Differential Expression of Proteoglycans by Corneal Stromal Cells in Keratoconus

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Purpose. Keratoconus is a heterogeneous disease associated with a range of pathologies, including disorders that involve proteoglycans (PGs). Some PG alterations, mainly in keratan sulfate (KS), occur in keratoconus. In this article, we studied the differential expression of the genes encoding PGs in cells isolated from keratoconus patients and healthy controls, as well as in corneal sections.

Methods. Human central corneal tissue was obtained from cadaver donors and patients undergoing penetrating keratoplasty surgery. A transcriptomic approach was used, employing quantitative PCR, to analyze both the expression of the enzymes involved in glycosaminoglycan (GAG) biosynthesis and the PG core proteins. The expressions of the differentially expressed core proteins and of the GAG chains were also analyzed by immunocytochemistry in the cultured cells, as well as by immunohistochemistry in corneal sections.

Results. The mRNA levels of most major matrix PG mRNAs in the cultured keratoconic stromal cells decreased except collagen XVIII, which increased. At keratocyte surfaces, some heparan sulfate PGs were down-regulated. With respect to GAGs, there were changes in gene expression for the polymerization of the GAG chains, mainly KS and chondroitin sulfate, and in the modification of the saccharidic chains, pointing to an alteration of the sulfation patterns of all GAG species.

Conclusions. Most of the PG core proteins underwent significant changes in cultured keratoconic cells and corneas. With regard to GAG chains, the polymerization of the chains and their chemical modification was modified in way that depended on the specific type of GAG involved.

Keywords: keratoconus, proteoglycan, glycosaminoglycan, keratan sulfate, chondroitin sulfate, heparan sulfate

Keratoconus is a corneal ectasia that displays many abnormal features affecting different layers of the cornea.[1–3] Keratoconus frequently occurs in isolation, but it is often associated with a wide range of ocular disorders, including various chromosomal abnormalities and connective tissue disorders.[2,4,5]

The central stroma constitutes 90% of the corneal thickness and consists of an extracellular matrix (ECM) interspersed with keratocytes. The corneal stroma is composed primarily of collagen fibrils that are coated by different proteoglycans (PGs).[6] Proteoglycans are a diverse group of glycoconjugates composed of various core proteins posttranslationally modified with anionic polysaccharides called glycosaminoglycans (GAGs) such as chondroitin sulfate/dermatan sulfate (CS/DS), keratan sulfate (KS), and heparan sulfate (HS). There are five major PGs located in the corneal ECM; three of them, lumican, keratocan, and osteoglycin, are KS PGs, whereas decorin and biglycan are CS PGs.[7,8] Moreover, keratinocytes express HS PGs, which are ubiquitously present in tissues.[9–11]

Various eye diseases are related to changes in PGs, such as the reduction of highly sulfated KS levels associated with corneal pathologies and inflammatory conditions.[12–14] A progressive opacification of the cornea is characteristic of some mucopolysaccharidosis.[15,16] Moreover, at least two corneal dystrophies, cornea plana and macular corneal dystrophy, result from the failure of normal KS production.[17,18]

There are few studies examining alterations in corneal PGs and GAGs in connection with keratoconus. With respect to PG core proteins, biochemical approaches have identified heterogeneity in both CS/DS PGs and KS PGs, although the samples analyzed in these studies included other diseases besides keratoconus.[19,20] More recently, analysis of the keratoconus corneal proteome showed a decrease in lumican, decorin, biglycan, and keratocan.[1] In terms of GAG chains, it has been described as an accumulation of GAG polyanions in keratoconic corneas.[21] However, in the central, thinned region of the keratoconic cornea, the KS antigenicity is decreased,[19] and in severe keratoconic corneas, KS organization is markedly different from that of controls.[22] Interestingly, some connective tissue disorders that appear in association with keratoconus also involve molecular alterations in PGs.[23–25]

In this study, we analyzed for the first time the differential expression of PGs by stromal cells from keratoconic and normal corneas. The study covers the genes encoding the PG core proteins and the enzymes involved in the biosynthesis of the GAG chains. We then examined keratoconic and normal corneas by immunohistochemistry to analyze the core proteins and GAG chains that were differentially expressed by the cultured cells.

Patients and Methods

Tissue Samples

All of the corneas used in this study were obtained from the Instituto Oftalmológico Fernández-Vega (IOFV, Oviedo, Spain) and from the Central University Hospital of Asturias (HUCA, Asturias, Spain). Informed oral and written consent of the patients was obtained, in accordance with the guidelines of the Tenets of the Declaration of Helsinki.

Isolation and Culture of Corneal Stromal Cells

Human central corneal tissue was obtained from cadaver donors and from penetrating keratoplasty interventions on patients suffering from keratoconus. Healthy donor tissues were screened and tested negative for HIV, hepatitis B and C viruses, and syphilis and were not usable for human corneal transplantation.

The epithelium was removed with ethanol (70%, 30 seconds) and a spatula, and the endothelium by Descemet membrane endothelial keratoplasty, after which the absence of epithelial and endothelial cells was assessed by microscopy. Corneal stromal cells were obtained by digesting 2-mm-diameter pieces from the central cornea in 0.25% trypsin/EDTA solution for 30 minutes at 37°C. After centrifugation, the supernatant was discarded, and the pellet was resuspended in a Dulbecco's modified Eagle's medium + F12 culture medium containing nonessential amino acids, RPMI 1640 Vitamin Solution 100×, 1% antibiotics (penicillin/streptomycin), and 10% fetal bovine serum (Gibco, Waltham, MA, USA). When cultures reached 80% confluence, they were replated at a density of 2×10^5 cells/mL in 75-cm^2 flasks. As a control to evaluate if the cells maintained a stable phenotype, we performed α-smooth muscle actin (SMA) immunostaining. Smooth muscle actin staining remained negative at least until the fifth passage, suggesting that the stromal cells did not readily differentiate to myofibroblasts. As a result, only cultures in passages 1 through 5 were used. In all studies carried out in this work, the different cell lines were analyzed separately.

Ribonucleic Acid Isolation and cDNA Synthesis

Ribonucleic acid was isolated from primary stromal cultures derived from three healthy corneal and three keratoconic cultures using the RNeasy kit (Qiagen, Hilden, Germany) and processed as previously described.[26] Complementary DNA synthesis was carried out using the High Capacity cDNA Transcription Kit (Applied Biosystems, Foster City, CA, USA). The reactions were performed and the products cleaned and stored as previously described.[26]

Quantitative Real-Time PCR Reactions and Data Analysis

Primer design (Supplementary Table S1), quantitative real-time PCR (qRT-PCR) reactions, and analysis of amplimer products was carried out as previously described.[26] Actin was used as the control gene to normalize individual gene expression.

The efficiencies of amplification were calculated using the program LinRegPCR (http://www.gene-quantification.de/download.html). The values of differential expression of the genes were expressed as described previously.[27] The nonparametric Wilcoxon test was used for the statistical analysis of the results. All analyses were performed using the program Statistics for Windows (Statsoft, Inc., Tulsa, OK, USA).

Immunocytochemistry and Immunohistochemistry

Tissue sections from the central cornea, prepared as previously described,[28] were dewaxed, and rehydrated sections were rinsed in PBS containing 1% Tween-20. Sections were heated in high pH Envision FLEX target retrieval solution (Dako, Glostrup, Denmark) at 65°C for 20 minutes and then incubated for 20 minutes at room temperature in the same solution. Endogenous peroxidase activity and nonspecific binding were blocked with 3% H_2O_2 and 33% fetal calf serum, respectively. The sections were incubated overnight at 4°C with 1:100 dilutions of the primary antibodies. Secondary antibodies were also used at a 1:100 dilution, and 3-3′ diaminobenzidine was used as a chromogen. Finally, samples were counterstained with hematoxylin, dehydrated, and mounted in Entellan (Merck Millipore, Billerica, MA, USA). The sections were studied and photographed under a light microscope (Eclipse 80i; Nikon Corporation, Tokyo, Japan).

Cell cultures were fixed in acetone at −20°C for 20 minutes, washed with PBS, and incubated with primary antibodies as described for the tissue sections. Fluorescent-conjugate secondary antibodies (Alexa 488 and Cy3) were used at 1:100 dilution and incubated with the fixed cells for 1 hour at room temperature in a dark humid chamber. Finally, samples were counterstained and mounted with 4′,6-diamidino-2-phenylindole (10 ng/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) diluted 1:1000 in fluoromount G (SouthernBiotech, Birmingham, AL, USA). Confocal immunofluorescence micrographs were captured with a TCS-SP2-AOBS confocal microscope (Leica Microsystems, Wetzlar, Germany).

We used the following antibodies: mouse monoclonal anti–syndecan-1 (CD138) from DakoCytomation (Carpinteria, CA, USA); rabbit polyclonal anti–syndecan-2 (M-140) and anti-keratocan (H-50) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse anti-endostatin monoclonal antibody and rabbit anti–glypican-4 polyclonal antibody from Thermo Scientific (Waltham, MA, USA); mouse anti-biglycan, rabbit anti-lumican monoclonal antibodies, rabbit anti–glypican 6, and rabbit anti–α-SMA polyclonal antibodies from Abcam (Cambridge, UK); rabbit anti-osteoglycin polyclonal antibody from USBiological (Salem, MA, USA); mouse anti-CS (CS-56) monoclonal antibody from Sigma-Aldrich Corp.; and mouse anti-HS epitopes 10E4, JM403 and 3G10 monoclonal antibodies from amsbio (AMS Biotechnology, Abingdon, UK). Anti-mouse (sc-2020) and anti-rabbit (sc-2004) secondary antibodies were also from Santa Cruz Biotechnology. Alexa fluor 488–conjugated goat anti-rabbit IgG was from Serotec (Oxford, UK), and CyTM3-conjugated donkey anti-mouse antibody was from Jackson-ImmunoResearch (Baltimore, MD, USA). Detection of HS epitope 3G10 required previous digestion with heparinase III (Sigma-Aldrich Corp.).

Results

Differential Expression of Genes Encoding Extracellular KS PGs

There are three KS PGs in the adult stromal ECM of the cornea: keratocan, lumican, and osteoglycin. All three genes were

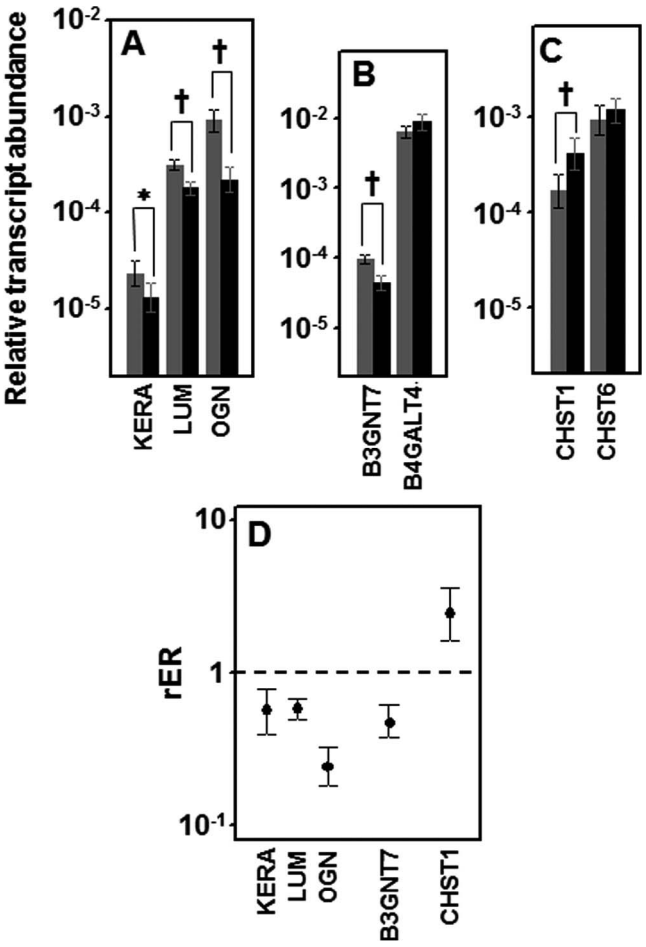

Figure 1. Differential transcription of genes encoding the core protein and the glycosaminoglycan chains of KS PGs by keratoconic and normal cells in culture. (**A**–**C**) Relative transcript abundance of mRNA. Relative abundance for cultured healthy stromal cells (*gray bars*) and keratoconic stromal cells (*black bars*) are plotted on a log scale for each gene assayed: (**A**) PG core proteins—keratocan (*KERA*), lumican (*LUM*), and osteoglycin (*OGN*); (**B**) KS glycosyltransferase genes—β-1,3-*N*-acetylglucosaminyltransferase 7 (*B3GNT7*) and β-1,4-galactosyltransferase (*B4GALT4*); and (**C**) KS sulfation genes—Gal-6-sulfotransferase (*CHST1*) and Gal-1-sulfotransferase (*CHST6*). (**D**) Relative expression ratios (rER) of genes that were significantly different between the keratoconic and normal cells in culture. Values on the *y*-axis on a logarithmic scale. $^*P < 0.02$; †$P < 0.001$. *Error bars* denote SDs.

underexpressed by the keratoconic primary stromal cultures. For keratocan and lumican, the transcription levels were approximately 50% of those in cultured cells from healthy corneas and approximately 25% for osteoglycin (Figs. 1A, 1D).

With regard to the enzymes involved in the synthesis of the GAG chains, no differences were apparent between the cultured keratoconic and normal stromal cells in the transcript levels of *B4GALT4*, which encodes a β-1,4-galactosyltransferase (Fig. 1B). On the other hand, *B3GNT7*, which encodes a β-1,3-*N*-acetylglucosaminyltransferase, was down-regulated more than 50% in the cultured keratoconic cells (Figs. 1B, 1D). Moreover, one of the two enzymes involved in the sulfation of saccharide chains, *CHST1*, was up-regulated approximately 2.4-fold in the cultured keratoconic stromal cells (Figs. 1C, 1D), whereas no alterations were detected for *CHST6*.

Determination of KS PG expressions by immunohistochemistry also displayed a decrease in the levels of the three core proteins (i.e., keratocan, lumican, and osteoglycin) in cultured stromal cells of keratoconus corneas (Figs. 2B, 2D, 2F) compared with cultured normal ones (Figs. 2A, 2C, 2E). The same results were obtained when the analyses were carried out using sections of keratoconus (Figs. 2H, 2J, 2L) and normal corneas (Figs. 2G, 2I, 2K).

Differential Expression of Genes Encoding Glycosyltransferases Involved in the Synthesis of the Common Linkage Region of HS and CS Chains

The first step in the synthesis of CS/DS and HS chains requires an array of glycosyltransferases (GTs) that generate a tetrasaccharide glycan linker shared by both GAGs (Fig. 3).[28] The transcription of three of the genes involved in the biosynthesis of the linker was altered in keratoconic stromal cells. The mRNA levels of genes involved in the transfer of the first xylose residue were deregulated, albeit in opposite directions: *XYLT1* was decreased more than 3.5-fold, whereas *XYLT2* was increased by a similar magnitude (Figs. 4A, 4B). Moreover, the transcription of *B3GALT6*, responsible for the transference of the second galactose residue, increased approximately 2-fold in the cultured pathologic cells. In addition, transcripts for only one of the three isoforms involved the transfer of the first glucuronate residue (GlcA), *B3GAT3*, could be detected, although there was no significant difference between the cultured keratoconic and normal cells (Fig. 4A). Moreover, neither *FAM20B* nor *PXYLP1*, both involved in the control of GAG chain formation through the phosphorylation and dephosphorylation of the xylose residues, appeared differentially transcribed.

Differential Expression of Genes Encoding Extracellular CS PGs

The CS PGs decorin and biglycan are important in the corneal stroma.[29] In the cultured keratoconic cells, the expression of decorin mRNA was not altered, but there was a 4-fold down-regulation of biglycan (Figs. 5A, 5D). With regard to the polymerization of CS chains, two GTs were significantly up-regulated. *CSGALNACT1*, which is involved in the transfer of the first *N*-acetyl-galactosamine residue (GalNAc), was increased approximately 3-fold, and *CHPF*, a chondroitin polymerizing factor, was increased approximately 7-fold (Figs. 5B, 5D).

Chondroitin sulfate/DS chains are modified to a lesser extent than those of HS (Fig. 2).[28] Two of the four genes encoding sulfotransferases for the sulfation of C4 on the GalNAc residue, *CHST12* and *-13*, were up-regulated approximately 2- and 6-fold, respectively, in the cultured keratoconic cells (Figs. 5C, 5D). More important seems to be the change that pathologic stromal cells undergo in the sulfation of GalNac C6. The genes involved in this reaction (i.e., *CHST15*, *-3*, and -7) were each overexpressed (Figs. 5C, 5D). For genes encoding enzymes that act on the GlcA residue, there was no difference in the expression of *UST*; however, there was a greater than 2-fold down-regulation of *DSE* in the cultured keratoconic cells (Figs. 5C, 5D).

Analysis of biglycan expression by immunohistochemistry showed a reduction of the staining in cultured cells (Figs. 6A, 6B) and in sections of the keratoconic corneas (Figs. 6E, 6F), indicating a correlation between the protein levels and the transcription data. Immunostaining with anti-CS antibodies showed a diffuse increase of staining in the pathologic corneas (Figs. 6G, 6H). This increase was also detected in the cultured cells (Figs. 6C, 6D).

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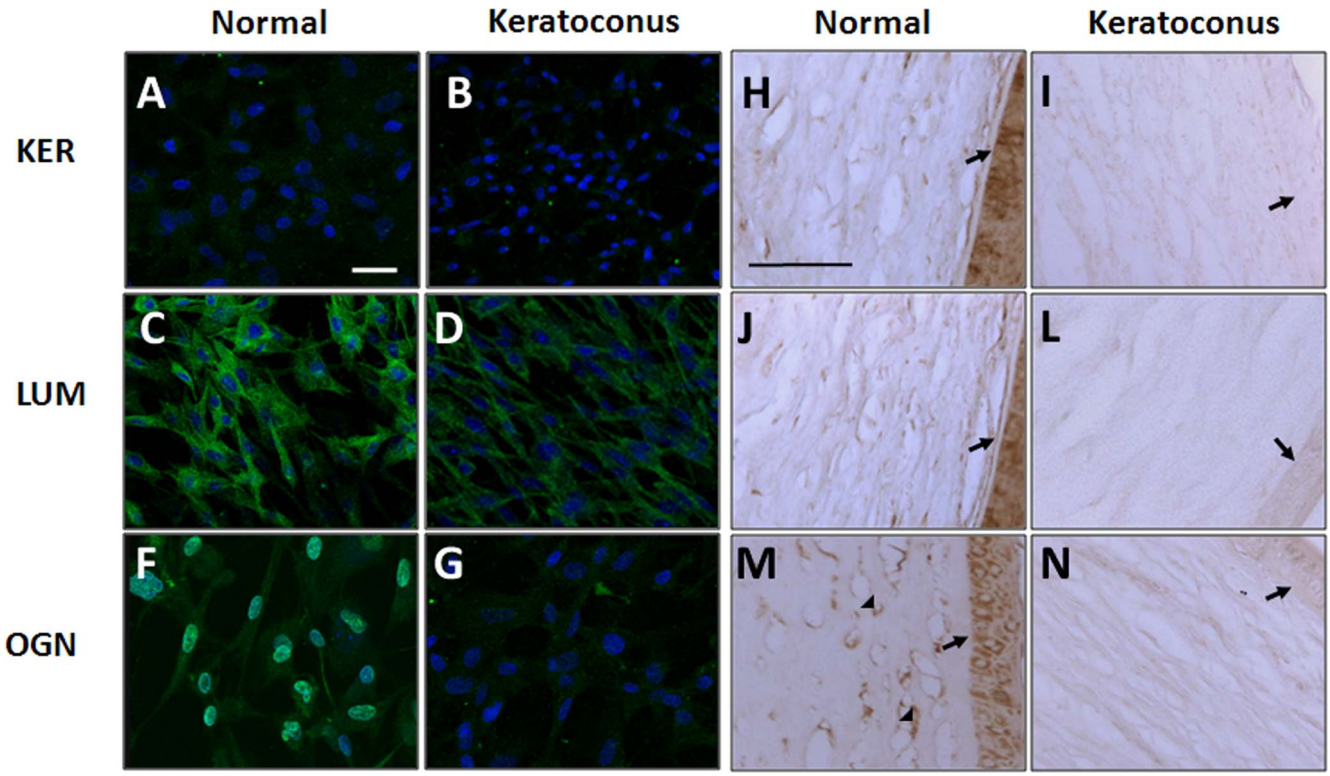

FIGURE 2. Immunohistochemistry of KS PG core proteins. The two columns on the *left* show the results obtained using cultured corneal stromal cells (*Scale bar*: 50 μm), whereas the two columns on the *right* show the results using corneal sections (*Scale bar*: 100 μm). (**A**, **B**, **G**, **H**) Keratocan. (**C**, **D**, **I**, **J**) Lumican. (**E**, **F**, **K**, **L**) Osteoglycin. *Arrows* show the epithelium. *Arrowheads* show increased staining.

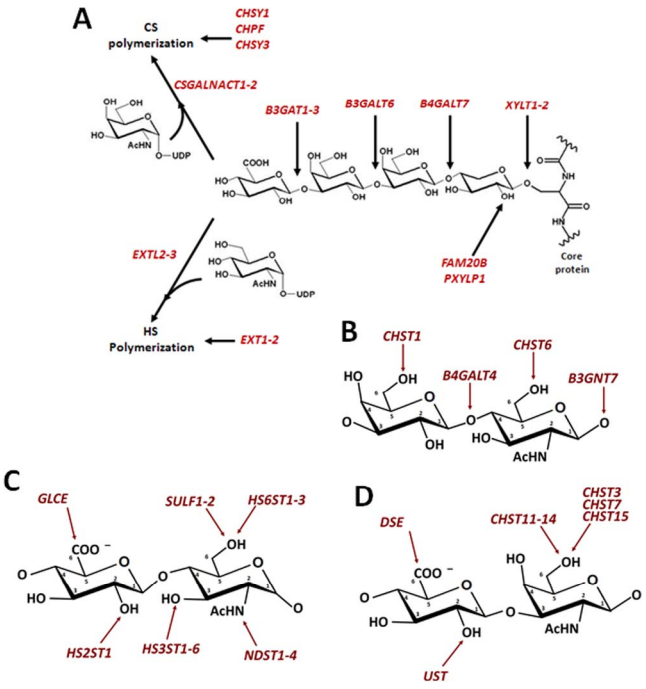

FIGURE 3. Genes involved in GAG biosynthesis. (**A**) Initiation and elongation of HS and CS/DS chains; (**B**) KS disaccharide; (**C**) HS disaccharide; and (**D**) CS disaccharide. The different genes for enzymes that catalyze the specific reactions are highlighted in *red*.

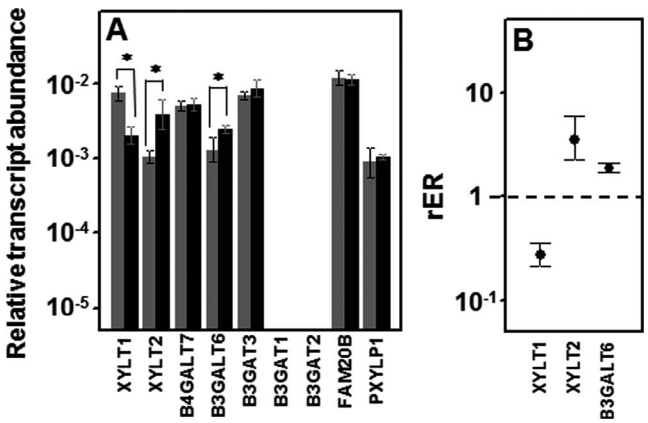

FIGURE 4. Differential transcription of genes involved in the biosynthesis of the tetrasaccharide glycan linker of HS and CS/DS saccharidic chains. (**A**) Relative transcript abundance of mRNA. Relative abundance for cultured healthy stromal cells (*gray bars*) and keratoconic stromal cells (*black bars*) are plotted on a log scale for each gene assayed. Xylosyltransferase I (*XYLT1*), xylosyltransferase II (*XYLT2*), β-1,4-galactosyltransferase I (*B4GALT7*), β-1,3-galactosyltransferase II (*B3GALT6*), β-1,3-glucuronyltransferase 3 (*B3GAT3*), β-1,3-glucuronyltransferase 1 (*B3GAT1*), β-1,3-glucuronyltransferase 2 (*B3GAT2*), xylosylkinase (*FAM20B*), and 2-phosphoxylose phosphatase 1 (*PXYLP1*). (**B**) Relative expression ratio of genes that were significantly different between the keratoconic and normal cells in culture. Values on the *y*-axis are on a logarithmic scale. $^*P < 0.001$. *Error bars* denote SDs.

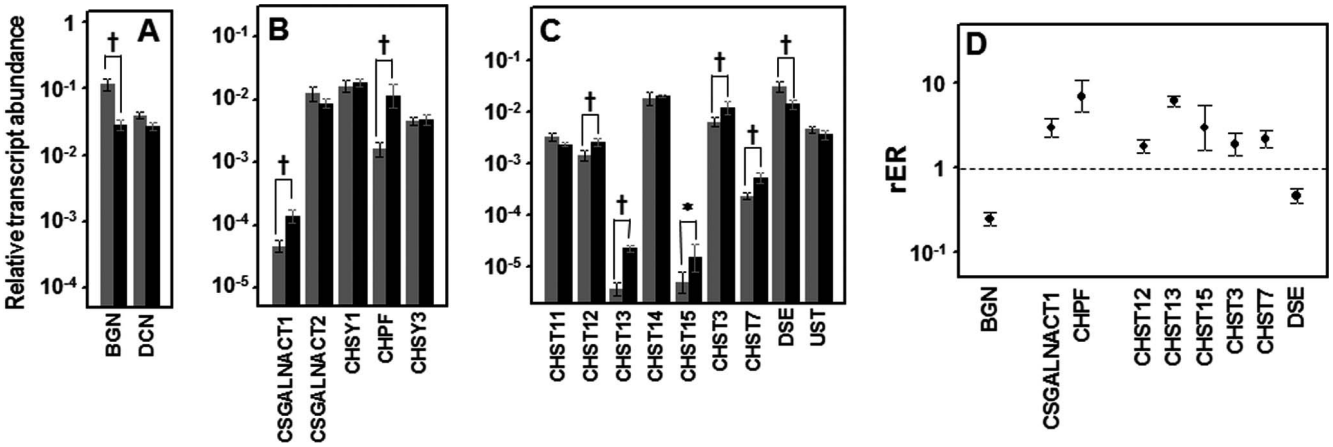

Figure 5. Differential transcription of genes encoding the core protein and the glycosaminoglycan chains of CS PGs. (**A**–**C**) Relative abundance for cultured healthy stromal cells (*gray bars*) and keratoconic stromal cells (*black bars*) are plotted on a log scale for each gene assayed. Genes that displayed significant differences in their transcription levels are highlighted: (**A**) PG core proteins—biglycan (*BGN*) and decorin (*DCN*); (**B**) CS chain polymerization genes—*N*-acetylgalactosaminyltransferase 1 (*CSGALNACT1*), *N*-acetylgalactosaminyltransferase 2 (*CSGALNACT2*), chondroitin sulfate synthase 1 (*CHSY1*), chondroitin polymerizing factor (*CHFP*), and chondroitin sulfate synthase 3 (*CHSY3*); and (**C**) genes involved in the modification of CS chains—chondroitin 4 sulfotransferase 11 (*CHST11*), chondroitin 4 sulfotransferase 12 (*CHST12*), chondroitin 4 sulfotransferase 13 (*CHST13*), chondroitin 4 sulfotransferase 14 (*CHST14*), *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (*CHST15*), chondroitin 6 sulfotransferase 3 (*CHST3*), *N*-acetylglucosamine 6-*O* sulfotransferase 7 (*CHST7*), dermatan sulfate epimerase (*DSE*), and uronyl-2-sulfotransferase (*UST*). (**D**) Relative expression ratio of genes that were significantly different between the keratoconic and normal cells in culture. Values on the *y*-axis are on a logarithmic scale. $^*P = 0.004$; †$P < 0.001$. *Error bars* denote SDs.

Differential Expression of Genes Encoding Cellular and Pericellular HS PGs

Heparan sulfate PGs occur both in cells and in the pericellular matrix.[30] The syndecan and the glypican gene families (*SDC1-4* and *GPC1-6*) account for most cell surface HS PGs. In the cultured keratoconic stromal cells, syndecan-1 and -2 were underexpressed approximately 2- and 2.4-fold, respectively (Figs. 7A, 7D). Within the glypican group, two isoforms, *GPC4* and *GPC6*, were down-regulated in the cultured keratoconic stromal cells approximately 50- and 2.6-fold, respectively (Figs. 7A, 7D). Other cell-associated HS PGs include betaglycan (*TGFBR3*), CD44v3, and neuropilin-1 (*NRP1*) at the cell membrane and serglycin, located intracellularly.[8] None of these showed any detectable differences between the cultured keratoconic and normal stromal cells. Of the pericellular HS PGs, only collagen XVIII was significantly up-regulated in the cultured keratoconic cells, approximately 2-fold (Figs. 7A, 7D).

Only one of the enzymes involved in HS chain polymerization, *EXT2* (Fig. 3), was modified in the keratoconic cells, with a more than 2-fold up-regulation (Figs. 7B, 7D). As the HS chain polymerizes, it undergoes a series of modifications. The first reaction involves N-deacetylation and N-sulfation of *N*-acetylglucosamine residues, catalyzed by four different N-deacetylase/N-sulfotransferase (NDST) isoforms (NDST1-4).[10,11] *NDST2* was up-regulated approximately 2.8-fold in the keratoconic corneas, whereas *NDST3* was overexpressed more

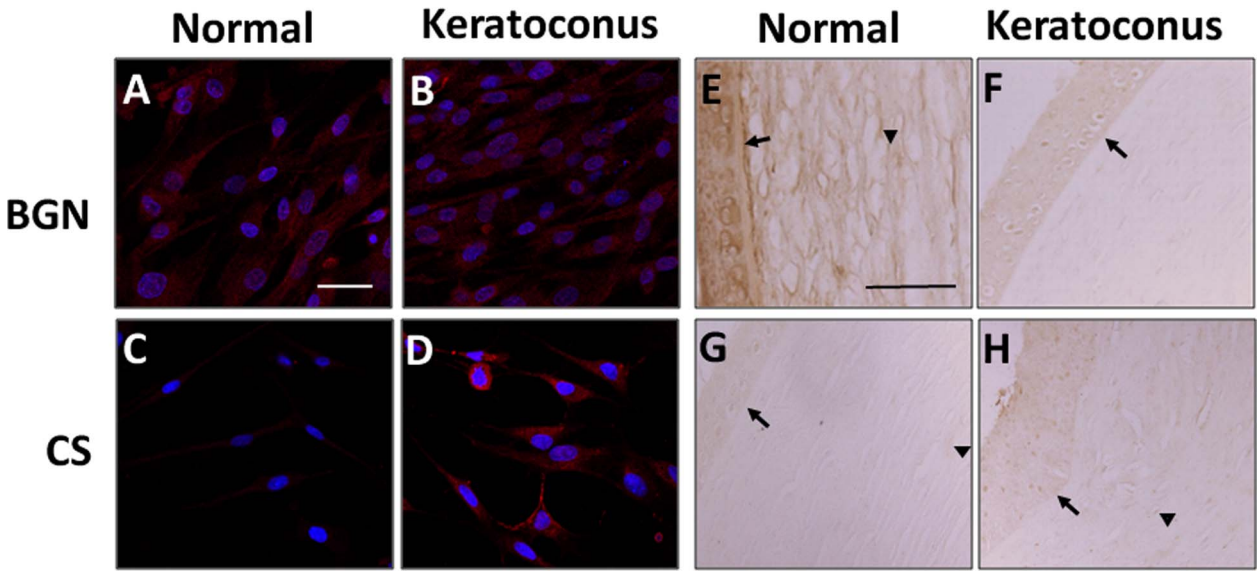

Figure 6. Immunohistochemistry of CS PG core proteins and CS saccharidic chains. The two columns on the *left* show the results obtained using cultured corneal stromal cells (*Scale bar*: 50 μm), whereas the two columns on the *right* show the results using corneal sections (*Scale bar*: 100 μm). (**A**, **B**, **E**, **F**) Biglycan. (**C**, **D**, **G**, **H**) Chondroitin sulfate. *Arrows* show the epithelium. *Arrowheads* show increased staining.

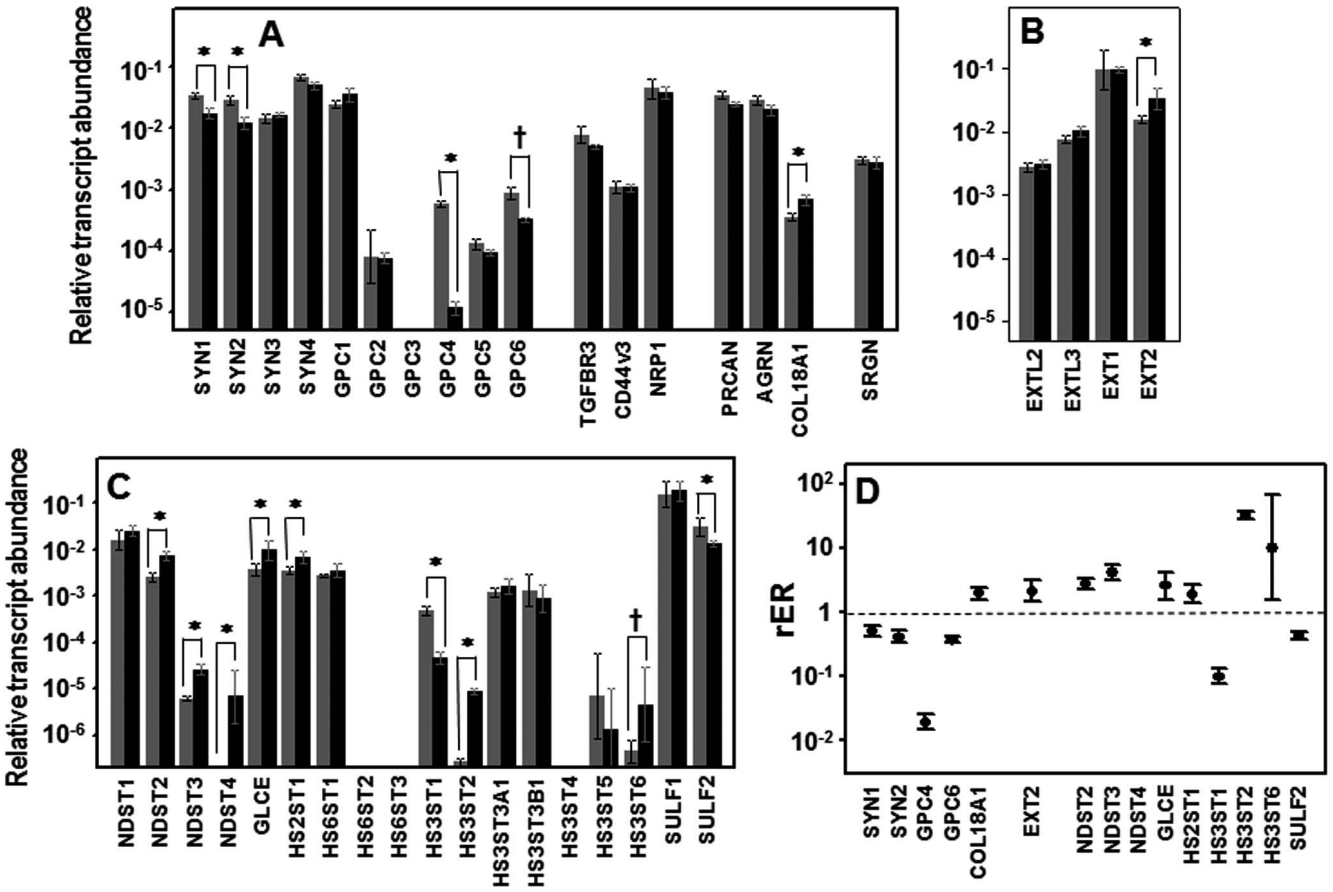

Figure 7. Differential transcription of genes encoding the core protein and the glycosaminoglycan chains of HS PGs. (**A**–**C**) Relative abundance for cultured healthy stromal cells (*gray bars*) and keratoconic stromal cells (*black bars*) are plotted on a log scale for each gene assayed: (**A**) PG core proteins—syndecan-1 (*SYN1*), syndecan-2 (*SYN2*), syndecan-3 (*SYN3*), syndecan-4 (*SYN4*), glypican-1 (*GPC1*), glypican-2 (*GPC2*), glypican-3 (*GPC3*), glypican-4 (*GPC4*), glypican-5 (*GPC5*), glypican-6 (*GPC6*), betaglycan (*TGFBR3*), CD44 isoform 3 (*CD44v3*), neuropilin 1 (*NRP1*), perlecan (*PRCAN*), agrin (AGRN), collagen XVIII (*COL18A1*), and serglycin (SRGN); (**B**) HS chain polymerization genes—xylosylkinase (*FAM20B*), exostosin-like glycosyltransferase 2 (*EXTL2*), exostosin-like glycosyltransferase 3 (*EXTL3*), exostosin glycosyltransferase 1 (*EXT1*), and exostosin glycosyltransferase 2 (*EXT2*); and (**C**) genes involved in the modification of HS chains—*N*-deacetylase/*N*-sulfotransferase 1 (*NDST1*), *N*-deacetylase/*N*-sulfotransferase 2 (*NDST2*); *N*-deacetylase/*N*-sulfotransferase 3 (*NDST3*), *N*-deacetylase/*N*-sulfotransferase 4 (*NDST4*), glucuronic acid epimerase (*GLCE*), 2-*O*-sulfotransferase 1 (*HS2ST1*), 6-*O*-sulfotransferase 1 (*HS6ST1*), 6-*O*-sulfotransferase 2 (*HS6ST2*), 6-*O*-sulfotransferase 3 (*HS6ST3*), 3-*O*-sulfotransferase 1 (*HS3ST1*), 3-*O*-sulfotransferase 2 (*HS3ST2*), 3-*O*-sulfotransferase 3A1 (*HS3ST3A1*), 3-*O*-sulfotransferase 3B1(*HS3ST3B1*), 3-*O*-sulfotransferase 4 (*HS3ST4*), 3-*O*-sulfotransferase 5 (*HS3ST5*), 3-*O*-sulfotransferase 6 (*HS3ST6*), sulfatase 1 (*SULF1*), and sulfatase 2 (*SULF2*). (**D**) Relative expression ratio of genes that were significantly different between the keratoconic and normal cells in culture. Values on the *y*-axis are on a logarithmic scale. $^*P < 0.001$; $\dagger P = 0.003$. *Error bars* denote SDs.

than 4-fold (Figs. 7C, 7D). Interestingly, *NDST4* was undetectable in normal cultured cells but was expressed at low levels in the cultured keratoconic cells.

Further modifications of the HS chain include the epimerization of GlcA into iduronate and the addition of sulfate groups at C2, catalyzed by enzymes encoded by *GLCE* and *HS2ST1*. Both genes were up-regulated approximately 2.5- and 2-fold, respectively (Figs. 7C, 7D). The subsequent reaction involves the sulfation of glucosamine residues at C6, catalyzed by 6-*O*-sulfotransferase isoforms 1-3 (*HS6ST1-3*).[10,11] Only transcripts for *HS6ST1* could be detected, and there were no significant differences between the two groups. Interestingly, however, the transcription of one of the two cell surface sulfatases that remove GlcN-6S groups from specific regions, *SULF2*, was down-regulated in the keratoconic corneas (Figs. 7C, 7D).

The last step in the synthesis involves the addition of a sulfate group at C3 of glucosamine, catalyzed by 3-*O*-sulfotransferases (*HS3ST1-6*).[10,11] Significant differences in expression were detected for *HS3ST1*, which was down-regulated approximately 10-fold in the cultured keratoconic cells, and for *HS3ST2*, which was barely detectable in the cultured stromal cells from healthy corneas but strongly up-regulated in cultured keratoconic cells (Figs. 7C, 7D). Additionally, *HS3ST6*, despite having high variability between the different cultured cells, was significantly up-regulated, approximately 12.5-fold, in keratoconic cells (Figs. 7C, 7D).

The PG core proteins that displayed differential transcriptions were analyzed by immunohistochemistry using cultured cells and corneal sections. Both syndecan-1 and -2 had a weak focal expression in the normal cultured stromal cells (Figs. 8A, 8C) and in the corneal stromas (Figs. 8K, 8M). This level of expression was reduced in the cultured keratoconus samples (Figs. 8B, 8D) and in the corneas (Figs. 8L, 8N), although the low intensity of the signal made it difficult to detect in the cornea. A diffuse reduction of glypican-4 expression was present in the cultured keratoconic cells (Fig. 8F) and in the corneas (Fig. 8P) compared with controls (Figs. 8E, 8O, respectively). In contrast, glypican-6 was sharply labeled in cultures of healthy stromal cells (Fig. 8G) and corneas (Fig. 8Q), but it was absent in keratoconic ones (Figs. 8H, 8R,

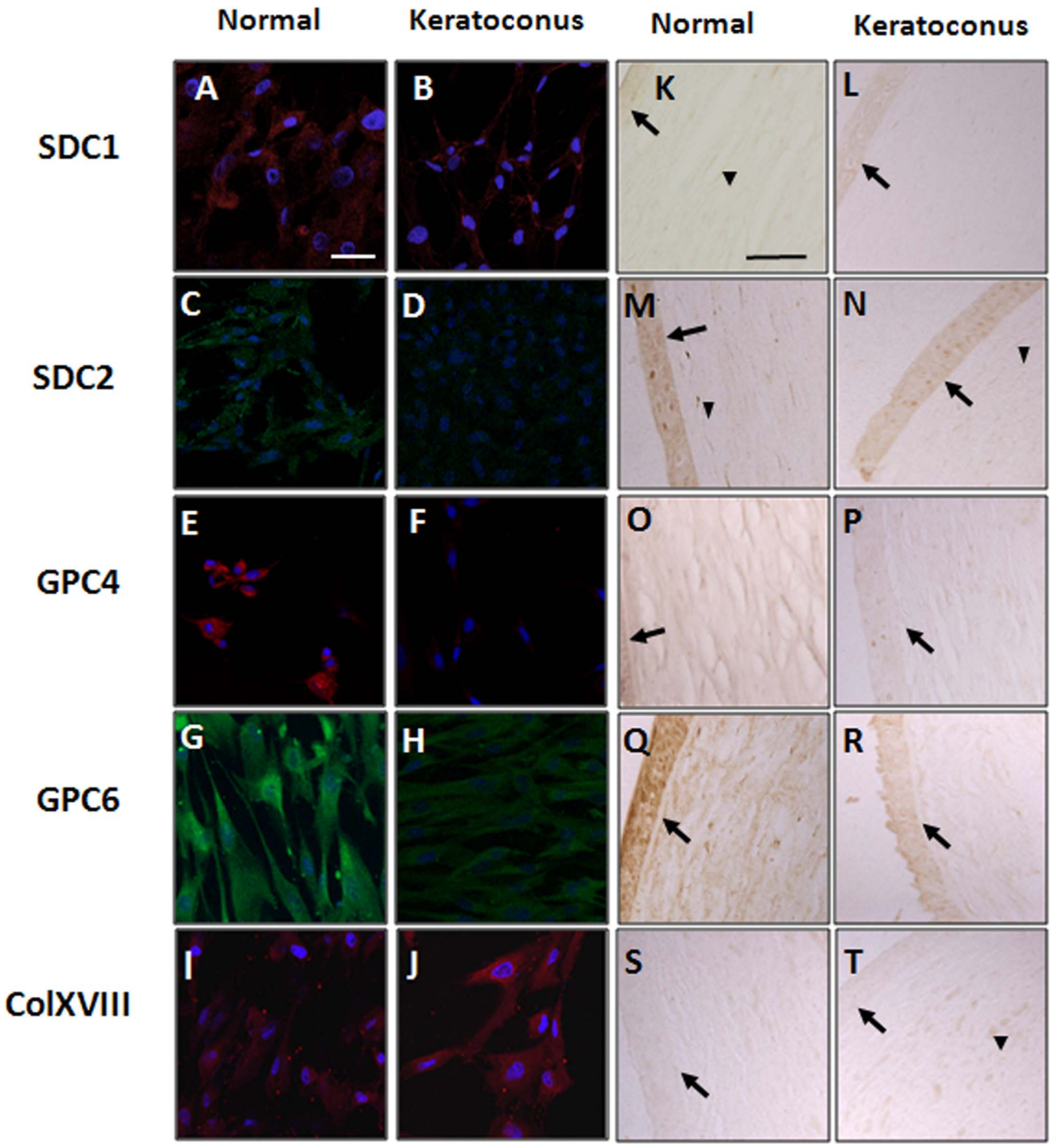

Figure 8. Immunohistochemistry of HS PG core proteins and HS saccharidic chains. The two columns on the *left* show the results obtained using cultured corneal stromal cells (*Scale bar*: 50 μm), whereas the two columns on the *right* show the results using corneal sections (*Scale bar*: 100 μm). (**A**, **B**, **K**, **L**) Syndecan-1. (**C**, **D**, **M**, **N**) Syndecan-2. (**E**, **F**, **O**, **P**) Glypican-4. (**G**, **H**, **Q**, **R**) Glypican-6. (**I**, **J**, **S**, **T**) Collagen XVIII. *Arrows* show the epithelium. *Arrowheads* show increased staining.

respectively). Furthermore, collagen immunostaining was higher in the keratoconic stromal cultures (Fig. 8J) and corneas (Fig. 8T) than in the controls (Figs. 8I, 8S), consistent with the differential transcription data.

Because the results showed changes in the transcription levels of several enzymes involved in HS biosynthesis, we deduced that the levels and fine structure of HS chains could also be altered. We therefore analyzed the distribution of different HS epitopes in cultured cells and corneal sections using immunohistochemistry. Immunodetection of 3G10 epitope was higher in both the keratoconic stromal cultures (Fig. 9B) and corneas (Fig. 9H) than in the respective controls (Figs. 9A, 9G). Epitope JM403 immunostaining was reduced in the cultured keratoconus samples (Figs. 9C, 9D), although the corneas displayed low intensity of the signal (Figs. 9I, 9J). Finally, immunodetection of the 10E4 epitope was higher in the keratoconic stromal cultures (Fig. 9F) and corneas (Fig. 9L) than in their respective controls (Figs. 9E, 9K).

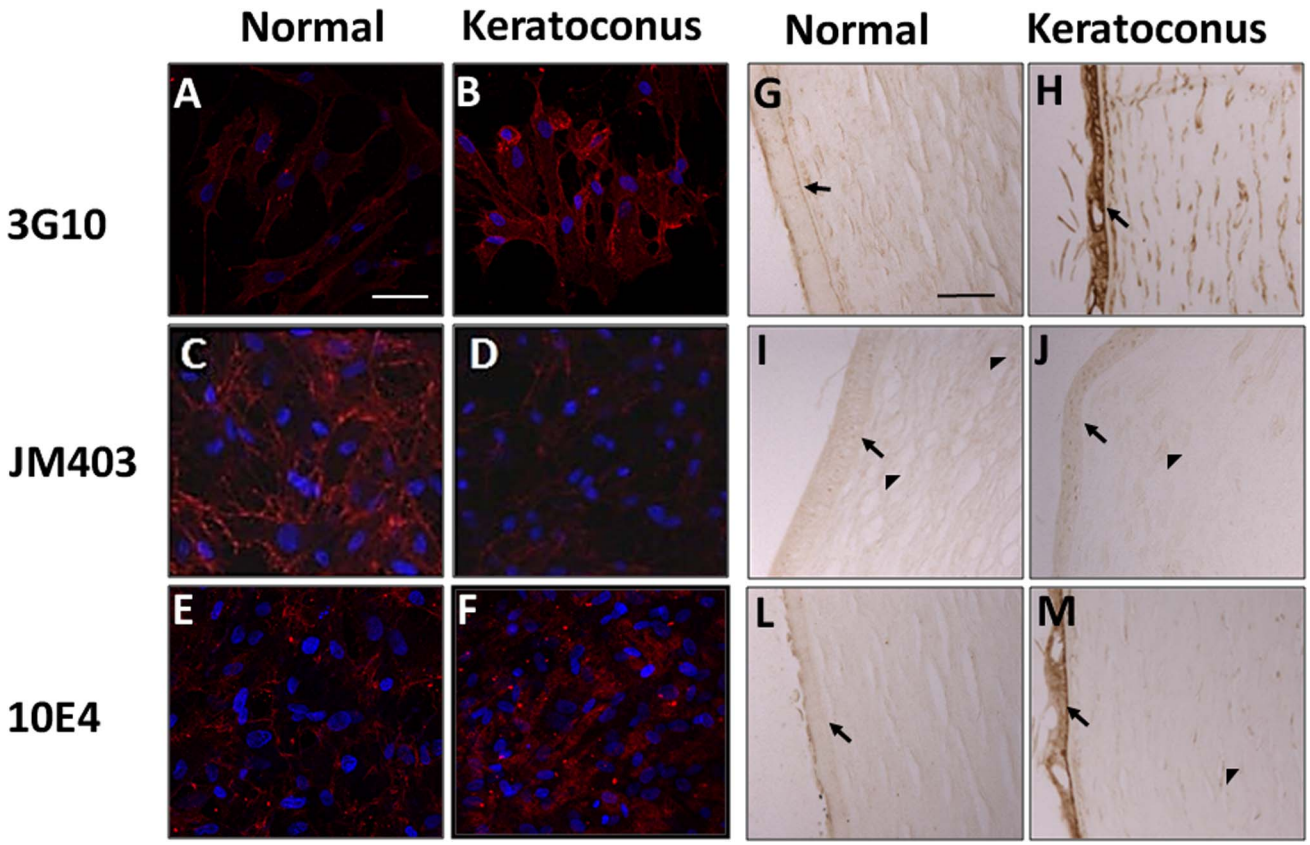

FIGURE 9. Immunohistochemistry of HS saccharidic chains. The two columns on the *left* show the results obtained using cultured corneal stromal cells (*Scale bar*: 50 μm), whereas the two columns on the *right* show the results using corneal sections (*Scale bar*: 100 μm). (**A**, **B**, **G**, **H**) Epitope 3G10 (neo-epitope generated by digesting HS with heparinases). (**C**, **D**, **I**, **J**) Epitope JM403 (includes N-unsubstituted glucosamine residues). (**E**, **F**, **K**, **L**) Epitope 10E4 (includes N-sulfated glucosamine residues). *Arrows* show the epithelium. *Arrowheads* show increased staining.

DISCUSSION

In this study, we quantified the transcription levels of the genes encoding all of the PGs and GAGs present in the cornea. This analysis was conducted in cultured corneal stromal cells from keratoconus patients compared with similarly prepared cells from normal corneas. Although some keratocytes cultured in media containing fetal bovine serum can become myofibroblasts,[6] neither healthy nor keratoconic cells in our cultures stained positively for α-SMA until the fifth passage, suggesting that the stromal cell phenotype remained stable through at least four passages. The expression products of the genes that displayed transcription changes, including core proteins and GAG chains, were analyzed by immunohistochemistry in cultured cells and in paraffin-embedded corneal sections for in situ comparisons.

Keratan sulfate is the major GAG in the cornea, and the expression of the three core proteins to which it is complexed (i.e., keratocan, lumican, and osteoglycin) was down-regulated in cell cultures derived from keratoconic corneas and in the corneas themselves as shown by immunohistochemistry. This decrease has also been described previously in some proteomic studies.[1] It is consistent with the thinning and ectasia of the cornea due to ECM degradation that itself may be due to the transcriptional down-regulation of core protein production.[3] Notwithstanding, some studies have detected increased levels of certain KS PGs in keratoconus,[31] probably due to methodologic differences in the analysis.[32] Interestingly, keratocan and lumican have been described to strongly inhibit collagen cross-linking, a clinical treatment that targets the stroma in progressive keratoconus.[33]

Chondroitin sulfate/DS are present mainly in the corneal stroma as part of decorin and biglycan, although some HS PGs may also carry them.[34,35] In this study, biglycan was down-regulated, as also found in previous studies,[20] and although, decorin mRNA levels remained unaltered here, other works have described increased[31] or decreased[3] levels in keratoconus.

With regard to the synthesis of KS chains, there was a decrease in mRNA levels of *B3GNT7*, along with an increase in *CHST1*, suggesting the existence of alterations in the chains that affect both the extension and the sulfation of the polysaccharide (Fig. 3). Although there are no previous studies specifically focusing on the transcript levels of these genes, the changes in *B3GNT7* and *CHST1* expression could be related to previously reported changes in the KS in keratoconus.[19,22,36]

For CS, there were different alterations that affect the polymerization and the chemical modification of the chains. The initiation of CS chain polymerization is shared with the synthesis of HS and involves the formation of a tetrasaccharide linkage. The mRNA levels of the two genes involved in the transfer of the xylose residue, *XYLT1* and *XYLT2*, displayed opposite alterations in the cultured keratoconic cells. The two xylosyltransferases encoded by these genes differ in their catalytic efficiency and expression patterns,[37] and modulation of their expressions is a regulator of GAG synthesis in some pathologies.[38] Moreover, *B3GALT6*, *CSGALNACT1*, and *CHPF*, which encode, respectively, the transferase for the second

galactose residue of the linker, the transferase that directs the pathway toward CS synthesis, and a chondroitin polymerizing factor, were all increased in the cultured keratoconic cells.

The CS backbone can be chemically modified in different ways. The GalNAc residue can be sulfated at C4 or at C6, and the uronic acid residue can be epimerized and sulfated at C2. Transcription of the gene encoding the epimerase (*DSE*) was down-regulated, which could affect the conversion of CS into its steroisomer DS. Moreover, the three genes involved in C6 sulfation were overexpressed, as were two of the four genes implicated in C4 sulfation. Interestingly, 4-O-sulfation has been related to the control of the CS chain length because cooperation between the enzymes encoded by *CSGALNACT1* and *CHST12* increases the number of CS chains.[39] Both genes were overexpressed in the cultured keratoconic cells.

Taken together, these results, for the first time, provide evidence that keratoconic cells possess modified CS chains. These changes affect expression levels and epimerization and sulfation patterns. In support of these data, immunochemical detection of the CS chains was increased in cell cultures and in corneal sections from keratoconic corneas.

Small leucine-rich PGs are essential to maintain the corneal structure, because they interact with collagen fibrils via their protein cores. They also extend the GAG chains, interacting with other GAG chains; thus, they organize the matrix and preserve corneal transparency.[40] Making stable antiparallel connections between GAG chains by means of noncovalent bonds requires domains that are long enough and is dependent on structural features like epimerization and sulfation.[40] Additionally, positively charged ions drawn by the charges on the GAG chains may create local concentration gradients that attract water molecules that participate in the control of corneal structure.[40] Therefore, the alterations described may constitute the molecular basis for some of the features of keratoconus such as changes in collagen fibril orientation and corneal clouding.[2]

At the cell surface or in the pericellular matrix, HS PGs are the most prevalent molecules, and although it has been suggested that they are required for maintaining corneal homeostasis,[41,42] there are no previous studies we are aware of which focus on them in keratoconus.[8] HS PGs are ubiquitous in tissues and mediate a variety of normal and pathologic functions.[10,11,43] Two gene families, syndecans and glypicans, account for most cell surface HS PGs.[30] In our study, mRNA levels of syndecan-1 and -2 and glypican-4 and -6 were down-regulated in cultured keratoconic cells, whereas transcripts for other cell-associated HS PGs did not appear to suffer any alteration. In contrast, collagen XVIII mRNA and protein, mainly located at the pericellular matrix, were up-regulated.

Analysis of the transcription levels of the GTs involved in the polymerization of the saccharidic chain showed up-regulation for only *EXT2*. However, 50% of the genes responsible for the HS fine structure displayed extensive alterations. NDSTs are essential for the creation of sulfated functional S-domains,[10,11] and the transcription of *NDST2*, *-3,* and *-4* was increased in cultured keratoconic cells. Notably, NDST3 and NDST4 are expressed primarily during embryonic development and in some pathologic conditions.[26,44] Epimerization and C2 sulfation of uronic acid residues are up-regulated, as well as sulfation at C6 of glucosamine due to under expression of the endosulfatase *SULF2*. Interestingly, the transcription of three genes responsible for C3 sulfation, *HS3ST1*, *-2*, and *-6*, involved in the formation of rare HS motifs that interact in a selective manner with protein ligands,[45] experienced specific alterations.

Analysis of the HS structure by immunohistochemistry showed that in both keratoconic stromal cultures and corneas, the expression of epitopes 3G10 and 10E4 increased, whereas the epitope JM403 decreased in the cultured cells and appeared weakly expressed in the corneas. 3G10 is a neo-epitope generated by digestion with heparinase III, which includes a desaturated uronate residue,[46] 10E4 is a native HS epitope that includes N-sulfated glucosamine residues,[46] and the JM403 epitope includes N-unsubstituted glucosamine residues.[47] These data therefore suggest the existence of an increase in heparan sulfate chains and their levels of sulfation.

Collectively, these results suggest that HS PGs undergo significant changes in the cells of the keratoconic corneal stroma. These changes could affect specific core proteins, mainly located on the cell surfaces, as well as the HS chains. The mechanism of action of HS includes specific, noncovalent interaction with many different ligands.[11] Through this variety of interactions, HS PGs control many physiological functions.[11] The observed differences can constitute the basis for the abnormalities described in stromal keratocytes,[48] as well as affect the interactions between keratocytes and matrix components that contribute to the regulation of stromal matrix assembly.[49]

Conclusions

In summary, analysis of differential transcription of the genes involved in the biosynthesis of PGs in cultured keratoconic stromal cells identified important changes affecting both core proteins and GAG chains. The changes were in genes that regulate the polymerization of the chains and the modifications by epimerization, N-sulfation, and O-sulfation. Interestingly, analyses of the proteomic and gene expression patterns of keratoconic corneas have detected the deregulation of several signal molecules that function through specific interactions with HS PGs.[1,30] These data suggest that a relationship exists between the alteration in the expression of ligands and their receptors during the development of keratoconus.

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