Role of Collagen-Binding Heat Shock Protein 47 and Transforming Growth Factor-β1 in Conjunctival Scarring in Ocular Cicatricial Pemphigoid

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PURPOSE. Submucosal fibrosis due to excessive accumulation of collagens is an important histologic feature in the pathogenesis of ocular cicatricial pemphigoid (OCP). Heat shock protein 47 (HSP47), a collagen-binding protein, plays an important role in the biosynthesis of procollagens. In the present study, we examined the role of HSP47 in conjunctival scarring in patients with OCP.

METHODS. Biopsy specimens of the conjunctiva of 15 patients with OCP and 5 normal subjects were studied for the expression of HSP47, transforming growth factor (TGF)-β1, type I collagen, and type III collagen. The role of TGF-β1 on the induction of HSP47 and type I collagen by conjunctival fibroblasts was studied by immunostaining, Western blot analysis, and quantitative real-time PCR.

RESULTS. Compared with the control, increased accumulations of type I and type III collagens were detected by immunohistochemistry in fibrotic conjunctiva of patients with OCP. Weak and sparse expression of HSP47 was detected in the epithelial cells and stromal fibroblasts in control conjunctival tissues. In contrast to the control, the expression of HSP47 was markedly increased in the stromal fibroblasts in conjunctival tissues obtained from patients with OCP, as detected by immunohistochemistry. By quantitative real-time PCR, compared with control conjunctival tissues, a 3.4-fold increase in the expression of HSP47 was noted in the conjunctival tissues obtained from patients with OCP. Similar to conjunctival tissues, fibroblasts isolated from conjunctiva of patients with OCP exhibited 4.8-fold increase in the expression of HSP47, compared with control fibroblasts. When conjunctival fibroblasts were treated with various concentration of TGF-β1, upregulation in the expression of HSP47 and type I collagen was detected.

CONCLUSIONS. This study demonstrated increased expression of HSP47 and TGF-β1 by conjunctival fibroblasts in biopsy specimens obtained from patients with OCP. TGF-β1 induced the expression of HSP47 and type I collagen by conjunctival fibroblasts. Increased levels of TGF-β1 and HSP47 may regulate increased synthesis, assembly, and production of collagens and thereby could significantly contribute to the process of conjunctival scarring in patients with OCP. (Invest Ophthalmol Vis Sci. 2003;44:1616–1621) DOI:10.1167/iovs.02-0644

Recent research has greatly increased our knowledge concerning the molecular mechanisms of the synthesis and processing of collagen.1 Procollagen assembly is a complex process within the endoplasmic reticulum (ER), in which the C-propeptide domains of three polypeptide α-chains fold individually and subsequently interact and trimerize to form a triple helix. Heat shock protein (HSP) 47 kDa stress protein that resides in the ER of collagen-producing cells. It binds specifically to native collagens and acts as a collagen-specific molecular chaperone during the biosynthesis and intracellular processing of newly formed procollagen polypeptides and assists in the formation of triple helices within the ER and cis-Golgi vesicles. The crucial role of HSP47 in the collagen biosynthesis is well documented, and HSP47 gene-disrupted mice demonstrate molecular abnormalities in procollagen and embryonic death.4

A close association between the increased expression of HSP47 and increased accumulation of collagens has been demonstrated in various fibrotic diseases of the lung, liver, and kidney.5–9 In these disease processes, HSP47 is thought to exert an important biological effect on procollagen synthesis and subsequent fibrosis. Moreover, in a rat model of renal fibrosis, in vivo treatment with HSP47-specific antisense oligonucleotides has been shown to decrease the accumulation of collagen.10 Although several studies have elucidated the role of HSP47 in human and experimental fibrotic diseases, its role in conjunctival scarring in response to injury or in pathologic states such as ocular cicatricial pemphigoid (OCP) is not yet known.

Mucous membrane pemphigoid is a rare autoimmune vesiculobullous disease. When ocular involvement is present, it is referred to as OCP. It is usually characterized by recurrent episodes of inflammation in the conjunctiva with progressive subepithelial fibrosis, resulting in shortening of the fornix. In a number of patients, despite immunosuppressive therapy, it progresses and eventually leads to blindness.1,11 HSP47 is closely involved in the folding, assembly, and/or posttranslational modification of procollagen.1 Consequently, it has a potential role in conjunctival fibrosis. In the present study, we investigated the role of HSP47 and transforming growth factor (TGF)-β1 in conjunctival scarring in patients with OCP.

MATERIALS AND METHODS

Conjunctival Specimens

Samples of the conjunctiva were obtained from 15 patients with OCP. 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. Biopsy specimens from conjunctiva of normal individuals, to be used as the control, were obtained from five patients who underwent routine cataract surgery. Conjunctival biopsy specimens were also obtained from three subjects...
with atopic diseases of the conjunctiva during episodes of inflammation. The study adhered to the guidelines of the Declaration of Helsinki for research involving human subjects.

**Immunohistochemistry**

Immunohistochemistry was performed on paraffin and frozen sections as described previously. Briefly, biopsy sections of the conjunctiva 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: HSP47 (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada), type I collagen (Sigma Chemical Co., St. Louis, MO), type III collagen (Fuji Chemical Industries, Tokyo, Japan), and TGF-β1 (R&D Systems, Minneapolis, MN). After a wash with PBS, sections were processed further using a kit (Histostain; Nichirei, Tokyo, Japan), and reaction products were developed with a mixture of 3,3′-diaminobenzine-4 HCl (DAB) and H2O2. Preabsorption of the primary antibody with excess recombinant HSP47 peptide (StressGen Biotechnologies) and normal mouse or goat IgG was used as the negative control. The staining pattern was graded semiquantitatively according to the intensity and distribution of the staining, as described in our earlier reports.

**Isolation of Fibroblasts from Conjunctiva**

Fibroblasts from conjunctiva of normal control subjects and patients with OCP were isolated as described earlier. Briefly, conjunctival tissue was cut into explants of approximately 2 × 2 mm², placed into tissue culture dishes, and covered with DMEM containing FCS, gentamicin, and amphotericin B and incubated overnight, at 37°C with 95% humidity and 5% CO2. 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⁺-free minimum essential medium (MEM) at 80% to 90% confluence. Fibroblasts isolated from conjunctiva of normal control subjects and patients with OCP were grown on the glass slides, fixed with methanol, and used for immunostaining for HSP47 and TGF-β1, as just described. In addition RNA and proteins extracted from fibroblasts isolated from conjunctiva of normal control subjects and patients with OCP were used for real-time PCR and Western blot analysis. Cells at passages 3 to 7 were used in this study.

**Effects of TGF-β1 on Expression of HSP47 in Conjunctival Fibroblasts**

Conjunctival fibroblasts were treated with various concentration (1, 10, and 100 ng/ml) of recombinant TGF-β1 (R&D Systems), for 24 hours, in an incubator at 37°C with 95% humidity and 5% CO2. The total RNA and proteins were extracted from conjunctival fibroblasts and were used for quantitative real-time PCR and Western blot analysis. Conjunctival fibroblasts, grown on the glass slides and treated with various concentrations of TGF-β1 for 24 hours and then fixed with cold methanol, were used for the detection of HSP47 by immunohistochemistry.

**Blocking the Effects of TGF-β1 on the Expression of HSP47 in Conjunctival Fibroblasts**

Conjunctival fibroblasts were incubated with various concentrations (2 and 20 ng/ml) of TGF-β type II receptor-neutralizing antibody (R&D Systems) for 12 hours at 37°C with 95% humidity and 5% CO2. The fibroblasts were then treated with 10 ng/ml recombinant TGF-β1 for 24 hours, and the extracted total RNA and proteins were analyzed by quantitative real-time PCR and Western blot analysis.

**Western Blot Analysis of Protein Extracted from Conjunctival Fibroblasts**

Protein extracted from TGF-β1-treated and nontreated conjunctival fibroblasts were electrophoresed on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, as described earlier. The membrane was blocked with 20 mg/ml bovine serum albumin dissolved in PBS solution for 2 hours at room temperature. The expression of HSP47 was detected with a mouse monoclonal antibody to HSP47 (overnight at 4°C). Blots were then washed and incubated with horseradish peroxidase (HRP)–conjugated antibody against mouse IgG for 1 hour. Immunoreactive protein was visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Blots were also reacted with anti-β-actin antibody (Sigma Chemical Co.), to assess the variations in protein loading between various samples. As a positive control, recombinant HSP47 was loaded along with samples, electrophoresed, transferred to the membrane, and reacted with anti-mouse antibody to HSP47. The negative control consisted of incubating the blots with mouse IgG, instead of primary antibody.

**Quantitative Real-Time PCR**

The total RNA isolated from conjunctival tissues and conjunctival fibroblasts of control subjects and patients with OCP was used to detect relative expression of HSP47, TGF-β1, and type I collagen mRNA. The principle of quantitative real-time PCR has been described elsewhere. The quantification of transcription of real-time PCR takes advantage of the 5′ nuclease activity of Taq DNA polymerase. Total RNA was extracted from conjunctival tissues and conjunctival fibroblasts using a RNA isolation kit (Qiagen, Valencia, CA). The primers and probe used for detection of HSP47, TGF-β1, and type I collagen are listed in Table 1. Each PCR reaction contained equivalent amounts of total RNA. Real-time PCR was always performed in duplicate with a PCR reagent kit (TaqMan; Applied Biosystems, Foster City, CA). All the reactions were controlled by standards (nontemplate control and standard positive control). The quantity of mRNA was calculated by normalizing the cycle threshold (Ct) of HSP47, TGF-β1, or type I collagen to the Ct of the housekeeping gene 18S or GAPDH of the same RNA sample, according to the following formula: the average 18S or GAPDH Ct (for each multiplex PCR was performed in duplicate) was subtracted from the average HSP47, TGF-β1, or type I collagen Ct. This result represents the ΔCt, which is specific and can be compared with the ΔCt of a calibration sample (for example control conjunctival tissues or control conjunctival fibroblasts). The subtraction of control ΔCt from the ΔCt of OCP samples or fibroblasts of OCP samples was referred as ΔΔCt. The relative expression of HSP47, TGF-β1, or type I collagen (in comparison to the control) in tissues and fibroblasts obtained from conjunctiva of patients with OCP was determined by 2−ΔΔCt. For all the probes the quencher dye was 6-carboxy-tetramethylrhodamine (TAMRA), the reporter dye was 6-carboxy fluorescein (FAM) for HSP47, TGF-β1, and type I collagen, and VIC for 18S or GAPDH.

**RESULTS**

**Expression of HSP47 in Conjunctival Tissue**

The expression of HSP47 was weakly and sparsely detected in the conjunctival epithelium and stromal cells in sections from
normal control conjunctiva (Fig. 1A). In contrast, in sections of conjunctiva from patients with OCP, both intensity and number of stromal cells expressing HSP47 were increased (Fig. 1B). Moderately increased expression of HSP47 was also noted in the epithelial cells, in comparison to control conjunctival sections. Moreover, compared with control conjunctival tissue, a 3.4-fold increase in the expression of HSP47 was detected by quantitative real-time PCR in the conjunctival tissue obtained from patients with OCP. (Fig. 2). However, no such increased expression of HSP47 was noted by quantitative real-time PCR in conjunctival tissues obtained from patients with atopic conjunctivitis.

Expression of Type I and Type III Collagens in Conjunctival Tissue

Interstitial type I and type III collagens was weakly expressed in the submucosal stroma and around the blood vessels in the conjunctival sections of normal control subjects (Figs. 3A, 3C). Compared with the control, an increased deposition of type I and type III collagens was detected in the fibrotic interstitium in conjunctiva of patients with OCP (Figs. 3B, 3D).

Expression of HSP47 in Conjunctival Fibroblasts

Fibroblasts isolated from conjunctiva of normal control subjects and patients with OCP were studied by immunostaining and real-time PCR, to examine the expression of HSP47. Compared with control conjunctival fibroblasts (Fig. 4A), increased cytoplasmic immunostaining for HSP47 was observed in the fibroblasts isolated from conjunctiva of patients with OCP (Fig. 4B). Similarly, a 4.8-fold increase in the expression of HSP47 mRNA was detected in the fibroblasts isolated from conjunctiva of patients with OCP (Fig. 2).

Expression of Type I Collagen and TGF-β1 in Conjunctival Fibroblasts

The expression levels of type I collagen and TGF-β1 in conjunctival fibroblasts was examined with quantitative real-time PCR and immunohistochemistry. Compared with control conjunctival fibroblasts, increased expression of type I collagen and TGF-β1 was detected by real-time PCR in the fibroblasts isolated from conjunctiva of patients with OCP. Compared with control conjunctival fibroblasts, increased expression of TGF-β1 was detected by immunohistochemistry in fibroblasts isolated from conjunctiva of patients with OCP (data not shown).

Induction of HSP47 and Type I Collagen by TGF-β1 in Cultured Conjunctival Fibroblasts

Conjunctival fibroblasts were treated with various concentrations of TGF-β1 (1, 10, and 100 ng/mL) for 24 hours to eluci-
date the effect of TGF-β1 on the expression of HSP47. Compared with the nontreated fibroblasts (Fig. 4A), increased cytoplasmic staining for HSP47 was noted in the TGF-β1–treated conjunctival fibroblasts (Fig. 4C). When the conjunctival fibroblasts were incubated with anti-HSP47 antibody, preabsorbed with recombinant HSP47, no specific staining was detected (Fig. 4D). This elimination of staining demonstrated the specificity of the HSP47 staining. In addition, compared with nontreated fibroblasts, TGF-β1–treated fibroblasts showed upregulated expression of HSP47, at both the mRNA level, detected by quantitative real-time PCR (Fig. 5) and the protein level, detected by Western blot analysis (Fig. 6). The increased expression of HSP47 in TGF-β1–treated conjunctival fibroblasts was correlated with the increased expression of type I collagen, as detected by quantitative real-time PCR (Fig. 7).

**Inhibition of TGF-β1–Induced HSP47 and Collagen Expression in Cultured Conjunctival Fibroblasts by TGF-β Type II Receptor–Neutralizing Antibody**

Conjunctiva fibroblasts were treated with two concentrations (2 and 20 ng/mL) of TGF-β type II receptor–neutralizing antibody (for 12 hours) and then treated with 10 ng/mL TGF-β1 (for 24 hours). Compared with the TGF-β1–treated conjunctival fibroblasts, TGF-β type II receptor–neutralizing antibody–treated fibroblasts demonstrated relatively less expression of HSP47 by quantitative real-time PCR (data not shown).

**DISCUSSION**

In this study, we have shown a possible role of HSP47 in the pathogenesis of conjunctival scarring in patients with OCP. Our results indicate that the expression of HSP47 was increased, at both the mRNA and protein levels, in conjunctiva obtained from patients with OCP. Increased expression of HSP47 was accompanied with an increased deposition of type I and type III collagens in the fibrotic conjunctiva of patients with OCP. The staining patterns and distribution of both type I and type III collagens was essentially similar. HSP47 is a collagen-specific chaperone that was originally identified by Kurkinen et al. from murine parietal endoderm.
The development of mouse conjunctival fibrosis, and mitomycin-C has been shown to reverse this fibrosis in mice. Moreover, increased expression of TGF-β1 and β3 has been reported in conjunctiva of patients with OCP. Epithelial cells and fibroblasts have been shown to produce increased levels of TGF-β in conjunctiva of patients with OCP, as detected by in situ hybridization.

In our present study, compared with the control conjunctival fibroblasts, increased expression of TGF-β1 was detected in conjunctival fibroblasts isolated from patients with OCP. Furthermore, when conjunctival fibroblasts were treated with recombinant TGF-β1, upregulation in the expression of both HSP47 and type I collagen was noted. Hence, the observation of this study suggests that TGF-β1 is one of the essential molecules that may regulate the expression of HSP47 and type I collagen in conjunctiva of patients with OCP. Our results are in accord with earlier studies that have shown activation and/or induction of HSP47 by TGF-β1.

Both in vitro and in vivo studies have shown that suppression of the expression of HSP47 modulates collagen production and consequently diminishes the production of type I procollagen. In a rat nephritis model, the inhibition of HSP47 overexpression by administration of HSP47 antisense oligonucleotides resulted in the suppression of collagen production and the attenuation of glomerulosclerosis. Similarly, modulation of the expression of HSP47 by caloric restriction has been shown to delay age-associated renal scarring in Fischer 344 rats. Hence, the multistep, multifactorial scarring process could be molecularly modulated, to reduce, inhibit, and possibly reverse the conjunctival scarring process, which may ultimately prevent blindness in some patients with OCP.

In conclusion, the present study demonstrates increased expression of HSP47 with excessive accumulation of collagens in the conjunctiva of patients with OCP. This upregulation of HSP47 and collagens appears to be induced by TGF-β1. We realize that it is possible that other molecules may also be involved in the process of this upregulation, and, if that is the case, then TGF-β1 may be working synergistically with them. A detailed study of the sequential events and factors that facilitate or enhance matrix remodeling in the conjunctiva, could be important in understanding the molecular mechanism of OCP and could provide specific sites for molecular intervention for treatment or arrest of the progression of disease.

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