

Characterization of the Collagen in the Hexagonal Lattice of Descemet's Membrane: Its Relation to Type VIII Collagen

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Abstract. To investigate the nature of the hexagonal lattice structure in Descemet's membrane, monoclonal antibodies were raised against a homogenate of bovine Descemet's membranes. They were screened by immunofluorescence microscopy to obtain antibodies that label Descemet's membrane. Some monoclonal antibodies labeled both Descemet's membrane and fine filaments within the stroma. In electron microscopy, with immunogold labeling on a critical point dried specimen, the antibodies labeled the hexagonal lattices and long-spacing structures produced by the bovine corneal endothelial cells in culture; 6A2 antibodies labeled the nodes of the lattice and 9H3 antibodies labeled the sides of the lattice. These antibodies also labeled the hexagonal lattice of Descemet's membrane in situ in ultrathin frozen sectioning. In immunofluores-

cence, these antibodies stained the sclera, choroid, and optic nerve sheath and its septum. They also labeled the dura mater of the spinal cord, and the perichondrium of the tracheal cartilage. In immunoblotting, the antibodies recognized 64-kD collagenous peptides both in tissue culture and in Descemet's membrane in vivo. They also recognized 50-kD pepsin-resistant fragments from Descemet's membranes that are related to type VIII collagen. However, they did not react either in immunoblotting or in immunoprecipitation with medium of subconfluent cultures from which type VIII collagen had been obtained. The results are discussed with reference to the nature of type VIII collagen, which is currently under dispute. This lattice collagen may be a member of a novel class of long-spacing fibrils.

DESCEMET'S membrane (DM)¹ is the basement membrane of corneal endothelial cells. DMs of some animal species contain characteristic stacks of hexagonal lattices that are arranged parallel to the surface of the membrane (16). The lattice is composed of electron-dense nodes at the vertexes and rodlike structures that connect the nodes. This highly regular arrangement has aroused the interests of many researchers. Linearly-arranged long-spacing (LS) structures with similar periodicities are often found in the vicinity of various basement membranes (for review, see reference 11). In an in vitro culture experiment, it was shown that the lattice consists of dumbbell-shaped unit structures 160 nm in length (33). They adhere to each other at their round ends to form hexagonal lattices or linear LS structures of ~150 nm in periodicity. The component of the lattice has been regarded as collagenous (16, 32). Type II collagen (15), type IV collagen (41), type VI collagen (6, 7), and type VIII collagen (18) have been postulated as candidates for the component of the lattice. However, in our previous immunoelec-

tron microscopic study, we showed that type II, type IV, and type VI collagens are not the essential component of the lattice structure (33, 35). Type VIII collagen remained to be tested as a possible candidate.

Type VIII collagen was first identified in the culture medium of bovine aortic endothelial cells and therefore was called endothelial cell (EC) collagen (28). Type VIII collagen possesses several characteristics distinct from other collagen species such as lack of interchain disulfide cross-links, extreme sensitivity to pepsin digestion, and low affinity for the DEAE cellulose column (1, 2, 3, 28). It can be secreted in the absence of prolyl or lysyl hydroxylation (29, 30). Chains of three different molecular masses (177 k [EC1], 125 k [EC2], and 100 k [EC3]) were identified. Digestion with pepsin originated 50-kD pepsin-resistant collagenous domains. On the basis of these observations Sage and co-workers proposed a cassette model in which three 50-kD collagenous domains are linked in tandem by noncollagenous domains smaller than 10 kD to form a 177-kD chain. EC2 and EC3 are regarded as differently processed forms which contain two tandem 50-kD collagenous domains (27, 30). Type VIII collagens were also detected in the culture of various normal and transformed cells (31). These include rabbit and bovine corneal ECs that are responsible for the production of DMs (2, 31). They were also found in DMs in vivo

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1. *Abbreviations used in this paper:* BAPN, β -aminopropionitrile; CBB, Coomassie brilliant blue; CS-PBS, PBS containing 50% calf serum; DM, Descemet's membrane; ECM, extracellular matrix; LS, long-spacing; NEM, *N*-ethylmaleimide; OA-PBS, PBS containing 1% ovalbumin.

as one of their major collagenous components (22, 23). Kapoor and co-workers raised both polyclonal and monoclonal antibodies against 50-kD pepsin digest (50-K DM) of DMs (17, 18). In immunofluorescence microscopy type VIII collagen was localized not only in DMs but also in various other tissues, some of which derived from neural crest cells (18). There has been, however, no report showing a direct correlation between the hexagonal lattices in the DM and type VIII collagen.

On the basis of careful biochemical analysis, Benya and Padilla (3) postulated that the native chain of type VIII collagen is 61 kD, and that 120- and 170-kD components are β - and γ -components of the collagens. They presented an alternative model in which a homotrimer with a 61-kD chain makes up a type VIII collagen molecule (3). Kapoor et al. presented evidence that the 61-kD chain does not have immunological crossreactivity to EC2 (125 kD). Thus the molecular mass of type VIII collagen and its molecular model are currently under debate (3, 17, 18).

Recently, cDNA clones of one chain in VIII-3 ($\alpha 1$ [VIII]) were sequenced by Yamaguchi et al. (40). It has amino acid sequence similar to that of type X collagen. Based on the length of the domains, they suggested the $\alpha 1$ (VIII) is the backbone of the lattice.

Here we report monoclonal antibodies that label various domains of the hexagonal lattice of the DM both in vitro and in vivo. They also recognize the 50-kD pepsin-resistant fragment isolated from DMs that has been postulated to be derived from type VIII collagen. We show that the molecular mass of the undigested antigen molecule is ~ 90 k (64 k with collagenous standards).

Materials and Methods

Monoclonal Antibodies

Adult bovine eyes were obtained from a local slaughterhouse. DMs were removed from stromas by peeling with forceps, and endothelial cells were removed by sonication in PBS containing 1 mM PMSF, 2 mM N-ethylmaleimide (NEM), and 2 mM EDTA. The DMs were washed with PBS, homogenized, and used for immunization. Hybridomas were produced by fusing splenocytes from immunized BALB/c mice with X63-Ag8-6.5.3 myelomas with polyethylene glycol 1530 (Sigma Chemical Co., St. Louis, MO) according to the standard procedure (14). Screening was performed by immunofluorescence microscopy as described below. Five clones stained both DMs and fine fibrillar structures in the stroma. Two (6A2 and 9H3) of these clones were used for the study.

Antibodies

Ascites containing monoclonal antibodies was obtained following standard procedures. 50% ammonium sulfate precipitate of ascites was dissolved in 0.3 M NaCl, 10 mM phosphate buffer, pH 8.0, and dialyzed against the same buffer. Classes of antibodies were determined by the Ouchterlony method. 6A2 and 9H3 were shown to be IgGs. They were passed through a protein A-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden), and IgG was eluted with 0.1 M glycine-HCl buffer, pH 3.0. The eluate was adjusted immediately to pH 8.0, concentrated by 50% ammonium sulfate precipitation, dialyzed against 0.3 M NaCl, 10 mM phosphate buffer, pH 8.0, and stored either at 4°C or -80°C. 6A2 antibody was stored at OD 280 = 2.9 and 9H3 at OD 280 = 6.0.

Immunofluorescence Microscopy

6 μ m frozen sections of unfixed or 4% paraformaldehyde-fixed adult bovine corneas and other tissues (optic nerves, scleras, choroids, lens, lungs, livers, kidneys, cerebrums, spinal cords, aortas, and tracheas) were cut with a cryostat (II; Tissue Tek, Elkhart, IN). They were rinsed with PBS, blocked

with 1% ovalbumin in PBS (OA-PBS), and labeled with hybridoma culture medium for 2 h at room temperature without dilution. After rinsing with PBS three times for 5 min each, FITC-labeled rabbit anti-mouse IgG (MBL Co. Ltd., Nagoya, Japan) at a dilution of 1:100 in OA-PBS was applied for 2 h at room temperature. Then they were rinsed with PBS three times for 5 min each, mounted in 90% glycerol containing 0.1% paraphenylenediamine (39) and observed and photographed with a fluorescence microscope (BH-2; Olympus Optical Company Ltd., Tokyo, Japan). For tissues other than the cornea, 6A2 antibody diluted to 1:100 in PBS containing 50% calf serum (CS-PBS) or 9H3 antibody diluted to 1:100 in CS-PBS were used as primary antibodies.

Immunoelectron Microscopy of Matrices Produced in Culture

Bovine corneal endothelial cells were obtained and cultured on formvar-covered gold grids that had been coated with carbon as described previously (33, 35). After the cells were removed the grids with extracellular matrices (ECMs) were fixed with 2% formaldehyde in PBS for 2 h at room temperature.

The grids were treated with 0.02 M glycine in PBS for 5 min, then with CS-PBS for 5 min to quench residual aldehydes and to reduce background labeling. The grids were treated with various concentrations of antibodies for 2 h at room temperature, then with rabbit anti-mouse IgG (MBL Co. Ltd.) at a dilution of 1:1,000 in CS-PBS and then with 5 nm goat anti-rabbit IgG gold (Auro Probe GAR G5, Janssen Life Sciences Products, Olens, Belgium) diluted in CS-PBS (1:20) for 2 h at room temperature.

After labeling and rinsing three times in PBS, the grids were fixed, dehydrated, and critical point-dried as described before (35), and observed in a transmission electron microscope (JEM 2000 EX; Jeol Akishima, Japan). For calibration, a carbon grating replica (2,160 lines/mm, Ernest F. Fullam Inc., Schenectady, NY) and the labeled ECMs were photographed at 20,000 \times .

Immunoelectron Microscopy of DMs In Situ

DMs were fixed with periodate-lysine-paraformaldehyde fixative (25), infiltrated with a mixture of 10% polyvinylpyrrolidone (10,000 mol wt) and 1.6 M sucrose in 10 mM phosphate buffer (pH 7.4) overnight, placed on a specimen holder for Sorvall LTC-2 cryosection device (RMC Cryosystems Inc., Tucson, AZ), and frozen in liquid nitrogen. Ultrathin frozen sections were made, and immunolabeling was performed according to the method of Tokuyasu (37). The sections were treated with PBS containing 0.02 M glycine for 10 min, then with 50% normal goat serum in PBS for 10 min, and were labeled with a drop of antibody solution diluted in 50% normal goat serum. After rinsing, the sections were labeled with rabbit anti-mouse IgG and then with 5 nm goat anti-rabbit IgG gold as described. They were rinsed with PBS three times and fixed with 1% glutaraldehyde in PBS, postfixated with 1% osmium tetroxide in PBS, stained with uranyl acetate, dehydrated, and embedded in LR white resin (medium) (London Resin Co. Ltd., Surrey, UK) (19, 34). After polymerization at 60°C they were observed in a transmission electron microscope (JEM 1200EX; JEOL) operated at 80 kV. At the same time, the periodicity of the lattice was measured as described in Immunoelectron Microscopy of Matrices Produced in Culture.

Isolation of 50 k Pepsin-resistant Fragments from DMs (50 K DM)

50-kD pepsin-resistant collagenous fragments from DMs were obtained according to the method of Kapoor et al. (17).

HPLC of 50-kD Collagen

Final purification of the 50-kD fragment fractions obtained by salt fractionation was done by C18 (Micro Bondasphere, 5 μ 100Å, 3.9 mm \times 15 cm; Waters Chromatography Div., Millipore Corp., Milford, MA) reversed-phase HPLC as described by Kapoor et al. (17).

Amino Acid Analysis

Several μ g of specimens obtained from C18 reversed-phase HPLC were dissolved in 50 μ l of distilled water. 20 μ l of the solution was dried and hydrolyzed with 6 N HCl. The analysis was performed by standard procedures with an amino acid analysis system (PICO-TAG TM; Millipore Waters Chromatography Div., Milford, MA).

SDS-PAGE and Immunoblotting

Bovine corneal endothelial cell cultures were maintained in MEM with 10% FCS containing 200 µg/ml β-aminopropionitrile (BAPN) 75 µg/ml sodium ascorbate for 15–30 d. They were rinsed with PBS three times and dissolved in Laemmli's sample buffer (1% SDS, 1% β-mercaptoethanol, 20% glycerol, 10 mM Tris-HCl, pH 6.8) (cell-matrix fraction). 50 k DM, 50 K-A, 50 K-B, bovine type I collagen, type IV collagen, and human type VI collagen (a kind gift of Dr. Heinz Furthmayr, Yale University) were dissolved in the sample buffer. DMs were peeled off at 4°C and endothelial cells were solubilized in 0.5% Triton X-100 in PBS containing protease inhibitors (20 mM NEM, 10 mM ε-aminocaproic acid, 10 mM benzamide hydrochloride, 10 mM EDTA, 1 mM PMSF, 0.23 TIU/ml aprotinin, and 10 µg/ml each of leupeptin, antipain, and chymostatin). Then the membranes were homogenized in 2% SDS, 2% β-mercaptoethanol, and 8 M urea in 10 mM Tris-HCl (pH 6.8), and boiled for 5 min.

The specimens were electrophoresed on either 10% or 8% polyacrylamide gels (24). Some gels were stained with Coomassie Brilliant Blue (CBB). For immunoblotting the electrophoresed proteins were transferred to nitrocellulose membranes for 3 h at 4°C at a voltage of 10 mV. The membranes were stained with Ponceau S (10) and photographed to show the position of major bands and to check the efficiency of blotting. The gels, after blotting, were also occasionally stained with silver nitrate according to the method of Merrill et al. (26) for the same purpose.

Next the membranes were cut into strips, blocked with CS-PBS, and incubated with monoclonal antibodies diluted in CS-PBS. 6A2 was used at 1:400 for materials from culture and at 1:400,000 for 50 K DM and its components. 9H3 was used at 1:1 for materials from culture and at 1:100 for 50 K DM and its components. After rinsing, they were labeled for 2 h at room temperature with anti-mouse IgG tagged with alkaline phosphatase (Bio-Rad Laboratories, Cambridge, MA) diluted to 1:100 in CS-PBS. Detection of the antibodies was performed according to the method of Blake et al. (5).

For comparison with EC2, semiconfluent cells were cultured in Dulbecco's MEM containing 200 µg/ml BAPN and 75 µg/ml sodium ascorbate for 24 h, and the medium was collected and precipitated with 20–50% ammonium sulfate in the presence of the protease inhibitors. The DEAE cellulose unbound fraction was obtained (28) and dissolved in the sample buffer and subjected to electrophoresis and blotting.

For molecular mass standards, myosin heavy chain (200 kD), α-actinin (95 kD), serum albumin (67 kD), catalase (60 kD), lactic dehydrogenase (36 kD), and ferritin (19 kD) were used as noncollagenous standards, and α1(I) (100 kD), α2-CB3-5(58 kD), α1-CB7(25.5 kD) and α1-CB8(23 kD) were used as collagenous standards (38).

Immunoprecipitation and Autoradiography

Semiconfluent corneal endothelial cells were labeled with L-2,3-[³H]proline, and 20–50% ammonium sulfate fraction and then the DEAE cellulose unbound fraction was obtained essentially as described by Sage et al. (28). 20 µl of the 6A2 antibody was added to 100 µl of the DEAE unbound fraction and incubated for 2 h. Then 40 µl of rabbit anti-mouse IgG antibody was added and incubated for an additional 2 h at 4°C. The precipitate was centrifuged and washed three times with PBS containing 2.5 mM EDTA, 0.5 mM PMSF, and 50 µg/ml NEM. The specific activity of specimen from each step was checked in a liquid scintillation counter (LS 4001; Beckman Instruments, Inc., Palo Alto, CA). Then the specimens were mixed with sample buffer and SDS-PAGE was performed. After electrophoresis, the gels were fixed, soaked in 1.1 M sodium salicylate, and processed for fluorography.

Digestion with Enzymes

In some experiments, cultured endothelial cells were solubilized at 4°C for 5 min with 0.5% Triton X-100 and the protease inhibitors. Then the matrix layer with cytoskeletons was washed with PBS containing the protease inhibitors, and collected with a rubber spatula. They were either washed with 0.1 N acetic acid and digested with 200 µg/ml pepsin in 0.1 N acetic acid at 4°C overnight, or washed with 0.025 M Tris-HCl, pH 7.0, 6.6 mM calcium acetate containing 2 mM NEM and 0.1 mM PMSF and digested with 100 µl of 50 µg/ml collagenase in the buffer for 2 h at room temperature (28). The specimens were lyophilized, dissolved in hot sample buffer, and used for SDS-PAGE and immunoblotting.

Results

Antibodies

There were several groups of clones that labeled the DM. A group which labeled the DM and fine fibrillar structures in the stroma was selected (Fig. 1 *a*). Of this group, antibodies designated as 6A2 and 9H3 were used for most of the study.

Immunofluorescence

In the cornea, these antibodies labeled intensely the whole thickness of DMs and the fine fibrillar structures coursing roughly parallel to the surface of the cornea. There was no labeling in the epithelial basement membrane. There was also positive immunofluorescence in other tissues. In the outer and inner sheath and septal connective tissue of the optic nerve, fine fibrillar immunofluorescence was observed (Fig. 1 *b*). In the sclera and choroid, there was diffuse fluorescence (Fig. 1 *c*). Concentric fibrillar immunofluorescence was observed in the dura mater of the spinal cord (Fig. 1 *d*), but not in the arachnoidea, pia mater, or spinal cord itself. The perichondrium of the tracheal cartilage was also labeled in a fibrillar fashion (Fig. 1 *e*). No immunofluorescence was detectable in the cartilage matrix. There was little reaction in the liver, lungs, cerebrum, lens, or kidney cortex. In the aorta, only the autofluorescence from elastic fibers was evident and no specific reaction was recognizable.

Immunoelectron Microscopy

Bovine corneal endothelial cells can produce ECM containing hexagonal lattice similar to that observed in situ (33). We labeled the matrix with the antibodies and with 5-nm gold probes and observed it after critical point drying. 6A2 antibody showed reactions only on the nodes of the lattice and the LS structure (Fig. 2 *a*). In a linear LS structure, all the nodes seemed to be labeled, including both terminals. Aggregates that may have contained lattice structures often had labels, though the lattice was not always visible. No other structures were labeled in a specific manner with the antibody. On the other hand, 9H3 antibody labeled the middle of the sides of the lattice (Fig. 2, *b* and *c*). In these preparations, the periodicity was 112 ± 12 nm ($n = 68$). This somewhat shorter value than our previous observation (33) may partly be because of the shrinkage during specimen preparation especially during critical point-drying (4) (compare also with the periodicity in cryosections).

The 6A2 antibody also recognized the lattice structure in situ. In immunogold labeling on ultrathin cryosections cut parallel to the surface of the DM, the gold particles were preferentially localized on the nodes of the lattice (Fig. 3 *a*). This indicates that the antigens are really the component of hexagonal lattices in situ and confirms that the lattice produced in the culture system contains the same components as the lattice in situ. The node to node distance was 161 ± 14 nm ($n = 74$). As a control, most of the corneal stroma was devoid of label (Fig. 3 *b*) except for an occasional linear array of gold reminiscent of the immunofluorescence pattern.

Immunoblotting on ECM Produced in Culture

The 6A2 antibody recognized three peptides of 90, 83, and 67 kD by immunoblotting, judging from noncollagenous

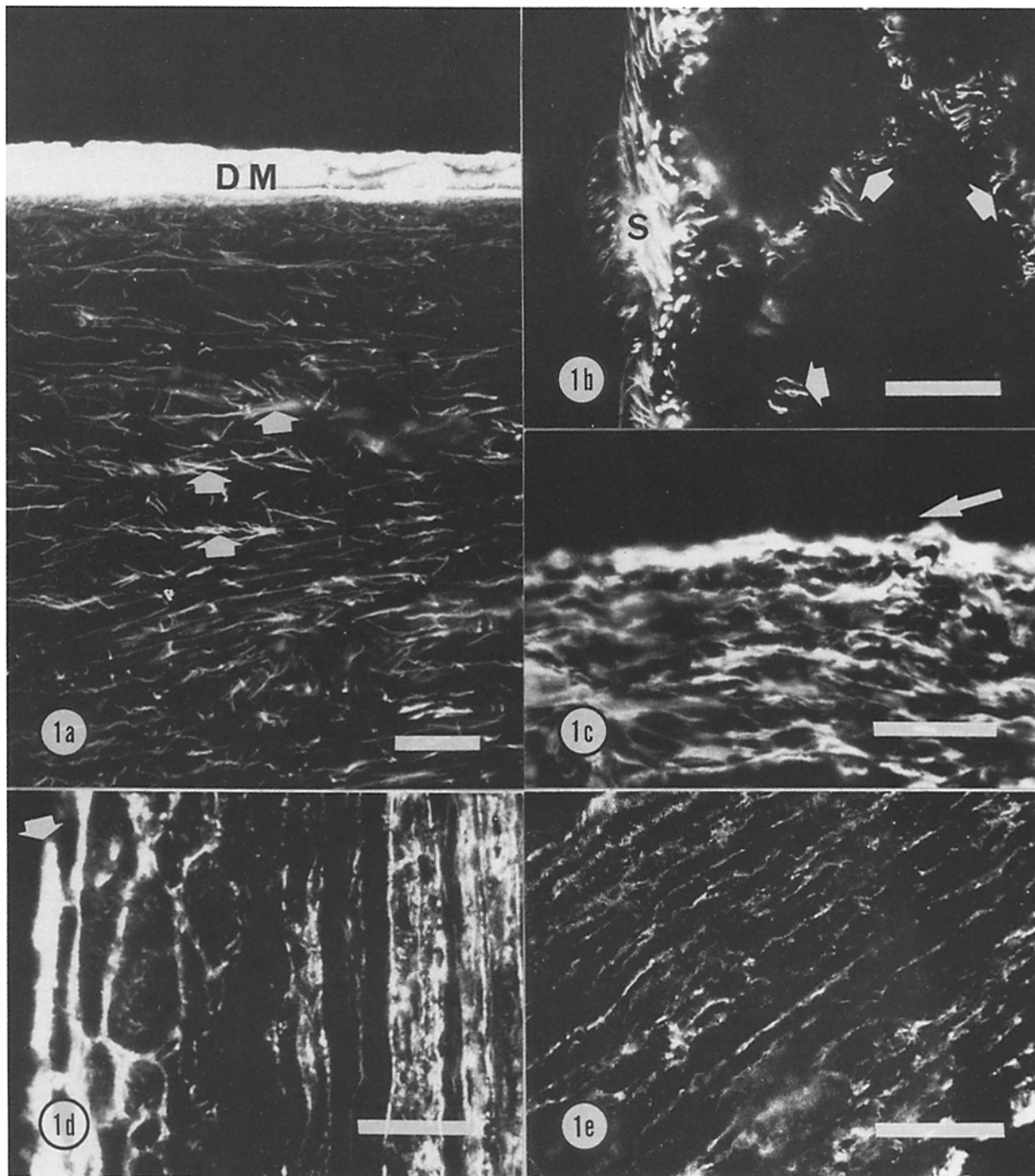


Figure 1. Immunofluorescence micrograph of the reaction on bovine tissues produced by 6A2 and 9H3 monoclonal antibodies. (a) 9H3 antibody stains the DM intensely. It also shows positive reaction on fine filaments in the stroma parallel to the surface of the cornea (*white arrows*). Other monoclonal antibodies including 6A2 showed identical reactions on the cornea. (b) In the optic nerve, fine filaments in the optic nerve sheath(s) as well as in the septums (*white arrows*) are labeled by 6A2 antibody. (c) In the choroid all the extracellular spaces are diffusely labeled by 6A2 antibody. Dark areas are occupied by pigmented cells. An arrow indicates the layer of pigment epithelium. Sclera and retina have been removed during preparation. (d) In the spinal cord, the dura mater is positive to 6A2 antibody. (e) In the tracheal cartilage, the perichondrium shows fibrillar immunofluorescence. 6A2 antibody. Bar, 100 μm .

standards (Fig. 4, lane *b*). With collagenous standards they were estimated to be 64, 57, and 50 kD, respectively. In the following sections, we use the molecular masses from collagenous standards for ease of comparison with the results of other investigators. The 64-kD peptide was the strongest

in reaction, and the 50 kD was a rather broad band whose intensity varied from experiment to experiment. The 57-kD band always showed only a weak reaction.

The 9H3 antibody required a far higher concentration to get a positive reaction. It showed bands at the same position

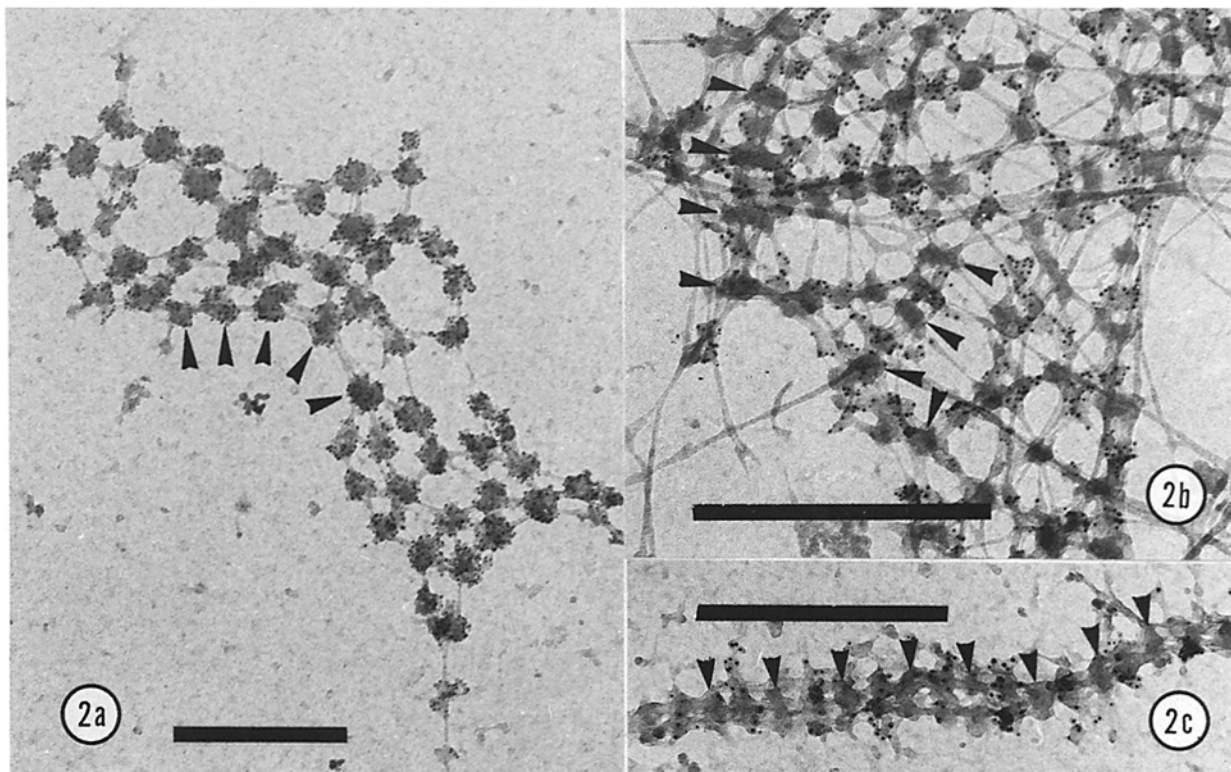


Figure 2. (a) Immunoelectron microscopy of the extracellular matrix produced by cultured bovine corneal endothelial cells with 6A2 antibody and immunogold labeling. The labels are preferentially located at the nodes (*arrowheads*) of the lattice structures. (b and c) Immunoelectron microscopy with 9H3 antibodies. The labels were located at internodes on the rod portion of the lattices and LS structures. *Arrowheads* indicate the nodes of the lattice. Because of heavy labeling, the labeled internodes are thickened in appearance. Fine filaments in the background are not labeled. Bar, 500 nm.

as the 6A2 antibody (Fig. 4, lane c). Since no bands were observed when normal mouse serum was substituted for antibodies, and these bands were not major components in CBB-stained material (Fig. 4, lane a), we regarded these labelings as specific. Possibly the 9H3 antibody recognizes a native triple-helical configuration much better.

These antibodies did not show crossreactivity to either type IV or type VI collagen by immunoblotting (Fig. 5). This shows that the cross-reactivity to 50 kD peptide was not because of the proteolytic product of these collagens.

Immunoblotting on 50-kD Fragment (50 K DM) from DMs

Type VIII collagen-related 50-kD fragment (50 K DM) was obtained from DMs. In 8% gel, it showed a mobility almost identical to that reported by Kapoor et al. (17). It was split into two peaks by HPLC (Fig. 6). The amino acid composition of these two peaks was also very similar to that reported by Kapoor et al. (17) (Table I).

Both 6A2 and 9H3 antibodies reacted only with the peak of longer retention time (designated 50 K-B; the other peak with shorter retention time was designated 50 K-A following reference 17) (Fig. 7). The concentration of the antibodies required was more than 100 times more dilute than that used to detect the intact antigens produced in culture (6A2 1:400,000 and 9H3 1:100). This suggests that the epitopes may be unmasked by the pepsin treatment or that the propor-

tion of antigen molecules to total protein is very small in the cell matrix fraction. The two antibodies showed different staining patterns. 6A2 reacted rather broadly with the lower half (faster mobility) of 50 K-B (Fig. 7, lane e), whereas 9H3 reacted narrowly with the upper half (slower mobility) of 50 K-B (Fig. 7, lane g). This basic pattern of labeling did not change even though various concentrations of antibodies were tried. Both showed a weak reaction to a band with the same mobility as the $\alpha 1(I)$ chain (designated as 95,000 M_r in reference 18), where a dimeric form of 50 kD was expected, if large amounts of specimens were applied to the gel (data not shown).

To check if our antibody recognized EC2 of type VIII collagen, we immunoblotted the medium precipitated with 20–50% ammonium sulfate and unbound to DEAE cellulose. No immunoreactive bands were detected although several bands including one band of 125,000 M_r were observed in a CBB stained gel (Fig. 8, lanes a and b). We also tried to immunoprecipitate the antigen from the medium. Although the DEAE unbound fraction contained 50,940 cpm/100 μ l of radioactivity, only 700 cpm precipitated with 6A2 antibody (average from two separate experiments). In fluorography, DEAE unbound fraction showed a prominent band at 125 kD whereas no band was observed in 6A2-precipitated material (Fig. 8, lanes c and d).

When ECMs from the culture system were digested with pepsin, materials from tissue culture yielded one major band

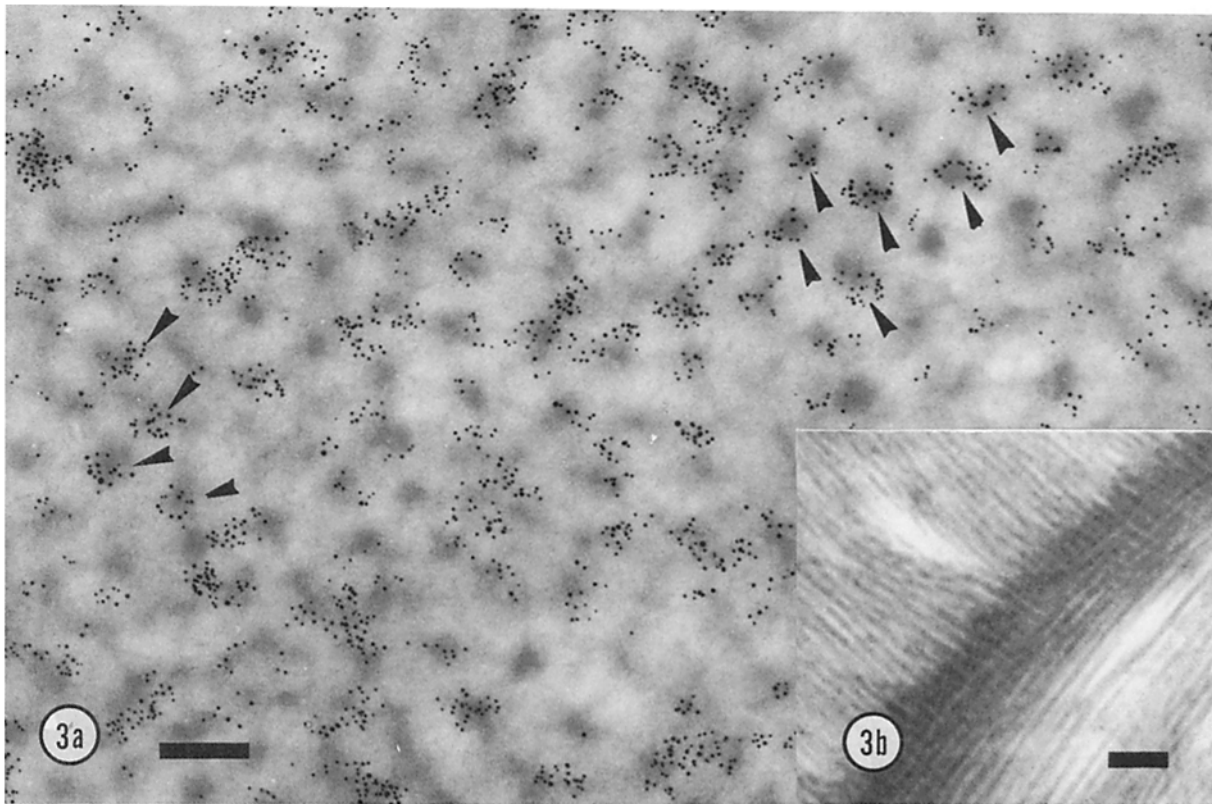


Figure 3. (a) Immunoelectron microscopy of DMs in situ with 6A2 monoclonal antibody on an ultrathin frozen section. Gold labels are located preferentially on the nodes of the lattice (arrowheads). (b) Corneal stroma labeled with 6A2 on the same grid as a. Note that most of the area is devoid of labels. Bar, 200 nm.

at ~50 kD (Fig. 9, lane b). After collagenase digestion, only a band at the front of the gel was immunoreactive (Fig. 9, lane c).

Two immunoreactive bands were obtained from DMs in situ after hot SDS-mercaptoethanol-urea extraction (Fig. 9, lane d). These were 64 kD and ~50 kD, which were similar in mobility to the corresponding bands from tissue culture.

Discussion

The monoclonal antibodies described here recognized hexagonal lattices in the DM in situ and also hexagonal lattices and LS structures deposited in vitro in a regular periodic pattern. The antibodies all reacted with molecules of ~64 kD together with 50-kD species both in tissue culture and in DMs in vivo. Although a 57-kD band was observed in the cell matrix fraction, it always showed only a weak reaction. Since type VIII collagens are highly susceptible to proteases, it is probable that the 57- and 50-kD bands were proteolytic degradation products during preparation of the specimen or during prolonged culture, though we dissolved the specimen in hot sample buffer as quickly as possible. It is also possible that 64-, 57-, and 50-kD components are differently processed forms of the same molecule. Apparently this 64-kD collagenous molecule is the major constituent of the hexagonal lattice of the DM.

The pepsin-resistant 50-kD fragments isolated from DMs have been postulated to be derived from type VIII collagen (3, 17, 22). Recently, Yamaguchi et al. isolated and se-

quenced cDNA clones which encode a type VIII collagen chain (40). The predicted polypeptide sequence from the cDNA sequence contains the same sequence as one of the CNBr peptides (CB3) of type VIII collagen from corneal endothelial cell culture (VIII-3). It also contains a highly homologous sequence to one of the pepsin-resistant 50-kD fragments from bovine DM (50K-B), which our antibodies recognized. The molecular mass of the chain deduced from cDNA is ~60,000 and that of its collagenous portion is ~50,000. This is in very good accordance with the molecular mass obtained in the present study. Thus the suggestion by Yamaguchi et al. that one of the type VIII collagen chains (50 K-B) has a molecular mass of ~60 kD and that it is a major component of the hexagonal lattice structure of DM is confirmed.

The EC2 molecule that was first postulated to be type VIII collagen has a much higher molecular mass (125 kD) than these antigens. The immunolocalization of type VIII collagen (EC2) by Kapoor et al. and that of our antigen are very similar in that both antibodies stain the DM, sclera, perichondrium, optic nerve sheath, and dura mater of the spinal cord, but not the lungs, liver, kidneys, or lens. However, there are some discrepancies in immunolocalization between anti-EC2 antibodies and ours. The antibodies used by Kapoor et al. did not stain corneal stroma or septal connective tissue of the optic nerve, whereas ours did. Instead, their antibodies stained the pia mater of the brain and spinal cord, whereas ours did not (18). However, it is also known that epitopes of type VIII collagen are masked on various oc-

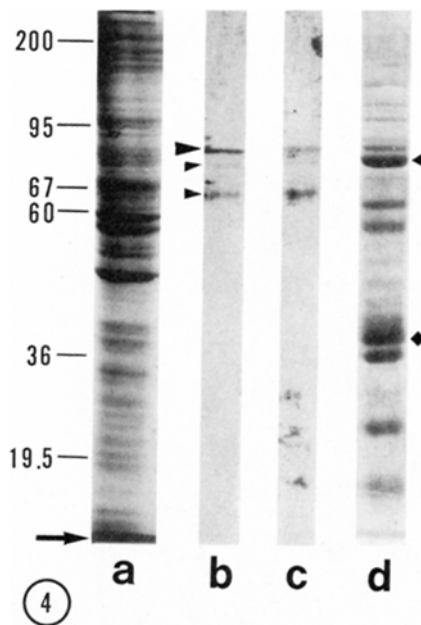


Figure 4. Corneal endothelial cell culture electrophoresed and immunoblotted with monoclonal antibodies. Lane *a*, the cell-matrix fraction from a corneal endothelial cell culture was stained with CBB. Lanes *b* and *c* show immunoblotting of the same material as lane *a* with 6A2 antibody (*b*) and with 9H3 antibody (*c*). Calibrations on the left were done with noncollagenous standards. CBB-stained CNBr peptides of human type I collagen are shown in lane *d* for molecular mass standards. The arrowhead on the right indicates α -2 CB3-5 (58 kD) and the rectangle indicates α -1 CB7 (25.5 kD). Both 6A2 and 9H3 antibodies show a similar reaction at 90 kD (large arrowhead), 83 and 67 kD (small arrowheads). These values register as 64, 57, and 50 kD with collagenous standards. 10% gel.

cations (18, 21), and these differences may also represent differences in age of the animals used.

There has been a preliminary report that in immunoelectron microscopic localization with an anti-type VIII collagen antibody, fine linear filaments were labeled in corneal endothelial cell culture (Keene, cited in reference 8). Though we do not know the culture conditions used in the study,

Table I. Amino Acid Composition of 50K-A and 50K-B Collagenous Fragments Isolated from Descemet's Membrane (Residues/1,000 Residues)

	50K-A	50K-B		50K-A	50K-B
Asx	30.7	12.0	Cys	ND	ND
Thr	22.5	7.7	Ile	18.3	37.3
Ser	25.4	10.5	Leu	66.6	63.3
Glx	70.2	78.3	Tyr	10.2	7.0
Pro	101.5	113.0	Phe	14.6	17.7
Hyp	143.5	156.1	Hyl	22.0	30.4
Gly	325.1	336.8	Lys	13.3	22.2
Ala	58.3	36.0	His	11.3	9.2
Val	30.1	32.3	Arg	36.4	17.4
Met	trace	12.8	Trp	ND	ND

Amino acid composition of peaks A and B of HPLC (Fig. 6). Their patterns are very similar to those reported by Kapoor et al. (17). Thus, we denote these two peaks 50K-A and 50K-B, respectively, according to their nomenclatures.

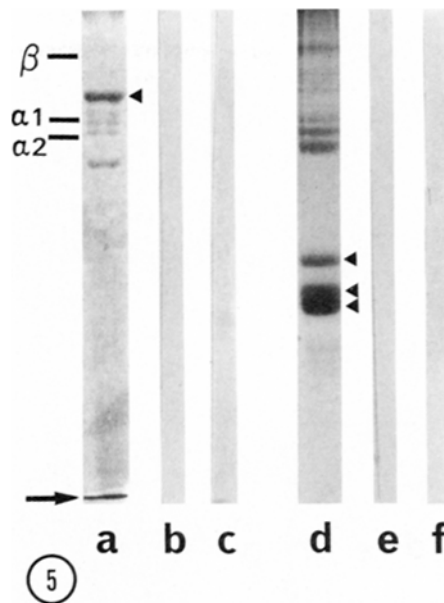


Figure 5. Cross-reactivity of 6A2 and 9H3 antibodies to type IV and type VI collagens. Lane *a*, CBB-stained type IV collagen. Lanes *b* and *c* show immunoblotting reactions with 6A2 and 9H3 antibodies, respectively, to the gel shown in *a*. Lane *d*, CBB-stained type VI collagen. Lanes *e* and *f* show immunoblotting with 6A2 and 9H3 antibodies, respectively, on the gel shown in *d*. No cross-reactivity is observed either to type IV or type VI collagen. Calibrations on the left show the point of migration of chains of type I collagen. The arrow on the left shows the position of the tracking dye. The molecular masses of the major bands are 130 kD for type IV collagen and 43–52 kD for type VI collagen (arrowheads); 8% gel.

these antibodies (possibly anti-EC2) may not recognize the lattice structure.

Kapoor et al. showed evidence that 125 kD species (EC2) and ~60-kD species (VIII-3) in the culture medium do not have immunological cross-reactivity. Our antibody against ~60-kD species in tissues did not cross-react with 125-kD species either by immunoblotting or by immunoprecipitation. It is very likely that EC2 and 50 K-B are immunologically unrelated peptides.

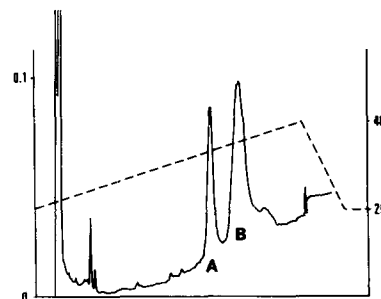


Figure 6. 100 μ g of 50 K DM was chromatographed on a Micro Bondasphere C18 HPLC column at room temperature with a linear gradient of acetonitrile (20–40%) and monitored by absorption at 220 nm. Two peaks, A and B, were subsequently analyzed by SDS-PAGE and amino acid analysis.

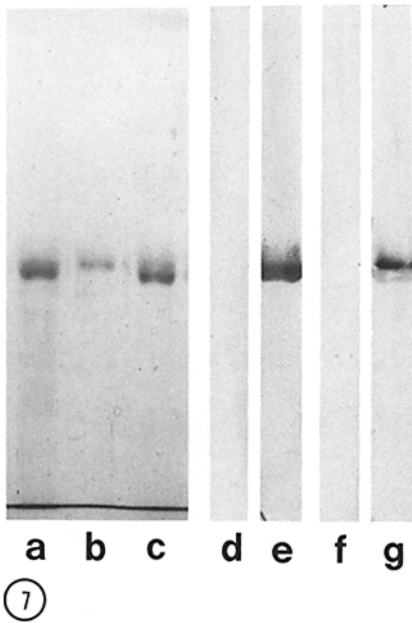


Figure 7. SDS-PAGE and immunoblotting analysis of 50-kD pepsin-resistant fragments isolated from DM on 8% gel. Lane *a*, CBB-stained pepsin fragment isolated from DMs (50 K DM); lane *b*, CBB-stained 50 K-A isolated by HPLC; lane *c*, CBB-stained 50 K-B; lane *d*, reaction of 6A2 antibody with 50 K-A; lane *e*, reaction of 6A2 antibody with 50 K-B; lane *f*, reaction of 9H3 antibody with 50 K-A; lane *g*, reaction of 9H3 antibody with 50 K-B. Only 50 K-B shows strong reaction at ~50 kD with both antibodies.

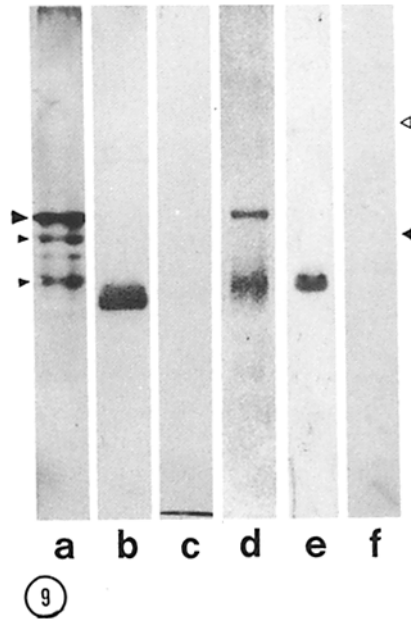


Figure 9. Comparison of the antigens deposited in vitro and those in vivo and their susceptibility to proteases. All the lanes were shown by SDS-PAGE (8%) followed by immunoblotting with 6A2 antibodies. Lane *a* shows the matrix deposited in culture. 64 kD (large arrowhead), 57, and 50-kD bands (small arrowheads) are seen. Lane *b* shows the pepsin-treated cytoskeleton-matrix fraction deposited in culture. The 64-kD band disappeared and the band ~50 kD is prominent. Lane *c* shows the cytoskeleton-matrix fraction treated with collagenase. Only a band at the front is immunoreactive. Lane *d* shows the material released by a hot SDS-mercaptoethanol-urea treatment from DMs in situ. Bands at 64 kD and ~50 kD are seen. Lane *e* shows the material obtained from DMs treated with pepsin (50 K DM). Lane *f* shows a control with pepsin alone. No reaction is seen. A control with collagenase alone showed the same results. White arrowhead on the right indicates the position of $\alpha 1(I)$. Black arrowhead indicates the position of $\alpha 2$ -CB3-5.

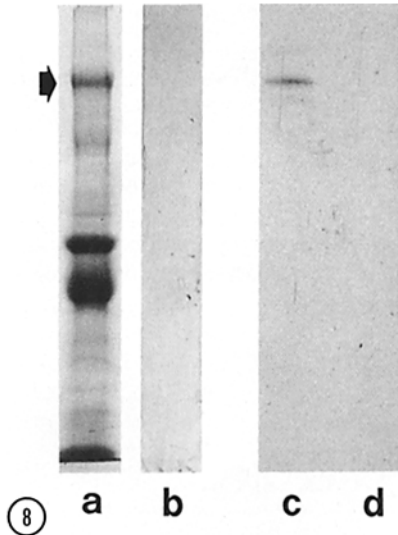


Figure 8. CBB-stained SDS-PAGE (*a*) and immunoblotting (*b*) of DEAE unbound fraction from medium of semiconfluent corneal endothelial cell culture. Although several bands, including one around 125 kD, are observed, no immunological reaction was observed on the nitrocellulose membrane. EC2 is expected to migrate to the position indicated by the arrow on the left. Fluorography of DEAE unbound fraction from the medium of semiconfluent corneal endothelial culture labeled with ^3H proline (*c*), and immunoprecipitated material with 6A2 antibody from the fraction (*d*). Although a band at the position of EC2 is observed in the fluorogram from the medium fraction, no band immunoprecipitated with the antibody; 8% gel.

There is evidence that more than one 50-kD chain is released by pepsin treatments from these tissues or from the corneal endothelial cell culture. 50 K-A has not been fully identified yet. The amino acid sequences of some of the CNBr peptides from VIII-3 (CB2 and CB4) were not found in the cDNA sequence determined by Yamaguchi et al., either (40). It is possible that EC2 gives rise to one of these peptides upon digestion with pepsin. There is also a possibility that type VIII collagen is a heterotrimer. However, the possibility that type VIII collagen consists of both an ~60-kD chain and a 125-kD chain is not likely, since immunoprecipitation with 6A2 antibody did not precipitate the 125-kD chain from the medium and the stoichiometry of 60-kD and 125-kD chains varies according to culture density (18).

Bruns postulated that many long-spacing collagens of ~100 nm in periodicity may be composed of type VI collagens (6, 7). As described, Descemet's lattice collagen can assemble into other LS fibrils with a longer periodicity (35). LS fibrils are often observed in nerve or glial tissues (for review, see reference 11; 12, 13, 36). They are often longer than 100 nm in periodicity. Long-spacing fibrils have also been observed in the vicinity of basement membranes (9, 11,

12, 13, 20, 36). The lattice collagen (50 K-B) very possibly contributes to another category of LS structures in these locations that may be assembled in that form under some special environment.

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