Ultrastructural Analyses of Enzyme-Treated Microfibrils in Rabbit Corneal Stroma

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Microfibrils have been identified within and between corneal collagen lamellae in a number of vertebrate species in a variety of developmental and pathological conditions, but they are relatively rare in normal adult animals. The present study was undertaken to analyze corneal microfibrils in adult rabbits using enzymatic digestion techniques. Transmission electron microscopy (TEM) showed clusters of 10–15 nm microfibrils arranged in quasi-parallel bundles within or between orthogonally arranged stromal collagen lamellae. When corneas were fixed with tannic acid/glutaraldehyde, the entire stroma showed increased electron density and microfibrillar bundles were heterogeneously stained. Peripheral fibrils were more electron-dense than those located more centrally. Following sequential detergent solubilization of unfixed corneas, all cellular elements were removed and collagen lamellae were distorted. Microfibrillar bundles remained intact, however, and resembled untreated controls. Subsequent treatment with pepsin, trypsin or elastase resulted in swollen corneal tissues in which collagen lamellae were no longer distinguishable but individual collagen fibrils maintained their morphological integrity. In these tissues microfibrillar bundles were rarely identifiable and were reduced to randomly oriented fragments or clusters of filamentous material. Testicular hyaluronidase or chondroitinase ABC did not affect the fibrils. These data indicate that rabbit corneal microfibrils are proteinaceous and that the tannic acid-staining component of the bundles is not glycosaminoglycan. The fibrils are indistinguishable from those identified as oxytalan in cornea and other ocular tissues. Moreover, their sensitivity to elastase and preferential staining with tannic acid/glutaraldehyde strongly suggest they may be related to the elastic system of fibrils. Invest Ophthalmol Vis Sci 29:578–585, 1988

Extracellular matrix comprises most of the vertebrate corneal stroma. At the level of transmission electron microscopy (TEM), matrix components include basal lamina, Bowman's layer, fibrillar collagen, microfibrils and various amorphous materials surrounded by an electron-lucent ground substance. The type and proportions of these components vary with age in normal individuals and are altered considerably in pathological conditions.

Microfibrils do not appear in the normal human adult cornea, although fibrils which appear to be components of the elastic system of fibers (oxytalan fibrils) may be seen by histochemical techniques in the stroma and Descemets membrane of juveniles.1 They have also been identified by light microscopy (LM) and TEM in pathological conditions such as keratoconus2 and Fuchs' endothelial dystrophy.2

More recently Carrington and coworkers4 demonstrated by LM and TEM the presence of oxytalan fibrils in newborn kitten corneas, though they disappeared from most parts of the cornea with maturity. Similar microfibrils have been identified in the corneal stroma of developing rabbits and rabbit corneal scars.5 Bundles of corneal microfibrils have also been described in adult rabbit corneas6 and, more recently, microfibrils similar to oxytalan fibrils were identified by Pratt and Madri7 within corneal collagen lamellae of adult mice. By LM and TEM immunohistochemical studies, they demonstrated that collagen types III and IV, and laminin were associated with the fibrils.

Oxytalan fibrils are believed to be the first type of elastic fiber to form in embryonic life.8 They are demonstrable by LM histochemical techniques9 and by TEM where they appear as 10–15 nm microfibrils with electron-lucent cores and are usually aggregated in bundles.10,11 As the bundles mature, individual fibrils are surrounded by amorphous elastin to form eluanin fibers which are believed to be intermediate in elastic fiber formation.12 Ultimately, in mature elastic fibers, microfibrils are peripherally situated around a central core of elastin.9 Oxytalan fibrils per-
sist in a variety of normal\textsuperscript{13-15} and pathological\textsuperscript{2,16} tissues, where their distribution has been described by LM and TEM techniques. These fibrils, however, have not been identified unequivocally as normal components of adult corneal stroma.

Although there is general agreement that oxytalan fibrils bear a strong morphological resemblance to microfibrils of the general connective tissue space,\textsuperscript{7,17-19} there is little consensus as to whether they should be regarded as identical. At the TEM level, oxytalan fibrils usually are described as beaded or periodic 10–15 nm microfibrils with electron-lucent cores.\textsuperscript{9} The more generic species of microfibrils, on the contrary, has been described variously as 10–12 nm tubular structures with 10–12 nm striations,\textsuperscript{19} 8–11 nm nonbeaded and dense-cored fibrils,\textsuperscript{7} or 12.8–13.8 nm pentagonal fibrils with electron-lucent lumens containing a 1–2 nm bead and surrounded by helical “ribbon-like structures.”\textsuperscript{17}

On the basis of their morphological similarities to developing elastic fibers, oxytalan fibrils are believed to contain elastin or elastin-related molecules.\textsuperscript{5,20} Other studies indicate, however, that these fibrils are comprised of a 350 kD glycoprotein called fibrillin.\textsuperscript{21} This molecule has been localized both to oxytalan fibrils and to the more general microfibril which is believed to contain microfibrillar core protein associated with fibronectin,\textsuperscript{22,23} microfibrillar protein either composed of or associated with collagen types III and IV and laminin,\textsuperscript{7} or a column of amyloid P units reinforced by an unidentified band-like or helical structure.\textsuperscript{17} This latter study is particularly intriguing since amyloid P is known to be localized at the surface of elastic fibers.\textsuperscript{24}

In the current study we reexamine the corneal microfibrils of adult rabbit cornea by TEM. The fibrils are located within and between lamellae of collagenous fibrils and are distributed throughout the full thickness of the cornea. We show the ultrastructure of the fibril in situ and demonstrate their relative digestibilities in proteolytic and glycosidic enzymes following detergent extraction. The data are discussed in light of recent studies of microfibrillar structure and function.

### Materials and Methods

New Zealand white rabbits were maintained at the University of North Dakota Animal Resources Facility and were treated in accordance with the ARVO Resolution on the Use of Animals in Research. Eyes were enucleated from male rabbits (approximately 2 kg) following sacrifice by overdose injection of sodium pentobarbital in the ear vein. A full-thickness 4 mm diameter corneal button was removed from each eye with care taken to exclude scleral tissue. The buttons were minced with a razor blade into pieces approximately 1 mm\textsuperscript{3}. Some of the minced corneal tissue was fixed\textsuperscript{25} immediately and served as onset controls. The remaining tissue was divided into fractions for treatment as described below.

Sequential passes through detergent were carried out to remove all cellular components. Freshly minced corneas were washed 2 hr with distilled water. This was followed by 4 hr treatment with 3% Triton X-100 and extensive distilled water rinsing. Samples were then incubated 4 hr with 0.025% DNAse (Type I, Sigma Chemical Co., St. Louis, MO). This was followed by a 4 to 6 hr treatment with 4% sodium deoxycholate and extensive rinsing in distilled water. All solutions contained 0.05% sodium azide and all treatments were carried out at room temperature. Samples were either fixed\textsuperscript{25} for microscopy (zero-hour controls) or further treated with enzymes.

Samples of detergent-treated corneal tissue were divided into five series and placed in small glass vials for enzyme digestions. These were rinsed three times in appropriate buffers (Table 1) and then placed in enzyme solution. Enzymes (Table 1) included pepsin (Sigma), trypsin (Sigma type II, elastase (Elastin Products, St. Louis, MO), testicular hyaluronidase (Sigma type IV-s), and chondroitinase ABC (Miles, Elkhart, IN). Incubations were carried out at room temperature (22°C) or 37°C (4°C only for pepsin) for 2, 4, 8, 24 or 48 hr.

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<th>Table 1. Conditions of enzyme treatment</th>
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<td>Testicular hyaluronidase</td>
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<td>Chondroitinase ABC</td>
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* Earle's balanced salt solution.
Our previous ultrastructural studies showed that several extracellular matrix components in the renal cortex were partially digested when they were treated with enzymes at concentrations of 0.1% to 0.4% (22°C) for 30 min to 24 hr. In the current investigation, similar concentrations (usually 0.1%) were used. Alternatively, appropriate concentrations were determined by pilot experiments. Control samples were treated with buffer (or 0.5 M acetic acid) only for 48 hr. Following appropriate treatment times, enzymetreated samples were washed extensively in their respective buffers prior to fixation for microscopy.

Onset controls, zero-hour controls, buffer controls and enzyme-treated samples were fixed for 1 hr at room temperature (pH 7.4) with 0.2 M sodium cacodylate/HCl buffered paraformaldehyde-glutaraldehyde25 with or without 0.25% tannic acid. The samples were rinsed and post-fixed in 2% OsO₄ buffered
with 0.15 M sodium cacodylate/HCl. Some tissues were subsequently treated 1 hr in 1% tannic acid prior to dehydration in a graded series of ethanol and propylene oxide. All samples were embedded in a mixture of Epon and Araldite and cured 48 hr at 60°C. One micron thick sections were cut and stained with toluidine blue (1% in 1% sodium borate) for determination of corneal orientation. Thin sections were cut on a Dupont-Sorvall (Newtown, CT) MT2-B Ultramicrotome provided with a diamond knife. These were mounted on 200 mesh copper grids, stained with lead citrate and uranyl acetate (5% in absolute ethanol) and observed in a JEOL (Medford, MA) 100S electron microscope at original magnifications of 4000–20,000 diameters.

**Results**

Low magnification electron micrographs show that normal rabbit corneal stroma is comprised of layers (lamellae) of unit collagenous fibrils with alternating orientations (Fig. 1a). The lamellae average 1 to 4 µm in thickness and are demarcated at their borders by keratocytes and electron-dense patches. At higher magnifications (Fig. 1b) these patches appear as parallel arrays of microfibrils located adjacent to interfibrillar keratocytes (Fig. 1c) or interposed between adjacent corneal lamellae, or they may be surrounded by collagen fibrils within a single layer. The microfibrils average 10–15 nm in diameter and are arranged in discrete parallel bundles.

In an effort to elucidate the substructural features of corneal microfibrils, corneas were fixed in glutaraldehyde/tannic acid (Fig. 2a–c). By this technique microfibrillar bundles are less distinct (Fig. 2a), but exhibit heterogeneous staining patterns. In general, the periphery of the bundles stain more intensely than their central portions. This disparate staining pattern is evident in longitudinal (Fig. 2b) or tangential section (Fig. 2c).

To eliminate cellular components and to improve the image of individual microfibrils, corneal preparations were subjected to a sequential detergent solubilization scheme prior to further experimentation. Following this procedure, stromal cells are completely removed and collagen lamellae become less regular. Microfibrillar bundles are more prominent, appear less rigid and frequently display wavy patterns (Fig. 3a). Longitudinal sections (Fig. 3b) of these specimens at higher magnification show that individual microfibrils are not striated or beaded, and that bundles are surrounded by masses of fine perimicrofibrillar filaments. In cross-section (Fig. 3c) the fibrils average 12 nm in diameter and most show electron-dense cores. They are separated from each other by approximately 25 nm (center-to-center spacing).

Specimens prepared in this way are designated zero-hour controls for the enzyme digestion studies described below.

Pepsin, trypsin and elastase are the proteolytic enzymes chosen for this investigation. Since pepsin is active only at low pH, it is important to control the experiment by determining the ultrastructural effects of 0.5 M acetic acid alone. Following 48 hr in acetic
Fig. 3. (a) Rabbit corneal stroma following sequential detergent treatment (see text for details). All cellular elements are solubilized and collagen lamellae are more irregular. Corneal microfibrillar bundles (CMF) are prominent within and between collagen lamellae cut in longitudinal (COL_L) and cross (COL_X) section (×5700). (b) Higher magnification of corneal microfibrillar bundle in longitudinal section (CMF_L) from specimen prepared as in (a). The removal of lipid and nucleotides from the sample does not alter the size or morphology of the bundles (×81,000). (c) Corneal microfibrillar bundle similar to that seen in (b) but cut in cross-section (CMF_X). Most fibrils show dense cores though some (arrows) show electron-lucent centers (×78,900).

acid (Fig. 4a), the cornea swells markedly. Stromal lamellae are not distinguishable and unit collagenous fibrils are no longer present in parallel array. Corneal microfibrils are clearly identifiable in these preparations, but due to general corneal disorganization, they appear as electron-dense elongate masses of parallel microfibrils interposed among randomly oriented collagen fibrils with unwound terminal tufts. When corneal samples are incubated 2 hr with pepsin in the same medium, few collagenous fibrils are present and microfibrillar bundles are reduced to clusters of disassociated fibrillar fragments (Fig. 4b).

Following treatment with trypsin (Fig. 5), few microfibrils remain. Those present appear as disorganized and wispy filaments. The effect of elastase (Fig. 6) is even more dramatic and results in nearly complete digestion of microfibrils.

Two mucopolysaccharidases (testicular hyaluronidase and chondroitinase ABC) are used in this study (Figs. 7, 8). Following treatment with these enzymes, corneas swell and collagen lamellae are disrupted but the integrity of individual collagenous fibrils or microfibrils are not affected.

Discussion

The current ultrastructural study of adult rabbit corneal microfibrils shows that they are distributed throughout the full thickness of the corneal stroma. They are clustered within and between collagen lamellae and are often associated with keratocytes. Individual fibrils range from 10–15 nm in diameter, show electron-dense cores, are aggregated in roughly parallel bundles and are ultrastructurally similar to oxytalan fibrils of the elastic fiber system. Microfibrils resembling oxytalan fibrils have been identified previously in several ocular tissues, most notably the zonules and the cornea.

Since no previous investigations have been carried out to characterize adult rabbit corneal microfibrils, the current study was designed to subject the fibrils to various extractions, enzymes and "staining" procedures in an effort to shed some light on their composition and to determine their possible relationships to oxytalan fibrils. By these procedures we have shown that they remain intact following treatments with detergents and endonuclease, indicating they are not comprised of lipids or nucleotides. Likewise, nonspecific mucopolysaccharidases do not alter their morphology, strongly suggesting little, if any glycosaminoglycan. On the contrary, a proteinaceous moiety may be inferred from the destruction of their integrity by nonspecific proteases such as trypsin or pepsin. The disorganization of the microfibrillar bundles by pepsin was particularly interesting since the digestion
Corneal microfibrils were carried out at 4°C, where interstitial collagens remain helical. Since individual fibrils remained identifiable following pepsin treatment, it seems possible that they may be pepsin-resistant but are surrounded and possibly linked together by pepsin-sensitive perimicrofibrillar filaments. The fragmentation of corneal microfibrils by elastase was somewhat unexpected since it has been reported that oxytalan fibrils require preoxidation to render them susceptible to this enzyme.\textsuperscript{13} Fullmer\textsuperscript{32} later suggested, however, that the necessity for fibril pretreatment in the early experiments may have been related to elastase impurities. Another explanation may be that the detergent extraction technique used in the current study "unmasks" the fibrils or otherwise renders them more susceptible to the enzyme without preoxidation. Finally, it is possible that the elastase, which is known to exhibit nonspecific proteolytic activity,\textsuperscript{33} may have digested a broad spectrum of microfibrillar proteins in addition to any available elastin.

Since oxytalan fibrils and other fibers of the elastic fiber system are clearly distinguished by tannic acid-glutaraldehyde fixation,\textsuperscript{11,20} we applied this procedure to rabbit corneal tissue prior to TEM observations. Tannic acid is a mordant which has been used to demonstrate the presence and ultrastructure of hyaluronic acid.\textsuperscript{34} Other studies, however, indicate that tannic acid effectively stains filamentous components of developing basement membranes.\textsuperscript{35} Moreover, these filaments are not removed by predigestion with hyaluronidase, suggesting that the stained materials may be more closely related to basement membrane collagens or noncollagenous glycoproteins than to glycosaminoglycan. In the current study, tannic acid treatment resulted in a general increase in staining of stromal collagen fibrils and often obscured microfibrillar bundles rather than rendering them more evident. Moreover, those identified consistently exhibited a preferential increase in electron density at their periphery, strongly suggesting compositional heterogeneity.

These data are particularly interesting in light of studies which show that tannic acid treatment increases the electron density of fibrillar structures within the lamina densa of glomerular basement membranes.\textsuperscript{36} Since this basement membrane layer consists partially of Type IV collagen and other basement membrane glycoproteins,\textsuperscript{37} the dense staining pattern seen at the periphery of corneal microfibrillar bundles following treatment with tannic acid may also indicate the presence of collagen IV. This is consistent with recent immunoelectron histochemical studies which have demonstrated Type IV collagen surrounding corneal microfibrillar bundles in the adult mouse.\textsuperscript{7} Moreover, the perimicrofibrillar fine filaments identified in our study may be a morphological manifestation of other glycoproteins such as fibronectin and laminin, also known to be associated with microfibrils.\textsuperscript{7,22}

With the techniques used in the current study, it is not possible to determine if adult rabbit corneal microfibrils should be regarded as oxytalan. It should be pointed out, however, that they are ultrastructurally indistinguishable from oxytalan fibrils previously identified in the cornea and other ocular tissues. Moreover, their sensitivity to elastase and preferential staining by tannic acid/glutaraldehyde suggests they may be related to the elastic system of fibrils.

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Fig. 4. (a) Rabbit corneal stroma following treatment with detergents (as in Figs. 3a–c) but exposed to 0.5 M acetic acid (pH 2.0) for 48 hr at 40°C. Corneal lamellae are disrupted and individual collagen fibrils begin to unwind. Microfibrillar bundles (CMF) do not disperse but are less orderly than untreated controls (×42,700). (b) Rabbit corneal stroma prepared as in (a) but treated 2 hr with 0.1% pepsin at pH 2.0. Few collagenous fibrils remain and corneal microfibrils (CMF) are highly fragmented (×44,800).
Fig. 5. Rabbit corneal microfibrils (CMF) following detergent treatment and incubation with 0.1% trypsin for 24 hr at room temperature. The fibrils are difficult to distinguish and only a few filaments remain (×29,300).

Fig. 6. Rabbit corneal microfibrils (CMF) following detergent treatment and incubation with elastase (250 IU/ml) for 24 hr at room temperature. Microfibrillar bundles are almost completely digested by this enzyme with only a few fibrillar "ghosts" remaining (×23,100).

Fig. 7. Rabbit corneal microfibrils (CMF) following detergent treatment and incubation with 0.1% testicular hyaluronidase for 48 hr at 37°C. Although the corneal stroma swells and collagenous fibrils are dispersed, microfibrillar bundles maintain their morphological integrity (×24,700).

Fig. 8. Rabbit corneal microfibrils (CMF) following detergent treatment and incubation with 0.1% chondroitinase ABC for 48 hr at 37°C. The microfibrillar bundles appear similar to those treated with hyaluronidase (compare with Fig. 7) and both are indistinguishable from controls (×30,100).

Key words: corneal stroma, rabbit, microfibrils, oxytalan, ultrastructure

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References