The Distribution of Myosin Heavy Chain Isoforms among Rat Extraocular Muscle Fiber Types

Neal A. Rubinstein¹ and Joseph Foon Yoong Hob²

PURPOSE. To determine the distribution of myosin heavy chain isoforms in each extraocular muscle (EOM) fiber type.

METHODS. Serial sections of adult rat EOMs were stained with isoform-specific monoclonal antibodies against an array of myosin heavy chains. Immunofluorescent antibody staining of whole adult rat EOMs, examined by confocal microscopy, demonstrated the longitudinal variations of isoforms along individual fibers.

RESULTS. Each global fiber type reacted predominantly with a single isoform-specific antibody and showed no longitudinal variation. Two major orbital fibers were defined, and both contained multiple myosin heavy chains. Both orbital singly and multiply innervated fibers stained proximal and distal to the neuromuscular junction with antibody to embryonic myosin heavy chain, but this isoform was sharply and completely excluded from the domain of the neuromuscular junction. Orbital singly innervated fibers also contained the EOM-specific isoform at the neuromuscular junction. Orbital multiply innervated fibers did not contain the EOM-specific isoform, but additionally contained a slow isoform along their entire length.

CONCLUSIONS. Adult rat EOMs show unique fiber types with arrangements of myosin heavy chain isoforms not seen in other skeletal muscles. Moreover, unique cellular mechanisms must exist to target each isoform to its proper domain along individual orbital fibers. (Invest Ophthalmol Vis Sci. 2000;41:3391–3398)

Cell diversity is a hallmark of vertebrate skeletal muscles. Adult muscles are composed of a variety of cell or fiber types, each characterized by distinct physiological and metabolic properties and by the synthesis of distinct myosin heavy chain (MyHC) isoforms. MyHC isoforms, however, are not just markers for fiber typing. Differences in ATPase activity, shortening velocity, force generation, and thermodynamic efficiency observed between individual fiber types correlate directly with the presence of specific MyHC isoforms.¹

Although vertebrate limb muscles have always been the paradigm for studies of myogenesis, several other muscle groups have functional specializations and patterns of MyHC gene expression distinct from those seen in limb muscles.² The patterns of gene expression in these muscle “allotypes” appear to be intrinsically controlled. Among these atypical muscle groups are the extraocular muscles (EOMs). EOMs differ in functionally significant ways from skeletal muscles. Most conspicuously, the morphologically, physiologically, or biochemically defined fiber types in EOMs do not correspond to those in skeletal muscles.³

For example, in addition to adult MyHC isoforms found in skeletal muscles, EOMs also synthesize a slow-tonic MyHC⁴ and an EOM-specific MyHC,⁵,⁶ as well as isoforms normally found only in developing fibers. Their fibers have shorter contraction times but lower tension generation than fibers in other skeletal muscles.⁷ Their combination of fast contractile properties and high oxidative capacity with high fatigue resistance is unusual among skeletal muscles and further emphasizes the singular complexity of these muscles. Moreover, in both the global and orbital layers of the EOMs, multiply innervated fibers are found, fibers unseen in other skeletal muscles. Finally, some EOM fibers synthesize multiple MyHC isoforms and may localize them differentially along the length of the fiber.⁸

The ability of EOMs to perform their wide range of required motions is dependent on the contractile properties of the individual fibers; these properties, in turn, are dependent to a large degree on the specific complement of MyHC isoforms expressed in each fiber. Before one can understand the contributions that unusual fiber types can make to both the physiology and disease susceptibility of EOMs, we must clarify the relationship between the MyHC isoforms and the three-dimensional architecture of the EOMs. Similarly, questions of gene regulation mechanisms make it important to determine whether EOM fibers synthesize one or more MyHCs, which isoforms are localized differentially along individual fibers, and whether they are responsive to external cues in a manner unique to these muscles. In this study we used a battery of monoclonal antibodies to investigate the localization of MyHC isoforms among rat EOM fiber types.

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METHODS

Immunohistochemistry

Isolated adult female rat orbits were sectioned and stained with isoform-specific antimyosin antibodies. Antibodies against the following MyHC isoforms were used: embryonic (2B6)\(^9\); Ila (SC-71)\(^10\); Iib (BF-F3)\(^10\); slow twitch (β) (NOQ7\(^{5–4D}\))\(^11\); slow tonic (HV11 and NA8), Everett Bandman, unpublished data; and aALD\(^12\); EOM-specific (4A6) \(^13\); α\(^2\)cardiac (F8812F8) \(^14\), and neonatal (BF-34, Stefano Schiaffino, unpublished data). Some muscles were also stained with Ta51, an antibody against neurofilaments (Virginia Lee, unpublished data). Frozen sections were preincubated with 5% goat serum, washed, and incubated in appropriate concentrations of primary antibody. After additional washing, sections were reacted with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG and examined in a fluorescence microscope (DM IRB Leica; Deerfield, IL). Some muscles were also stained with Texas-red labeled phalloidin, which reacts with actin filaments. Animals were maintained in approved accommodations at the School of Medicine, University of Pennsylvania and used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Confocal Microscopy of Whole EOMs

The protocol for staining whole EOM muscles was adapted from one provided by Patricia Laboski, modified from the procedure of Dent et al.\(^15\). Briefly, EOMs were exposed in isolated orbits and glycerinated in skinnning solution (140 mM KCl, 8 mM MgCl\(_2\), 5 mM Na\(_2\) adenosine triphosphate (ATP), 30 mM MOPS, and 4 mM EGTA, pH 7.1) for 48 hours. Muscles were then excised, rinsed in phosphate-buffered saline (PBS), and exposed to collagenase (type II, no. 6885; Sigma), 36° for 30 minutes, to increase permeability across the muscle. After washing with PBS, muscles were preincubated in PBS containing 2% nonfat dry milk and 0.5% Triton X-100 (PBSMT). Muscles were stained overnight in PBSMT containing the appropriate dilution of primary antibody, washed thoroughly, and exposed overnight to an FITC-labeled goat anti-mouse anti-body. Muscles were examined by a laser scanning confocal microscope, LSM 510 (Carl Zeiss, Oberkochen, Germany).

RESULTS

Distribution of MyHC Isoforms among EOM Fibers

The EOMs of adult rats expressed mRNAs for at least eight distinct skeletal MyHC isoforms: cardiac MyHCs (α and β), the three adult rat fast-twitch MyHCs (IIa, IIb, and IIx), the developmental isoforms (embryonic and neonatal), and a tissue specific EOM-MyHC\(^6,16\) (Rubinstein, unpublished data). We used isoform-specific anti-MyHC monoclonal antibodies to localize seven of these isoforms within adult rat EOM fibers.

MyHC Content of Global Fibers

Anti-MyHC immunohistochemistry distinguished four fiber types in the global region of rat EOMs. Each global fiber type stained predominantly with one antibody, although we cannot rule out small amounts of additional MyHCs undetectable by this method. In the global region, there did not appear to be any longitudinal variation in staining, and the antibodies showed a distinct pattern of distribution for each fiber type.

Antibody against the Iib MyHC stained a number of large fibers in the core of the global region (Fig. 1A). This antibody reacted with neither fibers in the orbital region nor fibers in the area bordering the orbital region. Antibody to the Ila MyHC, by contrast, stained smaller fibers that were scattered predominantly around the periphery of the global region and that surrounded the core of fibers containing the Iib MyHC (Fig. 1B). Few Ila positive fibers were found in the IIb “core” of the global region. As with the antibody to MyHC Iib, anti-Ila MyHC stained global fibers throughout their length. A small number of fibers in the orbital region, especially some small fibers close to the orbital-global boundary, also reacted with this antibody (see further discussion later).

A third set of fibers in the global region reacted with the antibody to slow-twitch (β-cardiac) MyHC (Fig. 1C). These
large fibers were scattered throughout the global region, without the distinct cross-sectional localization seen for both the IIa and IIb MyHCs. Similar to these other isoforms, however, the slow-twitch isoform showed no differential localization along the fiber. Although in the rabbit, these fibers contain the α-cardiac MyHC,17 this was not true in the rat (see later discussion). This antibody also stained a set of smaller fibers in the orbital region. Finally, staining the global region with a mixture of antibodies against the IIa, IIb, and slow-twitch isoforms resulted in a number of widely scattered fibers that failed to stain (Fig. 1D). These fibers also did not react with antibodies to embryonic, α2 cardiac, slow tonic, or EOM-specific MyHCs (see Fig. 4). Because polymerase chain reaction (PCR) and gel electrophoresis showed the presence of IIx MyHC and because our recent quantitative PCR studies have shown that IIx comprises approximately 15% of the MyHC isoforms in EOMs (unpublished data), we propose that these unreactive fibers seen in the global region of Figure 1D contain the IIx MyHC. It is possible, however, that these fibers contain a distinct, as yet unrecognized, isoform. Also, because this is a negative reaction, the possibility that the IIx isoform also exists in other fibers of the global and orbital region—in the presence of other isoforms—cannot be discounted.

MyHC Content of Orbital EOM Fibers

As others have described, the fibers of the orbital region were more complex. In most sections along the muscle, most orbital fibers reacted with the antibody to the embryonic MyHC (Fig. 4B). In one discrete area, however, most likely the region of the neuromuscular junction (see later discussion), almost all fibers failed to react with this antibody (Fig. 4A). Conversely, at this same area, most orbital fibers reacted with the antibody to the EOM-specific MyHC (Fig. 4C). Several sections distally,
however, staining with this antibody began to fade from most fibers (Fig. 4D). In general, fibers in the global region did not react with the antibodies to embryonic or extraocular MyHCs and neither did fibers in the levator palpebrae. Occasional scattered fibers in the global region showed some inconsistent reaction with one or the other of these antibodies. Because the great majority of the orbital fibers reacted with both these antibodies, we equate them with the orbital singly innervated fibers (oSIFs) which reportedly comprise 80% of orbital fibers.3

A smaller percentage of fibers did not react with anti-extraocular MyHC antibody, but instead reacted with the anti-slow-twitch MyHC antibody (Fig. 1C) and showed no longitudinal variation in staining with this antibody. Away from the neuromuscular junction, these reacted with the anti-embryonic MyHC antibody. These are presumably the oMIFs. A small number of fibers, mostly close to the orbital–global border, stained with antibody to the Ila MyHC (Fig. 1B). These fibers did not react with antibody to the slow-twitch MyHC, but reacted with embryonic MyHC in the same proximal–distal manner that other orbital fibers reacted and with antibody to the EOM-specific isoform near the motor endplate (Fig. 4D).

To confirm and to delineate more precisely the longitudinal variation of MyHC isoforms along orbital fibers, we combined fluorescent antibody staining of whole EOMs with confocal microscopy. Figure 5A shows the orbital region of a rat EOM stained with the antibody to embryonic MyHC. This antibody stained a majority of fibers in the orbital region, as seen in the cross sections; however, there was a sharp exclusion of staining at the putative neuromuscular junction region, although staining was present throughout the remainder of the fiber.

Staining a whole muscle with antibody to the extraocular MyHC gave an approximate complementary pattern of localization (Fig. 5B). Reactivity occurred most strongly at the putative neuromuscular junction and in a wide swath to either side, then rapidly faded toward the end of the muscle layer. The domain occupied by EOM-MyHC was not so sharply delineated as was the embryonic MyHC. A possible correlation of previously described orbital fiber types and their MyHC isoforms is shown in Table 1.

The area devoid of staining with the anti-embryonic MyHC antibody (Fig. 5A) was related to the neuromuscular junction.

<table>
<thead>
<tr>
<th>Fiber Type*</th>
<th>Predominant MyHC</th>
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<tbody>
<tr>
<td>gMIF</td>
<td>Slow</td>
</tr>
<tr>
<td>gSIF-red</td>
<td>Ila</td>
</tr>
<tr>
<td>gSIF-intermediate</td>
<td>IIX</td>
</tr>
<tr>
<td>gSIF-white</td>
<td>IIB</td>
</tr>
<tr>
<td>oSIF</td>
<td>Embryonic/EOM-specific</td>
</tr>
<tr>
<td>oMIF</td>
<td>Embryonic/Slow</td>
</tr>
</tbody>
</table>

* Spencer and Porter.18

**TABLE 1.** Correlation between MyHC and Histochemically Defined Fiber Types in Rat EOMs

![Figure 4. Indirect immunofluorescent staining of semi-serial sections of adult rat EOMs with antibodies (A, B) 2b6 to embryonic MyHC, (C, D) 4A6 to EO-specific MyHC, and (E) F8812F8 to α-cardiac MyHC. Sections were made near (A, C) the neuromuscular junction or distal (B, D, E) to the neuromuscular junction. Only orbital fibers react appreciably with these antibodies.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932910/ on 10/18/2018)
Under the light microscope, we could visualize the motoneuron entering this specific region. To confirm this, we double stained EOMs with FITC-labeled anti-embryonic MyHC antibody (Fig. 6A) and with Texas red-labeled Ta51, a monoclonal antibody to neurofilaments (Fig. 6B). Figure 6C is a superimposition of Figures 6A and 6B. This shows the putative neuromuscular junction area with embryonic MyHC positive fibers approaching from both sides. The great majority of neurofilaments were located in the region devoid of staining with the anti-embryonic MyHC antibody, strongly suggesting that this region is the location of the neuromuscular junction. Staining with Texas red–labeled α-bungarotoxin confirmed this finding.

The orbital fibers which were positive for embryonic MyHC continue into the neuromuscular junction region, and the cessation of anti-embryonic MyHC staining did not mark the end of the fibers. Figures 5C, 5D, and 5E show an enlargement of fibers at the edge of the neuromuscular junction region, approximately the area within the box in Figure 5A. Muscles were double stained with FITC-labeled anti-embryonic MyHC antibody (Fig. 5C) and with Texas red-labeled phalloidin (Fig. 5D). Figure 5E is a superimposition of both stains. Staining with anti-embryonic MyHC faded rapidly as the fibers entered the region of the neuromuscular junction. The fibers that reacted with the anti-embryonic MyHC antibody, however, continued into the neuromuscular junction region, which was devoid of staining with the anti-embryonic antibody, but remained positive with the phalloidin (long arrows).

Because others have shown that the orbital slow fibers may contain some slow tonic MyHC, we tested a number of polyclonal and monoclonal antibodies against the slow tonic MyHC. Unfortunately, all the slow tonic antibodies reacted with the same fibers stained by NOQ7-5–4D, the antibody to the slow-twitch MyHC. Therefore, we cannot differentiate between slow-twitch and slow tonic fibers. Moreover, staining sections with BF-34, an antibody against the neonatal MyHC did not give clean results. All fibers, except those reacting with the antibody to slow-twitch MyHC, reacted with BF-34. Unfortunately, other skeletal muscles showed the same pattern of staining, even though PCR did not identify the neonatal MyHC mRNA in those muscles. Therefore, it is also not possible to localize the neonatal isoform, although others have suggested a distribution similar to the one we have described for the embryonic MyHC.

Because PCR showed the presence of α-cardiac MyHC mRNA (Rubinstein, unpublished results), we used F8812F8 to stain sections and discovered a small population of cells, possibly oMIFs, which additionally reacted with that antibody (Fig. 4E). No global region fibers in the rat reacted with the anti-α-cardiac MyHC antibody.
DISCUSSION

Spencer and Porter\textsuperscript{18} have used the multiple criteria of location, pattern of innervation, and metabolic “color” to define six distinct fiber types in EOMs. This scheme is consistent in all mammals studied to date. In the orbital layer, 80% of the fibers are singly innervated (oSIFs). The remaining fibers in the orbital layer are multiply innervated (oMIFs). The global layer contains an additional four fiber types. Three are singly innervated fibers, which show differences in oxidative metabolic enzyme concentrations and size. These are the global red, intermediate, and white singly innervated fibers (gSIFs). Approximately 10% of the global fibers are multiply innervated (gMIFs).

We suggest that each of the global fiber types may correlate with the synthesis of one—or predominantly one—MyHC isoform synthesized through the length of the fiber (see Table 1), and evidence from the literature supports this correlation. Brueckner et al.\textsuperscript{19} have shown that the global red fibers have a pattern similar to our staining pattern with anti-IIa MyHC antibody. Both their work and ours have shown that the Iib isoform is the predominant one in the global region. Moreover, our pattern of staining with the anti-slow-twitch MyHC antibody parallels the pattern seen by a number of investigators for both gMIFs and oMIFs.\textsuperscript{5,19,20}

Both major orbital layer fiber types contain multiple MyHCs, which are arranged independently along the fiber. Both contain the embryonic MyHC proximal and distal to the motor endplate. The majority—presumably the oSIFs—contain the EOM specific MyHC at the motor endplate, while a smaller number—presumably the oMIFs—contain throughout their length MyHC(s) reactive against our anti-slow antibody. While the longitudinal variation of MyHC isoforms in orbital cells has been previously demonstrated, the precise isoforms involved and their domains have not previously been known. Our results are consistent with those of Brueckner et al.\textsuperscript{19} who used a cDNA probe to show that the EOM specific isoform was found only in the orbital region. They also showed that an antibody to developmental isoforms reacted proximal and distal to the neuromuscular junction with little reaction around the neuromuscular junction.

Our results for orbital fibers are also consistent with those described by Jacoby et al.\textsuperscript{8} for rat EOMs. In their study, all orbital fibers reacted with an antibody that recognized embryonic and/or neonatal MyHC away from the endplate region; oSIFs also reacted along the full length with an antibody that recognized all fast isoforms, while oMIFs also reacted with this antibody, but only in the endplate region. Presumably, the fast isoform reacting with their generic anti-fast MyHC antibody at the motor endplate was the tissue specific extraocular isoform.

\begin{figure}
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\includegraphics[width=\textwidth]{image.png}
\caption{Confocal images of the neuromuscular junction region of a rat EOM orbital layer. (A) Indirect immunofluorescence staining with FITC-labeled antibody to the embryonic MyHC; (B) Indirect immunofluorescence staining with Texas red labeled antibody to neurofilaments. (C) Superimposition of images from (A) and (B).}
\end{figure}
They dissected individual fibers and demonstrated a continuation of myofibrils from stained to non-stained regions across the putative neuromuscular junction region of orbital fibers. Their demonstration of a gap in staining with an antibody to developmental MyHCs in the presumptive neuromuscular junction area correlates exactly with our results.

In summary, the data support the notion that in the rat, oSIFs express EOM-specific MyHC principally in the endplate region, while the embryonic MyHC is excluded from the endplate region but is present in the rest of the fiber. The distribution of MyHC types along the length of oMIFs is less clear. This fiber type has ultrastructural characteristics of fast fibers at the endplate region, but slow-tonic characteristics in the rest of the fiber. While fast MyHC reactivity in oMIF is confined to the endplate region, we additionally found uniform staining of these fibers with our anti-slow antibody. This finding cannot be interpreted to mean that slow-twitch MyHC is uniformly distributed along the length; this antibody may cross-react with the slow-tonic MyHC which may be present away from the endplate region.

There are species differences in the distribution of MyHC isoforms in EOM fibers. In the rabbit, EOM MyHC is seen only in the global region, although it is localized predominantly in the orbital region in the rat. Thus, in the rabbit, some other fast isoform must take the place of the EOM-specific MyHC we found in the motor endplate region of rat oSIFs. In the rabbit, the slow-twitch or slow-tonic MyHC, with the alpha-cardiac MyHC present in only a few orbital fibers.

What is the significance of the considerable greater complexity of MyHCs in EOMs compared with limb muscles? Recent measurements on single EOM fibers have shown a very wide dynamic range of the mechanical parameter, fmin, which reflects the kinetics of cross-bridge cycling (Li, Rossmanith and Hoh, unpublished observations). Fmin values below and above those found in limb muscle fibers were obtained from EOM fibers and may be related to the presence of embryonic/neonatal and EOM-specific MyHCs, respectively, which are absent in adult limb muscle fibers.

A common feature of orbital fibers is the occurrence of a kinetically fast central segment (the endplate region) flanked by kinetically slower segments. In the case of oSIFs, the central segment contains EOM-specific MyHC associated with high speed and high fmin (Li et al.), while the flanking segments contain embryonic MyHC associated with slow speed and low fmin (Li et al.). For oMIFs, the central segment has fast-twitch electrophysiological properties and high sarcoplasmic reticulum volume which ensures rapid release and uptake of Ca2+. These arrangements in orbital EOM fibers may permit rapid changes in gage. Consider that these fibers are activated to hold the globe in a given position, and a need arises to change the gage in the direction which involves lengthening these fibers, as during a saccade in the ‘off’ direction. Without the fast central segment, the slow cross-bridges in these fibers would resist rapid lengthening by developing high tensions as in fibers undergoing eccentric contractions. By having a central segment which can relax more rapidly, lengthening can be accommodated initially by the sarcomeres within this region without stretching the slower segments.

Although longitudinal variation of isoforms is not the rule in skeletal muscles, several other examples do exist. The extrafusal nuclear bag fibers of the rat show similar longitudinal variations; denervation of the developing fibers results in the synthesis of the same MyHC isoforms, but an absence of longitudinal differences. It is also possible that the EOM MyHC might serve a purpose other than movement. The EOMs of billfish contain an outer layer of thermogenic cells, the heater cells, which are specialized to warm the overlying brain. Although they contain contractile proteins, they fail to assemble them into myofibrils and lack the highly organized contractile structures typical of other muscle cells. Moreover, in some of these heater cells the ends of the cells do contain assembled myofibrils while the centers do not. These cells, then, have a phenotype with a typical contractile appearance at one end and the thermogenic (few myofibrils, extensive SR and mitochondria) appearance at the other. This is analogous to the longitudinal variation we have seen in orbital fibers of mammalian EOMs; and these heater cells may be the forebears of unusual fibers seen in today’s EOMs.

In these thermogenic cells of the billfish as well as in the orbital fibers with their longitudinal variation, there must be unique mechanisms that regulate the synthesis and assembly of one set of muscle specific proteins at the neuromuscular junction and a distinct set in the extrajunctional area. Whether this is related to specific synthetic capacities of junctional versus non-junctional nuclei or to some other sorting mechanism remains to be evaluated.

Acknowledgments

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