High Expression of Chemokines Gro-α (CXCL-1), IL-8 (CXCL-8), and MCP-1 (CCL-2) in Inflamed Human Corneas In Vivo

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Objective: To investigate in vivo expression of chemokines in normal and inflamed human corneas, to determine whether chemokines are responsible for the recruitment of inflammatory cells.

Methods: In situ hybridization of the CXC chemokines growth-related oncogene-α (Gro-α) (CXCL-1), interleukin 8 (CXCL-8), macrophage interferon-γ inducible protein 10 (CXCL-10) and of the CC chemokines macrophage chemoattractant protein 1 (MCP-1) (CCL-2), macrophage inflammatory protein 1α (CCL-3), and regulated on activation, normal T-cell expressed and secreted (CCL-5) was performed to localize chemokine messenger RNA. Immunohistochemistry was used to identify the cellular infiltrate within the cornea. Three normal human eyes were compared with eyes enucleated because of chronic inflammation (n=10), secondary to perforating injuries.

Results: In normal corneas, no chemokine expression was detected. In inflamed lesions, a high intensity of signals from Gro-α (CXCL-1) and MCP-1 (CCL-2) messenger RNA was observed in limbal epithelium and from Gro-α (CXCL-1), interleukin 8 (CXCL-8), and MCP-1 (CCL-2) in corneal stroma. The Gro-α (CXCL-1) was the only chemokine expressed by central corneal epithelium. All other examined chemokines were only moderately expressed in limbus and corneal stroma, or barely detectable.

Conclusions: These cytokines are important agents in the cytokine network and contribute to the cell-specific and spatially restricted recruitment of neutrophils and mononuclear cells in acute inflammatory lesions of the human cornea.

Clinical Relevance: Understanding the role of chemokines in corneal inflammation may lead to the development of a selective receptor blockage of highly expressed chemokines to inhibit the recruitment of leukocyte subsets.

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One of the functions of the ocular surface is to protect the eye from infectious agents. The ocular surface comprises several tissues: the cornea, the conjunctiva, and the transition zone between the two, the limbus. The cornea consists of 3 distinct layers (a stratified epithelium, a dense stroma with a network of keratocytes, and a single-layered endothelium) and is characterized by an almost complete absence of white blood cells during physiologic conditions. In contrast, the limbus is known for its immunologic functions and harbors a variety of inflammatory cells. Although the infiltration of leukocytic cells is a common defense mechanism of the ocular surface, we do not know what factors are responsible for the presence of leukocytes in inflamed corneal tissue. Among the possible mediators for leukocyte recruitment are a subgroup of cytokines with chemotactic properties for leukocyte subsets, called chemokines (chemotactic cytokines). Chemokines are thought to play a crucial role in inflammatory diseases and are subdivided into 2 groups. Chemokines of the CXC subgroup (α-chemokines) are, according to in vitro studies, potent chemoattractants for neutrophils and T lymphocytes, and chemokines of the CC subgroup (β-chemokines) are known for their chemotactic properties toward macrophages and T-cell subsets.

Several studies have demonstrated the expression of CC and CXC chemokines in human corneal epithelial cells and keratocytes in vitro. However, few data exist examining the expression of chemokines in human cornea in vivo. We, therefore, examined the expression of 4 members of the α-chemokine family—macrophage inter-
feron-γ inducible gene (MIG, CXCL-9), interferon-γ inducible protein 10 (IP-10, CXCL-10), growth-related oncoprotein-α (Gro-α, CXCL-1), and interleukin 8 (IL-8, CXCL-8)—and of 3 members of the β-chemokine family—macrophage chemotactic protein 1 (MCP-1, CCL-2) regulated on activation, normal T-cell expressed and secreted (RANTES, CCL-5), and macrophage inflammatory protein 1α (MIP-1α, CCL-3)—in inflamed corneal lesions in vivo. These chemokines are well known for their chemotactic properties toward neutrophils, macrophages, and lymphocytes. We studied their chemokine profile in inflamed human corneas secondary to penetrating injuries of the eyeball (n=10) with an infiltrate consisting of neutrophils and mononuclear leukocytes.

RESULTS

IN SITU HYBRIDIZATION

Preparation of sulfur 35 (35S)–labeled RNA probes was performed as described. The complementary DNA probes used for in situ hybridization were kindly provided by Tiezo Yoshimura, PhD (National Cancer Institute, Frederick, Md; MCP-1), Thomas Schall, PhD (Divisions of Discovery Biology and Molecular Pharmacology, ChemoCentrxy, Inc, San Carlos, Calif; MIP-1β, RANTES), Genetics Institute (Cambridge, Mass; MIP-1α), Joshua M. Farber, PhD (National Institutes of Health, Bethesda, Md; MIG), Reinhard Kulke, PhD (University of Kiel, Kiel, Germany; IP-10), Charles Weissmann, PhD (University of Zurich, Zurich, Switzerland; IL-8), and Anthony Anisowicz, PhD (Dana Farber Cancer Institute, Boston, Mass; Gro-α). Sequence analysis of the Gro-α probe used and comparison with Gro-β and Gro-γ proved the probe to be subtype specific (data not shown). After linearization of plasmid DNA with appropriate restriction enzymes, 3S-labeled sense and antisense probes were obtained by in vitro transcription with the use of SP6, T3, or T7 RNA polymerases (Boehringer Mannheim, Mannheim, Germany) together with adenosine triphosphate, guanosine triphosphate, cytidine triphosphate (Boehringer Mannheim) (100 µM) as substrates. After elimination of the original linearized template complementary DNA, alkaline hydrolysis of labeled probes was performed for 30 to 30 minutes. After several ethanol precipitation steps, the radioactive riboprobe were adjusted to a specific activity of 2×105 cpm/µL. For hybridization to Tris hydrochloride, pH 7.5, containing 1 mM EDTA. Hybridization procedure was performed as described previously. Paraformaldehyde-fixed cryostat sections were treated with proteinase K (1 µg/mL; Boehringer Mannheim) for 30 minutes at 37°C, refixed in paraformaldehyde, acetylated with acetic anhydride in 0.1 M triethanolamine (pH 9.8, 10 minutes), dehydrated, and air-dried. The sections were overlaid with 20 µL of hybridization solution (50% formamide, 300 mM sodium chloride, 20 mM Tris hydrochloride, pH 8.0, 5 mM EDTA, 1× Denhardt solution, 10% dextran sulfate, 100 mM dithiothreitol, and 2× 105 cpm/µL heat-denatured radioactive probe). After hybridization for 12 to 16 hours, nonhybridized probes were removed by several high-stringency washing procedures. Nonspecific background RNA was digested with RNase A1 (20 µL/mL) and RNase T1 (1 U/mL; Boehringer Mannheim) for 30 minutes at 37°C. For autoradiography, slides were dipped in a photo-emulsion (Kodak NTB-2, 1:2 in 800 mM ammonium acetate; Eastman Kodak Company, Rochester, NY) and exposed for 4 weeks at 4°C.

EVALUATION OF SLIDES

For evaluation of the developed slides, a microscope (Axioskop; Carl Zeiss, Oberkochen, Germany) was used. Positive cells were counted with an ocular square grid at randomly selected areas (n=5-8) of all (n=10) corneal lesions (magnification ×200 and ×400) and related to the total number of cells. The average percentage of messenger RNA (mRNA)–expressing cells was determined as mean±SEM.

This study was performed according to the guidelines of the Declaration of Helsinki.

HISTOCHEMICAL CHARACTERIZATION OF LEUKOCYTE SUBSETS IN CORNEAL LESIONS

For identification and localization of infiltrating leukocyte subsets, immunohistochemistry was performed on inflamed (n=10) and normal (n=3) corneas by means of a panel of monoclonal antibodies that recognize T lymphocytes, macrophages, neutrophils, and Langerhans cells. The inflamed lesions showed a mild to moderate inflam-
mation corresponding to enucleation 2 to 4 days after trauma. None of the investigated specimens had stromal vascularization. All lesions had a more or less pronounced infiltrate localized below the limbal epithelium, and 3 lesions additionally demonstrated a leukocytic infiltration of central corneal stroma. Only single white blood cells were detected in corneal epithelium. The relative percentages of all leukocyte subtypes investigated depending on the location are summarized in Figure 1.

The subepithelial limbal infiltrate was found to consist predominantly of polymorphonuclear leukocytes and, to a lesser extent, of mononuclear cells (Figure 2A) (more polymorphonuclear leukocytes than CD68+ macrophages, and more CD68+ macrophages than CD3+ T lymphocytes). The histologic characterization of the 3 lesions with infiltrated central stroma showed 1 lesion predominantly of polymorphonuclear leukocytes and, to a more CD68+ macrophages than CD3+ T lymphocytes. A pronounced infiltrate localized below the limbal epithelium was paralleled in all lesions with a polymorphonuclear leukocyte infiltration (Figure 2B). In all investigated specimens, CD1+ Langerhans cells were barely detectable.

EXPRESSSion OF GRO-α, IL-8, AND MCP-1 IN INFAMEd CORNEA

On the basis of the immunohistologic findings, the chemokines suspected of exerting a chemotactic effect on T lymphocytes, macrophages, and neutrophils were examined. With the use of in situ hybridization, cell-associated silver grain precipitates were detected with radioactively labeled antisense probes, whereas, for control purposes, sense probes yielded consistently negative results. Furthermore, with the rare exception of single positive cells localized in the limbal epithelium, chemokine mRNA expression in normal corneas was not detectable.

In contrast, inflamed lesions showed expression of various chemokines. However, the expression pattern was not uniform but showed pronounced differences regarding the intensity of expression as well as the location. Figure 3 shows a detailed quantitation of the percentage of chemokine mRNA-expressing cells according to the location. The intensity and location changed, depending on the chemokine subgroup. The MCP-1 (CCL-2) showed the highest mRNA expression level of the CC chemokines, whereas Gro-α (CXCL-1) and IL-8 (CXCL-8) showed the highest expression levels of the CXC chemokines. All other chemokines investigated (MIP-1α [CCL-3], RANTES [CCL-5], MIG [CXCL-9], and IP-10 [CXCL-10]) were either moderately expressed or barely detectable.

CHEMOKINE EXPRESSION IN THE LIMBUS

With respect to the location, the regional distribution of inflammatory cells on the ocular surface as well as the presence of microvasculature corresponded to pronounced differences in the chemokine expression pattern between the limbus and the central stroma. In all lesions, chemokine expression was higher in the limbus (limbal epithelium more than limbal stroma) than central cornea (Figure 3). Furthermore, chemokine expression in limbal epithelium was paralleled in all lesions with a more or less pronounced subepithelial infiltrate. Of all examined chemokines, only Gro-α (CXCL-1) and MCP-1 (CCL-2) showed high expression levels in limbal epithelium (Figure 3). More than 27%±4.5% (mean±SEM) of total cells in limbal epithelium expressed Gro-α (CXCL-1) and 15%±2.6% expressed MCP-1 (CCL-2). The expression patterns of Gro-α (CXCL-1) and MCP-1 (CCL-2), however, differed: Gro-α (CXCL-1) mRNA-positive cells were restricted to suprabasal epithelial cell layers of the limbus (Figure 2C), whereas MCP-1 (CCL-2) mRNA expression was localized along the basal epithelium of the limbus and in the subepithelial infiltrate (Figure 2D and E). The MCP-1 (CCL-2) mRNA expression stopped at the transition from limbal to corneal epithelium (Figure 2E, see arrow). All other examined chemokines showed low expression levels in limbal epithelium, as shown in Figure 3. The RANTES (CCL-5) was expressed by basal limbal epithelium and adjacent leukocytes (Figure 2F). In contrast, MIG (CXCL-9) was expressed only by single keratinocytes in the basal limbal epithelium (Figure 2G).

EXPRESSsion OF ONLY GRO-α IN CENTRAL CORNEAL EPITHELIUM

The most conspicuous result was the strong expression of Gro-α (CXCL-1) in inflamed limbus. Sequence analysis of the Gro-α probe proved to be subtype specific. Expression of Gro-α (CXCL-1) was higher in limbal epithelium than in central cornea (limbal epithelium more than corneal epithelium, which was more than corneal stroma) (Figure 3). Furthermore, Gro-α (CXCL-1) was the only chemokine of all investigated chemokines expressed by corneal epithelium (Figure 4A). As mentioned in the previous section, MCP-1 (CCL-2) was not expressed by central corneal epithelium (Figure 2E). Furthermore, IL-8 (CXCL-8) message was not detected in corneal epithelium but in keratocytes in corneal stroma (Figure 4B). Finally, high amounts of Gro-α (CXCL-1) and MCP-1 (CCL-2) mRNA transcripts were localized in corneal stroma (Figure 4C and D). In contrast, only a few keratocytes expressed CXC chemokine MIG.

Figure 1. Leukocyte subsets in inflamed corneas determined by immunohistologic staining with a 3-step streptavidin-peroxidase method. Values shown are mean±SEM of 10 corneal lesions. PMN indicates polymorphonuclear leukocytes.

![Figure 1](https://www.archophthalmol.com/figure1.png)

![Figure 2A](https://www.archophthalmol.com/figure2a.png)

![Figure 2B](https://www.archophthalmol.com/figure2b.png)

![Figure 3](https://www.archophthalmol.com/figure3.png)

![Figure 4A](https://www.archophthalmol.com/figure4a.png)

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CXCL-9) (Figure 4E) and CC chemokine MIP-1α (CCL-3) mRNA (Figure 4F). According to the different mRNA expression levels and patterns, cross-hybridization between highly homologous chemokine probes could be excluded.

**NEUTROPHIL ATTRACTANT CHEMOKINE mRNA EXPRESSION IN ACUTE INFLAMED CORNEA**

If the composition of inflammatory cells is influenced by selective recruitment and trafficking through chemokines, expression of chemokines with neutrophil attractant properties should dominate acute inflamed corneas. To confirm this hypothesis, the number of cells expressing chemokines with neutrophil attractant properties (Gro-α [CXCL-1], IL-8 [CXCL-8]) were compared with cells that express mononuclear cell properties (MIG [CXCL-9], IP-10 [CXCL-10], MCP-1 [CCL-2], MIP-1α [CCL-3], and RANTES [CCL-5]). As shown in Figure 2, many more cells expressed neutrophil attractant chemokines than mononuclear cell chemokines. It is well known that MIG (CXCL-9) and IP-10 (CXCL-10) selectively recruit T lymphocytes, correlating with the low presence of CD3+ cells in inflamed le-
sions. Thus, the differential chemokine mRNA expression with dominance of neutrophil attractant chemokines (Gro-α [CXCL-1] and IL-8 [CXCL-8] over MIG [CXCL-9], IP-10 [CXCL-10], MCP-1 [CCL-2], MIP-1α [CCL-3], and RANTES [CCL-5]) clearly reflects the composition of the infiltrates showing a preponderance of neutrophil elastase–positive neutrophils.

**COMMENT**

The infiltration of leukocytes, particularly neutrophils, is an event common to various types of inflammation. Chemokines are known to mediate the migration of specific leukocyte subsets to an area of inflammation. In this study we attempted to elucidate which chemokines are responsible for the recruitment of neutrophils and mononuclear cells in inflamed corneas. Recent studies showed the expression of CC and CXC chemokines by keratocytes and corneal epithelial cells. These experiments were performed in cell culture. Experimental data about the expression of chemokines, especially in human corneas in vivo, is limited. It remains largely unknown which layers of inflamed corneas produce chemokines in vivo and what their expression patterns are. Furthermore, chemokine studies on inflammatory diseases show that cooperative efforts of a network of chemokines are important for leukocyte recruitment. However, a comprehensive repertoire of CXC and CC chemokines in corneal lesions. Values shown are mean±SEM of 10 corneal lesions. In situ hybridization was carried out with chemokine-specific sulfur 35–UTP–labeled RNA antisense probes as described in the “Methods” section. MCP-1 indicates macrophage chemoattractant protein 1; MIP-1α, macrophage inflammatory protein 1α; RANTES, regulated on activation, normal T-cell expressed and secreted; Gro-α, growth-related oncogene-α; IL-8, interleukin 8; IP-10, interferon-γ-inducible protein 10; and MIG, macrophage interferon-γ-inducible gene.

**Figure 3.** Expression of neutrophil and mononuclear cell attractant CC and CXC chemokines in corneal lesions. Values shown are mean±SEM of 10 corneal lesions. In situ hybridization was carried out with chemokine-specific sulfur 35–UTP–labeled RNA antisense probes as described in the “Methods” section. MCP-1 indicates macrophage chemoattractant protein 1; MIP-1α, macrophage inflammatory protein 1α; RANTES, regulated on activation, normal T-cell expressed and secreted; Gro-α, growth-related oncogene-α; IL-8, interleukin 8; IP-10, interferon-γ-inducible protein 10; and MIG, macrophage interferon-γ-inducible gene.

**Figure 4.** Chemokine messenger RNA (mRNA) expression by in situ hybridization with sulfur 35–uridine triphosphate–labeled RNA antisense probes in central inflamed cornea. There is high growth-related oncogene-α (Gro-α) mRNA expression in central corneal epithelium (A) and in corneal stroma (D). Interleukin 8 (IL-8) mRNA expression is absent in corneal epithelium but present in keratocytes in central stroma (B). There is a high number of macrophage chemoattractant protein 1 (MCP-1) mRNA-positive stromal cells in corneal stroma (C) and low macrophage interferon-γ-inducible gene (MIG; arrows) (E) and macrophage inflammatory protein 1α (MIP-1α) (F) mRNA expression in corneal keratocytes (original magnification, ×400 [A-C, E, F], ×320 [D]).
mokines has not been investigated in an acute inflamed human cornea with a predominant neutrophil infiltrate. We therefore studied the chemokine profile in inflamed human corneas (n = 10) with an infiltrate consisting of polymorphonuclear cells.

The current study demonstrated that human corneas after penetrating injuries express high levels of chemokines in limbal epithelium and limbal stroma, moderate levels in corneal stroma, and low expression levels in corneal epithelium. The most striking result in this study was the abundant expression of Gro-α (CXCL-1) and MCP-1 (CCL-2) in the limbus (epithelium more than stroma). Furthermore, the study showed the expression of MIG (CXCL-9), IP-10 (CXCL-10), and MIP-1α (CCL-3) in the human cornea, which has, to our knowledge, not been reported before in the literature. Other studies demonstrated the expression of MCP-1 (CCL-2), RANTES (CCL-5), Gro-α (CXCL-1), and IL-8 (CXCL-8) by keratocytes and corneal epithelium. These results are confirmed by other in vivo studies: Hong and colleagues demonstrated monocyte chemotactic and activating factor protein in keratocytes of rabbit corneas after epithelial scraping, and Elnor and colleagues showed the expression of IL-8 in endothelial and stromal cells of human corneas pretreated with IL-1β and tumor necrosis factor α (TNF-α). Taken together, these findings underscore the importance of the limbus and corneal stroma in the immune response of the anterior surface of the eye.

In contrast to the high chemokine expression levels in the limbal epithelium and corneal stroma, several studies have indicated a limited ability of the corneal epithelium to produce chemokines. They reported no expression of MCP-1 (CCL-2) and RANTES (CCL-5) and low expression levels of IL-8 (CXCL-8) in corneal epithelial cells. This study confirms these results. The Gro-α (CXCL-1) was the sole chemokine expressed by the central corneal epithelium. This result, however, is in contrast to recent in vitro studies that failed to detect message for Gro-α (CXCL-1) not only in normal cultures of corneal epithelium but also after stimulation with proinflammatory cytokines TNF-α and IL-1α. Although cultured corneal epithelial cells expressed very low levels of IL-8 (CXCL-8) mRNA, they could be significantly stimulated by TNF-α and IL-1α. In contrast, we could not detect IL-8 mRNA transcripts in corneal epithelium even in the context of significant inflammation. In our tissue, proinflammatory cytokines needed for the induction of IL-8 in corneal epithelial cells may be missing or a higher expression of this chemokine may occur in earlier or later stages of inflammation. In addition, a selective activation mechanism for high Gro-α mRNA expression or inhibiting factors for IL-8 mRNA expression have to be taken into consideration.

Chemokine studies in our laboratory of inflammatory skin diseases show very similar expression patterns between the skin and the limbus. This study demonstrated the expression of MCP-1 (CCL-2) by the basal limbal epithelium and the expression of Gro-α (CXCL-1) by suprabasal epithelial layers. In skin lesions of psoriasis, lichen planus, and erythema multiforme, MCP-1 (CCL-2) is expressed by the basal epithelium whereas Gro-α (CXCL-1) is expressed in clusters of keratinocytes. An explanation for the different expression patterns of Gro-α (CXCL-1) and MCP-1 (CCL-2) in the limbus might be that proliferating basal epithelial cells produce cytokines distinct from the postmitotic suprabasal cells.

In addition to their differential spatial distribution, studies have demonstrated that chemokines are expressed in a time-dependent manner. The study demonstrated that a broad array of chemokines are differentially expressed in inflamed corneas enucleated after a mean of 2 to 4 days. The neutrophil attractant chemokines Gro-α (CXCL-1) and IL-8 (CXCL-8) showed higher expression levels than the mononuclear cell attractant chemokines, MIG (CXCL-9), IP-10 (CXCL-10), RANTES (CCL-5), and MIP-1α (CCL-3). These findings are confirmed by wound healing studies performed by our laboratory. With the use of a skin repair model in adult humans, Gro-α (CXCL-1), IL-8 (CXCL-8), and MCP-1 (CCL-2) are maximally expressed and are associated with a neutrophil infiltration in the first 3 days. From day 4 on, MIG (CXCL-9) and IP-10 (CXCL-10) are highly expressed and paralleled by a mononuclear cell infiltration. It is, therefore, reasonable to suggest that, in the further time course of corneal inflammation, mononuclear cell attractant chemokines such as IP-10 (CXCL-10) and MIG (CXCL-9) will be up-regulated. This notion is confirmed by chemokine studies by our group on specimens with squamous cell carcinoma of the conjunctiva associated with chronic inflammation. The conjunctival epithelium showed loci with high expression levels of MIG (CXCL-9) in accordance with a high presence of mononuclear cells in the subepithelial epithelium (U.H.M.S., A.T., S.V., and R.G., unpublished results, 1999).

In conclusion, the differential chemokine production in limbus and cornea (limbus greater than corneal stroma greater than corneal epithelium) may be necessary to attract specific leukocyte subsets to different layers of the ocular surface. The corneal epithelium secretes significant levels of Gro-α (CXCL-1), to initiate an acute inflammatory response directed against superficial infections controllable by neutrophils. If the infection spreads to the corneal stroma, a more intense and longer-lasting immune reaction is needed to attract mononuclear cells to the cornea. Therefore, the expression of mononuclear cell attractant chemokines such as MCP-1 (CCL-2), MIP-1α (CCL-3), and MIG (CXCL-9) by corneal keratocytes, as shown in this study in vivo and other studies in vitro, may be triggered despite the risk of vision loss to protect the content of the eye.
leukocytes into corneal tissue. In addition, corticosteroids such as betamethasone inhibit cell infiltration into the aqueous humor and IL-8 expression in an endotoxin-induced uveitis rat model. The selective inhibition of a leukocyte subset, however, can only be achieved by means of selective chemokine antibodies such as anti–IL-8.

Further in vivo investigations are needed to demonstrate the expression of proinflammatory cytokines and chemokines in different inflammatory eye diseases. Selective receptor blockage of highly expressed chemokines or proinflammatory cytokines in inflamed eye tissue could then be explored as a reasonable strategy to inhibit the recruitment of leukocytes.

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