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

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PURPOSE. To determine whether changes in the expression of type IV α1, α2, or α3 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-β1–, 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 α1, α2, and α3 collagens were expressed in normal rabbit corneal stroma and in keratocytes cultured in serum-free and insulin-supplemented media. However, α3(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-β1. α3(IV) collagen mRNA levels were also diminished in the stromal cells cultured in these growth factor-supplemented media. KSPGs (lumican and keratan) 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 keratan mRNAs were downregulated by TGF-β1 and FBS. bFGF/HS promoted the downregulation of intracellular keratan but not lumican mRNA levels.

CONCLUSIONS. The loss in the expression of α3(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-β1, and it accompanies the downregulation of keratocyte expression. (Invest Ophthalmol Vis Sci. 2007;48:627–635) DOI:10.1167/iovs.06-0635

Corneal stromal cells (keratocytes) are responsible for the development and maintenance of the unique extracellular matrix of the transparent corneal stroma, which constitutes approximately 90% of the adult cornea. Although keratocytes are relatively quiescent in the 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-β1, platelet-derived growth factor (PDGF), bFGF, insulin-like growth factor (IGF)-1, and IL-1α, 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,13,14 and matrix metalloproteinases15–21 and significant decreases in the expression of keratan sulfate proteoglycans (KSPGs).1,3,5,22–25 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 perlecans, are also expressed in connective tissues including corneal stroma.29,30 Six genetically distinct chains of type IV collagen—α1(IV)–α6(IV)—have been identified and shown to have different cellular interactions and tissue distributions.30,31 Immunohistochemical analysis has indicated the presence of α3(IV), α4(IV), and α5(IV) collagens in normal human corneal stroma, epithelial BM, and Descemet membrane.52 The expression of different type IV collagen isoforms is altered in regenerated stroma after radial keratotomy.10 Interestingly, α1(IV) and α2(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. α3(IV) collagen was absent in regenerated BM. In the present study, we evaluated the expression of α1(IV) to α3(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 Jeser et al.6 Cornneas 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 μm; 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 μg/mL insulin, 2.0 ng/mL TGF-β1, or bFGF (20 ng/mL)/HS (5 μg/mL).

PKR 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 PKR eyes) were performed as described previously.34 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.35 Fixed and permeabilized cells or tissue sections on microscope slides were reacted with 10% heat-inactivated goat sera. After cells were rinsed with phosphate-buffered saline (PBS), they were fixed with paraformaldehyde-lysine-periodate and Alexa 488–conjugated or Alexa 546–conjugated goat IgG (H+L) and were mounted (Immuno-mount; Shandon, Pittsburgh, PA) and were maintained frozen at –85°C until staining was performed.

RESULTS

Distribution of α1(IV), α2(IV), and α3(IV) Collagen in Normal and Post-PKR Rabbit Cornea

Immunohistochemical analysis of cryostat sections of normal rabbit cornea indicated that the distribution patterns of α1(IV), α2(IV), and α3(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, α3(IV) collagen levels were highest in the stroma.
FIGURE 1. Immunohistochemical analyses of normal and wounded rabbit corneas. Cryostat sections of the normal rabbit cornea (left) were stained with rat anti–α1(IV) (A), α2(IV) (D), or α3(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 α1, α2, and α3(IV) collagens. Arrows: myofibroblasts in the subepithelial regions stained with anti–α-SMA (C, F, I), which also stain with α1(IV) (B) and α2(IV) (E) collagen but not with α3(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.
were lower in the anterior one third of the stroma (Fig. 1G) than in the posterior region (Fig. 1F). 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. Activated stromal cells expressing α-SMA (myofibroblasts) were detected in subepithelial regions of regenerated stroma (Figs. 1C, 1F, 1I; arrows in each). These α-SMA-positive cells also reacted with anti-α1(IV) and anti-α2(IV) collagen antibodies (Figs. 1Bm 1E; arrows in each). However, they did not react with anti-α3(IV) collagen antibody (Fig. 1H, arrows). Staining of α3(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 α3(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, 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, 3). bFGF/HS induced a 1.5-fold increase in cell number within 3 days. TGF-β1 induced stromal cells to differentiate to elongated larger cells containing a robust network of actin filament bundles (Figs. 2E, 2J) but did not undergo cell division (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 α1(IV), α2(IV), and α3(IV) collagen chains by corneal stromal cells cultured under the conditions described indicated that α1(IV) and α2(IV) collagens were synthesized by corneal keratocytes cultured in the medium without FBS or with insulin, FBS, bFGF/HS, or TGF-β1 (Fig. 4). However, though α3(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-β1-supplemented media (Figs. 4I, 4L, 4O). α3(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-β1 (Fig. 5).

Regulation of α3(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 α3(IV) collagen were also altered in stromal cells activated with FBS or growth factors. These analyses indicated that the levels of α3(IV) mRNA in the keratocytes cultured in DMEM/F12 supplemented with insulin were 90% ± 40% higher than in serum-free medium. However, in keratocytes cultured in FBS, TGF-β1, or bFGF/HS, α3(IV) mRNA levels were reduced to 2.5% ± 1.5%, 14% ± 6%, and 12% ± 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. KSPG Expression in Corneal Stromal Cells

Table 1. Primers Used in Quantitative RT-PCR

<table>
<thead>
<tr>
<th>Primer Names</th>
<th>Oligonucleotide Sequence (5′→3′)</th>
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<tr>
<td>18S</td>
<td>Forward: CCT AAC ACG GGA AAG CTC AC</td>
</tr>
<tr>
<td>GenBank X06778</td>
<td>Reverse: ACC ACC CAC AGA ATC GAG AA</td>
</tr>
<tr>
<td>α3(IV) collagen</td>
<td>Forward: GCT GTC AAC ACC AGC TCT GA</td>
</tr>
<tr>
<td>GenBank L47283</td>
<td>Reverse: CGG TGC ACC CTC TAA TGT AA</td>
</tr>
<tr>
<td>ALDH1</td>
<td>Forward: CC GTC TAT GAT GCT CAT TT</td>
</tr>
<tr>
<td>GenBank AY0988801</td>
<td>Reverse: TCT GCA GGT TTC AGC ATC AC</td>
</tr>
<tr>
<td>Lumican</td>
<td>Forward: TGC AGC TTA CCC ACA ACA AG</td>
</tr>
<tr>
<td>GenBank AFO20292</td>
<td>Reverse: TGA AGG TGA ACG AAG GTC AA</td>
</tr>
<tr>
<td>Keratocan</td>
<td>Forward: CTC ACG TGC TTA TGA TGT GT</td>
</tr>
<tr>
<td>GenBank DQ239829</td>
<td>Reverse: GAC CTT TGT GAG GCA ATT GT</td>
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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-β1 (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-β1, or bFGF/HS were 12% ± 8%, 33% ± 13%, and 90% ± 50%, 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-β1, or bFGF/HS were altered to 13% ± 7%, 8% ± 2%, and 22% ± 8% respectively, of those in serum-free medium. Insulin increased keratocan mRNA levels by 40% ± 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-trans-sketolase, and KSPGs such as keratocan and lumican. However, crystallin expression is significantly reduced when the stromal cells are cultured in media with FBS. A loss in KSPG expression has been reported in bovine keratocytes cultured with serum. 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 keratocytes allowed us to determine whether the changes in the expression of α1(IV) to α3(IV) collagen isoforms during wound healing in vivo are recapitulated in vitro. Growth factors, including bFGF/HS and TGF-β1, can induce keratocyte activation to fibroblast and myofibroblast phenotypes, respectively. We analyzed whether changes in the expression of α1(IV) to α3(IV) collagens are among the changes associated with this transition.
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 cornea. Normal adult human corneal epithelial BM and stroma have been shown to contain α3(IV), α4(IV), and α5(IV) collagen isoforms but not α1(IV) and α2(IV) collagen isoforms.32 However, in the present study, using the same antibodies, it was shown that α1(IV) and α2(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.16 In that study, α1(IV) and α2(IV) collagen isoforms not expressed by normal stromal and epithelial cells were detected in the regenerating stroma and epithelium, whereas the α3(IV) isoform was absent in the regenerated epithelial BM after radial keratotomy. In the present analyses of an earlier stage of corneal wound healing in rabbit, myofibroblasts were evident in the subepithelial regions 4 weeks after PRK. Significantly higher levels of α1(IV) and α2(IV) collagen and an absence of α3(IV) collagen were evident in these regions containing myofibroblasts. Similar distributions were also evident in the regenerated epithelial BM. Thus, although expression profiles of α1(IV) to α3(IV) collagens 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 α3(IV) collagen expression and an upregulation in α1(IV) and α2(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), α1(IV) to α3(IV) collagens were also expressed by rabbit keratocytes in culture (Fig. 4), and α3(IV) collagen expression was selectively diminished in fibroblasts and myofibroblasts induced by bFGF/HS and TGF-β1, respectively. Negligible levels of mRNA transcripts of α3(IV) collagen in the activated stromal cells indicated that the loss in the expression of the α3(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 α3(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 α3(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-β1 resulted in the downregulation of KSPG expression and of lumican and keratocan transcript levels. A similar loss in KSPG expression induced by TGF-β1 has been reported.

**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 μg/mL insulin, 2 ng/mL TGF-β1, or bFGF (20 ng/mL)/HS (5 mg/mL) compared with those in serum-free DMEM/F12. Ratios ± SD of mRNA levels from three separate experiments are presented.
in bovine corneal keratocytes cultured in the presence of a low concentration of horse serum. However, in the presence of ITS, TGF-β1 did not affect KSPG expression in bovine keratocytes for 6 days in culture. 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, 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 downregulated keratocan when keratocytes are activated in vitro. 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-β1, respectively.

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References


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