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Loss of Alpha3(IV) Collagen Expression Associated with Corneal Keratocyte Activation

Emily Guerriero¹, Jian Chen¹, Yoshikazu Sado², Rajiv R. Mohan³, Steven E. Wilson³, James L. Funderburgh¹, and Nirmala SundarRaj¹

¹UPMC Eye Center, Ophthalmology and Visual Science Research Center, Eye and Ear Institute, Department of Ophthalmology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

²Division of Immunology, Shigei Medical Research Institute, Okayama, Japan

³Cole Eye Institute, Cleveland Clinic Foundation, Cleveland, Ohio

Abstract

PURPOSE—To determine whether changes in the expression of type IV $\alpha 1$, $\alpha 2$, or $\alpha 3$ collagen isoforms are stringently associated with corneal stromal cell activation.

METHODS—Keratocytes isolated from rabbit corneal stroma by collagenase digestion were plated in serum-free or insulin-, bFGF/heparin sulfate (HS)-, TGF- $\beta 1$ -, or fetal bovine serum (FBS)-supplemented DMEM/F12 medium. Expression of type IV collagen isoforms and keratan sulfate proteoglycans (KSPGs) was evaluated by immunocytochemical analysis, Western blot analysis, or both. Concentrations of mRNAs were estimated by quantitative RT-PCR using SYBR Green RT-PCR reagents.

RESULTS—Immunohistochemical analysis indicated that type IV $\alpha 1$, $\alpha 2$, and $\alpha 3$ collagens were expressed in normal rabbit corneal stroma and in keratocytes cultured in serum-free and insulin-supplemented media. However, $\alpha 3$ (IV) collagen was not detectable in the regenerating stroma after photorefractive keratectomy (PRK) in rabbit or in corneal stromal cells cultured in media supplemented with FBS, bFGF/HS, or TGF- $\beta 1$. $\alpha 3$ (IV) collagen mRNA levels were also diminished in the stromal cells cultured in these growth factor-supplemented media. KSPGs (lumican and keratocan) were expressed and secreted in serum-free medium. Although the expression of KSPGs was promoted by insulin, the expression and intracellular levels of lumican and keratocan mRNAs were downregulated by TGF- $\beta 1$ and FBS. bFGF/HS promoted the downregulation of intracellular keratocan but not lumican mRNA levels.

CONCLUSIONS—The loss in the expression of $\alpha 3$ (IV) collagen is a stringent phenotypic change associated with activation of keratocytes in vivo and in vitro. This phenotypic change in activated corneal stromal cells is induced by bFGF/HS and by TGF- $\beta 1$, and it accompanies the downregulation of keratocan expression.

Corneal stromal cells (keratocytes) are responsible for the development and maintenance of the unique extra-cellular matrix of the transparent corneal stroma, which constitutes approximately 90% of the adult cornea. Although keratocytes are relatively quiescent in the

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Corresponding author: Nirmala SundarRaj, 1030 Eye and Ear Institute, 203 Lothrop Street, Pittsburgh, PA 15213; E-mail: sundarrajn@upmc.edu.

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normal adult cornea, they become activated to the fibroblast or myofibroblast phenotype after injury to the corneal stroma. Tissue culture models have been useful tools for studying keratocyte activation in vitro.^{1,2} When keratocytes isolated from the adult cornea are cultured in media without serum, they retain several features exhibited by the quiescent keratocytes in vivo.^{1,3–8} However, when cultured in media with serum, growth factors, or cytokines, including TGF- β 1, platelet-derived growth factor (PDGF), bFGF, insulinlike growth factor (IGF)-1, and IL-1 α , they attain phenotypic characteristics exhibited by activated stromal cells in vivo. Changes in the characteristics of keratocytes on activation in vivo and in vitro include assembly of actin stress fibers, de novo expression of extracellular matrix proteins such as type III collagen,^{9–12} tenascin,^{13–15} and matrix metalloproteinases^{16–21} and significant decreases in the expression of keratan sulfate proteoglycans (KSPGs),^{1,3,22–25} prostaglandin D synthase,²⁶ CD34,²⁷ and corneal crystallins, namely, aldehyde dehydrogenase and α -transketolase.²⁸

Although some phenotypic changes in activated stromal cells are transient, others may be permanent. When using in vitro tissue culture models for studying mechanisms of keratocyte activation, it is important to verify that the differential phenotypic characteristics exhibited by quiescent and activated keratocytes in vivo are recapitulated in vitro and to identify the extracellular factors that influence specific phenotypic changes in activated cells. It has become evident that macromolecules, which were once thought to be restricted to basement membranes (BMs) such as type IV collagen, laminin, and perlecan, are also expressed in connective tissues including corneal stroma.^{25,29} Six genetically distinct chains of type IV collagen— α 1(IV)- α 6(IV)—have been identified and shown to have different cellular interactions and tissue distributions.^{30,31} Immunohistochemical analysis has indicated the presence of α 3(IV), α 4(IV), and α 5(IV) collagens in normal human corneal stroma, epithelial BM, and Descemet membrane.³² The expression of different type IV collagen isoforms is altered in regenerated stroma after radial keratotomy.¹⁰ Interestingly, α 1(IV) and α 2(IV) collagens, not detectable in the normal human corneal stroma or epithelial basement membrane, were expressed in regenerated corneal tissues after laser-assisted in situ keratomileusis (LASIK) and radial keratotomy. α 3(IV) collagen was absent in regenerated BM. In the present study, we evaluated the expression of α 1(IV) to α 3(IV) collagen isoforms in the normal and regenerated rabbit stroma after photorefractive keratectomy (PRK). Differences were observed between expression patterns of type IV collagen isoforms in rabbit corneas and the reported findings in human corneas. An in vitro tissue culture model of corneal keratocyte activation was used to determine whether the phenotypic changes observed in vivo were recapitulated in vitro on the differentiation of keratocytes to fibroblasts or myofibroblasts.

MATERIALS AND METHODS

Cell Culture

Keratocytes were isolated and cultured according to a modification of the procedure of Jester et al.⁶ Corneas were excised from whole rabbit eyes (Pel Freez Biological, Rogers, AR) and cut into halves, the endothelium and Descemet membrane were removed under a dissecting microscope, and the epithelium and a thin layer of underlying stroma were removed with a scalpel. Stromal pieces, free of endothelium and epithelium, were then digested in 0.25% collagenase (Sigma-Aldrich Inc., St. Louis, MO) at 37°C for 16 to 18 hours. After centrifugation at 1200 rpm for 7 minutes, the pellets containing keratocytes were resuspended in DMEM/F12 with 0.021% L-glutamine (glutaMAX; Invitrogen/Gibco, Carlsbad, CA), 0.011% pyruvate, and penicillin/streptomycin and were filtered through a cell strainer (70 μ m; Becton-Dickinson, Falcon, Bedford, MA). Keratocytes were plated in 60-mm dishes (Falcon Primaria; Becton Dickinson, Lincoln Park, NY) in serum-free DMEM/F12. The medium in the dishes was changed after 24 hours to serum-free DMEM/F12 or to DMEM/F12

supplemented with 10% FBS, 10 $\mu\text{g}/\text{mL}$ insulin, 2.0 ng/mL TGF- β 1, or bFGF (20 ng/mL)/HS (5 $\mu\text{g}/\text{mL}$).

PRK and Tissue Preparation

Animal studies were performed in accordance with The National Institute of Health Office of Laboratory Animal Welfare (OLAW) guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Partial-thickness stromal ablations (-9 D correction identical with that used in the PRK eyes) were performed as described previously.³³ Rabbits were humanely killed, and corneoscleral rims (operated eyes and unoperated control eyes) were removed, frozen, and embedded in OCT compound (Tissue-Tek; Pelco International, Redding, CA). Central corneal sections (7- μm thick) were cut using a cryostat (HM 505M; Micron GmbH, Walldorf, Germany). Sections were placed on microscope slides (Superfrost plus; Fisher Scientific, Pittsburgh, PA) and were maintained frozen at -85°C until staining was performed.

Immunostaining

For immunocytochemical analyses, the cells were cultured on glass coverslips. After cells were rinsed with phosphate-buffered saline (PBS), they were fixed with paraformaldehyde-lysine-periodate and permeabilized with PIPES buffer containing 0.2% Triton X-100, as described previously.³⁴ Fixed and permeabilized cells or tissue sections on microscope slides were reacted with 10% heat-inactivated goat serum in PBS, pH 7.5, for 45 minutes to block the nonspecific binding of the secondary antibody, rinsed with PBS, and then treated with the primary and secondary antibodies as described previously.³⁴ Primary antibodies included monoclonal rat anti-human α 1(IV), α 2(IV), and α 3(IV) collagen (H11, H21, and H31, respectively),³⁵ monoclonal mouse anti-keratan sulfate (KS) antibodies (J19 or J36),³⁶ and mouse anti- α -smooth muscle actin (SMA; Sigma-Aldrich Inc.). Secondary antibodies were Alexa 488-conjugated or Alexa 546-conjugated goat anti-rat, anti-rabbit, or anti-mouse IgG (Molecular Probes Inc./Invitrogen, Eugene, OR) at 1:2500 concentrations. For double staining of actin filaments, Texas Red-X phalloidin (Molecular Probes) was included at 1:500 dilutions with the secondary antibody. Coverslips with stained cells were then mounted (Immunomount; Shandon, Pittsburgh, PA). Fluorescent Z-stack images were collected at 0.25- μm intervals using a confocal scanning laser system (Radiance 200; Bio-Rad, Hercules, CA) attached to an inverted microscope (IX70; Olympus, Tokyo, Japan) using the same acquisition parameters. The immunofluorescent images presented in Figure 1 to Figure 3 were projected from Z-stacks and then processed (Adobe Photoshop 7.0).

Western Blot Analyses

Keratocytes or fibroblasts grown under different culture conditions were extracted in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, pH, 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.03 TIU/mL aprotinin [Sigma-Aldrich], 1 mM sodium orthovanadate, and 100 $\mu\text{g}/\text{mL}$ phenylmethylsulfonyl fluoride [PMSF]) according to the protocol recommended by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). Aliquots of cell extracts containing 50 or 100 μg protein were subjected to SDS-PAGE. For Western blot analysis of type IV collagen, the cells were extracted in 4 \times SDS sample buffer (100 $\mu\text{L}/10^6$ cells, as estimated from microscopic analyses of several fields on the dish). Cell extracts in 4 \times sample buffer³⁷ were diluted twofold with distilled water and subjected to SDS-PAGE. For the analyses of secreted keratan sulfate, the volume of culture supernatants was adjusted with PBS to a final volume of 5 $\text{mL}/10^6$ cells. Between 50 μL and 80 μL culture supernatants were used per lane for Western blot analyses. Protein bands on SDS-PAGE were electrophoretically transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA), stained with 0.1% Coomassie

blue 250 in 40% methanol/1% acetic acid for 1 minute, destained one to three times with 50% methanol/1% acetic acid, scanned for densitometric comparisons of relative concentrations of proteins per lane, destained with methanol, and subjected to Western blot analysis. Immunoreactive bands were detected using chemiluminescence reagents (ECL reagent; Amersham) according to the manufacturer's protocols.

Quantitative RT-PCR

Cells were collected by scraping into cold working buffer (Buffer RLT; Qiagen, Valencia, CA), and RNA was isolated with an RNeasy Mini kit (Qiagen). Quantitative RT-PCR for the mRNAs of $\alpha 3(\text{IV})$ collagen, ALDH1, lumican, and keratocan was performed with reagents (SYBR Green RT-PCR; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reactions were performed (ABI 7700 Detection System; Applied Biosystems) for 45 cycles of 15 seconds each at 95°C and 60 seconds each at 60°C after initial incubation for 10 minutes at 95°C. The reaction mixture³⁸ contained 1× PCR buffer (SYBR Green), 3.0 mM MgCl₂, 100 μM each dATP, dCTP, dGTP, 0.05 U/μL polymerase (AmpliTaq Gold; Roche Molecular Systems, Indianapolis, IN), 1.0 μL cDNA, and forward and reverse primers (Table 1) at optimized concentrations. Gene-specific C_t values were standardized based on 18S C_t values obtained for each cDNA. Each experiment included triplicate sets, and their mean values were used to calculate the ratios of mRNA levels in the cells cultured under specific conditions and cells cultured in serum-free medium. Values shown represent the mean ± SD values of the analyses of the RNA samples (run in triplicate) from three separate experiments. After amplification, a dissociation curve for each reaction was generated to confirm the absence of nonspecific amplification. Gel electrophoresis confirmed the amplified product to be of the expected size. Primers used for these experiments are given in Table 1. Because the sequence of rabbit keratocan had not been reported, its partial sequence was determined by amplifying a short region of rabbit cDNA encoding keratocan by PCR (GenBank accession number DQ239829). Primers for generating the PCR product were designed from the conserved sequences of this gene in other animal species.

RESULTS

Distribution of $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, and $\alpha 3(\text{IV})$ Collagen in Normal and Post-PRK Rabbit Cornea

Immunohistochemical analysis of cryostat sections of normal rabbit cornea indicated that the distribution patterns of $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, and $\alpha 3(\text{IV})$ collagen in the normal cornea were similar though differences in the staining intensities of individual isoforms in the stroma were noted. All three isoforms were present in corneal epithelial BM (Fig. 1A), stromal cells (Fig. 1D), and stromal matrix (Fig. 1G), distributed linearly along the collagen lamellae and Descemet membrane (not shown). Based on the intensities of immunostaining, $\alpha 3(\text{IV})$ collagen levels were lower in the anterior one third of the stroma (Fig. 1G) than in the posterior region (Fig. 1G'). To evaluate the expression of type IV collagen isoforms in the activated stromal cells during wound healing, rabbit corneas removed at 4 weeks after -9 D PRK surgery were analyzed. These corneas have been shown to contain activated stromal cells.³³ Activated stromal cells expressing α -SMA (myofibroblasts) were detected in sub-epithelial regions of regenerated stroma (Figs. 1C, 1F, 1I; arrows in each). These α -SMA-positive cells also reacted with anti- $\alpha 1(\text{IV})$ and anti- $\alpha 2(\text{IV})$ collagen antibodies (Figs. 1Bm 1E; arrows in each). However, they did not react with anti- $\alpha 3(\text{IV})$ collagen antibody (Fig. 1H, arrows). Staining of $\alpha 3(\text{IV})$ collagen was either absent or discontinuous in the BM of the epithelium covering the regenerated stroma. In some subepithelial regions of regenerated stroma, the cells did not stain for α -SMA or $\alpha 3(\text{IV})$ collagen (not shown) suggesting that the activated cells in these regions were fibroblasts.

Morphologic Evaluation of Corneal Stromal Cell Activation In Vitro

Keratocytes isolated from rabbit corneas, when cultured in serum-free DMEM/F12, exhibited a dendritic morphology similar to that in vivo and contained only cortical actin filaments (Figs. 2A, 2F). These cells did not proliferate, as evidenced by the lack of change in cell number (Fig. 3) and the absence of Ki67-positive cells (not shown) after 3 days in culture. As expected, activation with FBS resulted in the transdifferentiation of keratocytes to highly proliferative spindle-shaped cells (fibroblasts) evidenced by more than a 10-fold increase in cell number within 3 days (Figs. 2B, 2G, Fig 3). Similarly, when keratocytes were cultured with bFGF/HS-supplemented DMEM/ F12, they differentiated to proliferative elongated spindle-shaped cells (fibroblasts) containing actin stress fibers (Figs. 2D, 2I, Fig. 3). bFGF/HS induced a 1.5-fold increase in cell number within 3 days. TGF- β 1 induced stromal cells to differentiate to elongated larger cells containing a robust network of actin filament bundles (Figs. 2E, 2J) containing α -SMA (not shown). TGF- β 1 induced limited cell division in 3 days with only a 1.2-fold increase in cell number (Fig. 3). In insulin-supplemented medium, the stromal cells attained spread morphology (Figs. 2C, 2H) but did not undergo cell division (Fig. 3).

Modulation of Type IV Collagen Isoform and KSPG Expression in Corneal Stromal Cells

Immunocytochemical analysis of the expression of α 1(IV), α 2(IV), and α 3(IV) collagen chains by corneal stromal cells cultured under the conditions described indicated that α 1(IV) and α 2(IV) collagens were synthesized by corneal keratocytes cultured in the medium without FBS or with insulin, FBS, bFGF/HS, or TGF- β 1 (Fig. 4). However, though α 3(IV) collagen was expressed in keratocytes in the serum-free and insulin-supplemented media (Figs. 4C, 4F), it was not expressed in keratocytes cultured in FBS-, bFGF/HS-, or TGF- β 1-supplemented media (Figs. 4I, 4L, 4O). α 3(IV) collagen (approximately 170-kDa band) was detected by Western blot analysis in the extracts of the keratocytes cultured in serum-free medium and insulin-supplemented media, but it was not detectable in the fibroblasts cultured in medium supplemented with FBS, bFGF/HS, or TGF- β 1 (Fig. 5).

Regulation of α 3(IV) Collagen mRNA Levels by Serum and Growth Factors

Real-time RT-PCR analysis was performed to determine whether the relative levels of mRNAs encoding α 3(IV) collagen were also altered in stromal cells activated with FBS or growth factors. These analyses indicated that the levels of α 3(IV) mRNA in the keratocytes cultured in DMEM/F12 supplemented with insulin were $90\% \pm 40\%$ higher than in serum-free medium. However, in keratocytes cultured in FBS, TGF- β 1, or bFGF/HS, α 3(IV) mRNA levels were reduced to $2.5\% \pm 1.5\%$, $14\% \pm 6\%$, and $12\% \pm 8\%$, respectively, of those in the serum-free medium (Fig. 6).

Effects of Culture Conditions on KSPG Expression in Corneal Stromal Cells

KSPGs were synthesized and secreted by the cells. Therefore, to evaluate the effects of the culture conditions on KSPG expression in rabbit keratocytes, culture supernatants were analyzed by Western blotting to detect secreted KSPGs. KS bands were present in the culture supernatants of serum-free medium but were not detectable in the supernatants of fibroblasts cultured in DMEM/F12 with FBS or with bFGF/HS and were significantly reduced in medium with TGF β -1 (Fig. 7). Stronger anti-KS reactive bands were present in the culture supernatants of keratocytes in medium supplemented with insulin. The identity of these bands as KSPGs was confirmed by their sensitivity to keratinase digestion (Fig. 7). To characterize the protein cores of KSPGs secreted by keratocytes, KSPGs were immunoprecipitated from culture supernatants, digested with keratinase, and analyzed by Western blotting. The KS-free protein cores thus derived from KSPGs in serum-free or insulin-supplemented medium consisted of lumican and keratocan.

Regulation of Intracellular Keratocan and Lumican mRNA Levels by Serum and Growth Factors

Lumican mRNA levels in fibroblasts cultured for 7 days in medium supplemented with FBS, TGF- β 1, or bFGF/HS were 12% \pm 8%, 33% \pm 13%, and 90% \pm 30%, respectively, of those in keratocytes cultured in the serum-free medium (Fig. 8). mRNA levels of keratocan in keratocytes cultured in medium with FBS, TGF- β 1, or bFGF/HS were altered to 13% \pm 7%, 8% \pm 2%, and 22% \pm 8% respectively, of those in serum-free medium. Insulin increased keratocan mRNA levels by 40% \pm 5% but did not significantly affect lumican mRNA levels.

DISCUSSION

Stromal keratocytes, normally quiescent in the healthy adult cornea, are activated during wound healing in response to various cytokines and growth factors. In vitro tissue culture models are useful to study the mechanisms of this stromal cell activation. Corneal keratocytes cultured in serum-free media or media with low concentrations of platelet-free horse serum retain their dendritic morphology and the ability to express high levels of corneal crystallins (ALDH1 and ALDH3), α -transketolase, and KSPGs such as keratocan and lumican.^{1,2,6,28} However, crystallin expression is significantly reduced when the stromal cells are cultured in media with FBS.^{6,22} A loss in KSPG expression has been reported in bovine keratocytes cultured with serum.²² It is unknown whether this phenotypic change in activated corneal stroma cells is specific to bovine cells. Previously reported methods for the culture of rabbit corneal keratocytes⁶ allowed us to determine whether the phenotypic changes in activated stromal cells reported in other animal species also occur in rabbit and whether the changes in the expression of α 1(IV) to α 3(IV) collagen isoforms during wound healing in vivo are recapitulated in vitro. Growth factors, including bFGF/HS and TGF- β 1, can induce keratocyte activation to fibroblast and myofibroblast phenotypes, respectively. We analyzed whether changes in the expression of α 1(IV) to α 3(IV) collagens are among the changes associated with the activation of keratocytes and whether these changes occurred concomitantly with any change in keratocan and lumican expression that have been observed in activated bovine keratocytes.^{1,4,39}

Differential expression of type IV collagen isoforms in human corneas is significantly altered in vivo in scar tissues after radial keratotomy.¹⁰ Given that the rabbit is a widely used animal model of corneal wound healing, it is important to determine whether the extracellular matrix modulations during human corneal healing process resemble those in the rabbit corneas. Normal adult human corneal epithelial BM and stroma have been shown to contain α 3(IV), α 4(IV), and α 5(IV) collagen isoforms but not α 1(IV) and α 2(IV) collagen isoforms.³² However, in the present study, using the same antibodies, it was shown that α 1(IV) and α 2(IV) collagens were present in the normal rabbit corneal epithelial BM and stroma. Alterations in the profile of type IV collagen isoforms in the corneal epithelium and stroma were noted in healed human corneal tissues at 6 months and 3 years after radial keratotomy.¹⁰ In that study, α 1(IV) and α 2(IV) collagen isoforms not expressed by normal stromal and epithelial cells were detected in the regenerating stroma and epithelium, whereas the α 3(IV) isoform was absent in the regenerated epithelial BM after radial keratotomy. In the present analyses of an earlier stage of corneal healing in rabbit, myofibroblasts were evident in the subepithelial regions 4 weeks after PRK. Significantly higher levels of α 1(IV) and α 2(IV) collagen and an absence of α 3(IV) collagen were evident in these regions containing myofibroblasts. Similar distributions were also evident in the regenerated epithelial BM. Thus, although expression profiles of α 1(IV) to α 3(IV) collagen in the normal rabbit cornea seemed to be different from those reported in human cornea, expression profiles of these collagens during corneal epithelial regeneration after partial-thickness penetrating wounds in rabbit were similar to those in human corneas after radial keratotomy. Furthermore, our results indicated a loss or a significant down-regulation in α 3(IV) collagen expression and an upregulation in α 1(IV) and α 2(IV) collagen

expression in differentiated myofibroblasts in regenerating corneal stromal tissue. These findings are particularly important in the selection of stringent phenotypic markers for validation of in vitro models of keratocyte activation.

As noted in vivo (Fig. 1), $\alpha 1(\text{IV})$ to $\alpha 3(\text{IV})$ collagens were also expressed by rabbit keratocytes in culture (Fig. 4), and $\alpha 3(\text{IV})$ collagen expression was selectively diminished in fibroblasts and myofibroblasts induced by bFGF/HS and TGF- $\alpha 1$, respectively. Negligible levels of mRNA transcripts of $\alpha 3(\text{IV})$ collagen in the activated stromal cells indicated that the loss in the expression of the $\alpha 3(\text{IV})$ collagen isoform was the result of decreased levels of its transcripts. The functional significance of type IV collagen, laminin, and perlecan, the components of BMs, in stroma is unclear. Recently, protein fragments from the C-terminal NC1 domain of $\alpha 3(\text{IV})$ collagen, including tumstatin,⁴⁰ have been identified as the inhibitors of angiogenesis. It is possible that tumstatin is produced in the cornea during the early stages of corneal wound healing by the proteolysis of $\alpha 3(\text{IV})$ collagen by metalloproteinases secreted by the stromal cells.

Reduction in the synthesis of KSPGs is another phenotypic change that occurs during early stages of wound healing in vivo.^{23,25} KSPG expression is eventually restored in scar tissue. As it is in vivo, KSPG expression is evident in bovine keratocytes cultured without serum, but it is downregulated on the activation of keratocytes to fibroblasts with serum.⁴ Similarly, in the present study, rabbit corneal keratocytes cultured without serum synthesized and secreted KSPGs (lumican and keratocan), and a loss in the expression was evident in serum-supplemented medium. The reduction in the serum-induced levels of KSPGs in fibroblasts demonstrated here was caused by decreased levels in the mRNA transcripts of lumican and keratocan. Reduced mRNA levels may result from the inhibition of transcription, posttranscriptional degradation, or both. Activation of rabbit keratocytes to fibroblasts with bFGF/HS led to a downregulation of KSPG expression and to a significantly reduced intracellular mRNA level of keratocan but not of lumican. Contrary to this finding, bFGF promoted KSPG expression in activated bovine keratocytes.⁴ However, bovine keratocytes were activated in insulin, transferrin, and selenium (ITS)-supplemented medium, which might have induced a different response than that seen with bFGF alone in rabbit keratocytes. Similarly, activation of rabbit keratocytes to myofibroblasts with TGF- $\beta 1$ resulted in the downregulation of KSPG expression and of lumican and keratocan transcript levels. A similar loss in KSPG expression induced by TGF- $\beta 1$ has been reported in bovine corneal keratocytes cultured in the presence of a low concentration of horse serum.^{3,22} However, in the presence of ITS, TGF- $\beta 1$ did not affect KSPG expression in bovine keratocytes for 6 days in culture.⁴ When keratocytes isolated from bovine corneas were cultured directly in FBS-supplemented medium, the loss in KSPG expression was reportedly restored on the withdrawal of FBS from the culture medium,⁴¹ which suggested that short-term transient changes in the environment may lead to reversible phenotypic alterations in keratocytes.

Insulin has been shown to have a variety of effects on different cell types, and the present study showed that it promoted the expression of $\alpha 3(\text{IV})$ collagen and keratocan in keratocytes. Therefore, insulin might have enhanced the wound healing process in the corneal stroma by stimulating the synthesis of normal stromal matrix components. Musselmann et al.³⁹ recently reported that insulin can promote bovine keratocyte proliferation and prevent keratocan degradation in low-density cultures. In the present study, we observed higher levels of keratocan and $\alpha 3(\text{IV})$ collagen mRNA in high-density cultures in insulin-supplemented culture medium than in serum-free medium. Thus, insulin may be a desirable growth factor for preventing loss in the expression of important matrix components during the regeneration of corneal stroma.

The present study demonstrated that a loss or a significant downregulation of $\alpha 3(\text{IV})$ collagen expression is one of the phenotypic changes in activated keratocytes in vivo. This stringent phenotypic change also occurs in vitro and accompanies the downregulation of keratocan when keratocytes are activated to fibroblast or myofibroblast phenotypes with bFGF/HS or TGF- $\beta 1$, respectively.

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References

1. Beales MP, Funderburgh JL, Jester JV, Hassell JR. Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture. *Invest Ophthalmol Vis Sci* 1999;40:1658–1663. [PubMed: 10393032]
2. Jester JV, Barry-Lane PA, Cavanagh HD, Petroll WM. Induction of alpha-smooth muscle actin expression and myofibroblast transformation in cultured corneal keratocytes. *Cornea* 1996;15:505–516. [PubMed: 8862928]
3. Funderburgh JL, Funderburgh ML, Mann MM, Corpuz L, Roth MR. Proteoglycan expression during transforming growth factor beta-induced keratocyte-myofibroblast transdifferentiation. *J Biol Chem* 2001;276:44173–44178. [PubMed: 11555658]
4. Long CJ, Roth MR, Tasheva ES, et al. Fibroblast growth factor-2 promotes keratan sulfate proteoglycan expression by keratocytes in vitro. *J Biol Chem* 2000;275:13918–13923. [PubMed: 10788517]
5. Jester JV, Budge A, Fisher S, Huang J. Corneal keratocytes: phenotypic and species differences in abundant protein expression and in vitro light-scattering. *Invest Ophthalmol Vis Sci* 2005;46:2369–2378. [PubMed: 15980224]
6. Jester JV, Ho-Chang J. Modulation of cultured corneal keratocyte phenotype by growth factors/cytokines control in vitro contractility and extracellular matrix contraction. *Exp Eye Res* 2003;77:581–592. [PubMed: 14550400]
7. Jester JV, Huang J, Barry-Lane PA, Kao WW, Petroll WM, Cavanagh HD. Transforming growth factor (beta)-mediated corneal myofibroblast differentiation requires actin and fibronectin assembly. *Invest Ophthalmol Vis Sci* 1999;40:1959–1967. [PubMed: 10440249]
8. Jester JV, Barry PA, Lind GJ, Petroll WM, Garana R, Cavanagh HD. Corneal keratocytes: in situ and in vitro organization of cytoskeletal contractile proteins. *Invest Ophthalmol Vis Sci* 1994;35:730–743. [PubMed: 8113024]
9. Kato T, Nakayasu K, Kanai A. Corneal wound healing: immunohistological features of extracellular matrix following penetrating keratoplasty in rabbits. *Jpn J Ophthalmol* 2000;44:334–341. [PubMed: 10974287]
10. Ljubimov AV, Alba SA, Burgeson RE, et al. Extracellular matrix changes in human corneas after radial keratotomy. *Exp Eye Res* 1998;67:265–272. [PubMed: 9778407]
11. Melles GRJ, SundarRaj N, Binder PS, et al. Immunohistochemical analysis of unsutured and sutured corneal wound-healing. *Curr Eye Res* 1995;14:809–817. [PubMed: 8529420]
12. SundarRaj N, Martin J, Hrinya N. Development and characterization of monoclonal antibodies to human type III procollagen. *Biochem Biophys Res Commun* 1982;106:48–57. [PubMed: 7049168]
13. Tuori A, Virtanen I, Aine E, Uusitalo H. The expression of tenascin and fibronectin in keratoconus, scarred and normal human cornea. *Graefes Arch Clin Exp Ophthalmol* 1997;235:222–229. [PubMed: 9143890]
14. Maseruka H, Bonshek RE, Zardi L, Tullo AB, Ridgway AEA. Tenascin-C variants in wound healing of the human cornea. *Vis Res* 1996;36(suppl):1326.

15. Vansetten GB, Koch JW, Tervo K, et al. Expression of tenascin and fibronectin in the rabbit cornea after excimer laser-surgery. *Graefes Arch Clin Exp Ophthalmol* 1992;230:178–183. [PubMed: 1374356]
16. Mulholland B, Tuft SJ, Khaw PT. Matrix metalloproteinase distribution during early corneal wound healing. *Eye* 2005;19:584–588. [PubMed: 15332107]
17. Sivak JM, Fini ME. MMPs in the eye: emerging roles for matrix metalloproteinases in ocular physiology. *Prog Retinal Eye Res* 2002;21:1–14.
18. Ye HQQ, Maeda M, Yu FSX, Azar DT. Differential expression of MT1-MMP (MMP-14) and collagenase III (MMP-13) genes in normal and wounded rat corneas. *Invest Ophthalmol Vis Sci* 2000;41:2894–2899. [PubMed: 10967042]
19. Azar DT, Hahn TW, Jain S, Yeh YC, Stetler-Stevensen WG. Matrix metalloproteinases are expressed during wound healing after excimer laser keratectomy. *Cornea* 1996;15:18–24. [PubMed: 8907376]
20. Fini ME, Girard MT, Matsubara M, Bartlett JD. Unique regulation of the matrix metalloproteinase, gelatinase-B. *Invest Ophthalmol Vis Sci* 1995;36:622–633. [PubMed: 7890493]
21. Fini ME, Girard MT, Matsubara M. Collagenolytic gelatinolytic enzymes in corneal wound-healing. *Acta Ophthalmol* 1992;70:26–33. [PubMed: 1557971]
22. Funderburgh JL, Mann MM, Funderburgh ML. Keratocyte phenotype mediates proteoglycan structure: a role for fibroblasts in corneal fibrosis. *J Biol Chem* 2003;278:45629–45637. [PubMed: 12933807]
23. Funderburgh JL, Chandler JW. Proteoglycans of rabbit corneas with nonperforating wounds. *Invest Ophthalmol Vis Sci* 1989;30:435–442. [PubMed: 2925315]
24. Carlson EC, Wang IJ, Liu CY, Brannan P, Kao CWC, Kao WWY. Altered KSPG expression by keratocytes following corneal injury. *Mol Vis* 2003;9:615–623. [PubMed: 14654769]
25. SundarRaj N, Fite D, Belak R, et al. Proteoglycan distribution during healing of corneal stromal wounds in chick. *Exp Eye Res* 1998;67:433–442. [PubMed: 9820791]
26. Berryhill BL, Beales MP, Hassell JR. Production of prostaglandin D synthase as a keratan sulfate proteoglycan by cultured bovine keratocytes. *Invest Ophthalmol Vis Sci* 2001;42:1201–1207. [PubMed: 11328728]
27. Espana EM, Kawakita T, Liu CY, Tseng SC. CD34 expression by cultured human keratocytes is downregulated during myofibroblast differentiation induced by TGF- β 1. *Invest Ophthalmol Vis Sci* 2004;45:2985–2991. [PubMed: 15326111]
28. Jester JV, Moller-Pedersen T, Huang J, et al. The cellular basis of corneal transparency: evidence for “corneal crystallins.” *J Cell Sci* 1999;112(pt 5):613–622. [PubMed: 9973596]
29. Hassell JR, Schrecengost PK, Rada JA, SundarRaj N, Sossi G, Thoft RA. Biosynthesis of stromal matrix proteoglycans and basement membrane components by human corneal fibroblasts. *Invest Ophthalmol Vis Sci* 1992;33:547–557.
30. Kalluri R. Discovery of type IV collagen non-collagenous domains as novel integrin ligands and endogenous inhibitors of angiogenesis. *Cold Spring Harb Symp Quant Biol* 2002;67:255–266. [PubMed: 12858548]
31. Hamano Y, Kalluri R. Tumstatin, the NC1 domain of alpha 3 chain of type IV collagen, is an endogenous inhibitor of pathological angiogenesis and suppresses tumor growth. *Biochem Biophys Res Commun* 2005;333:292–298. [PubMed: 15979458]
32. Ljubimov AV, Burgeson RE, Butkowski RJ, Michael AF, Sun TT, Kenney MC. Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. *Lab Invest* 1995;72:461–473. [PubMed: 7723285]
33. Mohan RR, Hutcheon AE, Choi R, et al. Apoptosis, necrosis, proliferation, and myofibroblast generation in the stroma following LASIK and PRK. *Exp Eye Res* 2003;76:71–87. [PubMed: 12589777]
34. Anderson SC, SundarRaj N. Regulation of a rho-associated kinase expression during the corneal epithelial cell cycle. *Invest Ophthalmol Vis Sci* 2001;42:933–940. [PubMed: 11274069]
35. Ninomiya Y, Kagawa M, Iyama K, et al. Differential expression of 2 basement-membrane collagen genes, *Col4A6* and *Col4A5*, demonstrated by immunofluorescence staining using peptide-specific monoclonal-antibodies 1. *J Cell Biol* 1995;130:1219–1229. [PubMed: 7657706]

36. SundarRaj N, Willson J, Gregory JD, Damle SP. Monoclonal antibodies to proteokeratan sulfate of rabbit corneal stroma. *Curr Eye Res* 1985;4:49–54. [PubMed: 2579771]
37. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]
38. Knudsen KA, Soler AP. Cadherin-mediated cell-cell interactions. *Methods Mol Biol* 2000;137:409–440. [PubMed: 10948557]
39. Musselmann K, Alexandrou B, Kane B, Hassell JR. Maintenance of the keratocyte phenotype during cell proliferation stimulated by insulin 1. *J Biol Chem* 2005;280:32634–32639. [PubMed: 16169858]
40. Maeshima Y, Colorado PC, Kalluri R. Two RGD-independent $\alpha\beta 3$ integrin binding sites on tumstatin regulate distinct anti-tumor properties. *J Biol Chem* 2000;275:23745–23750. [PubMed: 10837460]
41. Berryhill BL, Kader R, Kane B, Birk DE, Feng J, Hassell JR. Partial restoration of the keratocyte phenotype to bovine keratocytes made fibroblastic by serum. *Invest Ophthalmol Vis Sci* 2002;43:3416–3421. [PubMed: 12407151]

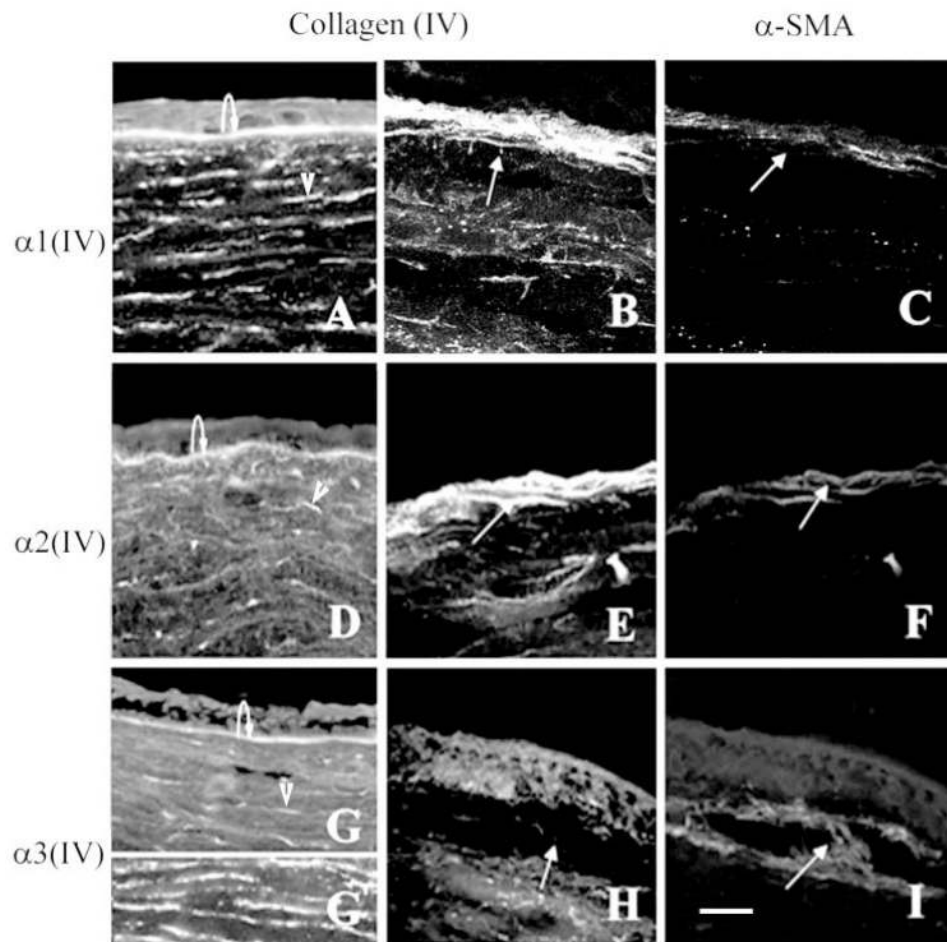


FIGURE 1. Immunohistochemical analyses of normal and wounded rabbit corneas. Cryostat sections of the normal rabbit cornea (*left*) were stained with rat anti- $\alpha 1$ (IV) (A), $\alpha 2$ (IV) (D), or $\alpha 3$ (IV) (G) collagen monoclonal antibodies. Wounded corneas at 4 weeks after -9 D PRK were double stained with rat anti-(IV) collagen antibodies (*middle*) and a mouse anti- α -smooth muscle actin (SMA) monoclonal antibody (*right*). Secondary antibodies were goat anti-rat Alexa Fluor 488 (*left and middle*) and goat anti-mouse Alexa 580 conjugated (*right*). Note staining of BM zone (*curved arrows*) and stromal cells (*arrowheads*) with antibodies to $\alpha 1$, $\alpha 2$, and $\alpha 3$ (IV) collagens. *Arrows*: myofibroblasts in the subepithelial regions stained with anti- α -SMA (C, F, I), which also stain with $\alpha 1$ (IV) (B) and $\alpha 2$ (IV) (E) collagen but not with $\alpha 3$ (IV) collagen (H).

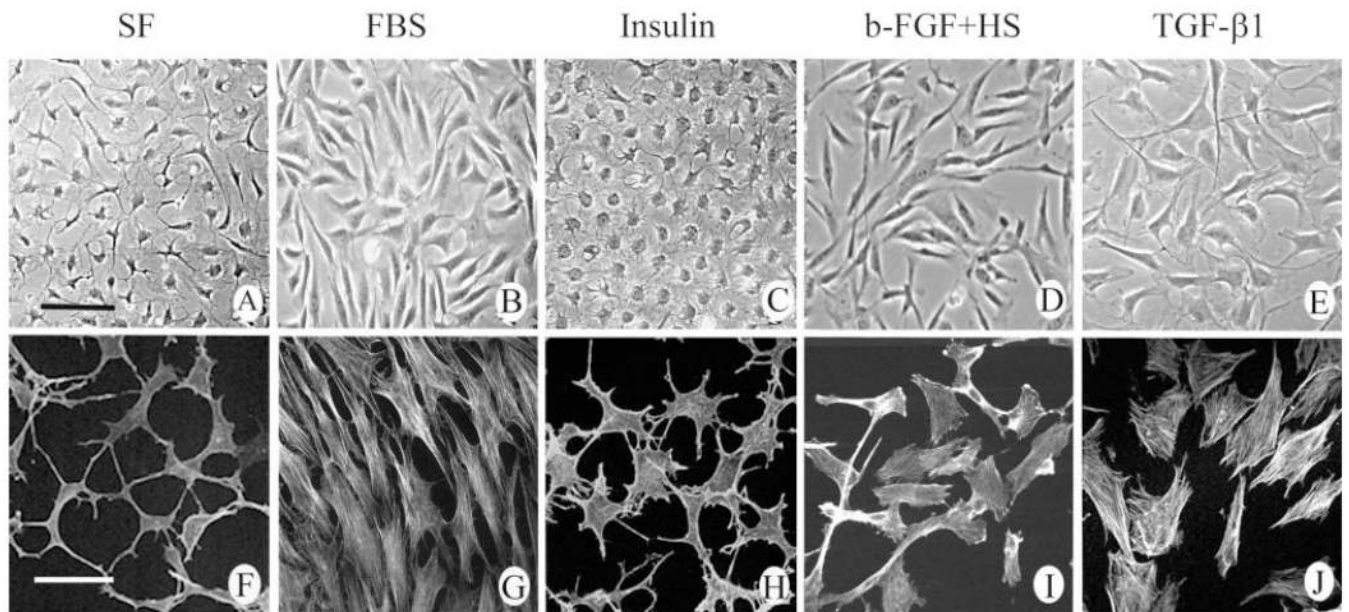


FIGURE 2.

Phase-contrast images (A–E) and actin filament staining patterns (F–J) of rabbit corneal stromal cells in culture. Keratocytes isolated from rabbit corneas were plated in serum-free DMEM/F12 and allowed to attach for 24 hours. The medium was then replaced with DMEM/F12 without serum (A, F), 10% FBS (B, G), 10 µg/mL insulin (C, H), 20 ng/mL bFGF + 5 µg/mL HS (D, I), or 2 ng/mL TGF-β1 (E, J). Media were changed every third day, and phase-contrast micrographs were taken at day 7 (A–E). For actin staining, cells were fixed, permeabilized, and stained with Texas Red-X-conjugated phalloidin (F–J). Bar, (A–E) 125 µm; (F–J) 60 µm.

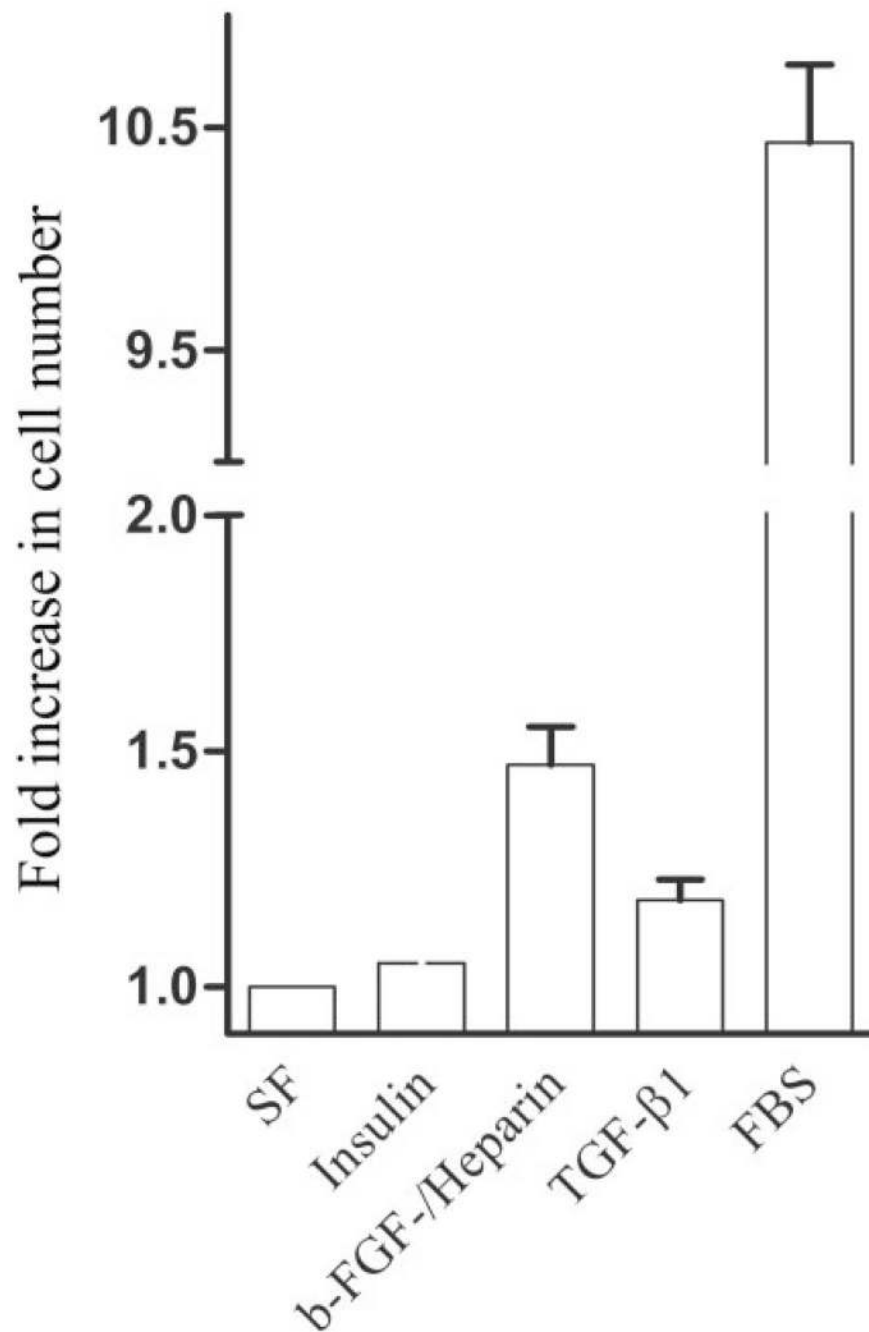


FIGURE 3.

Growth of rabbit corneal keratocytes under different culture conditions. Keratocytes isolated from rabbit corneas were plated in DMEM/F12 without FBS. After 24 hours, culture medium was replaced with DMEM/F12 without FBS or supplemented with 10 $\mu\text{g}/\text{mL}$ insulin, bFGF (20 ng/mL)/HS (5 $\mu\text{g}/\text{mL}$), 2 ng/mL TGF- β 1, or 10% FBS. Bars show the relative changes in cell number after 3 days (number of cells at day 3/number of cells at the start of the treatment).

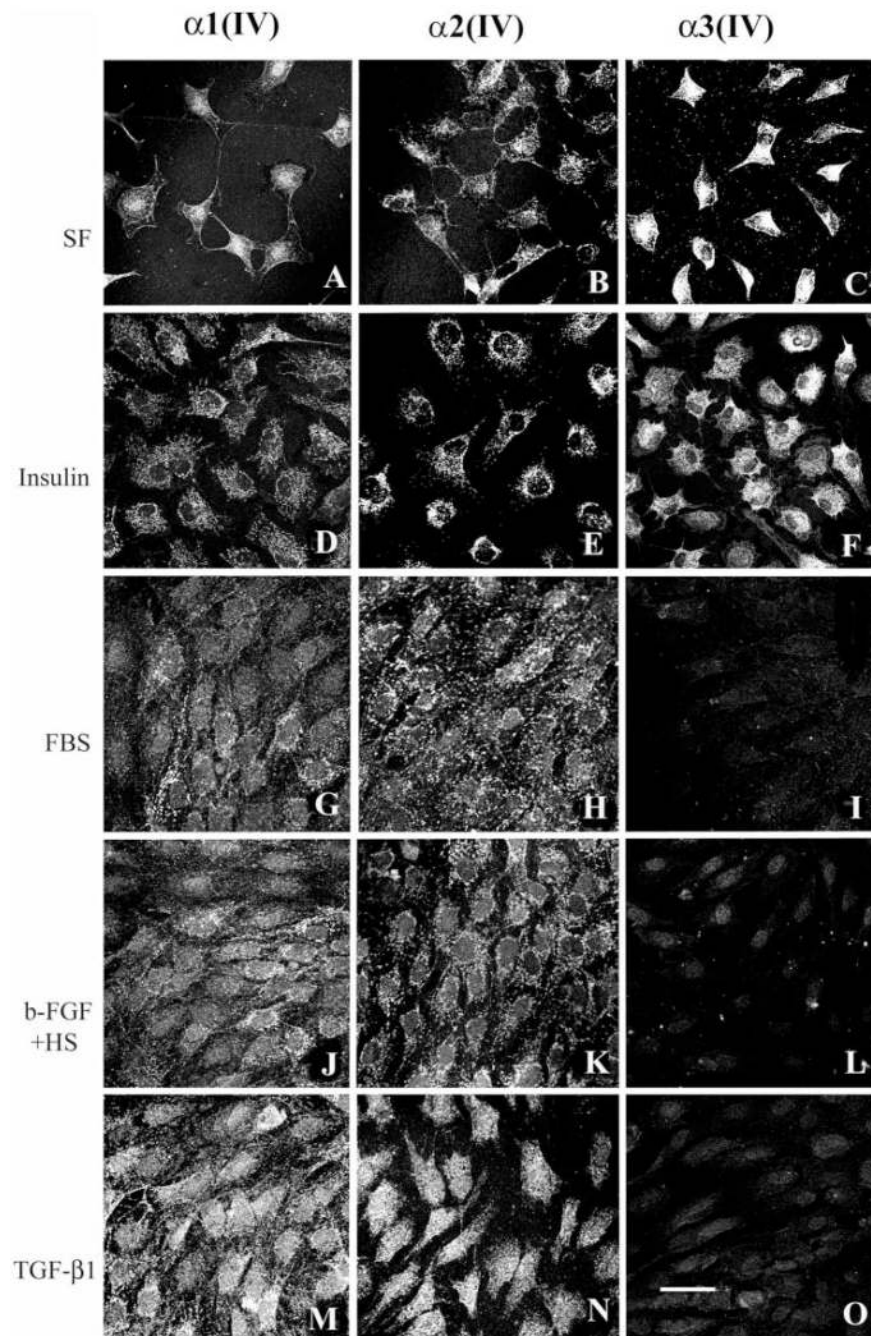


FIGURE 4. Immunocytochemical analyses of corneal stromal cells in culture. Stromal cells isolated from rabbit corneas were cultured in DMEM/F12 without FBS (A–C), with 10 $\mu\text{g}/\text{mL}$ insulin (D–F), with 10% FBS (G–I), with bFGF (20 ng/mL)/HS (5 $\mu\text{g}/\text{mL}$) (J–L), or with 2 ng/mL TGF- $\beta 1$ (M–O). After fixing and permeabilizing, the cells were immunostained with rat anti- $\alpha 1$ (IV) collagen (*left*), anti- $\alpha 2$ (IV) collagen (*middle*), or anti- $\alpha 3$ (IV) collagen (*right*) antibodies, followed by Alexa Fluor 488-conjugated goat anti-rat antibodies. Bar, 50 μm .

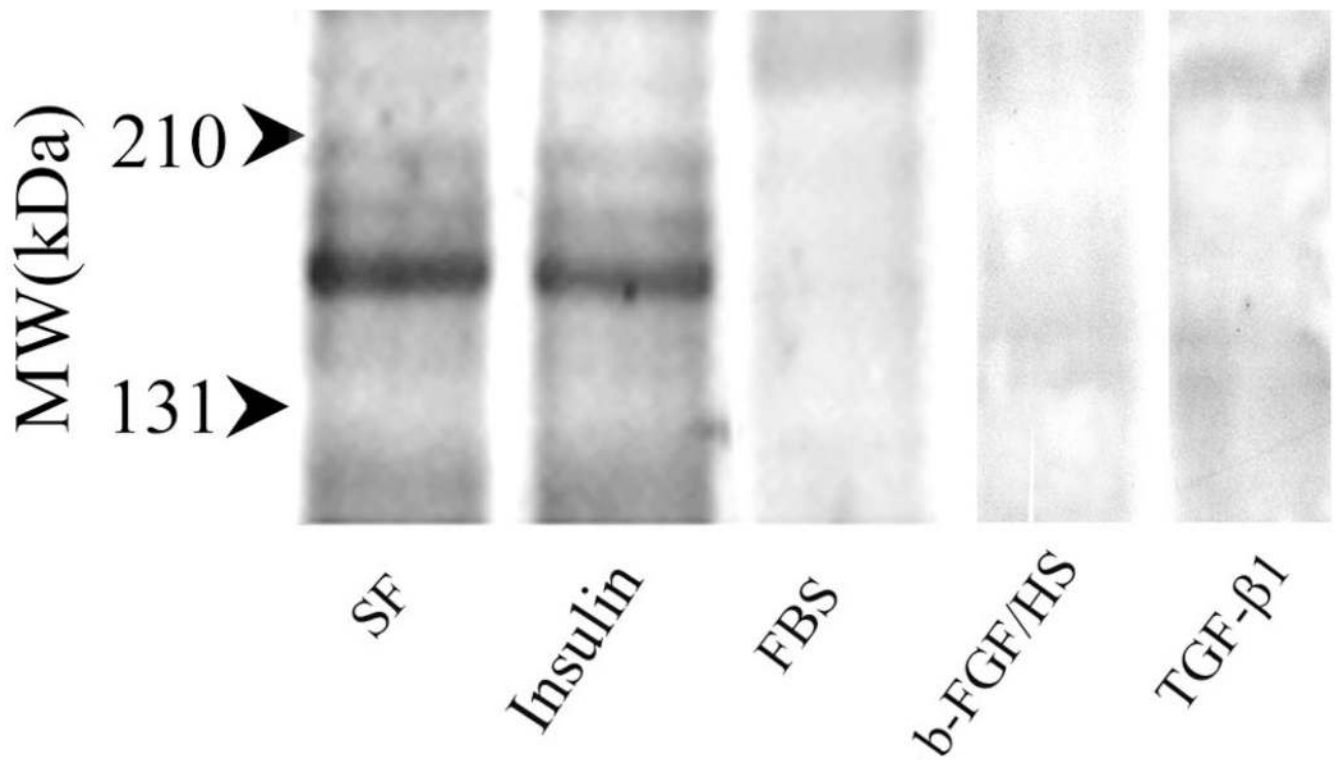


FIGURE 5.

Western blot analyses of the $\alpha 3(\text{IV})$ collagen in cell extracts of rabbit corneal stromal cells cultured in serum-free DMEM/F12(SF) or in DMEM/F12 supplemented with insulin, 10% fetal bovine serum (FBS), bFGF (20 ng/mL)/HS (5 $\mu\text{g/mL}$), or 2 ng/mL TGF- $\beta 1$. Blots were probed with rat anti- $\alpha 3(\text{IV})$ collagen antibody. Note an immunoreactive band of approximately 170 kDa was detected only in serum-free and insulin-supplemented media.

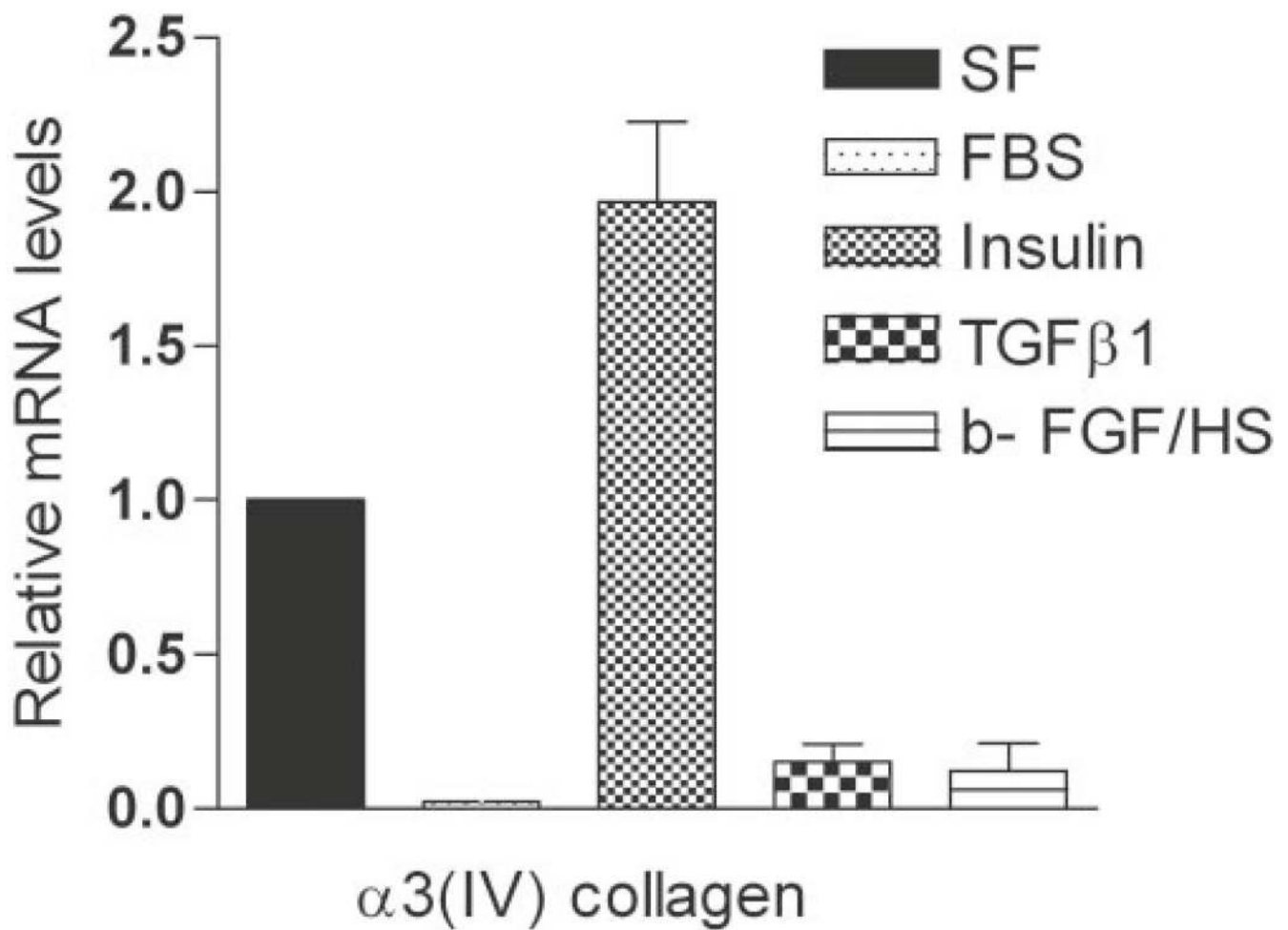


FIGURE 6.

Relative concentrations of mRNA transcripts of $\alpha 3(IV)$ collagen in corneal stromal cells. Relative abundance of mRNA transcripts for $\alpha 3(IV)$ collagen was analyzed with real-time RT-PCR using 18S rRNA levels to normalize values in each experiment. Triplicate samples were analyzed in each experiment, and the mean values were used to determine the relative levels of mRNAs in cells cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 10 $\mu\text{g}/\text{mL}$ insulin, 2 ng/mL TGF- β 1, or bFGF (20 ng/mL)/HS (5 $\mu\text{g}/\text{mL}$) compared with those in serum-free medium. Ratios \pm SD of mRNA levels from three separate experiments are presented.

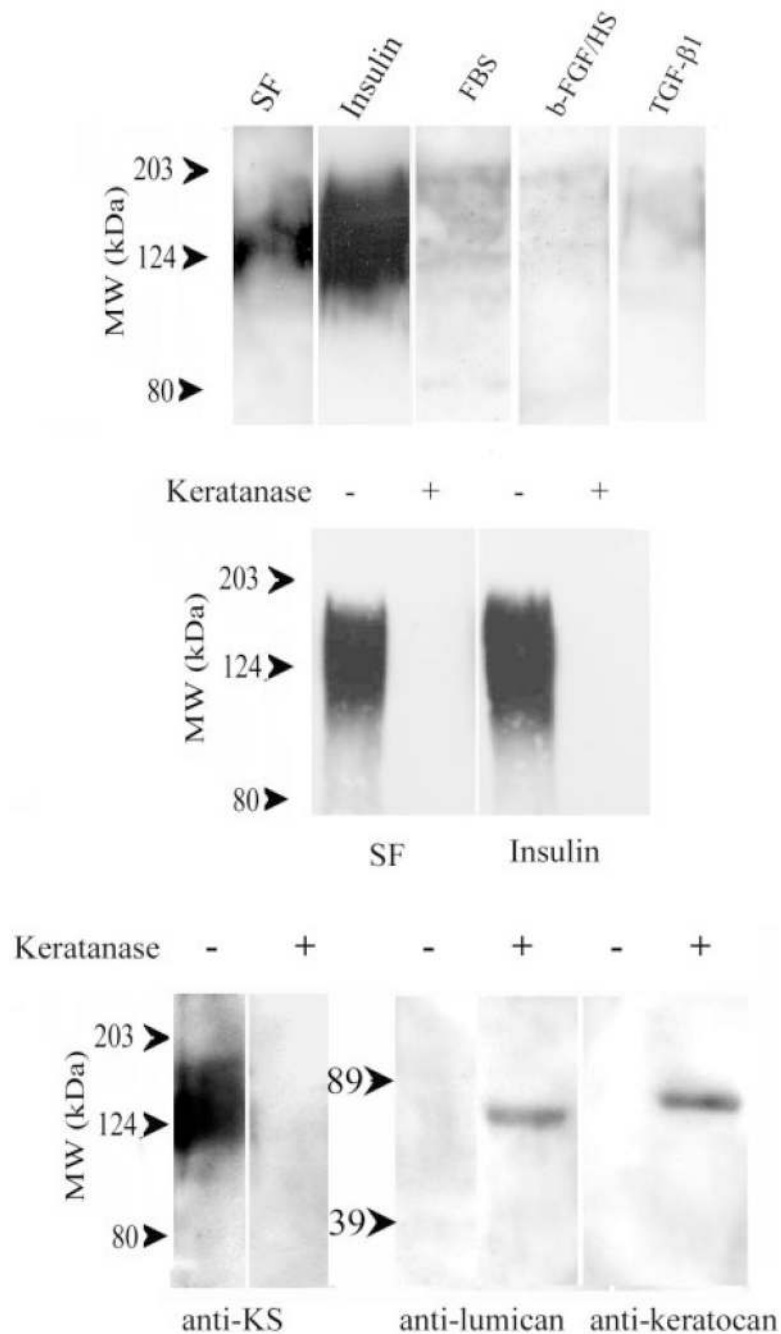


FIGURE 7.

Western blot analyses of KSPGs in the culture supernatants of rabbit corneal stromal cells. *Top:* Anti-KS reactive bands in the culture supernatants of rabbit corneal stromal cells cultured in DMEM/F12 without serum (SF) or with 10 $\mu\text{g}/\text{mL}$ insulin, 10% FBS, bFGF (20 ng/mL)/HS (5 $\mu\text{g}/\text{mL}$), or 2 ng/mL TGF- β 1. *Middle:* Western blot analysis of the keratinase-sensitivity of anti-KS-reactive bands secreted in serum-free and insulin-supplemented media. Culture supernatants were incubated at 37°C with or without keratinase before the analyses. *Bottom:* Analysis of protein cores of the KSPGs secreted in serum-free DMEM/F12. KSPGs in the culture supernatants were immunoprecipitated with anti-KS antibody and aliquots digested

with keratinase. Blots of undigested or keratinase-digested samples were reacted with anti-KS, anti-lumican, or anti-keratocan antibodies.

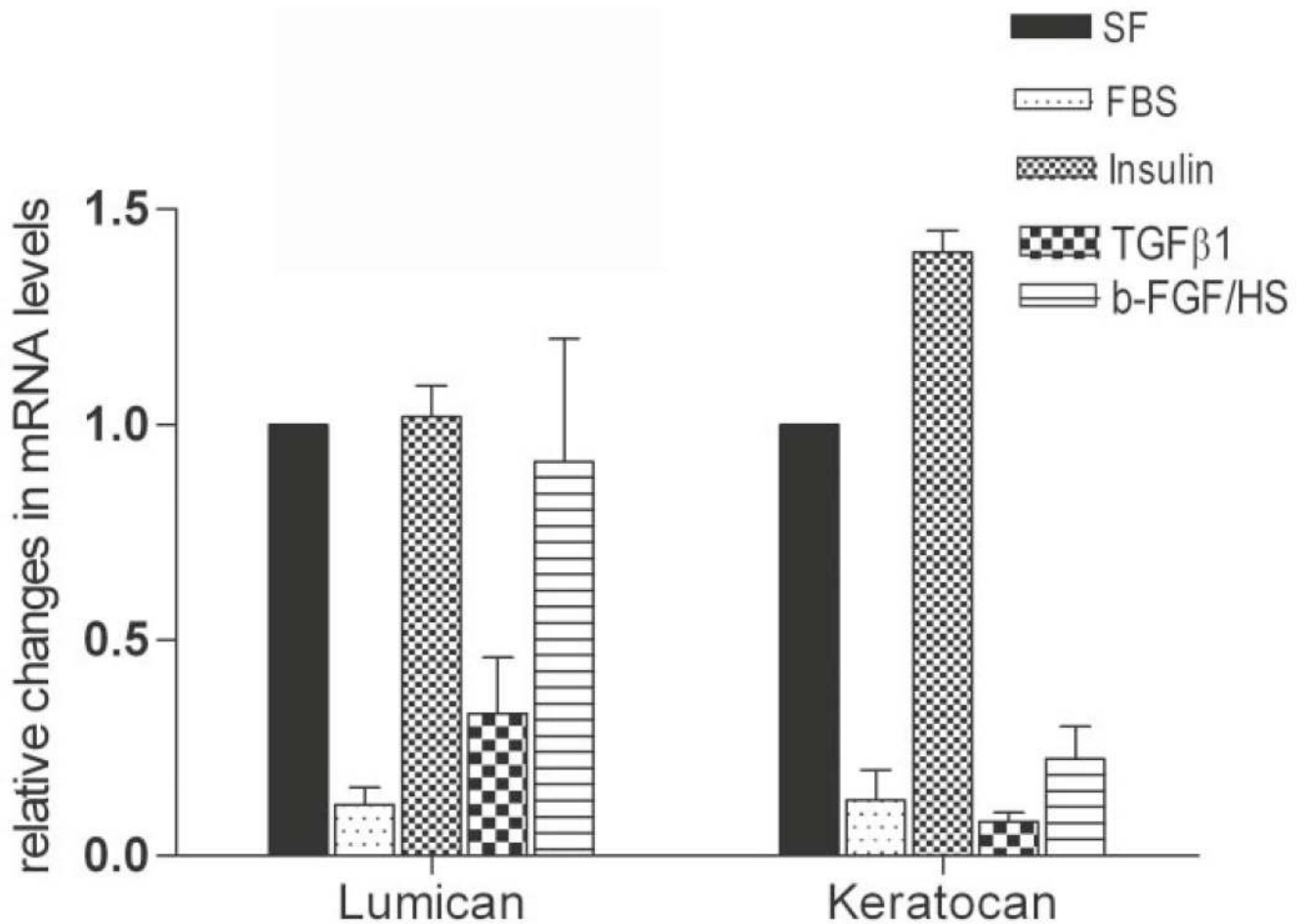


FIGURE 8.

Relative concentrations of mRNA transcripts of lumican and keratocan in rabbit corneal stromal cells. Relative abundance of mRNA transcripts for specific genes was analyzed by real-time RT-PCR using 18S rRNA levels to normalize values in each experiment. Triplicate samples were analyzed in each experiment, and the mean values were used to determine the relative levels of mRNAs in cells cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 10 μ g/mL insulin, 2 ng/mL TGF- β 1, or bFGF (20 ng/mL)/HS (5 mg/mL) compared with those in serum-free DMEM/F12. Ratios \pm SD of mRNA levels from three separate experiments are presented.

TABLE 1

Primers Used in Quantitative RT-PCR

Primer Names	Oligonucleotide Sequence (5'→3')
18S	Forward: CTC AAC ACG GGA AAC CTC AC
GenBank X06778	Reverse: ACC ACC CAC AGA ATC GAG AA
α 3(IV) collagen	Forward: GCT GTC AAC ACC AGC TCT GA
GenBank L47283	Reverse: CGG TGC ACC TGC TAA TGT AA
ALDH1	Forward: CC GTT GGT TAT GCT CAT TT
GenBank AY0388801	Reverse: TCT GCA GGT TTC ACG ATC AC
Lumican	Forward: TGC AGC TTA CCC ACA ACA AG
GenBank AF020292	Reverse: TGA AGG TGA ACG AAG GTC AA
Keratocan	Forward: CTC ACG TGG CTT TGA TGT GT
GenBank DQ239829	Reverse: GAC CTT TGT GAG GCG ATT GT