Alterations in Stromal Glycoconjugates in Macular Corneal Dystrophy

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Nine biotinylated lectins were used as histochemical probes to localize the carbohydrate residues of glycoconjugates in normal corneas and in corneas with macular and granular dystrophy. The lectin binding patterns of normal corneas and of corneas with granular dystrophy were indistinguishable from one another, but were distinctly different from those found in corneas with macular dystrophy. Concanavalin A reacted weakly with normal corneal stromal matrix, but stained stromal matrix of corneas with macular dystrophy intensely. Furthermore, unlike the normal corneal matrix, stromal matrix of corneas with macular dystrophy reacted positively with wheat germ agglutinin (WGA), Ricinus communis agglutinin I (RCA-I), Ulex europaeus I, Dolichos biflorus, Bandeiraea simplicifolia I, Bandeiraea simplicifolia II, and soybean and peanut lectins. This study demonstrates specific alterations in glycoconjugates which occur in the corneal matrix of patients with macular dystrophy, namely the presence of oligosaccharides with terminal α-fucose, β-galactose, N-acetylgalcosamine and N-acetylgalactosamine residues, and oligosaccharide chains with a β-galactose-N-acetylgalactosamine sequence. Invest Ophthalmol Vis Sci 27:1211-1216, 1986

Macular dystrophy is a rare autosomal recessive disease of the cornea. It is characterized by the gradual accumulation of irregular, poorly-defined grayish opacities in the stroma. The opacities usually commence during adolescence and gradually enlarge. As a result, vision is often impaired by the end of the second decade and it progressively worsens. Corneal transplantation may be required by age 30-40 yr. Histochemically, intra- as well as extracellular deposits are seen within the corneal stroma. These deposits react positively with colloidal iron and are often stained with periodic acid-Schiff reagent. They are resistant to digestion with testicular hyaluronidase, chondroitinase ABC, and neuraminidase. On the basis of these characteristics, the accumulated material was originally thought to represent keratan sulfate. However, recent biochemical studies have demonstrated that corneal explants from patients with macular dystrophy synthesize very little or no keratan sulfate. Instead these corneas were found to synthesize an abnormal glycoprotein. Normal glycoprotein reacts positively with antibodies specific for the protein core of the normal keratan sulfate proteoglycan.11,12 These studies also suggest that the abnormal glycoprotein in corneas with macular dystrophy is synthesized at a level similar to that found for keratan sulfate proteoglycan in normal corneas. This glycoprotein differs from the normal keratan sulfate proteoglycan in that the former lacks sulfate, exhibits resistance to digestion with keratanase and various exoglycosidases, and contains larger oligosaccharides. The present histochemical study was designed to identify the terminal sugar residues of abnormal oligosaccharides thought to be present in corneas with macular dystrophy.

Materials and Methods

Corneal buttons removed at keratoplasty from seven patients with macular dystrophy were examined. The ages of the patients at the time of the surgery ranged from 20–59 yr. Each patient had a family history of macular corneal dystrophy. Histologically, alcian blue positive deposits were present in each of the seven specimens. Control tissue consisted of five normal corneas and corneal buttons from seven patients with granular dystrophy. Normal human eyes were obtained from the New England Eye Bank. Ages of the donors ranged from 33–70 yr. Seven biotin-labeled lectins were purchased from Vector Laboratories (Burlingame, CA): Ricinus communis agglutinin I (RCA-I); soybean agglutinin (SBA); wheat germ agglutinin (WGA); Dolichos biflorus agglutinin (DBA); peanut agglutinin...
Fig. 1. Biotin-labeled WGA staining of a normal human cornea (N) and a cornea with macular dystrophy (M). In the normal cornea the epithelium and keratocytes stained, while the stromal matrix did not. In contrast, the stromal matrix of the macular cornea stained intensely. Arrows indicate abnormal deposits. (X60)

(PNA); *Canavalia ensiformis* agglutinin (Con A), and *Ulex europeus* agglutinin I (UEA-I). Two additional lectins, *Bandeiraea simplicifolia* agglutinin I (BSA-I) and *Bandeiraea simplicifolia* agglutinin II (BSA-II) were obtained from E.Y. Laboratories (San Mateo, CA). Six haptenic carbohydrates, used as blocking sugars, were obtained from Pfännstiel Laboratories (Waukegan, IL). These sugars included \( \beta \)-lactose, N-acetylgalactosamine, \( \alpha \)-methylmannopyranoside, \( \alpha \)-L-fucose, \( \alpha \)-methylgalactopyranoside, and N-acetylglucosamine. A kit consisting of avidin D and biotin-peroxidase was also obtained from the Vector Labs. Neuraminidase from *Clostridium perfringens* (Type VI) and mouse liver powder were purchased from Sigma Chemical Co. (St. Louis, MO).

**Tissue Sections**

Corneas previously fixed in 10% formalin-0.04 M sodium phosphate, pH 7.2 at room temperature for 24-48 hr and embedded in paraffin were processed according to established histological procedures.

**Staining With Biotin-Labeled Lectins**

The procedure employed was similar to that described by Hsu and Raine and has been described in detail elsewhere. Briefly, paraffin-embedded sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide (37°C, 10 min). Sections were subsequently incubated with biotin-labeled lectin (10 μg/ml, 1 hr), a freshly prepared complex of avidin D and biotin-peroxidase (30 min), and diaminobenzidine-

**Results**

**Corneal Epithelium**

Two lectins, WGA and Con A, reacted positively with epithelia of all five normal corneas (Fig. 1). Of the seven corneas with macular dystrophy, epithelia of five reacted with Con A and four reacted with WGA. In addition, epithelia of three of these corneas reacted with UEA-I and DBA (Table 1). In each case, WGA bound to the cell surface region whereas other lectins reacted with the cytoplasm of the cell.

**Keratocytes**

In normal corneas, keratocytes reacted positively with WGA and RCA (Figs. 1, 2). In most macular corneas, typical keratocytes could not be detected with light microscopy. Some positively stained structures referred to herein as abnormal deposits (see below) could have been engorged or disrupted keratocytes which had released their contents into the extracellular stromal matrix.

**Stromal Matrix**

The stromal matrix of normal corneas stained weakly with Con A and did not react with any other lectin, whereas matrices of corneas with macular dystrophy reacted positively with most lectins (Table 1). We also noted some variation in staining patterns of the stromal matrix among various corneas with macular dystrophy (Table 1).
Table 1. Lectin-binding patterns of normal corneas and corneas with macular dystrophy

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Epithelium</th>
<th>Stromal matrix</th>
<th>Abnormal deposits and/or engorged keratocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal (5)</td>
</tr>
<tr>
<td>WGA</td>
<td>++(1), +(2), +*(2), +(1)</td>
<td>++(2), +(5)</td>
<td>++(5), ++(2)</td>
</tr>
<tr>
<td>Con A*</td>
<td>++(4), +(1)</td>
<td>++(3), ++(1), +(3)</td>
<td>+*(3), ++(4)</td>
</tr>
<tr>
<td>RCA-I</td>
<td>-</td>
<td>++(3), +(3), ±(1)</td>
<td>++(6), ++(1)</td>
</tr>
<tr>
<td>SBA</td>
<td>-</td>
<td>+(2), +(2)</td>
<td>+<em>(1), +</em>(2)</td>
</tr>
<tr>
<td>DBA</td>
<td>+*(1), +(2)</td>
<td>++(1), +(3)</td>
<td>++(3), ++(1)</td>
</tr>
<tr>
<td>PNA</td>
<td>-</td>
<td>+(2), +(1)</td>
<td>+*(1), ++(2), +(1)</td>
</tr>
<tr>
<td>UEA-I</td>
<td>++(2), +(1)</td>
<td>++(1), +(2), ±(1)</td>
<td>+*(3)</td>
</tr>
<tr>
<td>BSA-II</td>
<td>-</td>
<td>+(2)</td>
<td>+*(2)</td>
</tr>
<tr>
<td>BSA-I</td>
<td>-</td>
<td>+(1), ±(1)</td>
<td>+*(1), +(1)</td>
</tr>
</tbody>
</table>

Numbers in parenthesis indicate the number of corneas. Corneas not graded specifically in table were negative. ++++ = very intense; +++ = intense; ++ = moderate; + = weak; ± = trace; - = negative.

* All normal and three macular corneas were also stained with Lens culinaris agglutinin, another glucose/mannose binding lectin. The staining pattern obtained with this lectin was similar to that found for Con A.

Abnormal Deposits

There was a parallel staining of stromal matrix and abnormal deposits in most corneas with macular dystrophy (Figs. 1-3), with two exceptions. In one cornea with macular dystrophy, (patient age 51 yr), SBA stained stromal matrix but not the abnormal deposits; in the other (patient age 33 yr), the stromal matrix was negative but deposits were seen following staining with PNA. In most cases, staining of the deposits was usually more intense than that of stromal matrix (Table 1).

Corneas With Granular Dystrophy

The lectin staining pattern of corneas with granular dystrophy was indistinguishable from that of normal corneas, except that the former demonstrated fewer keratocytes. In addition, corneas with granular dystrophy displayed equivocal staining of the superficial stroma with two lectins, WGA and RCA-I.

Control Sections

Control sections performed in the presence of specific blocking sugars were negative with each lectin. Among all six haptenic carbohydrates used for controls (see Materials and Methods), reactions with WGA, BSA I, and UEA were inhibited only by their specific blocking sugars, i.e., N-acetylglucosamine, α-methylgalactopyranoside and α-L-fucose, respectively, and none others; binding with RCA and PNA was inhibited by β-lactose and α-methylgalactopyranoside; reaction with SBA was inhibited by β-lactose and N-acetylglucosamine, and reaction with Con A was inhibited by α-methylmanno/glucopyranoside and N-acetylglucosamine. For each control, the concentration of specific sugar was 100 mM or less, whereas that of nonspecific sugar was 200 mM.

It is apparent that the results obtained in this study were due to specific lectin binding reactions because: (1) whenever staining of control sections was performed in the presence of specific blocking sugars with normal corneas or with corneas from patients with macular and granular dystrophy, no detectable staining was observed with any lectin, and (2) various lectins which stained corneas with macular dystrophy did not stain normal corneas or corneas with granular dystrophy. It is unlikely that nonspecific staining could differentiate between normal and dystrophic tissue as was demonstrated herein.

Discussion

The present study demonstrates that abnormal glycoconjugates are present in corneas with macular dystrophy. In this study, lectins were used as histochemical...
Fig. 3. Staining of a normal human cornea (N) and of a cornea from a patient with macular dystrophy (M) with biotin-DBA (top left), Biotin-BSA II (top right) and biotin-PNA (bottom left). Normal corneas did not stain with biotin-PNA (bottom right), biotin-DBA (not shown), or biotin-BSA II (not shown). In contrast, corneas with macular dystrophy were stained by all three lectins. Deposits in corneas with macular dystrophy are indicated by arrows. (×60)

probes and each cornea was analyzed individually. Unlike stromal matrices of normal corneas which stained weakly only with Con A, a glucose/mannose binding lectin, stromal matrices of all seven corneas with macular dystrophy stained moderately to intensely with Con A and WGA, and stromal matrices of six of these corneas reacted positively with RCA-I. WGA binds to N-acetylgalactosamine/sialic acid residues\(^\text{15}\) and RCA-I binds to terminal galactose residues.\(^\text{16}\) Of the six macular corneas which reacted positively with both RCA and WGA, four also reacted positively with SBA (N-acetylgalactosamine specific) and UEA (α-L-fucose specific); of these four, three reacted positively with PNA (binds to a disaccharide β-galactose-N-acetylgalactosamine), and of these three, two also reacted positively with BSA-I and BSA-II, lectins known to be specific for terminal α-galactose and N-acetylgalactosamine residues, respectively. These data suggest that, unlike normal corneas, stromal matrices of most macular corneas contain oligosaccharides with N-acetylgalactosamine/sialic acid and terminal galactose residues. In addition, stromal matrices of some corneas with macular dystrophy contain oligosaccharide with terminal fucose, N-acetylgalactosamine, and N-acetylgalactosamine/sialic acid and terminal galactose residues and chains with terminal β-galactose-N-acetylgalactosamine disaccharides. Staining of at least four of six RCA-positive macular corneas was most probably due to β-galactose and not α-galactose residues, because they reacted with RCA and not with BSA-I. RCA-I is known to bind to α- and β-galactose, whereas BSA-I binds to α-galactose residues only.\(^\text{16}\) Whether the oligosaccharides of the abnormal glycoprotein expected to be present in the corneas with macular dystrophy\(^\text{10}\) contribute to the abnormal lectin binding patterns we found in the present study remains to be established. It appears that the presence of abnormal keratan sulfate chains in this glycoprotein could not contribute to positive reaction of at least PNA, RCA, and BSA II, because Nakazawa et al\(^\text{12}\) have shown that these chains could not be digested with a mixture of exoglycosidases, including β-galactosidase and N-acetylgalactosaminidase.

With most lectins, there was a parallel staining of the stromal matrix and the abnormal deposits in corneas with macular dystrophy. The exact chemical nature of the abnormal deposits in macular corneas still remains to be established. Our data, together with results of previous studies,\(^\text{3-12}\) suggest that the deposits may consist of: (1) abnormal proteoglycans containing undersulfated keratan sulfate proteoglycan\(^\text{5-10}\) and oversulfated chondroitin sulfate proteoglycan,\(^\text{12}\) (2) proteoglycans containing abnormal N-linked and/or O-linked oligosaccharides, and (3) a mixture of both abnormal glycoproteins and proteoglycans.\(^\text{5-12}\) It has been established that the deposits stain with alcian blue in spite of pretreatment with neuraminidase,\(^\text{4}\) suggesting that the positive reaction of the deposits with the cationic dyes is probably not due to sialic acid residues.
Figure 4 depicts corneas with macular dystrophy arranged on the basis of the ability of their stromal matrix to bind with various lectins. There appears a variation in the lectin binding pattern among different corneas with macular dystrophy, ranging from one which bound to all nine lectins to one which reacted with only two lectins. There appears to be an ordered progression in the likelihood of binding with increasing numbers of lectins. For example, if a particular macular cornea reacted with a lectin depicted in the middle of the histogram, such as DBA, it always reacted with those lectins appearing to its left but not necessarily to its right. Such a trend was observed for each cornea analyzed in this study. Differences in the lectin binding pattern among various corneas with macular dystrophy may represent individual differences in the severity of the disease as manifested by severe, moderate, or mild abnormalities in corneal glycoconjugates. On the other hand, these differences may stem from differential receptor loss during fixation and subsequent tissue processing. Use of cryosections is preferable, especially when a negative reaction is obtained using paraffin sections. It has been shown that human corneal epithelium reacts with DBA, an α-N-acetylgalactosamine binding lectin, only when frozen sections are used. In the present study, we also found that DBA did not react with normal corneas and reacted with only three of seven corneas with macular dystrophy. Differences in lectin staining patterns among human corneal epithelia may be due to differences in the blood groups of the donors. It is well known that blood group antigens react with various lectins, and that such antigens are present on corneal epithelial cells in humans. On the other hand, the absence of DBA-staining in all normal corneas and five corneas with macular dystrophy could be due to the extraction of DBA reactive macromolecules during specimen fixation and subsequent processing. N-acetylgalactosamine residues are commonly found in glycosphingolipids, macromolecules likely to be leached during paraffin tissue processing because of their high solubility in organic solvents. In recent years, paraffin sections have been extensively used, with success in the study of lectin binding sites. Formalin fixation and paraffin embedding would not be expected to alter lectin binding sites chemically, because glycosidic bonds are stable at high temperatures (up to 100°C) and in organic solvents providing the pH is maintained close to 7.0. Differences in the lectin staining pattern between frozen and paraffin sections are probably related to differences in the solubility properties of various macromolecules. Lipid-like molecules are extracted in solvents used for deparaffinization, and are thus not expected to be retained. On the other hand, glycoproteins are often retained in the formalin-fixed paraffin sections.

Preliminary studies have suggested that, in corneas with macular dystrophy, there may be a deficiency in α-galactosidase, suggesting an accumulation of α-galactose-containing glycoconjugates. In the present study, only two of seven corneas with macular dystrophy were found to react with an α-galactose binding lectin (BSA-I). The reaction of these two corneas with BSA-I might be due to the presence of oligosaccharides containing terminal N-acetylgalactosamine residues rather than α-galactose residues because BSA-I reacts, though weakly, with terminal N-acetylgalactosamine residues second and both BSA I positive macular corneas stained intensely with DBA, an α-D- N-acetylgalactosamine binding lectin. Accumulation of α-galactose-containing glycoconjugates, if indeed it does occur in corneas of patients with macular dystrophy, would be expected in glycolipids and not in glycoproteins. Alpha-galactose residues commonly occur in glycolipids but have yet to be found in glycoproteins of human tissues. Abnormal glycolipids, if present in corneas with macular dystrophy, would have escaped detection in the present study because lipid-like molecules were likely to have been extracted in the solvents used to process our specimens. It is anticipated that future studies using frozen sections will further disclose
the chemical nature of abnormal glycoconjugates which accumulate in corneas with macular dystrophy.

**Key words:** cornea, macular dystrophy, lectin receptors, glycoconjugates

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**References**