Tissue Distribution of Type VIII Collagen in Human Adult and Fetal Eyes

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The type VIII collagen tissue distribution in human adult (32-78 yr of age) and fetal (16-27 weeks of gestation) eyes was studied immunohistochemically using a monoclonal antibody to type VIII collagen. Type VIII collagen was distributed in a linear or fibrous fashion in adult eyes in Descemet's membrane of the cornea, the trabecular meshwork, the walls of Schlemm's canal, Bruch's membrane, the choroidal stroma, the sclera, the cribriform plates of the optic nerve, and the intima of the central retinal artery. The staining in the central retinal artery was similar to that of type IV collagen. No distinct positive staining was seen in other blood vessels. When fetal eyes were examined, significant differences in positive staining were found between adults and fetuses in the sclera. In fetal eyes, the posterior sclera was strongly stained; however, the positive staining gradually decreased, and in the equatorial area it disappeared. The cribriform plates of the lamina cribrosa and the presumptive Bruch's membrane in fetal eyes did not react with the antibody. The trabecular meshwork and Descemet's membrane, but no other part of anterior section of fetal eyes, reacted with the antibody. Invest Ophthalmol Vis Sci 32:2636–2644, 1991

Collagen is one of the major components of the extracellular matrix, and at least eight genetically distinct collagen types have been detected in ocular tissues. For example, type I collagen is the predominant component in the cornea and sclera.1,2 Type II collagen is seen in the adult vitreous, and is synthesized by developing neural retinal cells3 and the corneal epithelium.4 Type III collagen is synthesized by developing corneal endothelial cells and fibroblasts obtained from healing wounds of cornea.5,6 Type IV collagen molecule, which is one of the important constituents of the basement membrane,7 is seen in Bowman's layer,8 the interface between Descemet's membrane and the posterior corneal stroma, and the posterior corneal stroma.9 Types V, VI, and VII collagens are distributed in the corneal stroma and Bowman's layer.10–15 Type IX collagen is synthesized by the developing corneal epithelium.16

Type VIII collagen was originally isolated from the culture medium of bovine aortic endothelial cells and designated as endothelial cell (EC) collagen.17,18 It is also produced by corneal endothelial cells,19,20 astrocytoma cells,21 and a limited number of transformed and normal cellular populations.22 Characteristic features of type VIII collagen, such as a lack of interchain disulfide bonds, low affinity for a diethylamino ethyl (DEAE) cellulose column, susceptibility to pepsin, and the existence of a 50-kD pepsin resistant collagenous domain, have been reported;18–21 however, the structure of type VIII collagen remains controversial. Sage et al reported three different chains with molecular masses of 180-kD (EC1), 125-kD (EC2), and 100-kD (EC3).19 They postulated the cassette model, in which 180-kD (EC1) chains contain 50-kD pepsin-resistant collagenous domains that are connected in tandem by noncollagenous sequences. On the other hand, Benya and Padilla showed that the three major components of type VIII collagen, VIII-1, VIII-2, and VIII-3, exhibited apparent molecular weights of 194-kD, 124-kD, and 61-kD (EC3).19 They postulated the cassette model, in which 180-kD (EC1) chains contain 50-kD pepsin-resistant collagenous domains that are connected in tandem by noncollagenous sequences. On the other hand, Benya and Padilla showed that the three major components of type VIII collagen, VIII-1, VIII-2, and VIII-3, exhibited apparent molecular weights of 194-kD, 124-kD, and 61-kD, respectively.20 They suggested an alternate model in which type VIII collagen would be composed of a homotrimer with a 61-kD chain of VIII-3. Yamaguchi et al isolated and sequenced cDNA clones that encode a 60-kD collagenous chain that they designated as the α1(VIII) chain of type VIII collagen.23 The triple-helical domain of

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this polypeptide comprises 454 amino acid residues with nontriple helical globular domains of 117 and 173 residues at the amino and carboxyl ends, respectively. This finding supported the short, triple-helical structure model proposed by Benya and Padilla.\(^{20}\)

In our previous study, we raised monoclonal antibodies against a homogenate of bovine Descemet's membranes.\(^{24}\) The antibodies recognized a 64-kD collagenous peptide and the 50-kD pepsin-resistant fragment in both a corneal endothelial cell culture and in Descemet's membrane in vivo, that are related to type VIII collagen; however, the antibodies showed no immunologic cross-reactivity with the 125-kD peptide (EC2). Kapoor et al reported that monoclonal antibodies to 125-kD (EC2) collagen showed no reaction with a 60-kD collagenous peptide from a corneal endothelial cell culture.\(^{25}\) These data suggest that type VIII collagen might include two distinct species of collagenous polypeptides.

Although several laboratories reported the immunohistochemical localization of type VIII collagen using monoclonal antibodies to EC2 or polyclonal antibodies to 50-kD collagenous peptides that contain at least two polypeptides,\(^{23-28}\) the tissue distribution of the \(\alpha_1\) (VIII) chain of type VIII collagen should be elucidated. There has been no report of the type VIII collagen tissue distribution in human eyes. We describe the tissue distribution of type VIII collagen in human adult and fetal eyes, determined using monoclonal antibodies to the 50-kD collagenous peptide that was shown to have identical protein sequences to the \(\alpha_1\) (VIII) collagen cDNA.

**Material and Methods**

**Preparation of Anti-Collagen Antibodies and type VIII Collagen**

To prepare anti-type VIII collagen antibody, the monoclonal antibodies against bovine Descemet's membrane were obtained. Finally, two clones (6A2 and 9H3) reacted with type VIII collagen (50-kD-B) from bovine Descemet's membrane (Fig. 1), and were used for further immunohistochemical studies.\(^{24}\)

Type VIII collagen was solubilized from bovine Descemet's membranes by limited pepsin digestion and purified. Characterization of pepsin-resistant 50-kD collagen (50-kD-A, 50-kD-B) as type VIII collagen was reported elsewhere.\(^{24}\)

**Gel Electrophoresis and Western Blotting Analysis**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Tris-glycine buffer system on 6% polyacrylamide gels, then stained with Coomassie brilliant blue.\(^{8}\) Western blotting analyses using anti-type VIII collagen anti-bodies were done according to the methods reported previously.\(^{8,24}\)

**Immunohistochemistry**

Human adult eyes, 32, 48, and 78 yr of age, were obtained at autopsy and the central cornea was used for keratoplasty. Human fetal eyes, 16, 20, and 27 weeks of gestation, were obtained at the time of spontaneous abortion or intrapartum death. There was no history of significant ocular disease or glaucoma. The eyes were enucleated and immediately frozen in dry ice–aceton and then stored at \(-70^\circ\) C. Frozen, 5–10-\(\mu\)m thick sections were treated with cold acetone for 5 min. Nonspecific reaction was blocked by overnight incubation in normal goat serum at 4° C. The tissue sections were incubated with antibodies to type VIII or type IV collagen, diluted 1:500 with phosphate-buffered saline (PBS) in a moist chamber for 2 hr at room temperature, and then rinsed with several
changes of PBS. The sections were incubated with biotinylated anti-mouse IgG for 30 min. After they were rinsed with PBS, the sections were incubated with avidin–biotinylated horseradish peroxidase complex (Vectors, Burlingame, CA) for 1 hr at room temperature. After they were rinsed with several changes of PBS, they were placed in 0.02% 3,3'diaminobenzidine in 50 mM Tris-HCl buffer (pH 7.5) for 20 min for increasing staining intensity. Then, they were immersed in the same buffer containing 0.005% H2O2 for 5 min.29 Counterstaining was performed with hematoxylin for 15 sec. As a negative control for the primary antibody, normal mouse serum, diluted 1:500, was used.

Results

Characterization of Anti-type VIII Collagen Antibodies

Two 50-kD collagenous fragments (50-kD-A and 50-kD-B) were obtained on high-performance liquid chromatography (HPLC) (Fig. 1, lanes 1–3). Western blotting analysis showed that both monoclonal clones (9H3 and 6A2) only reacted with the 50-kD-B collagen; no reaction with the 50-kD-A collagen was seen (Fig. 1, lanes 2–5). The 50-kD-B collagen was separated by SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After staining was done with Coomassie brilliant blue, the lower band of 50-kD-B collagen, which reacted with the monoclonal antibody, was cut out. Sequencing was then performed with a protein sequencer, ABI 477A/120A (Applied Biosystems, Foster City, CA). The amino-terminal 12 amino acids of the lower band material of 50-kD-B collagen were the same as those of the collagenous portion (COL1) of the α1(VIII) chain predicted from the α1(VIII) chain cDNA sequences reported by Yamaguchi et al23 (submitted for publication). From these results, we concluded that our anti-type VIII collagen monoclonal antibody recognized the α1(VIII) chain of type VIII collagen.

Immunohistochemical Distribution of type VIII Collagen in Human Adult Eyes

Table 1 shows the distribution of type VIII collagen tissue in human eyes.

In the cornea, the anti-type VIII collagen antibody stained the anterior one-third of Descemet's membrane in a linear fashion (Fig. 2); however, the corneal stroma and Bowman's layer did not react with the antibody. In sections taken from the area of the limbus, type VIII collagen was distributed in sclera, trabecular meshwork, and walls of Schlemm's canal, and the corneoscleral junction could be clearly identified (Fig. 3A). The staining pattern of type VIII collagen in the sclera and ciliary body was different from that in type IV collagen (Fig. 3B).

In sections of the equatorial retina, choroid, and sclera, the anti-type VIII collagen antibody reacted with the sclera in a fibrous fashion (Fig. 5A). Bruch's membrane of the choroid was clearly stained in a linear fashion (Fig. 5C, arrow) and the choroidal stroma showed a faint reaction (Fig. 5C). However, the retina was not stained with the anti-type VIII collagen antibody (Fig. 5A, C).

In sections of the optic nerve, the anti-type VIII collagen antibody stained the cribriform plates of the lamina cribrosa, the dural sheath of the optic nerve (Fig. 4A, arrow), and the intima of the central retinal artery (Fig. 4A, CRA). The staining pattern of type VIII collagen in the central retinal artery was similar to that of type IV collagen, one of the important structural elements of the basement membrane (Fig. 4B). On the other hand, the basement membrane of other blood vessels and the pial septa were not stained with the anti-type VIII collagen antibody. Longitudinal (Fig. 6A, B, C) and cross-sections (Fig. 6D, E, F) of lamina cribrosa studied at higher magnifications showed that anti-type VIII collagen antibody stained the cribriform plates in a fine fibrous fashion (Fig. 6A, D) that was distinct from the pattern of anti-type IV collagen antibody (Fig. 6B, E).

Type VIII Collagen Tissue Distribution in Human Fetal Eyes

Significant differences in the localization of type VIII collagen between adult and fetal eyes were found

### Table 1. Distribution of type VIII collagen in human adult and fetal eyes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Adult (32–78 yr of age)</th>
<th>Fetus (16–27 gestational weeks)</th>
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<tbody>
<tr>
<td>Cornea</td>
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<td>Bowman's layer</td>
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<tr>
<td>Stroma</td>
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<td>Descemet's membrane</td>
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<tr>
<td>Trabecular meshwork</td>
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<td>Sclera</td>
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<tr>
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<td>Iris</td>
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<td>Bruch's membrane</td>
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<tr>
<td>Stroma</td>
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<td>Optic nerve</td>
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<tr>
<td>Cribriform plates</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Central retinal artery</td>
<td>+</td>
<td>ND</td>
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ND, not determined.
Fig. 2. Adult cornea stained with the anti-type VIII collagen antibody. The anterior one-third of Descemet's membrane (De) was linearly stained; however, the corneal stroma (St) showed no reaction with the antibody. Bar = 100 μm.

Fig. 3. Adult eye stained with the anti-type VIII collagen antibody (A) and the anti-type IV collagen antibody (B). Descemet's membrane (A, De), the sclera (A, S), and the trabecular meshwork (A, TM) were stained with the anti-type VIII collagen antibody; however, the corneal stroma (A, St) and ciliary body (A, CB) showed no reaction with the anti-type VIII collagen antibody. The corneoscleral junction was clearly identified (A, arrowhead). Bar = 500 μm.

Fig. 4. Longitudinal sections of the adult optic nerve stained with the anti-type VIII collagen antibody (A) and the anti-type IV collagen antibody (B). Type VIII collagen was distributed in the cribiform plates (A, CP), the dural sheath of the optic nerve (A, arrow), and the central retinal artery (A, CRA). The linear staining of type VIII collagen in the central retinal artery (A, CRA) is similar to that of type IV collagen (B, CRA). Bar = 500 μm.
in the sclera. Anteriorly, the sclera terminates in the limbus. Posteriorly, it ends at the optic nerve. In fetal eyes, the posterior sclera was strongly stained by the anti-type VIII collagen antibody (Fig. 7A, B). However, the positive reaction gradually decreased (Fig. 7B), and in the equatorial position, it eventually disappeared (Fig. 7C). The cribriform plates of the lamina cribrosa were not stained by the antibody (Fig. 7A). In the anterior eye section, only the trabecular meshwork and Descemet's membrane showed reaction with the anti-type VIII collagen antibody (Fig. 7D). No differences were seen in the pattern of staining among the 16-, 20-, and 27-week-old fetal sclera and trabecular meshwork. We did not observe positive staining of the presumptive Bruch's membrane in fetal eyes (Fig. 7).

Discussion

Immunohistochemical staining using a monoclonal antibody to type VIII collagen showed that type VIII collagen was distributed in Descemet's membrane of the cornea, the trabecular meshwork, the...
Fig. 6. Longitudinal (A, B, C) and cross-sections (D, E, F) of the lamina cribrosa at higher magnification stained with the anti-type VIII collagen antibody (A, D), the anti-type IV collagen antibody (B, E), and normal mouse serum (C, F). The cribriform plates (CP) of the lamina cribrosa were stained with anti-type VIII collagen antibody in a fine fibrous fashion (A, D). On the other hand, anti-type IV collagen antibody stained the margin of the cribriform plates (CP) (B, E). In the postlaminar portion (A, B, C; post L), anti-type IV collagen antibody stained the edge of the pial septa (B, arrow), however, anti-type VIII collagen antibody had no reaction with the pial septa (A). Bar = 100 μm.
Fig. 7. Fetal eye (20 weeks of gestation) stained with the anti-type VIII collagen antibody (A–D) and normal mouse serum (E). In the sclera, the posterior lesion was strongly stained by the antibody (A, B; S). The positive reaction gradually decreased (B) and eventually disappeared in the equatorial position (C). In the anterior section, the antibody showed reaction with Descemet's membrane (D, De) and the trabecular meshwork (D, TM), but not with the corneal stroma (D, St). In longitudinal sections of the optic nerve, the dural sheath was stained with the antibody (A, arrow); however, no reactions with the cribriform plates were observed. The positions of the sclera tissues, shown in Figures A–E, were indicated in the schema of the eye. R, retina. Bar = 250 μm.

walls of Schlemm's canal, Bruch's membrane, the choroidal stroma, the sclera, the cribiform plates of optic nerve, and intima of the central retinal artery, in a linear or fibrous fashion, in the human adult eyes. In fetal eyes (16–27 weeks of gestation), however, we did not detect type VIII collagen in Bruch's membrane, the cribiform plates of the lamina cribrosa, or the equatorial sclera.

Our previous studies on bovine eyes showed that an anti-type VIII collagen antibody reacted with Descemet's membrane, fine fibers within the corneal stroma, the sclera, the choroid, and the optic nerve.
sheath and its septum. On immunoelectron microscopy, the antibody labeled the hexagonal lattice of Descemet's membrane. In the human eye, however, there are several differences of the type VIII collagen tissue distribution from that in the bovine eye. We did not detect a positive reaction in the corneal stroma or optic nerve septum, and only the anterior one-third of Descemet's membrane reacted with the antibody. This finding is consistent with the fact that the adult human Descemet's membrane has a hexagonal lattice in its anterior one-third.

The intima of the central retinal artery was linearly stained with the anti-type VIII collagen antibody just like with the anti-type IV collagen antibody. We examined other tissues, such as kidneys, livers, spleens, lungs, and brains, and tried to unmask the epitope with pepsin, however, type VIII collagen was not found in the blood vessels of these organs. Despite its original purification from aortic endothelial cells, the monoclonal antibody to type VIII collagen did not recognize vascular tissues. Kittelberger et al. reported that the affinity-purified anti-type VIII collagen (50-kD collagen) polyclonal antibody stained the subendothelial region of the sheep aorta and the carotid artery, especially after pretreatment with pepsin. Sage and Iruela-Arispe reported the immunohistochemical reaction of embryonic heart and placental capillaries with the anti-type VIII collagen (50-kD collagen) polyclonal antibody. As shown in Figure 1, however, the 50-kD collagen was composed of at least two different peptides (two 50-kD bands separated on SDS-PAGE, and 50-kD-A and 50-kD-B peaks on HPLC). With a protein sequence identical to that of the α1(VIII) chain cDNA reported by Yamaguchi et al., the lower band material of 50-kD-B collagen, which reacted with our monoclonal antibody, was identified as the collagenous portion of the α1(VIII) chain (submitted for publication). Our more recent studies involving a monospecific antibody to 50-kD-A collagen showed that the distribution of 50-kD-A collagen was distinct from that of 50-kD-B collagen (the α1 chain of type VIII collagen) in certain tissues (unpublished data). These data indicate that 50-kD collagen, which was considered to be type VIII collagen, might comprise more than two collagen species. So far, the only known blood vessel that definitely has type VIII collagen in its walls is the central retinal artery in the adult eyes. We do not know why only this vessel has type VIII collagen. The microdistribution of type VIII collagen in the central retinal artery, in terms of its relationship to the basement membrane and vascular endothelial cells, should be elucidated.

Our studies showed that in addition to intraocular tissues, type VIII collagen is widely distributed in dense connective tissues, such as the capsules of the liver, kidneys, adrenals and lungs, the Achilles tendon, and periodontal and perivertebral ligaments (submitted for publication). These portions need strengthening against external pressure. Therefore, together with other extracellular matrix components, collagen may help to strengthen the structures of these organs.

The α1 chain of type VIII collagen shows strong structural similarity to type X collagen which is developmentally regulated. We tried to clarify the developmental changes in type VIII collagen in human fetal eyes. Significant differences in type VIII collagen expression between adult and fetal eyes exist in the sclera; however, no differences in the pattern of staining among 16-, 20-, and 27-week-old fetal sclera were seen. Precise developmental changes of type VIII collagen distribution in the fetal and infantile equatorial sclera should be elucidated.

Collagen polymorphism in the normal trabecular meshwork and lamina cribrosa was studied in several laboratories. Types I, III, V, and VI collagen were distributed in the cribriform plates, and type IV collagen was localized in the margin of the cribriform plates. Types I, III, IV, and V collagen were distributed in the trabecular meshwork. In this report, the distribution of type VIII collagen in the trabecular meshwork from the early gestational period (at least 16 weeks of gestation) and the cribriform plates of the lamina cribrosa in adult eyes was shown. Although the function of type VIII collagen in these tissues is unknown, it may play an important structural role, as do some other collagens, in the facilitation of the flow of aqueous through the trabecular meshwork and the support of the optic nerve fibers through the wall of the eye ball. Alterations in type VIII collagen may result in structural changes in these tissues and concomitant changes in function.

Key words: collagen, type VIII collagen, human eyes, cornea, Descemet's membrane

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