Three-Dimensional Analysis of Collagen Lamellae in the Anterior Stroma of the Human Cornea Visualized by Second Harmonic Generation Imaging Microscopy

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PURPOSE. The structure of collagen lamellae in the anterior stroma of the human cornea is thought to be an important determinant of corneal rigidity. The three-dimensional structure of such collagen lamellae in normal human corneas was examined.

METHODS. The anterior portion of 27 normal human corneas was obtained from donor tissue for Descemet’s stripping automated endothelial keratoplasty (DSAEK) surgery, and blocks (~3-mm square) of the central cornea were examined by second harmonic generation (SHG) imaging microscopy. Each cornea was scanned from the surface of Bowman’s layer to a depth of 150 μm, and SHG forward signals were collected. The angles of collagen lamellae immediately below to a depth of 30 μm below Bowman’s layer (sutural lamellae) as well as of those at a depth of 50 or 100 μm were measured. The density and width of sutural lamellae were also evaluated.

RESULTS. Collagen lamellae in the anterior stroma were evenly distributed and randomly oriented. The angle of sutural lamellae relative to Bowman’s layer was 19.19 ± 4.34° (mean ± SD). The angles of collagen lamellae at depths of 50 or 100 μm were 8.91 ± 2.91 and 6.91 ± 2.11°, respectively. The density of sutural lamellae was 910.0 ± 480.4/mm², and their width was 13.14 ± 5.03 and 7.11 ± 3.00 μm in the region immediately beneath and 50 μm below Bowman’s layer, respectively.

CONCLUSIONS. Collagen lamellae in the anterior stroma of the normal human cornea are interwoven in three dimensions and adhere densely to Bowman’s layer. This structure may contribute to the rigidity and curvature of the anterior portion of the cornea.

The corneal stroma occupies ~90% of the entire thickness of the cornea, and is composed predominantly of collagen. The collagen molecules in the corneal stroma form triple-helix collagen fibers, and bundles of these collagen fibers form collagen lamellae.1 Approximately 250 to 300 collagen lamellae, each with a width of 10 to 320 μm and a thickness of 0.2 to 2.5 μm, occupy the entire thickness of the central corneal stroma in humans.2–4 Electron microscopy has revealed the three-dimensional (3D) arrangement or alignment of collagen fibers and lamellae in the cornea.4,5 Anatomic analysis has also shown that the structure of the collagen lamellae is not homogeneous, with the lamellae being interwoven in the anterior stroma and parallel in the mid to posterior stroma.6,7 In addition, x-ray scattering analysis of collagen structure in the cornea demonstrated that the orientation of stromal collagen changes with tissue depth.8,9 An interwoven microstructure of the anterior stroma is consistent with the results of both x-ray scattering analysis and microscopic observation.10 The structure of collagen lamellae in the corneal stroma is thus not uniform but varies, thereby contributing to the maintenance of corneal transparency.

We have previously characterized the structure of collagen lamellae in the human cornea with second harmonic generation (SHG) imaging microscopy,11–14 which allows the specific visualization of collagen in the cornea without sectioning.11 The collagen lamellae in the anterior stroma were found to be well interwoven (transverse lamellae). However, the extent of interweaving of the collagen lamellae was reduced in the mid stroma (interwoven lamellae), and the lamellae were arranged in a parallel manner in the deep stroma (orthogonal lamellae). We also observed that many collagen lamellae in the anterior stroma were anchored to Bowman’s layer (sutural lamellae).11–14 The distribution of the sutural lamellae was uneven and their density was decreased in the keratoconic cornea,12 whereas the sutural lamellae and other collagen lamellae in the anterior stroma were maintained in the edematous cornea.13 Our observations suggested that the structure of collagen lamellae in the anterior stroma is an important determinant of corneal rigidity and plays a key role in maintenance of anterior corneal curvature.

We have now further characterized the structure of collagen lamellae in the anterior stroma of the human cornea by SHG imaging microscopy. We found that the angle of sutural lamellae relative to the corneal surface is steeper than that of collagen lamellae located at greater depths of the anterior stroma. Our results show that the anterior stroma has a highly dense and interwoven structure, which likely contributes to the maintenance of corneal curvature and rigidity.

METHODS

Tissue Specimens

The study was approved by the Institutional Review Board of Yamaguchi University Hospital and adhered to the tenets of the Declaration of Helsinki. We collected the anterior segment of the corneal stroma remaining from 27 donor corneas after Descemet’s stripping automated endothelial keratoplasty (DSAEK). The tissue was obtained from 17 male (Caucasian) and 10 female (9 Caucasian, 1 black) donors.
(mean age ± SD, 63.5 ± 8.6 years; age range, 42 to 74 years) and was provided by Sight Life (Seattle, WA). After measurement of donor corneal thickness at a pressure of 70 mm Hg in an artificial anterior chamber, the anterior portion of the cornea with a thickness of ~350 μm was removed with a microkeratome. The corneal flaps were collected immediately after creation of the DSAEK graft.

Tissue Preparation

All corneal buttons were transferred to 4% paraformaldehyde immediately after their collection. The tissue was fixed overnight at 4°C, after which smaller (~3 mm square) blocks were dissected from the central region, washed with phosphate-buffered saline, mounted on glass coverslips with 50% glycerol in phosphate-buffered saline, and imaged.

SHG Imaging Microscopy

SHG imaging microscopy was performed as described previously. Samples were observed with a microscope (Axiovert 200; Zeiss, Jena, Germany) equipped with a 40× (numerical aperture = 1.2) water-immersion objective lens (Zeiss). Two-photon second harmonic signals from collagen were generated with a mode-locked titanium:sapphire laser (Mai Tai; Spectra-Physics Lasers Division, Mountain View, CA). The optimal wavelength for the generation of second harmonic signals from human corneal collagen was previously found to be 800 nm. Forward scatter signals or transmitted signals that passed through the tissue were collected with the use of a condenser lens (numerical aperture = 0.55) and a narrow bandpass filter (400/50) positioned in front of the transmission light detector.

The samples were mounted with the corneal surface parallel to the scanning plane and were scanned with a 1-μm step size in the z-axis, extending from the surface of Bowman’s layer to a depth of 150 μm into the anterior stroma. Twelve-bit, 512 × 512 images were recorded. The 3D data sets were analyzed with the use of an image browser (Zeiss LSM Image Examiner; Carl Zeiss MicroImaging). A minimum of three data sets was collected from different randomly scanned regions of each corneal block.

Measurement of the Angle of Collagen Lamellae

From the collected data sets, we identified Bowman’s layer on the basis of the characteristic punctate pattern in the SHG images (Fig. 1A). As the focus moved gradually deeper, collagen lamellae appeared as fine, short, and narrow SHG linear signals and subsequently passed out of the visualized field in the continuous images. The projection of these images from the base of Bowman’s layer to a depth of ~50 μm revealed the sutural lamellae (Fig. 1B). Three-dimensionally reconstructed projection images revealed adherence of the sutural lamellae to Bowman’s layer (Figs. 1C, D). The continuous SHG images with determined optical slices allowed the z-axis distance between two points to be given by the number of the plane (height a). The distance between the point at which collagen lamellae adhered to Bowman’s layer and that at which the lamellae disappeared was measured (length b). The angle (θ) of collagen lamellae adherence to Bowman’s layer was thus provided by: \( \theta = \tan^{-1} \left( \frac{a}{b} \right) \) (Fig. 2). The angle of five sutural lamellae was measured for each data set, resulting in the evaluation of 15 sutural lamellae for each subject. The angle of lamellae located 50 or 100 μm below Bowman’s layer was similarly measured for nine to 13 lamellae in each subject. For these measurements, we reviewed the data set around a depth of 50 or 100 μm, found collagen lamellae this depth, identified the initiation and termination points of these lamellae in the x-y data set, measured the x-y distance between the initiation and termination points of each lamella, and counted the number of x-y slices. The total number of evaluated lamellae was 405, 351, and 297 for sutural lamellae, lamellae 50 μm below Bowman’s layer, and lamellae 100 μm below Bowman’s layer, respectively.

Measurement of the Width of Sutural Lamellae

We measured the width of sutural lamellae at the point of their adherence to Bowman’s layer as well as at a depth of 30 μm below Bowman’s layer by moving the focal plane of the SHG data sets and with the use of an image browser (Zeiss LSM Image Examiner; Zeiss).

Measurement of the Density of Sutural Lamellae

We identified the adherence point of sutural lamellae with a length of >90 μm in three data sets of each subject and measured their density at the point of adhesion.

RESULTS

The distributions of the average angles of all sutural lamellae, all lamellae located 50 μm below Bowman’s layer, and all lamellae located 100 μm below Bowman’s layer are shown for the 27 study subjects in Figure 3. These distributions indicated that sutural lamellae are oriented at a steeper angle than are lamellae located 50 or 100 μm below Bowman’s layer. We also plotted the distributions of the angles of individual lamellae in the x-y plane.
Sutural lamellae appeared to widen at their point of adherence to Bowman’s layer (Figs. 1B, D). To quantify this observation, we measured the width of sutural lamellae at their adherence terminal and at a depth of 30 μm below these terminals (Fig. 6). The mean ± SD width of sutural lamellae at their adherence terminals and at a position 30 μm below these terminals were 13.14 ± 5.03 μm (range, 3.20 to 30.19) and 7.11 ± 3.00 μm (range, 1.86 to 16.42), respectively. These data thus confirmed that sutural lamellae widen at the point of their adherence to Bowman’s layer. On the basis of the identification of the adherence terminals of sutural lamellae, we calculated the mean ± SD density of the terminals of sutural lamellae longer than 90 μm to be 910.0 ± 480.4/mm².

The three locations (Fig. 4). The mean ± SD angle of sutural lamellae relative to Bowman’s layer was 19.19 ± 4.34° (range, 8.91 to 33.56), whereas the corresponding values for lamellae located 50 or 100 μm below Bowman’s layer were 8.91 ± 2.91° (range, 2.95 to 18.25) and 6.91 ± 2.11° (range, 1.87 to 13.66), respectively.

We next evaluated the relation between the angle of collagen lamellae in the anterior stroma and age (Fig. 5). The regression curve for the relation between the angle of sutural lamellae and age was $y = -0.1029x + 25.723$, with $R^2 = 0.0405$, whereas those for the relation between the angle of lamellae at 50 or 100 μm below Bowman’s layer were $y = -0.0199x + 10.351$ ($R^2 = 0.0029$) and $y = -0.0093x + 6.331$ ($R^2 = 0.0015$), respectively. This analysis thus did not reveal a correlation between the angle of collagen lamellae and age.

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DISCUSSION

In this study, we have revealed the 3D structure of collagen lamellae in the anterior stroma of the human cornea, paying particular attention to the sutural lamellae that adhere to Bowman’s layer and extend into the anterior stroma. The collagen lamellae were found to be highly organized, well interwoven, and densely packed, contributing to maintenance of the 3D structure of the anterior stroma. Our observations thus support the notion that the anterior stroma plays a key role in the physiological maintenance of the 3D structure of the entire cornea.

We found that the angle of collagen lamellae in the anterior stroma varied according to the distance below Bowman’s layer. The angle of the lamellae thus changed markedly within a distance (100 μm) corresponding to about one-fifth of the total corneal thickness. We previously showed that the collagen fibers in each lamella appeared longer in the mid or deep stroma than in the anterior stroma.11 In the current SHG imaging system, the optical slice thickness is thought to be very thin but to be equal at each focal plane. The change in the length of collagen fibers in individual images indicates that the angle of the collagen lamellae is smaller in the mid stroma and smaller still in the posterior stroma than in the anterior stroma. The extent of interweaving of collagen lamellae is thus high in the anterior stroma, likely resulting in high rigidity in the front-back direction. On the other hand, the extent of interweaving of collagen lamellae is lower in the posterior cornea, resulting in innate weakness in the front-back direction.

The high level of interweaving of collagen lamellae in the anterior stroma may underlie folding of Descemet’s membrane in stromal edema. The anterior stroma was found not to be swollen in an experimental model of edema.10 The keratometry value was also found not to differ between before and after DSAEK surgery,15 indicating that anterior curvature in the edematous cornea was not affected by stromal swelling. The increased volume of the stroma in the edematous cornea therefore extends to the anterior chamber, resulting in a decrease in the diameter of posterior curvature and folding of Descemet’s membrane as a consequence of its increased area. The non-swelling property of interwoven collagen lamellae in the shark cornea was described previously.16

We found that the width of sutural lamellae was increased at their point of adherence to Bowman’s layer, from which the lamellae narrowed and extended in random orientations into the stroma. We previously showed that collagen lamellae became wider and flatter with increasing depth of the stroma,11 a finding that may be explained by several lamellae in the anterior stroma, including sutural lamellae, combining to form assemblies of lamellae, with such assemblies corresponding to the textbook description of the lamellar structure of collagen in the mid stroma.17 Collagen fibrils in the anterior stroma were previously shown to adhere to Bowman’s layer.7 Our present observations, however, further reveal that the adherence terminals of sutural lamellae spread out at Bowman’s layer. Bowman’s layer, sutural lamellae, and other anterior collagen lamellae thus likely form a structural unit to maintain corneal rigidity and shape.

The role of Bowman’s layer is unknown. We now show that many sutural lamellae adhere to Bowman’s layer in the human cornea. We previously observed interwoven collagen lamellae in the anterior stroma of mammals such as mice and rabbits whose corneas do not possess a Bowman’s layer, although transverse lamellae, which include sutural lamellae, were detected only in the human cornea, not in that of mice or

![Figure 5. Relation between the angle of sutural lamellae or collagen lamellae at a depth of 50 or 100 μm below Bowman’s layer and age of the study subjects.](image)

![Figure 6. Distributions of the width of sutural lamellae at the point of adherence to Bowman’s layer as well as at a depth of 30 μm below Bowman’s layer.](image)
rattles.\(^4\)\(^5\) We speculated that the formation of sutural lamellae may be dependent on the development of Bowman’s layer during the fetal period. Examination of the relation between Bowman’s layer and sutural lamellae during fetal development by SHG imaging may provide further insight in this regard.

Photorefractive kerectomy (PRK) includes removal of the structural unit of Bowman’s layer and sutural lamellae and so might be expected to result in a loss of corneal rigidity and corneal ectasia. However, corneal ectasia after PRK is rare, occurring more frequently after laser in situ keratomileusis (LASIK) among refractive surgeries.\(^6\)\(^7\)\(^8\) This may be because only a central limited lesion of the anterior cornea is removed during PRK, with a large proportion of the structural unit of Bowman’s layer and sutural lamellae being left intact. Indeed, the refractive indication for PRK is limited compared with that for LASIK, with the result that a smaller volume of the corneal stroma is removed during PRK than during LASIK. LASIK involves a wide cut in the anterior stroma with a diameter of \(\sim 9\) mm, which results in weakening of \(x\)\(y\) directional rigidity around the flap hinge. In addition, wound adhesion at the interface between the flap and stromal bed may be weak as a result of the topical application of steroid to suppress postoperative inflammation and avoid haze. The structural weakness in the \(x\)\(y\) direction in the post-LASIK eye may therefore underlie the susceptibility to corneal ectasia.

Structural analysis of corneal collagen is important to provide insight into the basis of corneal shape and the pathogenesis of corneal disease. Collagen structure in the normal and diseased cornea has been examined by x-ray scattering. A recent x-ray scattering method revealed differences between stromal layers by sectioning of the cornea with the \(x\)\(y\) scattering technique. Such analysis provides information on bulk collagen lamellae but does not readily detect differences between stromal layers. In contrast, SHG imaging combined with confocal microscopy allows visualization of the detailed structure of collagen lamellae as well as the detection of differences between stromal layers. This approach does not allow analysis of larger fields such as the entire cornea, however. Current technology thus allows visualization of collagen microstructure by SHG microscopic imaging and of collagen macrostructure by \(x\)\(y\) scattering. A recent \(x\)\(y\) scattering method revealed differences between stromal layers by sectioning of the cornea with a femtosecond laser.\(^9\) Further improvements in both methods should provide more information on corneal collagen structure.

Conical stromal curvature is a major determinant of refraction and visual acuity. Collagen lamellae in the anterior stroma likely play an important role in maintenance of corneal curvature. Improvement in clinical examination methods, such as combining SHG imaging technology with in vivo confocal microscopy or topography, may allow observation of the structure of collagen lamellae or fibrils in patients, thereby providing the possibility of evaluation of corneal astigmatism, intraocular pressure, corneal haze, or corneal disease.

References