Role of Macrophage Migration Inhibitory Factor in Conjunctival Pathology in Ocular Cicatricial Pemphigoid

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PURPOSE. Macrophage migration inhibitory factor (MIF) is a pleiotropic, proinflammatory cytokine that mediates various immunoinflammatory processes. Ocular cicatricial pemphigoid (OCP) is an autoimmune disease in which affected conjunctiva show features of an immunoinflammatory disease. In this study, the role of MIF in the pathogenesis of OCP was examined.

METHODS. The expression of MIF in conjunctival tissues of patients with OCP (n = 10) and normal subjects (n = 5) was studied by quantitative real-time PCR and immunohistochemistry. The production of MIF by conjunctival fibroblasts of normal control subjects and patients with OCP was determined, by using quantitative real-time PCR and enzyme-linked immunosorbent assay (ELISA). In addition, the effects of interleukin (IL)-1, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β1 on the induction of MIF by conjunctival fibroblasts were studied by quantitative real-time PCR. To determine the relationship between conjunctival expression of MIF and accumulation of macrophages, in patients with OCP, a correlation study was performed.

RESULTS. An increased conjunctival expression of MIF was detected in patients with OCP, both at the mRNA (by real-time PCR) and protein level (by immunohistochemistry), compared with normal control patients. The expression of MIF was detected in the epithelial cells and occasionally in the stromal cells in control conjunctival tissues, by immunohistochemistry. In contrast, a statistically significant increase (P < 0.0001) in the expression of MIF was detected in the stromal cells of conjunctival tissues obtained from patients with OCP (control: 4.89 ± 0.5; OCP: 19.82 ± 1.34). By quantitative real-time PCR, compared with control conjunctiva, an increase in the expression of MIF was detected in the conjunctiva obtained from patients with OCP. A similar increase in the expression of MIF was also detected in conjunctival fibroblasts of patients with OCP, compared with control fibroblasts, by quantitative real-time PCR. A significantly increased (P < 0.001) level of MIF was also detected in supernatant collected from conjunctival fibroblasts of patients with OCP (186 ± 5.4), compared with supernatant collected from control fibroblasts (9.3 ± 7.6). Moreover, IL-1, TNF-α, and TGF-β1, known factors involved in the pathogenesis of OCP, were found to induce the expression of MIF by conjunctival fibroblasts. A statistically significant correlation (P < 0.0001, r² = 0.4465) was observed between the expression of MIF and accumulation of CD68-positive macrophages in conjunctiva of patients with OCP.

CONCLUSIONS. This study demonstrated an increased conjunctival expression of MIF in patients with OCP. MIF may be actively involved in the pathogenesis of OCP, possibly regulating the inflammatory events of the disease process. (Invest Ophthalmol Vis Sci. 2004;45:1174–1181) DOI:10.1167/iovs.03-1138

Monocytes and macrophages are considered to be important participants in the inflammatory events in various immunoinflammatory diseases, including ocular cicatricial pemphigoid (OCP).1,2 The factors that regulate the accumulation of macrophages in the conjunctiva of patients with OCP are not yet well understood. MIF plays an important role in determining the macrophage population in various immunoinflammatory diseases, by inhibiting the migration of macrophages from the affected sites and organs. For instance, MIF promotes macrophage accumulation in the skin in delayed-type hypersensitivity reaction.3,4 An increase in the level of MIF has been suggested to be responsible for the recruitment and local accumulation of macrophages in both experimental and human glomerulonephritis and in allograft rejection.5–7 Furthermore, in vivo blocking of the bioactivities of MIF results in the suppression of subsequent macrophage-mediated renal injuries and arthritis.6–10

OCP is one of the subsets of mucous membrane pemphigoid, characterized by T-cell dysregulation, and abnormal production of circulating autoantibodies directed against various components of basement membrane zone (BMZ), with generation of certain proinflammatory and fibrogenic cytokines.11,12 The process of immunoregulation in OCP and the subsequent conjunctival scarring are not yet completely understood. The initial trigger in OCP may be a process by which the target sites undergo a conformational change that provides antigenic stimulation, which may induce generation of specific B-cell clones that produce antibodies against various components of the BMZ. The antigen-antibody binding leads to the activation of the complement cascade, with generation of inflammatory mediators that facilitate migration of inflammatory cells, including macrophages, to the site of disease.1,2 The subepithelial BMZ separation observed may be the direct effect of the proteolytic enzymes. Fibroblast activation, which is secondary to initial immunoinflammatory events, may induce increased production of extracellular matrix (ECM) proteins and subsequent conjunctival scarring. Progressive conjunctival scarring causes profound tear insufficiency, meibomian gland dysfunction, and mucin deficiency. In the advanced stage of the disease process, symblepharon formation, trichiasis, distichiasis, and keratinization cause corneal ulceration, neovascularization, scarring, and possible blindness.1,2

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Although it is known that macrophages could intensify the immunoinflammatory responses and subsequent tissue damage in various organs,\textsuperscript{13–16} including those in patients with OCP, the factors regulating the conjunctival accumulation of macrophages are not yet completely defined. Because MIF has the potential to regulate the monocyte-macrophage population in certain pathologic conditions, we decided to study the role of MIF in the pathogenesis of OCP.

**Materials and Methods**

**Conjunctival Tissues**

Biopsy specimens of conjunctiva obtained from 10 patients with OCP were used in this study. The diagnosis of OCP was based on clinical presentation, histology, and direct immunofluorescence studies of the conjunctiva demonstrating IgG and C3 at the BMZ. Conjunctivae from five normal individuals who were undergoing routine cataract surgery were used as control samples. These individuals did not have any metabolic or systemic diseases. Both control subjects and patients with OCP used in this study were elderly and of similar age. The study followed the guidelines of the Declaration of Helsinki for research involving human subjects and was approved by the institutional review boards of our institutions.

**Immunohistochemistry**

Immunohistochemistry was performed on paraffin-embedded and on frozen sections of conjunctiva, as described in our earlier studies.\textsuperscript{17–20} Briefly, tissue sections were blocked with either 10% goat serum or 10% rabbit serum for 1 hour and then incubated overnight at 4°C with the following primary antibodies: rabbit anti-human MIF (FL-115; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-human CD68 (Dako, Glostrup, Denmark). After a wash in phosphate-buffered saline (PBS), the sections were treated with a secondary antibody for 30 minutes (biotinylated goat anti-mouse IgG for CD68; biotinylated goat anti-rabbit IgG for MIF), washed with PBS, and incubated further with streptavidin-peroxidase. The reaction products were developed with a 3,3-diaminobenzene-4 HCl (DAB) and H₂O₂. Both the secondary antibodies and streptavidin-peroxidase solution were from a staining kit (Histostain; Nichirei Co., Tokyo, Japan). Normal mouse serum was used as a negative control for the mouse anti-human CD68 antibody, and normal rabbit serum was used as a negative control for the rabbit anti-human MIF antibody. Conjunctival fibroblasts isolated from normal and OCP-affected conjunctiva were grown on glass slides, fixed with methanol, and immunostained for MIF, as described earlier.\textsuperscript{11,12} To quantitate the number of MIF- and CD68-positive macrophages, immunostained cells were counted randomly in at least three fields (magnification, ×20) in each biopsy section with the aid of a light microscope (Nikon Corp., Tokyo, Japan). The number of cells in each field from the control subjects and patients with OCP is expressed as the mean ± SEM.

**Isolation of Conjunctival Fibroblasts**

Fibroblasts from conjunctiva of normal control subjects and patients with OCP were isolated as described in the earlier studies.\textsuperscript{11,12,21,22} Briefly, available conjunctival tissue was cut into explants of approximately 2 × 2 mm², placed into tissue culture dishes, covered with Dulbecco’s Modified Eagle Medium (DMEM) containing fetal calf serum (FCS) and antibiotics, and left in an incubator overnight at 37°C with 95% humidity and 5% CO₂. The medium was changed three times weekly thereafter for 2 weeks. The isolated fibroblasts were subcultured with 0.1% trypsin and 0.02% EDTA in Ca²⁺/Mg²⁺-free minimum essential medium (MEM) at 80% to 90% confluence. Total RNA extracted from fibroblasts isolated from conjunctiva of control subjects and patients with OCP was used for quantitative real-time PCR analysis to determine the expression of MIF. In addition, control and OCP fibroblasts were grown on glass slides, fixed with methanol, and used for immunostaining for MIF, as described earlier.

**Effects of IL-1, TNF-α, and TGF-β1 on the Expression of MIF by Conjunctival Fibroblasts**

The conjunctival fibroblasts were subcultured and kept in serum-free medium for 24 hours and then were treated with various concentrations (1, 10, and 100 ng/mL) of recombinant IL-1, TNF-α, and TGF-β1 (R&D Systems, Minneapolis, MN) for 24 hours in an incubator at 37°C with 95% humidity and 5% CO₂. The total RNA was extracted from control and treated conjunctival fibroblasts and was used for quantitative real-time PCR to determine the induction of MIF.

**Enzyme-Linked Immunosorbent Assay**

Using supernatant collected from conjunctival fibroblasts obtained from patients with OCP and control subjects, the level of MIF were determined by an ELISA, as described in earlier studies.\textsuperscript{23,24} Briefly, immunoplates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with mouse anti-human MIF monoclonal antibody (catalog no. MAB289; R&D Systems) at a final concentration of 2 µg/mL in 0.1 M NaHCO₃ buffer (pH 8.2). PBS containing 0.5% Tween-20 and 3% BSA was used as a blocking solution for 2 hours at room temperature. Culture supernatants or recombinant human MIF (catalog no. 289-MF-002; R&D Systems) used as the standard, were added and incubated overnight at 4°C. Bound MIF was detected with 1 µg/mL goat anti-human MIF detection antibody (catalog no. BAF289; R&D Systems). All assays were performed by incubation with 0.5 µg/mL streptavidin-peroxidase (Amersham, Aylesbury, UK) for 30 minutes at 37°C and then with O-phenylenediamine dihydrochloride (OPD) (0.4 mg/mL; Merck, Darmstadt, Germany) and 0.01% H₂O₂ in 0.1 M citrate buffer. The absorbance of each sample was recorded at 492 nm in an automated plate reader. The level of MIF in the supernatant was determined from the standard curve generated by recombinant human MIF.

**Figure 1.** Immunostaining of MIF in conjunctival biopsy specimens. Immunohistochemistry for MIF on human conjunctival section prepared from a normal control specimen (A) and specimens from patients with OCP (B, C). There was an increased number of MIF-expressing cells in conjunctival sections of patients with OCP, located mainly in the subepithelial stromal areas (B, C).
Real-Time PCR

Total RNA isolated from conjunctival fibroblasts was used to determine the relative induction of MIF in IL-1β-, TNF-α-, and TGF-β1-treated fibroblasts by real-time PCR, as described in earlier studies. The primers and probe used for detecting mRNA for MIF are as follows: forward, AGC CCG GAC AGG GTC TAC A; reverse, GGA GTT GTT CCA GCC CAC A; probe (TaqMan; Applied Biosystems, Foster City, CA), FAM-CAA CTA TTA CGA CAT GAA CGC GGC CA-TAMRA. Each PCR reaction contained equivalent amounts of total RNA. Real-time PCR was performed in duplicate with a kit used according to the manufacturer’s recommendation (TaqMan One-step RT-PCR Master Mix Reagents kit; Applied Biosystems). All the reactions were controlled by standards (nontemplate control and standard positive control). While extracting total RNA from conjunctival fibroblasts, we routinely used DNase to prevent DNA contamination. When real-time PCR was performed without adding reverse transcriptase, no PCR product was detected either for the target gene or the housekeeping gene, eliminating any DNA contamination. The quantity of mRNA was calculated by normalizing the CT (threshold cycle) of MIF to the CT of the 18S housekeeping gene in the same sample, according to the following formula: The average 18S CT (each multiplex PCR was performed in duplicate) was subtracted from the average MIF CT; the result represents the ΔCₜ. This ΔCₜ is specific and can be compared with the ΔCₜ of a calibration sample (for example, control conjunctival fibroblasts). The subtraction of control ΔCₜ from the ΔCₜ of OCP fibroblasts is referred as ΔΔCₜ. The relative quantification of expression of MIF (in comparison to control) was determined by using 2⁻ΔΔCₜ. For all the probes, the quencher dye was 6-carboxy-tetramethylrhodamine (TAMRA). The reporter dye was 6-carboxyfluorescein (FAM) for MIF and VIC for 18S. The relative expression of MIF was also detected as described earlier, using total RNA extracted from control conjunctival tissues and from selected conjunctival tissues of patients with OCP.

Statistical Analysis

Data are expressed as the mean ± SEM. Differences between groups were examined for statistical significance using the t-test or one-way ANOVA. Correlations were examined by linear regression analysis. P < 0.05 denotes a statistically significant difference.

RESULTS

Expression of MIF in Conjunctival Tissues

The expression of MIF was mostly detected in the basal epithelial cells and occasionally in the stromal cells of the control conjunctiva (Fig. 1A). In contrast to the occasional stromal infiltration of CD68-expressing macrophages in the conjunctival section of patients with OCP, mainly accumulating in the subepithelial stromal areas (B, C).

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expression of MIF in conjunctival sections, a significantly increased (P < 0.0001) number of stromal cells expressed MIF in sections of conjunctiva obtained from patients with OCP (control: 4.89 ± 0.5; OCP: 19.82 ± 1.34; Figs. 1B, 1C). Furthermore, compared with control conjunctiva, an increase in the expression of MIF was detected by quantitative real-time PCR in the conjunctiva obtained from patients with OCP (Fig. 2).

Correlation between the Conjunctival Expression of MIF and Macrophage Accumulation

To examine the relationship between the conjunctival expression of MIF and macrophage populations in the conjunctiva affected by OCP, we counted the number of MIF-positive cells and CD68-positive macrophages in the conjunctival sections obtained from patients with OCP and performed a correlation analysis. Compared with the control (4.2 ± 0.42), an increased accumulation of CD68-positive macrophages was detected in conjunctival tissues of patients with OCP (26.94 ± 2.1; Fig. 3). A positive correlation between the conjunctival expression of MIF and accumulation of macrophages was detected in the conjunctival tissues obtained from the patients with OCP (r² = 0.4465, P < 0.0001; Fig. 4).

Expression of MIF in Conjunctival Fibroblasts

Compared with control conjunctival fibroblasts (Fig. 5A), an increased cytoplasmic expression of MIF was detected by immunostaining of the fibroblasts isolated from conjunctiva of patients with OCP (Fig. 5B). An increase in the expression of MIF at the mRNA level was detected, by quantitative real-time PCR, in the fibroblasts isolated from conjunctiva of patients with OCP, in comparison with control fibroblasts (Fig. 2). A significant increase (P < 0.001) in the level of MIF was detected by ELISA in the supernatant of fibroblasts isolated from conjunctiva of patients with OCP (186 ± 5.4 pg/mL), compared with the level in the supernatant from control fibroblasts (9.5 ± 7.6 pg/mL; Fig. 6).

Effects of IL-1, TNF-α, and TGF-β1 on the Expression of MIF by Conjunctival Fibroblasts

When conjunctival fibroblasts were treated with various concentrations (1, 10, or 100 ng/mL) of TNF-α, IL-1, and TGF-β1 for 24 hours, an induction in the expression of MIF was detected by quantitative real-time PCR. Although the induction was noted in all three different concentrations of TNF-α, IL-1, and TGF-β1, the maximum induction of MIF was noted in cells treated with TNF-α (Fig. 7). As for IL-1 (Fig. 8) and TGF-β1 (Fig. 9), the maximum induction of MIF was found in the cells that were treated with 10 ng/mL for 24 hours.

Discussion

In the present study, we investigated the expression of MIF in conjunctival tissues obtained from the patients with OCP, in an attempt to elucidate its role(s) in conjunctival disease. Our results indicate that the expression of MIF is increased in conjunctival tissues obtained from patients with OCP and suggest that this expression is probably associated with the increased stromal accumulation of macrophages. It appears that macrophage accumulation in the conjunctiva is an important event in the pathogenesis of OCP.1,2,11 Macrophage-derived cytokines and growth factors, including IL-4, platelet-derived growth factor (PDGF), and TGF-β1 could be actively involved in the subsequent conjunctival disease and in the irreversible scarring.
MIF is one of the earliest identified lymphokines, with potent macrophage-regulatory functions, and has been shown to be involved in inflammatory events in various human and experimental diseases. Subsequent studies have demonstrated expression of MIF in various human tissues. A critical role of MIF has been demonstrated in the pathogenesis of septic shock, adult respiratory distress syndrome, chronic glomerulonephritis, allergic lung diseases, and rheumatoid arthritis. A pathogenic role of MIF has also suggested in corneal wound healing and in patients with uveitis. Furthermore, the therapeutic potential of MIF has been suggested in various immunoinflammatory diseases. For instance, suppressing the bioactivities of MIF by using recombinant adenovirus bearing the antisense MIF gene reduced the rate of mortality in mice with acute liver failure that was induced by a combined treatment with Bacille Calmette-Guerin (BCG) and lipopolysaccharide (LPS).

In the present study, an increased expression of MIF in conjunctiva from patients with OCP was associated with the increased conjunctival accumulation of macrophages. These results are similar to those in earlier studies in which a role of macrophage colony-stimulating factor (m-CSF) has been suggested in the conjunctival accumulation of macrophages, possibly regulating local proliferation and increased recruitment. Because MIF also helps in local aggregation of monocytes-macrophages, an elevated level of MIF in conjunctiva of pa-

**Figure 7.** Effects of TNF-α on the expression of MIF. Conjunctival fibroblasts were treated with various concentrations of TNF-α (1, 10, and 100 ng/mL) for 24 hours, and the induction of MIF was determined at the mRNA level by quantitative real-time PCR. Real-time PCR was always performed in duplicate, and the mean level of the relative expression of MIF is shown. There was an increased expression of MIF in the TNF-α-treated fibroblasts, with a maximum induction at a concentration of 100 ng/mL.

**Figure 8.** Effects of IL-1 on the expression of MIF. Conjunctival fibroblasts were treated with various concentrations of IL-1 (1, 10, and 100 ng/mL) for 24 hours, and the induction of MIF was determined at the mRNA level by quantitative real-time PCR. All samples were run twice, and the mean level of the relative expression of MIF, in comparison with the control is shown. There was an increased expression of MIF in the IL-1-treated fibroblasts, with a maximum induction at a concentration of 10 ng/mL.
tients with OCP probably plays an important role in the local accumulation of macrophages in the early stages of the disease process. Moreover, MIF not only plays a role in the local macrophage accumulation by inhibiting migration of macrophages, but also helps in additional recruitment of macrophages. In addition, in the current study, fibroblasts isolated from conjunctiva of patients with OCP produced significantly increased amounts of MIF, in comparison to control fibroblasts. It is therefore likely that fibroblast-derived MIF contributes to the regulation of the macrophage population in the conjunctiva of patients with OCP.

To determine the factors regulating increased synthesis of MIF, we have studied the role of TNF-α, IL-1, and TGF-β1 on the induction of MIF by conjunctival fibroblasts. Because TNF-α, IL-1, and TGF-β1 have already been demonstrated to play roles in the early inflammatory and late scarring events in various chronic human diseases, including OCP, we choose these molecules to determine their effects on the expression of MIF. When conjunctival fibroblasts were treated with various concentrations of TNF-α, IL-1, or TGF-β1, an induction in the expression of MIF was detected. The presence of macrophages in conjunctival tissues is a frequently observed histologic feature in OCP, although the density appears to be more in the early inflammatory stages than in the late fibrotic stages. In this study, we have shown that both early inflammatory (TNF-α and IL-1) and late fibrogenic (TGF-β1) molecules induce MIF expression by conjunctival fibroblasts. Molecular interactions between MIF and TNF-α, IL-1, or TGF-β1 may play an important role in conjunctival injury and in disease progression in patients with OCP. Our results are in accord with the earlier studies, in which TNF-α was shown to mediate the induction of MIF in 3T3-L1 adipocytes by a tyrosine-kinase-dependent pathway. Similar induction was also detected in a murine colon carcinoma cell line (colon 26), where TGF-β1 was the mediating factor in the expression of MIF.

Intravenous immunoglobulin (IVIg), used as monotherapy, has been shown to be effective in treating patients with OCP. IVIg therapy arrested disease progression and produced a sustained clinical remission in patients with OCP. Recent studies have shown that IVIg exerts its effects, partly by inhibiting the differentiation and maturation of dendritic cells. It will be interesting to know whether IVIg exerts the clinical response in patients with OCP by modulating macrophage-regulating molecules such as MIF.

In summary, the expression of MIF was increased in the conjunctiva of patients with OCP. Because MIF plays a crucial role(s) in determining the population of macrophages, it is possible that increased conjunctival expression of MIF regulates conjunctival accumulation of macrophages in patients.
with OCP and thereby could enhance inflammatory responses in these patients. In addition, macrophage-derived fibrogenic factors could enhance subsequent formation of irreversible conjunctival scarring. Further studies are needed to determine the role of other inflammatory cell-regulating factors, such as chemokines and adhesion molecules, to gain a comprehensive understanding of the conjunctival inflammatory process (Fig. 10) in OCP. Such studies will provide the information necessary to develop novel therapeutic strategies to modulate the disease process by targeting molecules that regulate both inflammatory and fibrogenic events in OCP.

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