

Surface Topology of Collagen Fibrils Associated with Proteoglycans in Mouse Cornea and Sclera

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Purpose: To clarify the histological basis for the optical difference in the cornea and sclera, we investigated the surface ultrastructure of D-periodic collagen fibrils.

Methods: The fibril arrangement and topology of D-periodic collagen fibrils from corneas and scleras of mice were examined by transmission and scanning electron microscopy, as well as by atomic force microscopy (AFM). The banding patterns were estimated by densitometry and compared with the profile made by computer simulation from the protein sequence of amino acids.

Results: Considerable association of proteoglycans/glycosaminoglycans on XI bands of corneal D-periodic collagen fibrils was seen with ruthenium red staining. Atomic force microscopy imaging showed that the depth of the groove in the D-periodicity of corneal collagen fibrils was shallow compared with that of the sclera.

Conclusions: Corneal collagen fibrils are associated with many extracellular matrix components on both elevated and depressed surfaces of D-periodic bands, which may serve to maintain interfibrillar spaces, resulting in corneal transparency. **Jpn J Ophthalmol 2000;44:591–595** © 2000 Japanese Ophthalmological Society

Key Words: Atomic force microscopy, cornea, D-periodic collagen fibrils, sclera, surface ultrastructure.

Introduction

The cornea and sclera together form the outer fibrous tunic of the eye and withstand both internal and external forces to help maintain the shape of the eyeball. Although both of these tissues consist mainly of collagen fibrils, their optical properties are different; only the cornea is transparent. In addition to parallel arrangement in the cornea, collagen fibrils must have the same diameter to keep the cornea transparent.¹ This parallel arrangement of collagen fibrils can be maintained by organization of the interfibrillar spaces, which mainly consist of proteoglycans (PGs) or gly-

cosaminoglycans (GAGs).^{2,3} To clarify the histological basis for the difference in the cornea and sclera, we focused on the surface structure of D-periodic collagen fibrils, because the interaction of collagen fibrils with other extracellular matrix (ECM) components occurs on the surface of the fibrils.⁴

It is important to determine the three-dimensional ultrastructure of the fibril surface of D-periodic collagen fibrils. Atomic force microscopy (AFM) is a recently developed imaging technique that produces an image of the surface topography of a specimen by measuring the response of a tip attached to a cantilever that is raster-scanned over the sample.⁵ The surface topography of D-periodic collagen fibrils in the cornea and sclera may show some difference according to function. In this study, we examined the surface structure of D-periodic collagen fibrils, as well as their interaction with other ECM components.

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Materials and Methods

Methods of securing animal tissues complied with the Guide for Animal Research, Nagoya University School of Medicine, the Declaration of Helsinki, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation and Scanning Electron Microscopy

Corneas and scleras from 6 eyes of 3 mice (strain ddY, 8–10 weeks, female) were used. Immediately after enucleation, the corneas and scleras were cut into small fragments about 1-mm thick in the meridional direction for further experiments. The small fragments of cornea and sclera were fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer at 4°C for 1 day. They were immersed in 10% aqueous solution of NaOH for 5 days at room temperature, and then rinsed in distilled water for 1 day.⁶ Next, they were processed by conductive staining with 1% OsO₄ and 1% tannic acid for 1 hour each. Then the specimens were dehydrated in a series of graded concentrations of ethanol, and freeze-cracked in liquid nitrogen. They were then coated with osmium plasma coater (NL-OPC80N-S; Nippon Laser and Electronics Lab, Nagoya) and observed under a scanning electron microscope (S-800S; Hitachi, Tokyo) with an acceleration voltage of 10 kV.

Transmission Electron Microscopy

Small pieces of tissue from the cornea and sclera were incubated in phosphate-buffered saline (PBS) containing 20 mM Adenosine Tri-Phosphate (ATP) · 2 Na⁴ to eliminate the interaction of type VI collagen

and PGs. Cornea and sclera incubated in PBS only served as a control. A portion of the fragments was fixed in Karnovsky's fixative containing ruthenium red (RR) according to the method of Nakamura et al⁷ for the detection of surface PGs. They were dehydrated and embedded in Quetolmixture (Nissin EM, Tokyo). Ultrathin sections were observed under transmission electron microscopy with or without electron staining.

Densitometry and Computer Simulation

High magnification banding patterns of D-periodic collagen fibrils were digitized with an LA-525 personal image analyzing system (PIAS Company, Osaka), and the density across the fibril axis in the range of 50–130 pixels was computer-averaged. Banding patterns were expressed as densitometric profiles using the program P2C (PIAS-to-COL, Version 3).⁸ The distribution of polar amino acids along the D-periodic fibrils was computer generated by COL (Version 4.61) using human type I collagen sequence and a stagger of 234 residues, and averaging 12 consecutive residues as described elsewhere.⁹

Atomic Force Microscopy

Specimens macerated in NaOH were rinsed with distilled water. Collagen fibrils were dispersed finely with forceps and dried on a cover glass. The fibrils were scanned under normal atmosphere with an Auto Probe CP (Park Scientific Instruments, Sunny Vale, CA, USA). Atomic force microscopy was operated in the contact mode using silicon nitride cantilevers with a spring constant of 0.03 N/m. Images were taken at a scan rate of 1 Hz over a range of 1 to 4 μm² with an information density of 512 × 512 pixels. Atomic force microscopy profiles of D-periodic

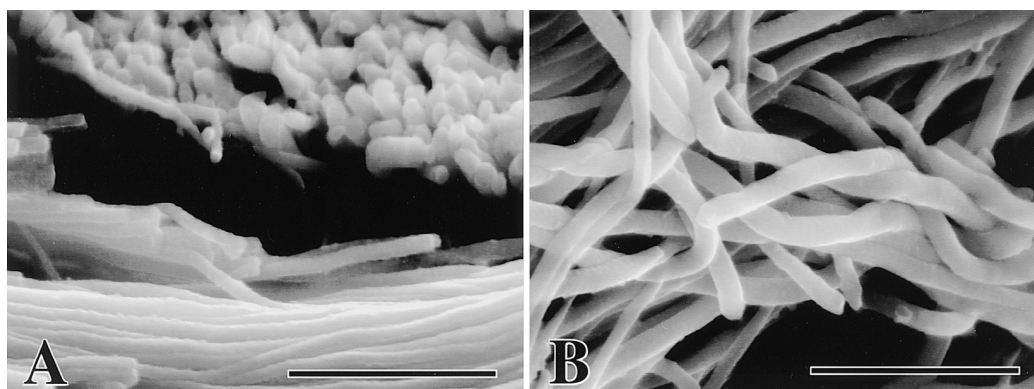
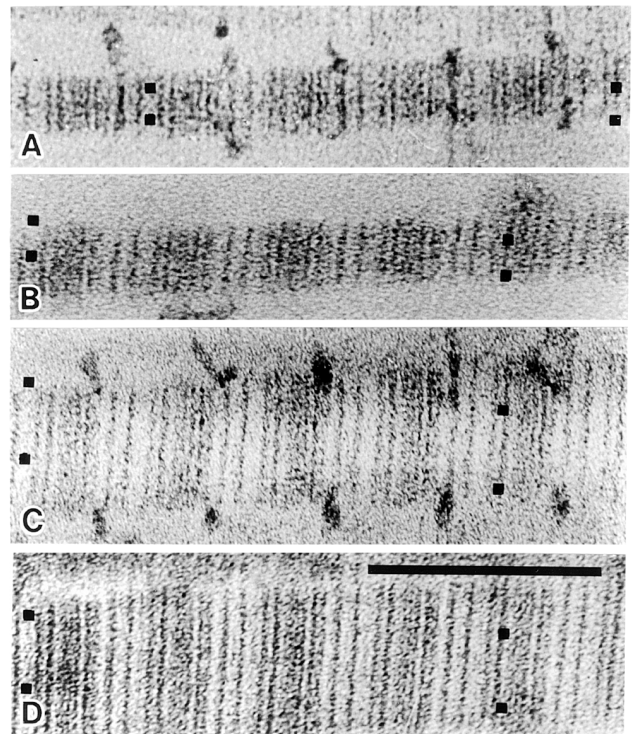


Figure 1. Scanning electron microscopic profiles of D-periodic collagen fibrils in mouse cornea (A) and sclera (B). Bar = 1

Figure 2. Higher magnification of D-periodic collagen fibrils in cornea (**A,B**) and sclera (**C,D**), with (**B,D**) and without (**A,C**) Adenosine tri-phosphate treatment (ATP). Ruthenium red (RR) staining. RR-stainable thick short filaments in cornea are associated with band XI of fibrils (**A**), and completely disappear after ATP treatment (**B**). RR stainable short filaments or dots in the sclera are localized around band XI of fibrils (**C**), and disappear after ATP treatment (**D**). Each area cornered by black squares was scanned to obtain densitometric profiles. Bar = 0.1 μm .



repetition of elevated and depressed surfaces along the fibrils were analyzed by line profile. To express the height, the baseline where the lowest grooves attached is taken as 0.

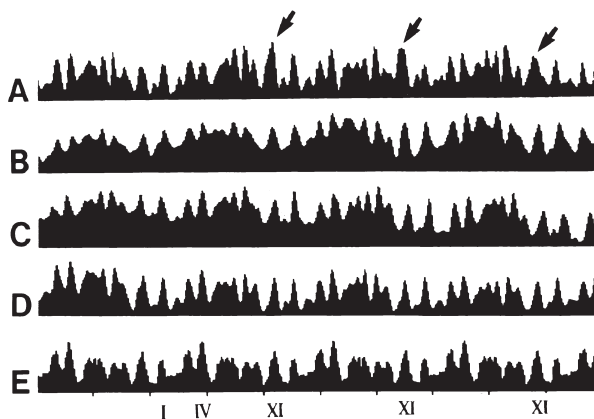


Figure 3. Densitometric profiles of D-periodic collagen fibrils in cornea (**A,B**) and sclera (**C,D**). Patterns (**A**)–(**D**) were obtained by scanning marked area of periodic fibrils in Figures 2A to 2D. Untreated fibrils in cornea show high and broad-based peaks at band XI (**A**, arrows), while in sclera, RR-filaments do not affect densitometric peak (**C**). Adenosine tri-phosphate-treated fibrils in sclera (**D**) show pattern typical of distribution of polar amino acids in D-periodic collagen fibrils (**E**) obtained by computation program COL (Ver. 4.61). Roman numerals I, IV, and XI represent intraperiodic bands of D-periodic collagen fibrils.

Results

Three-Dimensional

Arrangement of D-Periodic Collagen Fibrils

Maceration of the tissues with NaOH solution effectively removed the cellular elements, exposing the D-periodic collagen fibrils. Collagen fibrils in the cornea were regularly arranged, forming layers in transverse and longitudinal sectional profiles. They were thin and uniform in diameter (Figure 1A). In the sclera, collagen fibrils were randomly arranged, with rather thick and nonuniform diameters (Figure 1B).

Densitometry of Collagen Fibril Surface

Figures 2A–D show the enlarged banding pattern of D-periodic collagen fibrils stained with RR in the cornea and sclera with or without ATP treatment. Corresponding densitometric profiles of fibril surfaces are shown in Figures 3A to 3D. We reported previously that ATP served to dislocate RR-stainable PGs/GAGs from the surface of D-periodic collagen fibrils.² Without ATP treatment, RR-stainable short filaments were associated with D-periodic collagen fibrils on band XI (d) in the cornea (Figure 2A) and sclera (Figure 2C), but they completely disappeared after ATP incubation (Figure 2B, cornea; Figure 2D, sclera). To make an accurate determination of the banding pattern of D-periodic collagen

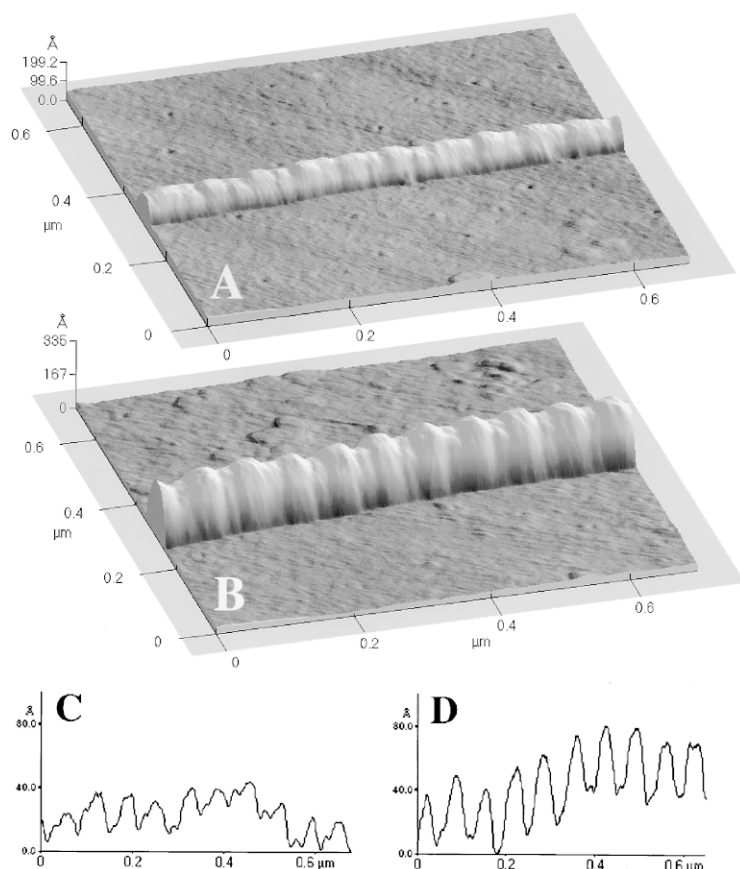


Figure 4. Atomic force microscopy (AFM) images of D-periodic collagen fibrils in cornea (**A**) and sclera (**B**). D-periodic repetition of elevated and depressed surfaces along fibrils is seen. Axial profiles show peaks at distances of about 61–64 nm. The surface of scleral collagen fibrils has prominent grooves. Line profiles of AFM images in cornea (**C**) and sclera (**D**) are shown. To express height, baseline where lowest grooves are attached is taken as 0.

fibrils, a densitometric estimation was performed. Densitometric profiles of untreated fibrils showed high and broad-based peaks at band XI (Figure 3A) in the cornea, which disappeared after ATP treatment (Figure 3B). In the sclera, no prominent peaks were observed (Figure 3C). The densitometric profile of ATP-treated fibril in the sclera (Figure 3D) was almost identical to the distribution profile of polar amino acid residues in the D-periodic collagenfibrils (Figure 3E).

Comparison of Surface Structure of Collagen Fibrils

Atomic force microscopy imaging was performed on D-periodic collagen fibrils from the cornea (Figure 4A)

and sclera (Figure 4B). The fibrils were not treated with ATP nor stained with RR. D-periodic repetition of elevated and depressed surfaces along the fibrils was clearly seen. The surface of collagen fibrils in the sclera has more prominent grooves than the fibrils in the cornea (Figures 4C,D). The depth of the grooves in the cornea was 2.20 ± 0.76 nm ($n = 26$), while in the sclera it was 3.53 ± 1.09 nm ($n = 31$) (Table 1). Line profiles of AFM images showed peaks at about 64 nm in the cornea and 61 nm in the sclera (Table 1).

Discussion

The surface topology of D-periodic collagen fibrils in mouse cornea and sclera was compared by AFM, which can be used to visualize the surface of biopolymers, including proteins.¹⁰ Mature collagen fibrils are formed by the polymerization of collagen molecules staggered by D (about 67 nm, 1/4.4 of the molecular length) from each other, and hence show D-periodicity. The D-periodic repetition of elevated and depressed surfaces along the collagen fibrils, corresponding to overlap (materially dense) and gap (materially sparse) regions, respectively, has been

Table 1. Surface Structure of Collagen Fibrils in Cornea and Sclera

Average Value	Cornea	Sclera
D periodicity (nm)	64.78 ± 4.50	61.43 ± 7.25
Depth of groove (nm)	2.20 ± 0.76	3.53 ± 1.09

Values are mean \pm SD.

revealed by AFM.^{11,12} In the present analysis by AFM imaging, the depth of groove in corneal collagen fibrils was shallower than that in scleral collagen fibrils. It shows probable interaction of some other ECM components in the groove. Atomic force microscopy can topologically show the interaction. Maceration of the tissues with NaOH solution effectively exposed the collagen fibrils. This makes it possible to examine the surface topology of collagen fibrils by AFM.

We have reported previously that type VI collagen interacts with collagen fibrils via PGs/GAGs in the mouse cornea.^{13,14} In the present study, we further examined the relationship of those molecules in the mouse sclera and compared it with that in the cornea. For ultrastructural observations of PGs/GAGs in various tissues, cationic dyes such as Alcian blue,¹⁵ cupromeronic blue,¹⁶ and RR¹⁷ have been widely used. Our present RR method also successfully stained PGs/GAGs on the surface of collagen fibrils in the cornea and sclera. RR-stainable PGs/GAGs caused the high and broad-based peaks in the cornea. In the sclera, however, PGs/GAGs did not cause these densitometric peaks. The results as a whole indicate that PGs/GAGs associated in XI bands of D-periodic collagen fibrils in the cornea. This association may be expressed by the spacers in the arrangement of collagen fibrils in the corneal stroma.

The difference in ultrastructure between cornea and sclera, revealed in the present study, could explain the differences in the number of molecules in these organs. Other ECM components associated with the depressed groove on the surface of corneal collagen fibrils should be further investigated in combination with the enzyme digestion method.

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