

Differential Regulation of ENA-78 and GCP-2 Gene Expression in Human Corneal Keratocytes and Epithelial Cells

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PURPOSE. To determine whether interleukin (IL)-1 α - and tumor necrosis factor (TNF)- α -stimulated human corneal epithelial cells (HCECs) and human corneal keratocytes (HCKs) produce the α -chemokines epithelial cell-derived neutrophil attractant (ENA)-78 and granulocyte chemotactic protein (GCP)-2.

METHODS. Cultures of HCECs and HCKs were stimulated with either human recombinant IL-1 α or TNF- α . At selected times after stimulation, culture supernatants were harvested and assayed for ENA-78 and GCP-2 by enzyme-linked immunosorbent assay. RNA was extracted from cell cultures to measure steady state levels of intracellular ENA-78 and GCP-2 pre-mRNA and mRNA by the reverse transcription-polymerase chain reaction.

RESULTS. Exposure of HCECs to either IL-1 α or TNF- α stimulated a more than 4.5-fold increase in ENA-78 RNA and protein synthesis without stimulating a significant increase in either GCP-2 RNA synthesis or protein production. Exposure of HCK to IL-1 α stimulated a 10-fold increase in ENA-78 and GCP-2 RNA synthesis and a more than 300-fold increase in ENA-78 and GCP-2 protein production. In contrast, exposure of keratocytes to TNF- α significantly enhanced ENA-78 RNA synthesis, resulting in a more than 68-fold increase in ENA-78 protein synthesis without significantly enhancing either GCP-2 gene expression or protein secretion.

CONCLUSIONS. ENA-78 gene expression is significantly enhanced in both HCECs and HCKs in response to either IL-1 α or TNF- α stimulation. In contrast, GCP-2 synthesis is only inducible in IL-1 α -stimulated HCKs. The results suggest that GCP-2 gene expression is more tightly regulated in diseased or injured corneal tissue than is ENA-78 gene expression. (*Invest Ophthalmol Vis Sci.* 2003;44:3432-3437) DOI:10.1167/iovs.03-0095

The cornea is a wet epidermal surface composed of an outer layer of epithelial cells, a middle layer of stromal tissue containing specialized fibroblasts termed keratocytes, and an inner layer of endothelial cells, which are in direct contact with the aqueous anterior chamber.¹ Although the epithelial layer serves as the primary barrier to ocular infection or injury, the highly regular arrangement of collagen fibrils in the gel-like stromal layer serve to maintain the transparency of the cornea. In addition, transparency is maintained by the endothelial layer, which functions to maintain proper stromal hydration.¹

After infection or injury to the eye surface, keratocytes and epithelial cells initiate acute inflammatory processes within the cornea by releasing members of a family of small neutrophil-chemotactic peptides called α -chemokines. These molecules play an important role in recruiting neutrophils into the stromal and epithelial layers of the cornea from limbal blood vessels.² Even though the infiltration of neutrophils into corneal tissue is an important mechanism of host resistance to infection, accumulation of these cells at eye surfaces can cause extensive damage to cells surrounding sites of injury by releasing a number of tissue-damaging molecules.³⁻⁶ Fibrosis and scarring generated during the healing process can interfere with light transmission through the cornea and may lead to blindness.⁷⁻⁹ The major inducers of chemokine synthesis in tissue are the proinflammatory mediators interleukin (IL)-1 α and tumor necrosis factor (TNF)- α .¹⁰ Both of these cytokines can be found in damaged or infected epithelial surfaces.¹¹⁻¹⁶

We have demonstrated that the synthesis of the α -chemokines interleukin-8 (IL-8) and growth-related oncogene α (GRO- α) is induced in human corneal keratocytes (HCKs) by both IL-1 α and TNF- α .^{17,18} In contrast, the two cytokines stimulate synthesis of IL-8 but not GRO- α in human corneal epithelial cells (HCECs).^{17,18} Epithelial cell-derived neutrophil attractant (ENA)-78 and granulocyte chemotactic protein (GCP)-2 are neutrophil chemotactic chemokines related to IL-8.¹⁹⁻²⁰ Many cell types that produce IL-8 also have the capacity to produce ENA-78 and GCP-2 in response to IL-1 α and TNF- α .²¹⁻²³

Therefore, it was of interest to know whether human corneal cells also synthesize ENA-78 and GCP-2 in response to the two cytokines. This is an important question, because it will be difficult to develop strategies to reduce innate immune responses and accompanying neutrophil infiltration into diseased or damaged corneal tissue until the spectrum of α -chemokines produced by corneal cells in response to proinflammatory mediators is known.

MATERIALS AND METHODS

Preparation of HCECs

Human corneas were received from the National Disease Research Institute Interchange (Philadelphia, PA). Pure primary cultures of HCECs were established in keratinocyte serum-free medium (K-SFM; Invitrogen/Gibco, Grand Island, NY) within 4 days of enucleation, as described previously.^{17,18} The medium was replaced every other day until the cells reached 90% confluence, at which time the medium was replaced with 2.0 mL of K-SFM containing either IL-1 α (rIL-1 α ; R&D Systems, Minneapolis, MN) or recombinant TNF- α (rTNF- α ; Genzyme, Cambridge, MA). Supernatants were then collected at selected time points after stimulation and stored at -80°C until ready for analysis.

Pure primary cultures of human corneal keratocytes were established in 75-cm² flasks in Dulbecco's modified Eagle's medium (Invitrogen/Gibco) containing 20% fetal bovine serum, 1 \times antibiotic-antimycotic (Invitrogen/Gibco), 10 mM HEPES, 1.5% sodium bicarbonate (Invitrogen/Gibco), 0.004N sodium hydroxide, and 0.2 $\mu\text{g}/\text{mL}$ kanamycin (Sigma-Aldrich, St. Louis, MO), as described previously.^{17,18}

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Once the cells reached confluence, they were harvested and seeded into 25-cm² flasks in medium containing 10% fetal bovine serum. Once the cells reached 80% confluence, the medium was replaced daily with serum-free medium (Opti-MEM 1; Invitrogen/Gibco) supplemented with 5 µg/mL gentamicin (Invitrogen/Gibco). After 3 days in serum-free medium, the medium was replaced with 2.0 mL of serum-free medium containing either rIL-1α or rTNF-α. Supernatants were then collected at selected time points after stimulation and stored at -80°C until analysis.

RNA Isolation

RNA was extracted from cell cultures by the acid guanidium thiocyanate-phenol-chloroform method, as described previously.²⁴ The amount of total RNA resuspended in nuclease-free water within each sample was determined by spectrophotometric analysis. An aliquot from each sample was DNase-treated using RQ1 RNase-free DNase (RQ1; Promega, Madison, WI) to degrade contaminating genomic DNA. The amount of purified DNase-treated RNA remaining was again determined by spectrophotometric analysis, and the RNA pellet was resuspended in nuclease-free water to obtain a final concentration of 0.5 µg/µL. To verify RNA integrity, a 1% agarose gel containing ~3 × 10⁻⁴ µg/mL ethidium bromide was prepared. Gel electrophoresis was performed at 100 V for 30 minutes with 1.0 µg of purified DNase-treated RNA per well in 1× Tris-acetate-EDTA (TAE) electrophoresis buffer containing approximately 6 × 10⁻⁴ µg/mL ethidium bromide. Gels were visualized with an electronic dual light transilluminator (Ultra-Lum; Ultra-Lum, Inc., Paramount, CA), and digital images of the gels were obtained with a zoom digital camera (Digital Science DC120; Eastman Kodak Co., Rochester, NY).

ELISA Assay

Supernatants collected from both stimulated and nonstimulated epithelial and keratocyte cultures were analyzed for human ENA-78 or GCP-2 by ELISA assays according to the manufacturer's protocols (R&D Systems, Inc). The minimum level of detection for human ENA-78 was 15 pg/mL, and the minimum level for human GCP-2 was 1.6 pg/mL. To eliminate background, the results were read at 450 and 550 nm on a microplate reader (MRX; Dynatech Laboratories, Chantilly, VA.). Significant differences between chemokine levels were evaluated by using a small sample paired *t*-test.

PCR Primers

Polymerase chain reaction (PCR) primers were selected with the program Primer Quest (<http://www.idtdna.com>). Reverse primers were selected to be complementary to exon sequences within the human ENA-78, human GCP-2, or human glyceraldehyde-3-phosphate dehydrogenase (GAPD) coding regions. Forward primers for mRNA amplification spanned at least one exon-exon boundary, whereas primers for pre-mRNA amplification were selected to be complementary to sequences in an intron (forward primer), as previously described.¹⁸ All primer sequences were analyzed by using BLAST (www.ncbi.nlm.nih.gov/blast/) provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), to ensure that the primers were specific and thus amplified only the gene of interest during PCR.

The primer sequences selected are listed, followed by the expected RT-PCR product size in parentheses: human ENA-78 primers (mRNA 493-bp product, pre-mRNA 275-bp product) mRNA forward oligonucleotide 5'-ATCTCCGCTCCTCCACCCAGT-3', pre-mRNA forward oligonucleotide 5'-GCAATTACACACAGCTTGTC-3', and mRNA and pre-mRNA reverse oligonucleotide 5'-TTCTGTCTCCCTGGGTT-CAGA-3'; human GCP-2 primers (mRNA 179-bp product, pre-mRNA 333-bp product): mRNA forward oligonucleotide 5'-CTGGTCCTGTCTCTGCTGTGCT-3', pre-mRNA forward oligonucleotide 5'-AGG-GAACTCTCCAGCAACCT-3', mRNA and pre-mRNA reverse oligonucleotide 5'-GCTTCCGGGTCCAGACAACT-3'; human GAPD primers (mRNA 417-bp product, pre-mRNA 286-bp product): mRNA forward oligonucleotide 5'-CCAAAGGGTCATCATCTCTGC-3', pre-mRNA for-

ward oligonucleotide 5'-AGAGGGGTGATGTGGGGAGTA-3', mRNA and pre-mRNA reverse oligonucleotide 5'-ATTTGGCAGGTTTTCTA-GACGG-3'. The RT-PCR products were verified by size (agarose gel electrophoresis, described below) and sequence with an automated sequencer (model 373XL; Applied Biosystems, Inc., Foster City, CA).

RT-PCR Analysis

cDNA strands to total cellular RNA were made with an RNA PCR core kit (GeneAmp; Applied Biosystems/Roche, Branchburg, NJ) according to the manufacturer's instructions. One microgram of total cellular RNA was added to a PCR master mix containing random hexamers and Moloney murine leukemia virus (MuLV) reverse transcriptase for a 20-µL total volume reaction. A negative control was created by adding all the above reagents excluding reverse transcriptase to confirm that no contaminating genomic DNA was present in the purified total RNA samples. For the PCR reaction, a master mix containing DNA polymerase (AmpliTag; Applied Biosystems/Roche) and appropriate primers was prepared according to the manufacturer's instructions. Twelve-microliter aliquots were then dispersed into PCR tubes along with 3 µL of cDNA, for a total volume of 15 µL. RT-PCR products were amplified using an initial thermocycle of 2 minutes at 95°C followed by thermocycles of 30 seconds at 95°C, 30 seconds at 65°C, and 2 minutes at 72°C. Preliminary experiments established that 20 to 22 cycles of amplification were within the exponential amplification phase of both GAPD and ENA-78 mRNAs, whereas 32 cycles of amplification were needed to amplify detectable levels of GCP-2 mRNA. Detection of ENA-78 and GCP-2 pre-mRNA required 30 cycles of amplification, whereas detection of GAPD pre-mRNA required 40 cycles of amplification.

Analysis of RT-PCR Products

Equal volumes (15 µL) of the PCR-amplified product was added to individual lanes of a 1.5% agarose gel containing 0.5 µg/mL ethidium bromide and subjected to electrophoresis at 100 V in 1× TAE electrophoresis buffer containing 0.1 µg/mL ethidium bromide. The gel was then viewed with a transilluminator, and the image was captured with a zoom digital camera (Digital Science DC120; Eastman Kodak Co.). The RT-PCR band intensities were analyzed by using the accompanying software (ID Image Analysis Software for Windows ver. 2.0.3; Eastman Kodak Co.). The constitutively expressed gene GAPD served as the positive RT-PCR control to ensure that each experimental sample contained equal amounts of purified mRNA. The relative density of each band was obtained by dividing the density of the band of interest by the density of the corresponding GAPD/pre-GAPD band. Significant differences between cytokine-stimulated and control mRNA and pre-mRNA levels were evaluated with a small-sample paired *t*-test.

RESULTS

ENA-78 and GCP-2 Gene Expression in IL-1α- and TNF-α- Stimulated HCECs

Cultures of HCECs were stimulated with either IL-1α or TNF-α. At selected intervals after stimulation, steady state levels of ENA-78 and GCP-2 pre-mRNA and mRNA were determined by RT-PCR. It was found that constitutive levels of ENA-78 pre-mRNA and mRNA are synthesized in unstimulated HCECs (Fig. 1). However, when HCECs were stimulated with either IL-1α or TNF-α, both ENA-78 pre-mRNA and mRNA levels increased more than fivefold (Fig. 1). In contrast, GCP-2-specific RNA was not detected in IL-1α- and TNF-α- stimulated HCEC cultures, even though the cDNA generated in the RT reaction was amplified for 40 cycles (data not shown).

Culture supernatants harvested from IL-1α- and TNF-α- stimulated HCEC cultures were then analyzed for the presence of ENA-78 and GCP-2 protein. It was found that nonstimulated cultures of HCECs synthesized detectable levels of both

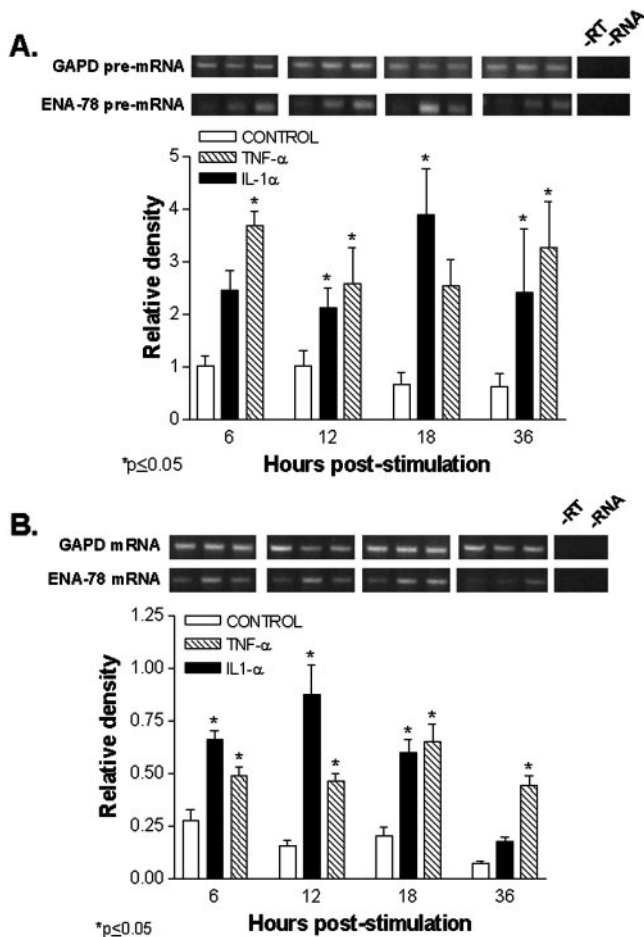


FIGURE 1. ENA-78 pre-mRNA and mRNA levels in human corneal epithelial cells after stimulation with IL-1 α and TNF- α . **(A)** Total RNA was extracted from HCEC cultures stimulated with either 1000 U/mL IL-1 α or 500 U/mL TNF- α . Specific pre-mRNAs were amplified by RT-PCR. The amplified products were separated on 1.5% agarose gels and photographed. A representative experiment performed on cultures established from one of at least four corneal donors is shown. The quantitative results for all four donors is shown as the mean \pm SEM. **(B)** Total RNA was extracted from HCEC cultures stimulated with either 1000 U/mL IL-1 α or 500 U/mL TNF- α . Specific mRNAs were amplified by RT-PCR. The amplified products were separated on 1.5% agarose gels and photographed. A representative experiment performed on cultures established from one of at least four corneal donors is shown. The quantitative results for all four donors is shown as the mean \pm SEM.

ENA-78 and GCP-2 protein (Fig. 2). After 6 hours of stimulation with either IL-1 α or TNF- α , significant increases in ENA-78 synthesis were detectable (Figs. 2A, 2C). By 18 hours after stimulation, cultures induced with the two proinflammatory cytokines were producing more than 4.5 times more ENA-78 than that produced by nonstimulated cultures. In contrast, we did not detect significant increases in GCP-2 protein synthesis (Figs. 2B, 2D). These results suggest that GCP-2 synthesis, within the limits of the assay, is quiescent in response to IL-1 α and TNF- α .

ENA-78 and GCP-2 Gene Expression in IL-1 α - and TNF- α -Stimulated HCKs

RNA was extracted from IL-1 α - and TNF- α -stimulated HCK cultures at selected intervals after stimulation to analyze changes in steady state levels of ENA-78 and GCP-2 pre-mRNA

and mRNA in this corneal cell type. Cultured HCKs synthesized low constitutive levels of both ENA-78- and GCP-2-specific RNA (Fig. 3). When cells were stimulated with either IL-1 α or TNF- α , steady state ENA-78 pre-mRNA and mRNA levels increased by more than two logs (Figs. 3A, 3C). Steady state levels of GCP-2 mRNA also increased by more than two logs in IL-1 α -stimulated HCKs (Fig. 3D). In contrast, GCP-2 pre-mRNA and mRNA levels increased by less than fivefold when the cells were stimulated with TNF- α (Figs. 3B, 3D).

Culture supernatants harvested from stimulated and nonstimulated HCKs were then assayed for ENA-78 and GCP-2 proteins. It was found that keratocytes stimulated with IL-1 α produced approximately 400-fold more ENA-78 and 300-fold more GCP-2 than did nonstimulated cultures (Figs. 4A, 4B). In contrast, TNF- α stimulation of HCKs increased ENA-78 synthesis less than 100-fold and failed to induce any significant increase in GCP-2 synthesis (Figs. 4C, 4D).

DISCUSSION

A major finding in this study was that exposure of HCECs to either IL- α - or TNF- α - enhanced ENA-78 gene expression without significantly stimulating expression of the gene for GCP-2. In a previous study, it was observed that GRO- α gene expression is not upregulated in IL-1 α - and TNF- α -stimulated HCECs, even though the cells could synthesize approximately 28 ng/10⁶ cells of IL-8 in response to proinflammatory mediators.¹⁸ Similar levels of ENA-78 were produced in this study in response to IL-1 α and TNF- α . Taken together, the two studies suggest that even though HCECs can produce IL-8 and ENA-78, they are probably not major producers of GRO- α and GCP-2.

The inability of corneal epithelial cells to produce GCP-2 along with ENA-78 was surprising because the promoters of the two genes are 95% homologous, sharing identical binding sites for several transcriptional factors, including AP-1, C/EBP, and NF- κ B.¹⁹⁻²⁰ There are, however, two areas of sequence variability that exist between the two promoters: Site A has been shown to regulate gene expression through its capacity to bind positive-acting transcriptional factors such as Sp-1 and Sp-3 or negative-acting transcriptional factors such as ZBP-89.²⁵ Because the ENA-78 site A differs from GCP-2 site A by four nucleotides, it is possible that site A on the two promoters has affinity with different combinations of positive and negative regulatory factors. Thus, HCECs may be able to synthesize ENA-78 without synthesizing GCP-2 by coproducing positive regulatory factors that possess exclusive affinity with the ENA-78 promoter and/or negative regulatory factors that possess exclusive affinity with the GCP-2 promoter. A second area of sequence variability between the two promoters is the presence within the GCP-2 promoter of a long interspersed DNA element (LINE-1), which is missing in the ENA-78 promoter.²⁰ LINE-1 has been shown to bind negative regulatory factors that downregulate gene expression by competitively overcoming the binding of positive transactivating transcriptional factors.²⁶ Thus, it is possible that HCECs can selectively inhibit GCP-2 gene expression in response to the two proinflammatory mediators by producing negative regulatory factors with affinity to GCP-2-specific LINE-1.

We have also found in the current study that TNF- α -stimulated HCKs had the capacity to upregulate ENA-78 gene expression without significantly upregulating GCP-2 expression. Unlike HCECs, however, keratocytes were capable of upregulating synthesis of both ENA-78 and GCP-2 in response to IL-1 α . Thus, GCP-2 is likely to play an important role in chemoattraction of neutrophils into human corneal tissues under conditions that result in the release of IL-1 α into stromal layers of the cornea. IL-1 α -stimulated HCKs are also capable of

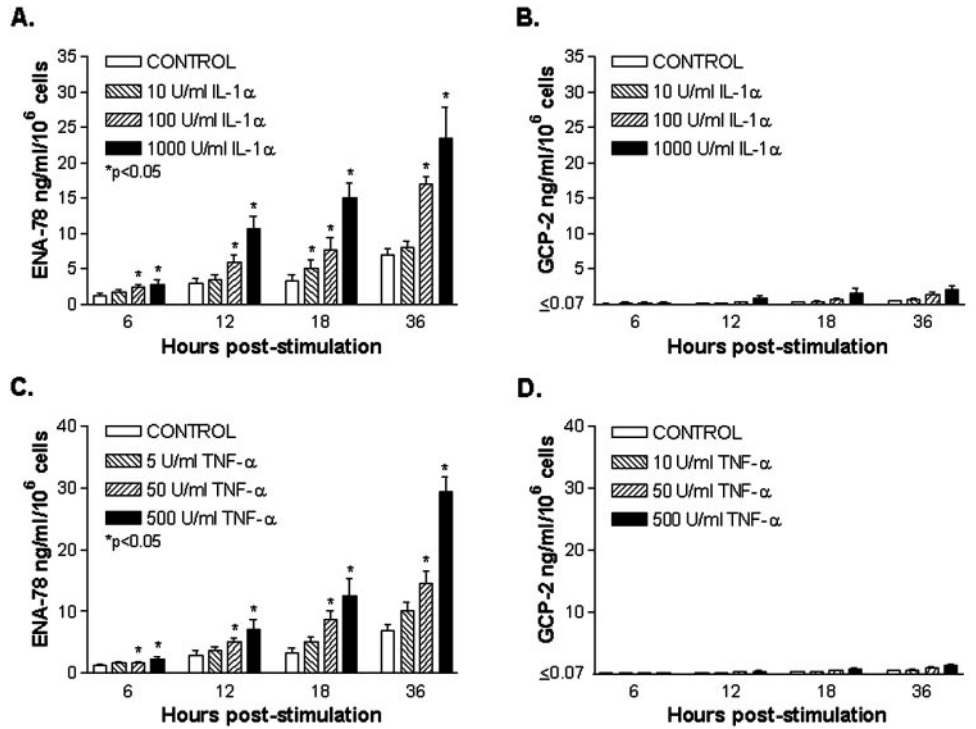


FIGURE 2. ENA-78 and GCP-2 synthesis in HCECs after stimulation with IL-1 α or TNF- α . Cultures of HCECs were exposed to increasing concentrations of human recombinant IL-1 α (A, B) or TNF- α (C, D). At selected time points, supernatants were removed and assayed for ENA-78 (A, C) and GCP-2 (B, D) by ELISA. Data are the mean \pm SEM ($n = 4$).

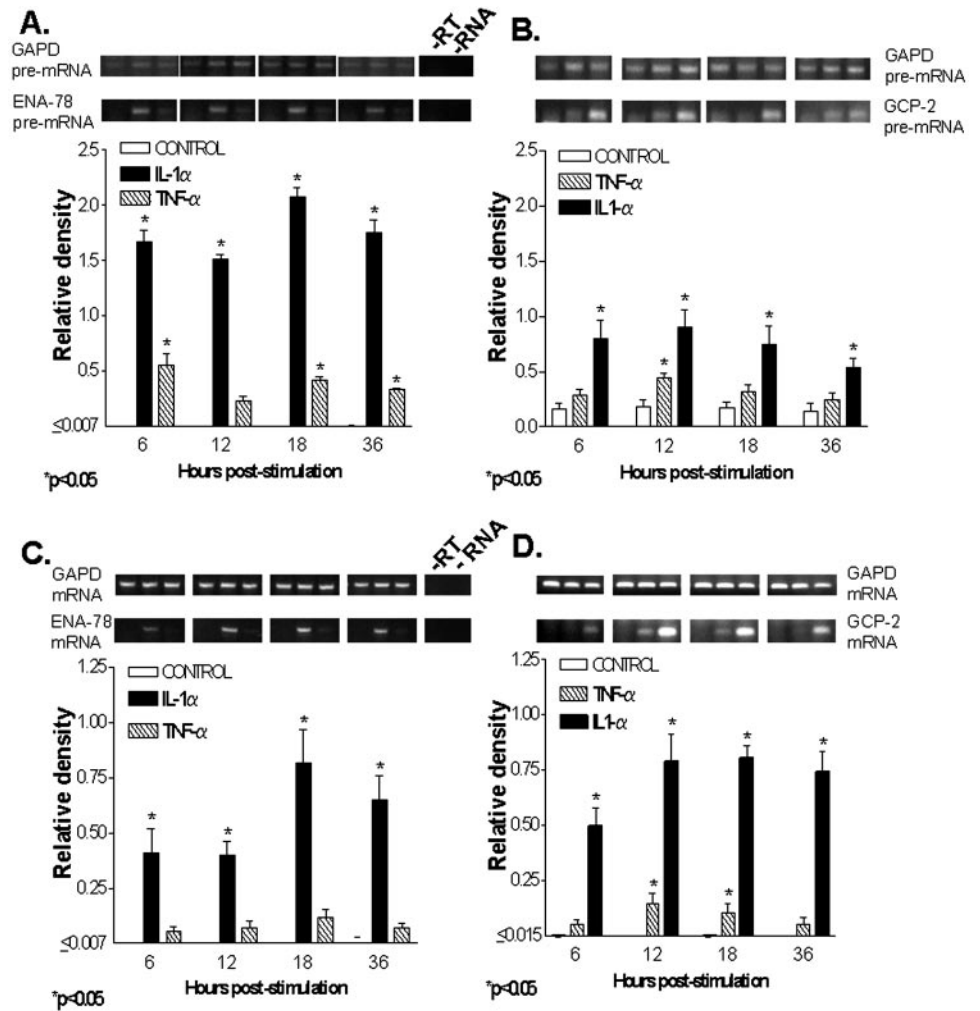


FIGURE 3. ENA-78 and GCP-2 pre-mRNA and mRNA levels in HCKs after stimulation with IL-1 α and TNF- α . Total RNA was extracted from HCK cultures stimulated with either 1000 U/mL IL-1 α or 500 U/mL TNF- α . (A) ENA-78- or (B) GCP-2-specific pre-mRNAs were amplified by RT-PCR. The amplified products were separated on 1.5% agarose gels and photographed. A representative experiment performed on cultures established from one of at least four corneal donors is shown. The quantitative results for all four donors is shown as the mean \pm SEM. (C) ENA-78- or (D) GCP-2-specific mRNAs were amplified by RT-PCR. The amplified products were separated on 1.5% agarose gels. A representative experiment performed on cultures established from one of at least four corneal donors is shown. The quantitative results for all four donors is shown as the mean \pm SEM.

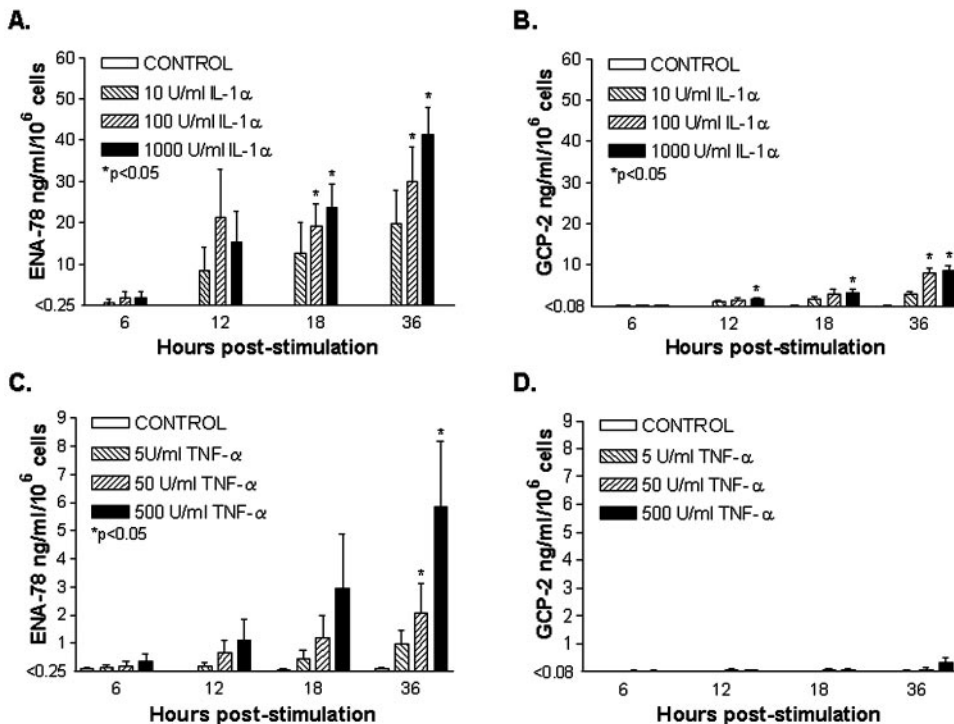


FIGURE 4. ENA-78 and GCP-2 synthesis in HCKs after IL-1 α and TNF- α stimulation. Cultures of HCKs were exposed to increasing concentrations of human recombinant IL-1 α (A, B) or TNF- α (C, D). At selected time intervals, supernatants were removed and assayed for ENA-78 (A, C) and GCP-2 (B, D) by ELISA. Data are the mean \pm SEM ($n = 4$).

producing up to 450 ng/10⁶ cells of IL-8.¹⁷ This compares with the 40 ng/10⁶ cells of ENA-78 and 10 ng/10⁶ cells of GCP-2 that was produced by HCKs in this study. IL-8 may be produced in larger amounts in comparison with other α -chemokines because CXCR1 is the only α -chemokine receptor that binds to IL-8 with high affinity. In contrast, CXCR2 possesses high affinity with all α -chemokines that attract neutrophils.²⁷ Because neutrophils possess an abundance of both receptors, the greater levels of IL-8 produced may ensure that the CXCR1 receptor, in addition to the CXCR2 receptor, is activated during inflammatory responses.²⁸

Even though IL-1 α -stimulated HCKs produced both ENA-78 and GCP-2, our results suggest that different mechanisms contribute to their enhanced expression in response to the proinflammatory mediator. In IL-1 α -stimulated cells, steady state levels of ENA-78 pre-mRNA rose to levels that were three times greater than steady state levels of ENA-78 mRNA. This suggests that IL-1 α activates ENA-78 gene expression primarily by stimulating transcription of the ENA-78 gene. In contrast to ENA-78 mRNA levels, in HCKs treated with IL-1 α , steady state levels of GCP-2 mRNA rose to levels that were 10 times greater than steady state levels of GCP-2 pre-mRNA. Thus, it is highly unlikely that increased transcription of the GCP-2 gene accounted for the 10-fold difference between pre-mRNA and mRNA levels. GCP-2 transcripts carry AU-rich elements serving as binding sites for proteins that can increase mRNA stability.²⁰ Thus, the 10-fold disparity between steady state GCP-2-specific pre-mRNA and mRNA levels in IL-1 α -stimulated keratocytes is likely to exist because IL-1 α stimulation not only increases transcription of the GCP-2 gene but also significantly increases the stability of GCP-2 transcripts, thereby making it possible for GCP-2 mRNA levels to exceed those of its pre-mRNA precursor. It has been reported that GRO- α mRNA stability is also enhanced in IL-1 α -stimulated HCKs but not in TNF- α -stimulated cells.¹⁸ In addition, IL-1 α -stimulated HCKs produced 250 ng/10⁶ cells of GRO- α whereas TNF- α -stimulated cells produced approximately 20 ng/10⁶ cells of the chemokine.¹⁸ The capacity of IL-1 α , but not TNF- α , to stabilize GRO- α and GCP-2 mRNA molecules may not account for the fact that IL-1 α -stimu-

lated HCKs can produce more of the two chemokines than can TNF- α -stimulated cells.

Although it is not known from our results whether the pattern of IL-8, GRO- α , ENA-78, and GCP-2 gene expression observed in cultured HCECs and HCKs is similar to that occurring in vivo, we can speculate as to how differences in the responsiveness of the four chemokine genes to IL-1 α and TNF- α may be advantageous to an infected host. Both IL-1 α and TNF- α can be synthesized by diseased or damaged corneal epithelium.¹⁰⁻¹⁵ Even though HCECs can synthesize IL-8 and ENA-78, their inability to synthesize significant quantities of GCP-2 and GRO- α in response to proinflammatory mediators may limit neutrophil-mediated damage to immediate corneal surfaces by reducing the number of neutrophils that infiltrate the corneal epithelium after infection of the eye surface. The capacity of IL-1 α -stimulated keratocytes to produce GCP-2 and GRO- α in addition to IL-8 and ENA-78 would be expected to lead to a more vigorous nonspecific immune response after an injury that results in penetration of infectious microorganisms into the stroma. Even though the heightened innate immune responses could lead to corneal damage, the accumulation of large numbers of neutrophils at this site could reduce the chance of further spread of an infectious agent, thereby protecting the host from a possible life-threatening systemic infection. However, the demand for neutrophils within stromal tissue would be reduced once cell-mediated immune responses are generated. Because T cells and macrophages are a major source of TNF- α at sites of cell-mediated immune responses,²⁹ the inability of TNF- α to stimulate synthesis of GRO- α and GCP-2 in keratocytes may provide a mechanism to reduce the amount of α -chemokines released into the stroma when a large number of neutrophils is no longer needed.

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