Distinct Myosin Heavy Chain Isoform Transitions in Developing Slow and Fast Cat Hindlimb Muscles

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Key Words
Development · Fiber types · Muscle · Soleus · Tibialis anterior · Kitten · Cat

Abstract
The expression of myosin heavy chain (MHC) isoforms leading to adult fiber phenotypes in the tibialis anterior (TA) and soleus muscles of the cat were investigated from embryonic day 35 to 1 year after birth. Electrophoresis and immunoblotting of myofibrils demonstrated the expression of 5 different MHC isoforms, i.e. I, IIA, IIx, embryonic, and neonatal, during development. Based on electrophoresis, the adult-like MHC composition of the soleus and TA were not observed until postnatal day 40 (P40) and 120 (P120), respectively. In contrast, immunohistochemical analyses revealed that the adult-like fiber phenotype composition was attained much later (P120) in the soleus. The existence of multiple MHC isoforms in individual fibers suggested that transitions occurred until P120 in both muscles. Adult type I fibers were first observed at P1. Adult IIA fibers were first observed at P30 in the TA and P40 in the soleus. IX fibers were not identified until P40 in the TA. The transition to the predominantly slow phenotype of the soleus involved a gradual loss of embryonic and fast isoforms accompanied by an accumulation of slow MHC. In contrast, the expression of slow and fast MHC in the fast TA muscle was relatively unchanged throughout development. These results show that the establishment of a given MHC-based fiber phenotype varies significantly between slow and fast muscles in the kitten.

Abbreviations used in this paper
mAb monoclonal antibody
mATPase myofibrillar adenosine triphosphatase
MHC myosin heavy chain
TA tibialis anterior
TBS Tris-buffered saline
TTBS Tris-buffered saline plus 0.2% Tween 20
Introduction

An extensive amount of the research on the physiological, morphological and biochemical properties of skeletal muscle in vertebrates has been carried out using hindlimb muscles of adult cats. Detailed analyses of flexor and extensor muscles of the hindlimb [Ariano et al., 1973; Burke, 1981] have demonstrated a diversity in their contractile profiles which is determined largely by the enzymatic properties of the muscle fiber compositions. In the cat, the three generally recognized muscle fiber types, i.e. fiber types I, IIA and IIB [Brooke and Kaiser, 1970; reviewed in Burke, 1981], express distinct isoforms of myosin heavy chain (MHC) [Unguez et al., 1993a; Talmadge et al., 1996b] and different amounts of other contractile and metabolic proteins. Type I and IIA fibers express type I and IIA MHC isoforms, respectively [Talmadge et al., 1996b]. The MHC expressed by IIB fibers has been previously referred to as either IIB [Talmadge et al., 1996a] or Fast-2 [Unguez et al., 1993a]. However, recent biochemical and histochemical studies demonstrated that this MHC is more closely related to the IIX isoform found in rodents. Hence, the MHC previously identified as IIB in hindlimb muscles of adult cat is now referred to as IIX and the fibers that express it as IIX [Talmadge et al., 1996b].

Although there has been much interest in assessing the MHC isoform transitions in hindlimb muscles of the cat during development, much remains unknown about the MHC transformations that occur prior to the establishment of the adult muscle fiber type composition. For examples: (1) to what extent does an immature muscle fiber express different MHC isoforms?, (2) do fibers in different muscles follow the same MHC isoform transitions to attain their adult phenotype?, and (3) do adult fiber types emerge simultaneously in predominantly 'slow' or 'fast' muscles?

Most of the early published work on developing feline muscle fibers [Dubowitz, 1965; Nystrom, 1968; Hammarberg, 1974] used the myofibrillar adenosine triphosphatase (mATPase) method [Brooke and Kaiser, 1970]. However, conclusions from these results are limited. First, the staining for mATPase is an indirect method that does not unequivocally discriminate between fiber phenotypes [Gorza, 1990; Talmadge et al., 1995a]. Second, this technique cannot identify embryonic or fetal MHC isoforms [Fitzsimons and Hoh, 1981]. Lastly, mATPase staining is unable to accurately or consistently detect the presence of more than one type of adult MHC isoform in the same fiber [Talmadge et al., 1995b].

More recently, Hoh et al. [1988] provided the first study of myosin changes during the postnatal development of feline fast extensor digitorum longus and slow soleus muscles using immunohistochemistry supplemented by gel electrophoretic techniques. These authors were first to identify a nonadult 'embryonic/fetal' MHC in the cat hindlimb. Furthermore, this work reported differences in the proportion of fiber types identified between both fast and slow muscles during the first 50 days after birth. However, although this study represents the most complete immunological data available on MHC isoform transitions in feline muscles to date, it did not determine the time at which adult fiber types emerge. Further, the extent to which slow versus fast muscles undergo similar MHC isoform transitions to attain their adult phenotype was not investigated.

The present study was undertaken to specifically characterize the temporal changes in expression of developmental, slow and fast MHC isoforms leading to the emergence of the adult fiber phenotypes in two hindlimb muscles in cats. The determination of the MHCs present in hindlimb muscles of the cat during development is of great interest because of the large amount of data that we have amassed on the plasticity of contractile protein profiles of hindlimb muscles of the cat under a wide variety of experimental perturbations that alter neural input and loading [see Roy et al., 1991; Edgerton et al., 1996; for reviews]. Further, this study significantly extends the findings of Hoh et al. [1988]. We have used monoclonal antibodies (mAbs) that identify the two distinct fast fiber types in adult hindlimb muscles of the cat [Unguez et al., 1993a] to determine the time course of their differentiation. Parallel studies with sodium dodecyl sulfate-polyacrylamide gel electrophoresis of specific MHCs were used to further elucidate the pattern of emergence of the MHC isoforms expressed at different embryonic and postnatal stages of development. The soleus and tibialis anterior (TA) were chosen as representative slow extensor and fast flexor muscles of the hindlimb, respectively. These data are novel in that two nonadult MHC isoforms corresponding to an embryonic and a neonatal MHC were identified. Further, the stage at which embryonic and neonatal MHC isoforms are no longer expressed and adult fiber types emerge during postnatal development were compared in the soleus and TA muscles. Preliminary results have been reported in abstract form [Unguez et al., 1995b].
Table 1. Specificity of antibodies for MHC isoforms in cat muscle

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Emb</th>
<th>Neo</th>
<th>Type I</th>
<th>Type IIa</th>
<th>Type IIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dev&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1:50</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Slow&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1:100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BA-F8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:10,000</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fast&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1:50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BF-13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:10,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BF-35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:10,000</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

All antibodies were monoclonal, immunoglobulin G class and originally generated in mice. The dilutions listed were used for immunohistochemical analysis. The specificities listed were determined for the cat according to the present study, Talmadge et al. (1996b), and the supplier’s instructions. The specificities of some of these antibodies for rat MHC isoforms have been previously published [Schiavino et al., 1988; Gorza, 1990] and are consistent with those reported for the cat. Dev = Developmental; Emb = embryonic; Neo = neonatal; + = positive reaction between a specific MHC isoform and a specific antibody; = no reaction.  
<sup>a</sup> Antibodies obtained from Novocastro Laboratories (Vector Laboratories, Burlingame, Calif.).  
<sup>b</sup> Antibodies obtained from Dr. S. Schiaffino (Institute of Pathology, Padova University, Padova, Italy).

Materials and Methods

Tissue Preparation

mAbs BA-F8, BF-13 and BF-35 (all IgG) specific to different MHC isoforms in rat skeletal muscle were provided by Dr. S. Schiaffino (Institute of Pathology, Padova, Italy). The specificity of these antibodies has been previously determined in rat [Gorza, 1990] and cat skeletal muscle (Table 1) [Talmadge et al., 1996b]. In cat, monoclonal antibody BA-F8 (1:10,000 dilution) reacts specifically with slow or type I MHC, whereas BF-13 (1:10,000 dilution) reacts with neonatal and all adult fast MHC isoforms. BF-35 (1:10,000 dilution) recognizes an epitope shared by embryonic, neonatal, slow and IIa MHCs. Also used in this study were mouse monoclonal (IgG) antislow (1:100 dilution), antifast (1:50 dilution), and antidevelopmental (1:50 dilution) myosin antibodies obtained from Novocastra Laboratories (Vector Labs, Burlingame, Calif.). Since similar staining patterns were obtained when fibers were reacted with BA-F8 or antiaslow and with BF-13 or antifast, the nomenclature used to refer to these antibodies throughout the remainder of the paper will be antiaslow and antifast, respectively. The antidevelopmental antibody was specific to embryonic MHC as shown in the Results section.

By examining the soleus and TA muscles, we could compare the development of muscles having different adult fiber type compositions and distributions. These muscles were removed from fetal cats at embryonic days 35, 45 and 55 in utero (E35, E45, E55; gestational period is ~60 days) and from cats at 1 (P1), 10 (P10), 15 (P15), 20 (P20), 30 (P30), 40 (P40), 60 (P60), 90 (P90), 120 (P120) and >300 (adult) postnatal days. Pregnant cats were anesthetized (ketamine 65 mg/kg, xylazine 10 mg/kg), a cesarean section was performed, and fetuses removed from their uterus at E35, E45 or E55. The abdominal cavity of the mother was sutured and she was returned to her cage to recover. All procedures used in this study followed the American Physiological Society Animal Care Guidelines and were approved by the Animal Use Committee at UCLA. Muscles from each fetus were removed under anesthesia (sodium pentobarbital, 30 mg/kg i.p.), weighed, cut into blocks, mounted on cork, frozen in isopentane cooled in liquid nitrogen and stored at −70°C. At each embryonic stage, the muscles were taken from one kitten. At each postnatal time point the muscles were taken from one kitten from each of two litters (n = 2).

Immunohistochemistry

Serial sections (12 µm thick) were cut from the midportion of each muscle and air-dried for immunohistochemistry. Tissue sections were incubated for 30 min in blocking solution and then overnight at 4°C in primary antibody diluted with phosphate-buffered saline. Sections incubated without primary antibody were used as controls to visualize nonspecific labeling. A Vectastain ABC kit (Vector Labs) was utilized to amplify the MHC antigen-antibody complex and this was visualized by reacting with diaminobenzidine and hydrogen peroxide. Sections were washed with distilled water, dehydrated in 70, 95 and 100% ethanol, cleared in xylene and mounted in Permount.

At each developmental stage, adjacent tissue sections of the soleus and TA were examined and muscle fibers were characterized according to their pattern of staining with the anti-MHC antibodies. For all muscle samples, photographic montages were constructed of each of the serial sections reacted with the different MHC antibodies. A total of 100 fibers from each TA and each soleus muscle were followed in serial sections and evaluated according to their reactivity with each antibody at all developmental stages from E55 to adulthood. Regional differences in both muscles were studied at these developmental stages by sampling 50 fibers from the deep (approximately one fourth of the muscle cross section closest to the bone) and 50 fibers from the superficial (approximately one fourth of the muscle cross section farthest from the bone) portions of the muscles at their midlength.

Biochemical Analyses

The immunohistochemical analyses were supplemented with immunoblot analyses performed on polyacrylamide gels of cat skeletal muscles. Purified myofibrils were prepared [Talmadge et al., 1995b] and MHCs separated by high resolution polyacrylamide gels [Talmadge and Roy, 1993]. The gels were stained with Coomassie blue. Western blotting of the gels was performed according to Towbin et al. [1979]. In succession, the nitrocellulose filters were blocked with 3% gelatin in Tris-buffered saline (TBS), incubated overnight in primary antibody diluted 1:10,000 with TTBS (TBS plus 0.2% Tween 20), rinsed with TTBS, incubated in secondary antibody (Sigma A9044, rabbit antimouse IgG conjugated to peroxidase, diluted 1:8,000 with TTBS), washed with TTBS, and then rinsed with TTBS. Peroxidase activity was visualized with the use of a peroxidase substrate kit (SK-4100, Vector Laboratories). The polyacrylamide gels and nitrocellulose filters were photographed with an Alpha Innotech IS-1000 digital imaging system and Seikosha VP-1500 videoprinter.

Unguez/Talmadge/Roy/Dalponte/Edgerton
Results

Cat Muscle MHC Isoforms

Five different MHC isoforms were identified in the hindlimb muscles of cats during the course of development, i.e. I, IIa, IIx, embryonic and neonatal. These five MHC isoforms were separated into three bands on polyacrylamide gels (Fig. 1). The top band contained both type IIa and embryonic MHCs. The middle band contained both IIx and neonatal MHCs. The bottom band contained only type I MHC. At P1, the soleus (lane 1) had two bands. The bottom band labeled with antilow MHC mAb and was identified as type I MHC. The top band labeled with antidevelopmental and BF-35 antibodies, but not with antifast or antilow MHC mAb and represented embryonic MHC. Therefore, P1 soleus contained both embryonic and type I MHCs. The adult TA (lane 3) was composed of three MHC bands (top, middle and bottom). The bottom band labeled like the bottom band in P1 soleus and was type I MHC. The middle band labeled with antifast MHC mAb, but not mAb BF-35 nor antidevelopmental, and was identified as type IIx MHC as previously determined [Talmadge et al., 1996b]. The top band which labeled with antifast MHC and mAb BF-35, but not with antidevelopmental, was determined to be type IIa MHC [Talmadge et al., 1996b]. Therefore, adult TA contained type I, IIa and IIx MHCs. The TA at P1 (lane 2) also had three bands (top, middle and bottom). The bottom band labeled like the bottom band in P1 soleus and adult TA and represents type I MHC. The middle band in P1 TA labeled positively with mAb BF-35 and, therefore, was not type IIx MHC. The middle band did not label with antifast, antidevelopmental nor antilow. This middle band in the neonatal TA, therefore, was identified as neonatal MHC since it was only present in fast muscle (not soleus) at the time of birth (note: it migrated like MHC IIx in the adult TA, but reacted differently to mAb 35). The top band contained both embryonic and type IIa MHCs since it labeled with antifast, antidevelopmental, and mAb BF-35, but not antilow. The comigration of embryonic and IIa MHCs was confirmed by coelectrophoresis of adult TA and E35 hindlimb muscle and subsequent Western blotting (Fig. 2). Western blotting also was used to confirm that embryonic MHC was expressed only at the early developmental time points for both the soleus and the TA, i.e. embryonic MHC was present from E35 to P40 in the soleus and from E35 to P30 in the TA (Fig. 3).

Adult Cat Muscle Fiber Phenotypes

Three predominant fiber phenotypes were found in hindlimb muscles of the adult cat (Fig. 4) according to the binding of the MHC specific mAbs used in this study (Table 2). Each of these fiber phenotypes contained a single MHC isoform, either type I, IIa, or IIx, and were designated as fiber types I, IIa or IIx, respectively. Within the adult cat TA muscle, 19% of the fibers were type I, 37% were IIa, and 44% were IIx. The deep region of the TA showed a higher incidence of type I fibers and a lower incidence of IIx fibers (22 and 39%, respectively) than the superficial region (12 and 51%, respectively). The percentage of IIa fibers was similar in both regions of the TA, i.e. 39% in the deep and 33% in the superficial regions. The soleus in adult cats contained primarily (>99%) type I fibers. However, a few (≤1%) type IIa fibers were also observed. No type IIx fibers were found in the adult.
soleus. A few adult cat soleus and TA fibers were observed to stain positively for both type I and II MHC isoforms; however, the proportions of these fibers were less than 1%. Further, no fibers containing development MHC were found in either muscle in the adult cat.

**Immunohistochemical Staining Patterns of Developing Fibers**

Immunoreactivity of muscle fibers with each of the antibodies used in this study (Table 1) resulted in the identification of seven distinct fiber phenotypes in the TA and soleus muscles of the developing kitten (Figure 2). The

**Fig. 2.** The MHC regions of a Coomassie blue-stained polyacrylamide gel (A) and Western blots (B-D) of myofibrils isolated from E35 kitten hindlimb muscle (lane 1), adult cat TA (lane 2), and a 50:50 mixture of E35 kitten hindlimb and adult cat TA (lane 3). Biot B was stained with the developmental (Dev) mAb (antiembryonic MHC mAb), biot C with mAb BF-13 (antifast MHC mAb), and biot D with mAb BF-35 (anti-Il MHCs except IIx). The embryonic MHC band from the E35 kitten muscle comigrated with the type IIa MHC band of the adult cat muscle. This band was strongly stained by mAb Dev, weakly stained by mAb BF-35, and not stained by mAb BF-13. Abbreviations are the same as in figure 1.

**Fig. 3.** The MHC regions of Western blots stained with mAb Dev of myofibrils isolated from soleus (A) or TA (B) muscle from cats at various stages of development. Lane 1 for both A and B contains myofibrils isolated from E35 whole hindlimb muscle; all other lanes contain myofibrils isolated from either the soleus (A) or the TA (B). Note the complete lack of mAb Dev staining at ages P60 and beyond. Very light staining was observed at P40 for the soleus.
three predominant fiber types observed in the adult cat have been described above and in Talmadge et al. [1996b]. In addition, four other fiber phenotypes were observed during development of the soleus and TA. For the present paper, we have designated these as types A, B, C and D. Type A fibers contained both type I and II (Iia, IIX and/or neonatal) MHCs; type B fibers contained embryonic MHC in addition to types I and II (Iia, IIX and/or neonatal); type C fibers contained embryonic MHC and type II (Iia, IIX and/or neonatal), and type D fibers contained embryonic MHC in addition to type I (table 2). The nomenclature used to identify fibers with mixed MHC composition, i.e. fibers A, B, C and D, is not intended to be used as a new classification scheme. To classify these nonadult MHC-based fiber phenotypes as type I, IIA or IIX fibers would be inaccurate. Similarly, to refer to these fibers as simply ‘fast’ or ‘slow’ would be inappropriate and misleading.

**Qualitative Assessment of MHC Isoform Composition of Soleus and TA Muscle Fibers during Early Muscle Development**

Quantitation of the proportions of distinct MHC patterns present at both E35 and E45 could not be obtained due to the difficulty in following individual fibers through serial cross sections. However, at E35 all fibers in both the soleus and TA muscles were labeled with antidevelopmental (fig. 5), thus containing embryonic MHC. Further, most fibers in both muscles also reacted with both antifast and antislow mAbs, indicating the presence of type I

**Table 2. Fiber types, MHC profiles, and antibody reactivity in seven distinct cat soleus and TA muscle fiber types**

<table>
<thead>
<tr>
<th>Fiber type designation</th>
<th>MHC profile</th>
<th>Antibody reactivity</th>
<th>Slow</th>
<th>Fast</th>
<th>BF-35</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>I only</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IIA</td>
<td>Iia only</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IIX</td>
<td>IIX only</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A</td>
<td>I and I1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>Emb, I and II</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>Emb and II</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>Emb and I</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: The nomenclature used for some fiber types (i.e. A, B, C and D) is not intended as a new classification scheme, but merely to more succinctly identify fibers composed of multiple MHC isoforms. + = Positive reaction between a specific MHC isoform and a specific antibody; - = no reaction.
MHC along with the presence of type IIa, IIx and/or neonatal MHC. However, polyacrylamide gels of the hindlimb muscles at E35 did not show the IIx/neonatal MHC band (fig. 6). Hence, either IIx or neonatal MHCs were expressed in small quantities (small enough to escape detection by polyacrylamide gel analyses), or type IIa MHC was the isoform that labeled histochemically with antifast mAb (fig. 5) at this stage.

At E45, all fibers of the soleus and TA muscles of the kitten maintained the expression of embryonic MHC (fig. 7). By this stage, a ‘rosette’-like spatial pattern was characteristic among fibers in all muscles (fig. 7), i.e. a larger myotube (presumably a 1° myotube) is surrounded by numerous, smaller myotubes (presumably 2° myotubes). The large fibers at the center of each ‘rosette’ also reacted with antislow, antifast and BF-35 antibodies,
Fig. 7. Serial sections of a portion of an E45 TA muscle reacted with antifast (A), antislow (B), BF-35 (C) and antidevelopmental (D) mAbs. The small fibers (arrows) did not stain with the antislow antibody (B), but were stained by all of the other antibodies. In contrast, the large fibers were stained by all four antibodies (*). The soleus muscle at E45 showed a similar pattern of staining (not shown). The scale bar in D represents 10 μm.

whereas the small fibers at the periphery of each ‘rosette’ did not stain with the antislow antibody, but did stain with antifast and BF-35. Since the polyacrylamide gels (fig. 6) of E45 hindlimb muscles showed little to no IIx or neonatal MHC, the immunohistochemical data is consistent with IIa MHC being present, particularly in the TA. Thus, in addition to embryonic MHC, the large fibers contained type I and IIa MHC, whereas the small fibers contained IIa MHC.

Quantitative Assessment of MHC Composition of Kitten Soleus and TA Fibers during Late Embryonic and Early Neonatal Stages

At E35, the earliest stage analyzed, most fibers in both muscles expressed embryonic, type I, and some form of fast MHC (fiber type B, table 2; fig. 8, 9). Some fibers, however, did not express type I MHC, but contained embryonic and fast MHC (fiber type C, table 2). The MHC profiles of individual fibers at E55 and P1 were remarkably similar between the two hindlimb muscles studied. For example, at E55, three fiber phenotypes, i.e., B, C and D, were identified in the TA (n = 1) and soleus (n = 1) muscles (fig. 8, 9). However, while similar proportions of type C (41%) and type D (36%) fibers were found in the TA, there were nearly twice as many C (60%) as D (37%) fibers in the soleus at this stage. At P1, TA (n = 2) and soleus (n = 2) muscles were comprised primarily of type I (TA = 15 and 17%; soleus = 24 and 34%) and type C (TA = 74 and 77%; soleus = 60 and 61%) fibers. Polyacrylamide gels showed similar MHC profiles in both the soleus and TA muscles at E55 and P1. However, the presence of the IIx/neonatal band was observed only in the TA at these stages (fig. 1, 6). Thus, some differences in MHC compositions do exist between the two muscles at early stages of development.

Quantitative Assessment of MHC Composition of Kitten Soleus and TA Fibers during Postnatal Development

Fiber phenotype C comprised a substantial percentage of fibers in both the TA and soleus muscles during the first 3 weeks after birth (fig. 8, 9). In the TA, C fibers increased from P1 (n = 2, 74 and 77%) to P20 (n = 2, 92 and 85%), whereas the proportion of C fibers in the soleus decreased between P1 (n = 2, 60 and 61%) and P20 (n = 2, 23 and 7%). During the postnatal stages, there were very low percentages of types A, B and D fibers in the TA (fig. 8) or soleus (fig. 9), except for a transient increase in type B fibers between P10 and P30 in the soleus muscle.
The largest number of fiber phenotypes (i.e. 7 phenotypes) in the TA was identified between P30 and P40 (fig. 8). In the soleus muscle, the greatest diversity (i.e. 6 phenotypes) was observed between P1 and P60 (fig. 9). By P120, the fiber phenotypes found in both muscles were similar to those found in adulthood. If the number of fiber phenotypes observed is indicative of the extent to which transitions in MHC expression are occurring within a muscle, then these data suggest that fibers in the TA were undergoing the greatest degree of MHC transitions between P30 and P40, whereas the soleus muscle maintained a similar degree of MHC adaptations throughout the first 2 postnatal months.

Adult type I fibers were first observed at P1 in the TA muscle (fig. 8). Type I fibers comprised between 10 and 20% of the total population in the TA muscles postnatally. The mean percent of adult type I fibers was similar in the deep and superficial compartments until P90. At P120, the deep and superficial compartments had 24 and 14% type I fibers, a proportion similar to that observed in the adult. Type IIA fibers were first observed in the TA muscles at P30, with the TA in one animal having 13% and the other 61% of this phenotype. This percentage increased to 56 and 84% for the two animals at P40 and then decreased to an adult level at P120, i.e. ~ 40%. Type IIX fibers were first observed in one TA muscle at P40 and by P60 ~ 40% of the fibers were type IIX. By P120, the percentage of IIX fibers was similar to that in the adult, i.e. ~ 45%. The percentage of both type IIA and type IIX fibers were similar in the deep and superficial regions of the muscle at all stages of development, except for P120 which had a high percentage of IIX fibers in the superficial region.

Similar to the TA, pure type I MHC fibers were first observed in the soleus at P1 (fig. 9). A mean of 29% (n = 2; 24 and 34%) of the fibers were labeled exclusively with antislow MHC, with a greater incidence in the deep (33%) than the superficial (14%) region of the muscle. The number of type I fibers in the soleus increased gradually with age in both the superficial and the deep compartments. By P120, the fiber type composition was similar to that found in the adult. A very small percentage of fibers (3%) in one soleus was still labeled by both antislow and antifast antibodies at P120 (fig. 9; A fibers). Type IIA fibers were first observed at P40 and comprised ~ 20% (n = 2; 8 and 33%) of the fiber population. Note that whereas one kitten soleus did not contain IIA fibers by P60, the soleus of the other kitten had IIA fibers through P120. IIX fibers were never observed in the soleus muscles.

**Discussion**

The temporal and spatial changes in the expression of distinct MHC isoforms were examined during the development of the cat TA and soleus muscles, hindlimb muscles destined to be predominantly fast or slow, respectively. Five MHC isoforms were identified during the course of development. Three MHCs, i.e. I, IIA and IIX, were expressed in the adult TA, whereas predominantly type I and small amounts of type IIA MHC were expressed in the adult soleus muscles. These three MHCs have been observed in other skeletal muscles of the adult cat [Talmadge et al., 1996b]. Two nonadult MHCs similar to those identified as embryonic and neonatal in skeletal muscles from other mammals [Hoh and Yeoh, 1979; Rubinstein and Kelly, 1981; Butler-Browne and Whalen, 1984; D'Albis et al., 1989; La Framboise et al., 1991; Cho et al., 1993] were also found in the TA and soleus muscles of kittens. Although a fetal MHC was previously identified in hindlimb muscles of the kitten [Hoh et al., 1988], whether this MHC corresponded to an embryonic or a neonatal MHC isoform was not determined. Combined polyacrylamide gel and Western blotting analyses used in the present study clearly revealed the presence of these two nonadult MHCs during development.

The immunohistochemical analysis of MHC expression among fibers of the soleus and TA muscles of the cat throughout development revealed some differences from the age-related changes in MHC expression observed with gel electrophoresis. For example, whereas immunoblots of the soleus muscle at P40 show the presence of only type I MHC, 24 and 39% of the fibers are colabeled with anti-developmental and antifast antibodies, respectively. Further, P40 soleus muscles also contain IIA fibers (8%) according to immunohistochemistry. In contrast, there is

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**Fig. 8.** Relationship between age and the percent distribution of seven fiber phenotypes in two TA muscles from different litters. Fiber MHC phenotypes A, B, C, D, I, IIA and IIX were defined according to the different staining patterns with the antibodies used in this study and correspond to those listed in table 2. Multiple MHC isoforms are found in individual fibers from E55 to P90. The first fibers with a single MHC isoform appear by E55 and they contain type I MHC. Fiber phenotype C represents a substantial percentage of the total fiber population from P1 to P30 and the frequency of these fibers is reduced after the 1st month after birth. Note that the decrease of C fibers precedes the emergence of IIA and IIX fibers in the TA. Each symbol represents a different animal (n = 2). The x-axis is not to scale.
little change in the banding pattern observed in the TA between P20 and P60. Yet, immunohistochemically, there is a marked decrease (85%) in the proportion of fibers that are labeled with antidevelopmental antibody, and adult IIA and IIX fibers emerge between P20 and P40. Discrepancies in the detection sensitivity of distinct MHCs between assays may be partly due to some antibodies reacting differently with nonadeninated proteins (i.e., tissue sections) than with denatured proteins (i.e., Western blots). Nevertheless, the electrophoretic analyses provided additional information on the number and types of MHCs present in the muscles: information that could not be resolved solely with immunohistochemistry.

Different combinations of MHCs were predominant among muscle fibers of the TA and soleus during the first 2 months after birth, suggesting that transitions occurred continuously during this developmental period. However, the MHC transitions identified differed between the two muscles and did not follow the same temporal sequence. For example, both muscles became devoid of embryonic MHC by P90, yet the rate of loss of embryonic MHC expression was markedly different between the TA and the soleus. Further, the TA maintained a high percentage of antifetal MHC fibers and a low percentage of antilsow MHC fibers from the 2nd week after birth through adulthood. In contrast, the soleus underwent an increase in the number of antialsow fibers with a concomitant decrease in the number of antifetal fibers during postnatal development. Thus, although a similar fiber type distribution was found in both TA and soleus muscles near birth, the differentiation process to the adult muscle fiber type composition varied significantly between the predominantly slow soleus and predominantly fast TA of the kitten.

The identification of at least three distinct fiber phenotypes in both hindlimb muscles near birth is in agreement with previous observations by Hoh et al. [1988]. We first observed adult type I fibers in both flexor and extensor muscles at birth, the postnatal age at which Hoh et al. [1988] also observed type I fibers. However, Hoh et al. [1988] reported the presence of a fiber type that labeled only with their antifetal MHC antibody during the first 2 postnatal weeks. In the present study, fibers reacting exclusively with the antidevelopmental antibody were not observed at any embryonic or postnatal age. It is possible that their fetal antibody recognized both embryonic and neonatal MHCs. If so, then this antifetal fiber type would correspond to fibers in our study which labeled with antidevelopmental, BF-35, and antifast antibodies suggesting the expression of both embryonic and neonatal MHCs within the fibers. However, the specificity of their fetal antibody was not determined. Further, these authors observed a complete absence of antifetal MHC fibers in the extensor digitorum longus by P50 whereas some antifetal fibers were still present in the soleus at 50 days of age. In contrast, we found that complete elimination of embryonic MHC in the TA and soleus did not occur until 90 days after birth. 1 month before both muscles had fully matured with respect to their adult fiber-type composition.

It is interesting to note that a considerable number of fibers in the TA and soleus muscles expressed embryonic MHC at postnatal stages in which a substantial percentage of adult fiber types had already emerged. For example, at 30 days after birth, one third (32%) of the fiber population in the soleus expressed embryonic MHC and nearly two thirds (61%) of the muscle fibers had attained their adult fiber phenotype (i.e., type I). Similarly, by P30, 38% of the fibers in the TA had fully differentiated into IIA fibers, yet 53% of the fiber population expressed embryonic MHC. However, by the time IIX fibers emerged in the TA, i.e. P40, less than 5% of the muscle fibers expressed embryonic MHC. Thus, adaptations in MHCs expressed by both soleus and TA muscles continued for some time after adult fiber phenotypes were established. Similar observations have been found in skeletal muscles from other mammalian species [Butler-Browne and Whalen, 1984; La Framboise et al., 1991; Picard et al., 1994]. A diagrammatic summary of the MHC transitions that we observed in the TA and soleus during development is presented in figure 10. The present data are consistent with a process in which three fiber MHC phenotypes, i.e., B, C and D, predominate in hindlimb muscles of the kitten early in development and give rise to the three fiber types found in the adult. In both soleus and TA muscles of the cat, type B and D fibers give rise to type I fibers in the adult. Type B fibers also may give rise to IIA fibers in the TA following the downregulation of embryonic and type I MHC.

**Fig. 9.** Relationship between age and the percent distribution of seven fiber phenotypes in two soleus muscles from different litters. Fiber MHC phenotypes A, B, C, D, I, IIA and IIX were defined according to the different staining patterns with the antibodies used in this study and correspond to those listed in table 2. Multiple MHC isoforms are found in individual fibers from E55 to P120. The first fibers with a single MHC isoform appear by E55 and they contain type I MHC. Fiber phenotype C represents a substantial percentage of the total fiber population from P1 to P30 in the soleus, and the frequency of these fibers is reduced after the 1st month after birth. The x-axis is not to scale.

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Fig. 10. A Diagram shows the postulated fate of the three fiber phenotypes identified in the soleus and TA muscles between E55 and P1. The three fiber types (B, C and D) correspond to those in table 2. Fiber phenotype A is not included since this fiber type represented a very low proportion of fibers in either TA or soleus at any stage. B Diagram shows the postulated MHC transitions occurring in the soleus and TA muscles of the cat during development. emb = Embryonic; neo = neonatal.

In the soleus muscle, type C fibers most likely stop expressing embryonic myosin, begin to express slow myosin and eventually stop expressing neonatal myosin to give rise to type I fibers in the adult. A few of these C fibers, however, stop expressing embryonic myosin, do not express slow myosin, and become IIA fibers in the adult. In contrast, the decrease in C fibers closely precedes the emergence of type II fibers in the TA (fig. 8). Further, since the percentage of type I fibers in the TA did not change appreciably throughout its postnatal development (fig. 8), the emergence of fiber types IIA and IIX appears to result primarily from C fibers downregulating their embryonic and/or neonatal MHCs and subsequently upregulating the expression of IIA and IIX MHCs. The differences in the age-related changes in MHC expression patterns reported here between the slow soleus and the predominantly fast TA muscle of the cat are similar to those from previous histochemical [Ho et al., 1983] and immunohistochemical [Dhoot, 1986; Narusawa et al., 1987] analyses for predominantly slow and fast contracting muscles of the developing rat. Hence, similar pathways of development of fiber type differentiation to adult phenotypes, at least with respect to MHC, may not be applicable to all muscles in the kitten. MHC transitions that precede the differentiation of a given fiber type were largely dependent on the type of muscle.

Coordinated neural control during postnatal growth may be mediated by muscular activity, and the resulting mechanical loading may assist in the regulation of fiber MHC differentiation [Gutmann et al., 1976]. In the present study, although the newborn kittens lacked the ability to stand and walk, they developed these functions during the first 4 postnatal weeks. For example, the kittens began to crawl at P14 and attempted to stand and walk between P15 and P20. During this time, about 65% of the fibers in the soleus were still expressing embryonic myosin, there was no change in the proportion of fibers labeled with antifast antibodies, and a sharp increase in the percentage of fibers expressing slow myosin occurred. In the TA, however, ~90% of the fibers expressed embryonic myosin and relatively little change was observed in the labeling with antislow and antifast antibodies between P15 and P20. During the subsequent 2–3 weeks, the animals were weaned (by P28) and became very active. The kittens walked proficiently and consistently at P25 and were able to climb and run between P35 and P42. The timing of the increased locomotor activity coincided with striking changes in the MHC expression patterns of the TA and soleus muscles. In the TA, an 89% decrease in the number of fibers expressing embryonic myosin, and the emergence of IIA (reaching a maximum of 68% by P40) and IIX fibers (comprising 15% of the fiber popula-
tion at P40) occurred. The soleus showed a 36% decrease in fibers expressing embryonic myosin, a 30% increase in type I fibers, and the emergence of few (8%) IIA fibers. Thus, the significant changes in the locomotor activity of kittens observed between P20 and P40 occurred coincidentally with changes in MHC expression patterns in both muscles studied.

Fluctuations in the composition of adult fiber types were noted in both hindlimb muscles during postnatal development. For example, the percentage of IIA fibers in the TA increased above adult levels between P30 and P120 (fig. 8). However, since the observed waves of transformation were age-dependent and occurred in the two TAs sampled, these cannot be explained entirely by inter-animal variability. A similar conclusion is made for the age-related changes that occurred in the soleus muscles. Thus, the fluctuations observed very likely represent true changes in the fiber-type composition of the two hindlimb muscles during postnatal development.

The mechanisms that regulate the appearance of different patterns of MHC isoforms at the transitions from prenatal to neonatal to adult stages in development are unknown. It is well established, however, that the expression of the genes corresponding to the various MHC isoforms can be regulated by thyroid hormones [Gambke et al., 1983; Izumo et al., 1986; Russell et al., 1988; D’Albis et al., 1990], the lineage of distinct myoblast populations [Miller and Stockdale, 1986; Vivarelli et al., 1988], innervation [Jolesz and Sreters, 1981], electrical activation [Pette and Vrbova, 1985], and load bearing [Roy et al., 1991]. No single regulatory mechanism appears adequate to explain all of the fiber-type adaptations observed during development. For example, all muscles do not respond in the same manner to thyroid hormone [Izumo et al., 1986; D’Albis et al., 1990], which might partly explain why the soleus and TA do not display the same myosin transition curves. Similarly, innervation alone does not specify such differences early in development since muscles denervated in neonatal rats maintain distinct profiles of MHC isoforms [Matsuda et al., 1984; Condon et al., 1990a, b].

In summary, this study provides information on the number of MHC isoforms expressed in feline hindlimb muscles and the changes in MHC expression that occur prior to the establishment of their phenotype distribution in the adult. Further, these data demonstrate that a difference in myosin composition between the TA and soleus muscles is established early in postnatal development, and the emergence of adult fiber types is preceded by age-related transitions of MHC isoforms that are specific to each muscle. Thus, hindlimb muscles with different contractile characteristics in the adult cat exhibit different developmental programs of fiber type differentiation, a process in which the roles played by genetic and epigenetic factors regulate MHC gene expression in a muscle-specific manner.

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References


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