The Zonules and the Elastic Microfibrillar System in the Ciliary Body

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It has been proposed that elastic fibers occur in some tissues as a three-part interconnecting system. The system includes two sizes of elastin-containing fibers surrounded by tubular microfibrils (elastic microfibrils), besides isolated bundles of tubular microfibrils without elastin (oxytalan fibers). This little-studied system was identified in the bovine ciliary body by light and electron microscopy. Its architecture varied regionally, suggesting different vectors of tractional force in the anterior and posterior ciliary body related to accommodation. Zonular fibers had the staining characteristics of oxytalan fibers, and their fibrils were ultrastructurally similar to the tubular microfibrils around elastic fibers and those composing oxytalan fibers. Antibodies to microfibrillar protein bound to zonules and to tubular microfibrils in all sites. This is the first evidence that tubular microfibrils both with and without elastin share antigenic determinants and confirms the close antigenic relationship of the zonules to this class of proteins. Invest Ophthalmol Vis Sci 24:667–681, 1983

The elastic tissue of the eye has seldom been studied in the modern era, so there is little knowledge of its architecture or its proportion of elastin and microfibrillar components. Ultrastructural evidence has established that typical elastic fibers are composed of a central elastin core surrounded by 10 nm tubular microfibrils. However, it has long been suggested that a more complex elastic system exists in many tissues. As described in the skin, this system consists of three elements. There is an outer layer of small elastic fibers, surrounded by many microfibrils ("elainin" fibers), connected to a deeper layer of large elastic fibers with few microfibrils. This elastic complex is connected to the epidermis by a third type of fiber composed solely of tubular microfibrils without elastin. These pure microfibrillar bundles have also been described in other tissues and correspond to the "oxytalan" fibers seen with the light microscopy. The 10 nm tubular microfibril, then, is a unit common to all three elastic tissue elements. Although it has been assumed from morphology and histological staining affinities that the microfibrils in these three fibers are identical in composition, this has not been proven.

The ocular zonular fibrils are also 10 nm tubular microfibrils, which resemble in morphology the microfibrils of the elastic system as first noted by Ravio. We have recently reported that the zonules and the microfibrils surrounding elastic fibers are similar biochemically and immunologically. The inference may be drawn that the zonules are part of the elastic system and are elements comparable to the pure microfibrillar bundles, although the latter point has not yet been demonstrated.

The possibility that the ocular zonules are part of the elastic system is an important one for understanding the linkage between lens dislocation and systemic connective tissue diseases. To examine this hypothesis further, we have studied the elastic system in the ciliary body, a structure that contains all of the elastic elements in convenient juxtaposition. The zonules and the microfibrils in the three elastic elements have been compared by histological staining, ultrastructural techniques, and an antibody to microfibrillar protein. The bovine eye was used as a model since...
the antibody was raised to the plentiful elastic microfibrils in this species.

Materials and Methods

Bovine eyes were obtained fresh from the slaughterhouse. Portions of the bovine ciliary body with adherent zonule were fixed in 4% neutral buffered formaldehyde and processed in paraffin for light microscopy. Standard elastic stains\textsuperscript{11} used were Weigert's resorcin-fuchsin, Tanzer's orcein, Gomori's aldehyde fuchsin, and Verhoeff's iron hematoxylin.\textsuperscript{11b} These stains were performed with and without prior oxidation with peracetic acid\textsuperscript{12} or 0.3% acidified potassium permanganate, as used in Gomori's chrome alum hematoxylin stain.\textsuperscript{11b} Small portions of the ciliary body from different regions were fixed in 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, and embedded in Mollenhauer's medium. Sections were examined by electron microscopy after staining with uranyl acetate and lead citrate, preceded in some instances by 0.3% orcein for 30 minutes to demonstrate elastin.\textsuperscript{13}

For the immunohistochemical studies, antisera to bovine elastic microfibrillar protein (MFP) were prepared. MFP from fetal calf ligamentum nuchae was obtained as described previously\textsuperscript{14} and kept freeze-dried until used. Antibodies to MFP were raised in 2.5 kg male New Zealand white rabbits by injecting subcutaneously a 1:1 mixture of MFP and complete Freund’s adjuvant, sonicated in 0.01 M phosphate buffered saline (pH 7.3). Five bi-weekly injections containing 0.2 mg of MFP each were given in divided sites. A sixth injection of 0.1 mg was given intravenously. One major band was shown to result from reacting MFP antisera against the antigen by immunoelectrophoresis.\textsuperscript{14} No reaction was found between MFP antisera and fibronectin prepared from rat or human plasma.

Fresh bovine tissues, as described above, were examined for antibody binding by electron microscopy, using the indirect immunoperoxidase technique and employing peroxidase-labeled staphylococcal protein A as an immunoreagent, as we have described previously.\textsuperscript{10} The antisera were used in 1:1 to 1:20 dilutions. The immuno-reacted tissues were postfixed in 2% osmium tetroxide and embedded in Mollenhauer’s medium. Sections were examined both unstained and stained with uranyl acetate and lead citrate. Controls included sections with no added serum and blanks containing only diaminobenzidine.

Results

The bovine ciliary body differed from the human in having the major ciliary processes derive from the periphery of the iris and, more accurately, the cilioiris (Fig. 1). The ciliary muscle was flat rather than tri-
angular in profile, most of it lying posterior to the major ciliary processes. Accessory processes were frequent in the mid pars plana region. Abundant zonular fibers originated from the pars plana, attaching to the accessory processes and posterior portion of the major ciliary processes on their way to the lens.

With all of the elastic fiber stains, the largest elastic fibers were found in the superficial stroma of the posterior pars plana (Fig. 2a). These fibers were 0.5–2.0 μm in diameter, mostly 0.5–0.7 μm, layered horizontally between the collagen fibers. The largest band lay just under the pigment epithelium, becoming thicker toward the ora serrata. Within the accessory processes, there was a network of elastic fibers, which began as sub-epithelial, brush-like fibrils under the epithelium (Fig. 2c). The fibers then extended down
toward the vessels and ciliary muscle perpendicularly or angled posteriorly. The outer edge of the pigment epithelial basement membrane contained a profusion of fine fibrils. The stroma under the ciliary epithelium more anteriorly had scanty elastic fibers that were much finer than those posteriorly, from 0.1–0.3 μm, and often showed an incomplete or dotted appearance (Fig. 3a). In the stroma of the main ciliary processes, the scanty and incomplete elastic fibers passed primarily to the walls of the blood vessels (Fig. 3c).

The zonules generally were negative with elastic stains (Fig. 3a) when these were fully differentiated,
Figs. 3, a–b. Anterior pars plana and ciliary processes. a, Zonules (Z) are unstained. Stroma has a few fine subepithelial elastic fibers (EF) and dotted extensions to an underlying blood vessel (BV) (Orcein, original magnification ×240). b, After oxidation with potassium permanganate the zonules (Z) are intensely stained, and many more subepithelial and deeper fibrils are seen (OXY). The pigment epithelium was bleached by the processing (Orcein, original magnification ×240).

except for aldehyde-fuchsin, which gave an irregular, faint pink stain. Few elastic fibers were seen among the ciliary muscle fibers, but many medium-sized ones (0.5 μm) linked the inner ciliary muscle and the pectinate ligament and were frequent in the outer ciliary stroma, pectinate ligament, and inner sclera, as these three tissues converged to form the filtration angle. Characteristic elastic fibers were present in the walls of arteries and the larger veins. The diameters of elastic fibers in all areas were smallest after orcein staining. Small elastic fibers in the stroma were shown poorly by Verhoeff's stain.

Oxidation prior to staining with elastic stains was carried out to see whether any previously invisible fibers would become apparent, thus fulfilling the criteria for "oxytalan" fibers. Prior oxidation resulted in excellent staining of the zonules by all of the previously negative or faint elastic stains (Fig. 3b), except for Verhoeff's, which is also characteristic of oxytalan fibers. In the ciliary body, oxidation demonstrated few additional stromal fibrils posteriorly (Figs. 2b, 2d) but did increase the number under the epithelium (Figs. 3b, 3d). The average fiber appeared broader by about a third (Fig. 2b), and more were complete (Figs. 3b, 3d). Elastic fibers in the region of the pectinate ligament were especially enhanced (Fig. 4). Aldehyde fuchsin and orcein stains after oxidation gave the most definitive staining of the zonules, besides fine fibrils around capillaries and under the basement membrane of the pigment epithelium, the other stains producing a muddy appearance.

By electron microscopy, the zonules lying over the bovine ciliary body were highly oriented, 10–12 nm fibrils with a 12–14 nm microperiodicity and an electron-dense tubular profile when cross sectioned (Fig. 5). A macroperiodicity of 40–45 nm was sometimes discerned, especially where the fibrils were closely aggregated.

As noted by light microscopy, the largest elastic fibers formed a series of rows in the posterior pars plana, beginning just under the ciliary epithelium (Fig. 6a). One-third to one-half of each elastic fiber was composed of a loosely arranged mesh of tubular microfibrils after oxidative staining, correlating well with the degree of fiber thickening described previously. The microfibrils were of the same dimensions
as the zonular fibrils (Fig. 6b). The cores were electron lucent and compartmentalized by clumps of microfibrils and granular material. After staining of the grids with orcein, the elastin in the cores became electron dense (Fig. 6 inset). The small and medium elastic fibers in the deeper stroma also had plentiful microfibrils around them.

Bundles of similar tubular microfibrils, which appeared to be free of elastin cores, were seen in many regions of the ciliary body. Their electron density compared to that of the usually poorly staining collagen fibers made them stand out sharply. In the simple architecture of the ciliary processes, these bundles could be seen extending down from the basement membrane, joining with others to form larger bundles before passing to blood vessel walls (Fig. 7). Micro-
Fig. 4. Profuse elastic and oxytalan fibers (arrows) among the ciliary muscle fibers (M) as they insert in the obliquely sectioned leaflets of the pectinate ligament (P) (Potassium permanganate oxidation, aldehyde fuchsin, x520).

Fig. 5. Zonular fibrils blending into the basement membrane (BM) of the ciliary nonpigment epithelium (NPE), pars plana. Cross-sectioned zonules have a tubular profile (arrow) (x65,500). Inset: 14-nm microperiodicity is visible (arrow) (x62,000).
Figs. 6, a and b. a. Elastin (EL) core in the normal human ciliary body (×18,500). Insert: (1) linearly under the basement membrane (BM) of the ciliary pigment epithelium. Its elastin (EL) core shows multiple electron-lucent compartments like an elasin fiber. Surrounding microfibrils compose more than one-third of its diameter. Edge of a second elastic fiber (2) and a connecting elastic fiber bundle (asterisk) are seen (×18,500). Insert: After orcein staining, the elastin (EL) aggregates of the core appear dense (×23,800). b. A 14-nm microperiodicity is seen on the 10-12 nm elastic microfibrils (arrow) (×63,200).
fibrillar bundles were found especially adjacent to vascular basement membranes (Fig. 8) and in the ciliary muscle. In the mid pars plana, they formed a meshwork-like layer beneath the basement membrane of the pigment epithelium (Fig. 9a). However, orcein showed a surprising amount of small elastin clumps in this subepithelial meshwork (Fig. 9b). Fibroblasts often were close to the microfibrillar bundles. Throughout the stroma, tubular microfibrils occurred also as isolated fibrils.

After reacting with microfibrillar protein (MFP) antisera the zonular fibril, its periodicity and tubular core were all obscured by antibody aggregates (Fig. 10). A macroperiodicity of 40–45 nm was seen on aggregated fibrils (Fig. 10 inset).

MFP antisera had an equally strong affinity for elastic microfibrils, causing the unstained elastin cores to stand out prominently (Fig. 11a). The antibody obscured the microfibril (Fig. 11a inset) and accentuated a 40–45 nm macroperiodicity here, too. In unstained grids, there was no consistent staining of collagen fibers compared with normal serum controls. With counterstain to identify tissue elements more clearly, a suggestive, dot-like stain was seen irregularly around small (40–60 nm) collagen fibers but not often around larger (90–300 nm) fibers (Fig. 11b).

The MFP antisera demonstrated some bundles of heavily stained, linear fibrils, which appeared to be pure microfibrillar bundles in the subepithelial and perivascular regions (Fig. 12). They had the same globular staining with 40–45 nm macroperiodicity as the microfibrils around elastin fibers. Likewise,
stained, single fibrils could be identified sporadically in the stroma.

Discussion

The complete elastic fiber system in the ciliary body has not been studied previously, and classical elastic stains were somewhat erratic in demonstrating its elements. Most were consistent in showing large and medium elastic fibers, but there was greater variability in demonstrating small fibers. The dotted appearance of stain in some fine elastic fibrils by light microscopy correlated with the ultrastructural suggestion that elastin may be intermittently present along the course of microfibrillar bundles, making these fibers a mixture of oxytalan and elaunin fibers. Demonstration of the system three-dimensionally will be necessary to prove this point. As shown by electron microscopy, orcein is deposited very selectively on elastin and was especially helpful for demonstrating small amounts of elastin ultrastructurally. Orcein is not electron dense in itself but is thought to serve as a mordant for more electron-dense stains.

Most of the ciliary body elastic fibers were elaunin in type, that is, associated with many microfibrils, as shown by their greater width after oxidative treatment and by their ultrastructural appearance. This characteristic was not seen in young adult human ciliary bodies, which had, instead, a sparse microfibrillar component, probably because the humans were relatively older, and microfibrils around elastic fibers decrease with age. This variability in microfibrillar content with age makes it unlikely that large and small elastic fibers are intrinsically different structures. Pure microfibrillar (oxytalan) fibers were more common anteriorly. They passed especially from the basement membrane of the pigment epithelium to the basement membrane of vessels and were generally associated with small vessels elsewhere. This prominence of oxytalan fiber association with vessels differed from that in the skin, where they usually appeared to be links between elastic fibers and epithelial basement membranes. The function of prior oxidation in making “oxytalan” fibers of microfibrillar and zonular types visible with elastic stains may be related to their high cysteine and disulfide bond content, since it can be abolished by methylation.

The prominence of small elastic and oxytalan fibers under the epithelium in the anterior and midpars plana and passing backward toward the horizontally disposed elastic fibers in the posterior pars plana was of interest. This distribution suggests that mechanical traction, most likely from the zonules, is primarily vertical in the inner part of the anterior and...
Figs. 9, a and b. Tubular microfibrils (MF) under the basement membrane (BM) of the pigment epithelium, mid pars plana. a. Meshwork-like arrangement of the microfibrils with intervening vacuolar spaces and irregular densities (×60,000). b. Orcein staining on a more obliquely cut section, same area. Darker clumps of elastin (EL) and collagen fibers (CO) replace many of the dense and vacuolar spaces (×26,000).
Fig. 10. Bovine zonular fibers reacted with microfibrillar protein antibodies. Globular staining and accentuation of a macroperiodicity on zonular fibrils (arrow). Inset: Macroperiodicity on aggregated fibrils. Immunoperoxidase technique, otherwise unstained (×48,000; Inset: ×62,000).

mid-ciliary body, becoming maximal as anteroposterior traction towards the ora serrata. The paucity of elastic fibers in the anterior ciliary processes is consistent with an absence of zonular traction in this region, especially in the cow, whose processes are so anteriorly placed. A similar distribution also was seen in the human.15

The distribution of staining produced by MFP antisera on the microfibrils around elastic fibers in this study was identical to that shown by the same antigen in 17-day-old chick aorta reported by Kewley, Steven, and Williams.14 There was accumulation of globular stain, which obscured the fibril completely, and accentuation of a macroperiodicity not actually measured in the previously cited study. Staining was identical on apparent oxytalan fibers and on the ocular zonules. We have found a similar pattern of staining in these tissues when a zonular antibody was used,16 although the zonular antibody showed a stronger affinity for the macroperiodicity sites. These results support the conclusion that one or more immunologically similar antigens are shared by the zonules and all the microfibrils of elastic tissue, and that they belong to the same family of fibrils. It is not known whether the antigenicity resides in the protein core of the molecule or whether it is associated with carbohydrate moieties in these glycoprotein structures. The antigen is not species specific, as we have ob-
Fig. 11, a and b. Elastic fibers stained with microfibrillar protein antibodies. a, Immune-reacting microfibrils outline many small elastic fibers (EF) under the pigment epithelial basement membrane (BM), showing their confluence into two long fibers. Macroporadicity is visible (arrow) on lower fiber. Inset: Globular staining on microfibrils around aggregates of elastin (EL). Surrounding collagen fibers (CO) show no consistent reaction product. Immunoperoxidase technique, light counterstain (X11,000; Inset: X41,500). b, Heavy staining of microfibrils around a deep stromal elastic fiber (EF). Suggestion of irregular dotted stain on some collagen fibers (CO). Immunoperoxidase technique, light counterstain (X55,000).

A peripheral faint stain was seen on some collagen fibers near elastic fibers in all the tissues examined to date, whether using MFP antibody as in the present study and that of Kewley et al., or antizonular antibody. This stain may represent a diffusion artefact.
Fig. 12. Large number of microtubuli (MF) under the pigment epithelial basement membrane (BM), stained by microfibrillar protein antibodies, mid pars plana. Arrow indicates a pure microfibrillar bundle. Collagen is (CO). Immunoperoxidase technique, light counterstain (x36,800).
or a minimal amount of similar antigenic material present on the surface of small collagen fibers. Pericollagen staining was found also by Kewley et al., using monospecific MFP antibody, but was absent around monomeric collagen. The staining, thus, may be related to carbohydrate moieties on the surface of collagen fibers, which are well described ultrastructurally. Whatever its nature, this scanty pericollagen material differs considerably in quantity and pattern from that associated with the microfibrils.

We wonder whether the strong elasticity shown by the ocular zonules in situ is a characteristic of all microfibrillar bundles. It is apparently independent of the presence of elastin, since no characteristics of classical elastin have been reported biochemically or ultrastructurally in the zonule. The difference in quality of elasticity shown by the delicate zonular fibers compared to true elastic fibers adds versatility to the elastic fiber system and could contribute to the rapid stretch necessary for accommodative movements.

Key words: ocular zonules, elastic microfibrils, oxytalan fibers, microfibrillar antibody, elastic fibers, ultrastructure, ciliary body

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References