Corneal Collagen Fibrils: Dissection With Specific Collagenases and Monoclonal Antibodies

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To investigate the relationship of collagen types I and V within corneal fibrils, and the collagenolytic mechanisms potentially involved in corneal development and remodeling, we have incubated cryostat sections of avian corneas with collagenases that specifically degrade collagen types I or V to digest selectively the collagen in situ. These preparations were then analyzed by immunofluorescence histochemistry and immunoelectron microscopy using anti-collagen type-specific monoclonal antibodies. Digestion of corneal sections with the type I-specific collagenase ("I'ase") exposed antigenically masked type V collagen, indicating that epitopes on type V collagen in heterotypic fibrils are inaccessible to the antibody due to their interaction with type I collagen. Sections digested with the type V collagen-degrading enzyme ("V'ase") showed no removal of type V collagen. However, after the fibrillar structure was disrupted by acetic acid treatment before enzyme digestion, the type V collagen was then degraded. Likewise, prior digestion of type I collagen by I'ase also rendered type V collagen susceptible to digestion by V'ase. These results suggest that the cleavage sites on type V collagen are buried within heterotypic fibrils and therefore inaccessible to the enzyme. They also document, for the first time, V'ase activity against type V collagen in situ. Electron microscopic observations of sections partially digested with the I'ase revealed many short striated fibrillar segments from which smaller filaments protrude. Both the striated regions and some of the filaments were labeled by an antibody against type I collagen; anti-type V antibody reacted preferentially with the thin filaments. Thus avian corneal fibrils contain type I collagen, in which filaments of type V collagen are embedded. Complete removal of the fibrillar stromal matrix in the course of normal or pathological remodeling requires at least two different collagenases acting in concert. Invest Ophthalmol Vis Sci 29:1125-1136, 1988

In previous immunofluorescence histochemical studies of connective tissues of embryonic chickens, we observed that epitopes on type V collagen in stromal matrices from a wide variety of ocular and extraocular tissues are normally unavailable to monoclonal antibodies that specifically recognize that collagen type. Reactivity of such matrices within tissue sections could be achieved by pretreatment of the sections with dilute acetic acid; treatments with a variety of neutral proteases and glycosaminoglycan-degrading enzymes failed to unmask the epitopes on type V.1 Since acetic acid interferes with acid-labile intramolecular crosslinks and causes disaggregation (swelling) of compact collagen fibrils (unpublished observations), we proposed that type V collagen was masked by its supramolecular fibrillar organization, perhaps due to an association with type I collagen, with which it might be coassembled into heterotypic fibrils. This hypothesis was tested in the embryonic corneal stroma using other methods to perturb fibrillar organization, such as: (1) experimental lathyrism, in which collagen fibrils deficient in aldehyde-derived crosslinks become disaggregated when cooled2-4; and (2) digestion with a mammalian collagenase which degrades type I collagen but not type V.5-8 As predicted by this hypothesis, both methods exposed the epitopes, as determined by immunofluorescence histochemistry.9,10 More recently, immunoelectron microscopic analysis of corneal sections from lathyritic embryos labeled with antibodies coupled to electron-dense markers have provided direct evidence that each 25 nm fibril in the avian corneal stroma is a heterotypic structure composed of molecules of type I and type V collagens.11,12

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The molecular arrangement of collagen types I and V within these fibrils, the mechanism of their coassembly, and the means by which such fibrils are degraded are unclear. For the fibrillar structure, a variety of models can be posed. For example, such fibrils in the corneal stroma might contain a central core of type V collagen insulated by type I; alternating concentric layers of collagen types I and V; intermixed molecules; or multiple microfibrillar units consisting of homogeneous aggregates of type I or V molecules, or of heterogeneous aggregates of both collagen types. The possibility even exists that only a portion of each type V collagen molecule is embedded within a fibril, the remainder being exposed on the surface.

For degradation of heterotypic fibrils, one can envision the need for several different collagenases, since it is known that type V collagen is refractory to degradation with the enzyme that degrades type I collagen.7,8

In the present study, we have investigated the relationship of these two collagens within fibrils, and the degradation of such fibrils, by performing digestions with two different collagen type-specific collagenases and analyzing the results using a combination of immunofluorescence histochemistry and immunoelectron microscopy. The two enzymes we have employed are a rabbit macrophage collagenase (termed "I'ase") which degrades type I collagen in solution but not type V, and a metalloprotease (termed "V'ase") produced by cultured MDCK cells which we have recently characterized9 as degrading native type V collagen molecules in solution but having no detectable activity against native type I molecules.7,15-18 This latter enzyme is also a gelatinase which digests the α-chains of denatured interstitial collagens. We have purified these enzymes, and have used them as tools to dissect the structure of collagen fibrils in corneal tissue sections. By immunofluorescence analyses, we have found that type V collagen in situ is not normally available for digestion by the V'ase, but can be degraded only after it has been exposed by prior treatment with dilute acetic acid or active I'ase. Our electron microscopic analyses revealed that, following partial digestion of corneal fibrils with I'ase, antibodies against type V collagen preferentially bind to thin filaments that appear to be derived from more intact fibrillar regions. These results directly confirm the heterotypic nature of avian corneal fibrils, suggest that type V collagen is largely buried within the fibril, and indicate that there is no subpopulation of type I collagen molecules that itself is masked by type V. They also show that removal of corneal fibrils must involve the concerted activities of at least two collagenases.

Materials and Methods

Tissue

White leghorn chicken embryos (Spafas, Norwich, CT) were incubated at 37°C in a humid atmosphere. Embryos were removed at 14 days of incubation, and staged according to Hamburger and Hamilton.19 Corneas with adjacent sclera and lens were removed in Hanks BSS, and immersed in phosphate buffered saline (PBS) with 7% sucrose for 10 min. They were then embedded in Tissue Tek OCT (Miles Laboratories, Elkhart, IN) and frozen in liquid nitrogen.

Cryostat sections (8 μm) of such unfixed material were mounted on 12-spot glass slides (Shandon Scientific, Sewickley, PA) coated with polylysine (MW ~ 350,000, Sigma Chemical Co., St. Louis, MO), air-dried, and stored dessicated at -20°C until used. The investigations using animals, as described in this manuscript, conform to the ARVO Resolution on the Use of Animals in Research.

Enzymes

Type I (interstitial) collagenase (I'ase) that specifically degrades collagen type I but not type V was purified from serum-free supernatants of "in vivo" activated rabbit alveolar macrophages. Cultures were prepared as previously described,7 and the pooled supernatants concentrated 50-fold using a hollow-fiber filter apparatus (Amicon). The concentrated supernatants were subjected to DEAE chromatography to separate the collagenase from other metalloproteases as previously described13 using conditions under which the enzyme passes through the column unretarded. The unbound fraction which contained all of the collagenase activity against type I collagen was dialyzed against 0.05 M Tris-acetate, 5 mM CaCl2, pH 6.8 containing 0.05% (v/v) Brij. The sample was pumped onto a MonoS column (Pharmacia, Piscataway, NJ) which had been equilibrated with the same buffer using a Hewlett-Packard 1090 HPLC apparatus equipped with a diode array detector. The column was washed with 20 volumes of the above buffer and the bound proteins were eluted with a stepwise linear gradient to 0.5 M NaCl in the same buffer. The flow rate was 1 ml/min and the gradient was to 0.1 M NaCl at 10 min, 0.4 M NaCl at 80 min, and 0.5 M NaCl at 90 min. Under these conditions collagenase was eluted as a single peak with a retention time of 52 min. The enzyme was further purified by gel filtration using a Superose 12 column equilibrated with 0.05 M Tris-HCl, 0.05 M NaCl, 5 mM CaCl2, 0.05% Brij, pH 7.6 on the same apparatus. The enzyme activity eluted as a single homogenous peak on this column.
Activation of latent collagenase was not necessary as the enzyme totally autoactivated with purification.

Type V collagenase (V'ase) was purified from culture supernatants of canine kidney epithelial cells by affinity chromatography on gelatin-Sepharose. The bound enzyme was washed with 0.5 M NaCl in 0.05 M Tris-HCl pH 7.4 containing 5 mM CaCl₂ and eluted with 15% DMSO in the same buffer. The enzyme was characterized as a metalloprotease which would degrade native type V collagen, but showed no activity against native types I or IV collagen.

The enzymes were stored at -70°C until used. The V'ase was activated with 0.01 M 4-aminophenylmercuric acetate just before use. The I'ase was spontaneously active.

Samples of each enzyme were checked for typespecific collagenolytic activity in vitro by mixing 100 µg of chicken type I or type V collagen with 0, 2.5 µl, 5 µl, 10 µl or 20 µl of enzyme in a total volume of 150 µl of 0.05 M Tris-HCl buffer, pH 7.4, with 5 mM CaCl₂ and 0.02% NaN₃. The reaction mixtures were incubated at 36°C in a temperature-controlled heat block for 18-20 hr. They were analyzed by SDS polyacrylamide (7%) gel electrophoresis by the method of Laemmli.

Antibodies

Monoclonal antibodies against collagen types I (I-BA¹²), V (V-DH2 and V-AB12¹¹), and IV (IV-IA822) were produced and characterized as described previously. All antibodies recognize helical epitopes that are type-specific; none of these antibodies cross-reacts with any other known collagen. For immunofluorescence histochemistry, the antibodies were used as hybridoma culture supernatants. For immunoelectron microscopy, monoclonal IgG was employed (see below).

Enzyme Digestions

Slides containing unfixed 8 µm cryostat sections of 14-day embryonic anterior eyes were rinsed in Tris/NaCl buffer (50 mM Tris, 150 mM NaCl, pH 7.4). Unmasking of type V collagen in situ was then accomplished by pretreating sections in 0.2 M acetic acid for 20 min at room temperature. Control sections, in which type V collagen remained masked during subsequent enzyme digestions, were immersed in Tris/NaCl for 20 min. The slides were rinsed in Tris/NaCl x3, and the sections were incubated for 20 min with a drop of 0.1% BSA in Tris/NaCl containing 5 mM CaCl₂. This was removed by aspiration, and replaced with a drop (30-35 µl) of either I'ase or V'ase. Control sections received either enzyme inhibited with 25 mM EDTA, or buffer without enzyme. (Sections incubated with either control solution were identical, showing no degradation of any collagen type, as measured by immunofluorescence histochemistry.) Incubations were carried out in humid chambers for 6-74 hr. In experiments involving long incubations, enzyme solutions were replaced with fresh enzyme after about 48 hr of incubation. The course of collagen degradation in situ was monitored by indirect immunofluorescence histochemistry with the monoclonal antibodies.

Indirect Immunofluorescence Histochemistry

Cryostat sections of unfixed anterior eyes treated in various ways (±acetic acid; ±active collagenase(s)) were rinsed twice in Tris/NaCl and once in PBS. In some experiments the sections were then fixed in 4% paraformaldehyde in PBS for 15 min at 4°C, washed in PBS, and treated with 0.05% NaBH₄ in PBS for 1 hr at 4°C to quench free aldehydes. In other experiments, the fixation/quenching steps were omitted. (Essentially the same results were obtained from both the fixed and unfixed sections, except that the fluorescent signals observed in the unfixed sections were noticeably greater. In the experiments pictured in Figures 1, 4 and 5, fixation was not done.) Sections were incubated with 1% BSA/PBS for 20 min, and then reacted with a monoclonal anti-collagen antibody followed by a rhodamine-labeled goat antimouse second antibody (Cappel, Malvern, PA) as described previously. Sections were observed and photographed using a Nikon Fluophot microscope equipped for epifluorescence, as described previously.

Pre-Embedding Immunoelectron Microscopy

Cryostat sections treated with active I'ase or V'ase buffer for 6-49 hr were rinsed in Tris/NaCl and PBS, and then fixed with 4% paraformaldehyde in PBS for 15 min at 4°C. The sections were quenched with 0.05% NaBH₄ as described above, washed in PBS, and incubated with 1% BSA in PBS for 90 min at room temperature to block nonspecific binding of colloidal gold particles. The sections were then incubated overnight at 4°C with colloidal gold particles coated with monoclonal anti-collagen IgG. In some cases sections were pretreated with testicular hyaluronidase (type IV-S, Sigma Chemical Co.) before application of antibody-gold conjugates. Just before use, the antibody-gold complexes were diluted with PBS/1% BSA (1/20-1/50) to give a pale pink color on visual examination, and large aggregates were removed by centrifugation (9000g, 5 min). After reaction with antibody-gold, the sections were washed.
with four changes of PBS containing 0.05% Tween-20, once with PBS without Tween-20, and once with 0.1 M sodium cacodylate, pH 7.4. All washes were for 10-15 min, with gentle agitation. The sections were fixed for 1 hr with 4% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and post-fixed in 1.25% osmium tetroxide in 0.1 M cacodylate. Following dehydration in graded alcohols, the sections were infiltrated overnight with epon-araldite, covered with BEEM capsules filled with epon-araldite, and polymerized. Embedded sections were popped from the glass slide after briefly heating them on a hot plate.

Sections with a pale gold interference color were picked up onto copper grids and stained with 2% aqueous uranyl acetate and either 1% lead stain or 1% phosphotungstic acid, pH 3.2. Sections were observed and photographed with either a Philips 200 or CM-10 transmission electron microscope.

Preparation of Colloidal Gold-ANTIBODY Complexes

Monoclonal anti-collagen IgG was purified from ascites fluid or concentrated hybridoma culture supernatant by affinity chromatography on Protein A-agarose columns using the Affi-Gel System (Bio-Rad, Richmond, CA). Purified IgG was dialyzed into PBS with 0.05% sodium azide, and stored at 4°C until used.

Colloidal gold particles with diameters of 5 or 10 nm were obtained from Janssen Pharmaceuticals (Piscataway, NJ). They were coated with purified monoclonal IgG in 10 mM phosphate buffer at pH 8-9, as described. Concentrated gold-antibody complexes were stored at 4°C until used. Just before being applied to tissue sections, the antibody-gold colloids were diluted with 1% BSA in PBS, and aggregates were removed by centrifugation (9000g, 5 min).

Results

Digestion With Type I-Specific Collagenase ("I'ase")

The specificity of the I'ase used in these experiments for type I collagen in solution is demonstrated in Figure 1A. Collagen types V or I were mixed with increasing amounts of I'ase and incubated for 18 hr at 36°C. Visualization of the reaction mixtures after polyacrylamide gel electrophoresis showed that the characteristic α1(V) and α2(V) bands migrated as expected for intact α-chains, indicating that no degradation of the native type V collagen molecules had occurred. The enzymatic digestion of native type I collagen is demonstrated by the cleavage of the α1(I) and α2(I) chains into smaller fragments. The extent of type I collagen degradation was proportional to the amount of I’ase added.

This enzyme was applied to unfixed frozen sections of 14d embryonic corneas and incubated for 12-49 hr at 37°C. Controls received either buffer without enzyme or enzyme inhibited with EDTA. Sections were then reacted with anti-collagen monoclonal antibodies, and the bound antibody was visualized by indirect immunofluorescence. A representative experiment is shown in Figure 1B-G. Corneal sections incubated with buffer or inhibited enzyme exhibited type I collagen-specific immunofluorescence throughout the corneal stroma and Bowman's membrane (Fig. 1B). Type V collagen-specific fluorescence was largely confined to Bowman's membrane (arrows, Fig. 1C), a site in which type V collagen is normally available for antibody binding. Sections incubated with active I'ase for various time intervals showed a progressive loss of the type I collagen as demonstrated by a decrease in the fluorescence signal for this collagen (Fig. 1F, G). The disappearance of type I collagen-specific immunoreactivity always proceeded more slowly in the interface between the stroma and Descemet's membrane (asterisk, Fig. 1G). The decrease in type I collagen was mirrored by a corresponding increase throughout the cornea in detectable type V collagen immunoreactivity (Fig. 1D, E). Thus, as previously reported, epitopes on type V collagen in the corneal stroma become available for antibody binding as type I collagen is enzymatically removed. Treatment with I'ase did not affect the pattern of immunofluorescence elicited by antibodies against collagen type IV (not shown).

Fine Structure of Sections Partially Digested With I'ase

The structure of the corneal stromal type V collagen exposed by digestion with I'ase and identified by anti-collagen antibody-coated gold particles was examined by electron microscopy. For analysis of the structural relationship between collagen types I and V in corneal fibrils, sections partially digested (for 12-24 hr) with I'ase, containing both essentially intact (striated) segments along with variably degraded fibrillar regions, revealed considerably more information than did more completely digested preparations, which will not be described here. The observations pictured in Figures 2 and 3 are of sections digested for 24 hr, containing partially unmasked type V collagen along with abundant type I (see Fig. 1D, F). Control sections incubated with I'ase buffer contained the small-diameter (25 nm) striated fibrils characteristic of the avian corneal stroma. These were well labeled by antibody against type I.
Fig. 1. Digestion with macrophage collagenase (I'ase). (A) Activity against purified collagens in solution. Aliquots of type V collagen (the five lanes on the left) or type I collagen (the five right-hand lanes) were mixed with increasing amounts of active I'ase, and incubated for 18 hr at 36°C. Analysis of the reaction mixtures by SDS-PAGE showed persistent α1(V) and α2(V) bands in all the left-hand lanes, indicating that the enzyme did not degrade native type V collagen in solution. In contrast, increasing amounts of I'ase (lanes on the right) degraded progressively more type I collagen into smaller fragments. The band at the bottom of each lane represents BSA, a component of the reaction mixtures. (B-G) show unfixed sections of embryonic chicken cornea incubated with I'ase or buffer without enzyme at 37°C for 24 hr or 49 hr and then reacted with an antibody against collagen type I or V. Bound antibody was visualized by indirect immunofluorescence. Bar = 100 μm. (B) and (C) show control sections incubated with buffer without enzyme for 49 hr. A section reacted with an antibody against type I collagen bound antibody almost exclusively within Bowman’s membrane (arrows). In (C), a section reacted with an antibody against type V collagen bound antibody almost exclusively within Bowman’s membrane (arrows). (D-G) document the emergence of anti-type V collagen immunoreactivity (D, E) and the concomitant loss of anti-type I immunoreactivity (F, G) with increasing time of digestion with active I’ase. (D) and (F) show sections digested for 24 hr; the sections in (E) and (G) were digested for 49 hr.
Fig. 2. Fine structure of corneal stromal fibrils partially digested with l'ase. (A–E) show cryostat sections of corneal stroma incubated for 24 hr at 37°C with active l'ase or buffer without enzyme, and then reacted with 10 nm gold particles coupled to a monoclonal antibody against collagen types I, V, or IV. The sections were then fixed and processed for electron microscopy. Bars = 100 nm. (A) and (B) show control sections incubated with buffer before treatment with immunogold particles that recognize collagen type I (A) or type V (B). In (A), the characteristic 25 nm corneal collagen fibrils are decorated with type I collagen-specific antibody. In contrast, a similar section reacted for type V collagen (B) contains very little gold label within the corneal stroma. (C–E) show enzyme-digested sections reacted with antibody against collagen types I, IV, or V. These preparations contained a network of small filamentous strands, with diameters of 10 nm or less, along with thicker segments of more intact striated collagen fibrils. In some cases, the thinner filaments appear to be derived from a contiguous striated segment. (C) shows a partially-digested section labeled with an antibody against type I collagen. Gold particles are associated with both the more intact fibrillar segments as well as some of the thinner filaments that surround them. In enzyme-treated sections reacted for type IV collagen (D), the corneal stroma is devoid of gold particles. In (E), a section reacted with the type V collagen-specific antibody shows gold particles preferentially bound to the thinner strands liberated by the enzyme treatment. The thicker fibrillar segments are only lightly labeled by this antibody.

collagen (Fig. 2A) but not by antibody against type V collagen (Fig. 2B). After digestion with the enzyme, sections of corneal stroma showed a mixture of thin filamentous strands and regions of relatively intact striated fibrils, from which the thinner strands could sometimes be seen to emerge (Figs. 2C–E, 3). Antibody against type V collagen preferentially bound to the thin filaments which were only seen after hydrolysis with l'ase, and labeled striated regions only lightly (Fig. 2E and lower-power overview in Fig. 3). The
filaments in such preparations had diameters of 10 nm or less (Fig. 2C-E).

In contrast, type I collagen-specific antibodies labeled both the thicker striated regions and the filamentous strands (Fig. 2C). Antibody against type IV collagen decorated neither striated nor filamentous collagen in the stroma (Fig. 2D).

Digestion With Type V-Specific Collagenase (V'ase)

We then determined the conditions under which the V'ase could degrade type V collagen. The activity against purified type I and V collagen in solution is shown in Figure 4A. After 19 hr at 36°C, increasing amounts of enzyme degraded a progressively greater portion of the type V collagen. This is seen as a decrease in the amount of α1(V) and α2(V) chains, and an increasing amount of a limited number of smaller fragments. Type I collagen was unaffected by this enzyme.

Sections of 14-day corneas were incubated with V'ase at 37°C for various time intervals, reacted with anticollagen antibodies, and examined by indirect immunofluorescence histochemistry. A representative experiment is shown in Figure 4B-G. When sec-
change in type I collagen-specific immunoreactivity of such acid-treated sections caused no detectable membrane-stroma interface (asterisk, Fig. 4E), some type V collagen remained (see Particulars). Particularly in the vicinity of the Descemet's membrane-stroma interface (asterisk, Fig. 4E), some type V collagen remained (see Discussion). V'ase digestion of such acid-treated sections caused no detectable change in type I collagen-specific immunoreactivity (Fig. 4G) or in type IV collagen-specific immunoreactivity within the lens capsule, Descemet's membrane, and iris (not shown).

Sequential Digestion With I'ase and V'ase

We also examined whether type V collagen, when exposed by digestion of type I collagen with the I'ase, is degradable by the V'ase. Corneal sections were incubated with active I'ase until the immunoreactive type I collagen was removed (24-48 hr), as determined by indirect immunofluorescence on parallel sections. Those then were incubated with V'ase and monitored at various time intervals by indirect immunofluorescence with the anticollagen antibodies. Controls received buffer without enzyme. The endpoint of such an experiment is shown in Figure 5. In sections incubated with the active enzymes, we observed a slow loss of type V collagen-specific immunoreactivity with time, until, in some sections, very little type I (Fig. 5C) or type V (Fig. 5D) collagen could be detected. Other sections retained somewhat more residual type V collagen within the corneal stroma even after three days exposure to V'ase (data not shown). Control sections showed the characteristic patterns of type I and V collagen immunoreactivity after more than five days in buffer (Fig. 5A, B). Parallel sections sequentially digested with the enzymes until the endpoint was reached retained type IV collagen associated with ocular basement membranes (not shown).

Discussion

We have used collagen type-specific collagenases and monoclonal antibodies to probe the molecular arrangement of corneal fibrils composed of collagen...
Fig. 5. Sequential digestion with I'ase and V'ase. (A–D) show sections of unfixed embryonic cornea incubated with active I'ase, or buffer without I'ase, for 48 hr and then active V'ase, or buffer, for 74 hr. In this experiment, the active V'ase was replenished with freshly activated V'ase after 48 hr of digestion. Bar = 100 μm. (A) and (B) show sections incubated with the buffers without enzyme. Reaction with antibodies against collagen types I (A) or V (B) reveal no change in the patterns of corneal immunoreactivity characteristic of these collagens. In (C) and (D), sections were sequentially digested with I'ase and V'ase. Subsequent indirect immunofluorescence histochemistry with antibodies against type I (C) or type V (D) collagen documents the virtually complete removal of immunoreactivity for these collagen types by this treatment.

types I and V. Using a collagenase that degrades type I collagen but not type V, we have confirmed and extended our earlier observation that concealed epitopes on type V collagen in situ can be unmasked by enzymatic cleavage of type I collagen molecules. This suggests that type I collagen in fibrils renders those epitopes on type V unavailable to anti-type V collagen monoclonal antibodies. Electron microscopic observations of corneal stromas partially digested with I'ase showed type V collagen in thin filamentous strands. These filaments frequently were observed to emerge from regions of thicker striated collagen fibrils. While anti-type V collagen antibody preferentially labeled the filamentous strands in these preparations, antibody against type I collagen labeled both the striated regions and many of the filaments. Since the thin filamentous strands could be labeled by antibodies against both collagen types, we believe that at least some of the filaments liberated by I'ase activity may themselves be heterotypic, being composed of collagen types I and V.

The differential pattern of collagen labeling reported here leads us to the tentative conclusion that much of the type V collagen in stromal fibrils is completely buried within the fibril. Otherwise, the thicker regions of partially digested fibrils should have bound more type V antibody (also see below). In addition, molecules of type V collagen appear to be diffusely distributed throughout the fibrils, since one could find examples of multiple, closely associated filaments containing type V collagen protruding from the same contiguous segment of a striated fibril. Such images are of partially digested structures, however, and thus do not necessarily exclude the possibility that type V collagen forms a central core in intact fibrils. The elucidation of the precise molecular arrangement of collagen types I and V will require further ultrastructural studies on fibrils dissected in a variety of ways.

Whatever the configuration of collagen types I and V in these fibrils, any model that entails a population of type I collagen molecules that is itself sequestered by type V (for example, an arrangement of alternating layers of the two collagen types), is largely eliminated. Such a model would predict that the sequential enzymatic digestion of the accessible molecules of type I and type V collagen would then unmask an inner population, possibly a core, of type I collagen. This was not detected in any of the sections sequentially digested with the collagenases. In such sections, regions of the corneal stroma devoid of type V collagen-specific immunofluorescence also lacked type I; the small amount of residual immunoreactivity for type I collagen (see below) also contained type V.
Type IV-specific immunofluorescence was essentially unchanged.*

The assertion that type V collagen molecules are largely buried beneath the fibril surface is strengthened by our experiments with the type V-specific collagenase purified from MDCK cell cultures. We found that type V collagen in undisrupted corneal fibrils is not degraded by this enzyme. This indicates that the cleavage sites on the type V collagen molecule, of which there are probably two or more, are also normally inaccessible to the enzyme in intact fibrils. Thus, several loci along the length of the type V molecule are hidden when the molecule is organized into heterotypic fibrils. These sites become available to the enzyme or antibodies only after the corneal fibrils are disrupted by treatment with acetic acid or enzymatic removal of type I collagen. The implication that type V collagen is largely buried beneath the fibrillar surface does not exclude the existence of surface domains of type V collagen in heterotypic fibrils. Indeed, in Bowman’s membrane, epitopes on at least some of the type V collagen molecules are normally exposed in corneal sections, and yet this collagen is also largely resistant to digestion with V’ase in sections of normal corneas and becomes available for digestion after fibrillar disruption. Perhaps the organization of type V collagen in the smaller diameter (18 nm) fibrils within Bowman’s membrane is such that the region containing the epitopes, which reside near one end of the molecule, is relatively more exposed than those regions bearing the collagenase cleavage sites.

In corneal sections in which type V collagen was unmasked by treatment with acetic acid or I’ase, the removal of type V collagen from the stroma was sometimes complete, but usually some residual immunoreactivity persisted. In acid-treated sections, this might have been due to insufficient swelling of some fibrils, such that epitopes on type V collagen were exposed but cleavage sites were not. Alternatively, some reformation of compact fibrils at 37°C may have protected some of the type V collagen from V’ase digestion, although whole-fibril reformation in such preparations certainly did not occur (see Fig. 4D). In sections treated sequentially with I’ase and V’ase, most of the persistent type V (as well as some type I) collagen was observed in a region of interfacial matrix bordering Descemet’s membrane that contains especially dense deposits of collagen types I, II, V, VI, and perhaps IV, as well as proteoglycan (preliminary observations). Digestion of this region with type I collagen-degrading enzymes proceeds considerably more slowly, and the collagen in this region has a higher thermal stability, than elsewhere in the cornea.

These experiments show that removal of the fibrillar collagenous matrix of the cornea requires both I’ase and V’ase acting in concert. The production of type I/II/III-specific animal collagenase by corneal stromal cells has been documented; type V-specific collagenase activity has not yet been identified in the cornea, but it is possible that corneal cells do possess such an enzyme in their repertoire. In the context of heterotypic fibrils composed of collagen types sensitive to different degradative enzymes, the differential control of enzyme activity (at the level of production, activation, or inhibition) would produce quite different extracelluar matrices in the course of normal or pathological processes of remodeling. In the corneal stroma, for example, the coordinate expression of both I’ase and V’ase would produce a loose filamentous matrix of uncertain orientation composed, perhaps, of type VI collagen, noncollagenous microfibrils, and proteoglycans. Conversely, the differential expression of I’ase without V’ase activity would also result in a loose filamentous matrix, but one with a spatial organization (imposed by the persistent type V collagen) that would precisely mirror that of its origin. This matrix might then serve as a template for directing the assembly of a new connective tissue matrix with the appropriate organization.

Key words: cornea, collagen, fibril structure, collagenases, immunocytochemistry

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References


* In addition, the persistence of type IV collagen in ocular basement membranes in these preparations suggests that this V’ase, unlike some other type V collagen-degrading enzymes, does not degrade type IV collagen in situ.


