Corneal Epithelial Cells and Keratocytes

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**Purpose.** To determine whether interleukin-6 (IL-6) can be synthesized by human corneal keratocytes and epithelial cells.

**Methods.** Epithelial cells and keratocytes isolated from the same donor corneas were grown in vitro. After 2 to 3 passages, the cultures were exposed to varying concentrations of recombinant human interleukin-1 (IL-1α) or tumor necrosis factor (TNF-α). Culture supernatants subsequently underwent enzyme-linked immunosorbent assays for cytokine content. The levels of cytokine mRNA in cell lysates were monitored by the reverse transcription-polymerase chain reaction.

**Results.** Cultured human keratocytes stimulated with 100 U/ml IL-1α for 18 hours produced more than 160 ng IL-6 per 10⁶ cells. Under the same conditions 500 U/ml TNF-α induced approximately 5 ng IL-6. IL-6 mRNA, evident by 3 hours after exposure to either cytokine, accumulated and persisted through 18 hours. Exposure of epithelial cells to IL-1α or TNF-α induced minimal and transient expression of IL-6 mRNA and < 0.5 ng protein product per 10⁶ cells. The poor production of IL-6 did not reflect an inability of epithelial cells to respond to IL-1α and TNF-α because both cytokines induced these cells to make copious amounts of IL-8.

**Conclusions.** These results demonstrate that both IL-1α and TNF-α could induce keratocytes to produce nanogram levels of IL-6 but IL-1α was a 30-fold more effective inducer. In contrast, neither cytokine could stimulate epithelial cells to make more than picogram quantities of IL-6. The abundant IL-6 synthesized by keratocytes may promote various activities including specific immune responses in surrounding lymphoid tissues. Invest Ophthalmol Vis Sci. 1995; 36:330–336.
tion within the cornea, we initiated this study to investigate the regulation of IL-6 secretion in human corneal epithelial cells and keratocytes after exposure to IL-1α and TNF-α. It was found that both IL-1α and TNF-α enhanced the synthesis of IL-6 in keratocytes. However, these cytokines were only minimally active at stimulating corneal epithelial cells to make IL-6. These results suggest that keratocytes play a more active role in amplifying specific immune responses at the eye surface than do corneal epithelial cells.

**MATERIALS AND METHODS**

**Preparation of Corneal Epithelial Cell and Keratocyte Cultures**

Human corneas were obtained from the National Disease Research Interchange (Philadelphia, PA) and processed within 4 days of enucleation. The previously described protocol was used to establish human corneal epithelial cell and keratocyte cultures in 25-cm² flasks. The reactivity of corneal epithelial cells but not keratocytes with anti-cytokeratin antibodies in immunofluorescence tests was used to establish the purity of each cell type in culture.

**Cytokine Assays in Epithelial Cell and Fibroblast Cultures Stimulated with rTNF-α and rIL-1α**

Epithelial cell cultures were used when they were approximately 90% confluent. The medium was aspirated and replaced with 2 ml K-SFM (Gibco, Grand Island, NY) medium containing the desired concentration of either human rTNF-α (Genzyme, Cambridge, MA) or human rIL-1α (R&D Systems, Minneapolis, MN). The endotoxin level was <0.0025 EU/1000 U of IL-1α and <0.063 EU per 500 U of TNF-α. At selected times after stimulation, medium was removed and frozen at −20°C for subsequent cytokine analysis. Keratocyte cultures were grown in Dulbecco’s minimum essential medium containing 10% fetal calf serum until approximately 90% confluent. To eliminate possible stimulation of cytokine production by fetal calf serum, Dulbecco’s minimum essential medium was replaced with Opti-MEM (Gibco) 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 time intervals and frozen at −20°C for subsequent cytokine assay. IL-6 and IL-8 levels in supernatants were quantified using enzyme-linked immunosorbent assay kits obtained from R&D Systems (Minneapolis, MN) as previously described. Significant differences between cytokine levels were evaluated by small sample paired t-statistics.

**Total RNA Isolation**

Epithelial cell and keratocyte cultures in 25 cm² flasks were established from individual corneal donors. After each culture was treated with the desired cytokine for the indicated times, total cellular RNA was isolated from cell cultures by the acid guanidinium thiocyanate-phenol-chloroform extraction method as previously described. Approximately 53 μg RNA/10⁶ cells was obtained from epithelial cells, and 9.0 μg RNA/10⁶ cells was obtained from keratocytes.

**Primer Selection**

Polymerase chain reaction (PCR) primers were selected with the aid of OLGIO primer selection software (Eccles Institute for Human Genetics and Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT) running on the National Cancer Institute-Frederick Cancer Research Center Vax 6620 (Frederick, MD). The primers were complementary to mRNA sequences within the human IL-6, IL-8, or human glyceraldehyde-3-phosphate dehydrogenase (GAPD) coding regions. The cDNA sequences in these analysis were obtained from the Wisconsin Genetics Computer Group program and are as follows:

- Human IL-6 primers (516 bp product)
  - Sense 5'CCACACAGACGCACCTG3′
  - Anti-sense 5'CTACATTGCCGAGAAGGCT3′

- Human IL-8 primers (217 bp product)
  - Sense 5'CTTGCTGTGCGCTCTGGATT3′
  - Anti-sense 5'AACTTCTCCACAACCCTCTGCAC3′

- Human GAPD primers (878 bp product)
  - Sense 5'GTAAGTGATATTGTTGCAAT3′
  - Anti-sense 5'AAATCGTTGTCACATCCAGGAAAT3′

**Analysis of mRNA by Reverse Transcription–Polymerase Chain Reaction**

Complementary DNA strands to total cellular RNA were made using a GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, CT) according to manufacturer specifications by adding 1 μg total cellular RNA, random hexamer primers, and M-MLV reverse transcriptase in a 20 μl total volume. Each reverse transcription reaction was then divided to four tubes. To one set of tubes, the PCR reagents including AmpliTaq DNA polymerase and primers specific for human IL-6 or IL-8 were added bringing the total reaction volume to 25 μl. The complementary DNA to cytokine mRNA was amplified for 35 thermocycles of 30 seconds at 95°C, 30 seconds at 65°C, and 2 minutes at 72°C. Preliminary experiments established that 35 cycles was within the exponential amplification phase of the IL-6 and 30 cycles for the IL-8 PCR. PCR reagents and primers specific for human GAPD were added to the other three sets of tubes. Primers for the constitutively

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expressed gene GAPD served as the internal reverse transcription–polymerase chain reaction (RT-PCR) control to confirm that each sample had equal amounts of purified RNA. These three sets of GAPD primed reactions were thermocycled for three different numbers of cycles to ensure that the amount of product among samples was equal throughout the exponential phase of the PCR. After completion of the PCR amplifications, a 10 μl volume of each PCR reaction was added to each lane of a 1.5% agarose gel. The gel was then stained with 1 μg/ml ethidium bromide, viewed with a transilluminator and then photographed using Polaroid (Cambridge, MA) 665 instant film.

RESULTS

IL-6 Gene Expression in Corneal Keratocytes

Primary human corneal keratocytes were cultured in serum-free medium in the presence of increasing concentrations of IL-1α or TNF-α. The quantity of IL-6 released by the cells into the media was then determined by enzyme-linked immunosorbent assay (Fig. 1). It was found that exposure of keratocyte cultures to either IL-1α or TNF-α significantly enhanced IL-6 synthesis in a dose-dependent fashion. By 18 hours after exposure, IL-1α stimulated cultures synthesized >1,100-fold more IL-6 than was produced by identical cultures of unstimulated keratocytes (Fig. 1A). During this same period, TNF-α stimulated cultures synthesized some 38-fold more IL-6 than that produced by identical cultures of unstimulated cultures (Fig. 1B).

RNA was also isolated from keratocyte cultures established from each donor so that IL-6 gene expression could be analyzed by RT-PCR amplification. IL-6 mRNA was not amplified from any of the RNA preparations derived from unstimulated cultures (Fig. 2). However, IL-6 mRNA was readily amplified in RNA preparations harvested from cultures stimulated with either IL-1α or TNF-α. In these cultures, mRNAs for both cytokines were detectable 3 hours after stimulation and persisted through 18 hours.

IL-6 Gene Expression in Corneal Epithelial Cells

IL-6 gene expression in epithelial cell cultures established from the same donors used in the above experiments was also studied by enzyme-linked immunosorbent assay and RT-PCR analysis after exposure of these cells to IL-1α and TNF-α. Epithelial cells stimulated with increasing concentrations of either cytokine did produce IL-6 (Figure 3). However, the amount produced was quite modest being on average only fivefold in IL-1α stimulated cells and tenfold in TNF-α-treated cells above that seen in unstimulated cultures. We previously reported that IL-1α and TNF-α stimulated epithelial cell cultures could produce nanogram quantities of IL-8.13 Therefore, to determine whether the present epithelial cultures were metabolically active and possessed the capacity to respond to these two cytokines culture supernatants were also examined for the presence of IL-8. Figure 4 shows that the cultures could produce nanogram levels of IL-8 even though they only synthesize picogram amounts of IL-6.

RNA was extracted from both IL-1α and TNF-α stimulated epithelial cell cultures to compare IL-6 and IL-8 gene expression. Figure 5 shows the results of one such experiment. Faint bands of IL-6 mRNA products were seen only at two time points, namely 3 hours poststimulation in IL-1α treated cultures and at 6 hours poststimulation in TNF-α treated cultures. All of the RNA samples had approximately equal amounts of amplified products generated from GAPD mRNA, indicating that equivalent amounts of RNA were analyzed. In contrast, RT-PCR products amplified from IL-8 mRNA produced bands that were most intense...
FIGURE 2. Effect of IL-1α and TNF-α on steady state levels of IL-6 mRNA in human corneal keratocytes. Cell cultures from an individual donor were established in 25 cm² flasks. Media from the cultures were then replaced with fresh medium or medium containing either 1000 U/ml IL-1α or 500 U/ml TNF-α. At specific times after cytokine treatment, total RNA was extracted from each culture. IL-6 and GAPD mRNA levels were determined as described in Materials and Methods. The GAPD primed reaction thermocycled for 30 cycles is shown. Two additional experiments performed with keratocyte cultures established from two additional corneal donors yielded similar results.

at 5 hours poststimulation and persisted for at least 12 hours.

DISCUSSION

In this study, we found that both human corneal epithelial cells and keratocytes can synthesize IL-6 and that production of this cytokine is enhanced in both cell types after exposure to either IL-1α or TNF-α. However, several lines of evidence suggest that IL-6 gene expression in corneal keratocytes is far more responsive to IL-1α and TNF-α stimulation than it is in epithelial cells. First, IL-1α stimulated keratocytes produced more than 160 ng of IL-6 whereas TNF-α stimulated keratocytes produced approximately 5 ng of this cytokine. This represented an increase in IL-6 synthesis above background levels by almost 1,100-fold in IL-1α treated cells and by more than 38-fold in TNF-α stimulated cells. In contrast, exposure of epithelial cells to TNF-α or IL-1α resulted in very modest increases (5 to 10-fold) in IL-6 synthesis. In experiments where IL-6 mRNA levels were assayed, both IL-1α and TNF-α stimulated keratocytes maintained elevated levels of IL-6 mRNA for at least 18 hours after exposure. In epithelial cells, IL-6 mRNA was detected at only a single time point after exposure in cultures treated with both TNF-α and IL-1α. RT-PCR products generated from this mRNA produced a much fainter band on agarose gels than did the RT-PCR products generated from stimulated keratocytes. These results indicate that both IL-1α and TNF-α induce a more intense and prolonged increase in steady state IL-6

FIGURE 3. Comparison of IL-6 production in epithelial cell cultures after IL-1α or TNF-α stimulation. Cultures of the two cell types were established from six corneal donors and then incubated in the presence of either IL-1α (A), TNF-α (B) or with medium alone. Culture supernatants were collected at times indicated and assayed for IL-6 content by enzyme-linked immunoassay. Each point represents the mean ± SEM of six independent assays.
Epithelial Cells

• Medium
• 1,000 U/ml IL-1α
• 500 U/ml TNF-α

FIGURE 4. IL-8 synthesis after IL-1α and TNF-α stimulation of human corneal epithelial cells. Supernatants collected from epithelial cell cultures used for the experiments in Figure 3 were assayed for IL-8 by enzyme-linked immunosorbent assay. The results shown are the averages ± SEM of cells established from the six corneal donors.

mRNA levels in keratocytes than in epithelial cells. It is therefore likely that the enhanced levels of IL-6 mRNA in stimulated keratocytes accounted for their capacity to synthesize more IL-6 than comparably stimulated epithelial cells.

The reason for the disparity in IL-6 gene expression between epithelial cells and keratocytes is not known. However, it was observed that even though epithelial cells exposed to IL-1α and TNF-α synthesized less than 0.5 ng of IL-6, they were capable of producing up to 18 ng of IL-8. This suggests that epithelial cells have receptors for both inducing cytokines and that these receptors can transduce an appropriate activating signal. IL-6 gene expression is regulated both transcriptionally through the cooperative interaction of specific transcriptional factors at the IL-6 promoter and posttranscriptionally by sequences within the 3′-untranslated region of mRNAs, which regulate translation. Thus, it is possible that the interaction of IL-1α and TNF-α with keratocytes leads to accumulation of larger amounts of the appropriate transcriptional activators within the nucleus than does the interaction of these cytokines with epithelial cells, or alternatively, that IL-6 mRNA turns over faster in epithelial cells than in keratocytes.

IL-6 is a pleiotropic cytokine and its abundant production could influence a number of immunologic activities within the eye. For example, IL-6 produced by cells within the epithelial and stromal layers of the cornea could play a role in activating T-lymphocyte subpopulations that accumulate within stromal tissues during inflammation. IL-6 made by corneal cells could also play an important role in driving the differentiation of B-cells into IgA secreting cells within mucosal-associated lymphoid. Interestingly, it has been shown that IL-6 can negatively regulate both IL-1α and TNF-α production by mononuclear cells. Because mononuclear cells found at inflammatory sites are a major source of IL-1α and TNF-α it is possible that IL-6 may help to dampen inflammatory responses mediated by these two proinflammatory cytokines thereby reducing damage to the eye surface.

FIGURE 5. Effect IL-1α and TNF-α on steady state levels of IL-6 mRNA in human corneal epithelial cells. The protocol was the same as that described for Figure 1 except that corneal epithelial cells were used in place of corneal keratocytes. IL-6, IL-8 and GAPD mRNA levels were detected as described in Materials and Methods. The GAPD primed reaction thermocycled for 30 cycles is shown. Studies using epithelial cell cultures established from two additional corneal donors gave comparable results.
IL-6 Synthesis in Human Corneal Cells

It is not clear from our in vitro studies if the observed difference in IL-6 synthesis between epithelial cells and keratocytes reflects the situation occurring in vivo. However, it can be speculated that the capacity of keratocytes to synthesize greater amounts of IL-6 than epithelial cells in vivo could be advantageous to the host. For example, the synthesis of smaller amounts of IL-6 by epithelial cells may lead to reduced development of humoral and cell-mediated immune responses after infection or injury to the immediate corneal surface. In contrast, the capacity of keratocytes to synthesize large amounts of IL-6 could lead to a more vigorous immune responses after an injury that results in penetration of infectious microorganisms into the stroma. Even though the resulting inflammation could lead to corneal damage, development of strong specific immune responses at this site could prevent further spread of an infectious agent thereby protecting the host from the risk of a life-threatening systemic infection.

Key Words
epithelial, keratocyte, IL-6, IL-1α, TNF-α

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