Role of Connective Tissue Growth Factor in the Pathogenesis of Conjunctival Scarring in Ocular Cicatricial Pemphigoid

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PURPOSE. Conjunctival fibrosis due to excessive accumulation of collagens is an important histologic feature in ocular cicatricial pemphigoid (OCP). Studies have suggested a role of transforming growth factor (TGF)-β1 in conjunctival fibrosis in patients with OCP. Connective tissue growth factor (CTGF) is an important downstream mediator of TGF-β1-induced collagen synthesis. CTGF usually acts synergistically with TGF-β1 during the process of fibrosis in various organs. Hence, studying the mechanism by which CTGF influences TGF-β1-induced synthesis of collagen in conjunctiva of patients with OCP would provide insight into the mechanism of conjunctival fibrosis in patients with OCP.

METHODS. Biopsy specimens from conjunctiva of 10 patients with OCP and 5 normal subjects, were studied, with immunohistochemistry and real-time PCR, for the expression of CTGF and interstitial type I collagen. Using fibroblasts cultured from conjunctival biopsies we determined the effects of TGF-β1 on the induction of CTGF and type I collagen by immunostaining, and quantitative real-time PCR. The effects of blocking the bioactivity of TGF-β1 on the expression of CTGF and type I collagen were determined in TGF-β1-stimulated fibroblasts, before and after treatment with type II receptor neutralizing antibody.

RESULTS. An increased stromal accumulation of interstitial type I collagen with an increased expression of CTGF was observed in biopsy sections of patients with OCP, compared with the control. By quantitative real-time PCR, a 3.2-fold increase in the expression of CTGF was detected in conjunctival tissues obtained from patients with OCP, compared with control conjunctiva. Fibroblasts isolated from conjunctiva of patients with OCP expressed 4.4-fold more CTGF, compared with control conjunctival fibroblasts, by real-time PCR. When these cultured fibroblasts were immunostained, an increased expression of CTGF was detected in fibroblasts isolated from patients with OCP, compared with control. Furthermore, when conjunctival fibroblasts were treated with TGF-β1, an approximately ninefold increase in the expression of CTGF and an approximately threefold increase in the expression of type I collagen were detected by real-time PCR, compared with unstimulated fibroblasts. Finally, when antibody to TGF-β type II receptor was added before TGF-β1 treatment of these fibroblasts, the expression of type I collagen and CTGF was significantly reduced.

CONCLUSIONS. In the present study, an increased expression of CTGF was recorded in conjunctiva of patients with OCP. TGF-β1 can induce production of CTGF and type I collagen by fibroblasts obtained from conjunctiva in OCP. This induction of CTGF by TGF-β1 can be blocked by antibody to TGF-β type II receptors. The findings lead to the conclusion that CTGF is one of the molecules involved in the pathogenesis of conjunctival fibrosis in patients with OCP. (Invest Ophthalmol Vis Sci. 2003;44:1998–2003) DOI:10.1167/iovs.02-0967

Ocular cicatricial pemphigoid (OCP) is an autoimmune disease, usually characterized by recurrent episodes of inflammation that gradually progress to subepithelial conjunctival fibrosis.1,2 In spite of available immunosuppressive therapies, a significant number of cases of OCP eventually lead to blindness.1 Recently, our understanding of the molecular mechanisms of fibrotic diseases in various tissues and organs has significantly improved.3–8 The unavailability of similar information on OCP in part is due to its relatively rare occurrence when compared with other fibrotic diseases, such as in the liver or lung. Recently, a number of important fibrogenic molecules have been identified and shown to play crucial roles in wound healing and in certain fibrotic diseases.9–12 CTGF is one of those fibrogenic factors. It exerts its biological effects on proliferation and differentiation of mesenchymal cells and on synthesis of matrix proteins. Recent studies have demonstrated that increased expression of CTGF is paralleled by increased synthesis and accumulation of collagens in fibrotic diseases in lung, skin, and kidney.13–15 Moreover, experimental studies have shown that blocking the expression of CTGF could modulate fibrotic processes in the kidney.16

TGF-β1 significantly influences the matrix remodeling, by stimulating the transcription of genes encoding for extracellular matrix (ECM) proteins. CTGF is a downstream, profibrotic mediator of TGF-β1–induced matrix synthesis.17,18 CTGF usually acts synergistically with TGF-β1 to enhance its fibrogenic effects. In this study, we examined the role of CTGF and its relationship to TGF-β1 in the pathogenesis of conjunctival fibrosis in patients with OCP.

MATERIALS AND METHODS

Conjunctival Specimens

Conjunctival biopsy specimens were obtained from 10 patients with OCP. The diagnosis of OCP was based on clinical presentation, histology, and direct immunofluorescence of the conjunctiva demonstrating deposition of IgG and C3 at the basement membrane zone (BMZ). Specimens of conjunctiva from five individuals were obtained during routine cataract surgery and were used as the control. The study was supported by National Eye Institute Grant 1R01EY08379.

Submitted for publication September 20, 2002; revised November 19, 2002; accepted December 5, 2002.

Disclosure: M.S. Razzaque, None; C.S. Foster, None; A.R. Ahmed, None

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followed the guidelines of the Declaration of Helsinki for research involving human subjects.

**Immunohistochemistry**

Immunohistochemistry was performed on paraffin-embedded and frozen sections, as described previously. Briefly, conjunctival sections were blocked with 10% goat serum for 1 hour and then incubated overnight at 4°C with the following primary antibodies: CTGF (Abcam, Cambridge, UK) or type I collagen (Sigma, St. Louis, MO). After washing with PBS, sections were processed further with a histochemical 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 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**

Conjunctival fibroblasts were isolated as described earlier. Briefly, conjunctival tissue obtained from control subjects and patients with OCP was cut into explants of approximately 2 × 2 mm² and placed into tissue culture dishes and covered with Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc., Herndon, VA), containing fetal bovine serum (FBS; Mediatech, Inc.), gentamicin, and amphotericin B, and incubated overnight, in an incubator 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⁺-free minimum essential medium (MEM) at 80% to 90% confluence. Fibroblasts isolated from conjunctiva of the normal control and patients with OCP, were grown on the glass slides, fixed with methanol, and immunostained for CTGF, as just described. In addition, total RNA extracted from fibroblasts isolated from conjunctiva of normal controls and patients with OCP were used for quantitative real-time PCR. Cells at passages 3 to 7 were used.

**Effects of TGF-β1 on Expression of CTGF and Type I Collagen by Conjunctival Fibroblasts**

Conjunctival fibroblasts were treated with various concentration (1, 10, and 100 ng/mL) of recombinant 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 by standard methods from conjunctival fibroblasts and was used for quantitative real-time PCR.

**The Effects of TGF-β Type II Receptor–Neutralizing Antibody on the Expression of TGF-β1–Induced CTGF and Type I Collagen**

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 in an incubator at 37°C with 95% humidity and 5% CO2. The fibroblasts were then treated with 10 ng/mL recombinant TGF-β1 for 24 hours. The total RNA was extracted by standard methods, and the expression of CTGF and type I collagen was determined with quantitative real-time PCR.

**Real-Time PCR**

The total RNA isolated from conjunctival biopsy tissues and conjunctival fibroblasts of control individuals and patients with OCP were used to detect relative expression of CTGF and type I collagen mRNA. The principle of real-time quantitative PCR has been described elsewhere. Total RNA was extracted from conjunctival tissues and conjunctival fibroblasts with an RNA isolation kit (Qiagen, Valencia, CA). The primers and probe used for detecting mRNA for CTGF were, forward: GAG GAA AAC ATT AAG AAG GGC AAA, reverse: CGG CAC AGG TCT TGA TGA, probe (TaqMan; Applied Biosystems, Foster City, CA): FAM-TTT GAG CTT TCT GGC TGC ACC AGT GT-TAMRA; and type I collagen, forward: CCT CAA GGG CTC CAA CGA G, reverse: VIC-ATC ACT GTG CCC CA, probe (TaqMan; Applied Biosystems): FAM-ATG GCT GCA CGA GTC ACA CCG GA-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 PCR reagent kit; Applied Biosystems). All the reactions were controlled by standards (nontemplate control and standard positive control). The quantity of mRNA was calculated by normalizing the C_T (threshold cycle value) of CTGF or type I collagen to the C_T of the housekeeping genes 18S or GAPDH of the same RNA probe, according to the following formula: the average 18S or GAPDH C_T (each multiplex PCR was performed in duplicate) was subtracted from the average CTGF or type I collagen C_T, this result represent the ΔC_T. This ΔC_T is specific and can be compared with the ΔC_T of a calibration sample (for example control conjunctiva or control conjunctival fibroblasts). The subtraction of control ΔC_T from the ΔC_T of OCP samples is referred as ΔΔC_T. The relative expression of CTGF, or type I collagen (in comparison to controls) in conjunctival tissues and fibroblasts isolated from conjunctiva of patients with OCP was determined by 2^ΔΔC_T. Similar calculations were made for the expression of CTGF and type I collagen in TGF-β1–treated fibroblasts, in comparison to nontreated fibroblasts. For all the probes the quencher dye was 6 carboxy-tetramethylrhodamine (TAMRA), the reporter dye was 6 carboxy fluorescein (FAM) for CTGF, and type I collagen, and VIC for 18S or GAPDH.

**RESULTS**

**Expression of CTGF**

Compared with the control, conjunctival sections of patients with OCP demonstrated an increased expression of CTGF in the basal epithelial cells and stromal cells, by immunohistochemistry (Fig. 1). A 3.2-fold increase in the expression of CTGF was also detected in the conjunctival tissues of patients with OCP, compared with control conjunctival tissue, as determined by quantitative real-time PCR (Fig. 2).

Fibroblasts isolated from the conjunctiva of patients with OCP demonstrated an increased cytoplasmic immunostaining for CTGF, compared with control conjunctival fibroblasts (Fig. 3). By quantitative real-time PCR a 4.4-fold increase in the expression of CTGF was noted in fibroblasts isolated from conjunctiva of patients with OCP, compared with control fibroblasts (Fig. 2).

**FIGURE 1.** Immunohistochemistry on a section of human conjunctiva stained for CTGF in a normal control subject (A) and a patient with OCP (B). An increase in CTGF-expressing cells was observed in submucosal stromal tissues (arrows) in the OCP tissue (B) compared with the control.
Expression of Type I Collagen

The expression of interstitial type I collagen was present in the submucosal stroma and around the blood vessels in control conjunctival sections. An increased deposition of type I collagen was detected in the fibrotic interstitium of conjunctival tissues obtained from patients with OCP, compared with the control, as detected by immunohistochemistry (data not shown). Similarly, compared with control fibroblasts, an increased expression of type I collagen (1.9-fold) as detected in conjunctival fibroblasts, obtained from patients with OCP, as detected by quantitative real-time PCR.

Induction of CTGF 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, and the expression of CTGF and type I collagen was determined by real-time PCR. Compared with nontreated fibroblasts, TGF-β1-treated fibroblasts showed upregulated expression of CTGF (as high as ninefold), by quantitative real-time PCR (Fig. 4). TGF-β1-treated fibroblasts showed a similar increase (as high as threefold) in the expression of type I collagen, by quantitative real-time PCR (Fig. 5).

Figure 3. Immunostaining of fibroblasts for CTGF isolated from conjunctiva of a normal control subject (A) and from a patient with OCP (B). Note an increased cytoplasmic expression of CTGF in fibroblasts obtained from the OCP tissue, compared with the control.

Effects of Blocking TGF-β1 on the Expression of CTGF and Type I Collagen

 Conjunctival fibroblasts were treated with 2 and 20 ng/mL of TGF-β1 type II receptor-neutralizing antibody for 12 hours and then treated with 10 ng/mL TGF-β1 for another 24 hours. Compared with the TGF-β1-treated (10 ng/mL) conjunctival fibroblasts, TGF-β type II receptor-neutralizing antibody-treated fibroblasts exhibited lower levels of expression for both CTGF (Fig. 6) and type I collagen (Fig. 7).

DISCUSSION

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. Progression of subepithelial fibrosis results in shrinkage of conjunctiva, symblepharon formation, meibomian duct obstruction, and eventual lacrimal duct compression with reduced tear flow. In most patients with OCP, the disease is diagnosed during the fibrotic stage. They are usually treated with systemic corticosteroids with or without immunosuppressive agents, with the objective of reducing inflammation and decreasing the production of pathogenic autoantibodies. No specific therapy to prevent or reduce the progression of conjunctival scarring is as yet available. Despite aggressive systemic therapy, 25% of patients with OCP become blind.1 Identifying some of the candidate molecules involved in the process of conjunctival fibrosis will increase our understanding of conjunctival fibrogenesis and may help in determining the possible therapeutic potential of these molecules to block or delay the progression of conjunctival fibrosis in patients with OCP.

TGF-β is a family of multifunctional regulatory peptides involved in the control of cell growth and differentiation, morphogenesis, and the remodeling of connective tissues. Three isoforms of TGF-β have been identified in mammals, and all these isoforms have a high level of sequence conservation. Among these, TGF-β1 has been extensively studied and shown to affect matrix remodeling, by stimulating the transcription of genes encoding ECM proteins. A significant increase in the expression of TGF-β1 has been detected in the conjunctiva of patients with OCP.2

CTGF is a heparin-binding, 38-kDa, cysteine-rich peptide that helps in proliferation of fibroblasts and enhanced produc-
FIGURE 4. Effects of TGF-β1 on the expression of CTGF. Conjunctival fibroblasts were treated with various concentrations of TGF-β1 (1, 10, and 100 ng/mL) for 24 hours, and the induction of CTGF was determined at the mRNA level, by quantitative real-time PCR. The mean relative expression of CTGF in TGF-β1-treated fibroblasts, in comparison to untreated fibroblasts, shows an increased expression of CTGF in the TGF-β1-treated fibroblasts.

FIGURE 5. Effects of TGF-β1 on the expression of type I collagen. Conjunctival fibroblasts were treated with various concentrations of TGF-β1 (1, 10, and 100 ng/mL) for 24 hours, and the induction of type I collagen was determined at the mRNA level, by quantitative real-time PCR. The mean relative expression of type I collagen in TGF-β1-treated fibroblasts, in comparison with untreated fibroblasts, shows an increased expression of type I collagen in the TGF-β1-treated fibroblasts.

FIGURE 6. Effects of blocking TGF-β1 by receptor type II antibody on the expression of CTGF. Conjunctival fibroblasts were treated with various concentrations (2 and 20 ng/mL) of TGF-β type II receptor-neutralizing antibody for 12 hours and then treated with 10 ng/mL of TGF-β1 for another 24 hours. A reduced expression of CTGF was detected in TGF-β type II receptor-neutralizing antibody–treated fibroblasts, compared with the TGF-β1–treated (10 ng/mL) conjunctival fibroblasts.
tion of matrix proteins. The regulation of CTGF has been shown to be partly controlled by TGF-β1. A brief exposure of fibroblasts to TGF-β resulted in the induction of CTGF expression in human corneal and mouse NIH 3T3 fibroblasts.27,28 This upregulation is mainly dependent on sequences present in the 5′ upstream region of the CTGF promoter. An upregulated expression of CTGF has been detected in various fibrotic diseases in skin and other internal organs.29

In this study, we have shown a possible role of CTGF in the pathogenesis of conjunctival scarring in patients with OCP. The important observation of this study is that the expression of CTGF was increased in conjunctiva and in conjunctival fibroblasts of patients with OCP. This enhanced expression of CTGF correlated with an increased accumulation of interstitial type I collagen in the specimens from patients with OCP. In addition to type I collagen, an increased expression and deposition of several matrix proteins, such as type III collagen, versican, and tenasin have been detected in the submucosal stroma in tissue from patients with OCP, compared with normal control conjunctiva (Chu DS, Razzaque MS, Ahmed AR, Foster CS, ARVO Abstract 2523, 2001)30 Some of these molecules are important components of various human and experimental fibrotic diseases.31–34 We used type I collagen, which is a known component of conjunctival fibrosis, as a prototype molecule to study the effects of TGF-β1 on the expression of CTGF and matrix proteins.35

We have shown that conjunctival fibroblasts isolated from patients with OCP expressed an increased level of TGF-β1, compared with control conjunctival fibroblasts. Exogenous treatment of these fibroblasts with recombinant TGF-β1 resulted in the induction of interstitial collagens.30 Hence, it became important to study the mechanism by which CTGF influences the TGF-β1–induced fibrosis. In this study, when conjunctival fibroblasts were treated with various concentrations of recombinant TGF-β1, an upregulation in the expression of CTGF, compared with untreated cells, was observed. The use of TGF-β type II receptor–neutralizing antibody resulted in the reduction of TGF-β1–induced expression of CTGF and type I collagen. This observation suggests that TGF-β1 can regulate the expression of CTGF and type I collagen by conjunctival fibroblasts. We would have preferred to study the direct effects of CTGF on synthesis of collagens by conjunctival fibroblasts. The unavailability of recombinant CTGF prevented us from conducting such studies. Similarly, attempts to block the activity of CTGF by antibody to CTGF were not conclusive, because of the unavailability of an appropriate neutralizing antibody. In spite of some of the experimental limitations, based on these observations, it appears likely that increased levels of CTGF in the conjunctiva of patients with OCP plays an important role in increased production of collagens and subsequent conjunctival scarring in patients with OCP. These observations are similar to those of earlier studies, in which an upregulated expression of CTGF and enhanced synthesis of collagens has been demonstrated in human and experimental models of fibrosis.13,14,17,18,29

We realize that the scarring process in the conjunctiva of patients with OCP may involve multiple molecules. This process, in addition to being complex, is multistaged, interactive, and regulated in various levels. We have studied TGF-β1 and CTGF to demonstrate this as one such possible pathway. TGF-β1 is a pluripotent growth factor and exerts fibrogenic effects during wound healing and fibrosis. It not only has the ability to induce matrix proteins during wound healing and fibrogenesis, but it has also other essential effects on the cell, such as the suppression of the growth of epithelial cells, inhibition of keratinocyte proliferation, enhancement of neovascularization, chemoattraction for monocytes and fibroblasts, and immunosuppression.37–38 It would be advantageous if only the fibrotic effects of TGF-β1 could be blocked without affecting other essential physiologic functions. Otherwise, inhibiting TGF-β1 would most likely have an adverse impact on numerous other essential cellular functions.

In this study, we have demonstrated a role for CTGF in the pathogenesis of conjunctival scarring in OCP. Future studies will determine whether blocking the activity of CTGF would result in decreased synthesis of matrix proteins. Such a process may modulate conjunctival scarring in patients with OCP.

Acknowledgments

The authors thank Victoria Patkova, PhD, for technical assistance in performing real-time PCR.

References


