Effects of IL-4 on Conjunctival Fibroblasts: Possible Role in Ocular Cicatricial Pemphigoid

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PURPOSE. Increased stromal accumulation of macrophages and submucosal fibrosis due to excessive accumulation of collagens are central histologic features in ocular cicatricial pemphigoid (OCP). Interleukin (IL)-4 plays an important role in both the inflammatory and fibrotic events in several human and experimental diseases. In the present study, the possible role of IL-4 in the pathogenesis of OCP was investigated.

METHODS. Biopsy specimens from the conjunctivae of 10 patients with OCP and 5 normal subjects were studied for the expression of IL-4 by immunohistochemistry. The expression level of IL-4 was also examined in conjunctival fibroblasts of normal control subjects and patients with OCP. The effects of IL-4 in the induction of inflammatory and fibrogenic molecules was studied in IL-4–treated conjunctival fibroblasts, and the expression levels of macrophage colony-stimulating factor (m-CSF), heat shock protein (HSP)-47 and type I collagen was determined by quantitative real-time PCR. The level of IL-4 was also measured by enzyme-linked immunosorbent assay (ELISA) in serum samples obtained from patients with OCP during active stage and remission and were compared with the levels in control sera.

RESULTS. Compared with the weak expression of IL-4 in the normal conjunctival sections, an increased expression of IL-4 was noted in conjunctival sections of patients with OCP. A similar increase in the expression of IL-4 was also detected in fibroblasts isolated from conjunctiva of patients with OCP compared with control fibroblasts. Real-time PCR and ELISA detected a significantly increased level of m-CSF, at both the mRNA and protein levels in IL-4–stimulated cells. Similarly, IL-4 treatment resulted in the induction of type I collagen and collagen-binding HSP47 by conjunctival fibroblasts, as detected by real-time PCR. However, no apparent changes in the levels of IL-4 were detected by ELISA in serum samples of patients with OCP and control subjects.

CONCLUSIONS. Increased conjunctival expression of IL-4 may play an important role in the regulation of local accumulation of macrophages (by inducing m-CSF), and matrix accumulation (by inducing HSP47 and collagen) during conjunctival scarring in patients with OCP. IL-4, therefore, may augment or enhance both conjunctival inflammatory and subsequent fibrotic responses in patients with OCP. (Invest Ophthalmol Vis Sci. 2003;44:3417–3423) DOI:10.1167/iovs.02-1084

Ocular cicatricial pemphigoid (OCP) is a systemic autoimmune vesiculobullous disorder characterized by recurrent episodes of inflammation and progressive subepithelial conjunctival fibrosis, as a result of excessive deposition of matrix proteins.1,2 Progression of subepithelial fibrosis results in fornix foreshortening, symblepharon formation, meibomian duct obstruction, and eventual lacrimal duct compression with reduced tear flow.3 The fibrosis also causes deformity of the lid and global architecture. Corneal involvement is usually a result of trichiasis, abnormal blinking, and decreased tear production. Frequent abrasion of the cornea causes secondary infection or scarring due to trauma, directly resulting in keratopathy. Thus, the cornea can be completely scarred and keratinized, with resultant blindness.4 In most patients, OCP is diagnosed during the scarring stage of the disease. It is frequently treated with systemic corticosteroids, with or without immunosuppressive agents, with the objective of reducing inflammation and delaying the progression of scarring. In a series of 61 patients with OCP observed for a mean period of 86 months, approximately 25% became blind despite aggressive systemic therapy.5

Fibrogenesis is a process of excessive accumulation of extracellular matrix (ECM) components in the involved organs, possibly due to an increased production and/or decreased degradation of matrix proteins.6–8 Fibrotic diseases encompass a spectrum of tissue damage that progresses to end-stage organ failure in various tissues and organs, including lung, liver, kidney, skin, and eye.2,7–9 Fibrogenesis usually consists of an initiation phase, followed by inflammatory and fibrogenic phases.4,7 Determining the molecules involved in various phases of the fibrotic diseases is essential in designing therapeutic strategies to prevent and/or arrest the progression of mostly irreversible fibrotic lesions. Over the past few years, significant progress has been made in understanding the molecular mechanisms of fibrotic diseases in various tissues. However, conjunctival fibrosis in OCP has not been studied in similar depth or detail. Identifying some of the molecules involved in this multistep process of conjunctival scarring in patients with OCP will increase our understanding of conjunctival fibrogenesis. Such information will assist and guide in developing therapies in which the identified molecules may be blocked or inactivated, thus delaying the progression of conjunctival scarring.

Interleukin (IL)-4 is a pleiotropic cytokine that plays major roles in fibrogenesis by amplifying inflammatory responses and stimulating collagen synthesis. It is a 20-kDa glycoprotein produced by a wide range of cells, including mature T-helper (Th)2 cells, mast cells, and fibroblasts. It has been implied that IL-4 has a central role in normal wound healing.10 Topical administration of IL-4 on experimental wounds in mice significantly accelerates the rate of healing, whereas blocking the bioactivities of IL-4 by antisense oligonucleotides results in significant inhibition of the healing process.10 A higher level of expression of IL-4 has also been detected in the fibrotic skin tissues of patients with scleroderma.10 A similar increase in the expression of IL-4 has been documented in human fibrotic...
renal diseases. In addition, IL-4 transgenic mice show fibrotic changes in the kidneys. Changes in the expression of macrophage colony-stimulating factor (m-CSF) have been shown to be involved in several immunoinflammatory phenomenon that lead to scarring. Conjunctival fibroblast-secreted m-CSF is thought to play a major role(s) in determining the macrophage population in the conjunctiva of patients with OCP. However, the factors regulating the expression of m-CSF by conjunctival fibroblasts in patients with OCP are not completely understood. IL-4 has the ability to induce the expression of m-CSF in human endometrial stromal cells, human bone marrow stromal cells, and peripheral blood monocytes. IL-4 may therefore have the potential to regulate the expression of m-CSF by conjunctival fibroblasts.

Heat shock protein (HSP)-47 is a 47-kDa stress protein that acts as a collagen-specific molecular chaperone during the biosynthesis and intracellular processing of newly formed procollagen polypeptides. Earlier studies have identified a close association between the increased expression of HSP47 and distinct deposition of collagens in various human and experimental fibrotic diseases of the lung, liver, and kidney. In these fibrotic diseases, HSP47 is thought to exert biological effect(s) on procollagen synthesis and subsequent accumulation of collagens. Recently, a potential role for HSP47 has been suggested in conjunctival scarring in patients with OCP. Nevertheless, factors regulating increased synthesis of HSP47 and type I collagen in conjunctiva of patients with OCP are not yet fully elucidated. In the present study, we examined the possible role of IL-4 in conjunctival scarring and determine its ability to induce m-CSF, HSP47, and type I collagen by conjunctival fibroblasts.

MATERIALS AND METHODS

Conjunctival Tissues

Biopsy specimens of conjunctivae obtained from 10 patients with OCP were used to study the expression of IL-4. The diagnosis of OCP was based on clinical presentation, histology, and direct immunofluorescence of the conjunctiva demonstrating IgG and C3 at the basement membrane zone (BMZ). Conjunctivae of five normal individuals, who underwent routine cataract surgery, were used as control samples. These individuals did not have any associated metabolic, inflammatory, or systemic diseases. The study adhered to the guidelines of the Declaration of Helsinki for research involving human subjects and was approved by the review boards of our respective institutions.

Immunohistochemistry

Immunohistochemistry was performed on frozen sections as described in our earlier publications. Briefly, biopsy sections of the conjunctiva were blocked with 10% goat serum for 1 hour and then incubated overnight at 4°C with an antibody against IL-4 (R&D Systems, Minneapolis, MN). After a wash with PBS, the sections were processed further with a staining kit (Histostain; Nichirei, Tokyo, Japan), and reaction products were developed with a mixture of 3,3'-diaminobenzidine-4 HCl (DAB) and H2O2. Normal mouse serum was used as the negative control. The staining pattern was graded semiquantitatively according to intensity (mild, moderate, and severe), and the distribution of the staining was characterized, as described in our earlier reports.

Isolation of Conjunctival Fibroblasts

Fibroblasts from conjunctivae of normal control subjects and patients with OCP were isolated as described. Briefly, conjunctival tissues were cut into explants of approximately 2 × 2 mm², placed into tissue culture dishes, and covered with Dulbecco’s modified Eagle’s (DMEM) medium containing 10% fetal bovine serum (FBS; Mediatech, Inc., Herndon, VA) supplemented with antibiotics, as previously described, except that instead of amphotericin B and gentamicin, we used 500 U/mL penicillin G and 500 μg/mL streptomycin as the antibiotics supplement. The conjunctival tissues were incubated overnight at 37°C with 95% humidity and 5% CO2. The media were changed three times weekly thereafter for 2 weeks. At 80% to 90% confluence the isolated fibroblasts were subcultured with 0.1% trypsin and 0.02% EDTA in Ca2+-free minimum essential medium (MEM). Fibroblasts isolated from conjunctivae of normal control subjects and patients with OCP, were grown on glass slides, fixed with methanol, and used for immunostaining for IL-4, as just described. In addition, total RNA extracted from fibroblasts isolated from conjunctivae of normal control subjects and patients with OCP, were used for quantitative real-time PCR.

Effects of IL-4 on the Induction of m-CSF, HSP47, and Type I Collagen by Conjunctival Fibroblasts

Conjunctival fibroblasts were treated with various concentrations (0.1, 1, 10, and 100 ng/mL) of recombinant IL-4 (R&D Systems) for 24 hours, in an incubator at 37°C with 95% humidity and 5% CO2. Then, the total RNA, extracted by standard methods from conjunctival fibroblasts, was used for real-time PCR, to determine the expression of m-CSF, HSP47, and type I collagen. The supernatant was collected from IL-4–treated and untreated cells, and the level of m-CSF was determined by using an ELISA kit (R&D Systems), according to the manufacturer’s protocol.

Real-Time PCR

Total RNA extracted from conjunctival fibroblasts was used to determine the relative induction of m-CSF, type I collagen, and HSP47 in IL-4–treated fibroblasts, by real-time PCR, as described in earlier studies. The primers and probe used for detecting mRNA for m-CSF, type I collagen, and HSP47 are as follows: m-CSF, forward TGC AGC GCC TGA TTG ACA, reverse TTA AAC TGT TTC TGG TCT A; CACTCC, probe (Taquin; Applied Biosystems, Foster City, CA) FAM-TGA GAT GGA ACA CTC GTG CCA AAT ATT TAMRA; type I collagen: forward CCT CAA GGG CTC CCA CGA G, reverse TCA TAC ACT GTC TTG CCC CA, probe (Taquin) FAM-ATG GCT GCA CGA GTC ACA CCG GAT TAMRA; HSP47: forward CGC CAT GAT CCT CAA GGC A, reverse CAT GAA GCC AGC GTT GTC C, probe (Taquin) FAM-CCTG GGA TGA CCA ATT CCA CCA GAT GTG TAMRA. Each PCR reaction contained equivalent amounts of total RNA. Real-time PCR was performed in duplicate with a kit according to the manufacturer’s recommendation (Taquin, One-step RT-PCR master mix reagents kit; Applied Biosystems). All the reactions were controlled by standards (nontemplate control and standard positive control). After extracting the total RNA from conjunctival fibroblasts, we have routinely used DNase to prevent DNA contamination. Moreover, when real-time PCR was performed without adding reverse transcriptase, no PCR product was detected for the m-CSF, type I collagen, and HSP47, or housekeeping genes. The quantity of mRNA was calculated by normalizing the Ct (threshold cycle) of m-CSF, type I collagen, or HSP47 to the Ct of the housekeeping genes 18S ribosomal RNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the same RNA probe, according to the following formula: the average 18S or GAPDH Ct (each multiplex PCR was performed in duplicate) was subtracted from the average m-CSF, type I collagen, or HSP47 Ct, with the result representing 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 m-CSF, type I collagen, or HSP47 (in comparison with the control) was determined by using the value of 2−ΔΔCt. For all the probes, the quencher dye was 6-carboxy-tetramethylrhodamine (Tamra), and the reporter dyes were 6-carboxy-fluorescein (FAM) for m-CSF, type I collagen, and HSP47, and VIC for 18S or GAPDH.
Enzyme-Linked Immunosorbent Assay
The conjunctival fibroblasts were subcultured and kept in the serum-free medium before the treatment with various concentrations (0.1, 1, 10, or 100 ng/mL) of IL-4 (R&D Systems) for 24 hours. The supernatant was collected and the level of m-CSF was determined by ELISA (R&D Systems), in four samples in each concentration, according to the manufacturer's instructions. The serum levels of IL-4 in the patients with OCP were measured in 14 patients as well, during both the active and remission stages of the disease. Sera from seven normal individuals were used as a control. In addition, the level of IL-4 was determined in the seven samples of supernatant collected from control conjunctival fibroblasts and in five samples of supernatant collected from fibroblasts isolated from patients with OCP. In this study, the level of IL-4 was detected using an ELISA kit (eBioscience, San Diego, CA), according to the manufacturer's instructions.

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.

RESULTS
Expression of IL-4 in Human Conjunctival Tissues and Fibroblasts
The expression of IL-4 was mild and sparsely detected in the control conjunctival sections by immunohistochemistry (Fig. 1A). In contrast, both intensity (moderate) and number of stromal cells expressing IL-4 were increased in conjunctival sections of patients with OCP (Figs. 1B, 1C). When antibody to IL-4 was replaced with mouse IgG, no specific staining was detected. An increased expression of IL-4 was also noted in the epithelial cell in the diseased conjunctiva. Similarly, an increased cytoplastic immunostaining for IL-4 was detected in the fibroblasts isolated from conjunctiveae of patients with OCP, compared with control conjunctival fibroblasts (Fig. 2).

Levels of IL-4 in the Sera of Patients with OCP and Supernatant of Conjunctival Fibroblasts
The mean level of IL-4 in sera of 14 patients with active OCP was (45.05 ± 14.346 pg/mL). The mean level of IL-4 in the sera of 14 patients in clinical remission was (42.58 ± 10.868 pg/mL). The mean level of IL-4 in sera of seven normal control subjects was (63.77 ± 21.865 pg/mL). The difference between the active and inactive stages and the normal control was not statistically significant. However, the level of IL-4 in supernatants of fibroblasts collected from conjunctiva of patients with OCP (245.14 ± 79.3 pg/mL) were significantly (P < 0.05) higher than its level in supernatants collected from control conjunctival fibroblasts (79.98 ± 10.141 pg/mL), as detected by ELISA.

In Vitro Induction of m-CSF by IL-4
The induction of m-CSF in the conjunctival fibroblasts treated with various concentrations of recombinant IL-4 (0.1, 1, 10, 100 ng/mL) was determined by ELISA. The supernatant from fibroblasts treated with 100 ng/mL of IL-4 showed the highest level of m-CSF production.
and 100 ng/mL) for 24 hours was determined by quantitative real-time PCR and ELISA. The experiments were repeated twice to determine the reproducibility of the results. By quantitative real-time PCR, an approximate sixfold increase in the expression of m-CSF was detected in IL-4–treated fibroblasts, compared with untreated fibroblasts (Fig. 3). A statistically significant increase ($P < 0.001$) level of m-CSF was also detected in the supernatants collected from conjunctival fibroblasts treated with various concentrations of IL-4, compared with untreated fibroblasts, as detected by ELISA (Fig. 4).

**DISCUSSION**

IL-4 is a multifunctional cytokine that facilitates the recruitment of inflammatory cells and proliferation of fibroblasts and enhances the production of matrix proteins. Increased expression of IL-4 has been observed in human and experimental fibrotic diseases of the lung, kidney, and other internal organs.10–12,35,36 In IL-4 transgenic mice, glomerular hypertrophy develops, with progressive mesangial sclerosis leading to renal failure.13,14 In this study, we have demonstrated a possible role for IL-4 in the pathogenesis of conjunctival scarring in patients with OCP. The expression of IL-4 was increased in conjunctival biopsy specimens of patients with OCP during the active stage of the disease. Fibroblasts cultured from conjunctivae of patients with OCP expressed and secreted more IL-4 than did fibroblasts cultured from conjunctivae of normal control subjects. The levels of IL-4 in the sera of patients with active disease were similar to levels during remission or inac-

**FIGURE 3.** Effects of IL-4 on the expression of m-CSF. Conjunctival fibroblasts were treated with various concentrations of IL-4 for 24 hours, and the induction of m-CSF was determined at the mRNA level by using quantitative real-time PCR. Data are the mean relative expression of m-CSF. Compared with control untreated fibroblasts, an increase in the expression of m-CSF was detected in IL-4–treated fibroblasts.

**FIGURE 4.** Effects of IL-4 on the expression of m-CSF. Conjunctival fibroblasts were treated with various concentrations of IL-4 for 24 hours, and the induction of m-CSF was determined at the protein level by ELISA. Data are the mean relative expression of m-CSF. Compared with control untreated fibroblasts, a statistically significant ($P < 0.001$) increase in the expression of m-CSF was detected in the supernatant collected from IL-4–treated fibroblasts.

**FIGURE 5.** Effects of IL-4 on the expression of HSP47. Conjunctival fibroblasts were treated with various concentrations of IL-4 (0.1, 1, 10, and 100 ng/mL) for 24 hours, and the induction of HSP47 was determined at the mRNA level by using quantitative real-time PCR. Data are the mean relative expression of HSP47 in IL-4–treated fibroblasts compared with untreated fibroblasts. Expression of HSP47 was induced the IL-4–treated fibroblasts.

**FIGURE 6.** Effects of IL-4 on the expression of type I collagen. Conjunctival fibroblasts were treated with various concentrations of IL-4 (0.1, 1, 10, and 100 ng/mL) for 24 hours, and the induction of type I collagen was determined at the mRNA level by using quantitative real-time PCR. Data are the mean relative expression of type I collagen in IL-4–treated fibroblasts compared with untreated fibroblasts. Expression of type I collagen was induced in the IL-4–treated fibroblasts.
tive stages of the disease or in normal control sera. These observations suggest that the effects of IL-4 in the pathogenesis of OCP are due to its presence in the local microenvironment and probably are not a systemic effect. To evaluate the potential role of locally produced IL-4, measurement of its level in the tears would have been advantageous. However, this is not practically feasible, because all our patients had dry eye (xerophthalmia) and used artificial tears frequently. Despite the limitations, the observations of this study are valid and significant and may provide a foundation and basis for future studies on the diverse roles of IL-4 in OCP.

Local changes in the microenvironment may partly explain why the disease process in OCP is mostly confined to conjunctiva. We have demonstrated that conjunctival fibroblast-secreted factors, such as m-CSF, HSP47, transforming growth factor β1 (TGF-β1), connective tissue growth factor (CTGF), and others, may influence some of these changes in the microenvironment of the conjunctiva. Changes in microenvironment could eventually facilitate and/or intensify local immunoinflammatory responses and regulate matrix remodeling. In the present study, we have shown that the expression of IL-4 is increased in the conjunctiva of patients with OCP, compared with control conjunctiva. However, no such changes in the levels of IL-4 were detected in the sera of patients with OCP, compared with sera of control subjects. Our results emphasize an important role for locally produced IL-4 in the pathogenesis of conjunctival scarring in patients with OCP. To determine the effects of IL-4 in the induction of molecules involved in the inflammatory phase (m-CSF) and fibrogenic phase (HSP47, type I collagen), we have performed in vitro studies, using conjunctival fibroblasts.

m-CSF plays an important role(s) in the recruitment and local proliferation of macrophages in various human and experimental diseases. Local proliferation of alveolar macrophages under the influence of m-CSF and granulocyte macrophage (GM)-CSF is associated with chronic inflammation and pulmonary fibrosis. Similarly, an increased expression of m-CSF has been observed with increased accumulation of macrophages in the kidney, with resultant intensification of renal injuries. Recently, we have shown that increased expression of m-CSF is associated with increased accumulation of macrophages in the conjunctiva of patients with OCP. In the present study, when cultured conjunctival fibroblasts were treated with various concentrations of IL-4, an increased level of m-CSF was detected in the IL-4-stimulated fibroblasts, compared with untreated fibroblasts. These results indicate that IL-4 can induce the expression of m-CSF by conjunctival fibroblasts and it is likely that m-CSF may play a role in determining the local population of macrophages in patients with OCP. Similar induction of m-CSF by IL-4 has been reported in human endometrial stromal cells and bone marrow stromal cells.

HSP47 is a collagen-binding protein involved in the folding, assembly, and/or posttranslational modification of procollagens. Studies have identified a close association between the increased expression of HSP47 and increased accumulation of collagen, in various fibrotic diseases of the lung, liver, and kidney. Recently, it has been demonstrated that an increase in the expression of HSP47 in the conjunctiva of patients with OCP is paralleled with an increased deposition of interstitial collagen. In the present study, IL-4 induced the expression of HSP47 and type I collagen by conjunctival fibroblasts. Similar induction of HSP47 and collagen by IL-4 has been demonstrated in dermal fibroblasts of patients with scleroderma. Hence, it would seem possible that IL-4-induced overexpression of HSP47 might increase the assembly and synthesis of type I collagen and could partially contribute to the scarring process in the conjunctiva of patients with OCP. 

**Figure 7.** Based on the present study and other earlier research works, we have schematically summarized some of the essential molecules that are involved in various events of conjunctival scarring. Some other factors that may play significant roles in the scarring process are not included, to keep the diagram simple and relevant to this study. In addition to abbreviations defined in the main text, m-CSF, macrophage colony-stimulating factor; MMP, matrix metalloproteinase; NFκB, nuclear factor-κB; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumor necrosis factor-α.

Recently, a role of IL-4 has been suggested in the pathogenesis of autoimmune diseases. It has been demonstrated that IL-4 transgenic mice have higher levels of autoantibodies and autoantibody-producing B cells in the spleen. B cells from the spleen, lymph node, and bone marrow of IL-4 transgenic mice were hyperreactive with a higher level of expression of major histocompatibility complex (MHC) class II molecules and CD23. These cells also showed greater reactivity and proliferating activities in response to lipopolysaccharide (LPS) and anti-μF(ab′)2 stimulation compared with B cells from control animals.

It has been demonstrated that severe and progressive OCP clinically responds to high doses of intravenous immunoglobulin (IV Ig) treatment. Whereas there is no clear or direct evidence of how IV Ig produces clinical benefit in patients with OCP, it is worth noting that IV Ig can influence IL-4. In vitro studies have suggested that IV Ig is a potent suppressor of IL-2 and -4 production by T-cells. These observations may suggest that infused IV Ig may modulate the inflammatory and/or fibrogenic effects of locally produced IL-4 in the conjunctiva.

In conclusion, the results of this study suggest that IL-4 plays a role in the pathogenesis of OCP. It may contribute to the inflammatory process by inducing m-CSF, which helps in recruiting macrophages and thus augments and/or intensifies the inflammatory responses in the conjunctiva. By inducing the synthesis of collagen and collagen-binding HSP47, IL-4, may help in the progression of fibrotic process in the conjunctiva (Fig. 7). Preliminary serologic studies indicate that these influences of IL-4 are not the consequence of a systemic effect, rather a local phenomenon. In all likelihood, the effects of IL-4 are occurring in association or synergy with other cytokines, growth factors, and molecules that are involved in inflammatory and fibrotic phases of the disease. Once our understanding of this multifactorial phenomenon is clarified, therapeutic strategies to modulate the disease process can be designed. Thus, the clinical outcome in patients with OCP can be improved.
Acknowledgments

The authors thank Takashi Taguchi, Nagasaki University Graduate School of Biomedical Sciences, for kindly providing antibodies, immunostaining kits, and useful suggestions; Victoria Pertkova for help in performing real-time PCR analysis; and Mehmet S. Inan for helping in staining and photography.

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