Regulation of Collagenase, Stromelysin, and Urokinase-Type Plasminogen Activator in Primary Pterygium Body Fibroblasts by Inflammatory Cytokines

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PURPOSE. To examine the expression patterns of extracellular matrix degrading enzymes in cultured primary pterygium body fibroblasts activated by cytokines and growth factors potentially derived from ocular surface epithelial cells and tears.

METHODS. EGF, TGF-α, PDGF-BB, IL-1β, bFGF, TGF-β1, TNF-α, or IL-6 were added at 10 ng/ml to early passaged primary pterygium body fibroblasts (PBF) or normal human conjunctival fibroblasts (HJF) in a serum-free medium. Expression of transcripts and proteins of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, TIMP-2, and uPA was determined by Northern hybridization, ELISA, and Western blotting, respectively. Gelatin and casein zymographies were performed in their serum-free conditioned media with or without enzyme inhibitors to determine the activity of MMP-2 and -3, respectively.

RESULTS. IL-1β and TNF-α dramatically increased the mRNA and protein expression of MMP-1 and MMP-3 in cultured PBF when compared to normal HJF and to their nonstimulated counterparts cultured in a serum-free medium. EGF and TGF-α also upregulated MMP-3 in PBF when compared to HJF. The transcript levels of MMP-2 were high but stable for the two cell types regardless of the cytokine treatment. Both TIMP-1 and TIMP-2 expressions were not influenced by the cell type or the cytokine treatment. MMP-9 was not expressed in either of these two types of fibroblasts. Both IL-1β and TNF-α induced a significant decrease in uPA expression in PBF, whereas bFGF induced a slight increase in both HJF and PBF.

CONCLUSIONS. Chronic inflammatory stimulation by IL-1β and TNF-α, which potentially can be derived from the ocular surface and tears, may be responsible for increased expression of MMPs in cultured PBF. These data have clinical implications on progression of pterygium and recurrence associated with incomplete excision of primary PBF under the influence of ocular surface inflammation. Suppression of intraoperative and postoperative inflammation may be a new strategy to prevent pterygium recurrence. (*Invest Ophthalmol Vis Sci.* 2000;41:2154–2163)

Pterygium is characterized by the encroachment of a fleshy fibrovascular tissue from the bulbar conjunctiva onto the cornea. Although historically described as a degenerative disorder, it is more closely associated with inflammation and progressive fibrovascular proliferation.[1-4] The extent and severity of this fibrovascular growth has been found to be a reliable morphologic index for predicting pterygium recurrence after excision.[5] Recent data suggest the existence of intrinsic abnormalities in the pterygial fibrovascular tissue. Fibroblasts isolated from pterygial tissues exhibit a transformed phenotype.[6] In contrast to normal conjunctival fibroblasts, pterygial fibroblasts grow much better in a medium containing a low concentration of serum. Furthermore, they can grow in a semisolid agar, indicative of anchorage-independent growth, that is, a phenotype of transformed or neoplastic cells. This abnormality could be a result of defective DNA repair, as recently demonstrated by the presence of microsatellite instability in pterygium.[7]

Ocular dryness and environmental insults may contribute to the progression of pterygium and its recurrence. These stimuli, enhanced by UV irradiation, can trigger the secretion of such proinflammatory cytokines as interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor (TNF) α from the corneal[8] and conjunctival[9] epithelium. In addition, conjunctival epithelial cells can secrete TNF-α, IL-6, IL-8, and GM-CSF after inflammatory stimulation.[8] Certain proinflammatory cytokines, which originate from the ocular surface epithelium or the tear fluid, specifically TNF-α and IL-1β, can stimulate proliferation of cultured Tenon's capsule fibroblasts.[10] Furthermore, several potent fibroangiogenic growth factors such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF)-BB, and transforming growth factor (TGF)-β1 have been localized by immunohistochemistry in different cell types of the pterygium specimen.[11]

Tissue remodeling is a key process involved in normal development, wound healing, and pathologic conditions such as tumor invasion, metastasis, and angiogenesis. The degradation of the extracellular matrix that is associated with these processes is mediated by several families of extracellular proteinases. These families include the zinc-dependent matrix metalloproteinases (MMPs), and the serine proteases, such as the plasminogen–urokinase plasminogen activator (uPA) system. MMPs are a family of enzymes that act to modify or degrade the extracellular matrix (ECM).[12–14] These enzymes are synthesized and secreted by a variety of cell types including fibroblasts. At least 17 members of the MMP family have been identified.[15] MMPs are normally coexpressed with a family of tissue inhibitors of metalloproteinases (TIMPs), which inhibit active forms of MMPs. At least four inhibitors, have been characterized and are also produced by many cell types including fibroblasts.[12,13] The MMPs and TIMPs have important roles in the process of fibrovascular proliferation. For example, MMPs have been clearly implicated in angiogenesis, whereas their inhibitors are shown to inhibit the angiogenic responses.[16] Overexpression of MMP-1 in the skin has led to acanthosis and increased basal-cell proliferation, suggesting that such overexpression may be coupled with a phenotype capable of increased proliferation.[17] Besides MMPs and TIMPs, the other proteolytic cascade leading to tissue degradation and remodeling involves uPA, a serine protease. Overexpression of uPA is correlated with the invasiveness of human cancer cells.[18]

We hypothesized that certain environmental stimuli known to be associated with pterygium, induce secretion of proinflammatory cytokines by the ocular surface epithelium, inflammatory cells in the tear fluid, or both. These cytokines in turn activate pterygium body fibroblasts (PBF), resulting in a phenotype capable of expressing various proteinases associated with ECM remodeling, angiogenesis, and fibroblast proliferation. These traits are important for pterygium progression as a primary lesion and for its recurrence after incomplete surgical removal. Hence, the purpose of this study was to investigate the effects of ocular surface–derived cytokines and growth factors on the expression pattern of proteinases associated with matrix degradation in cultured primary PBF compared with normal conjunctival fibroblasts.

METHODS

Materials

DMEM, fetal bovine serum (FBS), fungizone (amphotericin B), phenol, DNA or RNA size markers, and random primers DNA labeling kit were purchased from GIBCO-BRL (Grand Island, NY). Cell culture dishes, 6-well plates, and 15-ml centrifuge tubes were from Becton Dickinson (Lincoln Park, NJ). Recombinant human EGF, TGF-α, PDGF-BB, IL-1β, bFGF, TGF-β, TNF-α, and IL-6 were from R & D systems (Minneapolis, MN). BCA protein assay kit was from Pierce (Rockford, IL). Zymogram-ready gels containing gelatin or casein, 4% to 15% Tris-HCl polyacrylamide gradient ready gel, SDS, and electrophoresis equipment were from Bio-Rad (Hercules, CA). Human MMP-1 and MMP-3 ELISA kits and the monoclonal antibodies against human MMP-1, MMP-2, MMP-3, TIMP-1, and TIMP-2 were from Oncogene Research Products of Calbiochem (Cambridge, MA). uPA ELISA kit was from Oncogene Science Diag-

nostics (Cambridge, MA). Vectastain Elite ABC peroxidase kit was from Vector Laboratories (Burlingame, CA). Nitrocellulose membranes were from Scheicher and Schuell (Keene, NH). GeneAmp RNA-PCR kit was from Perkin-Elmer Cetus (Norwalk, CT). Wizard PCR Preps DNA purification kit was from Promega (Madison, WI). [α-^{32}P]dCTP was from Du Pont NEN (Boston, MA). XAR-5 and BioMax MS-1 films and intensifying screens were from Eastman Kodak (Rochester, NY). All other reagents and chemicals came from Sigma (St. Louis, MO).

Human Conjunctival and Primary Pterygium Fibroblast Cultures

All procedures followed the tenets of the Helsinki declaration and were approved by the Bascom Palmer Eye Institute Review Board. Specimens of normal human conjunctiva were obtained from healthy donors who did not show signs and symptoms of an ocular surface disorder or of dry eyes. Conjunctival biopsies of 2 × 2 mm were taken from the superotemporal bulbar conjunctiva during cataract surgery, 2 to 3 mm from the limbus. Pterygium specimens were obtained after the surgical removal of primary pterygium. The central portion of the pterygium body was used for all cultures. All normal conjunctiva and pterygium specimens came from age-matched donors, whose ages ranged from 45 to 55 years old. These tissue samples were used for explant cultures to generate normal human conjunctival fibroblasts (HJF) and PBF, respectively. Each specimen was cut into explants of approximately 1 × 1 mm and placed onto 100-mm tissue culture dishes. Ten minutes later, each explant was covered with a drop of FBS and placed overnight in an incubator at 37°C under 95% humidity with 5% CO_2. Ten milliliters of medium (D-FBS) containing DMEM enriched with 10% FBS, 50 μg/ml gentamicin, and 1.25 μg/ml amphotericin B was added the next day, and the medium was changed every 2 days thereafter. Fibroblasts were subcultured with 0.05% trypsin and 0.85 mM EDTA in a calcium-free MEM medium at 80% to 90% confluence with 1:3 to 1:4 split for three passages. Three cell lines of HJF and three cell lines of PBF were used in this study. Of these, two cell lines of each were used to study mRNA expression, and one cell line of each was used for determination of protein expression.

Cytokine Regulation Experiments

Third passage fibroblasts of either HJF or PBF were seeded in 100-mm tissue culture dishes at a density of 10^6 cells per dish. After 5 days in culture, on confluence, cultures were switched to a serum-free medium (D-ITS) containing DMEM supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 50 μg/ml gentamicin, and 1.25 μg/ml amphotericin B for 48 hours before treatment with different cytokines. Some cultures were maintained in D-FBS. A single concentration of 10 ng/ml was used for all cytokines. This was based on preliminary dose–response experiments of cytokine regulation in ocular surface fibroblasts, using three concentrations of each cytokine (1, 5, and 10 ng/ml) and on our previous reports on cytokine regulation in these fibroblasts[19,20] as well as other dose–response data for TNF-α showing the 10-ng/ml concentration to be optimal for MMP-1 and -3 expression in human cervical smooth muscle cells.[21]

To study mRNA expression, 10 ng/ml of each of the following human recombinant cytokines or growth factors were added to the cultures for 4 hours: epidermal growth

factor (EGF), TGF-α, PDGF-BB, IL-1β, bFGF, TGF-β1, TNF-α, or IL-6. In parallel, control cultures with D-ITS alone or D-FBS alone were compared. Total RNA was then isolated and subjected to Northern hybridization.

Probe Preparation

Five of human DNA probes, including 185-bp fragment of MMP-1, 480 bp of MMP-2, 155 bp of MMP-3, 551 bp of TIMP-1, and 590 bp of TIMP-2, were kindly provided by Velidi H. Rao (University of Nebraska Medical Center, Omaha, NE). Two cDNA probes, 519 bp of uPA and 498 bp of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purified from RT-PCR products by electrophoresis through a 1.2% low melting agarose gel using a Promega Wizard PCR Prep DNA purification kit according to the manufacturer's protocol. The primers used for PCR were 487 to 506 (sense) and 982 to 1002 (antisense) for uPA (GenBank accession no. A18397), and 541 to 561 (sense) and 1018 to 1038 (antisense) for GAPDH (GenBank accession no. M33197). The ^{32}P-labeled cDNA probes (1 to 2 $\times$ 10^9 cpm/μg DNA) were prepared with [α-^{32}P]dCTP (3000 Ci/mmol) using a random primers DNA labeling system.

Total RNA Isolation and Northern Hybridization

After 4 hours of incubation with either one of the above treatments, cells were extracted for total RNA by acid guanidium thiocyanate–phenol– chloroform extraction with some modifications, as previously reported.[22] Total RNA was quantitated by measuring the absorption at 260 nm and stored at -80°C before use. Total RNA at 25 μg/lane was electrophoresed through 1.2% agarose containing formaldehyde, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled cDNA probes at 2 to 4 $\times$ 10^6 cpm/3 to 8 ng/ml in the hybridization solution. After visualization of the hybridization product in the x-ray film, the ^{32}P-label on the membrane was stripped by washing the membranes at 65°C for 1 hour twice in 5 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 0.05% sodium pyrophosphate, and 0.1$\times$ Denhardt's solution and rehybridized with other ^{32}P-labeled probes. The relative amount of each mRNA transcript of interest was determined by scanning its autoradiogram, analyzing the scanned image with Gel-Pro imaging software (Media Cybernetics, Silver Spring, MD) and normalizing the data as a ratio to that of the GAPDH mRNA band.

MMP-1, MMP-3, and uPA ELISA

Third passage HJF and PBF were seeded at a density of 3 $\times$ 10^5 in each plate of 6-well plates. Cells were cultured for 10 days until confluence in D-FBS, washed three times in serum-free D-ITS, and then kept in the serum-free D-ITS medium for 24 hours. Cultures were then treated with 10 ng/ml of either TGF-α, IL-1β, TNF-α, or bFGF in serum-free D-ITS and compared to those in D-ITS or D-FBS alone for 24 hours. Each of these treatments was performed in triplicate wells. After 24 hours' incubation with the different added treatments, the conditioned media were collected, centrifuged, and stored in -80°C until assayed. Cell lysis solution, containing 50 mM Tris-HCl, pH 7.6, 300 mM NaCl, and 0.5% Triton X-100, was added to the wells for 3 hours, and the cellular protein was collected, centrifuged, and stored in -80°C until assayed. Human MMP-1 or MMP-3 ELISA in each conditioned medium and human uPA ELISA in cell lysates were determined in duplicates using their respective double-sandwiched ELISA kits according to the manufacturer's protocol.

The total cellular protein content in the cell lysate was determined by the microBCA protein assay reagent kit (Pierce). The protein concentration of MMP-1, MMP-3, or uPA in the culture supernatant was adjusted by its corresponding total cellular protein content for any possible difference in cultured cell numbers. Thus, all ELISA results were expressed as picograms per micrograms of total protein and represented the mean of three triplicates of samples.

Western Blot Analysis

To identify MMP and TIMP proteins present in each fibroblast-conditioned medium, Western blot analysis was performed using their specific antibodies. Conditioned media from different fibroblast cultures were adjusted to a final volume of 25 μl to represent the same quantity of cellular protein (8.3 μg) and electrophoresed under reducing condition at 4°C in a 4% to 15% gradient polyacrylamide gel. After electrophoretic transfer to a nitrocellulose membrane at 4°C, the membrane was immersed with 0.1% (v/v) Tween 20 in Tris-buffered saline (100 mM Tris, 0.9% NaCl, pH 7.5; TTBS) for 30 minutes with agitation. The primary antibody (i.e., 1 μg/ml of mouse monoclonal antibody against human MMP-1, MMP-2, MMP-3, TIMP-1, or TIMP-2) in TTBS containing 1% horse serum was placed on each membrane and incubated at room temperature for 60 minutes with agitation. After being washed with three to four changes of TTBS over 15 minutes, each membrane was transferred to a 1:200 diluted solution of biotinylated second antibody (goat anti-mouse IgG; Vectastain Elite ABC kit) in TTBS containing 1% horse serum and incubated for 30 minutes. After three to four washes with the same solution, they were incubated with 1:50 diluted Vectastain Elite ABC reagent conjugated with peroxidase for 30 minutes and processed for color development in 0.5 μg/ml diaminobenzidine in 50 mM Tris-HCl, pH 7.2 containing 0.05% H$_2$O$_2$ for 10 to 20 minutes.

Zymography of Metalloproteinase Activity

To determine gelatinolytic and caseinolytic activities of the various fibroblast cultures, zymography was performed using a method similar to that previously described.[23] Each conditioned medium (40 μl), after being adjusted to represent the same quantity of cellular protein (13 μg) or cell number (8000 cells), was treated with sample buffer without boiling or reduction. SDS-PAGE was performed using a 10% polyacrylamide gel containing 0.1% gelatin or a 12% gel containing 0.1% casein. The gels were soaked in 2.5% Triton X-100 for 30 minutes at room temperature to remove the SDS and incubated in a reaction buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl$_2$, and 0.02% Brij-35) at 37°C overnight to allow proteinase digestion of its substrate. Gels were rinsed again in distilled water, stained with 0.5% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 1 hour, and destained with 40% methanol and 10% acetic acid. Proteolytic activities appeared as clear bands of lysis against a dark background of stained gelatin or casein. To verify that the detected gelatinolytic and caseinolytic activities were specifically derived from metalloproteinases, the gels were treated with Triton X-100 solution and Tris/NaCl/CaCl$_2$ reaction buffer containing 5 mM phenylmethylsulfonyl fluoride with or without 10 mM EDTA in the parallel experiments.
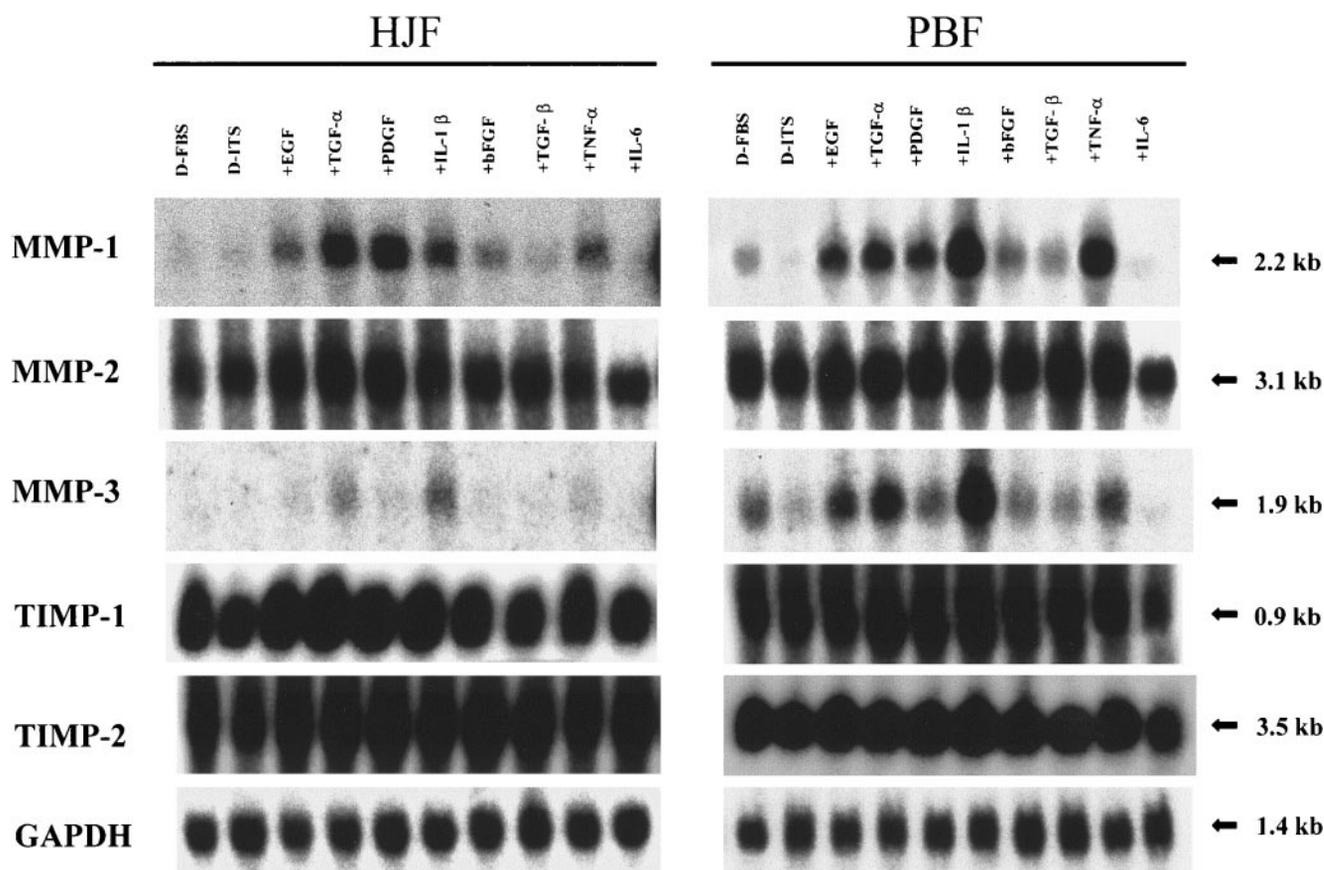
FIGURE 1. MMP-1, MMP-2, MMP-3, TIMP-1, and TIMP-2 mRNA expression by human conjunctival (HJF) and pterygium body fibroblasts (PBF) treated with different cytokines for 4 hours. HJF and PBF were grown to confluence in DMEM containing 10% fetal bovine serum (D-FBS) and switched to serum-free DMEM containing 1% ITS (D-ITS) for 48 hours, before treatment with 10 ng/ml of either EGF, TGF-α, PDGF-BB, IL-1b, bFGF, TGF-β1, TNF-α, or IL-6. Total RNA was isolated and individually hybridized with ^{32}P-labeled specific cDNA probes. The same filter was then hybridized with GAPDH probe as a control.

Statistical Analysis

Student's *t*-test was used for analyzing the ELISA results. The ELISA data are expressed as means $\pm$ SD, and the differences were considered statistically significant at $P < 0.05$.

RESULTS

Transcript Expression of MMPs and TIMPs in HJF and PBF

As shown in Figure 1 (lane 1), Northern blot analysis showed that the 2.2-kb MMP-1 transcript was very faintly expressed in D-ITS by normal HJFs, but upregulated by TGF-α and PDGF-BB (each 12-fold) and to a lesser extent by IL-1β (7-fold), TNF-α (4-fold), EGF (3-fold), and bFGF (2-fold). In cultured PBF, such MMP-1 expression was most markedly upregulated by the proinflammatory cytokines IL-1β and TNF-α (34-fold each, compared to D-ITS) and was also markedly upregulated by EGF and TGF-α (31-fold each) and by PDGF-BB (26-fold). A lesser increase was noted by bFGF (7-fold) and TGF-β1 (4-fold). For both HJF and PBF, IL-6 had no effect on MMP-1 expression.

The 1.9-kb MMP-3 transcript was not expressed by HJF in D-ITS and was only slightly upregulated by IL-1β and TGF-α but was not affected by other cytokines (Fig. 1, lane 3). In contrast,

the expression of MMP-3 transcript by PBF was most dramatically increased by IL-1β (12-fold) and to a lesser extent by TGF-α (11-fold), EGF (7-fold), TNF-α (5-fold), PDGF-BB (3-fold), and bFGF (2-fold).

The 3.1-kb MMP-2 transcript was uniformly expressed by both HJF and PBF without notable variation among different cytokine treatments (Fig. 1, lane 2). Likewise, there was no difference in the expression of the 0.9-kb TIMP-1 and the 3.5-kb TIMP-2 transcripts between HJF and PBF under these treatments (Fig. 1, lanes 4 and 5, respectively). The MMP-9 mRNA transcript was not expressed by either HJF or PBF (not shown).

Transcript Expression of uPA in HJF and PBF

The 2.3-kb transcript of uPA expressed by HJF was markedly upregulated by EGF (2.1-fold), TGF-α (2.6-fold), PDGF-BB (2.1-fold), and bFGF (3.2-fold) but was to a lesser extent by IL-1β (1.5-fold) and TGF-β1 (1.4-fold) (Fig. 2). A similar pattern was observed in PBF. The uPA transcript expression was upregulated by EGF (2.1-fold), TGF-α (2.2-fold), PDGF-BB (2.3-fold), and bFGF (2.5-fold) and to a lesser extent by IL-1β (1.3-fold). In contrast, uPA transcript expression was downregulated by TNF-α (0.5-fold) and by TGF-β1 (0.7-fold).
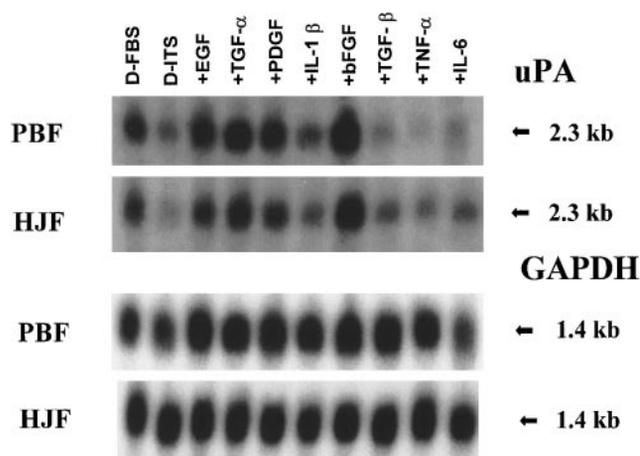
FIGURE 2. uPA mRNA expression by human conjunctival (HJF) and pterygium body fibroblasts (PBF) treated with different cytokines for 4 hours. HJF and PBF were grown to confluence in DMEM containing 10% fetal bovine serum (D-FBS) and switched to serum-free DMEM containing 1% ITS (D-ITS) for 48 hours, before treatment with 10 ng/ml of either EGF, TGF-α, PDGF-BB, IL-1β, bFGF, TGF-β1, TNF-α, or IL-6. Total RNA was isolated and individually hybridized with ^{32}P-labeled specific cDNA probes. The same filter was then hybridized with GAPDH probe as a control.

ELISA of MMP-1 and MMP-3 Proteins Secreted in Media of HJF and PBF

The protein levels of MMP-1 and MMP-3 were determined by their respective ELISAs in serum-free conditioned media of HJF and PBF after stimulation with 10 ng/ml of either TGF-α, IL-1β, TNF-α, or bFGF. The amount of MMP-1 in the conditioned medium of nonstimulated HJF (in D-ITS) was 10.9 ± 0.7 pg/μg protein and was significantly increased by addition of TGF-α (22.1 ± 3.3 pg/μg protein, $P = 0.005$), IL-1β (48.1 ± 10.7 pg/μg protein, $P = 0.004$), or bFGF (23.6 ± 6.3 pg/μg protein, $P = 0.026$) (Fig. 3A). However, in PBF, the level of MMP-1 was more dramatically increased by addition of IL-1β (229.8 ± 12.8 pg/μg protein or 7-fold increase, $P < 0.001$), TNF-α (158.6 ± 22.5 pg/μg protein, or 5-fold increase, $P = 0.001$), or bFGF (95.8 ± 7.7 pg/μg protein, or 3-fold increase, $P = 0.001$) compared with the baseline of D-ITS (30.8 ± 11.8 pg/μg protein) (Fig. 3B). When compared to the level in HJF, the upregulation by IL-1β, TNF-α, and bFGF in PBF was more notable.

A similar pattern was observed with the protein levels of MMP-3 in the conditioned media. In HJF, the only cytokine that markedly upregulated MMP-3 protein secretion was IL-1β (from 26.6 ± 8.4 pg/μg protein in D-ITS to 235.1 ± 93.7 pg/μg protein, or a 9-fold increase, $P = 0.06$) (Fig. 3C). However, MMP-3 in PBF was significantly upregulated by IL-1β (from
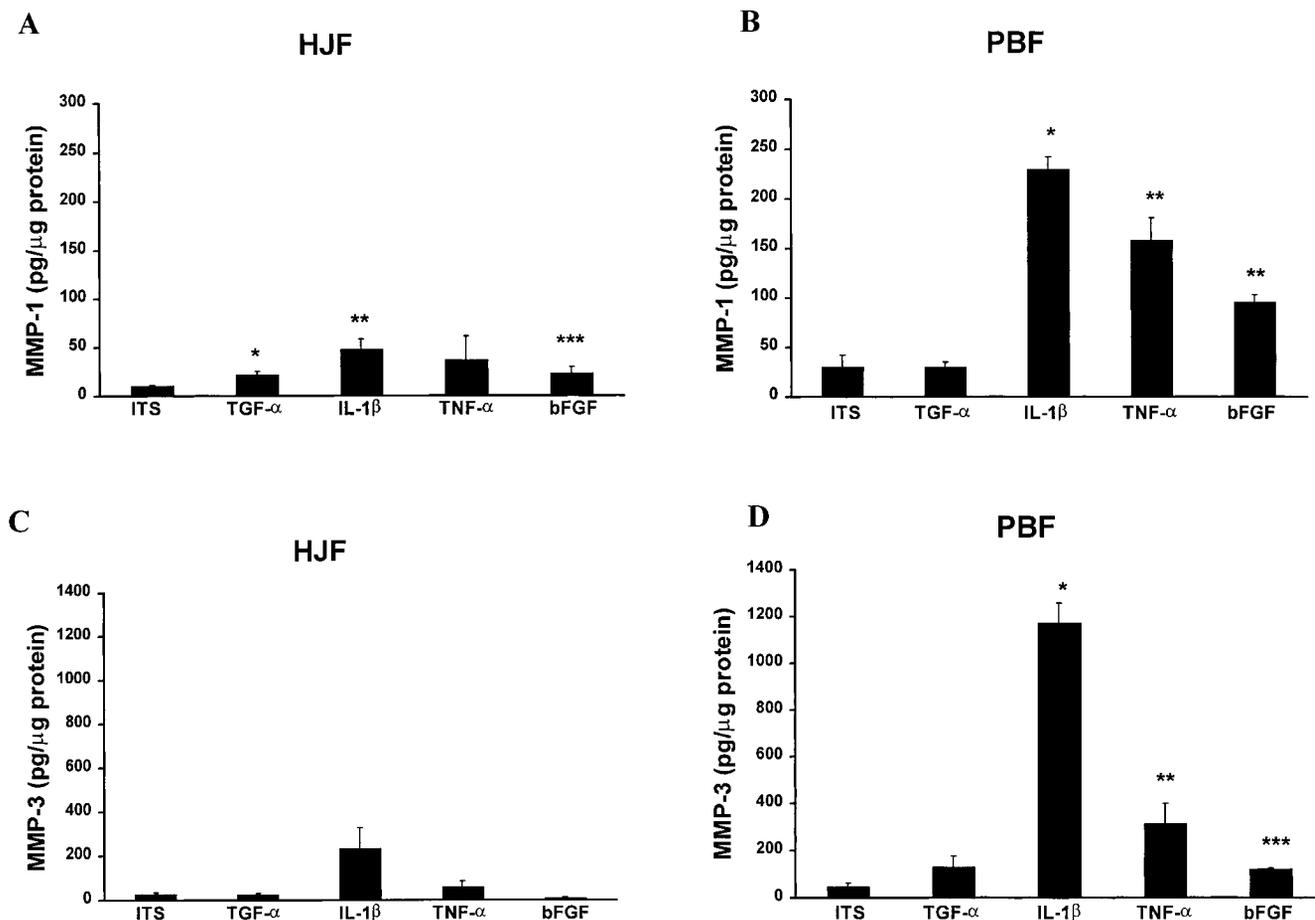

FIGURE 3. Levels of secreted MMP-1 (A, B) or MMP-3 (C, D) measured by their respective double-sandwiched ELISAs in conditioned media of HJF (A, C) or PBF (B, D) collected after 24 hours in serum-free DMEM containing 1% ITS (D-ITS), after stimulation with 10 ng/ml of either TGF-α, IL-1β, TNF-α, or bFGF. (A) *$P = 0.005$, **$P = 0.004$, ***$P = 0.026$. (B) *$P < 0.001$, **$P = 0.001$. (D) *$P = 0.002$, **$P = 0.027$, ***$P = 0.006$.
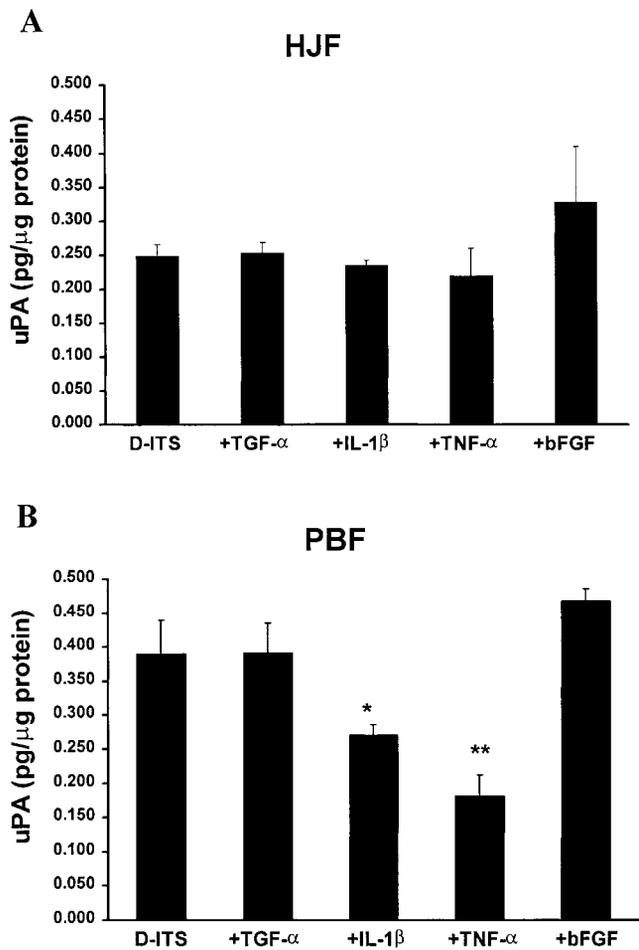
A

HJF

B

PBF

FIGURE 4. Levels of uPA measured by double-sandwiched ELISAs of PBF (**A**) or HJF (**B**) in cells lysates collected after 24 hours in serum-free DMEM containing 1% ITS (D-ITS), after stimulation with 10 ng/ml of either TGF-α, IL-1β, TNF-α, or bFGF. *$P = 0.018$, **$P = 0.004$.

49.3 $\pm$ 13.2 pg/μg protein in D-ITS to 1169.9 $\pm$ 86.3 ng/ml, or a 23-fold increase, $P = 0.002$), TNF-α (to 315.3 $\pm$ 84.6 pg/μg protein, or a 6-fold increase, $P = 0.027$), and bFGF (to 123.7 $\pm$ 3.1 pg/μg protein, or 2.5-fold increase, $P = 0.006$) (Fig. 3D). The protein levels measured by ELISA for both MMP-1 and -3 were well correlated with their relative amounts of transcripts expressed by both HJF and PBF under the treatment of these cytokines.

ELISA of uPA Protein in Cell Lysates of HJF and PBF

The uPA protein levels in cell lysates of HJF were not changed significantly by adding any of the cytokines (Fig. 4A). For PBF, however, a slight nonsignificant increase in uPA protein secretion was noted by adding bFGF (from 0.39 $\pm$ 0.05 pg/μg protein in D-ITS to 0.47 $\pm$ 0.02 pg/μg protein with bFGF, $P = 0.064$), whereas a significant downregulation was noted by TNF-α (to 0.18 $\pm$ 0.03 pg/μg protein, $P = 0.004$) and by IL-1β (to 0.27 $\pm$ 0.02 pg/μg protein, $P = 0.018$) (Fig. 4B). These data corresponded well with their respective mRNA data.

Protein Expression of MMPs and TIMPs by Western Blot Analysis

Western blot analysis was performed to identify and compare the protein expression of MMPs and TIMPs in serum-free conditioned media of HJF and PBF using their specific monoclonal antibodies, which recognize both latent and active forms. As shown in Figure 5, the intensity of the protein band of each MMP and TIMP expressed by these two fibroblasts was consistent with their mRNA expression and ELISA results. The 54-kDa MMP-1 band in PBF was in general more pronounced in cultures with D-ITS or D-ITS added with TGF-α, IL-1β, TNF-α, or bFGF than that of their corresponding cultures in HJF. Of note, the amount of MMP-1 protein expressed by PBF after stimulation with either IL-1β or TNF-α and to a lesser extent with bFGF was considerably higher than that of nonstimulated PBF (in D-ITS) or than that of corresponding cultures of HJF. A
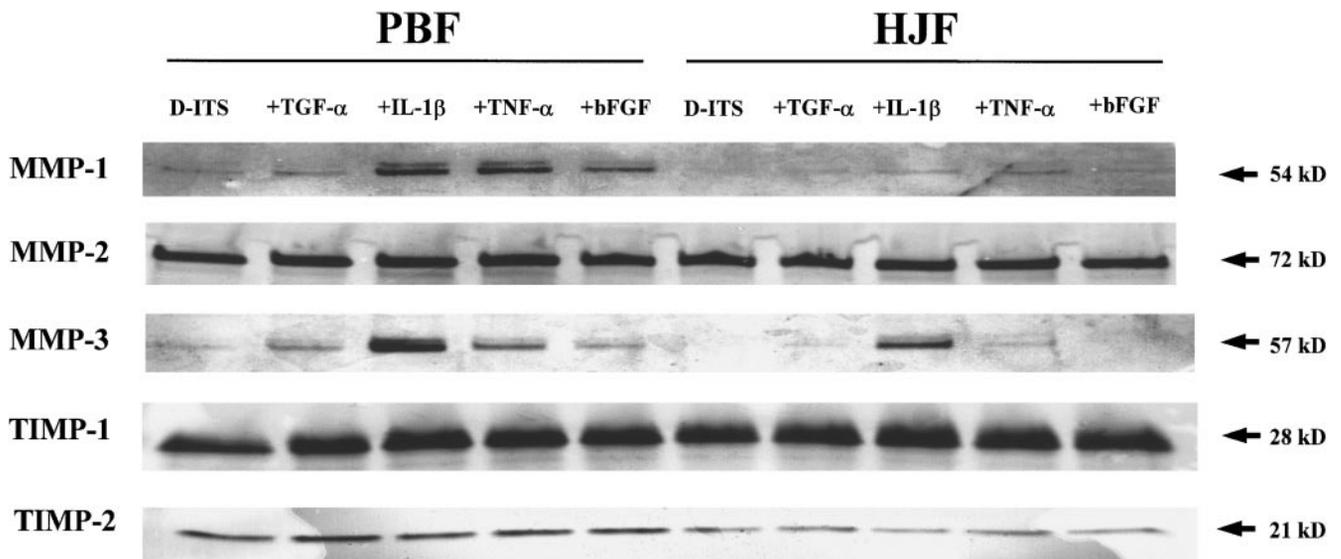

FIGURE 5. Western blot analysis of the protein expression of MMP-1, MMP-2, MMP-3, TIMP-1, and TIMP-2 expressed in the serum-free conditioned media of human conjunctival (HJF) and pterygium body (PBF) fibroblasts, collected after 24 hours in serum-free DMEM containing 1% ITS (D-ITS), after stimulation with 10 ng/ml of either TGF-α, IL-1β, TNF-α, or bFGF.

FIGURE 6.   Zymograms of MMP-2 and MMP-3 activity in HJF or PBF. The gelatinolytic activity of MMP-2 (*top two lanes*) and the caseinolytic activity of MMP-3 (*bottom two lanes*) in the serum-free conditioned media of human conjunctival (HJF) and pterygium body (PBF) fibroblasts, collected after 24 hours in serum-free DMEM containing 1% ITS (D-ITS), after stimulation with 10 ng/ml of either TGF-α, IL-1β, TNF-α, or bFGF.
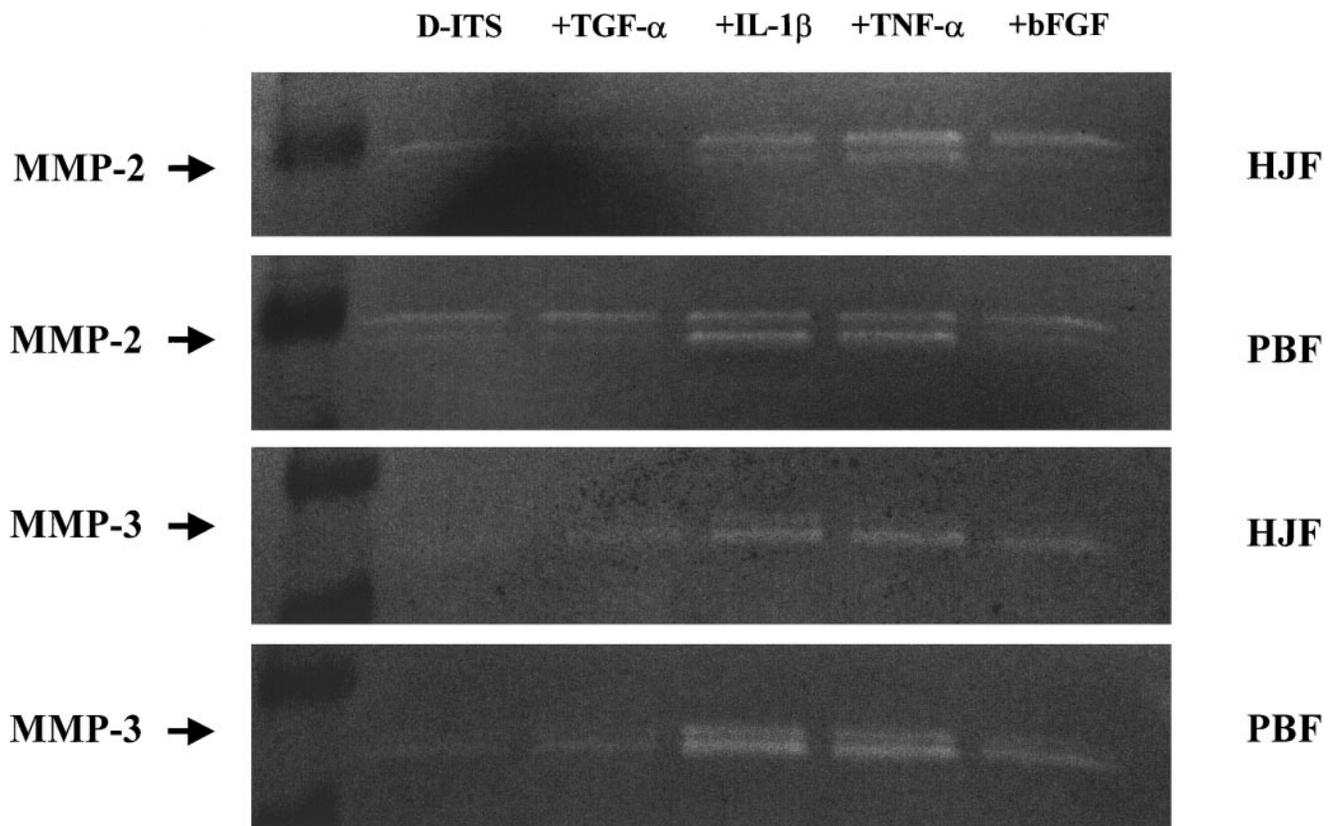
similar pattern was demonstrated for MMP-3. The 57-kDa band of MMP-3 expressed by PBF was more pronounced when stimulated by either IL-1β or TNF-α, and such levels were higher than their corresponding cultures of HJF. Similar to our observations for the respective mRNA transcripts in Northern blot analysis, the protein bands of 72-kDa MMP-2, 28-kDa TIMP-1, and 21-kDa TIMP-2 did not reveal any notable difference in both HJF and PBF under these treatments.

Zymography for Gelatinolytic Activity of MMP-2 and for Caseinolytic Activity of MMP-3

Zymography was performed on conditioned media of HJF or PBF grown for 24 hours in D-ITS alone or with addition of TGF-α, IL-1β, TNF-α, or bFGF, to verify the gelatinolytic and caseinolytic activities of MMP-2 and MMP-3, respectively. As shown in Figure 6 (top 2 lanes), the gelatinolytic activity of the 72-kDa band of MMP-2 was noted with both latent (predominantly) and active forms. This gelatinolytic activity was completely abolished by incubating the gel with solutions containing 10 mM EDTA (not shown). HJF produced the gelatinolytic activity of MMP-2, but PBF produced a higher gelatinolytic activity of the latent form of MMP-2 in all conditions and a higher active form under the treatment of IL-1β or TNF-α.

The casein zymogram also disclosed a strong caseinolytic activity of 57-kDa MMP-3 in these conditioned media (Fig. 6, bottom two lanes). The enhanced activity was demonstrated in HJF treated with IL-1β or TNF-α and to a lesser extent with

bFGF. A similar pattern was observed in PBF. The enhanced caseinolytic activity under the treatment of IL-1β or TNF-α in PBF also revealed two clear bands, corresponding to the glycosylated and unglycosylated forms of MMP-3, respectively.

DISCUSSION

The progressive nature of primary pterygium and its tendency to recur after inadequate removal lead us to speculate that pterygium fibroblasts play an important role in the pathogenesis of this lesion. This notion, first hinted by the documentation of a transformed phenotype of pterygium fibroblasts in tissue culture,[6] is now further supported by our finding that PBF may enhance their expression of such matrix degrading enzymes as collagenase (MMP-1), stromelysin (MMP-3), and uPA after stimulation of several cytokines and growth factors, which may derive from the ocular surface epithelia and potentially diffuse from the tear fluid. Because the majority of these modulators are products of inflammation, our data shed new insights in the pathogenic role of ocular inflammation in pterygium, allowing us to devise a new strategy of treating this lesion.

This study demonstrates that the expression of both MMP-1 and MMP-3 by PBF was preferentially promoted at both mRNA and protein levels after treatment with IL-1β and TNF-α. These two cytokines are potent proinflammatory mediators, which can be produced by macrophages and lymphocytes,

stimulate the proliferation of certain types of fibroblasts, and affect the production of MMPs by these cells. The expression of mRNA correlated well with that of the protein and the respective gelatinolytic and caseinolytic activities. Such levels were significantly higher than those expressed by HJF. These data resemble those previously reported for human synovial fibroblasts,[24] endometrial stromal cells,[25] and fibrochondrocytes.[26] Besides IL-1β and TNF-α, the two structurally related growth factors EGF and TGF-α also upregulated MMP-3 in PBF when compared to HJF in our study. EGF is a ubiquitous fibroblast mitogen that also stimulates the production of MMP-1 and MMP-3 in several types of fibroblasts.[27,28] Similarly, TGF-α upregulates MMP-1 and -3 in synergism with IL-1β but not alone.[29] Both EGF and TGF-α can be produced by ocular surface epithelial cells.[22,30,31]

The phenotype of MMP-1 and MMP-3 overexpression in fibroblasts is frequently associated with cellular proliferation. For example, TNF-α and IL-1β stimulate proliferation of cultured Tenon's capsule fibroblasts,[10] and TNF-α can stimulate the proliferation of other types of fibroblasts.[32,33] PDGF-BB and EGF induce rheumatoid fibroblasts proliferation while increasing production of MMP-1 and MMP-3.[34] IL-1 and PDGF-BB stimulate synoviocyte proliferation, whereas IL-1 stimulates collagenase transcription.[35] These data collectively show that a change in the regulation of the metalloproteinase genes may reflect a change in cellular proliferation. Further studies will be directed to correlating the higher expression of MMP-1 and MMP-3 by PBF than HJF with an intrinsic state of higher cellular proliferation. Furthermore, such upregulation of MMP-1 and MMP-3 was not accompanied by any change of expression in TIMP-1 and TIMP-2, which was constitutively expressed but not differentially affected by any of the cytokines and growth factors tested. This finding was also reported in rheumatoid synovial fibroblasts[24] and in endometrial fibroblasts.[28]

Besides the MMPs system, some cytokines and growth factors also modulated the uPA system. Expression of uPA is localized at discrete cell-matrix contact sites and at areas of cell–cell contacts in human fibroblasts.[36] Enhanced expression of uPA is correlated with local tumor proliferation.[37] Unlike the aforementioned inflammatory cytokines that preferentially upregulated MMP-1 and MMP-3, TNF-α and IL-1β were found to downregulate uPA expression at the mRNA and protein levels. On the contrary, bFGF was the only cytokine that upregulated uPA expression in HJF and PBF (Fig. 2). This finding resembles 3T3 fibroblasts in which the expression of uPA transcript is also induced by bFGF.[38] However, we did not note a significant difference in uPA expression under the influence of bFGF between HJF and PBF. Interestingly, UV irradiation enhanced the mRNA expression of uPA in human fetal fibroblasts.[39] Further studies are needed to determine whether the basal expression of uPA in nonstimulated PBF may result from chronic UV exposure.

Recently, our laboratory has discovered that pterygium head fibroblasts (cultured from the pterygium portion invading onto the cornea) intrinsically (i.e., without stimulation by any of these proinflammatory cytokines) overexpress MMP-1 and MMP-3 over TIMP-1 and TIMP-2.[40] Because enhanced expression of MMPs over TIMPs favors degradation of ECM, we thus speculate that this phenotype explains how progressive corneal invasion takes place in primary pterygium. Because the two proinflammatory cytokines IL-1β and TNF-α can render resting PBF a phenotype similar to that of pterygium head

fibroblasts, we believe that the progression of pterygium in the primary setting, or the recurrence after excision may be linked to ocular conditions that are associated with the production of these two cytokines.

The source of IL-1β and TNF-α can be the conjunctival epithelium,[8] stromal fibroblasts,[9] and inflammatory cells such as macrophages and lymphocytes.[41,42] When produced by these cells, these cytokines may appear in the tear fluid. Production of IL-1β and TNF-α is increased by surgical trauma,[43,44] lipopolysaccharide,[8] and UV irradiation.[9] Furthermore, when there is a decrease of tear clearance or turnover, IL-1β concentrations increase in the tear fluid.[45] Taken together, these data suggest that chronic inflammatory stimulation of the ocular surface, by such stimuli as UV irradiation, tear deficiency, and microtrauma from climatic or occupational environments, can lead PBF to overexpress MMPs over TIMPs.

The above findings have relevance to devising a new strategy of pterygium surgery. The pivotal concept is that if incompletely excised, residual PBF can be activated into proliferation and MMPs overexpression under inflammatory cytokines. Therefore, suppression or elimination of such ocular inflammation caused by any ocular surface insults should be desirable in managing pterygium. Hence, the ideal treatment of pterygium should include a combination of a thorough removal of the abnormal tissue, with an agent that reduces inflammation. Without being certain that all such tissues can be thoroughly removed, pterygium excision must be coupled with agents that reduce ocular inflammation to the minimum. One such strategy is the administration of intraoperative and postoperative injections of a long-acting corticosteroid to the margins of the excised tissue, to minimize inflammation and further activation of the residual fibroblasts. Subconjunctival corticosteroids injections have been used successfully in the treatment of corneal graft rejection,[46] and in nonnecrotizing anterior scleritis.[47,48] Cyclosporin A is another anti-inflammatory agent, previously shown to inhibit the expression of both IL-1[49] and TNF-α.[50] This drug has been administered topically for a variety of inflammatory disorders of the ocular surface,[51] or by a subconjunctival injection, as recently studied in a rabbit model.[52,53] Based on our results, a potent broad-spectrum anti-inflammatory treatment to the residual conjunctiva after pterygium excision may block the unwanted effects of ocular surface–derived cytokines on any remaining pterygium fibroblasts, thereby preventing further activation and proliferation of these cells.

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