Immunolocalization of Type VI Collagen in Developing and Healing Rabbit Cornea

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We have localized type VI collagen in normal developing and corneal scar tissue. Indirect immunofluorescence showed that type VI collagen was distributed throughout the normal stroma and most of the scar. No fluorescence was detected along the posterior margin of the scar and in a retrocorneal membrane continuous with the scar. Since the corneal endothelium in rabbits contributes to the formation of scar tissue and retrocorneal membrane, our observations suggest that the endothelium does not synthesize type VI collagen. Indirect immunoelectron microscopy showed that type VI collagen was located abundantly between collagen fibrils as fine filamentous structures containing beads with a periodicity of 100 nm, consistent with published observations of other tissues. Because these filaments are more prominent when stained with ruthenium red, and predigestion of tissue with Chondroitinase ABC enhances binding of monoclonal antibody to type VI collagen, proteoglycans probably are associated with this collagen in the cornea. Ultrastructural observations supported by previous biochemical analyses show that the proportion of type VI collagen to fibrillar collagen is smaller in scar tissue compared with fetal cornea. The abundance of type VI collagen and its distribution and association with proteoglycans in rabbit corneal tissues suggest that this macromolecule plays a role in the tensile strength and transparency of the stroma. Invest Ophthalmol Vis Sci 31:1096-1102, 1990

Collagen fibers are responsible for the functional integrity of tissues such as bone, tendon, and cornea and contribute to the structural framework of most organs. There are 12 different types of collagens,1,2 and the biosynthesis and molecular structures for many types have been described.3

A collagenous protein now termed collagen type VI was reported originally in 1976.4 Type VI collagen is a dumbbell-shaped molecule with two large globular domains linked by a short, collagenous triple helix.5,6 Monomers assemble into dimers, which in turn make tetramers by lateral association.7 Finally, tetramers are assembled end-to-end with overlapping between outer segments to produce a thin filament. Appearing in the electron microscope as beaded filaments with a characteristic periodicity of 100 nm,8,9 type VI collagen has been detected in many organs.10-13 Although its function is unknown, recent studies have suggested that it plays a role in anchoring basal-lamina-containing organs within connective tissues and in restricting movement of collagen fibrils relative to each other.14

Type VI collagen, described in human and avian corneas, is abundant in the stroma and present as filaments between collagen fibrils similar to other tissues.15-17 Recent studies have shown that rabbit neonate corneas and scar tissue from adult corneas deposit and synthesize type VI collagen during organ culture.18 The function and tissue distribution of this collagen in developing and wound-healing corneas, however, are unknown. In the current study we show that type VI collagen is located between the collagen fibrils of corneal stroma, forming an extensive filamentous network during development and wound healing of adult rabbit corneas. Furthermore, analyses of the ultrastructural distribution and relative abundance of type VI collagen in these tissues provide clues to its function in the cornea.

Materials and Methods

Tissue Preparation

All procedures involving animals were performed in compliance with the ARVO Resolution on the Use of Animals in Research and with National Institutes of Health 85-23, Guiding Principles in the Care and Use of Animals. Normal adult rabbits weighing approximately 2.5 kg were anesthetized with an intravenous injection of sodium pentobarbital and topical application of proparacaine drops to each eye before the corneas were wounded. A 2-mm diameter, full-
thickness wound was made in one eye each of four rabbits and allowed to heal for 2 weeks. Four rabbits with 2-week-old wounds, one pregnant albino rabbit carrying 24-day-old fetuses, two 2-week-old rabbits, and four normal adult rabbits were sacrificed with an overdose of sodium pentobarbital. Two fetuses were sacrificed by decapitation. Fresh whole fetal eyes and corneas were placed in OCT compound (Tissue-Tek; Ames Division Miles Laboratories, Elkhart, IN) and frozen at -20°C. Cryostat transverse corneal sections, 6 and 12 μm, were mounted on gelatin-coated microscope slides.

**Indirect Immunofluorescence Microscopy**

The 6-μm sections from 24-day-old fetuses, rabbits with 2-week-old wounds, and normal adult rabbits were washed for 10 min in three changes of phosphate-buffered saline (PBS), pH 7.2, containing 1% bovine serum albumin (BSA) at room temperature, and then incubated for 2 hr with undiluted conditioned medium containing monoclonal antibody 3G7 or 1G8 specific to human type VI collagen (a gift of Dr. Eva Engvall, La Jolla, CA). After two 10-min washes in PBS containing 1% BSA, antibody binding was detected by incubating the sections with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Zymed Laboratories, South San Francisco, CA) for 2 hr and photographed by fluorescence microscopy.

**Indirect Immunoelectron Microscopy**

The 12-μm tissue sections from 24-day-old fetuses and rabbits with 2-week-old wounds were rinsed in three changes in PBS for 30 min at 4°C, and then digested with 2.5 U/ml Chondroitinase ABC (ICN ImmunoBiologicals, Lisle, IL) in 0.05 M Tris HCl buffer, pH 8.0, for 3 hr at room temperature. Following incubation with monoclonal antibody 1G8, specific to human type VI collagen, for 10-15 hr at 4°C, the samples were washed four times in PBS for 2 hr at 4°C. Tissues were incubated in gold-conjugated goat anti-mouse IgG (5 nm in diameter; Janssen Life Science Products, Piscataway, NJ) overnight at 4°C, followed by an extensive rinse in PBS. The tissues were rinsed briefly in 0.1 M cacodylate buffer, pH 7.4; fixed in a freshly prepared mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 hr at 4°C; rinsed in 0.1 M cacodylate buffer; and postfixed in 1% buffered OsO4 containing 400 ppm ruthenium red for 1 hr, and dehydrated in ethanol without a buffer wash.

Control sections for immunofluorescence and immunoelectron microscopy were exposed to the same staining sequence, except that the primary antibody was replaced by PBS or nonimmunized mouse serum.

**Results**

**Indirect Immunofluorescence Microscopy**

Immunofluorescence analysis showed that type VI collagen was distributed throughout the stroma corresponding to stromal lamellae in the 24-day-old fetal and normal adult corneas (Figs. 1, 2). In addition, a meshwork pattern of fluorescence was seen in the anterior stroma beneath the epithelium in fetal cornea (Fig. 1). No significant fluorescence was detected in the epithelium, Descemet's membrane, or endothelium.

![Fig. 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933151/) Fluorescence micrograph of 24-day-old fetal cornea reacted with monoclonal antibody 3G7. Arrowheads point to meshwork pattern in anterior stroma. EP, epithelium; ST, stroma (X285).
Fig. 2. Fluorescence micrograph of normal adult cornea reacted with monoclonal antibody 3G7. No significant fluorescence was detected in epithelium (arrows, top), Descemet’s membrane, or endothelium (arrowheads, bottom). (X285)

Fig. 3. Fluorescence micrographs of 2-week-old (a) anterior scar (SC) and (b) middle scar (SC), both reacted with monoclonal antibody 1G8. The scar appears as a dense meshwork of fluorescence. Normal stroma (ST) adjacent to scar shows intense fluorescence. (c) Fluorescence micrograph of posterior region of scar (SC). (d) Phase-contrast microscopy of (c). Asterisk, same region of posterior scar as indicated by asterisk in (c), showing weak fluorescence. (X285)

Fig. 4. Fluorescence micrograph of Descemet’s membrane (DC) and retrocorneal membrane (RM) below the normal posterior stroma (ST) adjacent to scar reacted with monoclonal antibody 3G7. Marked fluorescence was detected in normal stroma, whereas no fluorescence was seen associated with Descemet’s membrane and retrocorneal membrane. (X285)

with the 2-week-old wound (Fig. 3a, b), similar to that seen in the fetal anterior stroma. Posterior regions of scar tissue showed weak fluorescence (Fig. 3c, d). In one case, a retrocorneal membrane, continuous with the 2-mm scar, showed no fluorescence (Fig. 4). All controls from each sample had negative staining of fluorescence (not shown).

Indirect Immunoelectron Microscopy

In the 24-day-old fetus, the anterior stroma beneath the epithelium contained a few randomly arranged collagen fibrils, whereas the posterior stroma had abundant collagen fibrils aligned parallel to each other (Fig. 5a, b). Binding of type VI collagen antibody in the tissue was improved when samples were predigested with Chondroitinase ABC before incubating the tissue with primary antibody (Fig. 5a-d). If tissue was not predigested with Chondroitinase ABC, fewer immunogold particles were detected (Fig. 5e). A filamentous structure between collagen fibrils was more apparent in tissues stained with ruthenium red (Fig. 5e). However, the pattern of ruthenium red staining was markedly different in later stages of de-
Fig. 5. Immunocytochemistry of type VI collagen in 24-day-old fetal cornea. (a) Anterior stroma and (b) posterior stroma of fetal cornea were predigested with Chondroitinase ABC before incubating tissue with primary antibody (bars = 0.3 μm). (c, d) High magnification of anterior and posterior stroma. Labels display 100-nm periodicity (arrowheads). (e) Posterior stroma of fetus without predigestion with Chondroitinase ABC. Filamentous structure of type VI collagen (arrowheads) is apparent when tissue is stained with ruthenium red after incubation with antibodies. Sparse staining with immunolabeled gold (arrows). (Bars = 100 nm)

Development (Fig. 6a, b). Immunogold-labeled beaded filaments with 100-nm periodicity were generally parallel to longitudinal sections of banded collagen fibrils in the anterior (Fig. 5c) and posterior (Fig. 5d) stroma. These labeled filaments were localized between collagen fibrils. In addition, this orientation and distribution of type VI collagen were also detected in 2-week-old neonate and adult corneas (Fig. 6a, b).

The sparsely distributed, randomly arranged collagen fibrils in 2-week-old scar tissue resembled those in the anterior stroma of fetal cornea, but collagen fibrils in scar tissue seemed more abundant than in the anterior stroma of 24-day-old fetal cornea (Figs.
Fig. 6. Immunocytochemistry of type VI collagen in (a) 2-week-old rabbit cornea and (b) adult cornea stained with ruthenium red but without predigestion with Chondroitinase ABC. Globular structures (arrowheads) are distributed along thin, flexible filaments (arrows) between collagen fibrils. (Bars = 100 nm)

5a, 7). Furthermore, the relative proportion of type VI collagen to fibrillar collagen seemed smaller in scar tissue compared with the fetal cornea. The distribution and orientation of type VI collagen in scar were also consistent with those in the developing cornea (Figs. 5c, d; 6a, b; 7 inset). The relative proportion of type VI collagen to fibrillar collagen was much smaller in scar tissue compared with the fetal cornea (Figs. 5a, 7).

No immunogold-labeled type VI collagen was detected in the posterior region of the scar and retrocorneal membrane (Fig. 8a, b) but was present in the middle region of the scar (Fig. 7). This observation was consistent with our immunofluorescence analysis (Figs. 3, 4).

Fig. 7. Immunocytochemistry of type VI collagen in middle region of 2-week-old scar tissue treated with Chondroitinase ABC (bar = 0.3 μm). Inset: Labels display 100-nm periodicity (arrowheads) (×137,000).

Discussion

In this study we observed that type VI collagen was present in the normal developing rabbit cornea and scar tissue from adult cornea. In addition, we showed that type VI collagen filaments in corneal tissues were located between collagen fibrils as fine, beaded structures. The presence of this macromolecule in the cornea and its structure are similar to those described in other species.8,9,14

In the current study, absence of type VI collagen staining in the posterior margin of scar tissue, which is produced by the endothelium,22,23 suggests that the endothelium does not synthesize this collagen. This is consistent with previous suggestions17 but is contrary to an observation of bovine endothelium cultures.24

Although scar tissue resembles the anterior stroma of fetal cornea ultrastructurally, the abundance of type VI collagen to banded collagen fibrils in fetal stroma seemed less evident in scar tissue. Although we did not analyze the collagen ratio quantitatively in electron microscopy, our observations agree with results from previous biochemical studies indicating a marked decrease in the ratio of type VI to type I (fibrillar) collagen in scar compared with the developing cornea.18 Networks of type VI collagen filaments may contribute to the mechanical strength of connective tissue by binding collagen fibrils to each other and restricting lateral displacement of fibrils.14 If this is true, decreased quantities of type VI collagen in the corneal scar may result in decreased tensile strength of this tissue, although this hypothesis remains to be tested.

We have shown that the labeled type VI filaments are found in increasing numbers when tissues are
predigested with Chondroitinase ABC. In addition, filamentous structures stained with ruthenium red appear more prominent when tissues are not predigested with Chondroitinase ABC. Similar observations have been made in cartilage and skin. Ruthenium red staining is not evident in later stages of development probably because of changes in the proportion and charge density of proteoglycans. Recent immunocytochemical studies in our laboratory have confirmed that proteoglycans associate with type VI collagen (unpublished observations). Corneal proteoglycans are hydrophilic, space-filling macromolecules located between collagen fibrils. Because corneal transparency depends on proper spacing of collagen fibrils, and type VI collagen filaments are located between collagen fibrils, these filaments through their interaction with proteoglycans may play an important role in maintaining the collagen fibril spacing so necessary for transparency.

**Key words:** type VI collagen, immunolocalization, cornea, corneal development, scar

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**References**