Overexpression of Insulin-like Growth Factor–Binding Protein-2 in Pterygium Body Fibroblasts

Abrabam Solomon,1 Martin Grueterich,1 De-Quan Li,1 Daniel Meller,2 Sao-Bing Lee,3 and Scheffer C. G. Tseng1,4

PURPOSE. To examine the expression pattern of insulin-like growth factor–binding protein (IGFBP)-2 in cultured primary pterygium fibroblasts and compare it with expression in normal conjunctival fibroblasts.

METHODS. Profile of gene expression by normal conjunctival and primary pterygium fibroblasts was performed by using a cDNA microarray. The overexpression of IGFBP-2 thus identified was further confirmed by RT-PCR and Western blot analysis of cultured cells and by immunohistochemistry on primary pterygium and normal conjunctival tissue sections.

RESULTS. A dramatically increased expression of IGFBP-2 mRNA was demonstrated in cDNA microarray membranes from two different pterygium fibroblasts. This finding was confirmed by RT-PCR in four additional different pterygium fibroblasts and by Western blot analysis of their culture supernatants. Immunohistochemistry of frozen sections from primary pterygium demonstrated increased staining in extracellular matrix of the stroma, compared with that of the normal conjunctiva. IGFBP-2 was also found in goblet cells of both normal conjunctival and pterygium epithelia.

CONCLUSIONS. The increased expression of IGFBP-2 mRNA and protein in pterygium fibroblasts is further strong evidence to support the transformed phenotype of these cells and helps explain why there is increased growth of fibrovascular tissue. This phenotype may be used as a marker to assess the malignant nature of pterygium growth and recurrence. (Invest Ophthal Mol Vis Sci. 2003;44:573–580) DOI:10.1167/iovs.01-1185

Pterygium fibroblasts overexpress gene products associated with proliferation, inflammation, and tissue invasion. Several potent fibrogenic and angiogenic growth factors, such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF)-BB and transforming growth factor (TGF)-β1 have been found by immunohistochemistry in pterygium tissues.1 Recently, several groups have demonstrated the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in normal cultured subconjunctival fibroblasts,2 in the conjunctival epithelium overlying the pterygium,3 and in cultured pterygium fibroblasts.4,5 Pterygium head fibroblasts obtained from the portion invading the cornea were shown to overexpress MMP-1 and -3 over TIMP-1 and -2 in culture more so than pterygium body fibroblasts and normal conjunctival fibroblasts.6 This finding helps explain why pterygium head fibroblasts may possess the capability of corneal invasion. The expression of MMP-1 and -3 by pterygium body fibroblasts can be markedly promoted when challenged with proinflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α.7 This phenotype of MMP-1 and -3 overexpression in fibroblasts has been found to be associated with increased cellular proliferation6,7 and tissue invasion, especially in tumors.

After the demonstration of overexpression of MMPs in pterygium fibroblasts, we sought to explore further the fundamental alterations of pterygium fibroblasts that may lead to such an invasive and proliferative phenotype. In this study, a survey of various candidate genes, by using microarray gene profiling, has helped to identify one such important gene, insulin-like growth factor binding protein type 2 (IGFBP-2). Insulin-like growth factor (IGF) types I and II and their binding proteins are responsible for cellular proliferation in many tumors and diseases.8,9 IGFs, especially IGF-II, and their receptors have been shown to play a central role in promoting tumor growth, inhibiting apoptosis, and inducing transformation and metastasis in many types of malignancies, such as breast cancer, Wilms tumor, colorectal carcinoma, and others.10 Increasing evidence indicates that both IGF-I and -II, their receptors, and all six IGFBPs, particularly IGFBP-2, are highly expressed in various tumors of the central nervous system.11

The effects of IGF-I and -II on cells are regulated by IGFBPs, a family of at least six proteins that are synthesized locally by most tissues, including cancer cells, and are capable of inhibiting or enhancing the actions of IGF-I or -II.12,13 Specifically, IGFBP-2 is expressed in fetal tissues that are highly proliferative, and its expression significantly decreases after birth.14 Increased expression of IGFBP-2 in tissue and high serum levels have been noted in various malignancies, including prostate carcinoma,15 malignant ovarian tumors,16 colon tumors,17 adenocortical carcinoma,18 leukemia,19 and glioblastoma multiforme.20

In this study we have extended our investigation to demonstrate an increased expression of IGFBP-2 in pterygium fibroblasts. Clinical implications of our finding regarding the pathophysiology and clinical characteristics of pterygium will be further discussed.

METHODS

Materials

Dubecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), amphotericin B, and a random primer DNA labeling kit were purchased from Gibco-BRL (Grand Island, NY). Cell culture dishes, six-well plates, and 15-mL centrifuge tubes were from BD Biosciences (Lincoln Park, NJ). Four percent to 15% Tris-HCl polyacrylamide gradient ready gel, SDS, and electrophoresis equipment were from Bio-
Table 1. PCR Primer Sequences

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<td>Downstream (853–873)</td>
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<td>GAPDH</td>
<td>5′-GCCAAGGTCATCCATGACAC-3′</td>
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<td>Sense (541–561)</td>
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<tr>
<td>Antisense (1018–1058)</td>
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Rad (Hercules, CA). An avidin-biotin complex (ABC) peroxidase kit (Vectastain Elite) was purchased from Vector Laboratories (Burlingame, CA), nitrocellulose membranes from Schleicher and Schuell (Keene, NH); an RNA-PCR kit (GeneAmp) from Applied Biosystems (Foster City, CA), and a DNA purification kit (Wizard PCR Prep) from Promega (Madison, WI). [α-32P]-dCTP was from NEN Life Sciences (Boston, MA). All other reagents and chemicals were from Sigma (St. Louis, MO).

**Human Conjunctival and Primary Pterygium Fibroblast Cultures**

All procedures followed the tenets of the Declaration of Helsinki and were approved by the Human Medical Science Committee of the University of Miami School of Medicine. Normal conjunctival specimens were obtained from 2×2-mm biopsy specimens from the superotemporal bulbar conjunctiva of healthy patients who did not show signs and symptoms of an ocular surface disorder or dry eyes while undergoing cataract surgery. Pterygium specimens were obtained from patients with a grade T3 pterygium (pterygium with a thick fleshy body, according to the Tan classification) after the surgical removal of primary pterygium. All normal conjunctiva and pterygium specimens came from age-matched donors, whose ages ranged from 45 to 55 years. Six different pterygium fibroblasts cell lines and four different normal conjunctival fibroblasts cell lines were tested. These tissue samples were used for explant cultures to generate normal human conjunctival fibroblasts (HJFs) and pterygium body fibroblasts (PBFs), respectively, according to a method previously described. Cultures were maintained in a medium containing DMEM supplemented with 10% FBS, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B and changed every 2 or 3 days. Fibroblasts were subcultured with 0.05% trypsin and 0.85 mM EDTA in a calcium-free MEM at 80% to 90% confluence with a 1:3 to 1:4 split for three passages.

**cDNA Expression Array**

Total RNA was extracted from third-passage HJFs or PBFs by the acid guanidium thiocyanate-phenol-chloroform method, with some modifications, as previously reported. A human cDNA expression membrane (Atlas Array I, PT3140-1; Clontech Laboratories, Inc., Palo Alto, CA) containing 588 human genes was used to screen genes that might be overexpressed by pterygium fibroblasts. 32P-labeled cDNA probes were generated by reverse transcription of each analyzed poly(A)+ RNA sample in the presence of [α-32P]-dATP. Labeled cDNA probes were purified (NAPTM-5 Sephadex G-25 Column, Amersham Pharmacia Biotech, Piscataway, NJ). cDNA probes were then hybridized to the cDNA expression array. After a high-stringency wash, the membranes were exposed to an x-ray film, and the autoradiographs were scanned and analyzed with image-analysis software (Gel-Pro; Media Cybernetics, Silver Spring, MD) to generate a value representing the relative density of each gene spot. After subtracting the background density, the density of each gene was divided by the density of the GAPDH gene, a housekeeping gene that was used as a control. The resultant value represented the expression of that gene in pterygium fibroblasts or in normal conjunctival fibroblasts, respectively. For each gene, the relative expression in pterygium fibroblasts and in normal conjunctival fibroblasts was calculated by dividing the pterygium expression value by the normal conjunctival value for that same gene. Overexpression of a gene in pterygium fibroblasts compared with normal conjunctival fibroblasts was defined as a relative expression higher than 2.21

**Reverse Transcription—Polymerase Chain Reaction**

Total RNA extracted from HJF or PBF cultures by acid guanidium thiocyanate-phenol-chloroform extraction was treated with DNase I for 15 minutes. RT-PCR was performed on 1 mg of total RNA samples. The RT-PCR was performed in a 100-μL volume using 0.2 mM of each dNTP, 1 mM MgCl2, 2 μl of reverse transcriptase/Tag polymerase mixture, 0.5 mM oligo-(dT)16, 0.25 mM of 5′ primer, and 0.25 mM of 3′ primer. The primers used in the PCR analysis are shown in Table 1 (GenBank is available at http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). RNA was reverse transcribed into cDNA by one cycle of 42°C for 50 minutes and 1 cycle of 95°C for 5 minutes and 5°C for 5 minutes. The cDNA was amplified for 22 cycles, each cycle consisting of 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute (last cycle at 72°C for 7 minutes). After amplification, 15 μL of each of the PCR products and 2 μL loading buffer were electrophoresed on a 1.5% agarose gel in 1× Tris-acetate-EDTA (TAE) containing ethidium bromide. Gels were photographed and scanned.

**Western Blot Analysis**

Conditioned media from both HJF and PBF cultures were adjusted to a final volume of 25 μL to represent the same quantity of cellular proteins and electrophoresed in a 4% to 15% gradient polyacrylamide gel. After electrophoretic transfer to a nitrocellulose membrane, the membrane was immersed with 0.1% (vol/vol) Tween 20 in Tris-buffered saline (100 mM Tris, 0.9% NaCl [pH 7.5]; TBBS) for 30 minutes. The primary antibody, 1:1000 of rabbit monoclonal antibody against human IGFBP-2 or -3 (Research Diagnostics, Inc., Flanders, NJ; in TBBS containing 1% horse serum, was placed on each membrane and incubated at room temperature for 60 minutes. After washing with TBBS, each membrane was transferred to a 1:200 diluted solution of biotinylated secondary antibody (goat anti-rabbit IgG from the Vectastain Elite ABC kit, Vector Laboratories) in TBBS containing 1% horse serum and incubated for 30 minutes. After a wash, the membrane was incubated with 1:50 diluted ABC reagent conjugated with peroxidase for 30 minutes, and processed for color development in diaminobenzidine (DAB) for 10 to 20 minutes.

**Immunohistochemistry**

Tissue specimens were placed in the DMEM (Life Technologies, Gaithersburg, MD) for transport, embedded in optimal cutting temperature...
compound (Tissue Tek; Miles Laboratories, Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −80°C. Within 72 hours, serial 4- to 5-μm sections were cut. The ABC technique (Vectastain ABC Elite; Vector Laboratories) was performed with a rabbit anti-human polyclonal antibody against IGFBP-2 (Research Diagnostics, Inc.). After endogenous peroxidase and avidin-biotin binding were blocked, tissue sections were incubated with the anti-IGFBP-2 antibody at a dilution of 1:1000 for 1 hour at room temperature. After three washes in PBS (each for 5 minutes), the slides were incubated for 30 minutes at room temperature with a secondary antibody (a 1:200 diluted solution of biotinylated goat anti-rabbit IgG from the Vectastain Elite ABC kit). After washing in PBS (three times, each for 5 minutes), sections were incubated with 1:50 diluted avidin-biotin-HRP reagent (Vectastain Elite ABC) for 30 minutes and processed for color development in diaminobenzidine (DAB). To determine nonspecific staining, parallel sections were incubated without the primary antibody, as the negative control, with the remainder of the protocol performed as just described.

Statistical Analysis

The two-tailed Student’s t-test was used for analyzing the Western blot and RT-PCR results. The data are expressed as the mean ± SD and the differences considered significant at P < 0.05.

RESULTS

The cDNA microarray for RNAs extracted from two different PBF cultures demonstrated a dramatic increase in the expression of the IGFBP-2 gene compared with that of HJFs (Fig. 1). After adjusting for the value of GAPDH, a housekeeping gene used as the control, the value for IGFBP-2 was 126.4- and 6.2-fold higher in two different PBF cultures, respectively, compared with that of HJFs. These ratios were ranked at the 1st and the 13th places, respectively, among all 588 genes surveyed in this type of differential array assay (Table 2).

To further confirm this cDNA array result, we performed RT-PCR for IGFBP-2 and -3 mRNAs in three different HJF and four different PBF cell lines. The mean amounts of IGFBP-2 mRNA from four different PBF cell lines were 20.8-fold higher than those of three different HJF cell lines (Fig. 2). No difference was found in the amount of IGFBP-3 mRNA between HJF and PBF.

Western blot analysis for IGFBP-2 and IGFBP-3 in the culture supernatants of four different PBF cell lines and three different HJF lines demonstrated a marked increase of the protein amount of IGFBP-2 in PBFs compared with HJFs (Fig. 3). The mean amount of IGFBP-2 protein of four different PBF cell lines was 4.15-fold higher than that of three different HJF cell lines (P = 0.0003). There was no significant difference in the amount of IGFBP-3 protein between HJF and PBF.

Immunostaining to IGFBP-2 was performed in normal and pterygium specimens. Compared with the control without primary antibody (Fig. 4A), a strong positive staining for IGFBP-2 was found in the goblet cells (cf. Figs. 4A, 4B; open arrows), whereas mild positive staining was found in the extracellular matrix of the stroma of the normal conjunctival specimen (Fig. 4B). In contrast, much stronger staining to IGFBP-2 was noted in the extracellular matrix of the stroma in pterygium specimens (Fig. 4C, black arrow) as well as in the basal epithelium, when compared with the normal conjunctival specimen (cf. Figs. 4B, 4C).

DISCUSSION

Pterygium, triggered by exposure to ultraviolet irradiation, is a proliferative disease with fibrovascular ingrowth into the cornea. We have reported an increased expression of MMP-1 and -3 in pterygium fibroblasts cultured from the head portion of the lesion and demonstrated upregulation of both MMP-1 and -3 of cultured pterygium fibroblasts from the body in response to stimulation with proinflammatory cytokines. These data explain how pterygium may destroy the corneal basement membrane and progressively invade the corneal stroma.

Besides abnormalities found in the basal epithelial cells of the pterygium, including overexpression of MMP-1, -2, -9, and -7; p53; and vimentin,3,24−27 abnormalities of pterygium fibroblasts have also been recognized. Fibroblasts isolated from pterygium tissues exhibit a transformed phenotype.28 In contrast to normal conjunctival fibroblasts, pterygium 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 (i.e., a phenotype of transformed or neoplastic cells). Furthermore, there is microsatellite instability in pterygium, suggesting intrinsic genetic abnormalities in the pterygium tissue.29 Further exploration of the molecular basis of such genetic changes is important to the understanding of the pathogenesis of this lesion and its tendency to recur after surgical removal.

As a first step to explore the basis of the aforementioned genetic abnormalities, we adopted a cDNA microarray to si-
multaneously survey the mRNA expression of 588 genes with a single hybridization. Using this screening method, we found a marked overexpression of several genes associated with regulation of cellular growth and proliferation, including transcription factors ETR 101 and ETR 103, DNA-binding protein (NF-E1), cAMP-dependent protein kinase catalytic subunit

**FIGURE 2.** RT-PCR analysis of IGFBP-2 and IGFBP3 by three different HJF (lanes 1–3) and four different PBF (lanes 1–4) for IGFBP-2 was found to be 20.8-folds (mean) overexpressed in PBF compared with HJF (*P = 0.005), whereas IGFBP3 did not show any statistical difference.

**TABLE 2.** Overexpression Ratios of Genes from Two Pterygium Fibroblast Cell Lines Compared to Normal Conjunctival Fibroblasts

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<td>X92669</td>
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<td>M62831</td>
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<td>D38505</td>
<td>Tob</td>
<td>52.78</td>
<td>2.02</td>
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<td>X07767</td>
<td>cAMP-dependent protein kinase catalytic subunit type α</td>
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<td>M76541</td>
<td>DNA-binding protein (NF-E1)</td>
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<td>B-cell specific transcription factor (BSAP)</td>
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The IGFBP-2 gene was one of the most expressed genes in RNA samples isolated from cultured pterygium body fibroblasts. We further confirmed that primary pterygium fibroblasts indeed overexpressed IGFBP-2 at both the mRNA and protein levels in culture and in tissue sections.

The IGFBPs are a family of six binding proteins that serve as circulating carriers for IGF-I and -II. By carrying the IGFs to various tissue sites, the binding proteins serve as regulatory proteins. The localization of the IGFBPs varies in different tissues, and as a result endowing a unique mechanism for tissue-specific modulation of IGF actions. Recent data show that the IGF-IGFBP system plays a central role in the pathogenesis of transformation and tumorigenesis. Many studies had demonstrated an important role for IGFBP-2 in human malignancies. Increased levels of IGFBP-2 were demonstrated in sera of patients with ovarian cancer, colonic cancer, and prostatic cancer and in cerebrospinal fluid of patients with malignancies of the central nervous system. A positive correlation was found between IGFBP-2 expression and the tumor grading of human gliomas. Using the same cDNA microarray membrane as used in our study, high expression of IGFBP-2 was found in several cell lines of highly malignant glioblastoma multiforme.

In ocular tissues, expression of IGFBP-2 has been demonstrated in the normal retinal pigment epithelium and in vitreous samples from patients with ischemic diseases such as proliferative diabetic retinopathy. The expression of IGFBP-2 in the anterior segment was demonstrated in the nonpigmented ciliary epithelium, the corneal germinal epithelium, and the corneal endothelium in the adult rat, and in the cornea, lens epithelium, ciliary body, iris, and aqueous of the bovine eye. None of these studies have revealed localization of this gene in fibroblasts.

IGFBP-3 was previously demonstrated in corneal extracts of the bovine eye. The protein was previously reported to be in the 50- to 60-kDa range, whereas in our study it was demonstrated as a 29-kDa fragment, by Western blot analysis. A similar 29-kDa proteolytically modified fragment was previously demonstrated in normal skin interstitial fluid and synovial fluid from healthy adults. In patients with insulin-dependent diabetes mellitus, IGFBP-3 immunoblot analysis revealed the presence of an 18-kDa fragment of IGFBP-3 in addition to a major 29-kDa fragment and an intact form of approximately 39 to 42 kDa. Immunoblot studies have revealed a major 29-kDa IGFBP-3 fragment in addition to intact 41- and 38-kDa IGFBP-3 forms in serum of patients with chronic renal failure. The 29-kDa fragment found in our study may have been a product of

**Figure 3.** Western blot analysis of IGFBP-2 and -3 proteins in supernatants of HJFs and PBFs. The mean amount of IGFBP-2 protein of four different PBFs were 4.15-fold higher than that of three different HJFs (*P = 0.0005). The mean amount of IGFBP3 in HJFs and PBFs revealed no statistical difference.
degradation by various MMPs. MMPs, specifically MMP-1, -2, and -3 are known to proteolytically degrade IGFBPs and have been found to be upregulated in pterygium fibroblasts. It is therefore possible that some of these MMPs, expressed by pterygium fibroblasts, have produced the 29-kDa fragment of IGFBP-3 demonstrated in our study by proteolytic degradation.

The relations between the various IGFBPs, specifically IGFBP-2 and -3, are complex and not well understood. We have found that whereas IGFBP-2 was markedly upregulated in pterygium fibroblasts, no differences were found in the expression of IGFBP-3 between normal conjunctival and pterygium fibroblasts. Different expression patterns were reported for IGFBP-2 and -3 in various diseases: IGFBP-2 and -3 were both increased in the sera of patients with hyperthyroidism and in patients with acromegaly and colonic neoplasia. Inverse relations were found in sera of patients with ovarian cancer and in chronic inflammatory conditions such as Crohn disease, idiopathic pulmonary fibrosis, and pulmonary sarcoidosis and in chronic hepatitis. Nevertheless, none of these reports showed localization of this gene in fibroblasts.

Therefore, we conclude that our finding is the first showing the strong association of overexpressed IGFBP-2 in abnormal pterygium body fibroblasts (i.e., in a lesion that is characterized by chronic inflammation, genetic alteration, and cellular transformation). This finding, along with other data showing increased expression of several fibroangiogenic growth factors and matrix-degrading enzymes in pterygium fibroblasts, provides the basis to explore the molecular mechanism by which pterygium is developed and may help develop new strategies to prevent its recurrence after surgical excision.

Although the increased expression of IGFBP-2 may be a result of an altered phenotype, indicative of some very early premalignant transformation, it may also be a result of an increased inflammatory, fibrogenic, or angiogenic activity. Indeed, we noted positive IGFBP-2 staining in the epithelium overlaying the pterygium, which may be secondary to the overall increased proinflammatory state of the ocular surface in pterygium. Moreover, different members of the IGFBP family were found to be expressed in several chronic inflammatory and fibrotic conditions such as Crohn disease, idiopathic pulmonary fibrosis, and pulmonary sarcoidosis and in chronic hepatitis. Nevertheless, none of these reports showed localization of this gene in fibroblasts.

**References**


49. Long L, Navab R, Brodt P. Regulation of the Mr 72,000 type IV collagenase by the type I insulin-like growth factor receptor. *Cancer Res.* 1998;58:3243–3247.


