Heterogeneity of Collagens in Rabbit Cornea: Type III Collagen

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Whole neonate rabbit corneas and adult corneas containing 2-week-old scars were incubated in the presence of [14C] glycine. Radiolabeled collagen extracted from the corneas and scar tissue were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and fluorography to determine the types and relative quantity of collagen polypeptides present and synthesized by these tissues. In addition to other collagen types, type III was found in both neonate cornea and scar tissue from adult cornea, albeit in relatively small quantities. Type III collagen in normal cornea was associated with the residue after pepsin digestion and formic acid extraction of the tissue, and the same type of collagen was extracted from scar tissue after similar treatment. Type III collagen-specific monoclonal antibody bound to developing normal corneas and healing adult tissue sections, as determined by immunofluorescence. Antibody binding was localized to the endothelium and growing Descemet's membrane in fetal and neonate corneas, and restricted to the most posterior region of the corneal scar tissue. Although monoclonal antibody to keratan sulfate, used as a marker for stromal fibroblasts, bound to most of the scar tissue, the antibody failed to bind to the posterior scar tissue positive for type III collagen. We conclude that endothelial cells from fetal and neonate rabbit cornea and endothelium-derived fibroblasts from healing wounds of adult cornea synthesize and deposit type III collagen. Moreover, this collagen appears to be incorporated into the growing Descemet's membrane of normal corneas and narrow posterior portion of the scar tissue. Although type III collagen remains associated with normal adult Descemet's membrane, subsequent disappearance of type III collagen-specific fluorescence in healing wounds may result from breakdown or masking of this collagen during scar remodelling. Invest Ophthalmol Vis Sci 29:767–775, 1988

Numerous immunohistochemical and biochemical studies have been conducted to determine the presence and location of type III collagen in corneal tissues. The apparently conflicting results may be a result of different techniques, animal species or tissue sources. For example, biochemical analyses of bovine corneas have shown the presence of type III collagen. Some studies claimed that type III collagen is found in calf as well as adult bovine corneas and constitutes up to 20% of the total collagen in the tissue.1,2 Others reported type III collagen in fetal bovine corneas as a minor component but not detectable in adult tissue.3,4 Type III collagen was also found in adult human corneas by biochemical and immunohistochemical techniques,5,6 but other studies showed type III collagen only in young fetal human corneas and not in later developmental stages or in adult tissue.7 Similarly, type III collagen was immunohistochemically detected in young chick embryonic corneas, associated with the corneal epithelium, but not in later stages.8,9 Although type III collagen in human keratoconus corneal tissue has been shown,6,10 other immunohistochemical studies failed to demonstrate this collagen in uninvolved keratoconus tissue.11 Instead these investigators showed evidence of type III collagen at the site of host-graft wound healing in a perforating keratoplasty. Biochemical analysis of pepsin-solubilized adult rabbit corneal collagens failed to detect type III collagen12; unfortunately, the insoluble tissue remaining was not examined for type III collagen.

Conflicting results of tissue culture studies have compounded the problem. To elucidate the source and regulation of collagen synthesis in corneal tissues, tissue cultures of stromal and endothelial cells were developed. Early studies indicated that type III collagen is not synthesized by human or bovine corneal stromal cells in culture,13–15 but later reports
showed that chick and human stromal fibroblasts can synthesize this collagen. Although no evidence was found for type III collagen synthesis in cultures of rabbit corneal endothelium, the bovine endothelium synthesizes this collagen as a major component.

Since the retrocorneal fibrous membrane and the posterior portion of the corneal scar in rabbits are believed to originate from corneal endothelial cells, analysis of collagen synthesized in these two tissues is of interest. Two independent studies show that the major collagen component synthesized by the retrocorneal fibrous membrane is type I collagen. The studies differed in the detection of minor quantities of type III, IV and V collagens. As far as we are aware, the collagens in the posterior portion of the corneal scar have not been studied.

It appears from this survey that at least some corneal tissues in most animal species can synthesize type III collagen. The age or physiological state of the cornea when this collagen is synthesized, the cell type(s) responsible for the synthesis and deposition, and the turnover of this type of collagen within the organ are still debatable.

Recent studies in our laboratory have shown that we can increase the sensitivity of detection of in vivo-type patterns of collagen synthesis by incubating whole rabbit corneas in vitro in the presence of radiolabeled precursors. Moreover, the availability of monoclonal antibodies specific to type III collagen has enabled us to test corneal tissues for the presence of this collagen. In the present study, we used these tools to determine the presence, relative rate of synthesis and localization of type III collagen in normal developing rabbit corneas and in healing adult corneal scars. The results indicate that type III collagen is synthesized during development of the rabbit cornea and formation of scar tissue in the adult. The quantity and location of type III collagen deposition suggest that this protein is synthesized by corneal endothelial cells.

Materials and Methods

Corneal Organ Culture, Collagen Extraction, and Analysis

The corneal organ culture technique, collagen extraction and method of analysis are described in detail in the preceding paper. Briefly, adult rabbit corneas with 2-week-old scars and neonate corneas were incubated in the presence of [3H]glycine, and the extracts and residues were run on sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE), without mercaptoethanol reduction. Part way through the run, electrophoresis was interrupted to incubate certain lanes with 5% 2-mercaptoethanol for 45 min before continuing electrophoresis. Gels were stained with Coomassie blue and subjected to fluorography.

Immunohistochemistry

Monoclonal antibody to human type III collagen was used to localize the deposition of this collagen in developing normal rabbit corneas and adult corneal scar tissue. Western blot analysis of type III collagen from fetal rabbit skin indicated that the antibody against the human collagen also recognizes the same type of collagen from rabbit (not shown). Inhibition ELISA indicate the antibody does not cross-react with type I or IV collagen. In the present study experiments were conducted to determine if type VIII collagen competes with type III for antibody binding. Type VIII collagen from bovine Descemet's membrane was a gift of Dr. Helene Sage (University of Washington, Seattle, WA). Inhibition ELISA was performed essentially as described in a previous study.

Six adult rabbits, about 2.5 kg each, were anesthetized and their corneas wounded as previously described. Wounds in four rabbits were allowed to heal for 4, 6 or 8 weeks. Wounds in two rabbits were allowed to heal for 2 weeks. These adult rabbits, fetal rabbits 21, 23 and 25 gestation days old, and neonate rabbits 4 days, 8 days, 2, 4 and 6 weeks old were killed with an overdose of sodium pentobarbital and their corneas processed for cryostat sectioning at 6 μm. All sections were placed on formalin-fixed, gelatin-coated slides and air-dried before storing in a −20°C freezer. Prior to incubation with antibody, frozen slides were placed in a 37°C oven overnight. The sections were then incubated with conditioned medium containing anti-type III monoclonal antibody overnight at room temperature. Sections were washed in phosphate-buffered saline (PBS) containing 3% bovine serum albumin and incubated in fluorescein isothiocyanate-conjugated (FITC) goat antimouse IgG, 20 μg/ml, for 30 min at room temperature. Controls were incubated with PBS or preimmune mouse IgG, 20 μg/ml, as the primary antibody. Sections were viewed and photographed with a Zeiss epifluorescence photomicroscope. Exposure times, film development and printing were kept constant. Because absence of fluorescence is not evidence for absence of antigen in immunohistochemical studies, we attempted to unmask type III collagen by treating cryostat sections of rabbit cornea with either 0.1 M acetic acid, 4M quanidine hydrochloride, or PBS, each for 30 min at room temperature. The samples were then washed extensively with...
buffer and processed for immunohistochemistry as described above.

Cryostat sections of 2-week-old scar tissue were incubated overnight with monoclonal antibody specific for keratan sulfate glycosaminoglycan (gift of Dr. James Funderburgh, Kansas State University, Manhattan, KS). Adjacent sections from the same scar tissue were incubated with monoclonal antibody against type III collagen as described above. FITC-conjugated goat anti-mouse IgG was then layered on all sections for 30 min, washed in buffer, and prepared for immunofluorescence. Photomicrographs were taken of the posterior portion of the scar by fluorescence or phase microscopy, or both, to localize the fluorescence attributed to the binding of each antibody. All procedures adhered to the ARVO Resolution on the Use of Animals in Research.

![Fig. 2. Fluorography of polyacrylamide gel electrophoresis shown in Figure 1. For identification of sample of each lane, see Figure 1.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933142/)

**Fig. 1.** Type III collagen from 3-day-old rabbit neonate corneas and 2-week-old scars in adult corneas. Pepsin-digested, formic acid-extracted and salt-fractionated collagen, was subjected to SDS-6% polyacrylamide slab gel electrophoresis. Part way through the run the current was interrupted to incubate certain lanes with 5% 2-mercaptoethanol before continuing electrophoresis. Gels were stained with Coomassie blue. Lanes 1-4, collagen standards, a. a1[III], b. a1[V], c. a1[II], d. SC3. Lanes 5-8, collagen polypeptides from scar tissues. Lanes 9-12, collagen polypeptides from neonate corneas. Lanes 5, 6, 9 and 10, unreduced polypeptides. Lanes 7, 8, 11 and 12, delayed reduction with mercaptoethanol. Lanes 5, 6, 9 and 10 contain the tissue fraction solubilized by pepsin. Lanes 6, 8, 10 and 12 contain residue remaining after pepsin digestion. Arrows point to position of type III collagen polypeptide [a1(III)] barely visible in this reproduction. Only the pepsin-soluble fraction of scar tissue and the pepsin-insoluble fraction of neonate corneas show a hint of type III collagen polypeptide.

**Results**

**Collagen Analysis**

Analysis of the polypeptides migrating into polyacrylamide gels after electrophoresis of pepsin-soluble and -insoluble fractions from scar tissue and neonate cornea indicates the presence of type I and V collagens in unreduced samples (Fig. 1). Delayed reduction with mercaptoethanol during an SDS-PAGE of collagen polypeptides allows us to determine if type III collagen is present in neonate and scar tissues (Fig. 1). Arrows point to regions where alpha 1 (III) polypeptide would be located. Although only a faint indication of this polypeptide is evident in Coomassie-blue stained gels (Fig. 1), the fluorograph of this gel clearly shows that radiolabeled material is associated with these bands (Fig. 2). Type III collagen polypeptide is evident only in the pepsin-soluble collagen fraction from scar tissue, and the pepsin-insoluble fraction from neonate tissue. Figure 2, lane 6 shows a skewed band positioned close to that located...
Fig. 3. Immunofluorescence of a cryostat section of cornea from normal adult rabbit. (a) Anterior portion of cornea showing epithelium (E) and stroma (S). (b) Posterior portion of cornea. Fluorescence is located at the interface of Descemet's membrane (D) and the stroma. The anterior chamber is at the lower right hand corner of the photograph. (c) Preimmune mouse IgG for control. Bar = 20 μm.

Fig. 4. Immunofluorescence of cryostat sections from fetal and neonate corneas. (a) 21-day, (b) 23-day, and (c) 25-day fetal corneas. (d) 4-day, (e) 8-day, (f) 2-week, (g) 4-week, and (h) 6-week neonate corneas. (i) Preimmune mouse IgG control for 21-day fetal cornea. Small arrows point to the endothelium of the cornea. The stromal cells show nonspecific fluorescence. Arrowheads and brackets point to the growing Descemet's membrane. Large arrow in d points to a capillary near the corneal limbus. Bar = 20 μm.
with type III polypeptide. This is due to the diffusion of mercaptoethanol from the well in lane 7 during reduction of type III polypeptides.

**Immunohistochemistry**

Immunohistochemical studies with monoclonal antibody to type III collagen show intense fluorescence in normal adult rabbit cornea, although restricted to the interface between Descemet's membrane and the stroma (Fig. 3). Attempts to unmask type III collagen in other regions of the tissue with acetic acid, guanidine hydrochloride or PBS failed (not shown). Corneal endothelial cells bind the antibody as early as 21 days of gestation and continue to fluoresce throughout fetal and neonate development at least up to 6 weeks after birth (Fig. 4). A faint line of fluorescence was seen between the endothelium and stroma on the fourth day after birth. This line became more intense and moved further away from the endothelium as development of the Descemet’s membrane progressed. At the corneal limbus, capillaries fluoresced, consistent with the known distribution of type III collagen (Fig. 4d). When preimmune mouse IgG was substituted for monoclonal antibody, the endothelium and Descemet’s membrane did not fluoresce (Fig. 4i).

At 1 week after wounding, the endothelial cells immediately adjacent to the wound and extending along the posterior wound margin above the anterior chamber showed discontinuous but intense fluorescence (Fig. 5a). Despite extensive growth of scar tissue between the first and second week of healing, fluorescence was still restricted to the posterior region of the tissue (Fig. 5b). In all four corneal specimens of 2-week-old scars, the fluorescence never appeared beyond the most posterior portion of the scar. In two of the specimens, fluorescence was present only along the posterior margin of the scar (not shown).

In the 4-, 6-, and 8-week-old scars the fluorescence was still restricted to the posterior margin and became less intense in progressively older scars (Fig. 6). Along the central posterior margin of the scar, the fluorescence often appeared as discontinuous lines, perhaps associated with cells in the scar (Fig. 6b, e, h).

The fluorescence in healing corneas suggested that endothelial cells migrate into the wound to participate in the deposition of scar tissue. The extent of this invasion, however, seemed to be limited to the most posterior portion of the scar, adjacent to the anterior chamber. To test this hypothesis, monoclonal antibody specific to keratan sulfate glycosaminoglycans, the major carbohydrate polymer component of the keratan sulfate proteoglycan synthesized by stromal cells, was used as an immunohistochemical marker of stromal cell involvement in scar tissue. In adjacent sections, monoclonal antibody against type III collagen was used as a marker for endothelial cells. The results show keratan sulfate-specific fluorescence in
Fig. 6. Immunofluorescence of cryostat sections from corneal scars. (a, b, c) 4-week-old scar. (d, e, f) 6-week-old scar. (g, h, i) 8-week-old scar. (a, d, g) Edge of the scars show the curled, cut surface of Descemet’s membranes (D). (b, e, h) The central posterior region of the scars. (c, f, i) Preimmune mouse IgG controls for each scar. Bar = 20 μm.

the 2-week-old scar throughout the tissue except for a narrow posterior margin adjacent to the anterior chamber (Fig. 7b, d). Type III collagen-specific fluorescence, however, was restricted to the posterior margin and was quite distinct from the scar region having keratan sulfate-positive fluorescence (Fig. 7a, c).

A test for the cross-reactivity of the antibody with type VIII collagen was carried out by inhibition ELISA. Antibody activity was inhibited by type III collagen but was unaffected by type VIII (Fig. 8).

Discussion

Genetically distinct alpha I(III) chain was established by an analysis of its cyanogen bromide peptides. Subsequently, the chain composition [alpha I(III)]3 was determined and the polypeptide chains were shown to be extensively cross-linked through cysteine-derived disulfide bonds which are located in the helical, pepsin-resistant portions of the native molecule. This unique crosslinking among the fibrous banded collagens allowed us to separate small
quantities of type III polypeptides from other collagens using delayed reduction of disulfide bridges during polyacrylamide gel electrophoresis. In this way, we have shown that type III collagen is synthesized in normal developing cornea and healing adult cornea. Moreover, the paucity of this collagen in these tissues, determined by the relative density of Coomassie blue-stained polypeptides on polyacrylamide gels, and the restriction of type III collagen-specific fluorescence to a local region in the tissues, suggest that this collagen is synthesized by the endothelial cells, which deposit it within the growing Descemet's membrane and the most posterior portion of the new scar tissue during wound healing. Finally, although type III collagen-specific fluorescence remains associated with Descemet's membrane in adult cornea, the gradual disappearance of fluorescence in healing tissue may be due to the subsequent breakdown of this collagen during remodelling. An alternative explanation may be masking of the epitope recognized by the monoclonal antibody. Although our observations are consistent with other studies that show a transient increase of type III collagen during development or healing,30-32 future experiments are needed to determine the fate of type III collagen.

The ubiquitous distribution of type III collagen in different organs makes it unlikely that it is exclusively involved in the maintenance of tissue linings, as has
been suggested. Since type III collagen is one of the earliest collagens to be laid down in areas of tissue growth and regeneration, one may speculate that the intermolecular disulfide crosslinks of this molecule could provide mechanical stability during the initial formation of scar tissue. It has been suggested, however, that these intermolecular bonds place constraints on the packing arrangement during fibrillogenesis, and may also be more readily degraded during the rapid growth and remodelling of the scar tissue. Moreover, type III collagen contains a collagenase cleavage region that is unusually susceptible to this enzyme. Unfortunately, no definitive evidence is available which clearly defines the role(s) of type III collagen in tissues.

Previous studies have implicated the endothelial cells as contributors to the deposition of scar tissue in corneal wounds. Our results are consistent with these studies but not with their suggestion that fibroblast-like cells, derived from the endothelium, occupy and deposit a substantial portion of the corneal scar tissue. Instead, our findings suggest that the extent of endothelial involvement is minimal and restricted to the posterior margin of the scar tissue adjacent to the anterior chamber. Knowledge of the type and quantity of cells involved in scar tissue is important for our understanding of the cellular source of macromolecules in healing wounds. Because types I, V and VI collagen are the major extracellular matrix macromolecules synthesized and deposited in the corneal wound, our results suggest that these collagens are synthesized by stromal-derived fibroblasts with minimal contributions from endothelial cells.

As noted in the preceding paper and consistent with the findings in this report, our organ culture system allowed the tissues to continue expressing the pattern of collagen synthesis found in vivo. Thus, the high rate of synthesis of type V collagen and the apparent synthesis of type I trimer in scar tissue during organ culture are consistent with the pattern of synthesis of these collagens in vivo. Moreover, the presence of type III collagen in growing corneal tissues, shown by immunohistochemistry, suggests new synthesis and deposition of this macromolecule, although limited in distribution and quantity within the tissues. In support of this fluorescence data, [¹⁴C] glycine incorporation into type III collagen in organ-cultured corneas suggests that this collagen is synthesized and deposited at a low rate relative to the other collagens within the tissues.

Type III collagen was found in pepsin-soluble fractions of corneal scar tissue and pepsin-insoluble fractions of normal neonate corneas. This difference in type III collagen solubility may be due to the fact that normal corneas incorporate the macromolecule into the highly insoluble Descemet's membrane, although this collagen does not appear to be present within a discrete membrane structure during early phases of healing. Although a more definitive explanation must await immunoelectronmicroscopy of this collagen in healing corneas, the recovery of radiolabeled type III collagen from distinct tissue fractions is consistent with continued normal synthesis and deposition of this macromolecule during organ culture.

Key words: cornea, immunohistochemistry, wound healing, development, type III collagen

References


