Protein-Related Abnormalities in Keratoconus

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Two-dimensional electrophoretic maps of extracts from eleven normal and eleven keratoconus corneas were compared. Of the eleven corneas analyzed, eight were pooled and the remaining three were analyzed individually. Several differences were demonstrated between electrophoretic patterns of normal and keratoconus corneas. In keratoconus corneas, 1) two abnormal components (MW 54kD and 26kD) were observed; 2) three normal corneal components (MW 12kD, 14kD, and 39kD) were present in significantly higher amounts; and 3) three normal corneal proteins (MW 66kD, 55kD, and 13kD) were present in reduced amounts. The molecular weight and isoelectric point of one of the normal corneal proteins that we found to be reduced in keratoconus corneas were close to that of a subunit of prolyl-4-hydroxylase, an enzyme required for hydroxylation of proline residues of collagen. The possibility the abnormal proteins detected in the keratoconus corneas were derived from those normal corneal proteins which were absent or were present in reduced amounts in the keratoconus corneas remains to be established. This study may provide protein markers for elucidation of the biochemical abnormality in keratoconus. Invest Ophthalmol Vis Sci 30:2481-2487, 1989

Keratoconus is characterized by thinning and scarring of the central cornea. It is slowly progressive and either bi- or unilateral. Although the clinical and ultrastructural characteristics of the disease are well established,1-3 the biochemical defect in keratoconus has not been elucidated. Histochemical as well as biochemical approaches have not detected significant differences in collagen types between normal and keratoconus corneas.4-6 It has been shown that media incubated with keratoconus corneas, as well as stromal cells in culture derived from keratoconus corneas, contain higher levels of collagenolytic activity compared to normal controls.7-9 a result which suggests a decrease in corneal collagen content in keratoconus. Attempts to demonstrate reduced collagen levels in keratoconus corneas, however, have provided conflicting results.5,7,9,13 Cannon and Foster14 reported abnormally high levels of lysinorleucine crosslinks of collagen in keratoconus; these findings, however, could not be confirmed.15 Biochemical approaches to detect differences related to proteoglycans and glycosaminoglycans have also provided variable data. Hassell et al16 reported that the pattern of proteoglycans synthesized by keratoconus corneas in organ culture was indistinguishable from that synthesized by normal corneas. In an earlier study, it was reported that the hexosamine concentration in keratoconus corneas was about 40% greater than normal.10 Anseth,17 however, found that the concentration of hexosamine was identical in keratoconus and normal corneas. Andreassen et al12 found no differences in uronic acid concentration in normal and keratoconus corneas; in contrast, in a recent histochemical study, an abnormally high amount of polyanions that include primarily glycosaminoglycans was found in keratoconus corneas when compared to normal controls.18 In another study, using a monoclonal antibody specific for keratan sulfate, it was shown that compared to normal controls, some keratoconus corneas contained less keratan sulfate.19

Thus, in spite of a number of well-designed studies, the exact biochemical abnormality responsible for the development of keratoconus has yet to be elucidated. A major obstacle in the study of keratoconus has been the unavailability of specific biochemical markers with which investigations can be designed. In various nonocular studies, comparison of two-dimensional gel electrophoretic patterns of normal and diseased tissue have led to the identification of the disease-related proteins.20,21 Two-dimensional electrophoresis is a powerful research tool permitting resolution of up to 1,000 proteins from a single human cell type.22 To our knowledge, two-dimensional electrophoretic patterns of the complete extracts of normal or keratoconus corneas have not been compared to date. The purpose of this study is to compare two-
dimensional maps of extracts of normal and keratoconus corneas and thereby identify protein markers that may be specific to the disease.

Materials and Methods

Tissue

Human keratoconus buttons were obtained at the time of keratoplasty. With the exception of the keratoconus, the patients undergoing keratoplasty were clinically normal; there was no evidence of generalized connective tissue disorders. The diseased corneas were rinsed three times with saline and kept frozen at −80°C or in liquid N₂ until used. Normal central human corneas were excised with an 8-mm trephine from frozen eyes supplied by the Lions Eye Bank (Seattle, WA) and were rinsed three times with saline prior to use. For the normal eyes, the average time period between death and enucleation was approximately 7 hr, and between enucleation and freezing, approximately 6 hr. Eleven normal corneas and eleven keratoconus buttons were used. Within each group, eight corneas were pooled and the remaining three were investigated individually. Ages of the normal corneal donors and of the patients with keratoconus are shown in Table 1. Information concerning the extent of scarring in keratoconus buttons is also shown in Table 1.

Extraction of Proteins from Normal and Keratoconus Corneas

The epithelium was scraped off the corneas while they were still frozen. The corneas were then minced into six to eight pieces with a scalpel, on dry ice. The minced tissue was thawed directly in ice-cold 4 M guanidine hydrochloride (1–1.5 ml/cornea), and extraction was carried out for 16–18 hr on a wrist action shaker in the presence of a solution of protease inhibitors, composed of 1 mM phenylmethylsulfonylfluoride, 1 mM benzamidine hydrochloride, 100 mM 6-aminohexanoic acid, and 10 mM N-ethylmaleimide. The extract was collected by decantation, and the residue was reextracted as described above for an additional 6–8 hr. Both supernatants were pooled, dialyzed extensively against cold water, and lyophilized. The lyophilized material was suspended in cold distilled water and aliquots were used for protein determination and two-dimensional gel electrophoresis.

Protein Determination

The protein concentration in the corneal extracts was determined by the bicinchoninic acid (BCA) method²³ (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard. Protein yield in the extract ranged from 400–650 µg/cornea.

Two Dimensional Gel Electrophoresis

Two-dimensional electrophoresis was performed according to the method of O’Farrell²² by Kendrick Laboratories (Madison, WI) as follows. Isoelectric focusing using ampholines at 1.5%, pH 5–7; 1.5%, pH 5–8; and 1.0%, pH 3.5–10 (LKB Instruments, Baltimore, MD) was carried out in 135-mm × 2-mm tubes at 400 V for 12 hr followed by 800 V for 30 min. Forty ng of an isoelectric focusing internal standard, vitamin D-dependent calcium-binding protein, MW 27,000 and pI 5.2, was added to the samples. The final tube gel pH gradient was estimated by a surface pH electrode (Biorad, Richmond, CA) and by colored acetylated cytochrome pI markers (Calbiochem-Behring, La Jolla, CA) run in an adjacent tube. Electrophoresis in the second dimension was performed in 135-mm × 150-mm × 0.75-mm slab gels containing 10% acrylamide. The following proteins (Sigma Chemical, St. Louis, MO) were added as molecular weight standards to the agarose that sealed the tube gels to the slab gels: myosin heavy chain (220 kD), phosphorylase A (94 kD), catalase (60 kD), actin (43 kD) and lysozyme (14 kD). Protein spots were visualized by silver staining according to the procedure of Oakley et al.²⁴

Table 1. Ages of normal corneal donors and of patients with keratoconus at the time of keratoplasty

<table>
<thead>
<tr>
<th>Group number</th>
<th>Cornea</th>
<th>Number of corneas</th>
<th>Age (yr)</th>
<th>Degree of scarring</th>
<th>% of button scarred</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>normal</td>
<td>8</td>
<td>28–57 (mean: 41)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>normal</td>
<td>1</td>
<td>47</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>normal</td>
<td>1</td>
<td>47</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>normal</td>
<td>1</td>
<td>47</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>keratoconus</td>
<td>8</td>
<td>26–41 (mean: 33)</td>
<td>– to ++</td>
<td>0-10</td>
</tr>
<tr>
<td>6</td>
<td>keratoconus</td>
<td>1</td>
<td>47</td>
<td>+</td>
<td>≤5</td>
</tr>
<tr>
<td>7</td>
<td>keratoconus</td>
<td>1</td>
<td>52</td>
<td>+++</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>keratoconus</td>
<td>1</td>
<td>40</td>
<td>+</td>
<td>≤5</td>
</tr>
</tbody>
</table>

Scarring: – none, + minimal, ++ moderate, +++ severe.
Two-dimensional electrophoretic maps of normal corneas were compared with each other and with those of keratoconus corneas by overlapping the gels on a light box. The spots which were present only in the diseased corneas, or which were present in the diseased corneas in higher concentrations, were desig-
The spots which were absent, or which were present in lower intensity, in the diseased corneas as compared to normal corneas were designated as N spots. Approximate intensities of the N and K spots were measured using an Ultrosan XL laser densitometer (LKB). The polypeptide densities in the respective gels were normalized against two reference proteins which had constant density ratios to each other in all gels. The densities of these two proteins in each gel were used to create a normalization factor for each gel compared to an arbitrarily selected standard gel.

**Results**

Comparison of two-dimensional maps of normal and keratoconus corneas revealed that 1) two components, K1 and K5, were detected in keratoconus corneas, but not in normal corneas (Figs. 1, 2); 2) three components, K2–K4, were present in significantly higher amounts in keratoconus corneas compared to those in normal corneas (Figs. 1, 2); and 3) three normal corneal proteins, N1–N3, were present in significantly lower amounts in keratoconus corneas (Figs. 1, 3). Molecular weights and isoelectric points of all N and K proteins are shown in Table 2. Intensities of various K and N spots are shown in Figures 2 and 3, respectively. As shown in Figure 1, N2 consisted of several spots. For density measurement of N2, the area representing several spots was analyzed as a whole, and the intensity of the various spots within the N2 area was not measured individually. The density of component N3 was not measured because in a number of gels this component was not resolved from lysozyme, which was used as an internal standard (Fig. 1). Difference in the intensity of N3 between normal and keratoconus corneas was more prominent in the two-dimensional maps of individual corneas (not shown) than in those of the pooled corneas (Fig. 1). The broad band migrating beneath the K3 position in the two-dimensional patterns of normal corneas comigrated with K3 in the two-dimensional patterns of pooled as well as two of the three individual keratoconus corneas.

A mixed extract of keratoconus and normal corneas was also analyzed to determine whether those N and K components which were found to be present in both normal and keratoconus comigrate. As shown in Figure 4, components N1–N3 and K2–K4, which remaining electrophoretic patterns were obtained from individual corneas. Ninety micrograms of protein was loaded on each gel, and polypeptide densities in the respective gels were normalized against two reference proteins as described in the text.
were present in both keratoconus and normal corneas, though in varying amounts, comigrate. Among all the keratoconus corneas we studied, the data derived from case no. 6 appeared to deviate significantly from those of the rest of the diseased corneas. As shown in Figure 4, in the case-6 cornea, components K1, K2, and K5 were present only in trace amounts. In contrast, these components were present in substantially greater amounts in all other keratoconus corneas studied. The cornea of case 6 was also dissimilar from the other keratoconus corneas studied in that it contained a higher amount of component N1, compared to all other diseased specimens (Fig. 3).

Discussion

We have demonstrated that in keratoconus corneas several abnormal proteins are present and that the amounts of several normal corneal proteins are reduced. Specifically, our data indicate that in keratoconus corneas, two abnormal components (K1 and K5) are present and three normal corneal components (K2–K4) are present in significantly higher amounts. In addition, three normal corneal proteins (N1, N2, and N3) are present in reduced amounts in the diseased corneas.

The molecular weight and isoelectric point of the normal corneal component N1, detected only in trace amounts in keratoconus corneas, is close to that of a subunit of prolyl-4-hydroxylase. It remains to be determined whether component N1 is a subunit of prolyl hydroxylase, but one study has suggested an elevated level of prolyl-4-hydroxylase activity in keratoconus corneas. Reports in the literature regarding hydroxyproline concentration in keratoconus conflict; some have reported reduced levels, whereas others have found identical concentrations of this imino acid in normal and keratoconus corneas.

It is possible that the abnormal proteins we detected in keratoconus corneas may have been derived from those normal corneal proteins which appear to be absent, or present in reduced amounts, in keratoconus corneas. These abnormal proteins may have an altered or truncated amino acid sequence, or they may be proteins with a normal amino acid sequence that may have undergone abnormal processing, such as insufficient or excessive glycosylation, sulphation, or phosphorylation. For example, abnormal protein K2 (MW 12kD, pi 5.35) could have been derived by alteration of the amino acid sequence of the normal corneal protein N3 (MW 12kD, pi 5.7), resulting in the decrease of the isoelectric point. It is unlikely that the abnormal K proteins are degradation products of N proteins, because all extractions were performed at low temperatures in the presence of various protease inhibitors. However, because the average time period between enucleation and freezing of the normal eyes was about 7 hr, it is possible, although unlikely, that K spots were detected in lesser amounts in normal corneas as a result of proteolytic degradation.

It remains to be determined whether the abnormal corneal proteins we detected in keratoconus corneas are involved in the development of the disease. It is

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### Table 2. Molecular weights and isoelectric points of various N and K components

<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular weight (kD)</th>
<th>Isoelectric point (pi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>54</td>
<td>5.25</td>
</tr>
<tr>
<td>K2</td>
<td>12</td>
<td>5.35</td>
</tr>
<tr>
<td>K3</td>
<td>39</td>
<td>6.45</td>
</tr>
<tr>
<td>K4</td>
<td>15</td>
<td>7.38</td>
</tr>
<tr>
<td>K5</td>
<td>28</td>
<td>6.75</td>
</tr>
<tr>
<td>N1</td>
<td>62–72</td>
<td>5.20 (4.9–5.3)</td>
</tr>
<tr>
<td>N2</td>
<td>52–60</td>
<td>5.5–5.95</td>
</tr>
<tr>
<td>N3</td>
<td>13</td>
<td>5.7</td>
</tr>
</tbody>
</table>
possible that the abnormal electrophoretic pattern we
found in keratoconus corneas is at least in part due to
the nonspecific scarring associated with keratoconus.
This study may provide marker proteins for the elu-
cidation of the biochemical abnormality in kerato-
conus.

Key words: keratoconus, two-dimensional gel electrophore-
sis, proteins, cornea.

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Fig. 4. A two-dimen-
sional electrophorogram of
a mixed extract of normal
and keratoconus corneas.
Equal amounts of kerato-
conus and normal corneal
extracts were mixed, and a
total of 90 μg protein was
applied on the isoelectric
focusing gel. For other ex-
perimental details, see leg-
end for Figure I. Note that
all N and K components,
which are present in both
normal and keratoconus
corneas, comigrate in the
mixed extract. IEF = iso-
electric focusing; SDS = SDS
polyacrylamide gel
electrophoresis.