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Observation of Human Corneal and Scleral Collagen Fibrils by Atomic Force Microscopy

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Purpose: We attempted to analyze the three-dimensional ultrastructure of human corneal and scleral collagen fibrils with an atomic force microscope (AFM).

Methods: A normal eye removed from a 66-year-old male patient during therapy was used in this study. Suspended corneal and scleral collagen fibrils were individually attached to glass slides by centrifugation. These collagen fibrils were air-dried and observed with a non-contact-mode AFM in air.

Results: AFM imaging provided information on the surface topography of both corneal and scleral collagen fibrils. The corneal collagen fibrils had a height of 11.9 ± 1.0 nm (mean ± SD) and scleral fibrils a height of 82.5 ± 35.6 nm. A periodic banding pattern of grooves and ridges was clearly found on both types of fibrils; the D-periodicity and the groove depth were 65.7 ± 0.8 nm and 1.46 ± 0.50 nm in the corneal fibrils, and 67.3 ± 1.1 nm and 6.16 ± 1.23 nm in the scleral fibrils.

Conclusions: Surface topographic images of human corneal and scleral collagen fibrils were clearly obtained with the AFM. This technique provides quantitative information on the surface morphology of the collagen fibrils at high resolution. **Jpn J Ophthalmol 2002;46:496–501**

Key Words: Atomic force microscopy, collagen, cornea, sclera, ultrastructure.

Introduction

The cornea and sclera are composed mainly of collagen fibrils. Although the major constituent is collagen type I,[1,2] the collagen fibrils in both tissues differ from each other in thickness and arrangement, as demonstrated previously by transmission electron microscopy (TEM) and/or scanning electron microscopy (SEM).[3–8] However, few reports have been published focusing on the structural difference between corneal and scleral collagen fibrils,[9] especially in relation to their three-dimensional ultrastructure. Thus, in order to clarify the structural difference, we recently investigated collagen fibrils in the bovine cornea and sclera with an atomic force microscope (AFM).[10]

The AFM was developed in 1986[11] and has been widely applied recently in the fields of biology and medicine because of its potential for observing samples at high resolution in vacuum and nonvacuum (ie, air or liquid) environments.[12,13] This microscope has a sharp probing tip that scans the sample surface at an atomic distance. By monitoring the interaction force between the tip and the sample surface, this instrument can create topographical images of the sample surface at high resolution (Figure 1).

Using this new microscope, in the present study we attempted to analyze the three-dimensional ultrastructure of human corneal and scleral collagen fibrils more precisely than has been possible before.

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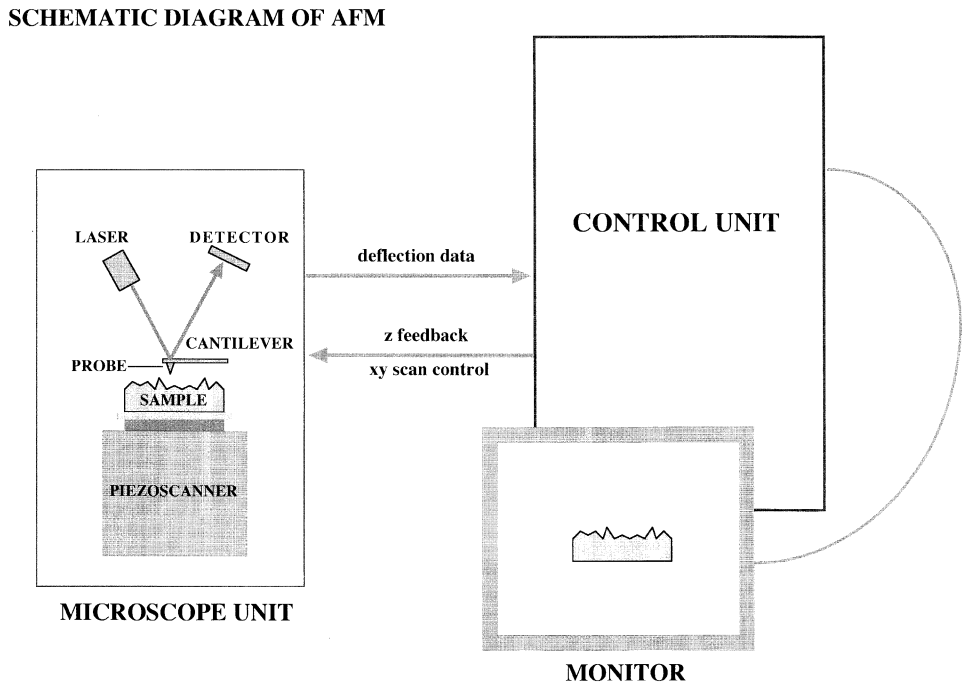

Figure 1. Schematic drawing of the principle of the atomic force microscope.

Materials and Methods

A normal eye, enucleated from a 66-year-old male patient during sinusitis therapy, was used in this study. Informed consent for investigating extirpated tissues was obtained before the enucleation. Isolated collagen fibrils were prepared as described previously.[10] Briefly, the central cornea and the equatorial part of the sclera were separately processed with a homogenizer (Polytron, Kinematica AG, Switzerland) in phosphate-buffered saline (pH 7.4) to suspend the collagen fibrils. Each sample was incubated with chondroitinase ABC (0.1 U/mL, Seikagaku, Tokyo) and keratanase (0.01 U/mL, Seikagaku) at 37°C for 12 hours. Collagen fibrils in suspension were adsorbed to glass slides by using a centrifuge (Auto Smear, Sakura, Tokyo) at 500*g* for 5 minutes, dried in air, rinsed briefly with distilled water, and air-dried again.

AFM imaging was carried out using an SPA-300 scanning probe microscope controlled by an SPI 3700 probe station (Seiko Instruments, Chiba) as described previously.[10] For statistical analysis, 20 collagen fibrils each of the cornea and sclera were imaged with a scan range of 1 μm. The scan direction was set along the fibril axis to avoid the influence of the sample drift. The width and height of each collagen fibril and its D-periodicity and depth between grooves and ridges (ie, the depth of the gap zone) were directly measured from the AFM images, because each image is composed of set pixels (eg, 256 × 256 pixels), which contain quantitative information on the height of the corresponding portion.

Results

Using AFM, the surface topography of isolated human corneal and scleral collagen fibrils were clearly analyzed for quantitative information. In these images, periodical grooves and ridges corresponding to the D-periodicity were observed on the surface of collagen fibrils. Corneal collagen fibrils were thin and uniform in width and height (Figure 2), while scleral collagen fibrils varied (Figure 3). Table 1 shows the width, the height, the D-periodicity, and the depth of the gap zone measured in AFM images of the corneal and scleral collagen fibrils. The difference of all data between the cornea and sclera was statistically significant at $P < .01$ (t test).

Figure 4 shows longitudinal section profiles of the corneal and scleral collagen fibrils obtained by AFM. The transition from ridges to grooves was abrupt and steep on corneal collagen fibrils; a prominent protrusion was often found at both ends of the ridge region. Another small protrusion was sometimes present in the middle of the groove region. In

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Vol 46: 496–501, 2002

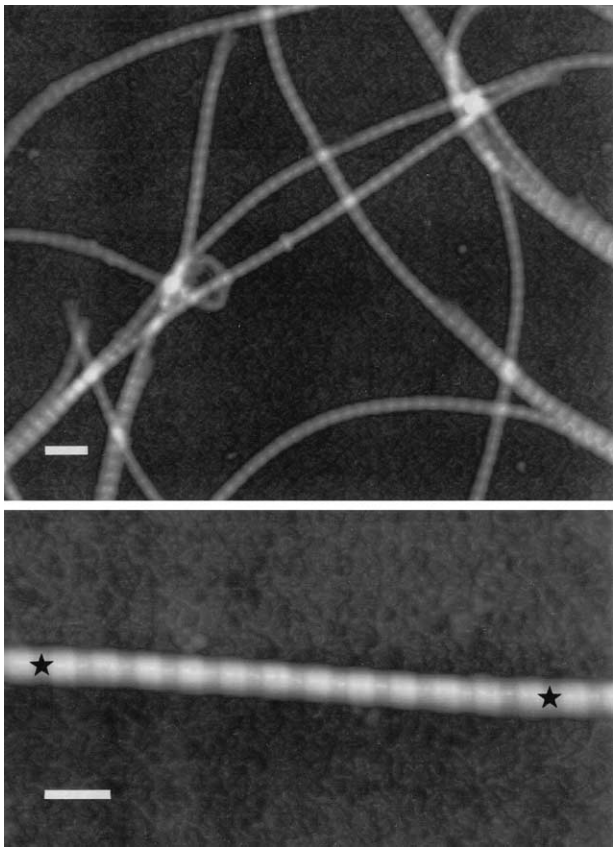

Figure 2. Atomic force microscope images of corneal collagen fibrils. Corneal collagen fibrils have a thin and uniform diameter. Periodical grooves and ridges are clearly observed on their surface. A section profile between the two asterisks is shown in Figure 4. Bar = 200 nm (top) and 100 nm (bottom).

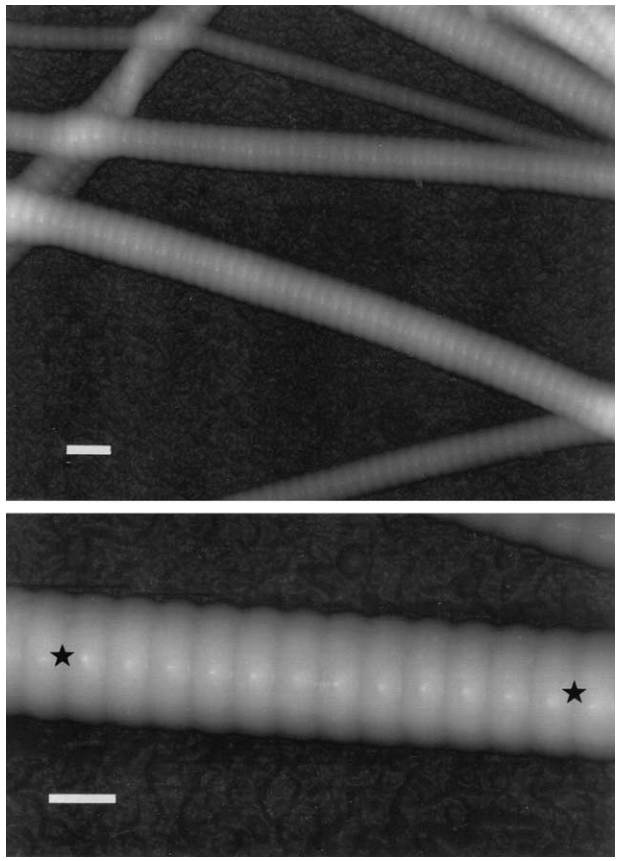

Figure 3. Atomic force microscope images of scleral collagen fibrils. Scleral collagen fibrils are thick and varied in diameter. Periodical grooves and ridges can be found on their surface. A section profile between the two asterisks is shown in Figure 4. Bar = 200 nm (top) and 100 nm (bottom).

contrast, the transitions between ridges and grooves were gentle on scleral collagen fibrils and no minor protrusions were present on their surface.

Tapered ends were sometimes observed on scleral collagen fibrils (Figure 5); this feature was quite different from that of cutting edges because the latter were obtuse or tuft-like in shape. Grooves and ridges with D-periodicity were observed even in the tapered region of scleral collagen fibrils.

Discussion

The AFM used in the present study is a new microscope developed by Binnig et al[11] in 1986. This microscope has been applied mainly to studies in the physical sciences because it can create images of the sample surface at high resolution (eg, the atomic arrangement can be easily observed on the surface of freshly cleaved mica). Compared with electron microscopy, AFM has the advantage of visualizing nonconductive samples in a nonvacuum (ie, air or liquid) environment with quantitative three-dimensional information. Thus, a number of recent investigators have attempted to apply AFM to the imaging of biological samples.[12,13]

Several papers have been already published on AFM observation of collagen fibrils. As to the cornea and sclera, Meller et al observed slices of the human cornea and sclera by AFM in a contact mode.[14] On the other hand, we previously investigated isolated bovine corneal and scleral collagen fibrils with a noncontact-mode AFM and succeeded in analyzing precisely the ultrastructure of the collagen fibrils.[10] In this previous study, we also postulated that the height of isolated fibrils should be measured as their diameter to avoid overestimation; under

Table 1. Data Obtained by Atomic Force Microscopy (in nm)

	Height	Width	D-periodicity	Depth of Gap Zone
Cornea	11.9 ± 1.0	87.4 ± 4.7	65.7 ± 0.8	1.46 ± 0.5
	(9.0–12.8)	(78.7–95.8)	(64.3–67.2)	(0.4–2.3)
Sclera	82.5 ± 35.0	204.2 ± 62.9	67.3 ± 1.1	6.16 ± 1.3
	(27.1–148.6)	(104.8–294.0)	(65.5–68.9)	(4.3–8.1)

AFM observation, the side of the probing tip first contacts the fibril during scanning and the tip draws a domed trajectory for tracing the fibril (Figure 6). In the present study, we thus have used the same observation method as in our previous study for analyzing collagen fibrils in human cornea and sclera. We consider our method using isolated collagen fibrils is more suitable than previous methods of using simply teased tissues[14–17] or tissue sections[18] in order to investigate the structure of individual collagen fibrils by AFM.

Corneal collagen fibrils are known to have a uniform diameter, unlike scleral collagen fibrils, which have various thicknesses.[1,2] In this study, human corneal collagen fibrils had a diameter of 11.9 ± 1.0 nm and scleral collagen fibrils had a diameter of 82.5 ± 35.5 nm. According to our previous study, bovine corneal collagen fibrils had a diameter of 15.6 ± 1.5 nm and scleral collagen fibrils had a diameter of 74.2 ± 55.7 nm,[10] indicating that the human collagen fibril is thinner than bovine corneal collagen fibrils. This result corresponds to a report on corneal collagen fibrils of various species analyzed by an x-ray diffraction method.[19] However, the diameter of human corneal and scleral collagen fibril in the present study was smaller than previous data from other methods, such as TEM of ultrathin sections and an x-ray diffraction method. For example, Borcherding et al analyzed thin sections of corneal and scleral collagen fibrils by TEM and reported that the diameter was 22.3–33.6 nm in the cornea and 10.7–233 nm in the

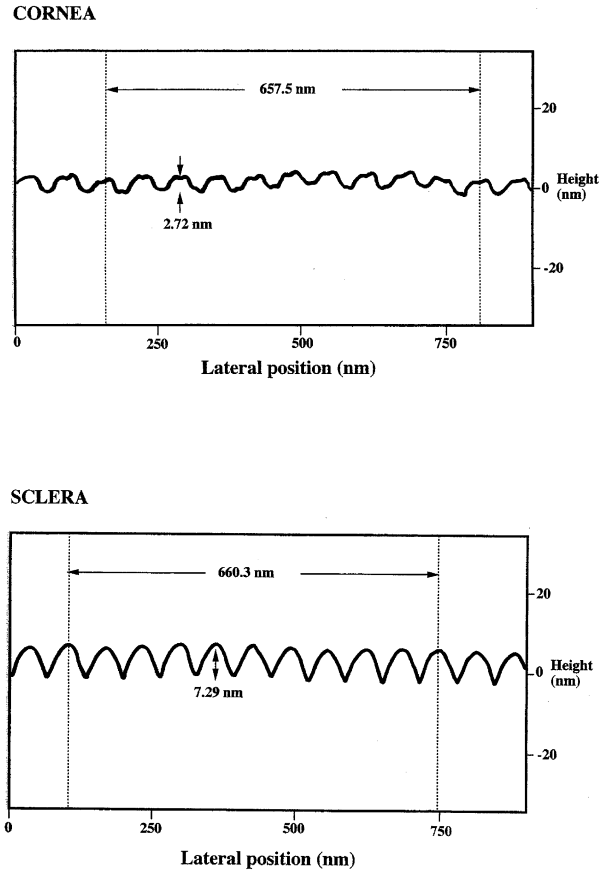

Figure 4. Diagrams of the atomic force microscope (AFM) screen for measurement. The longitudinal section profiles of corneal (top) and scleral (bottom) collagen fibrils obtained between the two asterisks in Figure 2 or Figure 3. AFM images can directly take a three-dimensional measurement because the images contain quantitative information.

Figure 5. The end of a scleral collagen fibril. Characteristic grooves and ridges are obvious even on the tapered end of the fibril. Bar = 200 nm.

Jpn J Ophthalmol
Vol 46: 496–501, 2002

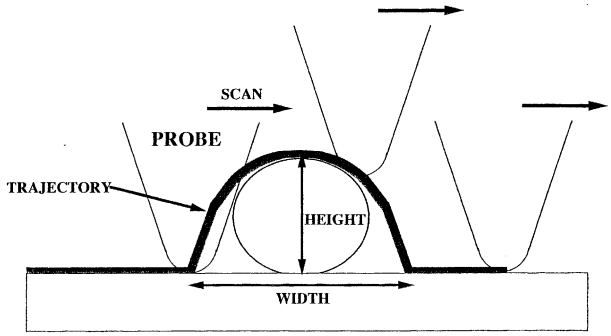

Figure 6. A trajectory of the atomic force microscope probing-tip. The width tends to be larger than the height when imaging cylindrical samples.

sclera.[4] Curtin et al also demonstrated by TEM that collagen fibrils had a diameter of 40–280 nm in the normal sclera.[5] Komai et al investigated human corneal and scleral collagen fibrils with the combined use of TEM and SEM and described that the diameter of collagen fibrils was 25–35 nm in the cornea and 25–230 nm in the sclera.[7] In x-ray diffraction analysis, corneal collagen fibrils were 30.8 nm in diameter.[19] The reason for the difference between current and previous data is unknown. One possibility is that collagen fibrils in the present study were deformed by the surface tension of water during air-drying.[20] It is also probable that collagen fibrils were compressed by the probing tip during AFM operation. Further studies are needed to resolve these differences.

D-periodicity of the collagen fibrils has been explained by the staggered arrangement of collagen molecules in each fibril.[21] Previous TEM studies showed the difference of D-periodicity among collagen fibrils in different tissues.[22] Several investigators interpreted the difference as artifacts of TEM preparation, while others proposed that the difference was produced due to the different internal structure of collagen fibrils in certain tissues. Marchini et al demonstrated the presence of subfibrils in collagen fibrils by TEM using a freeze-replica method, and stated that the different inclinations of subfibrils produce different the D-periodicity between corneal and tendon collagen fibrils. We have already shown that the D-periodicity of corneal collagen fibrils is shorter than that of scleral collagen fibrils in bovine tissues.[10] The present study has confirmed this phenomenon in human samples. Visualization of the subfibrillar structure in human corneal and scleral collagen fibrils is a subject for further studies.

AFM can measure the depth of the gap zone, which is impossible by TEM and SEM observation. The present study has shown that the depth of gap zones was 1.46 ± 0.50 nm in corneal fibrils and 6.16 ± 1.32 nm in scleral fibrils. On the other hand, Meller et al[14] reported a diameter of 0.23 ± 0.11 nm in corneal fibrils and 0.42 ± .014 nm in scleral fibrils. The difference in measurement between the two reports may be explained by the different preparation methods; we digested proteoglycans with chondroitinase ABC and keratanase while they observed fibrils without any enzymatic treatment. Scott and Haigh investigated rabbit corneal collagen fibrils by TEM in combination with cuprolinic blue staining and showed the presence of keratan sulfate on the overlap zone and dermatan sulfate on the gap zone of fibrils.[24] Raspanti et al demonstrated the difference in the surface structure of collagen fibrils between proteoglycan-digested and nondigested samples.[17]

In the present study we observed tapered collagen fibrils in scleral samples and demonstrated the presence of the characteristic banding pattern in the tapering region. This finding is similar to that found at the end of collagen fibrils in fibrillogenesis.[25] We assume this region to be the true end of scleral collagen fibrils.

In conclusion, we investigated the surface structure of human corneal and scleral collagen fibrils by AFM. Because this microscope has the potential for observing samples in liquid without metal coating, studies in additional physiological conditions are expected in the near future.

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