Three-Dimensional Scanning Electron Microscopic Study of Keratoconus Corneas

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Objective: To examine the 3-dimensional collagen fibril organization in the Bowman layer of keratoconus corneas.

Methods: Eight keratoconus corneas, 8 corneas with other diseases, and 5 normal human corneas were studied. A cell maceration method in combination with scanning electron microscopy was used to examine the collagen network in the Bowman layer.

Results: In normal corneas, the surface of the Bowman layer was smooth and collagen fibrils were regularly arranged. By contrast, sharply edged defects in the Bowman layer were found in keratoconus corneas. Lattice-like configurations of the ruptured Bowman layer and collagenous scar tissue were observed, to varying degrees, in all keratoconus corneas examined. None of the other diseased corneas exhibited the ruptures.

Conclusions: Scanning electron microscopy demonstrated alterations in the Bowman layer specific to keratoconus. Fragmentation of the Bowman layer may be an early change leading to keratoconus conditions.


Keratoconus is a non-inflammatory disease characterized by thinning and scarring of the central portion of the cornea. Histopathologic and ultrastructural studies have demonstrated that in early stages of the disease, fragmentation of the epithelial basement membrane occurs with disintegration of the Bowman layer and fibrillation of the anterior stroma. The central cornea then becomes thinned, with destruction of the Bowman layer and stromal scarring. A loss of collagen lamellae occurs, but the collagen fibrils are of normal diameter. The lamellae are surrounded by granular materials, which are shown to be rich in neutral polysaccharides and glycoproteins.

In advanced stages of the disease, the central portion of the Descemet membrane may rupture, resulting in acute hydrops.

Electron microscopic studies aimed at 2-dimensional examination of keratoconus corneas were performed more than 20 years ago. Since then, the technology, resolution, and machinery have vastly improved.

Our investigation sought to revisit the structure of keratoconus corneas, focusing particularly on the 3-dimensional collagen architecture in the Bowman layer. Direct observation of this structure by conventional techniques has been infeasible because of the overlying corneal epithelium. With a recently developed cell maceration and tissue conductive method, we removed the corneal epithelium and visualized the 3-dimensional collagenous architecture of the Bowman layer under scanning electron microscopy. Multiple ruptures in the Bowman layer of keratoconus corneas and the possible sequence of events were demonstrated.

The Table summarizes the clinical features of 8 patients with keratoconus, including age at onset, age at surgery, and ocular manifestations. Patients 5 and 6 had a history of acute hydrops and patients 4 and 8 had relatively mild cases. None of the patients had a family history of the disease except for patient 7, whose brother had keratoconus. Routine pathologic examinations showed the typical features of keratoconus, including breaks in the Bowman layer, scarring, and iron ring.
MATERIALS AND METHODS

TISSUES

Eight corneal buttons from patients with typical clinical features of keratoconus (Table) were obtained at the time of penetrating keratoplasty from the Cornea Service of the University of Illinois at Chicago College of Medicine or Seirei Hamamatsu Hospital, Shizuoka, Japan. Patients ranged in age from 18 to 46 years at the time of surgery. Five normal human corneas from donors (aged 2, 19, 43, 53, and 66 years) were obtained from the Illinois Eye Bank, Chicago, within 24 hours of death. The corneas were clear and the donors did not have known ocular disease.

To serve as another set of controls, corneas were obtained from 8 patients with other corneal diseases. They were from 2 patients (aged 69 and 70 years) with pseudophakic bullous keratopathy, 1 patient (aged 18 years) with bullous keratopathy associated with absolute glaucoma, 1 patient (aged 55 years) with corneal scar after ulceration, 2 patients (aged 55 and 58 years) with lattice corneal dystrophy, 1 patient (aged 48 years) with granular corneal dystrophy, and 1 patient (aged 51 years) with herpetic interstitial keratitis.

SCANNING AND TRANSMISSION ELECTRON MICROSCOPY

Corneas obtained were fixed immediately in 2.5% glutaraldehyde and 2% formalin in Sorensen phosphate buffer solution for 2 to 5 days and transferred to buffered formalin until processing.

The cell maceration method was carried out as previously described. Briefly, the fixed specimens were rinsed in distilled water overnight, immersed at room temperature for 5 days in a 10% sodium hydroxide solution, and rinsed thoroughly with several changes of distilled water for 24 hours. They were then subjected to a conductive staining method by soaking in 2% tannic acid for 3 hours, washed in distilled water for 1 hour, and postfixed in aqueous 1% osmium tetroxide for 3 hours. After dehydration through a graded ethyl alcohol series, the specimens were transferred to isooxyl alcohol, critical-point dried, mounted on aluminum stubs, and coated with gold in an ion coater (Hitachi, Tokyo, Japan). Scanning electron microscopy was performed with a scanning electron microscope (Hitachi S-2300) at an accelerated voltage of 25 kV.

To ensure that the corneal specimens were processed properly, pieces of the tissue were embedded in epoxy resin after cell maceration and conductive staining. Ultrathin sections were double stained with uranyl acetate–lead citrate and were observed under a transmission electron microscope (Hitachi H-7000).

As evidenced by transmission electron microscopy, treatment of corneas with 10% sodium hydroxide solution removed most of the cellular elements, basal laminae, and other extracellular materials, leaving collagen fibrils relatively intact (Figure 1). The scanning electron micrographs shown in Figure 2 depict the surface of the Bowman layer from normal human corneas at varying magnifications. At lower magnifications (Figure 2, A and B), smooth surface of the Bowman layer was observed. At higher magnifications (Figure 2, C and D), the Bowman layer displayed a honeycomb-like porous structure, made up of a dense meshwork of collagen fibrils. Similar features were found in all 5 normal human corneas studied.

In contrast, multiple sharply edged defects in the Bowman layer (Figure 3) were noted in all the keratoconus corneas examined. A lattice-like configuration of ruptured Bowman layer was found. Hypertrophic collagenous proliferation, which partially or totally replenished the ruptures, was observed. The fact that patients 5 and 6 (Figure 3, D and E) had a history of acute hydrops may explain why much more collagenous proliferation was observed.

Under a higher magnification, the proliferation of collagenous tissue and ruptures in the Bowman layer could be viewed more clearly (Figure 4, A). The ruptured areas of the Bowman layer were filled by proliferated collagenous tissue that appeared to derive from the anterior stroma just beneath the Bowman defects (Figure 4, B).

When specimens were observed from a lateral view, normal human corneas (Figure 5, A) showed well-packed collagenous lamellae with spindle-shaped remains of sodium hydroxide–digested keratocytes and the Bowman layer (Figure 5, A, between white arrows) uniformly covering the stroma. In keratoconus corneas, misaligned Bowman layer (Figure 5, B, arrow), irregularly thinned Bowman layer (Figure 5, C) and defects (Figure 5, D) were demonstrated. With increasing severity of damage in the Bowman layer, the arrangement of collagenous lamellae also seemed to be more distorted into the deeper stroma (Figure 5, B through D) of keratoconus corneas. Under a higher magnification, the well-packed and well-aligned collagenous lamellae observed in normal human corneas (Figure 5, E) were replaced by the loose and randomly oriented collagen fibrils in keratoconus corneas (Figure 5, F). Multiple pores (Figure 5, F, asterisks) were seen.

None of the other diseased corneas (Figure 6) exhibited these alterations in the Bowman layer under scanning electron microscopy. No multiple breaks were observed. The surface appearance was unlike that of the keratoconus cases even when prominent collagenous scar tissue could be seen, such as those in the cases of bullous keratopathy (Figure 6, A through C) and corneal scarring (Figure 6, D). Corneas from the patient with lattice corneal dystrophy (Figure 6, E) and the patient with granular corneal dystrophy (Figure 6, F) had a characteristic lattice or granular appearance, respectively.

The current scanning electron microscopic study after a cell maceration and tissue conductive procedure distinctly demonstrated the 3-dimensional organization of collagen fibrils in the Bowman layer of normal human, keratoconus, and other diseased corneas. This method,
previously used successfully for examination of peripheral nerve,\textsuperscript{8,9} pial septa,\textsuperscript{10} cornea,\textsuperscript{11-13} sclera,\textsuperscript{11} and the Bruch membrane,\textsuperscript{14} allows a direct visualization of the architecture of collagen fibrils, which, to our knowledge, had not previously been attainable for the Bowman layer.

Keratoconus specimens in this study, depending on disease severity, showed varying degrees of lattice-like, sharply edged ruptures and fragmentation of the Bowman layer. In more advanced areas, hypertrophic collagenous proliferation partially or totally filled the ruptures. The observed characteristic breaks and changes in the Bowman layer confirm and expand findings reported previously by light and electron microscopy.\textsuperscript{2-5,12,13} When the changes in the Bowman layer were minimal to mild, the corneal stroma underlying the Bowman layer correspondingly showed only minimal changes. However, the corneal stromal alteration advanced when damage in the Bowman layer became more evident in keratoconus.

In 2 earlier light and electron microscopic studies, Chi et al\textsuperscript{2} and Teng\textsuperscript{3} postulated that the ruptures of the Bowman layer were most likely to be replaced by tissue from stroma underneath the Bowman layer. Such a possibility was substantiated by this study. The ruptured areas appeared to be filled with proliferated collagenous tissue from the anterior stroma just beneath the ruptured Bowman layer.

By slitlamp microscopy, anterior clear spaces have been observed within the thin portion of the conical protrusion in both early and advanced cases of keratoconus.\textsuperscript{15} Histologic examinations further revealed that the clinical finding correlated with breaks in the Bowman layer. It was suggested that the clear zones observed may represent breaks in the Bowman layer before the scar formation. In the current study, we are unable to determine whether our scanning electron

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|c|}
\hline
Patient & Age at Onset/Surgery, y & Clinical Features & Family History of Keratoconus & Other Data* \\
No./Sex & & & & \\
\hline
1/M & 16/22 & Iron ring, scar & No & - - - \\
2/M & 20/41 & Iron ring, scar & No & - - - \\
3/M & 17/23 & Iron ring, scar & No & - - - \\
4/F & 29/46 & Iron ring, scar & No & Hay fever \\
5/M & 16/42 & Scar, acute hydrops & No & Atopy, eye rubbing \\
6/M & 15/18 & Scar, acute hydrops & No & Arthritis, atopic dermatitis, cataract \\
7/F & 35/45 & Iron ring, scar & Yes† & Asthma, atopic dermatitis, cataract \\
8/M & 35/42 & Iron ring, scar & No & Arthritis, atopic dermatitis, eye rubbing \\
\hline
\end{tabular}
\caption{Clinical Characteristics of Patients With Keratoconus}
\end{table}

* Ellipses indicate not applicable.
† The patient’s family history includes a brother with keratoconus and mild atopy and a father with atopy.

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Transmission electron micrographs of corneas from (A) a 19-year-old normal subject and (B) a 22-year-old keratoconus-affected patient (patient 1) after treatment with sodium hydroxide. Note that almost all cellular and extracellular elements appear to be removed and only collagen fibrils remain relatively intact. BM indicates Bowman layer; ST, corneal stroma. Arrowheads point to the edge of ruptured BM layer in keratoconus.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{Scanning electron microscopic observations of the Bowman layer from a 19-year-old normal subject at varying magnifications. At lower magnifications (A and B), the surface of Bowman layer appears to be slightly wavy. At higher magnifications (C and D), the regular collagen fibrillar network displays a honeycomb pattern.}
\end{figure}
Figure 3. Scanning electron microscopic findings in corneas from 7 patients with keratoconus (A through G, patients 2 through 8). Specimens show varying degrees of sharply edged ruptures in the Bowman layer. Hypertrophic collagenous scar tissue fills the gaps. More collagenous scar tissue covering the Bowman layer (asterisks) is found in specimens from patients 5 (D) and 6 (E), who had a clinical history of acute hydrops.

Figure 4. Scanning electron micrographs of the cornea from a patient with keratoconus (patient 1). A, Proliferated collagenous tissue (arrows) and relatively normal area of the Bowman layer (lower left) are demonstrated. Between the 2 areas, defects of the Bowman layer can be seen (asterisks). B, Area between the asterisks in A at higher magnification. Sharply edged ruptures (arrowheads) can be seen. Defects of the Bowman layer appear to be occluded by proliferated collagenous tissue of anterior corneal stroma (arrows). Note that the honeycomb pattern on the surface of the Bowman layer is distorted around the gaps.
microscopic findings can be correlated with the clinical finding, as slitlamp photographs were unavailable.

We\textsuperscript{16} and other investigators\textsuperscript{17,18} have reported that the collagen content in some cases of keratoconus is reduced compared with normal human corneas. In this study, loosely packed and randomly oriented collagen fibrils were demonstrated in some of the keratoconus corneal stroma, which may reflect the reduced collagen density. Consistent with the earlier histologic findings,\textsuperscript{4} abnormal chondroitin and dermatan sulfate-type proteoglycans were more recently found to accumulate in keratoconus corneas around collagen fibrils and collagen lamellae.\textsuperscript{19} The numerous pores noted in the stroma of keratoconus corneas in this study may thus represent

Figure 5. Lateral views of scanning electron microscopic findings from corneas obtained from a 43-year-old normal subject (A and E) and a patient with keratoconus (patient 1) (B through D and F). Compared with the regular Bowman layer (A), the keratoconus cornea showed irregularly thinned and partially disrupted Bowman layer (B through D). In the normal human cornea (A and E) and in areas at perhaps a very early stage of Bowman layer changes in keratoconus (B), collagen lamellae appear to be well packed and regularly organized. Only a few spindle-shaped defects are seen in B, compared with the increasingly distorted, loosely packed collagen lamellae with numerous irregular pores in moderate (C) to advanced (D and F) areas of the keratoconus specimen. Arrow in part B indicates the very early and mild changes of the Bowman layer in keratoconus; asterisks in part F, irregularly shaped stromal defects with irregularly and loosely arranged collagen fibrils in keratoconus.
areas occupied by keratocytes and by the abnormal proteoglycan molecules that are dislodged during the sodium hydroxide treatment. In nonkeratoconus diseased corneas, we noted no alterations in the Bowman layer characteristic of keratoconus changes.

The Bowman layer is an acellular matrix at the interface between the corneal epithelium and the stroma. It links the epithelial basement membrane and the stroma proper and may be crucial for the epithelial attachment and function. During human corneal epithelial development, a distinct Bowman layer is formed at 19 weeks. After birth, the thickness of the Bowman layer remains unchanged. Components of Bowman layer are believed to be synthesized by both corneal epithelial and stromal cells and the epithelial-stromal interaction may be a major factor in the formation of the Bowman layer. Several years after radial keratotomy in human corneas, a Bowman layer–like structure was formed underneath epithelial plugs that extended into the stroma. The collagen fibrils in the Bowman layer are of relatively small diameter and are randomly arranged. It is unclear however how these collagens are organized or how they are maintained. In the underlying corneal stroma, the resident stromal cells are responsible for the maintenance and organization of the collagens. However, the Bowman layer is acellular. One possibility is that the maintenance is performed by the sparse stromal cells that transverse into the Bowman layer. Alternatively, cytokines may also be

Figure 6. Surface views under scanning electron microscopy of other diseased corneas. Corneas were obtained from 70-year-old (A), 69-year-old (B), and 18-year-old (C) patients with bullous keratopathy, a 55-year-old patient with corneal scar (D), a 55-year-old patient with lattice corneal dystrophy (E), a 48-year-old patient with granular corneal dystrophy (F), and a 55-year-old patient with herpetic infection (G). Note that no findings such as multiple breaks of the Bowman layer seen in keratoconus (Figure 3) can be demonstrated. However, depending on disease conditions, different degrees of collagenous scar tissues are observed.
involved. In keratoconus, the maintenance function of the stromal cells or the cytokines may be disturbed both for the Bowman layer and for the stroma.

The etiologic mechanism for the development of keratoconus is not fully clear. One hypothesis, based on data from our laboratory, and others, is that the degradation processes in keratoconus may be aberrant. Supporting evidence includes elevated lysosomal hydrolase activities in keratoconus corneas. Through the course of enzyme and inhibitor studies, the corneal epithelium consistently shows the most dramatic biochemical abnormalities in keratoconus.

It thus has been suggested that even though the thinning in keratoconus occurs primarily in the stroma, the corneal epithelium may also be involved in the disease development.

The corneal epithelial theory was proposed based on an electron microscopic examination by Teng in the early 1960s. Lacking convincing evidence, however, this suggestion has since been considered as only conjecture. Our studies showing biochemical alterations mostly in the corneal epithelium provide more direct support for the theory. It is also corroborated by our finding that conjunctival epithelial cells from patients with keratoconus contain higher than normal lysosomal hydrolase activities.

The sequence of events noted in this study further indicates that the changes in the Bowman layer precede those in the corneal stroma, suggesting that the corneal epithelium may be an important factor at the initial or early stage of keratoconus development. One scenario may be that the increased degradative enzymes and reduced inhibitors in the corneal epithelium trigger rupture and fragmentation of the Bowman layer. The subsequent environmental changes or the interactions between the epithelial cells and the genetically predisposed stromal cells may ultimately induce the thinning and scarring manifested in keratoconus.

The epithelial-stromal interaction has been considered to be a factor involved in the development of keratoconus. Wilson et al. postulated that interleukin 1 (IL-1) may be a modulator of epithelial and stromal interactions, regulating the corneal cell proliferation, differentiation, and death. They have also proposed a role of the IL-1 system in the causes of keratoconus. Interestingly, cultured keratoconus stromal cells have been shown to contain 4-fold higher binding sites for IL-1. An enhanced expression of IL-1 receptor has also been noted in keratoconus corneas. The IL-1 hypothesis is consistent with the degradation hypothesis because IL-1 is known to regulate the expression of matrix metalloproteinases in the cornea.

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