Review

Immunohistochemical Classification of Skeletal Muscle Fibers

Aiko Hori¹, Akihiko Ishihara², Shigeo Kobayashi¹ and Yasuhiko Ibata³

¹Department of Bio-informatics, Graduate School of Informatics, ²Laboratory of Neurochemistry, Faculty of Integrated Human Studies, Kyoto University, Kyoto 606–8501 and ³Department of Anatomy and Neurobiology, Kyoto Prefectural University of Medicine, Kyoto 602–0841

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Mammalian skeletal muscle fibers have several types of myosin heavy chain (MHC) isoforms. Immunohistochemical techniques enable us to identify the MHC isoform type in a single muscle fiber, i.e., MHCI, MHCIIa, MHCIIx, or MHCIIb. In addition, hybrid single fibers having more than one MHC isoform type can be classified using immunohistochemistry. The presence of different MHC isoform(s) in a single fiber correlates

with the maximal contractile velocity and the enzyme histochemical reaction of the myofibrillar adenosine triphosphatase. The adaptive response of the skeletal muscle is the basis of the fiber-type transformation depending on the phenotypic expression pattern in several MHC isoforms. In this review article, we address the availability of the monoclonal antibodies which recognize specific MHC isoform(s) in a single fiber.

Key words: Enzyme histochemistry, Immunohistochemistry, Muscle fiber type, Myosin heavy chain isoform, Skeletal muscle

I. Introduction

Myosin heavy chain (MHC) in mammalian skeletal muscles has several types of isoforms [68, 69], which enables us to classify muscle fiber types depending on the MHC diversity. MHC isoforms of a muscle or a single fiber can be detected using electrophoresis or immunohistochemistry.

Immunohistochemistry has the advantage of being able to show the coexistence of multiple MHC isoforms within a single fiber in skeletal muscle. Although the regulation of muscle fiber-type transformation has not yet been clarified, immunohistochemical analyses will provide useful information for unsolved problems regarding muscle fiber-type plasticity. In this review article, we address the availability of the monoclonal antibodies which recognize specific MHC isoform(s) in a single fiber.

II. MHC Isoform of Muscle Fiber

Myosin consists of six subunits; two heavy chains and two pairs of light chains. MHC is the major myofibrillar protein, and its adenosine triphosphatase (ATPase) activity is required for the contractile activity of muscle fiber. MHC protein is a product of a multigene family. At present, nine isoforms are identified as skeletal muscle MHC isoforms at both the protein and mRNA levels; MHCemb (embryonic), MHCneo (neonatal), MHCI (MHCI β), MHCIIa, MHCIIx (or MHCIIId), MHCIIb, MHCEO (extraocular), MHCm (masticatory or mandibular), and MHC α (α -cardiac-like) [19, 62, 69, 94]. Among them, the major isoforms in adult skeletal muscles are MHCI, MHCIIa, and MHCIIb. These MHC isoforms have been separated using electrophoretic techniques [21] and coded by three distinct genes [41]. Thereafter, a fourth MHC isoform in adult skeletal muscles called MHCIIx (or MHCIIId) has been investigated [7, 48, 70]. There are also some reports regarding MHC isoform variants such as MHCItan (slow-tonic) [49] and MHCIIa [26, 28], which have not yet been cloned.

Myosin forms bipolar thick filaments that interdigitate with thin actin filaments in the sarcomere, forming

Correspondence to: Dr. Akihiko Ishihara, Laboratory of Neurochemistry, Faculty of Integrated Human Studies, Kyoto University, Sakyo-ku, Kyoto 606–8501, Japan.

the contractile unit of muscle fiber. In the myosin head near the ATP- and actin-binding sites, there are two regions which are flexible in structure and variable in amino acid sequence [6, 75]. These two regions in the motor domain of myosin are thought to produce the difference in the maximum velocity of the myosin molecule during movement along a single myosin head, which leads to the correlation between the ATPase activity and the speed of shortening in both the whole muscle [8] and the skinned muscle fiber [13, 15]. Therefore, the presence of specific MHC isoform(s) is related to muscle fiber contractile properties, such that the maximal contractile velocities are in the rank order of MHCI < MHCIIa < MHCIIx < MHCIIb [13]. Even in hybrid fibers in which multiple MHC isoforms coexist, the contractile property depends on the proportion of the MHC isoform types in the hybrid fiber [14]. Functional differences of MHC isoforms in skeletal muscle have been extensively investigated [38, 86].

Some other proteins such as myosin light chain, troponin, actin, sarcoplasmic (endoplasmic) reticulum Ca^{2+} -ATPase (SERCA), and parvalbumin contribute to the identification of specific fiber types and have different isoform expressions between slow- and fast-twitch fibers [56, 69, 71, 74, 91, 95]. The functional diversity of the skeletal muscle is, however, most adequately represented by the MHC isoform distribution of the muscle.

III. Immunohistochemical Classification of Muscle Fiber

Mammalian skeletal muscles are composed of heterogeneous types of fibers based on their different MHC isoform expressions. This heterogeneity is present in different muscles of the same species and in the same muscles of different species.

MHC isoforms expressed in a single fiber can be determined by immunohistochemical techniques. Monoclonal antibodies (MAbs) for the classification of the four major MHC isoform types (MHCI, MHCIIa, MHCIIx, and MHCIIb) are used for immunohistochemistry, and the specificity of the MAbs is either for a single MHC isoform or for multiple MHC isoforms. Prior to a study, however, the specificity of MAbs in the subject animal should be tested, because some MAbs have interspecies variability in their specificities.

Figure 1 shows serial transverse sections of the rat soleus muscle stained with different MAbs (panels A to D), as well as for ATPase (panels E to G) and succinate

dehydrogenase (SDH) (panel H) activities. The specificity of these MAbs for rat MHC isoforms is summarized in Table 1. Rat soleus muscle, which is a typical slow-twitch muscle, contains mainly MHCI and MHCIIa [22, 85], and the proportions of these types change with postnatal development [83]. In the adult rat, approximately 80% of the total fibers in the muscle are stained positively with MAb Slow (specific for MHCI), and those fibers are marked as 1 (panel A). The rest of the fibers are stained positively with both MAb Fast (specific for all types of MHCII) and MAb 71 (specific for MHCIIa), and marked as 2 in panels B and C, respectively. None of the fibers are stained positively with MAb F3 (specific for MHCIIb, panel D), and all of the fibers are stained positively with MAb 35 (specific for all types of MHCs except MHCIIx, not shown). The staining pattern of each MAb is almost homogeneous, implying the diffuse distribution of MHC isoform molecules in the fiber cross-sectional area. The distribution of the MHC isoform along the fiber length is, however, reported to be nonuniform even in the skeletal muscle under normal conditions [80], and the nonuniformity increases under muscle fiber-type transformation [29, 80]. Note that two fibers marked as 3 are stained positively with MAbs Slow (panel A), Fast (panel B), and 71 (panel C), indicating that they are hybrid fibers character-

Table 1. Monoclonal antibody specificity

MAb	MHC isoform			
	I	IIa	IIx	IIb
Slow	+	—	—	—
Fast	—	+	+	+
71	—	+	—	—
F3	—	—	—	+
35	+	+	—	+
D9	—	—	+	+
N1.551	—	++	+/-	—
A4.1519	—	++	—	+/-

The specificity of each monoclonal antibody (MAb) bound to specific myosin heavy chain (MHC) isoforms in rat muscle fibers was determined by Bottinelli *et al.* [14] and Schiaffino *et al.* [70] and according to the instructions of the supplier. +, positive reaction for that MAb with that specific MHC isoform(s); —, no reaction between MAb and MHC isoform(s); ++, positive reaction with higher intensity; +/-, positive reaction with lower intensity. The MAbs used in this study were types IgG (Slow, Fast, 71, and 35) and IgM (F3, D9, N1.551, and A4.1519).

Fig. 1. Serial transverse sections of the soleus muscle of an 8-week-old rat. The sections are stained with the specific monoclonal antibodies against specific myosin heavy chain (MHC) isoforms (panels A–D, see Table 1 for specificity) and for ATPase activities following preincubations at pH 10.3 (panel E), 4.5 (panel F) and 4.3 (panel G) and for SDH activity (panel H). To visualize the monoclonal antibodies in the sections, fluorescein-labeled avidin D and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) are used for the anti-mouse IgG antibodies (panel A, Slow; panel B, Fast; panel C, 71) and the anti-mouse IgM antibodies (panel D, F3), respectively. Labels for fibers containing each MHC isoform: 1, MHCI; 2, MHCIIa; 3, hybrid fibers containing both MHCI and MHCIIa; labels for fibers enzyme-histochemically identified: 4, type I; 5, type IIA; 6, type IIC. Scale bar on panel H indicates 50 μm for all panels.

Fig. 1

ized by the coexistence of both MHCI and MHCIIa in varying ratios [77]. The proportion of different MHC isoforms within a single fiber is uncertain, since the immunohistochemical analysis is not quantitative because of the difference in the sensitivity of each MAb and in the different amplification processes.

The rat plantaris muscle is a typical fast-twitch muscle, and its serial transverse sections are shown in panels A to H of Fig. 2. It contains four major MHC isoforms [60]. The isoform proportions are different between the deep and superficial portions of the muscle, and they also change with postnatal development [84]. In the plantaris

Fig. 2. Serial transverse sections of the plantaris muscle of an 8-week-old rat. The sections are stained with the specific monoclonal antibodies against specific myosin heavy chain (MHC) isoforms (panels A–H, see Table 1 for specificity) and for ATPase activities following preincubations at pH 10.3 (panel I), 4.5 (panel J) and 4.3 (panel K) and for SDH activity (panel L). To visualize the monoclonal antibodies in the sections, fluorescein-labeled avidin D and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) are used for anti-mouse IgG antibodies (panel A, Slow; panel B, Fast; panel C, 71; panel E, 35) and anti-mouse IgM antibodies (panel D, F3; panel F, D9; panel G, N1.551; panel H, A4.1519), respectively. Labels for fibers containing each MHC isoform: 1, MHCI; 2, MHCIIa; 3, MHCIIb; 4, MHCIIx; 5 and 6, hybrid IIxa and IIax fibers containing both MHCIIa and MHCIIx, respectively; labels for fibers enzyme-histochemically identified: 7, type I; 8 (and 8̄), type IIA; 9 (and 9̄), type IIB (see text for details). Scale bar on panel L indicates 50 μ m for all panels.

muscle of the adult rat, as shown in panels A and B, about 15% of the fibers have MHCI in the deep portion (marked as 1), while the proportion of MHCI is less in the superficial portion of the muscle. The fibers containing MHCIIb (marked as 3) or MHCIIx (marked as 4) are predominant, and some fibers have MHCIIa (marked as 2). At present, a MAb specific for MHCIIx alone is not available, and therefore, the fibers containing only the MHCIIx can be identified with the negative staining for MAb 35 (specific for all types of MHCs except MHCIIx, panel E). The inconvenience is that MHCIIx included in the hybrid fibers cannot be precisely detected with only MAb 35. The combination of several MAbs is necessary for fiber-type classifications including MHCIIx fibers.

Four hybrid fibers are shown in Fig. 2. Three fibers marked as 5 are stained weakly with MAb 35 (panel E). They are positive for MAbs Fast (panel B), 71 (panel C), D9 (panel F), N1.551 (panel G), and A4.1519 (panel H), and negative for Slow (panel A) and F3 (panel D). This means that they have MHCIIa in addition to MHCIIx. Another hybrid fiber marked as 6 cannot be verified as having MHCIIx by the staining with MAb 35 (panel E). The positive staining for D9 (panel F) and the negative staining for F3 (panel D) reveal the possibility that this fiber has MHCIIx, probably in a small amount. We decided to name these fibers IIxa (marked as 5) and IIax (marked as 6) according to the proportion of the isoforms within a single fiber. For example, the fiber name "IIxa" means that the percentage of the MHCIIx is higher than that of the MHCIIa in a single fiber, while the fiber name "IIax" means that the percentage of the MHCIIa is higher than that of the MHCIIx in a single fiber.

IV. MHC Isoform and ATPase Histochemistry

The histochemical ATPase activities of single muscle fibers based on the differential responses to alkali and acid preincubations correspond to their MHC isoform profiles. This correspondence was analyzed in studies combining the immunohistochemistry and/or electrophoresis of either the whole muscle or the single muscle fiber [45, 70, 77, 78, 81, 93, 94].

Muscle fibers containing MHCI correspond to type I fibers, and muscle fibers containing MHCIIa correspond to type IIA fibers. Type IIB fibers determined by the ATPase staining reaction include MHCIIb and/or MHCIIx, and some modifications of the staining method using ATPase activity have been developed in order to distinguish MHCIIx from MHCIIb [31, 35, 65]. Type IIC fibers, which are active at all pHs throughout alkali to acid ATPase preincubations, correspond to hybrid fibers containing both MHCI and MHCII (mainly MHCIIa) [78]. In general, alkali-ATPase measures the fastest MHC isoform present in the fiber, whereas acid-ATPase reflects the average ATPase activity associated with the proportion of MHC isoform types within the fiber [90]. Interspecies differences in the pH stability of ATPase

activity have been noted [16, 17].

V. MHC Isoform and Metabolic Enzyme Histochemistry

A correlation between metabolic enzyme activities and MHC isoforms has been reported histochemically [44, 59] and biochemically [22]. The linkage between MHC isoforms and the metabolic properties is supported by their coordinated alterations under muscle fiber-type transformation [25, 44]. However, there is a report that individual motor unit fibers have uniform SDH activity even if they are composed of mixed MHC isoforms [72]. As there is no direct relation between the MHC molecule and metabolic enzyme activity, they may influence each other to achieve the best match for functional demand. Generally, oxidative enzyme activities, i.e., SDH and citrate synthase activities are reported to be in the rank order of MHCIIa > MHCI > MHCIIx > MHCIIb, and glycolytic enzyme activity, i.e., α -glycerophosphate dehydrogenase (α -GPD) activity is in the rank order of MHCIIb > MHCIIx > MHCIIa > MHCI [25].

VI. MHC Isoform Analysis by Other Methods

MHC proteins of muscle homogenates are analyzed with electrophoresis. Four major MHC isoforms in adult skeletal muscle are completely separated [85], and the rank order of migration is MHCI > MHCIIb > MHCIIx > MHCIIa. It should be noted that the electrophoretic mobility shows interspecies differences even within identically labeled isoforms [36]. Single fiber electrophoretic studies support the coexistence of multiple MHC isoforms in individual fibers [51, 93]. Previous studies have shown that the hybrid fiber is not an exception but exists as a standard [36, 56, 69].

MHC isoforms are also identified at the mRNA level [12, 20, 23, 42]. Hybrid fibers are detected as the coexistence of multiple MHC mRNA by *in situ* hybridization [12, 23] and reverse-transcriptase polymerase chain reaction (RT-PCR) directly from single fibers [57]. Combined studies with immuno- or enzyme-histochemistry have confirmed the correspondence of MHC protein and mRNA [23, 57]. During muscle fiber-type transformation, however, the mismatch between the content of MHC protein and the expression of mRNA occurs in transform-

Table 2. Adenosine triphosphatase activity

Preincubation pH	Fiber type			
	I	IIA	IIB	IIC
10.3	—	+	+	+
4.5	+	—	+	+
4.3	+	—	—	+

The fiber type classification was determined by Brooke and Kaiser [16–18]. +, positive reaction; —, no reaction.

ing fibers [2, 12]. The distribution of MHC mRNA along the fiber length is also reported to be nonuniform [57].

VII. Classical Identification of Muscle Fiber

Based on the differences in the ATPase activity following preincubations at different pHs, skeletal muscle fibers are classified into two major types; acid-stable and alkali-labile type I (slow-twitch) and acid-labile and alkali-stable type II (fast-twitch) [34]. Type II fibers are further subclassified into type IIA and type IIB according to their ATPase stabilities in the acid range; type IIA fibers become ATPase-negative prior to type IIB fibers when the acidity of the preincubation medium increases [16–18] (Table 2).

Figure 1 shows the correspondence between the expression of MHC isoforms and the staining pattern of ATPase in the rat soleus muscle. The fibers containing MHCI marked as 1 (panels A to D) are negative at pH 10.3 (panel E) and positive at pH 4.5 (panel F) and 4.3 (panel G), and thus correspond to the type I fibers marked as 4 in panels E to G. The fibers containing MHCIIa marked as 2 are positive at pH 10.3 and negative at pH 4.5 and 4.3, corresponding to the type IIA fibers marked as 5 in panels E to G. The hybrid fibers containing both MHCI and MHCIIa marked as 3 are positive at all pHs examined, and correspond to the type IIC fibers marked as 6 in panels E to G. Type IIC fibers are exclusively found during regeneration processes including embryonic and neonatal stages. The SDH activities in the fibers containing MHCIIa (type IIA) and both MHCI and MHCIIa (type IIC) are higher than those in the fibers containing only MHCI (type I) (panel H).

In the plantaris muscle, the hybrid fibers are classified with ATPase and SDH staining; MHCIIax fibers determined by immunohistochemistry (panels A to H of Fig. 2) are classified as type IIA, marked as 8 (panels I to K) and MHCIIxa fibers are classified as type IIB, marked as 9 (panels I to K). Pure MHCIIb (marked as 3) and MHCIIx (marked as 4) fibers cannot be distinguished with ATPase staining, although SDH activity is higher in MHCIIx fibers than in MHCIIb fibers (panel L) as reported previously [22, 40, 67].

Another commonly used classification of skeletal muscle fibers is based on the differences in the energy utilization and metabolic demand [54, 55]. Oxidative enzyme activities, i.e., SDH and nicotinamide adenine dinucleotide dehydrogenase activities are related to the mitochondrial aerobic pathway for oxidative energy supply, whereas the glycolytic energy supply pathway is associated with the enzyme activity, i.e., α -GPD activity. By combining the ATPase-based and oxidative and glycolytic enzyme-based classification, skeletal muscle fibers are categorized into three types; fast-twitch oxidative glycolytic (FOG), fast-twitch glycolytic (FG), and slow-twitch oxidative (SO) [4, 54].

MHC isoforms of skeletal muscle fibers correspond to

certain types of muscle fibers determined by the ordinary enzyme histochemical classification [37, 39, 56, 76, 78, 79] as shown in Figs. 1 and 2. Types IIA (FOG), IIB (FG), and I (SO) fibers defined by enzyme histochemistry correspond to types MHCIIa, MHCIIb, and MHCI fibers, respectively. However, the variation of MHC isoforms is far more than we would expect from the results obtained by enzyme histochemistry. Thus, multiple MHC isoforms can coexist in a single muscle fiber [1, 11, 77], an aspect which further increases the diversity of muscle fiber properties. These hybrid fibers are found even in mature skeletal muscles under normal conditions [36], and they become more prominent during muscle fiber-type transformation [56].

The immunohistochemical method using monoclonal antibodies which are specific for different MHC isoforms provides useful information regarding muscle fiber-type plasticity which could not be obtained by the earlier enzyme histochemical methods.

VIII. MHC Isoform Transformation

The composition of MHC isoforms in skeletal muscle is dynamic, not static. The MHC isoforms are adaptive to the physiological changes such as development [24, 30, 43, 50, 58, 63, 96], aging [46, 66], increased [3, 9, 27, 64, 82] or decreased [10, 51, 88, 89] neuromuscular activity, hormones [32, 47, 50, 61], and other factors [73]. For example, all fibers in rat muscles have MHCemb at the perinatal stage, and then they differentiate to coexpress MHCneo and/or MHCI [50, 63]. The growth-related transformation of fast-to-slow and slow-to-fast MHC isoforms is observed in the slow- and fast-twitch muscles, respectively, and fibers containing MHCemb and MHCneo disappear during the first month of life [30, 58]. In contrast, the transformation of the fast-to-slow MHC isoform generally occurs during the aging process [44, 46]. For example, fibers containing MHCIIa, which are about 20% of the slow-twitch soleus muscle of the adult rat, disappear at the latest stage by transforming from MHCIIa to MHCI. Even in the fast-twitch muscle, fibers containing the fastest type (MHCIIb) are progressively transformed to those containing the second fastest type (MHCIIx) during aging. Neuromuscular activity is one of the important factors which affect MHC isoform profiles; increased neuromuscular activity and mechanical overloading for muscle cause the transformation in the fast-to-slow direction [3, 9, 64], whereas decreased activity and unloading result in the slow-to-fast transformation [10, 51, 88, 89]. Hyperthyroidism causes the transformation in the slow-to-fast direction, and hypothyroidism causes the transformation in the opposite direction [32, 50].

Muscle fiber-type transformation occurs in order to provide the contractile or metabolic property most adequate for the new condition. The MHC isoform profile in a single fiber is a good marker of muscle fiber-type transformation, and immunohistochemistry can

easily detect it. MHC isoform transformation has been reported to occur as a sequential conversion from MHC I $\leftrightarrow$ MHC IIa $\leftrightarrow$ MHC IIx $\leftrightarrow$ MHC IIb [5, 81, 93]. However, careful immunohistochemical observation revealed that a "jump" from MHC I to MHC IIx, for example, can occur under special conditions [87]. The proportion of the hybrid fibers apparently increases under muscle fiber-type transformation. Up to four different isoforms are found to be coexpressed in transforming fibers [93]. An immunogold electron microscopic study revealed that specific MHC isoforms are segregated in some distinct populations of myofibrils within individual fibers [29]. In isolated myosin thick filaments, the coexistence of two MHC isoforms has been observed [92].

Immunohistochemistry can qualitatively identify the MHC profile of hybrid fibers by combining several MABs. At present, the variety of specific antibodies for MHC isoforms is small, which limits the usage of the double-labeling technique on the same preparation. An investigator must prepare as many serial sections as the number of MABs to be used, and, therefore, many sections including the same fiber are needed. This is undesirable for analyzing MHC proteins, which have a nonuniform distribution along the longitudinal axis of the muscle fiber. Another problem is the quantification of the MHC isoform content on the histological preparation. There are some ongoing trials to quantify the immunoreactivity of individual muscle fibers [48, 53]. The development of easy and reliable quantification methods will be useful for immunohistochemical analyses of hybrid fibers. In addition, there may well be MHC isoforms as yet unidentified and histochemically undetectable.

Although muscle fiber-type transformation is one of the major topics in bioscience, the regulatory mechanism of the gene expression involved is not yet clear. The transformation is known to occur not only in MHC proteins but also in other proteins such as troponin and myosin light chain [69], and there may be a mechanism of coupling in the regulatory elements. Detailed histochemical observations of the transformation process in response to various stimuli and environmental changes are necessary. The genetic regulation in multinuclear cells such as skeletal muscle fibers is also a problem; whether a single nucleus produces mRNA of a single isoform or multiple isoforms. Does the MHC protein produced remain within the nuclear domain of the nucleus which has expressed its mRNA [49]? Does the transformation occur simultaneously along the longitudinal axis of a muscle fiber [52]? If not, where does it start, and how is it coordinated along the fiber's full length? Although mechanisms regulating muscle fiber-type diversity have been extensively investigated [33], advanced immunohistochemical approaches will help to answer these remaining questions.

IX. Conclusion

We have described the immunohistochemical methods

used for the classification of muscle fiber-types and their utility, especially for detecting hybrid fibers using different monoclonal antibodies against specific MHC isoforms. These methods are essential for studying how specific kinds and amounts of proteins are synthesized and expressed in single fibers. Immunohistochemical data will help to clarify the dynamic nature of muscle fibers and the mechanism of muscle fiber-type transformation.

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