Postnatal muscle fibre histochemistry in the rat

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SUMMARY

Histochemical techniques were used to study the postnatal muscle fibre differentiation patterns in the plantaris and soleus muscles of male Sprague-Dawley rats. Nine groups of animals (n = 6/group) were killed at 1, 6, 11, 16, 21, 26, 31, 36, and 140 days of age. Serial transverse sections of the two muscles were stained with H & E, NADH-D, and myofibrillar ATPase with acid (pH 4.35) or alkali (pH 10.4) preincubation. In each of the age groups, all available fibres across the muscle sections were classified. Obtained data show that fibre types are basically undifferentiated at birth in both muscles.

In the plantaris muscle there are about 99% type IIA and less than 1% type I fibres at 6 days of age. Type IIB fibres can be identified at 11 days of age. There are increases in the percentages of type I fibres (from 0.7% to 3.5%) and type IIB fibres (from 1.1% to 6.5%) between 6 and 11 days and between 16 and 21 days respectively. By 36 days of age the relative numbers of type IIA, IIB, and type I fibres in the plantaris are approximately 80%, 14%, and 6%, respectively. A gradual change in fibre-type composition continues until it becomes 47% for type IIA, 43% for type IIB, and 10% for type I at 140 days of age.

In the soleus muscle there are approximately 73% type IIA and 26% type I fibres at both 6 and 11 days of age. However, type IIA fibres decrease to 44% and type I fibres increase to 56% at 16 days of age. This rapid shift in fibre composition continues up to 31 days of age when the distribution becomes 25% for type IIA and 74% for type I fibres. Thereafter, the differentiation rate is much slower. At 140 days of age, there are 17% type IIA and 83% type I fibres in the soleus muscle.

The results of this study show that the fibre populations in the plantaris and soleus muscles of the rat undergo a postnatal differentiation process. In both muscles the adult fibre population is established by 140 days of age. Although relatively rapid increases of type I and type IIB fibres occur in the plantaris during the second and third weeks of life, differentiation in that muscle appears to be an essentially continuous process. There is a notable shift in the fibre composition of the soleus muscle during the second postnatal week. Differences between the patterns of differentiation in the two muscles are apparent.

INTRODUCTION

It is well known that skeletal muscles are functionally organized into motor units and that the muscle fibres in a given motor unit are homogeneous in terms of their metabolic properties, their recruitment patterns in performing various activities, and their potential work capacities (Burke & Edgerton, 1975). Many useful systems of fibre-type classification have evolved according to the morphological, physiological, biochemical, and histochemical characteristics of different

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muscle fibres, but the widely accepted nomenclature of Brooke & Kaiser (1970) consisting of type I, IIA, IIB, and IIC was adopted for use in this investigation.

Numerous studies have been conducted on the properties of mature muscle. However, the development of fibre types has not been fully understood. It is believed that skeletal muscle fibres in the rat are basically undifferentiated at birth (Engel & Karpati, 1968; Shafiq, Asiedu & Milhorat, 1972; Riley, 1977a) and that the fibre populations of muscles in the adult rat are determined by a postnatal differentiation process (Shafiq et al. 1972; Curless & Nelson, 1976; Riley, 1977a; Haltia, Berlin, Schucht & Sourander, 1978). Shafiq et al. (1972) reported that type I and type II fibres can be identified in the gastrocnemius and extensor digitorum longus (fast muscles in the adult rat) and in the soleus (a slow muscle in the adult rat) by 2–3 weeks of age. Haltia et al. (1978) found that type IIB and type IIA fibres were evident in the extensor digitorum longus muscle at 5 days and at 15–20 days of age, respectively.

The percentages of various fibre types in a muscle are dynamic during the period of postnatal development. In the extensor digitorum longus muscle, increases in type I fibres (from 10 % to 28 %) and type IIA fibres (from 0 % to 40 %) accompanied by decreases in type IIB fibres (from 41 % to 27 %) and in type IIC fibres (from 49 % to 5 %) have been observed between 5 and 180 days of age (Haltia et al. 1978). In the soleus muscle, a 10 % increase in type I fibres and a corresponding 10 % decrease in type IIA fibres between 11 and 26 days of age also were reported (Riley, 1977a). On the other hand, Curless & Nelson (1976) found that the relative number of type I fibres in the soleus held constant at about 60 % from birth to 29 days of age. The discrepancies in these studies may be due, in part, to strain differences and to the fact that only portions of muscle sections were used for fibre-type classification.

Because the patterns of postnatal muscle fibre differentiation are not clear at present, this study was designed to investigate developmental changes in the fibre-type populations of the plantaris and soleus muscles in the rat using two traditional histochemical procedures. To avoid sampling problems associated with the fact that fibre types are not evenly distributed across various regions of muscle in the rat (Ariano, Armstrong & Edgerton, 1973), all available fibres in transverse muscle sections were classified at specified times during the postnatal development period.

MATERIALS AND METHODS

Nine groups of male albino rats (Sprague-Dawley strain, Spartan Research Animal Inc., Haslett, Michigan) were killed at 1, 6, 11, 16, 21, 26, 31, 36, and 140 days of age. There were six rats per age group. The animals were anaesthetized by an intraperitoneal injection of sodium pentobarbital. The right plantaris, a fast muscle in the adult rat consisting mostly of type IIA and IIB fibres with only a small number of type I fibres, and the right soleus, a slow
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muscle in the adult rat consisting of a large percentage of type I fibres and a small percentage of IIA fibres, were immediately removed and frozen in isopentane precooled (−170°C) with liquid nitrogen.

Serial transverse sections (8 μm) were cut on a microtome in a cryostat at −20°C from the middle of each muscle belly. These muscle sections then were stained with haematoxylin-eosin (H&E), reduced nicotinamide adenine dinucleotide diaphorase (NADH-D) (Novikoff, Shin & Drucker, 1961), and myofibrillar adenosine triphosphatase (myo-ATPase) (Padykula & Herman, 1955) with acid (pH 4.35) or alkali (pH 10.4) preincubation (Guth & Samaha, 1969).

The histochemically stained serial sections from each muscle were projected with a Prado Universal Microprojector (Ernst Leitz Gmbh Wetzlar) in a darkroom. The staining intensity of each muscle fibre in each section was evaluated subjectively using a rating scale of dark, medium, and light (Peter et al. 1972). According to these ratings, each fibre was typed using the classification system of Brooke & Kaiser (1970). Mean values of the total numbers and percentages of different fibre types were calculated for each muscle in each age group. All histochemical evaluations were performed blind.

RESULTS

In newborn rats, fibres from the plantaris and soleus muscles cannot be differentiated with the two histochemical procedures used. That is, the staining intensity of NADH-D is homogeneous across each muscle section, and myo-ATPase reactions following acid (pH 4.35) or alkali (pH 10.4) preincubation show little selective activity among the myotubes.

At 6 days of age, the staining intensity of NADH-D is uniformly dark across all sections from the plantaris muscle. Using myo-ATPase, approximately 99% of the fibres stain dark with preincubation at pH 10.4, and 0.7% of the remaining fibres stain dark with preincubation at pH 4.35. Therefore, these fibres can be classified as type IIA and type I, respectively. Differences in stain intensity for NADH-D start to show in the plantaris by 11 days of age. At this time type IIB fibres become identifiable, and the relative frequency of type I fibres rises to 3.5%. By the 16th day, the distribution of type IIA, type IIB, and type I fibres is approximately 95%, 1% and 3%, respectively. These values change to 88%, 6.5% and 4% at 21 days of age and to 80%, 14% and 6% at 36 days of age. Thereafter, a gradual change in the fibre-type profile continues until the distribution becomes 47% for type IIA, 43% for type IIB and 10% for type I fibres at 140 days of age (Fig. 1). The fibre population at this time is similar to that previously reported for the plantaris muscle in adult rats (Ariano et al. 1973).

In the soleus muscle, the staining intensity of NADH-D is undifferentiated until 11 days of age, but slow and fast fibres can be identified by the two myo-ATPase stains at 6 days of age. There are approximately 73% type IIA and 26%
Fig. 1. Fibre type composition of the plantaris muscle as a function of age. Each point represents the mean and standard deviation of six rats. (Unshown standard deviations <1%.)

Fig. 2. Fibre type composition of the soleus muscle as a function of age. Each point represents the mean and standard deviation of six rats.

Type I fibres at both 6 and 11 days. However, the distribution changes rapidly between 11 and 16 days. The relative frequency of type IIA fibres drops to 44% while that of the type I fibres rises to 55%. This rapid shift in the fibre-type profile continues up to 31 days of age, at which time the distribution is 25% for
type IIA and 74% for type I fibres. Thereafter, the differentiation rate is much slower. At 140 days of age there are approximately 17% type IIA and 83% type I fibres in the soleus muscle (Fig. 2). This fibre population is almost identical to
Table 1. *Means and standard deviations for absolute frequencies of different fibre types in the plantaris and soleus muscles at various ages* *(n = 6 per age group)*

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Fibre type</th>
<th>6</th>
<th>11</th>
<th>16</th>
<th>21</th>
<th>26</th>
<th>31</th>
<th>36</th>
<th>104</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plantaris</td>
<td>Type I</td>
<td>29 ±34</td>
<td>162 ±178</td>
<td>151 ±48</td>
<td>179 ±56</td>
<td>143 ±64</td>
<td>98 ±41</td>
<td>229 ±39</td>
<td>252 ±16</td>
</tr>
<tr>
<td></td>
<td>Type IIA</td>
<td>4054 ±576</td>
<td>4470 ±1238</td>
<td>4667 ±362</td>
<td>3674 ±1061</td>
<td>3513 ±859</td>
<td>3695 ±485</td>
<td>3356 ±841</td>
<td>1244 ±100</td>
</tr>
<tr>
<td></td>
<td>Type IIB</td>
<td>7 ±8</td>
<td>53 ±39</td>
<td>269 ±122</td>
<td>245 ±91</td>
<td>488 ±212</td>
<td>606 ±262</td>
<td>1126 ±26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unclassified</td>
<td>5 ±13</td>
<td>5 ±7</td>
<td>68 ±62</td>
<td>38 ±34</td>
<td>59 ±36</td>
<td>190 ±65</td>
<td>27 ±16</td>
<td>20 ±14</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4088 ±596</td>
<td>4644 ±1405</td>
<td>4939 ±365</td>
<td>4160 ±1116</td>
<td>3960 ±974</td>
<td>4471 ±669</td>
<td>4218 ±1047</td>
<td>2642 ±125</td>
</tr>
<tr>
<td>Soleus</td>
<td>Type I</td>
<td>574 ±206</td>
<td>552 ±141</td>
<td>1499 ±193</td>
<td>1558 ±162</td>
<td>1673 ±253</td>
<td>1706 ±306</td>
<td>1942 ±180</td>
<td>1522 ±162</td>
</tr>
<tr>
<td></td>
<td>Type IIA</td>
<td>1577 ±369</td>
<td>1521 ±154</td>
<td>1193 ±158</td>
<td>1057 ±209</td>
<td>760 ±68</td>
<td>580 ±146</td>
<td>724 ±173</td>
<td>318 ±62</td>
</tr>
<tr>
<td></td>
<td>Type IIB</td>
<td>17 ±3</td>
<td>17 ±6</td>
<td>9 ±2</td>
<td>6 ±2</td>
<td>6 ±3</td>
<td>8 ±4</td>
<td>4 ±2</td>
<td>3 ±2</td>
</tr>
<tr>
<td></td>
<td>Unclassified</td>
<td>2169 ±574</td>
<td>2090 ±70</td>
<td>2702 ±332</td>
<td>2602 ±277</td>
<td>2438 ±234</td>
<td>2293 ±399</td>
<td>2670 ±216</td>
<td>1843 ±222</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4088 ±596</td>
<td>4644 ±1405</td>
<td>4939 ±365</td>
<td>4160 ±1116</td>
<td>3960 ±974</td>
<td>4471 ±669</td>
<td>4218 ±1047</td>
<td>2642 ±125</td>
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that previously reported by Ariano et al. (1973) for adult rats. Fig. 3 shows a temporal sequence of fibre profile changes in the soleus muscle at selected postnatal ages.

Table 1 gives the means and standard deviations for the absolute frequencies of the different types of fibres observed at various ages in the two muscles. It should be noted that the values presented may not represent the actual total numbers of fibres in the muscles. Occasional losses of small portions of tissue may have occurred during the histochemical preparation. Furthermore, Gollnick, Timson, Moore & Riedy (1981) recently pointed out the limitations of using histological methods for determining absolute fibre counts in muscles.

**Discussion**

The results of this study support the observation that traditional myo-ATPase and NADH-D histochemical procedures are unable to differentiate muscle fibre types in newborn rats (Engel & Karpati, 1968; Shafiq et al. 1972; Riley, 1977a; Haltia et al. 1978).

Several investigators have claimed that a population of type IIC fibres is present in rat muscles during early development and that these fibres may be precursors to the later appearing fibre types (Brooke & Kaiser, 1970; Curless & Nelson, 1976; Haltia et al. 1978). In the current study, the large number of fibres that reacted positively to both the myo-ATPase (pH 10.4) and the NADH-D stains in the plantaris muscle at 6 days of age, although classified as type IIA, may have included some of these transitional type IIC fibres. A definite pattern of fibre differentiation seems to start in the plantaris after 6 days of age which results in an essentially continuous decline in type IIA fibres and steady increases in type I and type IIB fibres until the adult fibre composition is attained (Fig. 1).

The differentiation pattern in the soleus muscle shows a noticeable shift in the fibre population between 11 and 16 days of age (Fig. 2). This is in contrast to the data of Curless & Nelson (1976) in which there was a non-significant change in the percentage (53–65 %) of type I fibres in randomly selected fields of the soleus muscle between 1 and 29 days of age in the same strain of rat. Likewise, Riley (1977a) found a change of only 10 % (49–59 %) in the relative frequency of type I fibres between 11 and 26 days of age in the soleus muscles of Long Evans rats. Although, Riley counted all fibres in each muscle, fibre types were determined on an average of only 395 fibres per muscle. It is well known that fibre types usually are not uniformly distributed across the muscle in lower animals. For example, a relatively high concentration of type IIA fibres can be found in the area next to the medial head of the gastrocnemius in a typical transverse section of the adult rat soleus. In the present study, all available fibres across the entire transverse section of the muscle were classified instead of only the fibres appearing in selected areas. The discrepancy in fibre distribution between the results of
this study and those previously reported by Curless & Nelson (1976) and Riley (1977a) may be due, in part, to this procedural difference.

Fluorescently labelled antibodies and/or electrophoretic techniques recently have been used to identify different polymorphic forms of myofibrillar proteins in skeletal muscles. Staining serial cross sections of adult muscles by the immunoperoxidase technique with antibodies to each of the purified proteins in turn has shown that slow and fast components of troponin are located in type I and type II fibres respectively and, furthermore, that $\alpha$-tropomyosin is restricted to type II fibres and $\beta$-tropomyosin exists only in type I fibres (Dhoot, Frearson & Perry, 1979; Dhoot & Perry, 1979). Different molecular forms of myosin also have been identified in type I and type II fibres (Lowey & Risby, 1971; Weeds, Hall & Spurway, 1975; Rubinstein et al. 1978). In addition, Gauthier & Lewey (1979) have suggested that another myosin isoenzyme may exist which is localized in the type IIA fibres. Therefore, the presence of a single polymorphic form of each of the myofibrillar proteins in a given fibre type appears to be a feature of adult skeletal muscle. However, this observation does not apply at all stages of muscle development.

It has been reported that in foetal and early postnatal muscles all cells can synthesize the fast forms of troponin. Cells that are identified as presumptive type I fibres also can synthesize the slow forms of troponin (Dhoot & Perry, 1980). The exact forms of myosin present in immature muscle fibres are not yet clear. Based upon cross reactivity with antibodies against both adult fast and slow myosin, Gauthier, Lowey & Hobbs (1978) claim that slow and fast forms of myosin coexist in all fibres during early development. Rowlerson (1979), using only antibodies to slow adult myosin, reports that all cells in foetal and newborn muscle contain some slow myosin; whereas, other investigators conclude that fast myosin is predominant (Chi, Rubinstein, Strahs & Holtzer, 1975; Rubinstein & Holtzer, 1979). There is other evidence for the existence of an embryonic form of myosin (Whalen, Butler-Browne & Gros, 1978; Hoh & Yeoh, 1979; Whalen et al. 1979). Any failure to completely purify the myosin that is used as an antigen and/or any failure to affinity purify the myosin antibodies could explain, in part, the conflicting results obtained in these studies (Rubinstein & Holtzer, 1979). In addition, Dhoot & Perry (1979) point out that polymorphic forms of troponins and tropomyosin are single polypeptide chains which presumably are the products of single genes. Therefore, specific antibodies can be used to identify the form(s) of each regulatory protein present in a given cell. The myosin molecule, however, consists of two large and four small subunits. Each polymorphic form is the product of at least three structural genes. Localization studies using antibodies against only part of the molecule, therefore, cannot provide definitive information about the exact nature of the whole myosin molecule.

Although the exact forms of myosin present in immature muscle cells are still to be determined, contractile proteins are known to be synthesized around the
time when myoblasts fuse to yield myotubes (Devlin & Emerson, 1978; Whalen et al. 1978). Regulatory proteins also can be identified in foetal muscle during early gestation (Dhoot & Perry, 1980) when the motor nerves have very little, if any, differential activity (Diamond & Miledi, 1962). Furthermore, there is much evidence now for the synthesis of these myofibrillar proteins in cultured embryonic tissue where neural innervation is absent (Whalen et al. 1978; Rubinstein & Holtzer, 1979; Whalen et al. 1979). Therefore, the initial expression of genes responsible for the synthesis of various myofibrillar proteins can be considered to be an intrinsically programmed event in developing muscle. However it is also hypothesized that the activity of these genes is under some kind of coordinated control during postnatal development (Rubinstein & Holtzer, 1979; Dhoot & Perry, 1980).

The fact is well established that both the contractile properties and the forms of myosin in adult muscle can be altered by the type of innervation provided. For example, either cross-reinnervation with a 'slow' nerve or prolonged low-frequency stimulation can convert a fast muscle into a slow muscle with transformed myosin properties (Buller, Eccles & Eccles, 1960b; Salmons & Vrbova, 1969; Weeds, Trentham, Kean & Buller, 1974; Salmons & Sreter, 1976). Transformation of the type of troponin complex also results from cross-reinnervation of adult muscle (Amphlett et al. 1975).

A similar nerve–muscle interaction is seen during early development. The normal differentiation of contractile activity of the muscle can be altered by denervation or cross-reinnervation (Buller et al. 1960a; Gordon & Vrbova, 1975). For example, it is known that fibre-type differentiation does not occur following neonatal denervation in rat skeletal muscle. Histochemical and electron microscopic results have shown that fibre types are well differentiated in normal gastrocnemius, extensor digitorum longus, and soleus muscles at 2–3 weeks of age, but differentiation is not seen in denervated muscles (Shafiq et al. 1972).

Even during the course of normal muscle development, there is indirect evidence to suggest the existence of a neural influence on muscle fibre differentiation. Fibres in neonatal rat muscles are polynervously innervated. During the first 2–3 weeks of postnatal life, however, there is a marked transition from polynervous to unineuronal innervation of muscle fibres (Redfern, 1970; Benoit & Changeux, 1975; Brown, Jansen & Van Essen, 1976; Korneliussen & Jansen, 1976). Riley (1977b) reported that the percentage of rat soleus muscle histologically innervated by two or more terminal axonal branches decreases from 73 % to 9 % between 11 and 15 days of age. While studying correlated morphological and physiological changes during the elimination of terminals, O’Brien, Östberg & Vrbova (1978) observed that polynervous innervation can no longer be detected electrophysiologically in the rat soleus at 15 days of age although some muscle fibres still show histological polynervous innervation. Rosenthal & Taraskevich (1977) also reported a rapid loss of polynervous
innervation in the rat diaphragm, a fast muscle, between 8 and 14 days of age with a roughly linear decline in the average number of axons innervating each muscle fibre from 2.4 to 1.1. These data suggest that by 2 weeks of age most muscle fibres in the rat have only a single functional junction and that extra terminals about to be retracted are in fact non-functional. It should be noted that these rapid changes in innervation pattern coincide with the most intense growth period of the spinal anterior horn cells in rats (Haltia, 1970). Of further interest is the fact that several investigators have observed the synthesis of fibre-type-specific regulatory and contractile proteins correlates well in time with the elimination of polyneuronal innervation (Gauthier et al. 1978; Dhoot & Perry, 1979; Dhoot & Perry, 1980). For example, Dhoot & Perry (1980) used Sprague-Dawley rats to show that although all cells in gestation synthesize fast forms of troponins, cells identified as presumptive type I fibres also can synthesize slow forms. In these presumptive type I fibres, the amount of fast troponins progressively decreases while the slow forms steadily increase after birth. By 10–11 days, the fast and slow forms of troponin components are completely segregated into separate muscle fibres.

If the synthesis of specific forms of myofibrillar proteins in a given muscle cell is under coordinated control during postnatal muscle development, one might expect that a marked alteration in the innervation pattern should be reflected by a somewhat parallel response in the fibre population of the muscle. The results of this study confirm such a relationship. It was shown that approximately 51% of the eventual type I fibre population appears in the rat soleus, a slow muscle, between 11 and 16 days of age. This time span corresponds exactly to the period of maximal polyneuronal elimination observed by Riley (1977b) and O'Brien et al. (1978) in the same muscle. As for the plantaris, approximately 30% of the adult type I fibre population appears between 6 and 11 days, a period which corresponds closely with that reported by Rosenthal & Taraskevich (1977) for rapid loss of polyneuronal innervation in the rat diaphragm, another fast muscle. Such concurrent changes suggest that a relationship exists between the polyneuronal elimination pattern and the muscle fibre differentiation pattern, but it would be premature to ascribe cause and effect to the relationship.

The role played by muscular activity in the coordinated control of postnatal muscle fibre differentiation is intriguing but even more tenuous. As early as 1970 Redfern suggested that activity might be an important factor in polyneuronal elimination because the period of maximum elimination coincides with a general increase in the activity of the animal. This theory was supported by the observation that the elimination process is delayed when muscle activity is reduced either by tenotomy (Benoit & Changeux, 1975) or by nerve block (Thompson, Kuffer & Jansen, 1979) and is accelerated by low-frequency electrical stimulation (8 Hz) of the motor nerve for 4–6 h per day (O'Brien et al. 1978). Of course, since the relationship between polyneuronal elimination and fibre-type differentiation has not been defined fully, the demonstrated influence of muscular activity on
polyneuronal elimination cannot be extrapolated to fibre-type differentiation at this time. On the other hand, Jones (1981) did show that slow-frequency stimulation (10 Hz), between 4 and 12 days of age, prevents the development of fast contraction times in the normally fast extensor digitorum longus and tibialis anterior muscles and yields a very large proportion of histochemically identified slow fibres in the extensor digitorum longus. Stimulation of the normally slow soleus muscle, however, produced inconclusive results in Jones' study.

Further evidence that muscular activity may assist in the regulation of muscle fibre differentiation has been provided by Gutmann, Melichna, Herbrychowa & Stichova (1976) who concluded that mechanical loading is needed for normal differentiation to take place. A compatible observation is that an intact afferent impulse from the muscle is essential (McArdle & Sansone, 1977).

The mechanism by which the fibre population is established in a muscle is not clear at present. During the initial stages of muscle development, the synthesis of various forms of myofibrillar proteins apparently is an intrinsically programmed event. However, the importance of coordinated neural control during postnatal growth is evident, and this neural control may be mediated by muscular activity.

The results of this study show that the adult fibre profiles in the plantaris and soleus muscles of the rat are developed through a postnatal differentiation process which is completed by 140 days of age. Although relatively rapid increases in the percentages of type I and type IIB fibres occur in the plantaris during the second and third weeks of life, the differentiation process in that muscle follows an essentially continuous pattern. A somewhat different pattern of differentiation is found in the soleus muscle in that a notable shift in fibre population occurs during the second postnatal week.

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