Fibrillin and Elastin Networks in Extrafusal Tissue and Muscle Spindles of Bovine Extraocular Muscles

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Purpose. Bovine extraocular rectus muscles were examined to map the distribution of elastin and fibrillin in extrafusal tissue and muscle spindles.

Methods. Immunohistochemical techniques and immunolocalization were employed to pinpoint the placement of molecules relative to muscle fibers.

Results. Strands containing elastin and fibrillin surrounded all extrafusal fibers. They also covered the external surface of intrafusal fibers, more extensively at the equator than at the pole. Within strands elastin was placed in the center, whereas fibrillin was located in microfibrils on the periphery.

Conclusions. The wide distribution in extrafusal tissue of elastin and fibrillin suggests that they are factors in determining the mechanical properties of extraocular muscles. Their placement in proximity to individual intrafusal fibers should affect the viscoelastic properties of these fibers and, thus, influence the dimensions of the afferent discharge. Invest Ophthalmol Vis Sci. 1994:35:3103-3110.

Connective tissues are important functional units of striated muscles. They securely link contractile elements to the muscle tendon and act as conduits for force transmission. Connective tissue macromolecules whose distribution in muscle have been investigated include collagen types I, III, and VI, basal lamina components, and fibronectin. Data obtained from previous investigations on connective tissue matrix have led to an understanding of how muscle fibers are bundled into fascicles and how they are affixed at the myotendinous junction. Yet, little information is available on the distribution in muscle of elastin and its associated microfibrils. One component of these microfibrils is a large (350 kD) protein called fibrillin. Recent evidence indicates that Marfan syndrome, a condition involving pathologic changes in elastic fibers, is associated with a mutation in the fibrillin gene located on chromosome 15. The current study employed antibodies against fibrillin and elastin to evaluate the distribution of these molecules in extrafusal tissue and muscle spindles. Extraocular muscles were selected because of the fine movements they perform in positioning the globe, a task that might require an extracellular matrix containing extensible and rigid components, as exemplified by elastin and collagen, respectively. Muscle spindles were examined because equatorial regions of intrafusal fibers, or structures immediately adjacent to them, require resiliency to produce the dynamic peak of the afferent discharge in response to phasic changes in muscle length. Elastin could be the vehicle that renders this resiliency.

MATERIALS AND METHODS

Extraocular rectus muscles were obtained from six healthy male or female domestic cattle of a local meat processing plant. Twenty muscles were examined. Immediately after death, globes were enucleated, packed in ice, and taken to the laboratory. No deterioration of muscle tissue was observed on subsequent microscopic examination. Muscles were detached from the sclera and frozen in melting isopentane, and they were held with tweezers to approximate their in situ length. Tissue was frozen under these conditions to avoid structural artefacts introduced by curling of muscle fibers. Serial cross-sections were cut (6 to 8 μm) in a cryostat and allowed to dry at room temperature for
30 minutes. Using standard immunohistochemical procedures, individual sections were then incubated with any one of the primary monoclonal antibodies against myosin heavy chains (MHC) or connective tissue macromolecules listed in Table 1. Repetitive series of three or more consecutive sections were processed to permit tracing of the same connective tissue structures and of extrafusal and intrafusal fibers for appreciable distances through the muscles. Before incubation, sections were fixed for 2 minutes in 2% phosphate-buffered paraformaldehyde and washed repeatedly in phosphate-buffered saline (PBS). Incubation with the primary antibodies lasted for 60 to 90 minutes (37°C). After several PBS washes, sections were incubated with the appropriate fluorescein-, rhodamine- or HRP-conjugated secondary antibodies for 30 to 45 minutes (37°C). Diaminobenzidine was used as chromogen on sections incubated with HRP-conjugated second antibodies. Sections were washed several more times with PBS and coverslipped with 30% glycerol in PBS (fluorochrome sections) or dehydrated and mounted in Permount (HRP sections). In some instances, sections used for demonstrating elastin or fibrillin were double labeled with rhodamine-conjugated phalloidin toxin. This compound combines with actin to facilitate identification of muscle fibers and pinpointing of the connective tissue matrix location relative to extrafusal and intrafusal fibers.

In a limited number of series, some sections were incubated for myosin (m) ATPase, acid, and alkaline preincubation, according to the method of Guth and Samaha.9 This was done to identify, in conjunction with MHC sections, muscle fiber types.

Tissue from several muscles was prepared for colloidal gold immunolocalization. Forty micro-thick sections of frozen blocks containing muscle spindles were cut, allowed to dry for 30 minutes (RT), and fixed for 10 minutes with 1% glutaraldehyde and paraformaldehyde in PBS. Sections were then incubated for 30 minutes (4°C) in 0.5% sodium borate in PBS to remove excess fixative. This was followed by washing in PBS, incubating for 2 hours in 0.1% BSA in PBS, and rinsing in PBS. Sections were incubated with the primary antibodies overnight (4°C), washed with PBS, and then incubated with 5 nm colloidal gold-conjugated second antibodies (Auroprobe, Amersham, Arlington Heights, IL) for 4 hours (4°C). The remaining procedure was a standard protocol for embedding in plastic and sectioning of electron microscopic specimens. Silver or gold sections were cut with a diamond knife on an ultramicrotome, stained with uranyl acetate and lead citrate, and inspected on a JEOL (Peabody, MA) 100X transmission electron microscope.

RESULTS

There were no noticeable differences between data collected from male and female animals.

Immunohistochemistry

Extrafusal fibers. Individual fiber types were identified to determine if the distribution of elastin or fibrillin differed around different fiber types. About 78% of the fibers reacted strongly with the anti-MHC MY32 antibody and stained dark after incubation for mATPase, pH 9.6 preincubation. Both reactivities are characteristic of fast-twitch muscle. All fibers also exhibited low to moderate amounts of slow MHC, as seen with antibody CA83, and stained moderately to strongly for mATPase, pH 4.6 preincubation. Thus, most fibers were at least to some extent fast/slow hybrids, also known to occur in other mammalian extraocular muscles.

Table 1. Antibodies Used

<table>
<thead>
<tr>
<th>Designation</th>
<th>Reactivity</th>
<th>Working Dilution</th>
<th>Reference or Manufacturers</th>
</tr>
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<tbody>
<tr>
<td>11C1</td>
<td>Fibrillin</td>
<td>1:100</td>
<td>Wright⁵</td>
</tr>
<tr>
<td>E-4013</td>
<td>Elastin</td>
<td>1:100</td>
<td>Sigma Chemical, St. Louis, MO</td>
</tr>
<tr>
<td>V-5255</td>
<td>Vimentin</td>
<td>1:100</td>
<td>Sigma Chemical</td>
</tr>
<tr>
<td>ALD58</td>
<td>Slow-tonic MHC</td>
<td>1:5</td>
<td>Shafig²</td>
</tr>
<tr>
<td>1975</td>
<td>Laminin</td>
<td>1:100</td>
<td>Chemicon, Temecula, CA</td>
</tr>
<tr>
<td>MY32</td>
<td>Neonatal/fast MHC</td>
<td>1:500</td>
<td>Sigma Chemical</td>
</tr>
<tr>
<td>2B6</td>
<td>Embryonic MHC</td>
<td>1:100</td>
<td>Gamoke⁹</td>
</tr>
<tr>
<td>CA83</td>
<td>Slow MHC</td>
<td>1:100</td>
<td>Sweeney⁶</td>
</tr>
</tbody>
</table>

* Diluent: 0.05% Triton X-100 and 0.05% bovine serum albumin in phosphate-buffered saline. MHC = myosin heavy chains.
Incubation of sections with anti-elastin (Fig. 1A) and anti-fibrillin (Fig. 1B) antibodies produced similar staining around all extrafusal fiber types; however, instead of a uniform reaction around each muscle fiber, there was for the most part alteration of thicker and thinner staining, interspersed with occasional unstained segments. It is unlikely that this pattern resulted from shortened muscle fibers because muscles were kept near their in situ length as they were frozen. When disregarding unstained segments, the pattern resembled in shape and location that seen for basal lamina macromolecules, such as laminin (Fig. 1C). Double labeling with phalloidin established that the anti-elastin and anti-fibrillin staining was external to muscle fibers, close to the basal lamina. Distribution of fibrillin and elastin extended into the perimysium and epimysium. Strong immunostaining for both molecules and for vimentin was observed in the walls of larger blood vessels and around intramuscular nerve trunks (not shown).

Muscle spindles. Most spindles were located in the orbital sections of the muscles. They displayed the same general structure as receptors in limb muscles, consisting of an intrafusal fiber bundle wrapped by an inner capsule. More peripherally, there was a connective tissue outer capsule that bulged at the equatorial region to accommodate an extensive periaxial space. Intrafusal fiber types could be classified with conventional mATPase histochemistry and MHC immunohistochemistry. Nuclear chain fibers presented contractile protein profiles resembling fast fibers; nuclear bag fibers appeared more like slow fibers. The nuclear bag fibers did not react with the antibody against neonatal/fast MHC, whereas the nuclear bag fibers reacted moderately. (Fig. 2A; Table 2).

The outer spindle capsule contained small to moderate amounts of fibrillin and elastin. Fibrils of these proteins situated within the outer capsule were seemingly continuous with similar structures in the adjacent perimysium (Fig. 2F).

Intrafusal fibers were routinely circled by thin and thick strands of fibrillin, which in cross-section appeared as dashes or dots, respectively (Fig. 2B, 2D, and 2F). Their location suggested that they were part of the inner portion of the inner capsule. When traced through serial sections, it became apparent that these strands were not of constant diameter nor did they pursue a straight course. Instead, they changed their size, shape, and course along the lengths of intrafusal fibers (Fig. 3A). Elastin was similarly distributed (Figs. 2C and 2G), except that thinner strands were more numerous. Even though not strictly applicable to each intrafusal fiber, on average elastin and fibrillin were more prevalent and occurred more often in distinct strands at the equator than at the pole (Figs. 2B and 2C versus Figs. 2F and 2G). None of the three intrafusal fiber types had significantly more elastin and fibrillin associated with it than the other two. There was strong immunostaining for the intermediate filament vimentin (Fig. 2E), comparable in location to that for elastin and fibrillin.

From the network of elastin and fibrillin around intrafusal fibers, slender extensions projected across the periaxial space in the direction of the outer cap-
sule. They did not traverse the space in a fixed plane but meandered in and out of the field of view. Ultimately, however, they appeared to connect to the outer capsule (Figs. 4A and 4B). Extensions of fibrillin were more frequently observed than were similar extensions of elastin.

**EM Immunolocalization**

**Extrafusal fibers.** Labeling was present just external to extrafusal fibers and their basal laminas. In support of findings obtained with the light microscope, immunoreactivity was also observed in the perimysium, epimysium and perineurium; however, labelling was often less extensive than in the immunohistochemical sections. Elastin label was relatively sparse and concentrated in the more interior of the elastin–fibrillin complex (Fig. 5A), whereas fibrillin label was abundant on, and principally restricted to, outlying microfibrils (Fig. 5B). There was a greater density of gold particles in tissue incubated with anti-fibrillin than in tissue incubated with anti-elastin.

**Muscle spindles.** Fibrillin label was located along the periphery of the inner capsule. It was deposited around oval patches of elastin (Fig. 5C), typically interconnected by narrower labeled strips (Figs. 3B). No instances were encountered in which the label clearly reached the basal lamina of intrafusal fibers. Labeling for elastin was largely unsuccessful, but elastin fibrils could always be recognized by their amorphous structure (Figs. 5A and 5C). As in the immunohistochemical sections, no difference was observed in the distribution and amount of the two molecules around different types of intrafusal fiber.

In accordance with immunofluorescence data, thin and curved labeled extensions were seen to project from immunopositive regions immediately adjacent to intrafusal fibers toward the outer capsule (Figs. 4A and 4B). In these extensions, fibrillin was more strongly represented than elastin.

**DISCUSSION**

With the immunohistochemical methods employed here, it has been demonstrated that elastin and elastin-
TABLE 2. Defining Criteria of Intrafusal Fibers

<table>
<thead>
<tr>
<th>Nuclear Fibers</th>
<th>mATPase pH 4.6</th>
<th>mATPase pH 9.6</th>
<th>Anti-MHC Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bag₁</td>
<td>++</td>
<td>-</td>
<td>MY32 (+)</td>
</tr>
<tr>
<td>Bag₂</td>
<td>++</td>
<td>-</td>
<td>2B6 (+)</td>
</tr>
<tr>
<td>Chain</td>
<td>-</td>
<td>+</td>
<td>CA83 (+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ALD58 (+)</td>
</tr>
</tbody>
</table>

In the muscles examined here, elastin and fibrillin formed cylindrical sleeves that clothed all extrafusal muscle fibers. They approximated in shape and location sleeves of endomysium and basal lamina. Electron microscopic immunolocalization indicated that the fibrillin-elastin network was positioned external to the basal lamina within the endomysium, but it probably had no extensive direct linkage with the basal lamina. There is evidence that at the dermal–epidermal junction, microfibrils do attach to basal lamina. Force transmission from contractile elements to connective tissues is not limited to the distal portions of muscle fibers that connect at myotendinous junctions but also occurs at lateral surfaces of muscle fibers via the endomysium, perhaps in association with costameres. Even though it is not known how elastin and fibrillin interconnect with the basal lamina and the endomysial layer, both are potential links in the chain of force transmission from muscle fiber to tendon. Differences in conformational states of cross-linking exist in elastin during length changes, and closed and open conformations have been postulated for fibrillin. Because of these properties, both molecules could dampen excessive force transfer.

Gold immunolabeling was more readily accomplished with anti-fibrillin than with anti-elastin. When present, label was confined to the periphery of the elastin fibril, neither entering its center nor extending to surrounding microfibrils. The lesser success with anti-elastin may be a penetration problem related to numerous cross-linkages. Moreover, fixation and subsequent preparation for electron microscopy may have damaged the epitope. Despite low levels of label, elastin could be always unequivocally identified at the ultrastructural level because of its constant association with microfibrils.

Cooper and Gladden found in histologic sections of mammalian spindles from nonextraocular muscles more elastic fibers around nuclear bag fibers than around nuclear chain fibers. Preferential location of elastin around nuclear bag fibers supports the notion that the rising and declining limbs of the dynamic phase of the afferent signal are imparted by the viscoelastic properties of this intrafusal fiber, aided by an external extensible connective tissue skele-

**Figure 3.** (A) Three-dimensional reconstruction from serial fluorescing cross sections of one ring of elastin–fibrillin surrounding one intrafusal fiber near the equatorial region. Black areas represent regions where strong staining predominates and most thicker strands are located. Light areas denote regions where staining is weak or absent and thinner strands prevail. To preserve clarity, the intrafusal fiber has not been drawn. (B) Schematic drawing illustrating in cross-section the spatial relations of intrafusal fiber (IF), basal lamina (BL), inner capsule (IC, stipple), and the elastin–fibrillin network (EFN). Arrows point to equivalent regions in A and B.
was a greater reactivity for fibrillin and elastin at the equator than at the pole. This observation is consistent with the thinning and eventual termination of the inner capsule in the polar region. The functional implication relating to this architecture is that after releasing them from a stretch, the greatest recoil potential for intrafusal fibers is available at the sensory region of the equator. As a corollary, release from stretch would remove tension from the primary after-

FIGURE 5. Electron microscopic immunolocalization (5 nm gold) of elastin (EL; A) and fibrillin (FI; B) around extrafusal fibers, and of fibrillin around intrafusal fibers (C). Label for elastin is confined to the periphery of an elastin-fibrillin strand and rarely reaches its core (x). Fibrillin label is concentrated in microfibrils (arrows) surrounding the elastin core. Inset in (C) is a fluorescing section that shows a ring of fibrillin surrounding a single intrafusal fiber. A small circle marks the location where the center of the intrafusal fiber would be. A double arrow points to a single elastin-fibrillin strand as it appears at the light microscope and electron microscope levels. Elastin-fibrillin complexes connect with neighboring like complexes via thinner lamellae of fibrillin and elastin. Bar in inset of panel C = 10 \mu m. Bar in (A) also applies to (B). IC = Inner capsule.

A condition that applied to most intrafusal fibers was a greater reactivity for fibrillin and elastin at the equator than at the pole. This observation is consistent with the thinning and eventual termination of the inner capsule in the polar region. The functional implication relating to this architecture is that after releasing them from a stretch, the greatest recoil potential for intrafusal fibers is available at the sensory region of the equator. As a corollary, release from stretch would remove tension from the primary after-

FIGURE 4. Panels on right show electron microscopic immunolocalization of fibrillin (5 nm gold) in the periaxial space (ps) and the outer spindle capsule (oc). Immunofluorescing sections to the left illustrate in each case the appearance at the light microscope level. Small circles mark the position where centers of intrafusal fibers would be. (A) Thin strands of fibrillin (arrowheads) extend from the inner capsule or from fibrillin—elastin complexes around intrafusal fibers (arrows) across the periaxial space. (B) Ultimately, the strands connect to the outer capsule. Frequently, the strands pursue a wavy course, thus passing in and out of the plane of view (arrowheads in periaxial space, right panel). Bar for fluorescing sections = 20 \mu m. pm = Perimysium.
ent and change its discharge from the higher level during the stretch to a basal rate. The strong staining in the inner capsule for vimentin, an intermediate filament occurring in fibroblasts, indicates the presence of a population of fibroblasts or specialized inner capsule cells. The coincidence in location of cells and microfibrils suggests that fibrillin and elastin are products of these cells.

Although the periaxial space in extraocular spindles of some species is diminutive, in the bovine muscles examined here it is voluminous. At the equator of these spindles, the intrafusal fiber bundle is separated from the outer capsule by as much as 100 \( \mu \)m. To be an effective sensor of muscle length, it would appear that intrafusal fibers and the associated sensory endings need to maintain a reasonable parallel alignment with extrafusal fibers. Hyaluronate within the periaxial space could orient the transducing elements to some extent. Anchorage of the intrafusal fiber bundle may be accomplished via connections from the inner to the outer capsules, involving modified inner or outer capsule cells, or fibroblasts. Our data suggest that struts containing fibrillin and elastin also play a role in suspending intrafusal fibers within the periaxial space (Fig. 6). There are no large amounts of fibrillin or elastin within the outer capsule, and it has yet to be determined how fibrillin bonds to other capsular matrix. The undulating nature of the struts indicates that the bracing is not rigid but allows for some movement in each plane. This arrangement could compensate for changing conditions, permitting the intrafusal fiber bundle to slide back and forth in relation to the outer capsule when the gross muscle is lengthened in response to an applied pull, or when lateral pressure is applied.

**Key Words**

extraocular muscles, muscle spindles, elastin–fibrillin network, electron microscopic immunolocalization, mechanical properties

**Acknowledgments**

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**References**

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