Endogenous Hyaluronan in Corneal Disease

Thomas D. Fitzsimmons,* Nils Molander,† Ulf Stenevi,‡ Per Fagerholm,* Mona Schenholm,‡ and Anette von Malmborg‡

Purpose. Hyaluronan (HA) is a disaccharide polymer capable of binding considerable amounts of water. It is present in trace amounts on the cornea endothelium, and it is not normally found in the epithelium or stroma. A specific histochemical stain was used to test for HA in a wide variety of corneal disorders.

Methods. Eighty-six human corneal tissue specimens were examined histochemically for HA. The material consisted of 84 full-thickness corneal buttons, one epithelium scraping, and one pterygium. Cases were analyzed according to the patient’s sex, age, diagnosis, and localization of HA staining.

Results. The corneal tissue specimens came from 47 women and 39 men, average age 59 years. Fifty-seven percent of the specimens displayed abnormal HA. HA was visualized in Fuch’s dystrophy, keratoconus, infections, regrafts, mechanical and chemical trauma, post-eximer ablations, dystrophies, degenerations, pseudophakic bullous keratopathy, congenital opacities, Stevens-Johnson syndrome, and others. Staining was variously seen in the epithelium, stroma, and endothelium, with intensity of staining ranging from trace amounts to extremely heavy.

Conclusion. Endogenous hyaluronan production is seen in virtually the entire spectrum of corneal disorders. The presence of HA was most often associated with dividing, migrating, or fibroblast-like cells and probably represents a nonspecific tissue response to wounding. Its production is biochemically distinct from that of normally present proteoglycans. The abnormal presence of HA may reduce corneal transparency by disrupting the normal spacing between collagen fibrils, creating focal changes in the index of refraction, and altering the normal flow of solutes through the cornea. Invest Ophthalmol Vis Sci. 1994;35:2774-2782.

Hyaluronan (HA), also known as hyaluronic acid and hyaluronate, is a disaccharide polymer composed of glucuronic acid and N-acetylglucosamine. It is a ubiquitous molecule, present in the connective tissue of all higher animals and even produced by some bacteria. Its very high molecular weight makes it capable of binding considerable amounts of water. High concentrations are present in the vitreous humor, where HA was first identified.¹

HA is usually classified among the proteoglycans, but it does not contain a protein component. Proteoglycans fill the space between collagen fibrils in the corneal stroma and are thought to play important roles in corneal hydration and transparency.² Although several groups have isolated HA experimentally in tissue cultures of rabbit and human stromal cells,³⁻¹ HA was previously thought to be completely absent from the adult cornea.² A histochemical stain specific for HA has recently been developed.¹⁰,¹¹ Trace amounts of HA and HA-specific receptors have subsequently been identified on the apical surface of the corneal endothelium.¹²⁻¹⁴

We used the HA-specific histochemical stain to visualize HA, for the first time, in a wide variety of corneal diseases. The significance of the findings is discussed.
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METHODS

Specimens

The research followed the tenets of the Declaration of Helsinki, informed consent was obtained, and approval for the study was granted by our institutional review committee. Eighty-six human corneal tissue specimens were examined histochemically for HA. The tissues consisted of 84 full-thickness corneal buttons removed during transplantation surgery, one corneal epithelium scraping from a patient with Meesman’s dystrophy, and one excised pterygium. Patients were examined and operated on by one of the authors (US or PF) at either Lund University Hospital, Lund, or St Eriks Eye Hospital/ Karolinska Institutet, Stockholm, Sweden, with four exceptions: three normal corneas were obtained from eyes enucleated for choroidal malignant melanoma, and a cornea with Reis-Bücklers’ dystrophy was obtained from another hospital. The endothelial surfaces of many of the corneal buttons were exposed to sodium hyaluronate (Healon, Pharmacia AB, Uppsala, Sweden) at the time of removal during transplantation surgery; 27 were not.

All tissue specimens were fixed in a solution of 4% buffered formaldehyde containing 1% (wt/vol) cetylpyridine chloride. They were dehydrated, fixed in paraffin, and then sectioned for histochemistry.

Histochemistry

The HA-binding region of cartilage proteoglycans used in the staining technique was prepared by affinity chromatography on HA sepharose, as described earlier. The purified protein was then linked to biotin according to the principles outlined by Ripellino et al. HA was used during the biotinylation to protect the HA-binding site on the HA-binding region and was then removed. After incubation with HA-binding region biotin for 2 hours at room temperature, the corneal tissue sections were rinsed in phosphate-buffered saline and then incubated for 1 hour with avidin-biotin-complex Vectastain Reagent (Vector Laboratories Inc., Burlingam, CA). The sections that now were labeled with peroxidase were visualized with ethyl carbazole. Control sections from two normal corneas and two corneas that stained heavily for HA were incubated for 2 hours in a humidified chamber at 37°C with 50 U/ml of Streptomyces hyaluronidase (Seikagaku Fine Biochemicals, Tokyo, Japan) before staining for HA. Sections from these corneas were additionally prepared absent one of the following reagents: HA-binding region probe, avidin-biotin complex, or ethyl carbazole. Some sections were counterstained in Mayer’s hematoxylin. The diagnosis of macular dystrophy was confirmed by appropriate staining with alizarin blue, Masson’s trichrome, PAS, and congo red. The sections were mounted under coverglass slips in Kaiser’s glycerin-gelatin.

The sections were examined with a Nikon Microphoto FX microscope (Tokyo, Japan). Photomicrographs were taken using brightfield optics. Cases were analyzed according to sex, age, diagnosis, and presence and location of hyaluronic acid.

RESULTS

General Comments

The 86 patients were comprised of 47 women and 39 men. The mean age was 59 years, and the range was 18 to 93 years. The specimens were divided into 15 diagnostic categories and were tabulated according to the total number in each group showing HA, along with a breakdown of where the HA was localized (Table 1). Forty-nine specimens, or 57% of the cases, showed abnormal HA staining. With the exception of the normal cornea group, all categories had at least one example with HA staining. HA was variously observed in the epithelium, stroma, and endothelium, with intensity of staining ranging from trace amounts to extremely heavy. In most sections, the HA staining appeared to be extracellular, but intracellular staining could not be ruled out in some heavily stained corneas. The intensity of staining on the endothelium in positive cases was uniformly light; comments about endothelial HA were made only in those cases not exposed to sodium hyaluronate during surgery. Thirty of the 34 pathologic corneas that did not show HA staining came from either the Fuch’s dystrophy or keratoconus groups. None of the control sections had HA staining.

HA shows up as reddish brown in the photographs (Figs. 1 to 12). Most photographs have a hematoxylin (blue) counterstain to highlight corneal details.

Analyzed by Diagnosis

Normal corneas. Three corneas were obtained from patients with choroidal malignant melanomas. No evidence of HA was seen in the stroma of sections stained with HA stain and hematoxylin (Fig. 1), or with HA stain alone. Normally present keratan sulfate and chondroitin sulfate proteoglycans did not stain. A minimal trace amount of normal HA on the endothelial surface was seen in 2 out of 20 sections examined.

Fuch’s dystrophy. Eleven out of 28 corneas in this group showed HA staining. When HA was observed, it was mild to moderate in intensity and was associated with subepithelial fibrosis (Fig. 2). Four specimens from patients with Fuch’s dystrophy showed faint but definitely increased endothelial HA deposition, one within the hypertrophic Descemet’s membrane. One
cornea with a healed, superficial wound extending through Bowman’s layer was notable for a complete absence of HA staining.

**Keratoconus.** Six out of 18 keratoconus corneas stained positively for HA. As in the Fuch’s dystrophy group, the staining was relatively mild and was associated with subepithelial fibrosis. One patient with keratoconus after acute hydrops did not have HA staining.

**Infection/Postinfection.** This group included three patients with remote, infectious keratitis (presumed bacterial, 29 to 60 years after infection) (Fig. 3), two patients with chronic herpes simplex keratitis (Fig. 4), and two cases of active bacterial keratitis. Moderate to very heavy HA staining was seen in six of the specimens; one cornea infected more than 50 years earlier showed no HA staining.

**Regrafs.** Six regraft specimens were obtained from patients originally grafted for the following reasons: herpes simplex keratitis (3), congenital syphilis (1), acne rosacea with perforation (1), and Fuch’s dystrophy (1). HA staining was seen in all six regraft specimens. The heaviest stromal staining of all the corneas in this report came from a 76-year-old man with a history of herpes simplex keratitis and multiple graft failures (Fig. 5).

**Dystrophies.** The dystrophy group included two patients with macular dystrophies, one with Reis-Bücklers’ dystrophy, one with granular dystrophy, and one with an epithelium scraping of Meesman’s dystrophy. All showed abnormal HA staining. The macular dystrophy specimens came from patients aged 35 and 39 years. HA was focally heavy in subepithelial and pre-Descemet’s areas (Fig. 6), and corresponded to similar areas that stained heavily with alcian blue. Diffuse staining of HA in the stroma was not observed. Moderately heavy HA staining was seen in areas of Bowman’s layer disruption and subepithelial membrane formation in the Reis-Bücklers’ specimen. Light, focal HA staining was present within a subepithelial membrane in the granular dystrophy cornea. A few spots of HA were seen in the epithelium from the Meesman’s dystrophy cornea.

**Trauma.** Two corneas were examined after excimer laser treatments. Both patients had undergone phototherapeutic keratectomy with the excimer laser in unsuccessful attempts to ameliorate visually disabling corneal scars. The first was treated with the laser 1 year after a contact lens-related *Pseudomonas* infection; the second was treated more than 60 years after a presumed bacterial infection. These corneas showed heavy subepithelial HA staining 7 months (Fig. 7) and 10 months, respectively, after laser surgery. The staining did not extend outside the excimer-treated areas, nor did it involve the deeper untreated areas of stromal opacification. The alkali-wounded cornea showed heavy stromal HA. This cornea was notable for displaying the heavy HA only in the anterior stroma and epithelium; the posterior half, containing a higher density of fibroblasts/keratocytes and blood vessels, was relatively free of HA (Fig. 8). A cornea from a 63-year-old man who was kicked in the eye by a horse 49 years earlier showed heavy HA staining anterior to and within an area of tremendous stromal thickening (Fig. 9). The cornea of a 58-year-old man who 20 years earlier had a full-thickness corneal lacer-

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**TABLE 1. Tissue Localization of Hyaluronan in Various Corneal Diseases**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number</th>
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<th>Epithelium</th>
<th>Stroma</th>
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<tr>
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<td>0</td>
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<tr>
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<td>11</td>
<td>1</td>
<td>10</td>
<td>4/9</td>
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<td>6</td>
<td>2</td>
<td>6</td>
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<tr>
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<td>0</td>
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<td>Trauma—mechanical</td>
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<td>—</td>
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<td>Post-acute glaucoma decompenasion</td>
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<td>0/1</td>
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<tr>
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<td>1</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>86</td>
<td>49</td>
<td>10</td>
<td>47</td>
<td>8/27</td>
</tr>
</tbody>
</table>

+HA, number in each disease group displaying hyaluronan.

All corneas exposed to sodium hyaluronate (Healon) during removal showed endothelial staining; endothelium results are tabulated only for corneas in each group not exposed to sodium hyaluronate. Endothelium column fraction shows number of abnormally intense endothelial HA specimens over total number specimens not exposed to sodium hyaluronate during removal.
FIGURE 1 (top left). Normal cornea from a patient with a choroidal melanoma. No HA is visible (HA stain + hematoxylin, original magnification X125).

FIGURE 2 (top right). Fuch’s dystrophy. HA has accumulated between epithelium and anterior stroma in an area of subepithelial fibrosis and disrupted Bowman’s layer (HA stain + hematoxylin, original magnification X500).

FIGURE 3 (middle left). Postinfection. A 30-year-old woman suffered a unilateral infection, presumed bacterial, as an infant. An irregular epithelium covers a vascularized anterior stroma. Very heavy HA is present throughout the stroma, especially anteriorly (HA stain + hematoxylin, original magnification X156).

FIGURE 4 (middle right). Chronic herpes simplex virus infection. Original infection occurred 38 years previously, then there were multiple recurrences during the 6 years before transplantation. Heavy HA throughout the stroma separates collagen lamellae (HA stain + hematoxylin, original magnification X200).

FIGURE 5 (bottom left). Transplantation failure. A 76-year-old man with a 28-year history of herpes simplex virus had multiple graft failures. This cornea displayed the heaviest amount of stromal HA of all corneas tested (HA stain + hematoxylin, original magnification X200).

FIGURE 6 (bottom right). Macular dystrophy. HA stain without counterstain shows two areas of heavy HA deposition subepithelially and in the deep stroma (HA stain, original magnification X125).
tion displayed HA throughout the wound area. One cornea removed because of a traumatic scar showed heavy subepithelial fibrosis but no HA.

**Pseudophakic bullous keratopathy.** All three corneas transplanted because of pseudophakic bullous keratopathy showed moderate to heavy HA staining (Fig. 10).

**Others.** Three corneal degenerations were examined. The first, a pterygium, showed heavy subepithelial HA. The second, a cornea with Salzman’s nodular degeneration, showed heavy stromal HA. The third, a degeneration of unknown type but with a heavy band keratopathy, did not show HA staining. Two congenital opacities were examined. The first, from an 18-year-old woman with multiple, bilateral congenital ocular abnormalities, including aniridia, strabismus, and nystagmus, showed diffuse epithelial and stromal HA. The second, from a patient with bilateral posterior stromal opacities, showed moderately heavy HA only in the pre-Descemet’s area. A cornea from a 48-year-old patient diagnosed with Stevens-Johnson syndrome (erythema multiforme) 15 years earlier showed heavy HA staining (Fig. 11) and was unique for its display of epithelial HA only within the spinous cell layer of a tremendously hypertrophied epithelium (Fig. 12). A cornea from a patient diagnosed with angle-closure glaucoma 18 years earlier, with subsequent band keratopathy and endothelial cell decompensation, showed heavy HA staining, notably within the basal layer of the epithelium and within a hypertrophied Descemet’s membrane. Two patients with corneal decompensation after posterior segment silicone oil administration were examined; one had mild, diffuse HA staining in the stroma.

**DISCUSSION**

This is the first histochemical survey of hyaluronan in diseased human corneas. Because HA is normally detectable only on the corneal endothelium, the presence of any amount of HA in the epithelium or stroma is likely indicative of altered tissue physiology. A 1986 study on the most common indications for penetrating keratoplasty listed 17 diagnostic categories; the present group of corneas includes at least one specimen with abnormal HA from each category and several others not listed. HA production is part of the pathophysiology of virtually the entire spectrum of corneal disorders.

The importance of finding HA in the cornea is related to the key role of the extracellular matrix in corneal physiology. The corneal stroma is largely an...
extracellular compartment, with keratocytes and nerve processes making up only about 5% of the volume. The principal components of this compartment are collagen type I and proteoglycans containing the glycosaminoglycans, keratan sulfate and chondroitin sulfate. Proteoglycans coat collagen fibrils and bind various amounts of water; their overall action accounts in large part for the tendency of the cornea to swell. It is thought that they regulate the precise spacing between collagen fibrils, which is key for corneal transparency.

HA is usually grouped among the proteoglycans, but several unique features distinguish it. The keratan sulfate and chondroitin sulfate components of the normally present corneal proteoglycans are sulfated, covalently bound to protein, and have relatively low molecular weights (4,000 to 50,000 daltons). HA, on the other hand, is not sulfated, is not bound to protein, and has a high molecular weight (several million daltons). Keratan sulfate and chondroitin sulfate proteoglycans are produced intracellularly through the Golgi apparatus. HA, on the other hand, is pro-
duced and extruded directly into the extracellular space through an enzyme located in the cell wall membrane. On a cellular level, then, the production of HA in various disease states represents a fundamentally distinct biochemical activity in the cornea, separate from an altered synthesis or replenishment of normally present molecules.

The physical properties of hyaluronan have important implications for the cornea. The high number of negative charges present in HA attracts osmotically active cations such as Na+, causing large amounts of water to be sucked in and effectively bound to the molecule. The result is that HA in solution occupies a huge volume relative to its mass. Conceivably, even small amounts of HA might diminish corneal clarity by disrupting the normal spacing between collagen fibrils, by creating focal changes in the index of refraction, or by altering the normal flow of solutes through the cornea. It is clear that HA is not the only factor in corneal clarity, however, because many opacified corneas (predominantly in the Fuch's dystrophy and keratoconus groups) did not show HA staining.

HA plays a significant role in the embryology of the cornea. The waves of neural crest cells into the stroma are associated with HA production; it is thought that HA facilitates cell movement and prevents precocious development. The action of hyaluronidase later leads to stromal dehydration and corneal clarity. The presence of HA after various types of trauma, including excimer ablation, alkali burns, and mechanical wounds, supports the notion that adult HA production in the cornea represents a recapitulation of an earlier embryologic event. Several cell types appear capable of producing HA. We have observed HA apparently produced by cells derived from ectoderm (corneal epithelium, lens epithelium), neural crest (keratocytes, endothelium), and neural ectoderm (iris-pigmented epithelium). Given that hyaluronan is an ontogenetically primitive molecule capable of being produced by many cells and given the multiplicity of stimuli that seemingly reactivates the production of HA in the adult cornea, one could conjecture that cellular trauma in general causes a derepression of the HA synthesize gene.

Fuch's dystrophy, keratoconus, and pseudophakic bullous keratopathy are the three most common indications for corneal transplantation. The presence of HA in the first two disorders is likely related to subepithelial fibrosis and accompanying breaks in Bowman's layer. The significance of HA in these groups is probably minor; nearly all of the corneas not stained for HA (30 out of 34) came from either the Fuch's dystrophy or keratoconus groups. It is interesting to note that although the primary defect in Fuch's dystrophy and pseudophakic bullous keratopathy is thought to be a degeneration of the endothelial cell layer, the causes of subepithelial membrane formation and HA production are unknown. The endothelial cells in Fuch's dystrophy have been described as occasionally assuming fibroblast-like characteristics, producing glycosaminoglycans. The finding of increased amounts of endothelial HA in several Fuch's dystrophy corneas supports this observation.

Regrafts and infections, the next most common indications for transplantation, had the heaviest overall HA staining. Just as HA production may represent a nonspecific tissue response to trauma, it may also be a nonspecific component of graft rejection and infection. Increased HA concentrations and edema have recently been described in rejected human kidney grafts and heart allografts in rats. All these conditions are characterized by cellular wound responses and fibroblastic activity.

No such cellular activity, however, is apparent in macular dystrophy corneas. In macular dystrophy, mature keratan sulfate proteoglycan is not produced. The disease may also be characterized by a primary, aberrant production of HA. Although HA staining corresponded to areas that stained heavily with alcian blue, alcian blue sections also characteristically show mild staining within fine lacunae scattered throughout the stroma. The HA stain might appear to be a more specific test for aberrant material produced in the disease, but, as is apparent from the other corneas in this study, the occurrence of HA is not specific to macular dystrophy.

The other corneal dystrophies showed variable amounts of HA. The Reis-Bücklers' dystrophy cornea stained heavily for HA in the anterior stroma, probably in connection with subepithelial membrane formation. The Meesman's dystrophy epithelium specimen displayed small amounts of HA, but it was not diffuse; we are unable to state that this represents staining of the "peculiar" substance described in the disease. The significance of HA in granular dystrophy probably is minor and is related to subepithelial fibrotic changes.

The trauma category included two corneas that stained heavily for HA 20 and 49 years after the original injuries. Two other mechanically wounded corneas showed no HA staining. It is unknown why some wounds continue to produce HA, whereas others appear to heal completely. Excimer laser trauma is particularly interesting, for the trauma is inflicted with minimal surrounding tissue damage. The signals for HA production and its discontinuation are unclear. How does the cornea "know" that it has been injured? Epithelial defects might allow the entry of various cells and growth factors, but we have not observed a reactive production of HA in rabbit corneas that received either a mechanical epithelial debridement or excimer laser ablation of the epithelium (unpublished data,
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91). Corneal nerves and keratocyte processes are certainly transected during the ablation process; perhaps they play a role in signaling HA production.

Abnormal epithelial and endothelial accumulations of HA were less common than stromal ones in the present group of corneas. The hypertrophied corneal epithelium of the patient with Stevens-Johnson syndrome was essentially identical to epidermis; the HA visualized between the cells of the spinous layer matches HA recently described in normal human epidermis. The endothelial cell staining of HA was extremely light in comparison to the epithelial and kidney, and mammary gland. Theoretically, high en-

syndrome was essentially identical to epidermis; the neal epithelium of the patient with Stevens-Johnson known if abnormal endothelial staining is a conse-

quence of HA production within the endothelium or possibly of increased HA concentration within the aqueous humor. A cornea obtained postmortem from a person who had exfoliation syndrome was remarkable for a tremendously thick layer of HA on the endo-

The histochemical stain used in this study resulted from research identifying the presence of proteins in bovine nasal cartilage that bind specifically and non-

covalently to HA. These proteins were isolated and linked to biotin, forming a specific marker for hyaluronic acid. This marker is then visualized by an enzyme complex that binds specifically to HA. Biotin, also known as vitamin B9, is important in several enzyme reactions in the body, especially in the liver, kidney, and mammary gland. Theoretically, high endo-
genous concentrations of tissue biotin could cause false-positive HA staining. One might predict faint, background staining in such tissue sections. For the majority of tissues, however, biotin levels are too low to cause unwanted binding. Examinations of the normal corneas and many normal rabbit corneas (unpublished data, 1992), furthermore, have not revealed any degree of background staining. We conclude that the present methodology is particularly applicable to the visualization of HA in the cornea.

The discovery of HA in various diseased corneas opens many new avenues of research. Little is known about the turnover of HA in the human cornea. We have observed that steroids reduce HA production in rabbit corneas after excimer wounding and that readministration of steroids in some patients after ex-
cimer surgery is associated with dramatic changes in corneal topography, but a cause-and-effect relationship between HA and topographic changes has yet to be established. Further corneal testing is needed for HA staining in conditions not found in our group, including metabolic errors, tumors, and other less common diseases. Genetics controlling HA production and catabolism are unknown, and the correlation between amount of HA present in the cornea and the degree of visual impairment is untested. The possibil-

Key Words
hyaluronan, wound healing, proteoglycan, cornea, transparency

References


