The Three-Dimensional Organization of Collagen Fibrils in the Human Cornea and Sclera

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The organization of collagen fibrils in the human cornea and sclera was studied by scanning electron microscopy, after digestion of cellular elements by sodium hydroxide, and by conventional transmission electron microscopy. The collagen fibrils in the cornea had a uniform diameter of about 25 nm. In Bowman's layer, individual collagen fibrils were interwoven densely to form a felt-like sheet. In the stroma, most of the collagen fibrils ran abreast in lamellae, with varying widths and thicknesses. These lamellae were arranged basically parallel to the corneal surface but often communicated with those of adjacent layers by interchanging their fibrils. In the innermost stromal region adjacent to Descemet's membrane, collagen fibrils were oriented in various directions and interlaced, forming loose fibrillar networks. The sclera, however, was composed of collagen fibrils with various diameters ranging from 25–230 nm. Although these collagen fibrils formed bundles, they were not parallel but were entangled in individual bundles. These collagen bundles varied in width and thickness, often gave off branches, and intertwined with each other. Invest Ophthalmol Vis Sci 32:2244–2258, 1991

The cornea and sclera together form the outer fibrous tunic of the eye and withstand both internal and external forces to maintain the shape of the eyeball. Although both of these structures consist mainly of collagen fibrils, their optical properties are different. The cornea is transparent; the sclera is not. To clarify the histologic basis for this difference, many investigators studied the cornea and sclera by focusing on the patterns of collagen fibril arrangement by light microscopy,1–3 transmission electron microscopy (TEM),4–15 light scattering,16–19 and x-ray diffraction.20,21 From his chemical and microscopical studies of the corneal stroma, Maurice22 framed the hypothesis that the corneal transparency is a result of the regular, lattice-like arrangement of collagen fibrils in the stroma. Such an arrangement, if the fibril spacing is less than half the wavelength, leads to destructive interference of the light except in the forward direction. Although this interpretation appears to have been accepted after some alterations by later investigators, The NaOH cell-digestion method24 is very effective in removing cellular elements and extracellular matrices (except collagen fibrils) from the tissues, making it possible to observe directly the three-dimensional architecture of collagen fibrils in the cornea and sclera.

We studied the human cornea and sclera by scanning electron microscopy (SEM), after cell digestion with sodium hydroxide, and by conventional TEM. The NaOH cell-digestion method24 is very effective in removing cellular elements and extracellular matrices (except collagen fibrils) from the tissues, making it possible to observe directly the three-dimensional architecture of collagen fibrils in the cornea and sclera.

Materials and Methods

Eight human eyes were provided from the Eye Bank of Iwate Medical University. They were obtained from subjects aged 63–82 yr. The eyeballs were immersed in a fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) under normal pressure for 5 days or more.

For the TEM study, the cornea and sclera were cut into small pieces about 1 × 2 mm and postfixed with...
1% OsO₄ in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 2 hr. They were dehydrated in a graded series of ethanol and embedded in Epon 812 (TAAB, Reading, England). Ultrathin sections were cut with a diamond knife on an LKB ultratome (LKB, Bromma, Sweden), stained both with tannic acid and uranyl acetate solution²⁵ and lead citrate, and then examined in a transmission electron microscope (H-700; Hitachi, Tokyo, Japan).

For the SEM study, the specimens, fixed similarly, were immersed in a 10% aqueous solution of NaOH for about 5 days at room temperature (20–25°C), and rinsed in distilled water for about 1 day.²⁴ They then were processed by a conductive staining method.²⁶ The specimens were immersed in a 2% aqueous solution of tannic acid for 3 hr, washed in distilled water for more than 1 hr, and postfixed in an aqueous solution of OsO₄ for 3 hr. The conductive-stained specimens then were dehydrated in a graded ethanol series. To observe the deeper layers of the corneal stroma, some of the dehydrated specimens were embedded in paraffin, cut with a microtome tangentially to the corneal surface, deparaffinized in xylene, and then put in absolute ethanol. All specimens were transferred to isoamyl acetate and critical point-dried using liquid CO₂. The dried specimens were mounted on aluminum stubs, coated with platinum in an ion coater (Eiko IB5; Eiko, Japan) and observed in by SEM (S-2300 or S-800; Hitachi) at an accelerating voltage of 10 kV.

Results

Cornea

The cornea usually is divided into five layers: epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium. Of these, only Bowman's layer and stroma contain collagen fibrils. Therefore, we will concentrate our description on these two layers.

TEM observations: Bowman's layer (Fig. 1) was composed of collagen fibrils with a diameter of 20–25 nm. These fibrils were not ordered in bundles; individual fibrils ran in various directions to form a sheet about 8–12 μm thick as a whole. The anterior surface
Fig. 2. Collagen fibrils in the corneal stroma observed by TEM. Collagen fibrils are arranged in a lamellar fashion. L = lamellae; K = keratocyte (X8600).

Collagen fibrils in the stroma had a uniform diameter of 25–35 nm (Figs. 2, 3). These fibrils ran parallel to each other with somewhat regular spacing to form flat lamellar bundles. In the anterior one third of the stroma, collagen lamellae were thin (about 0.2–1.2 μm thick), ran mostly obliquely to the corneal surface, and sometimes split into two or three sublayers that became interwoven. Moving posteriorly, most of the collagen lamellae tended to be arranged parallel to the corneal surface. The lamellae were thicker (0.2–2.5 μm) than those in the superficial layers. Flattened keratocytes with thin, long processes were located between these collagen lamellae.

Interposed between the stroma and Descemet's membrane was a thin layer (about 0.5 μm) consisting of irregularly disposed collagen fibrils (Fig. 4). Those in the innermost part apparently were embedded in Descemet's membrane.

SEM observations: Treatment of the cornea with 10% NaOH solution consistently removed cellular elements, basal laminae, Descemet's membrane, and interfibrillar substance of the stroma while leaving collagen fibrils undamaged. Although this method caused a slight swelling of the cornea, the original shapes and locations of the collagen fibrils were preserved well.

Bowman's layer was seen as a dense felt-like sheet with a thickness of 8–12 μm (Fig. 5). This sheet was composed of collagen fibrils intimately interwoven into a dense fibrous lattice. There were numerous microridges and shallow depressions, which gave a honeycomb appearance to the surface of Bowman's sheet (Figs. 5, 6). Small pores (about 0.5–1.5 μm in diameter), which appeared to penetrate Bowman's sheet, were seen on the surface. The collagen fibrils in the posterior layer of Bowman's sheet gradually were assembled in bundles and merged in the collagen lamellae of the stroma.
The stroma was composed of successively stacked layers of lamellae of collagen fibrils (Figs. 7, 8). These lamellae varied in width (about 0.5–250 μm) and thickness (about 0.2–2.5 μm), but they tended to be wider and thicker toward the posterior region. The lamellae in the anterior region had a flat, tape-like shape with 0.5–30 μm width and 0.2–1.2 μm thickness; those in the posterior region often looked like broad sheets, about 100–200 μm wide and 1–2.5 μm thick. Because keratocytes were removed completely by the cell-digestion treatment, the locations of keratocytes were left as spaces of various sizes within or between the lamellae. The arrangement of the collagen lamellae also showed regional differences as follows. The lamellae in the anterior region (Fig. 7) ran in random directions and were often branched and intertwined in an irregular manner. Those in the posterior region (Fig. 8) were piled up almost parallel to the corneal surface. When specimens were sectioned obliquely or tangentially, the lamellae in the posterior layers were shown to run across their neighbors at varying angles with very few lamellae crossing at a right angle. These lamellae often divided into smaller components that later merged with adjoining lamellae. At a higher magnification (Fig. 9), each lamella was composed of thin collagen fibrils running in parallel. This parallel array of fibrils was regular, although slight undulations were found in places probably as a result of artifacts caused by the chemical treatment. Loose meshworks of collagen fibrils were observed on the surface of some lamellar bundles.

Collagen fibrils in the innermost layer of the stroma (Figs. 10, 11) were not aligned in bundles. Individual
fibrils ran in various directions and were interwoven to form a thin sheet of collagen networks.

Sclera

Because the orientation of the scleral collagen fibers showed regional differences, our description was restricted to the equatorial region of the eye.

TEM observations: Collagen fibrils in the sclera (Figs. 12, 13) exhibited a wide range of diameters from 25–230 nm. Although these fibrils formed lamellar bundles, their arrangement in the individual bundles was more random than that in the cornea. The fibrils were grouped in subbundles occasionally separated from each other by large empty spaces. Collagen bundles varied from about 0.5–6.0 μm in thickness. They tended to be somewhat thinner in the outer than in the inner zone of the sclera. These bundles appeared to run almost parallel to the scleral surface and were interwoven with each other. Elastic fibers and microfibrils occasionally were found between (and sometimes within) these collagen bundles. Fibroblasts and their processes were interposed between collagen bundles.

SEM observations: The external region of the sclera (Fig. 14) was composed of collagen bundles narrower (about 1–5 μm) and thinner (about 0.5–2 μm) than those in the inner region. In the outermost layer, collagen bundles, consisting of thin (about 80 nm) collagen fibrils, intersected at various angles along the surface of the sclera. Beneath this layer, collagen bundles usually were oriented either meridionally or circularly; they were interwoven densely. In a still deeper layer (Fig. 15), although collagen bundles showed a wide range of widths (about 1–50 μm) and thicknesses (about 0.5–6 μm), most of them were 10–50 μm wide and 1–6 μm thick and tended to be wider and thicker toward the inner layers. These thicker bundles sometimes appeared to be assemblies of many thinner bundles. Thicker bundles ran in various directions, often giving off branches and intertwined in a complex fashion. At a higher magnification of tangential sections (Fig. 16), the collagen bundles in this region were composed mainly of thick (about 200 nm) collagen fibrils that did not run in a parallel fashion, but rather were organized in a wavy, intermingled fashion. Between such major bundles, there were very loose fibrillar net-
works consisting of entangled collagen fibers and fibrils, some of which obviously derived from surrounding major bundles.

Discussion

Our SEM study clearly and precisely demonstrated the three-dimensional organization of collagen fibrils in the human cornea and sclera using the NaOH cell-digestion method. Previous studies revealed that this digestion method is useful in various tissues for the observation of three-dimensional architecture of collagen fibrils by SEM. By NaOH treatment, cellular and some extracellular elements including interfibrillar matrices, elastic fibers, and basal laminae were removed effectively from the tissues without any appreciable damage to the collagen fibrils. Our study showed that this method is also useful for the SEM observation of the collagen fibrils in the cornea and sclera.

Collagen Arrangement in the Cornea

Previous TEM studies showed that Bowman's layer consists of uniform-sized collagen fibrils running in random directions. We clearly visualized the dense meshworks of collagen fibrils in this layer. In addition, we observed the presence of a honeycomb pattern and small, presumably penetrating, pores on the anterior surface. This honeycomb pattern appears to reflect the contour of the base of the epithelium and the attachment of anchoring fibrils to the basal laminae at the hemidesmosomes. The small pores probably represent pathways of nerve fibers innervating the corneal epithelium. The lamellar arrangements of collagen fibrils in the...
stroma also were demonstrated clearly by SEM. Previous TEM studies\textsuperscript{11-15} showed that collagen lamellae in the superficial one third of the stroma are much narrower and more irregularly interwoven than those in the deeper two thirds. Our study confirmed these regional differences in the collagen lamellae. This finding agrees with the fact that dissection is easier in the posterior than in the anterior stroma in lamellar keratoplasty.\textsuperscript{31} With a biomicroscope, the anterior third of the stroma appears slightly gray rather than whitish like the posterior two thirds, indicating differences in the tissue structure including the arrangement of collagen lamellae.

The relationship between adjacent lamellae also was clarified in our study. Using interstitial injecting methods, the lamellar bundles were found to cross irregularly and form interlaced meshworks in the central area of the human cornea.\textsuperscript{1} When corneas of the
Fig. 9. Higher magnification of the lamellar surface of the cornea. Collagen fibrils of each bundle are arranged in the same direction. Small bundles (arrows) extended between lamellae. Fine netlike patterns are observed on the lamellar surface (arrowheads) (×6200).

Fig. 10. The innermost region of the stroma adjacent to Descemet's membrane. (The endothelium and Descemet's membrane have been removed by digestion.) Collagen fibrils adjoining Descemet's membrane make a thin feltlike sheet (arrows), whereas collagen fibrils of the overlying layers are oriented in the same directions to form platelike bundles (×6000).
rabbit, dog, cattle, and human were studied by silver impregnation, a similar interweaving and branching pattern of the lamellae was found. Our study showed the three-dimensional features of lamellae crisscrossing at various angles to form interlaced meshworks in the anterior and posterior stroma. Furthermore, there were small collagen bundles extending between neighboring lamellae that formed closer relationships between lamellae. As shown in many TEM studies, each lamella consists of uniform-sized collagen fibrils arranged in parallel. This pattern of collagen fibril arrangement may be related to the transparency of the cornea, as discussed subsequently.

The collagen networks in the innermost layer of the stroma are considered to contribute to tight binding between the stroma and Descemet’s membrane.

Collagen Arrangement in the Sclera

Our study clearly demonstrated that, unlike the cornea, bundles of collagen fibrils in the sclera show no plate-like arrangement and they are interwoven in a more irregular and complex pattern than those in the cornea. In the TEM study of human sclera, it was noted that collagen bundles were mostly interwoven randomly in the inner region but were arranged almost in a lamellar fashion in the outer region. Our findings agree with these observations.

We found that the outer region of the sclera consists
of layers of slender collagen bundles. The elaborate intertwining of the collagen bundles may be functionally important because this structure is considered to be effective in giving the eyeball its rigidity and flexibility against changes in intraocular pressure. The most remarkable finding of our study was the three-dimensional demonstration of irregular and intermingled arrangements of collagen fibrils in individual bundles in the deeper layers. Such an arrangement of collagen fibrils is in striking contrast with the cornea and may be related to the opacity of the sclera.

**Some Considerations With Regard to Corneal Transparency**

Corneal transparency has been explained basically in two different ways: one is based on the assumption that the collagen and intervening ground substance have uniform refractive indices, and the other is the pattern of spatial arrangement of collagen fibrils that are responsible for destructive interference of the light except in the forward direction.

As shown in our study, the corneal stroma consists
Fig. 13. Higher magnification of collagen fibrils in the sclera. Scleral collagen fibrils display various diameters. They are much larger than those in the cornea. Mf = microfibril (×18,000).

of characteristic lamellae of collagen fibrils; each collagen lamella is composed of uniform-sized collagen fibrils running parallel to each other. It is true that the collagen fibrils are not disposed in a completely regular pattern like the crystalline lattice as described by Maurice.22 This may be a result partially of artificial distortion induced by tissue preparation techniques such as fixation, NaOH treatment, and dehydration. According to some investigators,18,33,34 collagen fibrils in the corneal stroma are arranged in a quasirandom order, which is sufficient for corneal transparency. One such ordering is short-range ordering; the fibril positions are correlated only over a few nearest-neighbor distances. The other is long-range ordering in which the fibril positions are disturbed slightly from ideal lattice sites. We, therefore, assume that the arrangement of uniform-sized fibrils might be important in corneal transparency. The intermingled pat-
tern of collagen fibrils in the sclera strikingly contrasts with the regular arrangement in the cornea. It is evident that such disordering of the fibrils causes the scleral opacity. The opacity also may be related to the diameter of the scleral collagen fibrils, because fibrils with large diameters scatter light strongly.

The spatial relationship of adjacent lamellae also is considered to be an important factor for the corneal transparency. Our study showed that the arrangement of the stromal lamellae is more complicated than expected. This finding opposes the generally accepted notion that corneal transparency is a result of the regular arrangement of collagen bundles. However, our TEM observations indicated that the collagen lamellae are attached closely to each other with interlamellar spaces smaller than the wavelength of light. Therefore, we consider that stacked lamellae, as a whole, optically act as a single uniform sheet, causing no significant reflections at the surface of individual lamellae.
Key words: collagen fibrils, cornea, sclera, scanning electron microscope, human

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