Research Article

Heparanase Overexpresses in Keratoconic Cornea and Tears Depending on the Pathologic Grade

Beatriz García,1,2 Olivia García-Suárez,1,3 Jesús Merayo-Lloves,1 Guilherme Ferrara,1 Ignacio Alcalde,1 Javier González,4 Carlos Lisa,1 Jose F. Alfonso,1 Fernando Vazquez,1,2,5 and Luis M. Quirós1,2

1Instituto Universitario Fernández Vega, Universidad de Oviedo & Fundación de Investigación Oftalmológica, Oviedo, Spain
2Department of Functional Biology, University of Oviedo, 33006 Oviedo, Spain
3Department of Morphology and Cell Biology, University of Oviedo, 33006 Oviedo, Spain
4Department of Organic and Inorganic Chemistry, University of Oviedo, 33006 Oviedo, Spain
5Department of Microbiology, Hospital Universitario Central de Asturias, Oviedo, Spain

Correspondence should be addressed to Luis M. Quirós; quirosluis@uniovi.es

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Background. Keratoconus has classically been defined as a noninflammatory disorder, although recent studies show elevated levels of inflammatory markers suggesting that keratoconus could be, at least in part, an inflammatory condition. Heparanase upregulation has been described in multiple inflammatory disorders. In this article, we study the differential expression of heparanase in cornea and tears from keratoconus patients and healthy controls. Methods. A transcriptomic approach was used employing quantitative polymerase chain reaction to analyze the expression of heparanase and heparanase 2 in stromal and epithelial corneal cells. The protein expression was analyzed by immunohistochemistry in corneal sections. Enzymatic activity in tears was measured using [3H]-labeled heparan sulfate as substrate. Results. Heparanase transcription was detected in stromal and epithelial cells and appeared upregulated in keratoconus. Overexpression of the enzyme was also detected by immunohistochemistry. Corneal expression of heparanase 2 was detected in some cases. Heparanase catalytic activity was found in tears and displayed a positive correlation with the degree of keratoconus. Conclusions. Heparanase overexpresses in keratoconic corneas, possibly reinforcing the inflammatory condition of the pathology. The presence of heparanase activity in tears allows us to propose its use as a biomarker for the diagnosis of the disorder.

1. Introduction

Keratoconus is a corneal ectasia that results in the cornea taking on a conical shape, causing severe astigmatism, scarring, and, for one in five patients, ultimate loss of vision and the need for corneal transplants [1].

Histologically, keratoconus displays many abnormal features which affect different layers of the cornea, including abnormal epithelial and stromal keratocyte shape, local thickening of the epithelium, Bowman’s layer breakage, and thinning of the stroma [2, 3]. Keratoconus is likely a multifactorial, multigenetic disorder with complex inheritance patterns, and environmental factors probably play an equally important role in disease causation [4]. Although keratoconus has traditionally been viewed as a noninflammatory disease, reports of the presence of certain inflammatory mediators in keratoconus patients has led some authors to suggest that inflammation plays a role in the onset or progression of the pathology [5].

Proteoglycans (PGs) are a diverse group of glycoconjugates composed of various core proteins posttranslationally modified with linear, anionic polysaccharides called glycosaminoglycans (GAGs), consisting of repeating disaccharides. Heparan sulfate proteoglycans (HSPGs) comprise a reduced...
molecule is modified N-acetylated-D-glucosamine. At various positions, the sulfate (HS) GAG chains. HS is a complex biopolymer ini-

Disease Markers

2. Methods

2.1. Materials. The following materials were purchased from the manufacturers indicated: RNaseasy Kit and RNase-Free DNase from Qiagen (Hilden, Germany); High-Capacity cDNA Reverse Transcription Kit and Power SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA); GenElute PCR clean-up kit, 3-3’-diaminobenzidine, heparinase I and III blend from Flavobacterium heparinum, and heparan sulfate from Sigma-Aldrich (St. Louis, MO); EnVision™ Flex/HRP and Envision FLEX target retrieval solution of high pH from Dako (Glostrup, Denmark); cellulose acetate filter paper with pore size of 0.22 μm was purchased from Sartorius Stedim (Göttingen, Germany); tetracaine hydrochloride and oixibuprocin hydrochloride were from Alcon Cusi (Barcelona, Spain); DMEM + F12 culture medium containing nonessential amino acids, RPMI 1640 Vitamin Solution 100x, 1% antibiotics (penicillin/streptomycin), and fetal bovine serum from Gibco (Waltham, MA); HiTrap Desalting column, superose 12, and [3H]Acetic anhy-

2.2. 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 virus, and syphilis and were not usable for human corneal transplantation.

The epithelium was removed with ethanol (70%, 30 s) 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/ethylenediaminetetraacetic acid solution for 30 min at 37°C. After centrifugation, the supernatant was discarded and the pellet resuspended in a DMEM + F12 culture medium containing nonessential amino acids, RPMI 1640 Vitamin Solution 100x, 1% antibiotics (penicillin/streptomycin), and 10% fetal bovine serum. When cultures reached 80% confluence, they were replated at a density of 2 × 10^5 cells/ml in 75 cm² polystyrene flasks and incubated at 37°C in a 5% (v/v) CO₂ atmosphere. As a control to evaluate whether cells maintained a stable phenotype, we performed alpha-smooth muscle actin immunostaining; this staining remained negative at least until the fifth passage, suggesting that the stromal cells did

2.2.1. Expression Patterns. In this paper, we investigated the expression patterns of heparanase genes in the keratoconus cornea in comparison to healthy controls. The study analyzes both the transcription and the protein levels in the corneal tissues using qRT-PCR and immunohistochemistry. Taking into account that the tear proteome displays a highly dynamic character, we investig-

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not readily differentiate to myofibroblasts. As a result, only cultures in passages 1–5 were used. In all studies carried out in this work, the different cell lines were analyzed separately.

2.3. Obtaining Corneal Epithelial Samples by Impression Cytology. All the samples used in this study were obtained from patients (n = 8) from the Instituto Oftalmológico Fernández-Vega (IOFV, Asturias, Spain) and from healthy volunteers (n = 5). Informed oral and written consent of the patients and volunteers was obtained under a protocol approved by the Ethical Committee of the IOFV in accordance with the guidelines of the Tenets of the Declaration of Helsinki.

Prior to impression, one drop of local anesthetic (0.1% tetracaine hydrochloride and 0.4% oxibuprocain hydrochloride) was instilled into the eye and excessive tear fluid and medication were wiped away. Next, a 6 mm disc of cellulose acetate paper was applied gently onto the corneal surface with the aid of sterile blunt forceps. The paper was allowed to remain in contact with the central cornea for 5 seconds, peeled off, turned over, and the other side applied for an additional period of 5 seconds. The paper was immediately transferred into RNase-free tubes and rapidly frozen and stored at −80°C.

2.4. Obtaining Tear Fluid Samples. Tear fluid was obtained from patients (n = 42) and healthy volunteers (n = 11) with the same range of ages by using a sterile capilar tube and applying gentle suction with a syringe. The fluid was immediately transferred into sterile tubes containing 20% of the final volume in glycerol and rapidly frozen and stored at −80°C. A minimum of 10 μl of tear fluid was required to be included as a valid sample.

2.5. RNA Isolation and cDNA Synthesis. Tissue fragments (20 and 30 mg in weight) were homogenized using a polytron PT 2100 (Kinematica Inc.; Bohemia, NY), and RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) and processed as previously described [18]. To ensure removal of residual contaminating DNA, samples were subjected to treatment with RNase-free DNase during the purification process itself. The concentration of RNA obtained was determined spectrophotometrically by measuring absorbance at 260 nm of a 1:50 dilution using a BioPhotometer (Eppendorf; Hamburg, Germany). cDNA synthesis was carried out using the High Capacity cDNA Transcription Kit (Applied Biosystems, Foster City, CA, USA). The reactions were performed using a thermocycler iCycler IQ (BioRad; Hercules, CA), using 2 μg of RNA as starting material. The reaction products were cleaned using the PCR Clean-Up GenElute Kit in line with the manufacturer’s instructions. Finally, the aliquots containing the cDNA were diluted 1:20 with water and used for qR-PCR assays or stored at −20°C until use.

2.6. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and Data Analysis. The primer sequences were HPSE (gene ID 10855) forward 5′-ATGCCTAGTGGCTCTGGAC-3′, reverse 5′-TCCTAATCTGCGACCATTG-3′ and HPSE2 (gene ID 60495) forward 5′-CACCCTGATGTCTGGAG-3′, reverse 5′-TCCAGGCAATCGACA AAGTTA-3′. qRT-PCR reactions, and analysis of amplimer products were carried out accordingly to the methods already detailed [19]. Actin was included on each plate as a control gene to compare run variation and to normalize individual gene expression. Statistical analysis of the data and expression of the values of differential transcription were performed as previously described [18].

2.7. Immunohistochemistry. Tissue sections from the central cornea, prepared as previously described [13], were dewaxed, and the rehydrated sections were rinsed in phosphate-buffered saline containing 1% Tween-20, and then immunostained as previously described [13].

2.8. Radioisotopic Labeling and Molecular Size Fractionation of HS. HS was partially de-N-acetylated and re-N-acetylated with [3H]acetic anhydride according to the method previously described [20]. Molecular size fractionation of HS was carried out using size-exclusion chromatography. 20 μCi of [3H]-HS was applied to a superose 12 column connected to an FPLC system (GE) and eluted with 50 mM of pH 8.0 Tris-HCl buffer and 130 mM of NaCl at a flow rate of 1 ml/min. 1 ml fractions were collected, and HS eluting from the column was determined by measuring the radioactivity in 200 μl aliquots of each fraction. Fractions including molecules of high molecular weight were pooled and precipitated with 85% ethanol for 2 h at −80°C. The identity of the polysaccharide was confirmed by exclusion chromatography in the same conditions after treatment with a mixture of heparinas I and III.

2.9. Heparanase Assay. The ability of the tears to fragment HS chains was monitored by ultrafiltration. The reactions were carried out in a total volume of 200 μl that included 100 mM citrate buffer pH 5.3, 1 mM CaCl2, 20 nCi of [3H]-HS and 10 μl of tears. The reactions were incubated for 16 h at 37°C and stopped by the addition of 220 μl of 2 M NaCl and 200 μl of chloroform. After vigorous shaking, the sample was centrifuged at 10,000 × g for 5 minutes, and the reaction was measured by determining the radioactivity in aliquots of the filtrate.

2.10. Statistical Analysis. All analyses were performed using the Statistics for Windows program (Statsoft Inc.; Tulsa, OK). Mean values were compared between two samples by the Mann–Whitney U test and between multiple samples by the Kruskal–Wallis test. Correlations were assessed by Pearson’s correlation coefficient. p < 0.05 was accepted as significant.

3. Results

Analysis of the differential transcription of the genes encoding heparanases in keratoconic corneas was carried out by qRT-PCR. The study was conducted independently for corneal stroma (using cultures of different stromal cells lines obtained from different healthy corneas and keratoconic
values found in healthy tissue did not display significant differences which were statistically significant ($p < 0.001$). HPSE2 transcripts were also detected, mainly in epithelial cells, although not in all the individuals analyzed, and the values found in healthy tissue did not display significant differences from keratoconic cornea (Figure 1).

HPSE is synthesized as a proenzyme of 61.2 kDa, which is cleaved by cathepsin L to generate the active form consisting of 8 and 50 kDa subunits that associate noncovalently [16]. We evaluated changes in the expression of the HPSE protein by immunohistochemistry using two different antibodies. L19 antibody recognizes peptide mapping near the C-terminus of the molecule and is consequently present in both the catalytically active form and in the latent precursor. Using this antibody on healthy corneas resulted in certain levels of staining, predominantly in the basal layers of the epithelium (Figure 2, A). In contrast, the corneas of keratoconus patients showed significant increases in staining, both in the epithelium and in the stroma; at the level of the epithelium, a homogeneous overexpression in keratoconus was observed, while the stroma showed a particularly intense overexpression, predominantly in the subepithelial region (Figure 2, B). H-80 antibody is produced against amino acids 101–180 so that it is able to recognize only the inactive precursor. Immunohistochemical analysis of corneas using this antibody resulted in faint stainings, mainly detectable in keratoconic epithelia (Figure 2, C and D). Furthermore, immunostaining of corneas with anti-heparanase 2 antibodies allowed the detection of certain levels of expression, especially in the epithelium, suggesting the presence of the protein in the corneas (Figure 2, E and F).

The presence of endo-β-D-glucuronidase activity in tears was tested for by determining the appearance of ultrafiltrable HS fragments of low molecular weight resulting from the hydrolysis of high molecular weight [3H]-labeled HS. The results showed that low levels of HPSE activity could be detected in tears of healthy individuals and that in keratoconic individuals the level progressively increased as the Rabinowitz grade of the keratoconus increased (Figure 3). Differences between tears from keratoconic and healthy individuals were statistically significant in all the cases, as they were between the different grades except for between grades 1 and 2. This case, though, approached significance ($p = 0.07$), suggesting that it may well be positive if a wider sample of patients were used. The data showed a strong positive correlation between HPSE activity and the grade of the keratoconus ($r = 0.89, p < 0.001$).

4. Discussion

HSPGs are present in all types of human cells, albeit that HS species from different sources differ in terms of molecular size and their overall patterns of chain modification. Based on their structural diversity, HS chains are able to selectively interact with many different types of soluble and insoluble proteins, lipids, and even microorganisms, thereby modulating numerous cellular activities, including cell adhesion and migration, organization of the ECM, regulation of proliferation, differentiation and morphogenesis, cytoskeleton organization, tissue repair, inflammation, vascularization, and cancer metastasis [6, 8]. Given the importance of the biological functions in which it participates, the enzymatic remodeling of HSPGs profoundly affects a wide variety of physiological and pathological processes [14, 16]. HPSE is the sole human endoglycosidase that cleaves HS, although this molecule also displays various nonenzymatic activities [16, 17].

Whereas HS is produced by virtually all cells in the body, HPSE expression is kept tightly regulated at the transcriptional and posttranslational levels, since any uncontrolled cleavage of HS could result in significant tissue damage [14, 16]. HPSE expression in noncancerous cells has been reported to be restricted to certain specific cases, such as in the placenta, activated immune cells, and keratinocytes [21]. With respect to its expression in ocular tissues, studies in murine eyes have reported constitutive expression in the corneal epithelium and several retinal layers [15]. In this article, we report the expression of certain levels of HPSE in healthy corneas, predominantly in the basal layers of the epithelium. It is of note that corneas of keratoconus patients significantly overexpressed HPSE at both the epithelial and stromal level. Upregulation of HPSE associated with pathological processes has been widely described, including tumors, inflammatory bowel disease, rheumatoid arthritis, diabetic nephropathy, or atherosclerosis [14, 16, 22]. Increased levels of HPSE have also been related to ocular pathologies, such as the overexpression in corneal epithelium and stroma during infection with Pseudomonas aeruginosa.
in the vitreous of patients with proliferative diabetic retinopathy [23].

The upregulation of HPSE in cornea associated with Pseudomonas infection has been related to HPSE-positive infiltrating cells, and it was not able to be detected in corneas from immunized mice since they had a lower inflammatory response [15]. However, in our study, we were able to detect an approximately 6- to 7-fold increase in the transcription of the gene using epithelial cells, obtained by impression cytology, and cultured stromal cells, clearly suggesting that the observed overexpression occurs as a result of increased transcription in the corneal cells themselves. The protein is first synthesized as a latent 65 kDa proenzyme that is secreted via vesicles that bud from the Golgi apparatus, which then interacts with cell membrane HSPGs and other receptors, accumulates in endosomes, and undergoes processing at two proteolytic cleavage sites, located at Glu109-Ser110 and Gln157-Lys158, yielding 8- and 50-kDa subunits that heterodimerize to form the active enzyme. Using an antibody (H80) that recognizes an internal region present only in the 65 kDa form of the protein, we were able to detect immunostaining in epithelial cells, particularly in keratoconic epithelial cells. These immunostainings showed faint labeling, as would be expected for a molecular species that is temporary, in contrast to the intense labeling obtained for the final, processed HPSE. Together, these results indicate that the transcriptional and posttranslational regulation of the expression of the molecule occurs in the corneal cells.

HPSE upregulation, locally expressed at the site of inflammation, has been shown in multiple organ systems as well as in several autoimmune and human autoinflammatory disorders, although its precise mode of action is not completely understood [14]. The enzymatic degradation of HS affects several aspects of inflammatory response, including the release of cytokines and chemokines, the activation of immune cells, and leukocyte recruitment [14]. Keratoconus has traditionally been defined as a noninflammatory disorder due to the lack of neovascularization and cellular infiltration [24]. However, recent studies have shown the
significant role of proteolytic enzymes, cytokines, and free radicals. Moreover, evidence increasingly supports the notion that thinning and ectasia of the cornea are related to a degraded extracellular matrix involving inflammatory events, which include increased levels of matrix metalloproteinases [24] and which might be reinforced by the action of HPSE. It has also been described that loss of corneal epithelial sulfate leads to corneal degeneration [25], and the health of the ocular surface involves soluble factors whose action may be strongly influenced by HPSE, as in the case of lacritin [26]. Some authors have proposed that the definition of inflammation should not necessarily be limited to the absence of neovascularization and lack of marked cellular infiltration [25]. Taking this line, the upregulation of HPSE in keratoconus could be added to the elevated levels of other inflammatory markers to suggest that keratoconus could be, at least in part, an inflammatory condition.

HPSE2 is a homologue of HPSE that lacks HS-degrading activity, although it is able to interact with HS with high affinity and is capable of modulating HPSE enzymatic activity and signaling properties, such that an antimitastatic character has been proposed for it [16]. Although it has previously been suggested that this molecule is not expressed in the eye [27], in the current work, it was indeed possible to detect transcripts in epithelial cells, although not in all the cases analyzed. Immunohistochemistry also detected certain levels of protein, although no significant differences were found between normal and keratoconic cells.

Mature HPSE is located in lysosomes, which are not considered typical secretory vesicles. Nevertheless, they may secrete their content in response to local or systemic cues, which releases the enzymatically active molecule and other molecules like cathepsins into the extracellular milieu [16, 28]. It has been reported that HPSE secretion increases in response to proinflammatory cytokines such as TNFα, although the effective stimuli vary among cell types and biological settings [28]. Altered levels of inflammatory cytokines, including TNFα, have been reported in tears of keratoconic patients, as well as elevated levels of cathepsins [5, 29]. Using [3H]-labeled HS, we were able to detect the presence of certain low levels of HPSE catalytic activity in tears from healthy individuals. This activity greatly increased in tears of keratoconic patients, and the values showed a strong positive correlation with the grade of keratoconus. The tear proteome displays a highly dynamic character that may reflect the altered states of specific eye disorders, as has been described in meibomian gland disease [30], autoimmune thyroid eye disease [31], pterygium [32], ocular rosacea [33], blepharitis [34], diabetes [35], and dry eye [36]. In the case of keratoconus, previous reports have shown differences in the tear protein profile [37], although the changes found do not include HPSE, probably because the methodology used does not directly detect the protein, but rather its catalytic activity, which allows higher sensitivity.

5. Conclusions

In summary, this work describes an overexpression of HPSE in keratoconic corneas that affects both the epithelium and the stroma. The presence of catalytic activity in tears is also reported, and this activity shows a positive correlation with the grade of keratoconus, thus allowing its use as a biomarker for the diagnosis of the disorder.

Conflicts of Interest

The authors declare no conflict of interest.

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