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Type VI Collagen Bound to Collagen Fibrils by Chondroitin/Dermatan Sulfate Glycosaminoglycan in Mouse Corneal Stroma

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Abstract: We investigated the ultrastructural localization of type VI collagen in mouse corneal stroma and its relationship to striated collagen fibrils and glycosaminoglycans, using chondroitinase ABC digestion and immunoelectron microscopy with colloidal gold particles. After chondroitinase ABC digestion, the arrangement of striated collagen fibrils was disrupted, and large spaces containing widely scattered fibrils appeared. The spaces were filled by filamentous networks that were stained by anti-type VI collagen IgG, which were apparently clumps of beaded filament. Interfibrillar type VI collagen beaded filaments and immunogold particles decreased. Our results indicate that type VI collagen is bound to the striated collagen fibrils by mediation of chrondroitin/dermatan sulfate glycosaminoglycan or proteoglycan. We believe that this interaction is essential to the orderly arrangement of the striated collagen fibrils, which results in corneal transparency. **Jpn J Ophthalmol 1997;41:71–76** © 1997 Japanese Ophthalmological Society

Key Words: Chondroitin/dermatan sulfate glycosaminoglycan, indirect immunoelectron microscopy, mouse corneal stroma, striated collagen fibril, type VI collagen.

Introduction

Corneal transparency depends on the regular arrangement of stromal D-periodic collagen fibrils (striated collagen fibrils) with uniform diameter[1,2] composed of types I and V collagen.[3] Most of the interfibrillar space is filled with proteoglycans[4] and type VI collagen,[5] and the relationships of these extracellular matrix components are critical factors in regulation of the stromal structure.

Bruns et al[6] reported that the type VI collagen fibrillar substances of 100 nm periodicity found in human foreskin fibroblast cultures increased in number and size when treated with low pH adenosine 5′-triphosphate (ATP). Following their procedure, we demonstrated that the ladder-like 100 nm periodic fibrils, which were composed of type VI collagen,[7] were experimentally formed in the mouse[8] and human[9] corneal stroma by the ATP treatment. These type VI collagen fibrils appeared in close association with the striated collagen fibrils,[9,10] indicating some interaction between them.

We also showed that the ATP-induced type VI collagen periodic fibrils could be isolated from the striated collagen fibrils when the tissues were digested by chondroitinase ABC prior to ATP treatment, but neither keratanase nor *Streptomyces* hyauronidase had the same effect.[10] These experiments indicate that chondroitin/dermatan sulfate glycosaminoglycans or proteoglycans mediate the interaction between type VI collagen and the striated collagen fibrils.[10] Ours was the first report showing the simultaneous interaction of the three extracellular corneal components: type VI collagen, glycosaminoglycans or proteoglycans, and striated collagen fibers.[10]

Because the ATP-induced type VI collagen fibrils had been experimentally produced, we wished to confirm their function under physiologic conditions: The present study examines the ultrastructural dis-

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tribution of type VI collagen in mouse corneal stroma and its relationship to the striated collagen fibrils and glycosaminoglycans, using chondroitinase ABC digestion and immunoelectron microscopy with colloidal gold particles.

Materials and Methods

Tissue

Corneas from 8-week-old female ddY-strain mice were used. Care of the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice eyes were enucleated immediately after death by carbon dioxide and the corneas were meridionally razor-cut into small fragments about 0.5 mm wide.

Enzyme Digestion

Chondroitinase ABC (EC 4.2.2.4, Seikagaku Kogyo, Tokyo, Japan; 5 or 10 units/mL) was used for the enzyme treatment. Buffer (pH 8.0) for the reaction, prepared according to Scott and Haigh,[11] contained 0.25 M Tris, 0.3 M sodium acetate, 0.24 M sodium chloride, 0.5 mg/mL bovine serum albumin with 2.5 mM benzamidine (Sigma B-6506) and 5 mM disodium EDTA as protease inhibitors. Soybean trypsin inhibitor (0.04 mg/mL) (Sigma T-9003) was also added. After enzyme digestion at 37°C for 3 hours, the specimens were examined by immunoelectron microscopy. Control specimens were incubated in enzyme-free buffer.

Indirect Immunoelectron Microscopy

A post-embedding method was used to determine the distribution of type VI collagen. The corneal fragments were rinsed in pH 7.4 phosphate buffered saline (PBS), fixed in Karnovsky's fixative (2% paraformaldehyde/2.5% glutaraldehyde in 0.05 M PBS) for 12 hours at 4°C. The fragments were then rinsed again in PBS and dehydrated in a graded series of ethanol concentrations up to 100% and embedded in Epon-Araldite (Epon 812 25%, Araldite M 15%, dodecenyl succinic anhydride 55%, dibutyl phthalate 3.5%, tri-dimethyl-aminomethylphenol 1.5%) to obtain cross-sections of the cornea. Sections were cut to a thickness of 1 micron and stained with toluidine blue (1% in 0.1 M sodium borate) to determine the corneal orientation. Ultrathin sections were cut on a Porter-Blum MT-1 ultramicrotome with a diamond knife and mounted on nickel grids. These were then etched with 10% hydrogen peroxide (H_2O_2) for 10 minutes, washed in PBS 3 times (30 minutes), and incubated with normal goat serum (1:100 dilution) for 1 hour at room temperature (RT) to avoid nonspecific reactions, followed by a triple washing in PBS. Next, the sections were immuno-stained for 4 hours at RT with rabbit anti-human type VI collagen IgG (HEY HY-0300-20, Heyl Vertriebs, Iserlohn, Germany; 1:30) as the primary antibody,[7] rinsed in PBS four times (2 hours), and incubated for 3 hours at RT with goat anti-rabbit IgG conjugated with colloidal gold particles (5 nm) (GAF-01205, E Y Labs, San Mateo, CA, USA; 1:100) as a secondary antibody, followed by four rinses (2 hours) in PBS. The next step was staining with uranyl acetate (15% in absolute methanol) for 15 minutes and Reynolds' lead citrate for 2 minutes, followed by examination under a JEM 1200EX transmission electron microscope (JEOL, Tokyo) at 100 kV. Control sections were exposed to normal rabbit serum (1:30) instead of the primary antibody.

Results

Ultrastructural Localization

Control samples that had not been subjected to enzyme digestion were examined first. After incubation in enzyme-free buffer at 37°C for 3 hours, the distribution of striated collagen fibrils was almost entirely uniform (Figure 1A).

High magnification revealed many fine filaments with beads (beaded filaments) in the interfibrillar spaces, connected to the collagen fibrils. The immunogold particles that label type VI collagen were present on these filaments (Figures 1B,C).

In other control samples that were fixed immediately after enucleation, there was a regular parallel arrangement of fibrils more closely spaced than in samples that had been incubated in enzyme-free buffer. Immunogold particles, labeling type VI collagen, were present on the interfibrillar filaments of these samples (data not shown).

Chondroitinase ABC Digestion

After chondroitinase ABC digestion (5 or 10 units/mL) at 37°C for 3 hours, the striated collagen fibrils were markedly disorganized. Interfibrillar spaces were not uniform; there were large spaces with widely scattered fibrils (Figure 2A). High magnification showed that these spaces were filled by filamentous networks looking like clumps of beaded filaments that were stained by the immunogold particles marking type VI collagen (Figure 2B). Interfibrillar beaded filaments and immunogold particles on the filaments decreased (Figures 2C and 2D): We counted fewer immunogold particles on the filaments in enzyme-treated samples (144: Figure, 2D) than in samples incubated in enzyme-

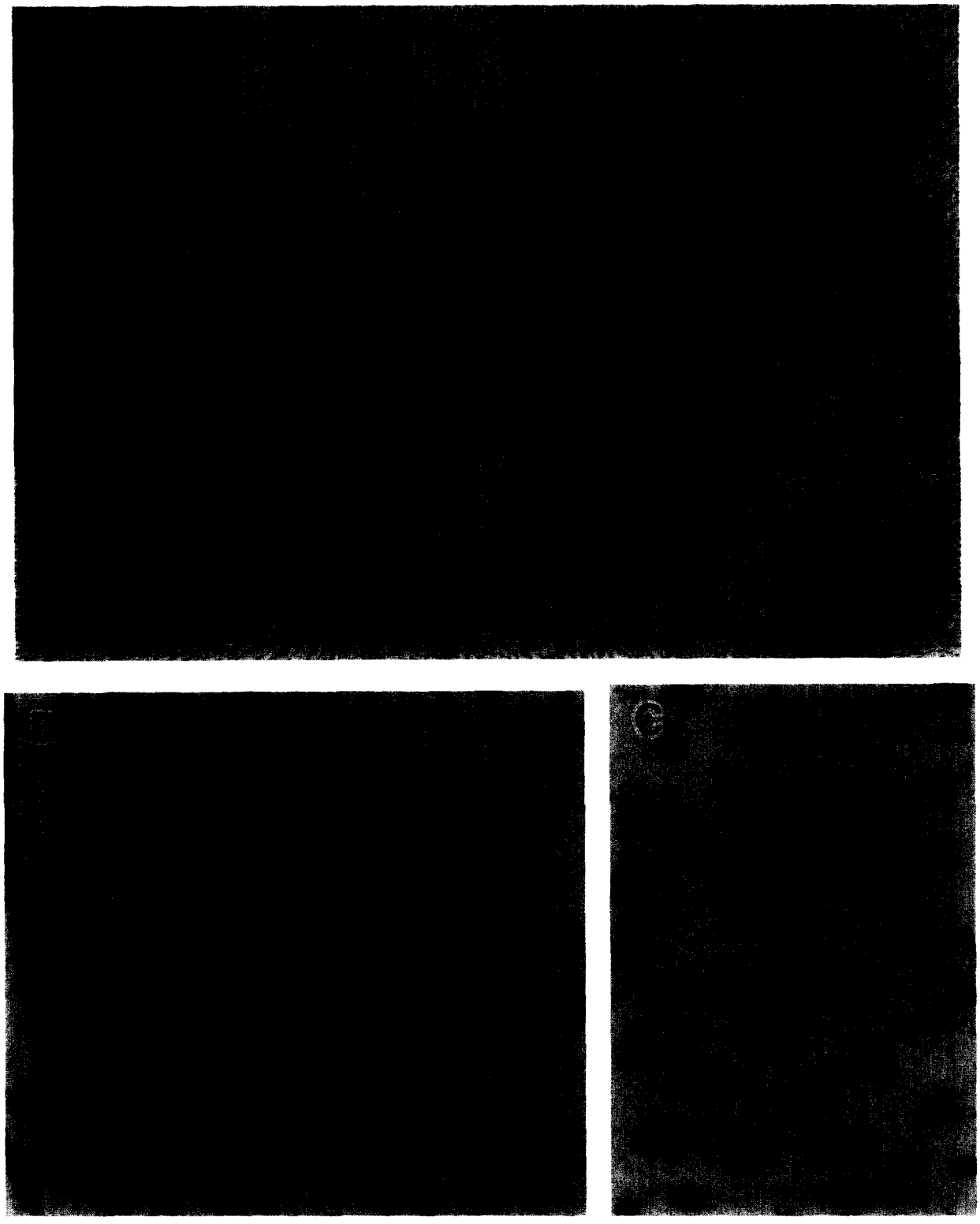

Figure 1. Immunoelectron micrographs of mouse corneal stroma after incubation in the enzyme-free buffer, pH 8.0, at 37°C for 2 hours. Ultrathin sections were immuno-stained with anti-type VI collagen IgG and gold particle-conjugated (5 nm) secondary antibody. (**A**) Regular arrangement of collagen fibrils (× 10 000, Bar = 1 μm). (**B**) Longitudinal section of these fibrils at high magnification. Immunogold particles labeling type VI collagen seen on interfibrillar beaded filaments (arrows) (× 120 000, Bar = 100 nm). (**C**) Fibril cross section at high magnification. Immunogold particles again observed on interfibrillar filaments (arrows) (× 120 000, Bar = 100 nm).

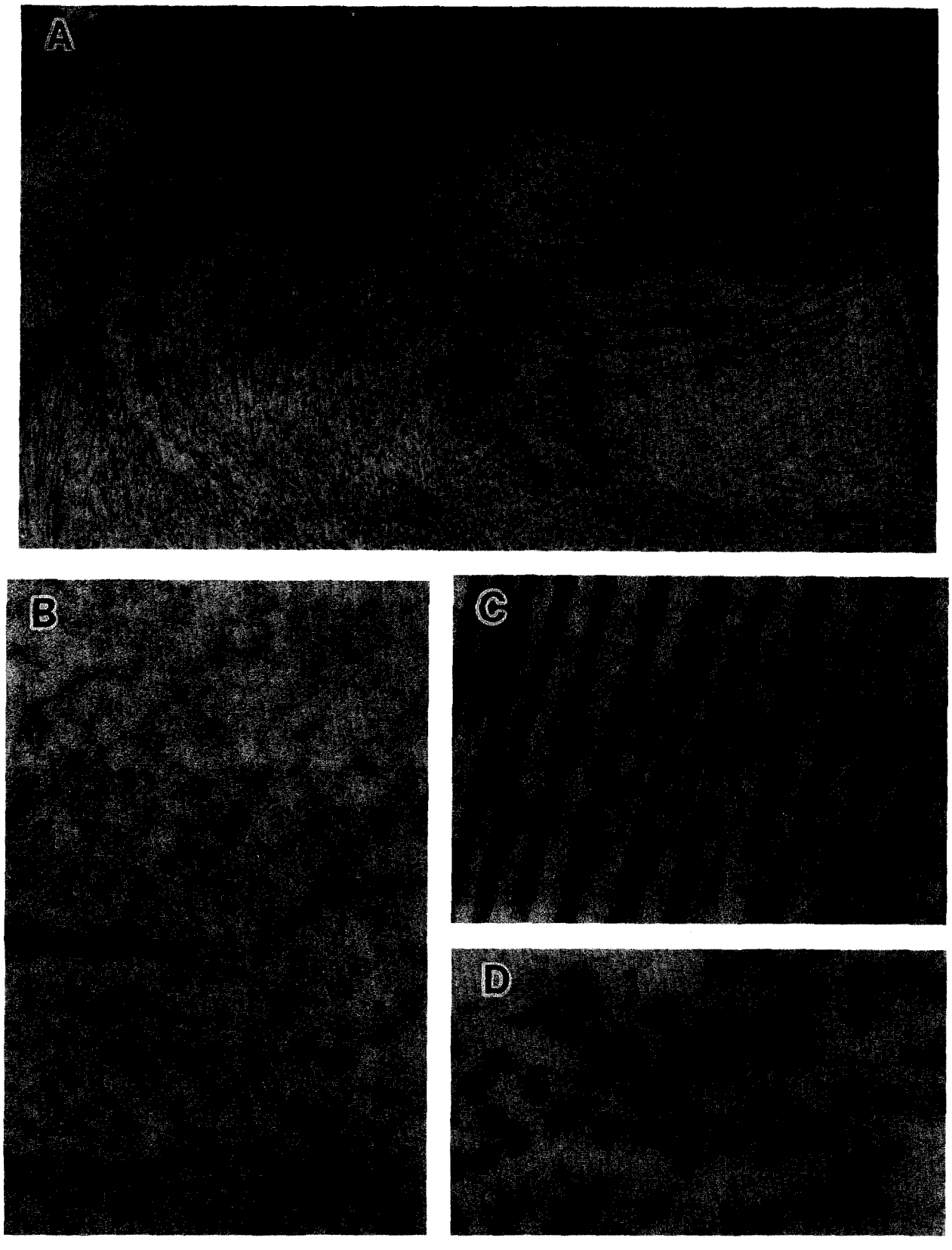

Figure 2. Immunoelectron micrographs of mouse corneal stroma after chondroitinase ABC (10 U/ml) digestion at 37°C for 3 hours. Ultrathin sections were immuno-stained with anti-type VI collagen IgG and gold particle-conjugated (5 nm) secondary antibody. (**A**) Disorganized arrangement of striated collagen fibrils with irregular spacing and scattering of fibrils (asterisks) (× 10 000, Bar = 1 μm). (**B**) High magnification of area near asterisks in (**A**). Few collagen fibrils; space filled with networks of clumped beaded filaments. Immunogold particles seen on the networks (arrows) (× 120 000, Bar = 100 nm). (**C**) Longitudinal section of striated collagen fibrils at high magnification: fewer immunogold particles seen on interfibrillar filaments (arrows) than in enzyme-untreated cornea (Figure 1B) (× 120 000, Bar = 100 nm). (**D**) Cross section of collagen fibrils at high magnification: fewer immunogold particles were seen on interfibrillar filaments (arrows) than in enzyme-untreated cornea (Figure 1C) (× 120 000, Bar = 100 nm).

free buffer (480; Figure 1C) among the same number of collagen fibril cross sections (895).

Our results indicate that type VI collagen filaments were bound to striated collagen fibrils by the activity of chondroitin/dermatan sulfate glycosaminoglycan or proteoglycan, which was sensitive to chondroitinase ABC digestion. The control samples exposed to normal rabbit serum instead of the primary antibody contained almost no gold particles (data not shown).

Discussion

Large amounts of type VI collagen[5] and proteoglycans[4] are found among the striated collagen fibrils of the corneal stroma. It is, therefore, necessary to investigate their distribution and relationships in order to understand the tissue organization. Type VI collagen is unique because of its globular structure, its beaded polymer filaments,[12] and its occurrence as fine periodic filaments or filamentous networks in the extracellular matrix of tissues.[6,13] In this study, we examined the immunoelectron microscopic localization of type VI collagen in mouse corneal stroma, and demonstrated that it was distributed on the interfibrillar beaded filaments. This is similar to previous reports describing the localization of type VI collagen in the corneal stromas of the developing avian[14] and rabbit,[15,16] and the older human.[17,18]

Corneal proteoglycan, another important stromal matrix component, includes chondroitin/dermatan sulfate proteoglycan and keratan sulfate proteoglycan.[4,19] The ratio of keratan sulfate proteoglycan to total proteoglycan varies in animals.[4] The mouse cornea, for example, has little keratan sulfate glycosaminoglycan: The undersulfated keratan sulfate content is <20% of total glycosaminoglycan and there is no oversulfated keratan sulfate.[4] The major chondroitin/dermatan sulfate proteoglycan in the corneal stroma is decorin, composed of one core protein and only one glycosaminoglycan chain of chondroitin/dermatan sulfate.[19–21] Decorin associates with interfibrillar type VI collagen beaded filaments in fetal rabbit[16] and senile human[18] corneal stromas. There are other biochemical studies of interactions between chondroitin/dermatan sulfate proteoglycan and type VI collagen[22,23]: a membrane-bound chondroitin sulfate proteoglycan bound type VI collagen,[22] and a core protein of decorin bound type VI collagen mediated by protein–protein interaction.[23]

Interactions between proteoglycans and collagen fibrils have been extensively studied.[4,11,24–26] By ultrastructural studies with Cupromeronic blue staining, Scott and Haigh[11,24,25] showed that chondroitin/dermatan sulfate proteoglycan and keratan sulfate proteoglycan were associated with specific bands of the collagen fibrils of various tissues, including the cornea. Some immunoelectron microscopic studies have shown that the core protein of decorin is located near the d and e bands of the bovine tail tendon[27] and senile human scleral[18] type I collagen fibrils. Brown and Vogel[26] revealed that a core protein of small decorin-like dermatan sulfate proteoglycan, synthesized by bovine tendon fibroblast, bound to type I collagen.

We previously reported that the ATP-aggregated type VI collagen periodic fibrils separated from the striated collagen fibrils after chondroitinase ABC digestion; we proposed the theory that type VI collagen would interact with the striated collagen fibrils by mediation of chondroitin/dermatan sulfate glycosaminoglycans or proteoglycans.[10] The present study examined this theory without ATP treatment, investigating the effect of chondroitinase ABC digestion on the localization of type VI collagen by immunoelectron microscopy. After digestion, the arrangement of collagen fibrils was disrupted, and large spaces containing widely scattered fibrils appeared. The spaces contained filamentous networks that were stained by anti-type VI collagen IgG and were apparently clumps of beaded filaments. Interfibrillar type VI collagen beaded filaments and immunogold particles decreased. Because chondroitinase ABC reacts with chondroitin/dermatan sulfate glycosaminoglycans, this indicated that type VI collagen bound to the striated collagen fibrils by mediation of this compound and that the association was destroyed by chondroitinase ABC digestion, supporting our hypothesis physiologically. However, because this may occur only in the mouse cornea (which has less keratan sulfate than other animals[4]) further study is needed in other species.

Using enzyme digestion and immunoelectron microscopy, we have identified the ultrastructural site of type VI collagen in the mouse corneal stroma and shown that at least a part of type VI collagen binds to the striated collagen fibrils by action of chondroitin/dermatan sulfate glycosaminoglycans or proteoglycans. This association appears influential in the orderly arrangement of the striated collagen fibrils, which is responsible for corneal transparency.

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