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THREE HIERARCHIES IN SKELETAL MUSCLE FIBRE CLASSIFICATION ALLOTYPE, ISOTYPE AND PHENOTYPE[1]

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ABSTRACT Immunocytochemical analyses using specific anti-myosin antibodies of mammalian muscle fibres during regeneration, development and after denervation have revealed two distinct myogenic components determining fibre phenotype. The jaw-closing muscles of the cat contain superfast fibres which express a unique myosin not found in limb muscles. When superfast muscle is transplanted into a limb muscle bed, regenerating myotubes synthesize superfast myosin independent of innervation. Reinnervation by the nerve to a fast muscle leads to the expression of superfast and not fast myosin, while reinnervation by the nerve to a slow muscle leads to the expression of a slow myosin. When limb muscle is transplanted into the jaw muscle bed, only limb myosins are synthesized. Thus jaw and limb muscles belong to distinct allotypes, each with a unique range of phenotypic options, the expression of which may be modulated by the nerve. Primary and secondary myotubes in developing jaw and limb muscles are observed to belong to different categories characterized by different patterns of myosin gene expression. By taking into consideration the pattern of myosins synthesized and the changes in fibre size after denervation, 3 types of primary (fast, slow and intermediate) fibres and two types of secondary (fast and slow) fibres can be distinguished in rat fast limb muscles. All primaries synthesize slow myosin soon after their formation, but this is

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withdrawn in fast and intermediate primaries at different times. After neonatal denervation, slow and intermediate primaries express slow myosin whereas fast primaries do not, and slow primaries hypertrophy while other fibres atrophy. In the mature rat, the number of slow fibres in the EDL is less than the number of slow primaries. Upon denervation, hypertrophic slow fibres matching the number and topographic distribution of slow primaries appear, suggesting that a subpopulation of slow primaries acquire the fast phenotype during adult life, but reveal their original identity as slow primaries in response to denervation by hypertrophying and synthesizing slow myosin. It is proposed that within each muscle allotype, the various isotypes of primary and secondary fibres are myogenically determined, and are derived from different lineages of myoblasts.

INTRODUCTION

Fibres of limb and trunk muscles of mammals have been classified phenotypically into slow, fast-red and fast-white types, each type containing a distinct type of myosin, and associated with a specific profile of metabolic enzymes. Consequently, these various types of fibres differ in intrinsic speed of contraction, power output and endurance. Such phenotypic diversity has been attributed to the myo-regulatory function of the motor nerve supply. According to this hypothesis, muscle fibres are considered to be "plastic", and fibre types interconvertible according to the pattern of impulses received from the nerve (1,2).

During myogenesis, myotubes are uniformly slow contracting and synthesize embryonic and foetal myosins before expressing adult fibre characteristics. In view of the profound influence motor nerves have on mature muscle fibres, it has generally been considered, since the work of Buller, Eccles and Eccles (3,4) that the emergence of muscle fibre heterogeneity during myogenesis is also brought about by the action of nerves on a common, undifferentiated myotube.

The experiments described in this paper were done to test the neural regulatory hypothesis for fibre type diversity. The jaw muscles of carnivores contain superfast muscle fibres which express a unique myosin not found in limb muscles. If the neural regulatory hypothesis is valid for these muscles, jaw muscles regenerating in limb muscle

beds should express limb muscle myosins and vice versa. During limb muscle development, muscle fibres are polyneuronally innervated. Emerging myotubes would be expected to co-express a mixture of adult myosins. The results of these experiments obtained with immunocytochemical techniques using monoclonal and polyclonal anti-myosin heavy chain antibodies do not confirm these expectations. They reveal two hierarchically distinct levels of myogenic influences affecting fibre phenotype. A hierarchical classification is proposed in which jaw and limb muscles belong to different allotypes which define their phenotypic options. Within each allotype, myogenically distinct isotypes emerge during development.

RESULTS

Nerve Independent Intrinsic Differences Between Cat Jaw and Limb Muscles

Strips of posterior temporalis muscle, a homogeneous superfast muscle, were treated with Marcaine to destroy mature muscle fibres and transplanted into limb muscle beds for regeneration and reinnervation by the host nerve (5). Early regenerates in the bed of either the fast extensor digitorum longus (EDL) or the slow soleus muscle react with antibodies against the heavy chain of foetal, slow or superfast myosins, but not with antibodies against fast myosin. In the long-term, regenerates innervated by the EDL nerve express only superfast myosin whereas in the regenerates innervated by the soleus nerve most fibres react only with the anti-slow myosin antibody, while some fibres react only against superfast myosin even after 213 days. In contrast, EDL and soleus muscles regenerating in their own beds express foetal, slow and fast myosins, but do not express superfast myosin. The isometric contraction times of the various types of regenerates reflect the types of myosin synthesized.

The ability of the regenerating superfast muscle to express the superfast myosin is independent of the nerve (6). This is shown in experiments in which reinnervation of the transplant in the EDL bed is prevented by cutting the common peroneal nerve and reflecting it back into the thigh. In these denervated beds the early temporalis regenerates are indistinguishable from innervated regenerates in expressing superfast myosin in addition to foetal and slow myosins.

Intrinsic differences between jaw and limb muscle cells

have also been shown by the transplantation of limb muscles into the jaw muscle bed. Jaw muscle beds are less satisfactory from the point of view of defining the innervation of the regenerates. Limb muscle strips were transplanted into the anterior temporalis bed after partial excision of this muscle. Fig. 1 shows the results of an EDL regenerated in the anterior temporalis muscle bed for 12 weeks. Staining for superfast (Fig. 1a) and foetal (Fig. 1b) myosins is negative, whereas nearly all fibres stain for fast myosin (Fig. 1d), and some fibres also stain for slow myosin (Fig. 1c). Although innervation of these fibres is not specifically demonstrated, their large size and the absence of staining for foetal myosin suggests that they are innervated.

It is concluded that jaw and limb muscle cells are two distinct types of muscle cells, each having a distinct repertoire for the expression of adult isomyosins, and that the particular isomyosin expressed can be modulated by the nerve.

Heterogeneity of Primary Fibres in Developing Limb Muscles

The postnatal development and the effects of neonatal denervation on muscle fibres in the EDL and tibialis anterior (TA) muscles of the rat were studied immunocytochemically using monoclonal antibodies against myosin heavy chains. Three types of primary myotubes (fast, intermediate and slow) with distinct topographic distributions can be distinguished perinatally. All primaries synthesize slow myosin initially, but in fast and intermediate primaries, slow myosin is no longer detectable in the neonatal period and at 2 weeks of age respectively. The fast primaries are localized principally in a superficial strip of the TA (Fig. 2B) where in the matured muscle slow fibres are absent. Slow primaries are located deep in the muscle while intermediate primaries lie in between. The distribution of slow and intermediate primaries at birth is shown in Fig. 2A.

Following neonatal denervation, the slow and intermediate primaries still express slow myosin, whereas the fast primaries do not stain for slow myosin. At three weeks after denervation, slow primaries are hypertrophic and intermediate primaries are atrophic, both staining with anti-slow antibody. The topographic distribution of these primaries is shown in Fig. 2C. These results show that the three different types of primary myotubes respond

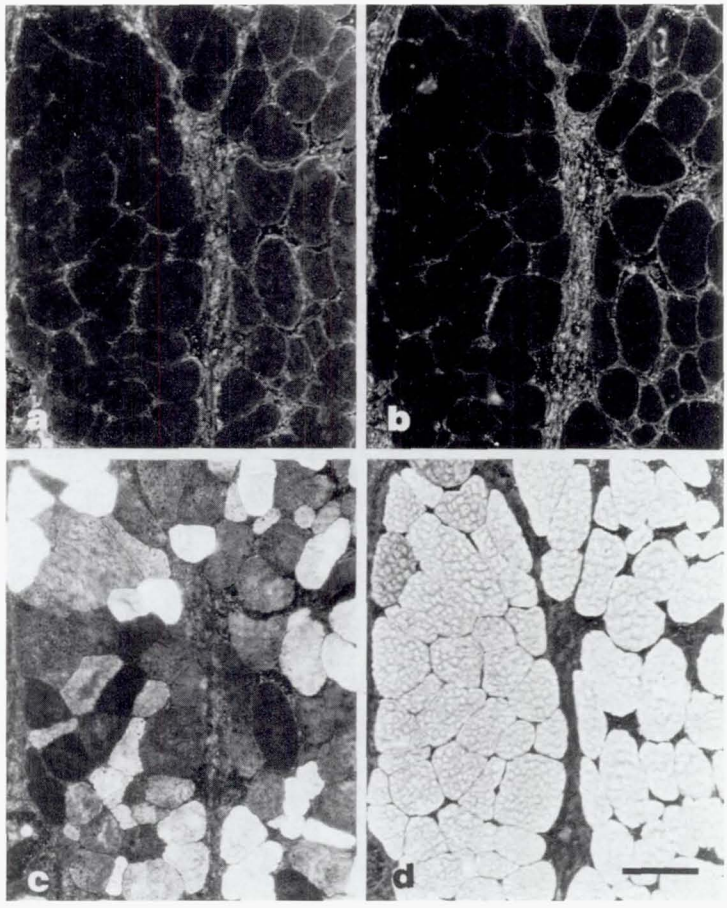

Fig. 1. Fluorescence photomicrographs of serial sections of cat extensor digitorum longus muscle regenerated in the anterior temporalis muscle bed for 12 weeks stained for superfast (a), foetal (b), slow (c), and fast/foetal (d) myosin heavy chains. The scale represents 100μm.

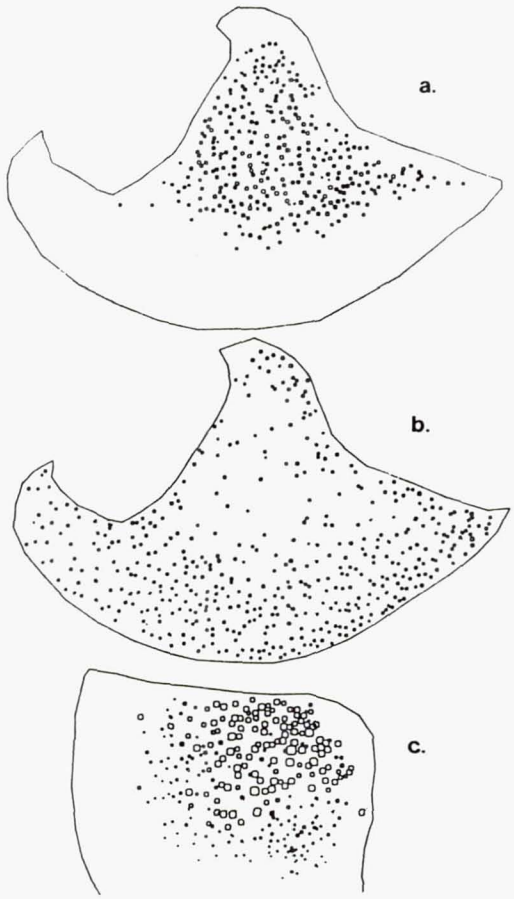

Fig. 2. Distribution of fibres which stain with anti-slow myosin heavy chain antibody in rat tibialis anterior (TA) muscle at birth (A, B) and TA three weeks after neonatal denervation (C). Fibres which stained strongly (slow and intermediate primaries) and those that stained faintly in the neonatal TA (fast primaries) are shown in (A) and (B) respectively. Note the hypertrophy of slow primaries and atrophy of intermediate primaries following denervation (C).

differently to denervation.

Heterogeneity of Secondary Fibres in Developing Limb Muscles

Immunocytochemical analysis of neonatal cat EDL and soleus muscles (7) has revealed that there are at least two distinct classes of secondary fibres. Both classes initially stain strongly for embryonic/foetal myosins. In one of these classes, developmental myosins are replaced by fast myosin. These fast secondaries do not stain for slow myosin nor react with anti-superfast myosin antibody at any stage. The other class of secondaries, the slow secondaries, are prevalent in the slow soleus muscle. These fibres acquired staining for slow myosin but not for fast or superfast myosins.

The vast majority of neonatal secondary myotubes in rat EDL and TA muscles are fast secondaries and stain with an anti-foetal/fast-red myosin antibody. These myotubes diverge at 9 days into a superficial fast-white region and a deep fast-red region. The majority of superficial secondaries no longer stain for foetal/fast-red myosins, presumably expressing fast-white myosin, whereas secondaries located in the region occupied by slow primaries predominantly express fast-red myosin. This topographical distribution of the two classes of secondaries is present in both the EDL and TA muscles, but is more conspicuous in the latter.

Following neonatal denervation in the rat, the divergence of fast-red and fast-white fibres in the EDL and TA muscles is not abolished, but delayed till three weeks post-operatively, suggesting that this divergence is neurally independent.

Effects of Denervation on Slow Primaries in Adult Rats

Immunocytochemical analyses of rat limb skeletal muscle fibres using specific anti-myosin antibodies have revealed that post-denervation changes of muscle fibres cannot be predicted by the fibre phenotype (8). The number of slow fibres in the EDL of a three month old rat is about half the number of slow primaries seen during development. Upon denervation of this muscle, the number of slow fibres increases to match the number of slow primaries at birth. These fibres hypertrophy while other fibres suffer denervation atrophy. These observations suggest that about half

of the slow primaries in mature rats undergo fibre type transformation into phenotypically fast fibres. Upon denervation, all slow primaries express slow myosin and hypertrophy, just as they do after neonatal denervation, irrespective of their phenotype at the time of denervation.

Heterogeneity of Primary and Secondary Fibres in Developing Cat Jaw Muscles

There are two phenotypes in jaw-closing muscle fibres in the cat: superfast and slow. In the posterior temporalis muscle of the mature animal, all fibres are superfast. During late foetal life, sections of this muscle stained for slow myosin appear very similar to those of fast limb muscle: slow staining primary fibres surrounded by rosettes of secondary fibres. Later, both primary and secondary fibres synthesize superfast myosin and the slow myosin in primary fibres is withdrawn (9). Primary fibres in the posterior temporalis are therefore analogous to fast primaries in limb muscles, and may be termed superfast primaries. Slow fibres are present in the anterior temporalis and the masseter muscles of adult cats, and these developmentally are derived from both primary and secondary fibres. The jaw slow primary fibres are analogous to slow primaries in limb muscles in which slow myosin synthesis persists to adult life. The jaw slow secondaries appear in early postnatal life, and some of these fibres stain also for superfast myosin during this period. At no time during the development of cat jaw muscle fibres does any fibre stain for fast myosin.

DISCUSSION

The results of these experiments reveal that the neural regulatory hypothesis cannot account for the difference between limb and jaw muscles. Each type of muscle has a specific repertoire for myosin gene expression, the limb muscles express slow, fast-red and fast-white myosins while jaw muscles express slow and superfast myosins. The ability to express these myosins is intrinsic to the muscle type, and can occur during regeneration in the absence of innervation. Innervation by limb nerves does not induce jaw regenerates to express fast myosins, nor does innervation by jaw nerve fibres lead to the expression of superfast myosin in limb regenerates. However, the specific form of myosin expressed by jaw or limb muscles is subject

to neural regulation within the constraints of the repertoire. The limited repertoires for myosin gene expression for jaw and limb muscles is also seen during developmental myogenesis.

It is useful to introduce the term allotype to describe different classes of skeletal muscle fibres with distinct intrinsic properties such as limb and jaw muscles. Jaw and limb allotypes probably arise from distinct lineages of myoblasts committed to differentiate along different paths. Extraocular muscles, which are isometrically faster than limb and jaw muscles (10) and which express a unique myosin heavy chain (11) may be another skeletal muscle allotype.

Immunocytochemical analyses of developing limb and jaw muscles reveal considerable heterogeneity in the pattern of myosin gene expression in both primary and secondary fibres. Such heterogeneity may be due to some extrinsic influence, such as innervation, acting upon a homogeneous population of myotubes. Alternatively, the myotubes may be intrinsically heterogeneous, being preprogrammed to express different types of myosin during subsequent development.

Evidence against the suggestion that fibre type diversity emerges as a result of neural regulation is the observation that divergence of fast and slow primaries is already apparent prenatally (12) whereas the impulse patterns of developing fast and slow motoneurons in the neonatal rat are very similar; differences emerge only at 3 weeks postnatally (13). Furthermore, the occurrence of polyneuronal innervation of muscle fibres (14) in the early postnatal period also argues against the neural regulatory hypothesis.

In support of the notion that myotubes are intrinsically heterogeneous may be cited the observations that clonal colonies of early myoblasts in chicken (15) and human embryos (16) are not homogeneous with respect to nutrient requirements and colony morphology. Miller and Stockdale (17) have isolated three types of clones from early chicken myoblasts which express fast, slow or a mixture of both myosins. These clones provide a nerve-independent mechanism for the generation of different muscle fibre types during myogenesis (18).

We propose that the emergence of diverse primary fibres in mammalian limb and jaw muscles is due to various lineages of myoblasts with intrinsicallydifferent properties. The characteristic responses to neonatal denervation of the three types of primaries in the rat limb clearly reveal their differences, the most spectacular feature of which being the

hypertrophy of slow primaries. This property of slow primaries is retained in the adult even though some of the slow primaries had apparently undergone, through the neural regulatory influence, a phenotypic change to become fast fibres. Thus, the hypertrophic response of denervated adult muscle fibres cannot be predicted on the basis of fibre phenotype, but can be so predicted according to the developmental origin of the fibres. Hence it is important to classify muscle fibres in accordance with their developmental origin in addition to their allotype and their phenotype. We propose to use the term isotype in this context. Thus there are at least three isotypes (slow, intermediate and fast) of primary fibres in limb muscles and two isotypes (superfast and slow) of primary fibres in jaw muscle.

The emergence of phenotypic characteristics of secondary fibres occurs relatively late during myogenesis, making it possible for neural regulatory mechanisms to have an impact on it. However, the possibility of there being various isotypes of secondary fibres cannot be discounted. Our neonatal denervation study shows that the divergence of fast-red and fast-white fibres is neurally independent, raising the possibility of the existence of two distinct isotypes of fast secondary myotubes. An interesting alternative mechanism for generating different phenotypes of secondary fibres is for primary fibres to influence the phenotype of the secondary fibres associated with them. The existence of gap junctions between primary and secondary fibres is well established (19). These junctions may provide the physical basis for the postulated myogenic influence on secondary fibres. The co-localization of slow primaries and fast-red fibres in the deep region of TA is consistent with the notion that slow primaries induce the expression of the fast-red phenotype in secondary myotubes associated with them.

The various myogenic and neurogenic influences on the phenotypic expression of myosin genes during myogenesis may now be summarized. The allotype defines the various phenotypic options available: superfast and slow myosins for the jaw allotype and fast-red, fast-white and slow for the limb allotype. Very early in myogenesis, diverse isomyoblasts emerge within each allotype. These fuse to produce myotubes of corresponding isotypes, each destined to undergo a particular pattern of myosin gene expression within the options defined by the allotype. Innervation may only play a trophic or permissive role on myogenesis up to this point.

Neural regulatory influences may operate after polyneuronal innervation has been eliminated and phasic and tonic nerve impulse patterns established. These influences may change the fibre phenotype within the range of options defined by the allotype, but do not alter the fibre isotype, nor transform the fibre allotype.

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