Heterogeneity of Collagens in Rabbit Cornea: Type VI Collagen

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Normal adult rabbit corneas were digested with 5% pepsin and their collagens extracted with acetic acid. Collagen extracts were fractionated by differential salt precipitation. The 2.5 M NaCl fraction was then redissolved with tris buffer and precipitated with sodium acetate. The precipitate contained a high-molecular-weight disulfide-bonded aggregate which, upon reduction with mercaptoethanol, was converted into three distinct polypeptides having molecular weights between 45 and 66 Kd. These physical characteristics, together with the susceptibility of these polypeptides to collagenase and their amino acid composition, identified the high molecular weight aggregate as type VI collagen. Corneas from neonate rabbits and adult corneas containing 2-week-old scars were organ cultured in the presence of [14C]glycine to incorporate radiolabel into collagen. Tissues were digested with 0.02% pepsin and their collagens extracted with formic acid. The total radioactivity of the extracts and tissue residues was determined before the collagens were separated by SDS-polyacrylamide slab gel electrophoresis. Radioactive collagen polypeptides bands were then stained with Coomassie blue, processed for fluorography, and analyzed by densitometry. The results show that: (1) type VI collagen is synthesized by neonate corneas and healing adult corneas; (2) it is not readily solubilized from either corneal tissue by 0.02% pepsin digestion and formic acid extraction; and (3) the proportion of type VI collagen deposited in scar tissue is markedly lower than that found in neonate corneas. Invest Ophthalmol Vis Sci 29:760–766, 1988

The predominant banded collagen fibrils of mammalian corneal stroma are composed of type I collagen.1–4 Other than a single report, there is no evidence indicating the presence of type II collagen in mammalian corneas.5 Although there is as yet some controversy as to the distribution and quantity of type III collagen within the cornea, it appears to be associated with neonate and healing adult tissues.3,4,6–10 Type IV collagen seems to be restricted to the basement membrane of the corneal epithelium and the Descemet's membrane of normal corneas.1,12 Type V collagen constitutes about 10% of the normal stromal collagen.13,14 The relative quantity of type V collagen within the stroma of fetal and healing cornea is markedly different from the adult, suggesting that this collagen may play a role in the morphogenesis of the corneal stroma. Although we do not know the ultrastructural distribution of type V collagen in mammalian corneas, recent reports have indicated that chick stromal collagen fibrils are copolymers of type I and V collagens.15 Type VI collagen is also located in the mammalian stroma and because of its unusual structure, it may be associated with fine filaments between the banded collagen fibrils.16–19 Anchoring fibrils associated with the basement membrane of the corneal epithelium and extending into the stroma have been identified with type VII collagen.20,21 Finally, type VIII collagen, originally designated "endothelial collagen,"22 has been described as a prominent collagen in bovine and rabbit Descemet's membranes.23,24 Because each corneal collagen has a unique structure, distinctive chemical properties, a specific ultrastructural distribution within the tissue, and different and varying abundance during morphogenesis, these macromolecules must play an important role in normal corneal development and healing.

Our studies have shown that developing neonate corneas and corneal scar tissue from adult corneas synthesize and deposit mostly types I and V collagens.13 The limitations in our analyses were partially due to the paucity of tissue and the low specific activity of radiolabeled collagen synthesized after in vivo labeling of the corneal proteins. To increase the specific activity, we developed an organ culture method that maintains the whole cornea deturgescant and ultrastructurally normal during incubation of the tis-
sues with radiolabeled precursors. This we believe also maintains a pattern of macromolecular synthesis similar to that in vivo.

In the present study, we show that normal adult rabbit corneas contain type VI collagen. Moreover, rabbit neonate corneas and scar tissue from adult corneas deposit types I, V and VI collagen and synthesize these collagens during organ culture. Semi-quantitative analyses of the collagens deposited and synthesized in these growing corneal tissues indicate that the relative quantity and solubility of collagens differ among different types of collagens as well as between tissues. These differences have significance to our understanding of scar formation.

Material and Methods

Type VI Collagen

Adult rabbit corneas, freed of Descemet’s membrane, endothelium and epithelium were homogenized in 0.1 M acetic acid with a blender at high speed for 1 min followed by limited pepsin digestion at 4°C for 24 hr with a 5% pepsin/tissue weight ratio in 0.1 M acetic acid. The digest was centrifuged at 60,000 rpm for 10 min and the supernate directly fractionated by differential salt precipitation at 0.7 M, 1.2 M and 2.5 M NaCl concentration in acid. The 2.5 M precipitate fraction was redissolved in 0.05 M 0.1 M acetic acid. The digest was centrifuged at 60,000 rpm for 10 min and the supernate eventually pooled with the first extract. The supernate was again treated with pepsin and centrifuged, and the supernate was treated with divalent cations and dialyzed against 0.05 M sodium acetate, pH 4.8, to precipitate the proteins. These precipitated proteins could not penetrate an SDS-8% polyacrylamide slab gel during electrophoresis. Disulfide reduction with 5% 2-mercaptoethanol and electrophoresis of the digestion products, generated by the high-molecular-weight disulfide-bonded aggregate (HMWDA), into small, Coomassie blue-stained proteins, which penetrated the polyacrylamide gel.

The HMWDA was hydrolyzed with 6 N HCl under nitrogen at 105°C for 24 hr to determine the amino acid composition with a Beckman amino acid analyzer, model 121 (Fullerton, CA). Susceptibility of HMWDA to bacterial collagenase was determined by incubating 100 μg of the HMWDA with 50 μg of Clostridium histolyticum collagenase, protease free (Cal Biochem, San Diego, CA), in 200 μl of 0.05 M Tris-HCl buffer, pH 7.5, containing 10 mM CaCl2 at 37°C for 2 hr. Controls contained HMWDA in appropriate buffer and incubated at the same temperature for the same amount of time. The hydrolysates and controls were then reduced with mercaptoethanol and electrophoresed in an SDS-8% polyacrylamide slab gel.

Corneal Organ Culture, Collagen Extraction, and Analysis

Rabbits weighing about 2.5 kg were anesthetized with intravenous injections of sodium pentobarbital and topical application of proparacaine drops to each eye before wounding the corneas. A 2 mm diameter, full-thickness wound was made in each eye of six rabbits and allowed to heal for 2 weeks. The wounded rabbits and three newborn rabbits, approximately 3 days old, were killed with an overdose of sodium pentobarbital. The whole cornea of each eye, with an attached 1–2 mm scleral rim, was removed and placed in ice-cold culture medium. One scarrd cornea was not used due to complications during healing. Our culture medium contained Hank’s basal medium, Coon’s concentrate, newborn calf serum and the antibiotics, penicillin and streptomycin. The medium was modified by the addition of 2% (w/v) chondroitin sulfate (Sigma, St. Louis, MO). Corneas were washed with 4 ml of fresh medium before incubation in Linbro tissue culture multi-well plates with a well capacity of 7.5 ml (Flow Labs, McLean, VA). Enough fresh medium was used to cover the explant with the corneal endothelium up. The medium contained 75 μCi of [14C] glycine per ml (New England Nuclear, Boston, MA) and the tissues were incubated at 37°C in a 5% CO2/air mixture for 18 hr. These procedures have been shown to maintain the rabbit cornea deturgescence and ultrastructurally normal for 48 hr.

After incubation, explants were washed in fresh nonradioactive medium, and the scleral rims removed from the corneas. The total neonate cornea and only the scar tissue of the adult cornea were weighed, frozen in liquid nitrogen and pulverized. Powdered corneas were digested with 0.02% (w/w) pepsin/tissue weight in 0.5 M formic acid at 4°C for 18 hr. Digests were centrifuged for 15 min at 12,000 rpm and the supernate decanted. The tissue residues were again treated with pepsin and centrifuged, and the supernate eventually pooled with the first extract. Collagens solubilized after the first and second pepsin treatments were termed first and second pepsin-soluble fractions, respectively. Similarly, the undigested residues after each enzyme treatment were referred to as the first and second pepsin-insoluble fractions. The first pepsin-soluble fractions, containing collagens, were precipitated with 1.2 M NaCl (pepsin-soluble, 1.2 M NaCl precipitate fraction). The second pepsin-soluble fraction was too scant to precipitate with salt. Precipitated collagens were re-
Results

Type VI Collagen

The HMWDA obtained in the 2.5 M precipitate fraction from pepsin-solubilized proteins of adult rabbit cornea were electrophoresed on an SDS-polyacrylamide gel with and without mercaptoethanol reduction (Fig. 1). Other than the intense Coomassie blue-stained material in the stacking gel, containing unreduced protein, no proteins were detected in the running gel when mercaptoethanol was not present. Upon reduction, three distinct bands, SC1, SC2 and SC3, migrated between 45 and 66 Kd. The predominant corneal collagens, types I and V, migrated much more slowly than the short-chain polypeptides.

Susceptibility of these polypeptides to bacterial collagenase indicated the presence of collagen-like proteins in the HMWDA (Fig. 2). Moreover, the dissoluted in 0.5 M formic acid, dialyzed against the solvent and lyophilized. Collagen extracts and insoluble tissues were reduced with 5% 2-mercaptoethanol and run on a 6% SDS-polyacrylamide slab gel. Proteins were stained with Coomassie blue and the relative quantity of collagen protein bands was measured by densitometry. Gels were then treated with Enhance (New England Nuclear, Boston, MA) and exposed to X-ray film for fluorography. Developed film was also subjected to densitometry to determine the relative quantity of [14C] incorporation into collagens. All procedures adhered to the ARVO Resolution on the Use of Animals in Research.

Fig. 1. Reduced and unreduced SDS-8% polyacrylamide slab gel electrophoresis of rabbit cornea type VI collagen stained with Coomassie blue. Pepsin-digested, acetic acid-extracted collagens from adult rabbit corneas were subjected to salt fractionation to obtain a 2.5 M NaCl-precipitated material which was then dissolved and reprecipitated at pH 4.8. The precipitate was called high-molecular-weight disulfide-bonded aggregate (HMWDA). Lane 1, HMWDA proteins remain in the stacking gel. Lane 2, HMWDA proteins, reduced with 2-mercaptoethanol, migrate into the gel. Three bands are identified as SCI, SC2 and SC3. Lane 3, type V collagen. Lane 4, type I collagen. Lane 5, molecular weight markers (myosin, 200 Kd; B-galactosidase, 116 Kd; phosphorylase b, 93 Kd; bovine serum albumin, 66 Kd; ovalbumin, 45 Kd; carbonic anhydrase, 31 Kd).

Fig. 2. SDS-8% polyacrylamide slab gel electrophoresis of mercaptoethanol-reduced bacterial collagenase-digested rabbit cornea type VI collagen stained with Coomassie blue. Lane 1, bacterial collagenase. Lane 2, HMWDA (see Fig. 1) after digestion with bacterial collagenase. Lane 3, Control HMWDA incubated with collagenase buffer. Lane 4, Molecular weight markers, as in Figure 1, with addition of lysosome, 14 Kd.
amino acid composition of the HMWDA indicated the presence of amino acids common to collagen, such as hydroxyproline, hydroxylysine and high levels of glycine and proline (Table 1). The high content of hydroxylysine, leucine, valine and isoleucine, and low content of alanine were similar to those in types IV and V collagen. However, a high cysteine content provided the most characteristic property of type VI collagen.

Corneal Organ Culture

Radiolabeled glycine incorporated into fractions of collagen extracts and residues from corneal tissue were measured to compare collagen synthesis in neonate and scar tissue. Although total incorporation of label was greater in neonate tissue, the proportions of label distributed among the soluble and insoluble fractions of neonate and scar tissue were similar (Table 2). No marked changes in the counts of the pepsin-insoluble fraction were noted between the first and second pepsin digestion. Conversely, the number of counts recovered from the tissue after the second pepsin digestion was only 10% of the first pepsin-insoluble tissue fraction. These observations indicate that most of the soluble collagens are extracted with two digestions of pepsin. No further digestions or extractions were performed on these tissues. The weights of the tissues before and after collagen extraction are consistent with our published findings, indicating that scar tissue collagen is less soluble than neonate corneal collagen.13

Polyacrylamide slab gel electrophoresis of mercaptoethanol-reduced pepsin-soluble and -insoluble collagen fractions from neonate corneas and 2-week-old scars shows the presence of types I, V and VI collagen polypeptides (Fig. 3). In neonate corneas, type I collagen was the predominant protein extracted with pepsin treatment, leaving most of the types V and VI in the insoluble fraction (Fig. 3, lanes 3 and 6). Very little collagen could be detected in the first pepsin-soluble, 1.2 M NaCl supernate, and in second pepsin-insoluble fractions (lanes 4 and 5). In scar, types I and V collagens were present primarily in the pepsin-soluble, 1.2 M NaCl precipitate fraction (Fig. 3, lane 7). As in the neonate cornea, type VI collagen in scar remained in the insoluble fraction (lane 9). Delayed mercaptoethanol reduction of these collagen fractions indicated the presence of type III collagen in these tissues (not shown; see following paper10).

The relative optical densities of collagen polypeptides in Coomassie blue-stained SDS-PAGE are indicative of the relative quantities of different collagens present in the tissues (Table 3). The a1:a2 ratios greater than 2.00 indicated contamination of type I collagen polypeptides with types II, III, V or type I trimer polypeptides. This was particularly evident in the insoluble fractions. The quantity of type V collagen relative to the type I collagen in the soluble and insoluble fractions, and the high ratio of type VI collagen in scar in comparison to neonate cornea, suggested marked differences in the molecular organization of these collagens in these tissues (Table 3).

To determine the relative proportion of collagens in the tissues, we added the densities of certain polypeptides.
The relative densities of silver deposits on autoradiographs of SDS-PAGE slabs containing radiolabeled collagen polypeptides are suggestive of the relative rates of collagen synthesis, the processing of the various collagens, or both in neonate and scar tissue (Table 4). As in the Coomassie blue-stained gel, the high ratio of \(a_1:a_2\) in scar tissue indicated synthesis of other types of collagen such as types II, III and I trimers, or contamination with \(a_2(V)\) polypeptide. The ratio of \(a_1(V):a_2(I)\) pepsin-soluble collagen polypeptides from scar is markedly different from that in neonate corneas. In contrast to deposition, no marked difference was found in the incorporation of label into type VI collagen in the insoluble collagen fractions (Tables 3, 4). However, the ratio of the autoradiographic band densities of type VI polypeptides to the sum of the band densities of \(a_2(I)\) from soluble and insoluble fractions of scar and neonate tissues are 0.376 and 0.606, respectively.

Discussion

Our studies confirm previous observations showing that rabbit corneal collagen is readily solubilized from normal tissue after pepsin digestion in comparison to scar tissue. Moreover, the results are consistent with the presence of marked amounts of type I trimer in scar tissue. That our corneal organ culture method maintains the pattern of collagen synthesis previously shown by in vivo labeling is evidenced by the higher proportion of type V collagen synthesized in scar tissue than in normal neonate corneas regardless of labeling method. Radiolabeling of collagen during organ culture has made it possible to see evidence of type VI and, as we show in the following paper, type III collagen deposition in corneal tissues, which has not previously been detected by the in vivo labeling method.

Our results show that the incorporation of \([^{14}C]\) glycine into collagen fractions from neonate corneas

### Table 3. Semiquantitative analysis of collagen present in neonate cornea and corneal scar

<table>
<thead>
<tr>
<th>Tissues and fractions</th>
<th>(a_1(I))</th>
<th>(a_2(I))</th>
<th>(a_2(V))</th>
<th>(VI^+)</th>
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<td>Corneal scar</td>
<td>2.54</td>
<td>0.70</td>
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<td>Pepsin-soluble†</td>
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<td>Pepsin-insoluble</td>
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<tr>
<td>Neonate</td>
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<td>3.60</td>
<td>1.69</td>
<td></td>
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<tr>
<td>Pepsin-soluble‡</td>
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<tr>
<td>Pepsin-insoluble</td>
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* Based on the relative band densities in Coomassie blue-stained SDS-PAGE of collagen polypeptides from 3-day neonate corneas and 2-week-old corneal scar tissue. Type I collagen polypeptides are calculated from \(a_1(I)\), \(a_2(I)\), and \(1/4\beta(I)\).† The sum of SC1, SC2 and SC3 polypeptides of type VI collagen.
‡ Pepsin-soluble, NaCl-precipitable collagen.
and scar tissue is about the same when expressed as cpm per mg wet weight of tissue. The tissues in these experiments, however, contain large quantities of water and extracellular matrix. A more meaningful way of expressing the data is to relate them to the number of cells. The DNA values from similar tissues in previous studies in our laboratory were 50 and 19 μg DNA for the neonate and scar tissues, respectively.27,30 Our calculations show that the incorporation of label per μg DNA into collagen fractions from scar tissue is about half of that from neonate corneas, suggesting that the rate of collagen synthesis is markedly lower in the scar.

Previous studies from our laboratory have indicated many morphological and biochemical similarities between healing adult cornea and the normal development of fetal and neonate cornea.27,30 If we consider a 2-week-old corneal scar as temporally equivalent to the first 14 days of fetal corneal development (27 days of gestation), scar tissue has deposited a greater amount of collagen per cell than has the 27-day fetal cornea.30 It is not until the first week of birth that the neonate cornea accumulates collagen at a rate comparable to the 2-week-old scar. These neonate corneal cells are morphologically well differentiated into keratocytes, albeit they contain enormous quantities of rough endoplasmic reticulum. Thus, the deposition of collagen in healing wounds is high during early stages of healing, whereas normal developing cornea accumulates collagen at a high rate only after cell morphological differentiation is almost complete. Since the present data suggest that scar tissue synthesizes collagen at a lower rate than that of the neonate cornea, assuming the specific activity of the precursor pools were the same and incorporations were constant during incubation, the similarity in accumulation of collagen in these tissues must be due to differences in the turnover of this macromolecule. Because the neonate cornea continuously remodels its extracellular matrix to accommodate growth, the collagen turnover is very high.31,32 The scar tissue, on the other hand, synthesizes collagen at a slower rate and remodels the scar gradually,13,33,34 indicating its collagen turnover is also gradual. Although our hypothesis is consistent with the results of this and previous studies, future studies are needed to test collagen turnover in these tissues more directly.

Type VI collagen has been extracted from numerous tissues and subjected to several analyses.16,35-38 Among the prominent features of this collagen are its abundance of interchain disulfide cross-links, which may be responsible for its resistance to bacterial collagenase.36,39 Its unique polymeric structure within the extracellular matrix, identifying it with microfibrillar structures,17,18,37; its unusual carbohydrate composition of mannose and fucose residues; and its large terminal nonhelical portions of the molecules in the completed structure, constituting over 50% of the molecular weight.40 Although structural details of type VI collagen are still not clear, chemical, ultrastructural and analytical ultracentrifugation studies have suggested that the monomer of type VI collagen, 110-145 Kd, is a 105-nm-long triple helix terminated by globular domains on each end. Monomers associate to form dimers with a lateral 30 nm stagger and align themselves in an antiparallel fashion. The outer segments of two parallel dimers are covalently linked to form tetramers. Finally, tetramers are assembled end-to-end with overlap between outer segments to form a microfibrillar structure.37,38,41

Although indirect immunofluorescence has demonstrated the presence of type VI collagen in many tissues and cell cultures,19,36,41,42 cartilage, basement membranes, elastin and cross-striated collagen fibrils do not appear to contain this collagen.16 The present results adds to the list of tissues containing type VI collagen and suggest that this collagen is deposited at a slower rate in corneal scars than in normal neonate tissue.

Current concepts of corneal transparency require that small distances between the collagen fibrils be maintained.43 If indeed type VI occupies the spaces between collagen fibrils,17,18,44 is it possible that the opacity of the scar is partially maintained by the space-filling type VI collagen? Clearly, further studies are required to determine the ultrastructural distribution of type VI collagen within scar tissue.

Key words: cornea, collagen, wound healing, development, type VI collagen

References


Table 4. Semiquantitative analysis of collagen synthesized in organ culture of neonate cornea and corneal scar*

<table>
<thead>
<tr>
<th>Tissues and fractions</th>
<th>a1(I)</th>
<th>a1(V)</th>
<th>V/I</th>
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<td>Corneal scar</td>
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<tr>
<td>Pepsin-soluble‡</td>
<td>2.52</td>
<td>0.76</td>
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<td>Pepsin-insoluble</td>
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<tr>
<td>Pepsin-soluble</td>
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<td>Pepsin-insoluble</td>
<td>1.98</td>
<td>1.18</td>
<td>1.35</td>
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</table>

* Based on the relative band densities in SDS-PAGE fluorographs of collagen polypeptides from 3-day neonate corneas and 2-week-old corneal scar tissue. Type I collagen polypeptides are calculated from a1(I) and a2(I) only.
† The sum of SCI, SC2 and SC3 polypeptides of type VI collagen.
‡ Pepsin-soluble, NaCl-precipitable collagen.


