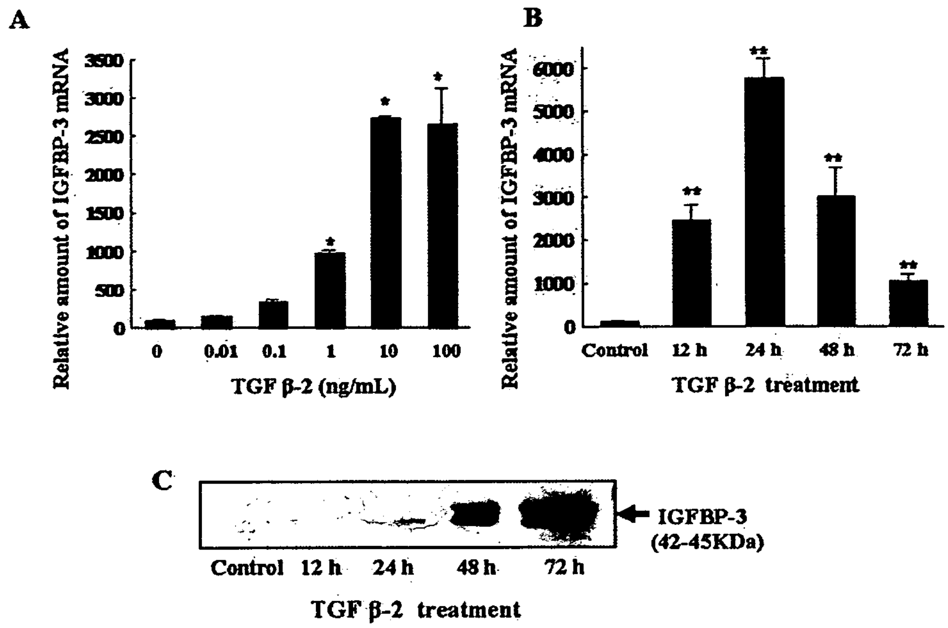


**Figure 1.** TGF-$\beta$2 stimulation of IGFBP-3 production by corneal fibroblasts. (A) IGFBP-3 mRNA expression in the presence of various concentrations of TGF-$\beta$2. Corneal fibroblasts were incubated for 24 hours in serum-free medium in the absence or presence of TGF-$\beta$2 at concentrations ranging from 0.01 to 100 ng/mL. The relative amount of IGFBP-3 mRNA compared with that in the untreated control was determined by real-time quantitative PCR. *Significantly different ($P < 0.05$) from the serum-free control. (B) Time course of IGFBP-3 mRNA expression in corneal fibroblasts after treatment with 1 ng/mL TGF-$\beta$2. The relative amount of IGFBP-3 mRNA compared with the untreated control was determined by real-time quantitative PCR. **Significantly different ($P < 0.05$) from 0-hour control. (C) Western blot of conditioned medium, performed to determine the time course of IGFBP-3 secretion from corneal fibroblasts. Media from untreated cells or cells treated for 12, 24, 48, or 72 hours with 1 ng/mL TGF-$\beta$2 were concentrated and subjected to immunoblot analysis with an antibody against IGFBP-3. Data are representative of results in three independent experiments.

ries, West Grove, PA) for 1 hour at room temperature. The membrane then was rinsed thoroughly with TBS-0.1% Tween. Bound antibody was detected with a chemiluminescence detection kit (Super Signal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology, Rockford, IL) and an imager (Lumi Imager; Roche Diagnostics, Mannheim, Germany).

## Assays of DNA Synthesis

The effect of TGF-$\beta$2, IGF-I, and IGFBP-3 on corneal fibroblast proliferation was assessed by BrdU incorporation using a BrdU enzyme-linked immunosorbent assay (ELISA; Cell Proliferation ELISA, BrdU colorimetric; Roche Diagnostics) according to the instructions of the manufacturer. Corneal fibroblasts were cultured in 96-well plates ($2.8 \times 10^3$ per well) for 24 hours in DMEM containing 0.1% bovine serum albumin (BSA), after which the culture medium was further supplemented with growth factors. Control cultures were incubated in the absence of growth factor. To study the effect of exogenous TGF-$\beta$2, IGF-I, and IGFBP-3 on DNA synthesis, we incubated corneal fibroblasts for 24 hours in the presence of TGF-$\beta$2 (0.01–10 ng/mL), IGF-I (50 ng/mL), IGFBP-3 (50–1000 ng/mL), and immunoneutralizing antibody against IGFBP-3 (10 $\mu$g/mL; R&D Systems). Cells were pulse labeled for 24 hours with 100 $\mu$M BrdU. All assays were performed in triplicate or quadruplicate and were replicated in at least two separate experiments.

## Statistical Analysis

Data are presented as the mean ± SD and were analyzed by one-way analysis of variance (ANOVA). Post hoc comparisons between groups used the Fisher protected least significant difference test. $P < 0.05$ was accepted as indicating statistical significance. All experiments in this study were repeated at least three times, in the same conditions.

# Results

## Induction of IGFBP-3 Expression by TGF-$\beta$2 in Human Corneal Fibroblasts

We began the present study by asking whether TGF-$\beta$2-treated human corneal fibroblasts express IGFBP-3 at the mRNA and protein levels, using real-time quantitative PCR and Western blot analysis. Figure 1A shows that treatment with TGF-$\beta$2 significantly stimulated the expression of endogenous IGFBP-3 in a TGF-$\beta$2 dose-dependent manner (Fig. 1B). As for time, 1 ng/mL TGF-$\beta$2 increased IGFBP-3 protein production within 24 hours of addition (Fig. 1C), after 72 hours, the degree of stimulation was much greater.

## Effect of IGFBP-3 on DNA Synthesis by Corneal Fibroblasts

We next asked how IGFBP-3 affects corneal fibroblast DNA synthesis. Addition of IGFBP-3 to corneal fibroblasts significantly inhibited basal DNA synthesis in a dose-dependent man-

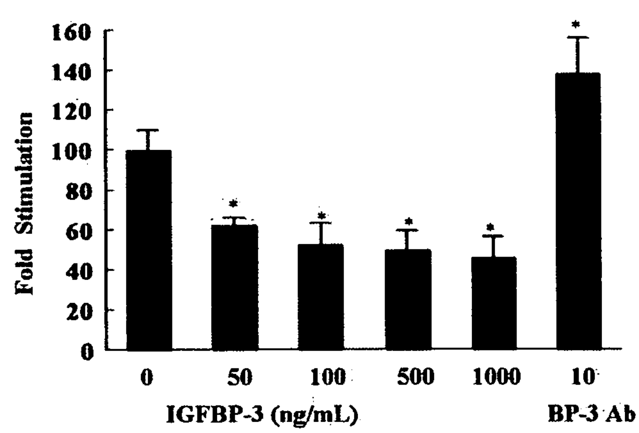


**Figure 2.** Effect of IGFBP-3 on DNA synthesis in human corneal fibroblasts. Cells were cultured for 24 hours in DMEM containing 0.1% BSA in the absence or presence of IGFBP-3 at concentrations ranging from 50 to 1000 ng/mL, or in the presence of 10 $\mu$g/mL IGFBP-3 neutralization antibody (BP-3 Ab). Cells were pulse-labeled with BrdU for 24 hours. Data represent the mean ± SD of results in three experiments, in which determinations were performed in triplicate. *Significantly different ($P < 0.05$) from the serum-free control.

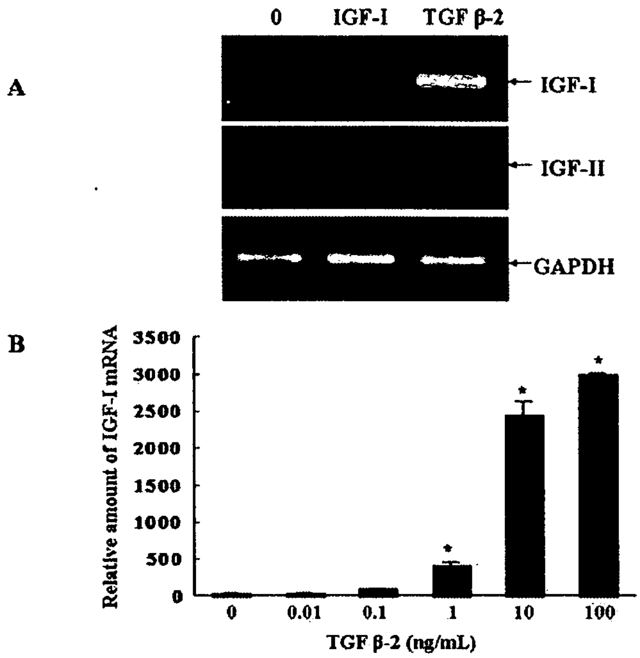


**Figure 3.** IGF-I and IGF-II mRNA expression in response to TGF-$\beta$2 (1 ng/mL) and IGF-I (50 ng/mL). (**A**) Ethidium bromide-stained agarose gels show PCR products for IGF-I and -II amplified from reverse-transcribed RNA isolated from human corneal fibroblasts treated with TGF-$\beta$2 or IGF-I for 24 hours. Note the induction of IGF-I, but not IGF-II, by TGF-$\beta$2. This experiment was replicated three times. (**B**) IGF-I mRNA expression in response to various concentrations of TGF-$\beta$2. The relative amount of IGF-I mRNA compared with that in the untreated control was determined by real-time quantitative PCR. *Significantly different ($P < 0.05$) from the serum-free control.

ner (Fig. 2), whereas basal DNA synthesis in corneal fibroblasts was increased in the presence of the IGFBP-3-neutralizing antibody. The result implied that endogenous IGFBP-3 directly inhibits corneal fibroblast proliferation.

## Induction of IGF mRNA Expression by TGF-$\beta$2 in Human Corneal Fibroblasts

Next, we investigated the effect of TGF-$\beta$2 on expression of other components of the IGF axis. RT-PCR was used to determine whether mRNA expression of IGFs is altered by TGF-$\beta$2. Treatment with TGF-$\beta$2 stimulated expression of endogenous IGF-I mRNA but not that of IGF-II mRNA (Fig. 3A). As shown in Figure 3B, TGF-$\beta$2 induced IGF-I expression in a dose-dependent manner.

## Effect of TGF-$\beta$2 and IGF-I on Type I Collagen and $\alpha$-SMA mRNA Expression

We next assessed the effect of IGF-I and TGF-$\beta$2 on type I collagen and $\alpha$-smooth muscle actin expression. As shown in Figure 4, in cells treated for 48 hours, IGF-I and TGF-$\beta$2 induced a similar increase in type I collagen mRNA. Furthermore, corneal fibroblasts treated with TGF-$\beta$2 and anti IGF-I neutralizing antibody also induced significant upregulation of COLIA1 mRNA compared with the untreated control. Treatment with TGF-$\beta$2 and anti-IGF-I neutralizing antibody significantly suppressed this expression compared with TGF-$\beta$2 treatment. In contrast, although TGF-$\beta$2 treatment resulted in a fivefold increase in $\alpha$-SMA mRNA expression, no $\alpha$-SMA mRNA increase was detected in response to IGF-I.

## Effect of TGF-$\beta$2 and IGF-I on DNA Synthesis by Corneal Fibroblasts

To determine whether addition of TGF-$\beta$2 or IGF-I to corneal fibroblasts affects DNA synthesis, we used a BrdU incorporation assay. As shown in Figure 5, incubation with IGF-I for 24 hours significantly stimulated DNA synthesis in cultured corneal fibroblasts compared with the untreated control. Whereas a low concentration of TGF-$\beta$2 enhanced corneal fibroblast DNA synthesis, a high concentration inhibited it.

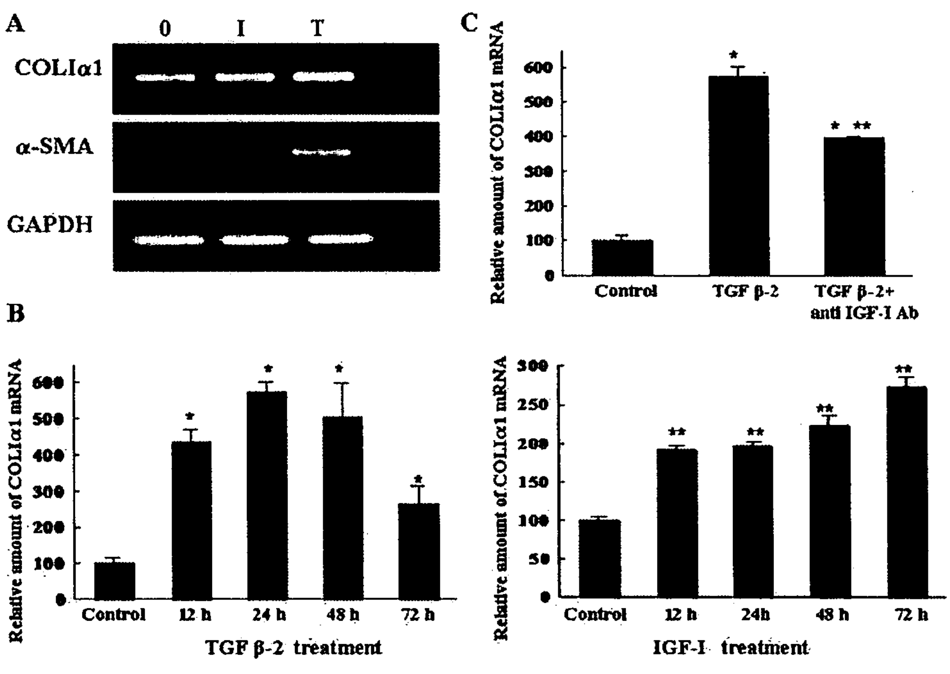


**Figure 4.** Procollagen-I$\alpha$1 and $\alpha$-SMA mRNA expression in response to TGF-$\beta$2 and IGF-I. (**A**) Ethidium bromide-stained agarose gels show PCR products for procollagen-I$\alpha$1 and $\alpha$-SMA amplified from reverse-transcribed RNA isolated from human corneal fibroblasts after treatment with TGF-$\beta$2 (T) or IGF-I (I) for 24 hours. (**B**) Time course of procollagen-I$\alpha$1 mRNA expression in corneal fibroblasts after treatment with TGF-$\beta$2 or IGF-I. The relative amount of procollagen-I$\alpha$1 mRNA compared with that in the untreated control was determined by real-time quantitative PCR. *Significantly different ($P < 0.05$) from 0-hour control. **Significantly different ($P < 0.05$) from 0-hour control. (**C**) COLIA1 mRNA expression in response to TGF-$\beta$2 or TGF-$\beta$2+anti-IGF-I neutralizing antibody for 24 hours. The relative amount of COLIA1 mRNA compared with that of the untreated control was determined by real-time quantitative PCR. *Significantly different ($P < 0.05$) from the serum-free control. **Significantly different ($P < 0.05$) from TGF-$\beta$2 treatment groups.

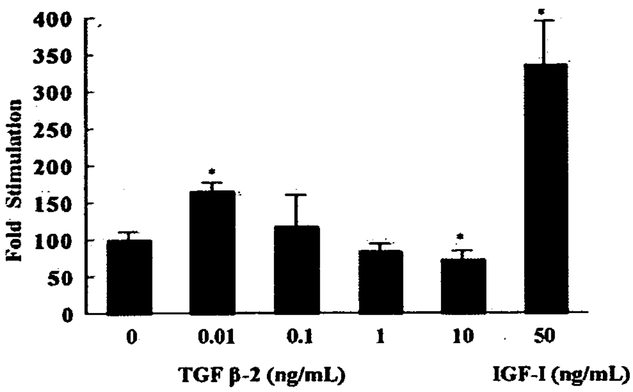


**Figure 5.** Effects of TGF-$\beta$2 and IGF-I on DNA synthesis in human corneal fibroblasts. Cells were cultured for 24 hours in DMEM containing 0.1% BSA in the absence or presence of TGF-$\beta$2 at concentrations ranging from 0.01 to 10 ng/mL or 50 ng/mL IGF-I. Cells were pulse labeled with BrdU for 24 hours. Data represent the mean ± SD of results in three different experiments performed in triplicate. *Significantly different ($P < 0.05$) from the serum-free control.

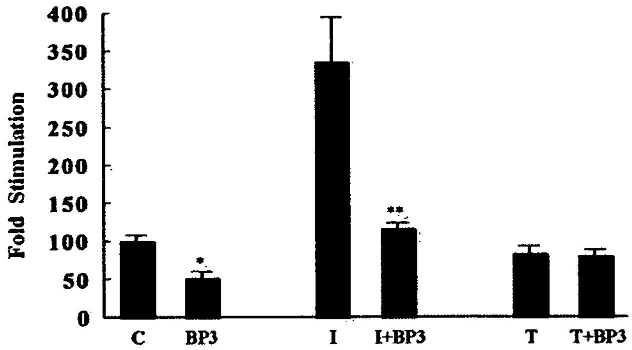


**Figure 7.** IGFBP-3 modulated basal and IGF-I-stimulated DNA synthesis. Human corneal fibroblasts were preincubated with 1000 ng/mL IGFBP-3 (BP3) for 24 hours before addition of medium supplemented with BrdU and 50 ng/mL IGF-I (I) or 1 ng/mL TGF-$\beta$2 (T). Data represent the mean ± SD of results in three different experiments performed in triplicate. *Significantly different ($P < 0.05$) from the serum-free control; **Significantly different ($P < 0.05$) from IGF-I-treated cells. C, serum-free control.

## Effect of IGF-I on Proliferation and Characteristics of Corneal Myofibroblasts Induced by TGF-$\beta$2

We next asked whether TGF-$\beta$2 and IGF-I act sequentially in regulating the activated myofibroblast phenotype or cell proliferation. Cells were pretreated with TGF-$\beta$2, followed by substitution of medium containing IGF-I but not TGF-$\beta$2. Cells pretreated with TGF-$\beta$2 for 7 days showed a significant increase in DNA synthesis when subsequently exposed to IGF-I instead (Fig. 6A). To determine whether IGF-I affects the activated phenotype induced by TGF-$\beta$2, cells pretreated with TGF-$\beta$2 for 7 days were exposed to serum-free medium, without or with IGF-I, for 3 days and then immunostained for $\alpha$-smooth muscle actin. A large percentage of corneal fibroblasts that expressed $\alpha$-smooth muscle actin after 7 days of TGF-$\beta$2 treatment maintained the expression of $\alpha$-SMA after exposure for 3 days to serum-free medium or IGF-I. The activated phenotype therefore was not reversed spontaneously or by IGF-I over this time frame (Fig. 6B).

## Modulation of Basal and IGF-I-Stimulated DNA Synthesis by IGFBP-3

Interaction between IGFBP-3 and IGF-I or TGF-$\beta$2 then was considered in terms of DNA synthesis by corneal fibroblasts. When added to medium together with IGF-I, IGFBP-3 significantly inhibited IGF-I-stimulated DNA synthesis. However, IGFBP-3 did not affect the inhibitory effect of 1 ng/mL TGF-$\beta$2 on basal DNA synthesis (Fig. 7) or TGF-$\beta$2-stimulated expression of $\alpha$-SMA and type I collagen (data not shown).

## Effect of PRK on IGFBP-3 mRNA and Protein Levels in Mouse Cornea

To investigate the involvement of IGFBP-3 in corneal wound healing in vivo, we studied IGFBP-3 expression in mouse cor-

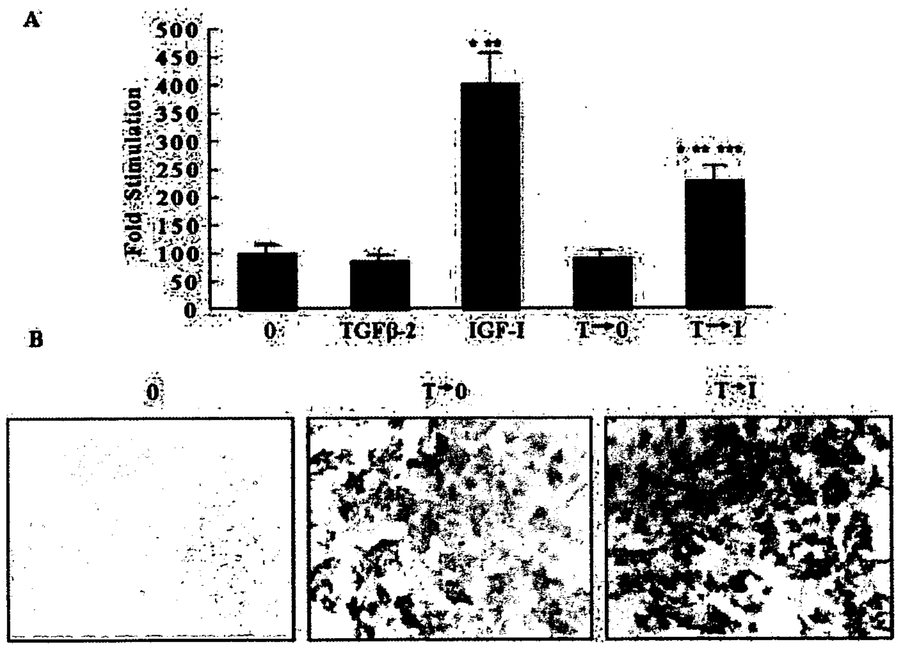


**Figure 6.** Effects of sequential treatment with TGF-$\beta$2 and IGF-I. (A) DNA synthesis in response to IGF-I (50 ng/mL) in human corneal fibroblasts pretreated with TGF-$\beta$2 (1 ng/mL). After pretreatment for 7 days, TGF-$\beta$2 was removed, and serum-free medium supplemented with only BrdU (T→0) or BrdU+IGF-I (T→I) was added for 24 hours. Data represent the means ± SD of results in three experiments performed in triplicate. *Significantly different ($P < 0.05$) from the serum-free control. **Significantly different ($P < 0.05$) from TGF-$\beta$2 treatment groups. ***Significantly different ($P < 0.05$) from the T→0 groups. (B) Immunostaining of human corneal fibroblasts with $\alpha$-SMA-specific antibody after pretreatment with TGF-$\beta$2 for 7 days, followed by removal of TGF-$\beta$2 and subsequent treatment with serum-free medium (T→0) or IGF-I (T→I) for another 3 days. 0, nonstimulated, negative control. Magnification, ×40.

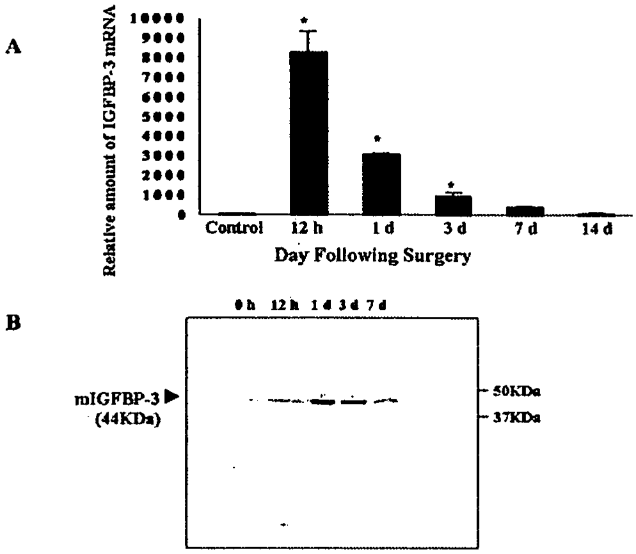


**Figure 8.** Expression of IGFBP-3 in mouse corneas after PRK. (**A**) The relative amount of IGFBP-3 mRNA compared with control corneas without surgery was determined by real-time quantitative PCR. Six mouse corneas were analyzed for this RNA at 0 or 12 hours, 1 day or 3, 7, or 14 days after PRK. *Significantly different ($P < 0.05$) from the untreated control. (**B**) Western blot analysis of IGFBP-3 expression in mouse corneas after PRK. Six mouse corneas were analyzed for this protein at 0 or 12 hours or 1 day or 3 or 7 days after PRK.

neas after PRK. As shown in Figure 8, amounts of IGFBP-3 mRNA and protein significantly increased in mouse corneas after PRK. In particular, IGFBP-3 mRNA dramatically increased in the cornea at 12 hours after PRK, compared with control corneas without PRK. IGFBP-3 mRNA then progressively decreased with time. IGFBP-3 protein increased significantly at days 1 and 3 compared with the untreated control corneas, declining to normal by day 7.

## IGFBP-3 Localization in Mouse Cornea after PRK

Localization of IGFBP-3 during mouse corneal wound healing after PRK was examined by immunostaining. As shown in Figure 9, IGFBP-3 was immunolocalized in paraffin-embedded sections of mouse corneas harvested at the same time points after surgery at which protein and RNA were measured. IGFBP-3 was detected in only slight amounts in the stromal matrix of normal corneas before PRK (day 0). On day 1 after surgical injury, intense staining was present in the deep layer of the corneal stroma. By day 7, staining in the deep stromal layer was reduced, in agreement with the results of Western blot analysis.

## Discussion

IGFBP-3, the major serum transport protein for IGFs, also is active in the cellular environment, where it acts as a potent antiproliferative agent.[22,23,28] We found that in human corneal fibroblasts, TGF-$\beta$ induced expression of IGFBP-3 mRNA and protein, whereas IGFBP-3 inhibited DNA synthesis in corneal fibroblasts. In addition to its effect on IGFBP-3, TGF-$\beta$ induced IGF-1 mRNA expression. IGF-I promoted proliferation of myofibroblasts without reversing the activated phenotype. We conclude that during corneal wound healing, IGF axis components are likely to regulate corneal mesenchymal overgrowth and suppress corneal stromal wound contraction.

TGF-$\beta$ is a well-established mediator of wound healing and fibrosis in several organs.[36] In the cornea, it potently activates keratocytes to a myofibroblast phenotype expressing $\alpha$-SMA and also induces expression of type I collagen.[3–5,9] Previous reports of potent upregulation of IGFBP-3 by TGF-$\beta$ in subconfluent fibroblasts[34,35] were confirmed in our corneal cells in subconfluent, serum-free culture. In accord with evidence that IGFBP-3 plays a role in antiproliferation,[23,28] IGFBP-3 appeared to suppress proliferation of myofibroblasts induced by TGF-$\beta$.

Potentiation and inhibition of IGF action by IGFBP-3 have been demonstrated in many cell culture systems.[17,18,22] It is thought that cotreatment of cells with IGFBP-3 and IGF-I causes

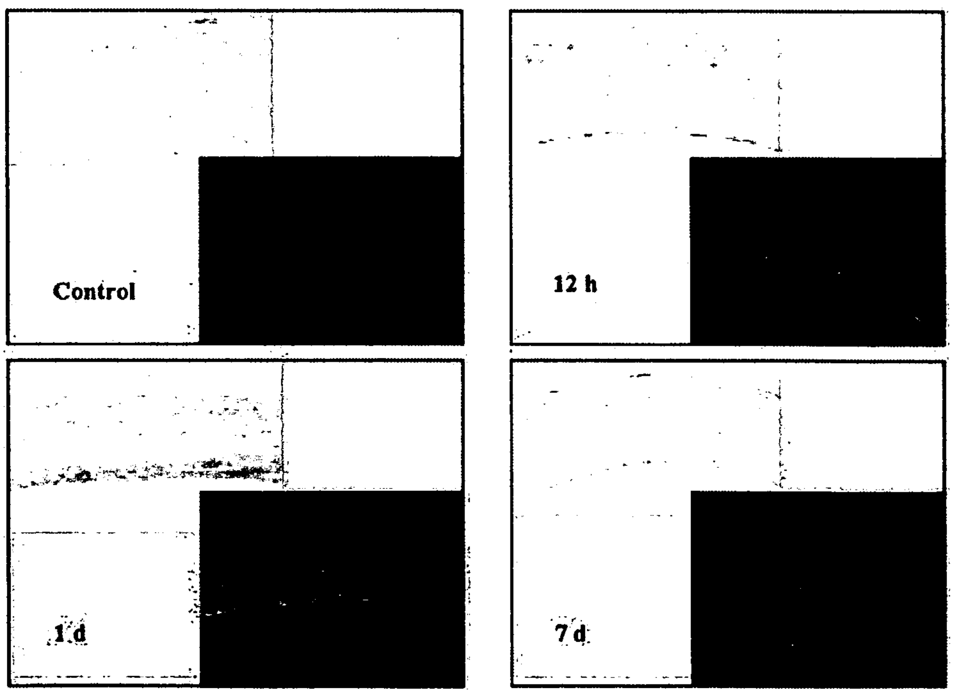


**Figure 9.** Immunolocalization of IGFBP-3 in mouse corneas at 0 or 12 hours or 1 day or 7 days in mouse corneas after PRK. IGFBP-3 was localized using an alkaline phosphatase visualization substrate. Images were obtained from the same field by fluorescence microscopy. Mouse corneas were subjected to immunofluorescent staining with antibodies to IGFBP-3 (*green*) and the nuclear marker propidium iodine (*red*). Magnification, ×200.

IGFBP-3 to inhibit IGF-I-mediated effects via high-affinity sequestration of the ligand, presumably leading to prevention of IGF-I-induced IGF-RI autophosphorylation and signaling.[22,23] In cornea, epithelial cells and fibroblasts express IGF-I, IGF-II, and IGF-IR.[37] IGF-I is suggested to play a critical role in the maintenance of the keratocyte phenotype[38] and has been shown to be mitogenic for human corneal fibroblasts[39] and protective against apoptosis.[40] IGF-I also has been shown to be chemotactic for human corneal fibroblasts,[41] and to enhance epidermal growth factor stimulated collagen gel contraction.[42] The effects of TGF-$\beta$ on IGF-I and IGFBP-3 mRNA observed in our present experiments have important implications for regulation of corneal mesenchymal overgrowth during corneal wound healing. Our new observations that TGF-$\beta$2 induces expression of IGF-I in corneal fibroblasts and that IGF-I stimulates growth of corneal fibroblasts activated to a myofibroblast phenotype by TGF-$\beta$2 suggest that such regulation may take place during corneal stromal wound healing. The myofibroblast phenotype was not reversed by IGF-I. Furthermore, our study supports a role for IGF-I, together with TGF-$\beta$2, as an upregulator of extracellular matrix (ECM) synthesis during corneal stromal wound healing. IGF-I, then, is critical not only to maintenance of the keratocyte phenotype in intact cornea, but to regulation of myofibroblast behavior in injured cornea. These aspects of IGF-I activity in corneal wound healing currently are being studied in corneal cell culture.

Our findings that TGF-$\beta$ induced upregulation of IGFBP-3 mRNA by 12 hours after TGF-$\beta$ treatment and that immunoneutralization of endogenous IGFBP-3 increased basal DNA synthesis in corneal fibroblasts suggest possible IGF-independent effects of IGFBP-3. In several carcinoma cell lines and in some normal cells, IGFBP-3 regulates cell growth independent of IGF-I.[23,43] Two mechanisms for this effect have been identified[23]: The first involves the interaction of IGFBP-3 with TGF-$\beta$ receptors and TGF-$\beta$-dependent signaling mechanisms[23]; the second involves the interaction of IGFBP-3 with nuclear retinoid X receptor-$\alpha$ (RXR-$\alpha$).[44,45] Furthermore, recent studies have shown that endogenous IGFBP-3 directly inhibits proliferation of human intestinal smooth muscle cells by activation of TGF-$\beta$RI and Smad2.[46] Although this IGFBP-3-dependent inhibition of growth is mediated via TGF-$\beta$ receptors, these effects are independent of endogenous TGF-$\beta$ because immunoneutralization of endogenous TGF-$\beta$ does not diminish IGFBP-3-dependent Smad2 activation or IGFBP-3-dependent inhibition of [$^3$H] thymidine incorporation.[46] Therefore, one may postulate that IGFBP-3 also inhibits corneal fibroblast growth directly, helping to prevent excessive proliferation of fibroblasts before their differentiation to the activated phenotype in the wound cornea.

The ability of IGFBP-3 to bind other molecules has been demonstrated previously.[47-49] Recent studies have shown that plasminogen binds IGFBP-3 and the binary IGF-I/IGFBP-3 complex with high affinity by interacting directly with the IGFBP-3 heparin-binding domain.[47] In vitro studies have shown that hypertrophic scar fibroblasts produce elevated levels of IGFBP-3 and type I$\alpha$ collagen and that TNF-$\alpha$ treatment reduces IGFBP-3 and collagen expression in a dose-dependent fashion.[49] A recent report indicated that physiologic effects of IGFBP-3-collagen interaction may include modulation of cell adhesion and migration because they characterized type I$\alpha$ collagen as one of the IGFBP-3 binding proteins.[50] In our present in vivo experiments, strong IGFBP-3 immunoreactivity was found in the extracellular matrix of mouse corneal stroma at an early time point during wound healing after PRK. This IGFBP-3 may bind to the collagen matrix and contribute to regulation of corneal stromal wound healing.

After refractive surgery, a corneal subepithelial haze develops in some patients as a wound healing response.[1,2] This reaction has been reported to be associated with increased myofibroblast transformation.[3-6] When it follows PRK, the corneal haze develops in the subepithelial lesion,[1,2] not in the deep stromal layer. We found immunostaining for IGFBP-3 at an early time point after PRK to be much stronger in the deep stromal than subepithelially. IGFBP-3, then, may act to suppress formation of haze by inhibiting the proliferation of corneal myofibroblasts. Pathologic fibrosis and myofibroblast formation induced by TGF-$\beta$ within the eye represents a significant pathophysiologic problem and may lead, not only to a subepithelial corneal haze, but to various other adverse effects, such as posterior capsular opacification,[51] anterior subcapsular cataract,[52,53] and trabeculectomy bleb failure.[54] As yet, no report has characterized the activity of IGFBP-3 in these conditions. Our study may expand the possibilities for preventing these adverse effects, because IGFBP-3 has hidden potential to become a key factor in various fibroses and wound contraction.

We present evidence of the induction of IGFBP-3 by TGF-$\beta$ treatment of corneal fibroblasts. We found that the combined actions of TGF-$\beta$ and IGF-I would stimulate collagen synthesis in healing, whereas proliferation would be limited by IGFBP-3 induced by TGF-$\beta$. Persistent expression of IGF-I in cells exposed to TGF-$\beta$ would permit proliferation of myofibroblasts, resulting in fibrosis. It is noteworthy that the effect of TGF-$\beta$2 to induce both IGF-I and IGFBP-3 indicates that, if such an effect occurs in vivo, the spatial and temporal distribution of IGF-I and IGFBP-3 may have major effects on the degree to which fibrogenic populations of myofibroblasts are expanded. The current report demonstrates that IGFBP-3 induced by TGF-$\beta$ may be critical in the suppression of mesenchymal overgrowth after corneal injury.

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難治性疾患克服研究事業

# 黄斑変性カニクイザルを用いた補体活性抑制剤による加齢黄斑変性の予防・治療法の確立と情報収集システムの開発

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