Granulocyte Macrophage Colony–Stimulating Factor Expression in Human Herpetic Stromal Keratitis: Implications for the Role of Neutrophils in HSK

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PURPOSE. Granulocyte macrophage colony–stimulating factor (GM-CSF) is thought to play a key role in chronic inflammatory diseases by governing the survival and function of infiltrating neutrophils. The objective of this study was to determine the putative role of GM-CSF in the pathogenesis of human herpetic stromal keratitis (HSK).

METHODS. Primary human corneal fibroblast (HCF) cultures and a telomerase-immortalized human corneal epithelial (HCE) cell line representative of native HCE were stimulated with the known HSK-inducing cytokines interferon (IFN)-γ, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α. Alternatively, the T-cell cytokine IL-17 was added solely or simultaneously. Human neutrophils were incubated with conditioned medium (CM) of the HCF and HCE stimulated with the aforementioned cytokines, or recombinant GM-CSF, and their viability or activation status was determined by flow cytometry. GM-CSF and IL-8 secretion levels in the CM were determined by ELISA. The antibody-dependent cellular cytotoxicity (ADCC) of neutrophils toward herpes simplex virus (HSV)-infected HCFs was determined by flow cytometry. The expression of GM-CSF was determined in HSK and control corneal buttons by real-time RT-PCR and immunohistochemistry.

RESULTS. Compared with IFN-γ, CM of either cell type stimulated with IL-1β, or in the case of HCE cells, stimulated with TNF-α or IL-17, delayed neutrophil apoptosis significantly. Only in HCFs did IL-17 exhibit a synergistic effect with TNF-α. The antipapoptotic activity was attributable in part to the GM-CSF secreted by the activated HCFs and HCE cells. GM-CSF stimulation of neutrophils induced their activation and the secretion of IL-8. GM-CSF did not increase significantly the ADCC reaction of neutrophils toward HSV-infected HCFs. Finally, GM-CSF was expressed in corneas of the patients with HSK but not in control subjects.

CONCLUSIONS. The data suggest that GM-CSF, expressed by cornea-resident cells such as HCFs and HCE cells, may play a role in the immunopathogenesis of HSK by prolonging the survival and modulating the effector function of corneal infiltrating neutrophils. (Invest Ophthalmol Vis Sci. 2007;48:277–284) DOI:10.1167/iovs.06-0055

Herpes simplex type (HSV)-1 infection of the cornea can induce keratitis clinically classified into herpetic epithelial keratitis (HEK) and herpetic stromal keratitis (HSK).1 HEK is an acute inflammation and results from viral toxicity in infected corneal epithelial cells. In contrast, HSK is characterized as a chronic immunopathogenic disease in which tissue injury and eventually blindness are due to the complex interplay between cells of the innate and adaptive immune response to antigens expressed in the corneal tissue.1,2 Studies performed in an experimental HSK mouse model have greatly improved our understanding of the pathogenesis of HSK. Although dendritic cells, macrophages, and CD4+ T cells play a pivotal role in the induction of the disease, neutrophils are considered to be the main cell type directly involved in the destruction of corneal architecture.2 The extravasation and function of neutrophils is coordinated by cytokine- and chemokine actions within the cornea.3–5 The cells that secrete these immune modulatory factors remain ill defined. Until recently, corneal infiltrating inflammatory cells have been advocated as the main source. Evidence is accumulating that tissue-resident cells such as fibroblasts play an important role as well.6–8 Activated human corneal epithelial (HCE) cells and fibroblasts (HCFs) secrete key cytokines such as interleukin (IL)-6 and -8.9–11 We have recently extended these studies by demonstrating that HCFs secrete a broad variety of chemokines after stimulation with proinflammatory cytokines.6 Moreover, we have shown that the T-cell cytokine IL-17, expressed within affected corneas of patients with HSK, have a modulatory effect on the secretion of these chemokines.6 Neutrophils normally live for less than 24 hours within the peripheral circulation. They undergo constitutive spontaneous cell death, referred to as apoptosis, as a mechanism to facilitate normal cell turnover and immune system homeostasis. Their rate of apoptosis is delayed on entry into tissues and subsequent exposure to specific cytokines.1,2 Conversely, extended neutrophil survival within tissues can result as persistent inflammation and tissue damage if these cells are stimulated to secrete their cytotoxic molecules such as proteases and reactive oxidants.1,2 Besides being indispensable for the growth and development of granulocyte-macrophage progenitors, granulocyte macrophage colony stimulating factor (GM-CSF) is a major regulator governing the effector function of both mature macrophages and neutrophils.14 It delays apoptosis and induces the release of proteolytic enzymes and oxygen free radicals, the latter referred to as the oxidative burst of neutrophils.14,15 There is mounting evidence of a proinflammatory role of GM-CSF in chronic inflammatory diseases.17,18 Rheumatoid arthritis is associated with sustained overproduction of cytokines such as IL-1, tumor necrosis factor (TNF)-α, IL-6, and GM-CSF.19 Neutralization of IL-1, TNF-α, and GM-CSF has been shown to ameliorate the disease symptoms in representative experimental animal models.19–21 Moreover, mice deficient in GM-CSF are
largely disease resistant.\textsuperscript{22} Considering the similarities between the pathogenic mechanisms and cell types involved in experimental arthritis and HSK, we explored the putative role of GM-CSF in human HSK by determining the intracorneal expression and induction of GM-CSF secretion by HCF and HCE on stimulation with the known HSK-inducing cytokines IFN-γ, TNF-α, and IL-1β. The combination effect of IL-17 and the role of GM-CSF on human neutrophil function were emphasized. The data show that GM-CSF is expressed in corneas of patients with HSK and has a regulatory effect on neutrophils by prolonging neutrophil survival and function.

**Materials and Methods**

**Cytokines and mAb Treatment**

Human recombinant IL-1β, IL-17, TNF-α, and IFN-γ were obtained from PeproTech (London, UK) and recombinant human (rh)GM-CSF R&D Systems (Abingdon, UK). For blocking experiments, a neutralizing mouse monoclonal antibody (mAb) directed to human GM-CSF (clone 32091; 5 μg/mL; R&D Systems) and an isotype-matched control mAb was used (clone 107.5; BD Biosciences, Erembodegem, Belgium). The optimal concentration of the anti-GM-CSF mAb was predefined by examining GM-CSF and neutrophils in pilot experiments (data not shown). Secretion levels of GM-CSF (U-CyTech, Utrecht, The Netherlands) and IL-8 (Biosource, Etten-Leur, The Netherlands) in cell-free conditioned media (CM) of cytokine-stimulated HCFs, HCE cells, and neutrophils were measured according the manufacturer’s instructions. The detection limit of both ELISAs was 10 pg/mL.

**Human Corneal Cell Cultures**

The local ethics committee approved the study and informed consent was obtained from all subjects who donated clinical specimens. The study adhered to the tenets of the Declaration of Helsinki. Primary HCF cultures were generated from four individual donor corneas, control corneas obtained from the Dutch Cornea Bank (Amsterdam, The Netherlands) that had been rejected for transplantation use because of low endothelial cell counts, and from two corneas of patients with HSK who underwent therapeutic keratoplasty to restore sight. The corneas were finely minced and digested with collagenase (Sigma-Aldrich, Zwijndrecht, The Netherlands) essentially as described elsewhere.\textsuperscript{6} Adherent cells were cultured in six-well plates in medium consisting of a 1:1 ratio (vol/vol) of Dulbecco’s modified Eagle’s medium (DMEM) and F-12 nutrient mixture (Ham F12; Invitrogen, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (referred to hereafter as HCF medium). HCFs and HCE cells were grown in six-well plates in HCF and SFM medium, respectively. At confluence, approximately 3 × 10^6 cells/well for both cell types, medium of the HCF cultures was replaced with a serum-free medium (referred to as SF-HCF medium) consisting of DMEM and Ham F12 (1:1; vol/vol) supplemented with insulin-transferrin-selenium-X supplement and 0.5% bovine serum albumin (BSA; all obtained from Invitrogen). HCF was left for 5 days on SFM before stimulation with cytokines. SFM was used to maintain a more native biosynthetic phenotype and appearance and to reduce background levels of cytokine and chemokine production.\textsuperscript{6} Analogously, the SFM of the HCE cultures was replaced before addition of the cytokines. The HCF and HCE cultures were incubated in triplicate for 48 hours at 37°C with stimulatory cytokines added at previously defined optimal concentrations: IL-17 (100 ng/mL), IL-1β (100 ng/mL), TNF-α (50 ng/mL), and IFN-γ (100 U/mL) in a total volume of 1 mL.\textsuperscript{6} Subsequently, cell-free CM was collected and frozen in aliquots at −70°C. Experiments were repeated at least three times.

**Assessment of Cytokine-Induced Viability and Activation of Human Neutrophils**

Human neutrophils were isolated from heparinized venous blood of healthy individuals (Polymorphprep; Axis-Shield, Oslo, Norway), and residual erythrocytes were lysed (BD Pharm Lyse; BD Biosciences), according to the manufacturer’s instructions. The isolated cell fraction typically contained at least 90% to 95% granulocytes with a viability of >95%, as determined by May-Grünwald/Giemsa and trypan blue exclusion staining, respectively. The granulocytes obtained consisted almost exclusively of neutrophils with traces of eosinophils (<1%) as assessed by differential CD16 (fluorescein-conjugated anti CD16; clone 3G8; BD Biosciences) expression by both granulocyte subsets using flow cytometric analyses.\textsuperscript{25} Moreover, the latter technique confirmed the frequency of granulocytes in the isolated cells, as defined by May-Grünwald/Giemsa staining, judged on the differential forward and side-scatter pattern of mononuclear cells versus granulocytes (data not shown). The neutrophils were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS and antibiotics (referred to as R10F; 5 × 10^6 neutrophils in 500 μL medium) and incubated with diluted SF-HCF or SFM or CM of mock- and cytokine-stimulated HCFs and HCE cells for 18 hours at 37°C in a CO₂-incubator. The total assay volume was 0.5 mL and the dilution, with R10F, of the HCF (1:100) and HCE (1:20) CM was defined in pilot experiments for optimal distinction between neutrophil survival by using the CM of the cells stimulated with the different cytokines. For blocking experiments, the CM was similarly diluted before the addition of anti-GM-CSF and the isotype control mAb. The neutrophils were stained with 20 μg/mL 7-aminocinomycin D (7AAD; Sigma-Aldrich) for 20 minutes at 37°C and examined by flow cytometry. Based on a study by Philpott et al.,\textsuperscript{26} 7AAD-negative cells were considered to be viable nonapoptotic cells (Vcells). Samples were acquired on a flow cytometer (FACSCalibur; BD Biosciences). The forward scatter (FSC) and side scatter (SSC) acquisition threshold was set to include all neutrophils, including dead neutrophil events (Dcells), but to exclude mononuclear cells. Debris was excluded by gating in FSC-7AAD dot plots during data analyses. Percentages of viable 7AAD-negative events (%)Vcells were calculated with the formula 100 × (number of Vcells)/(number of Vcells + number of Dcells).

Alternatively, neutrophils were incubated, in a total assay volume of 0.5 mL, with increasing doses of rhGM-CSF, the known neutrophil-activating mitogen lipopolysaccharide (LPS; 1 μg/mL; Sigma-Aldrich) or solely R10F for 30 minutes or 18 hours at 37°C in a CO₂-incubator to determine their activation status or viability, respectively. Neutrophils stimulated for 30 minutes were stained with fluorochrome-conjugated mAbs directed to CD11b (fluorescein-conjugated; clone M1/70; BD Biosciences) and CD62L (phycoerythrin-conjugated; clone Dreg56; BD Biosciences), or their respective isotype controls A95-1 and A95-2 (BD Biosciences), respectively. Cell surface expression was analyzed by flow cytometry (FACSCalibur device; BD Biosciences). The CM of stimulated neutrophils, collected after 6 hours that was defined as the optimal time point in pilot experiments (data not shown), was analyzed for IL-8 secretion levels by a commercial ELISA (R&D Systems).

**Antibody-Dependent Cellular Cytotoxicity Assay**

The HCFs were infected with a recombinant HSV-1 virus (strain v44), expressing VP16 linked to the green fluorescent protein (GFP), at a multiplicity of infection of 0.02, and incubated overnight. This recombinant HSV-1 strain replicates with virtually normal kinetics and yields and incorporates the fusion protein into the virion, resulting in autofluorescent particles.\textsuperscript{27} As the control, HCFs were treated similarly.
but without addition of virus, referred to as mock-infected HCF. The HSV-1- and mock-HCF (i.e., target cells) were trypsinized, extensively washed, and incubated at 4°C for 1 hour in HCF medium with 1:200 (vol/vol) diluted heat-inactivated human pooled serum of donors serologically identified as HSV seronegative or -positive. Subsequently, neutrophils (i.e., effector cells) were added at different effector:target ratios and incubated for 4 hours at 37°C. Alternatively, rhGM-CSF was added to a final concentration of 100 pg/mL. During the last 20 minutes of incubation TO-PRO-3 iodide (TP3; final concentration at 25 nM; Invitrogen) was added to discriminate between viable and nonviable cells.28 Samples were acquired on the flow cytometer (FACSCalibur; BD Biosciences). The FSC acquisition threshold was set to include nonviable events. Debris was excluded by gating in FSC-TP3 dot plots during data analyses. A region to exclude GFP-negative events was defined in GFP-TP3 or GFP-fluorescence channel 3 (FL3) dot plots of the data acquired from cultures that contained mock-infected HCF. GFP-positive events derived from HCF cultures, infected with the GFP expressing HSV-1 strain, were displayed in FSC-TP3 or GFP-TP3 dot plots for the definition of viable HSV-1-infected GFP-positive (GFP+Vcells) events (i.e., GFP+; TP3-negative events) and nonviable or dead GFP-positive (GFP+Dcells) events (i.e., GFP+; TP3-positive events). Percentages of dead GFP-positive cells (% GFP+Dcells) were calculated with the formula 100 × (number of GFP+Dcells)/(number of GFP+Vcells + number of GFP+Dcells).

RNA Isolation and Real-Time Reverse Transcription–Polymerase Chain Reaction Analyses

Total cellular RNA was extracted from HSK and control corneas (TRizol LS reagent; Invitrogen) and subsequently purified (RNeasy kit; Qiagen, Crawley, UK) according to the manufacturer’s protocol. The cornea buttons analyzed were obtained from patients with severe HSV-induced HSK after therapeutic penetrating keratoplasty. Donor corneas, replaced for transplantation purposes, were included as control corneas. For real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analyses, RNA was converted into single-stranded copy DNA using random primers and reverse transcriptase (all from Invitrogen) according to the manufacturer’s protocol. Relative expression levels of GM-CSF and the housekeeping gene β-actin were measured with the 5’ fluorogenic nuclease assay in real-time quantitative PCR (TaqMan chemistry; 7700 Prism real-time PCR system; Applied Biosystems, Inc. [ABI] Warrington, UK). The GM-CSF and β-actin primer probe sets were obtained from Applied Biosystems (Assays-on-Demand, ID No. Hs00171266 and Hs99999903, respectively). PCR was conducted using the following cycle parameters: 95°C, 12 minutes for 1 cycle (95°C, 20 seconds; 60°C, 1 minute), for 45 cycles. Analysis was conducted using the sequence-detection software supplied with the real-time PCR system (model 7700; ABI). The software calculates the threshold cycle (Ct) for each reaction and this was used to enumerate the amount of starting template in the reaction. The Ct for each set of duplicate reactions were averaged for all subsequent calculations. A difference in Ct (∆Ct) was calculated for each gene by taking the mean Ct of the gene of interest and subtracting the mean Ct for β-actin for each cDNA sample. Relative mRNA expression levels were calculated using the formula 2−∆∆Ct.

Immunohistochemistry

Corneal buttons, obtained within 3 hours after surgery, were fixed with formalin, embedded in paraffin, and cut into 4-μm-thick sections. After antigen retrieval by pronase (1 mg/mL; Sigma-Aldrich) treatment at 37°C for 12 minutes, sections were treated with an avidin-biotin blocking kit (Vector Laboratories, Peterborough, UK) to block nonspecific binding sites of the avidin-biotin system reagents. Consecutive slides from each cornea were stained with the unconjugated mAbs anti-human GM-CSF (clone 5209.1; R&D Systems) or IgG1 isotype control (clone 107.3; BD Biosciences). Subsequently, the streptavidin–biotin immunoperoxidase method was used. The reagents used for the subsequent steps, such as biotinylated goat anti-polyvalent immunoglobulin, peroxidase-labeled streptavidin and 3-aminob-9-ethyl carbazole, were obtained from Laboratory Vision (Fremont, CA).

Statistical Analyses

The data are presented as the mean ± SEM. A two-tailed paired t test or a one-tailed ANOVA with the Bonferroni posttest was performed (Prism; GraphPad Software, San Diego, CA). P < 0.05 was taken as indicative of statistical significance.

RESULTS

Effect of CM from Cytokine-Stimulated HCFs and Epithelial Cell Lines on Spontaneous Neutrophil Cell Death

Studies in the experimental HSK mouse model have demonstrated that neutrophils are the main cell type–associated with collateral tissue damage.1,2 Apoptosis is the physiologic cell death of neutrophils. However, neutrophil apoptosis within inflamed tissue can be delayed when neighboring cells like tissue resident cells generate survival factors.1,2 We determined whether HCFs and a human corneal epithelial cell line, closely resembling native HCE cells,23,24 secrete neutrophil survival factors in vitro on stimulation with cytokines known to be involved in the development of HSK; TNF-α, IL-1β, and IFN-γ. Compared with mock-treated cells, CM of IL-1β and TNF-α-treated HCE significantly delayed in neutrophil cell death at the same level. In contrast, only CM of IL-1β-treated HCF improved neutrophil survival significantly (Fig. 1). For both cell types IFN-γ stimulation had no effect. Subsequently, we determined the combinatorial effect of IL-17, a proinflammatory T-cell cytokine potentially involved in the immunopathogenesis of HSK.6 In contrast to HCF, the CM of IL-17-treated HCE showed a significant delay in neutrophil cell death (Fig. 1). However, in contrast to HCE, incubation of HCF with combinations of the aforementioned cytokines showed that IL-17 had a synergistic effect only on the TNF-α-induced secretion of a neutrophil survival factor(s) (Fig. 1).

Contribution of GM-CSF Secreted by Cytokine-Treated HCF and Epithelial Cell Lines to Neutrophil Survival

Delayed neutrophil apoptosis has been associated with several acute and chronic inflammatory diseases and appears to be largely mediated by the production of GM-CSF.16,17 To determine whether GM-CSF contributes to the observed delay in neutrophil cell death, we determined the GM-CSF secretion levels in CM of the cytokine-treated HCF and HCE cell cultures. For both cell types, only IL-1β and TNF-α stimulation induced GM-CSF secretion, and IL-17 exerted a synergistic effect with TNF-α. Overall, HCFs secreted approximately three times more GM-CSF than did HCE cells (Fig. 2).

To assess the potential role of GM-CSF in contributing to neutrophil survival directly, the CM obtained from the HCF and HCE cultures was immunodepleted of GM-CSF by using a specific mAb. First, the kinetics of neutrophil survival in relation to increasing amounts of rhGM-CSF was determined. rhGM-CSF inhibited neutrophil cell death, even at the lowest concentrations, during the first for 6 hours of incubation. Only at 20 hours was a clear, dose-dependent decline in the number of viable neutrophils observed (Fig. 3A). Second, we noted that the anti-GM-CSF mAb, at a concentration of 5 μg/mL, inhibited the neutrophil ant apoptotic effect of 100 pg/mL rhGM-CSF significantly (Fig. 3B).

Finally, the CM of both cell types displaying high neutrophil survival activity were analyzed. Compared with the isotype
control or untreated CM, pretreatment of the CM with a neutralizing anti-GM-CSF mAb significantly blocked neutrophil survival (Figs. 3C, 3D). However, in all anti-GM-CSF–treated CM analyzed, residual neutrophil survival factor activity remained present.

Effect of GM-CSF Stimulation on the Expression of Adhesion Molecules and Secretion of IL-8 by Neutrophils

The functional activity of neutrophils is modulated on several levels. Two particularly important aspects are the priming of neutrophils to undergo an oxidative burst (i.e., release of reactive oxygen derivatives, and simultaneously the mobilization of granule contents).16 Examples of the latter are CD11b, a component of the integrin Mac-1 (i.e., CD11b/CD18-dimer), and the adhesion molecule L-selectin (CD62L).16,29 Consequently, these adhesion molecules have been advocated as surrogate markers for the neutrophil oxidative burst.29 In addition, these molecules are important for neutrophil recruitment and transmigration through the endothelial layer into tissues. Both adhesion molecules are expressed by resting neutrophils. On neutrophil activation, CD11b is mobilized from granules to the cell surface and membrane-bound CD62L is proteolytically shed.29 To evaluate the role of GM-CSF on the expression of these neutrophil activation markers, neutrophils were incubated with increasing amounts of GM-CSF, and the cell surface expression of CD11b and CD62L was analyzed by flow cytometry. GM-CSF treatment induced, in a dose–response fashion, the up- and downregulation of CD11b and CD62L, respectively (Fig. 4A).

In addition to intralesional mononuclear cells such as macrophages and T cells, tissue-infiltrating neutrophils have been identified as a prominent source of proinflammatory cytokines and chemokines. Herein, we demonstrate that GM-CSF treatment of neutrophils induced IL-8 secretion in a dose–response fashion (Fig. 4B). Neutrophils stimulated with LPS, used as positive control, responded accordingly in both assays.

Effect of GM-CSF on Neutrophil Antibody-Dependent Cellular Cytotoxicity of HSV-1–Infected HCFs

Anti-HSV antibodies, both secretory IgA and IgG, are commonly detected in the tear film of patients with HSV keratitis.30,31 In contrast to the experimental HSK mouse model, HSV-infected corneal fibroblasts have been detected in corneas of patients with HSK.1,2 Lysis of HSV-infected corneal fibroblasts by means of ADCC, considered to be the major antiviral mechanism of neutrophils, may play a role in the

FIGURE 1. Conditioned medium of cytokine stimulated HCFs and an HCE cell line differentially delay neutrophil cell death. Neutrophils were incubated overnight in diluted CM, generated by stimulating HCF or HCE for 48 hours with the indicated cytokines, and the percentage of viable neutrophils was assessed. The CM was diluted with assay medium: 1:100 (HCF) and 1:20 (HCE). The results, expressed as the mean ± SEM of three independent experiments, are the net values of cytokine minus medium stimulation. The probabilities were calculated with one-way ANOVA with the Bonferroni posttest.

FIGURE 2. HCFs and an HCE cell line secrete GM-CSF on cytokine stimulation. The HCFs and HCE were stimulated for 48 hours with the indicated cytokines, and the concentration of GM-CSF present in the conditioned medium was assessed by ELISA. Results are expressed as the mean ± SEM of a representative experiment performed in triplicate. The probabilities were calculated with one-way ANOVA with the Bonferroni posttest.
pathogenesis of HSK. Accordingly, we analyzed the killing of HSV-1-infected HCFs by neutrophils in the presence of serum of HSV seropositive versus seronegative donors. Infected HCFs were not killed on incubation with serum only and mock-infected HCFs were not killed by the neutrophils on incubation with either serum sample (data not shown). Neutrophils lysed HSV-1 infected HCFs preincubated with serum of HSV-seropositive donors to a greater extent—but only significantly at the highest neutrophil (effector; E)/HCF (target; T) ratio: E/T of 100—than did cells incubated with HSV-seronegative serum (Fig. 5). Addition of rhGM-CSF to ADCC assays has been shown to amplify the neutrophil cytolytic efficiency. Conversely, rhGM-CSF tended to increase, although not significantly, the neutrophil-mediated killing of HSV-1-infected HCFs in the presence of HSV-seropositive serum only at the highest E/T ratio (Fig. 5).

FIGURE 3. GM-CSF secreted by cytokine-stimulated HCFs and an HCE cell line contributed to neutrophil survival. (A) Kinetics of neutrophil cell death in relation to increasing concentrations of rhGM-CSF. Neutrophils were incubated with increasing amounts of rhGM-CSF, or with control medium only, for the indicated times at 37°C. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with medium. (B) Anti-GM-CSF mAb specifically neutralized rhGM-CSF-mediated neutrophil survival. rhGM-CSF (100 pg/mL) was preincubated with anti-GM-CSF (GM-CSF + aGM-CSF) or isotype control mAb (GM-CSF + isotype), both at 5 μg/mL, for 30 minutes and neutrophils subsequently added for overnight culture at 37°C. (C, D) Dilted conditioned medium from HCFs (C; 1:100 diluted with assay medium) or HCE (D; 1:20 diluted) cultures, stimulated with the indicated cytokines, were preincubated with 5 μg/mL anti-GM-CSF (u) or an isotype control mAb (f) and neutrophils subsequently added and incubated overnight at 37°C. (□) Untreated conditioned medium. All results are expressed as the mean ± SEM percentage of viable neutrophils of representative experiments performed in triplicate. Probabilities were calculated with one-way ANOVA with the Bonferroni posttest.

FIGURE 4. GM-CSF stimulation induces the activation and IL-8 secretion of neutrophils. Freshly isolated neutrophils were incubated with increasing amounts of GM-CSF, LPS (1 μg/mL), or control medium only, for 30 minutes (A) or 6 hours (B) at 37°C. The expression of CD11b and CD62L on neutrophils was analyzed by flow cytometry. The secretion levels of IL-8 were determined by ELISA in cell-free CM. Results are expressed as the mean ± SEM of a representative experiment performed in triplicate. Probabilities were calculated with one-way ANOVA with the Bonferroni post test. *P < 0.01 compared with medium.
GM-CSF in Corneas of Patients with HSK

Demonstrating that HCF secretes GM-CSF on cytokine treatment in vitro, challenges the question of whether GM-CSF is expressed in corneas of patients with HSK. We first determined the expression of GM-CSF transcripts in HSK corneas and compared it with control corneas. The relative transcript levels of GM-CSF were significantly higher in cornea buttons of patients with HSK compared with the control (Fig. 6).

Second, to determine the cell type expressing GM-CSF, we performed immunohistologic staining on cornea sections of patients with HSK. Compared with the isotype control, staining of HSK corneas with a GM-CSF-specific mAb revealed marked GM-CSF expression in the corneal epithelial cell layer. In contrast, limited GM-CSF expression within the corneal stroma was detected which, based on their cell morphology and localization, could be addressed to infiltrating inflammatory cells. The corneal fibroblasts did not appear to express GM-CSF. No GM-CSF expression was detected in control corneas rejected for transplantation purposes (data not shown). Representative stainings for GM-CSF and an isotype control on corneas of two patients with HSK are shown in Figure 7.

DISCUSSION

The deposition of functional neutrophils in inflamed tissue is essential in the host defense to curtail infections, but a subsequent controlled elimination of these cells is of similar importance to prevent the development of a chronic potentially pathogenic inflammation. Current knowledge of the pathogenic processes involved in chronic inflammatory responses challenges the previous unappreciated role of tissue-resident cells. Such cells impose a stromal address code on tissue-infiltrating inflammatory cells that governs their accumulation, survival, activation, and differentiation. It is generally accepted that mesenchymal cells, on activation with proinflammatory cytokines of macrophage/monocyte (IL-1β and TNFα) and T-cell (IFN-γ) origin, are involved in the recruitment of inflammatory cells like neutrophils via the expression of adhesion molecules and chemoattractants. However, the factors involved in prolonging survival and modulating the effector function of these pathogenic neutrophils in HSK remain ill-defined. Our laboratory is engaged in an ongoing effort to define the possible role of corneal resident cells, in addition to infiltrating inflammatory cells, in the disease process. This report evaluates the potential role of GM-CSF in relation to the role of neutrophils in the pathologic course of human HSK.

The results presented herein demonstrate that GM-CSF is expressed in corneas of patients with HSK, but not in control corneas. The proinflammatory cytokines TNF-α and IL-1β induced GM-CSF secretion by HCFs and HCE cells. These findings are concordant with those in similar studies of other cell types. However, the data are in contradiction to a study by Cubitt et al. of corneal epithelial cells and HCFs. Whereas both corneal cell types secreted copious amounts of GM-CSF on IL-1α stimulation, GM-CSF secretion could only be induced in HCE cells by TNF-α. This puzzling discrepancy between our data and findings in the latter study remains inexplicable, in that in both studies similar amounts of TNF-α and cell types were used. Possibly, differences in culture conditions or donors from whom the HCFs were obtained are responsible for the discrepancy. Unfortunately, efforts to culture corneal epithelial cells from the cornea donors to compare both cell types in our study were unsuccessful. The similarities in GM-CSF secretion levels after TNF-α and IL-1α/β stimulation of the immortalized HCE cell line described herein (Fig. 2), and the primary HCE cultures applied by Cubitt et al. once more underlines the close resemblance of this HCE cell line to native HCE.

We have recently shown that IL-17, expressed in corneas of patients with HSK, modulates the secretion of various chemokines by cytokine-stimulated HCF. Herein, we demonstrated a new aspect of the T-cell-specific cytokine IL-17 in terms of its stimulatory action on both HCF and HCE. Whereas IL-17 itself had no effect, IL-17 selectively enhanced TNF-α-induced GM-CSF secretion by both cell types, consistent with previous studies on different cell types. The expression of GM-CSF is regulated both by transcriptional and posttranscriptional mechanisms. IL-17 shares properties mainly with IL-1β and TNF-α, in that these three cytokines activate the transcription factor NF-κB in a variety of cell types including mesenchymal cells like fibroblasts. In human colonic myofibroblasts, the synergistic effect of IL-17 to TNF-α in GM-CSF release was mediated both by the additive effect of IL-17 on TNF-α-induced NF-κB DNA-binding activity as well as the effect of IL-17 in stabilizing the rapid degradation of the GM-CSF transcript in TNF-α-stimulated cells. Of interest, the IL-1β-induced GM-CSF mRNAs were stable and were not affected by IL-17 in colonic myofibroblasts. Given the similarity between colonic myofi-
broblasts and HCFs, these mechanisms may have contributed to the synergistic effect of IL-17 on TNF-α-induced HCF secretion of GM-CSF. In the case of HCE cells, the mechanisms involved are unknown and necessitate additional research.

In contrast to corneal epithelial and infiltrating inflammatory cells, corneal fibroblast do not appear to be the cellular source of GM-CSF in cornea buttons of patients with severe HSK (Fig. 7). However, the in vitro data on primary HCF cultures proved otherwise (Fig. 2). Penetrating keratoplasty (PKP) is the last option to treat patients with severe HSK to restore sight. Immunosuppressive treatment of these patients, often for many months, before PKP may have differentially inhibited GM-CSF production by the corneal fibroblasts compared with the epithelial cells in situ. For obvious reasons, corneal buttons from patients with untreated HSK are not available. Studies on the experimental HSK mouse model may provide more insight into this discrepancy.

We demonstrated that the production of GM-CSF is functionally significant. CM from both cell types stimulated with TNF-α or IL-1β, with or without addition of IL-17, prolonged the survival of human neutrophils, and this effect was blocked significantly by a neutralizing GM-CSF specific mAb. However, given the relatively low GM-CSF levels in the aforementioned CM of cytokine-treated HCFs and HCE cells, compared with the combination of TNF-α and IL-17, other neutrophil survival factors besides GM-CSF are most likely involved (Fig. 1). Notably, the CM of IL-17 that stimulated HCE cells was devoid of GM-CSF and exerted sustained neutrophil survival activity. The potential fibroblast factors involved may be cytokines like G-CSF or IL-6.12 Previously, others and our group have demonstrated that these proinflammatory cytokines induce IL-6 secretion by HCFs and HCE cells.5,10 Studies are needed to determine the total spectrum of neutrophil survival factors differentially secreted by cytokine-stimulated cornea-resident cells.

In contrast to the HSK mouse model, HSV-infected corneal fibroblasts have been demonstrated in corneas of patients with HSK.31,42 The presence of HSV-specific antibodies within tears of these patients poses the possibility of a neutrophil ADCC response, as part of their pathogenic role in HSK.50,51 The in vitro data presented herein underline this option and are in agreement with other studies in which different target cells were used.43,44 Neutrophil ADCC depends on the expression of both Fc γ-receptors and Mac-1.45 GM-CSF has been shown to improve mAb-dependent cellular cytotoxicity of neutrophils toward various tumor cell lines.32,45 This effect was largely attributable to the upregulation of Mac-1, mediating adhesion and subsequently the neutrophil oxidative burst once neutrophil-target cell conjugates have been established by mAb-antigen complexes on the target cell.45 Conversely, addition of GM-CSF did not significantly enhance killing of the HSV-infected HCFs (Fig. 4)—this, despite the ability of GM-CSF to induce the early upregulation of CD11b and shedding of CD62L (Fig. 3A), described as surrogate markers for the neutrophil oxidative burst response, similar to that described by others.29 Possibly cell type differences (e.g., expression of Mac-1 ligands) and/or the use of serum antibodies compared with mAbs may have accounted for this incongruity.32,45

The present study is the first to detect GM-CSF expression in corneas of patients with HSK. In vitro studies showed that cytokine-stimulated HCFs and HCE cells secreted GM-CSF capable of inducing the survival, activation, and even secretion of the major neutrophil chemoattractant IL-8 (Fig. 3B). Because chronic inflammation is critical to the loss of corneal function in the setting of HSK, our data suggest that GM-CSF may represent a promising therapeutic target as an antagonist of neutrophil-mediated tissue destruction. In addition, cytokine-induced expression of GM-CSF may initiate the entry and activation of macrophages into HSV-infected corneas, as has been demonstrated in GM-CSF transgenic mice.46 Studies are in progress to investigate the putative proinflammatory role of GM-CSF in the experimental HSK mouse model.

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References


