Developmental Progression of Myosin Gene Expression in Cultured Muscle Cells

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Summary

Myosin heavy chains are encoded by distinct members of a multigene family at different stages of muscle development. Study of the underlying regulatory mechanisms has been hindered because transitions in myosin expression have not been readily attained in tissue culture. Here we show a transition from early (fetal) to late (perinatal/adult) myosins defined by two monoclonal antibodies, F1.652 and N3.36, in the myotubes of mouse C2C12 cells. On day 1 of differentiation, essentially all myosin was early myosin. By day 8, early myosin dropped to 25% of its day 1 value and was replaced by late myosin. The transition occurred without neural contact, connective tissue components, or complex substrates, suggesting that its regulation may be intrinsic to the muscle cell. Our results demonstrate that a developmental progression in myosin gene expression, which occurs rapidly, with high frequency, and under relatively simple conditions, is now amenable to molecular analysis in cultured muscle cells.

Introduction

We demonstrate here that an established muscle cell line is capable of expressing a sequence of myosin isoforms characteristic of distinct stages of muscle development. The discovery five years ago that different myosin isoforms were present in the contractile apparatus of muscle in the embryo, newborn, and adult mammal first demonstrated that in muscle, like red blood cells, there are closely related but distinct proteins that perform similar functions at different stages of development (Whalen et al., 1981). This finding provided the first means of monitoring muscle development at the molecular level.

A major limitation in the further study of the mechanisms controlling the expression of developmentally regulated muscle genes such as myosin was that changes in myosin gene expression could only be detected in the complex environment of the intact organism. Over the past years, we and others have made several attempts to obtain transitions in myosin gene expression under the more well-defined conditions of tissue culture. Numerous reports suggested that cultured muscle alone could not undergo these transitions and that only fetal forms of myosin were produced (Whalen et al., 1981; Bandman et al., 1982; Bador et al., 1982; Periasamy et al., 1984). However, recently, a progression of developmentally distinct myosin isoforms was detected when muscle was cultured together with nerve explants and connective tissue components for prolonged periods of as much as a month or more (Ecob et al., 1983).

We demonstrate here that a shift in myosin gene expression from early (fetal) to late (perinatal to adult) isoforms can be achieved in muscle alone. This change is rapid and occurs in the majority of colonies containing myotubes within a 1 week period under relatively simple culture conditions that do not use complex substrates or other cell types. These results open the door to the in vitro study of the expression of developmentally regulated multigene families in muscle and the modulation of that expression by hormones and nerves in a manner previously not possible.

Results

To analyze the mechanisms underlying transitions in myosin isoform expression during mammalian muscle development, we produced a library of monoclonal antibodies. Studies with two of these reagents, F1.652 and N3.36, are described here. Hybridomas secreting monoclonal antibodies F1.652 and N3.36 were isolated following immunizations of mice with myosins prepared from human limb muscles at fetal and neonatal stages of development, respectively (Silberstein and Blau, 1986). The antibodies are specific to the myosin heavy chain, since they recognized a component with a molecular weight of 200 kd in muscle cell extracts separated by SDS gel electrophoresis (Figure 1) and reacted with the myosin-containing A-band striations of the sarcomere in isolated myofibrils of mouse and human muscles (data not shown). In addition, the antibodies distinguished developmentally regulated myosins in Western blots: F1.652 reacted only with fetal muscle extracts, whereas N3.36 did not react with extracts until around the time of birth and this reactivity persisted into adulthood (Figure 1).

The time course of expression and distribution of the two antigens was characterized by double immunofluorescent staining of transverse sections of mouse muscle tissues. To permit direct comparisons and eliminate differences due to processing, tissues from two different stages, from limb muscle at 17 or 19 days of gestation and from adult limb or diaphragm muscle, were frozen, sectioned, reacted with antibodies, and photographed together (Figure 2). At 17 days of gestation, F1.652 myosin was present in all fibers and N3.36 myosin was not detectable, whereas in adult limb the converse pattern of staining was observed (Figures 2A and 2B). In perinatal muscle at 19 days of gestation, both myosins were present (Figures 2C and 2D), however, F1.652 myosin had already markedly decreased in amount in a proportion of the fibers (Figure 2C) and by two weeks after birth was further reduced (data not shown). This is in good agreement with the results obtained in Western blots (Figure 1). The stain-
pressed both F1.652 and N3.36 myosins. Myoblasts were plated at limiting dilution so that the progeny of individual cells could be followed in well-separated colonies (Blau and Webster, 1981; Blau et al., 1983b; Webster et al., 1986). Differentiated multinucleated myotubes began to appear within 2 days after exposure of the colonies to low serum medium. A colony was scored as positive for a given type of myosin if it had more than 5 myotubes that reacted with either the F1.652 or the N3.36 antibody. In three separate experiments, the proportion of colonies that contained F1.652 myosin remained relatively constant: 91% ± 4% (SEM) and 98% ± 4% (SEM) on day 2 and day 6, respectively. The colonies that did not react with F1.652 were relatively small and had not yet differentiated to form myotubes, probably because they proliferated after a longer lag time or had a longer generation time than their neighbors (Blau et al., 1983b). The presence of N3.36 was determined during the same time period. A total of 6% ± 3% (SEM) of individual colonies scored after 2 days in low serum medium contained detectable amounts of N3.36 myosin (Figure 3A). In contrast, 88% ± 6% (SEM) of colonies scored 4 days later contained N3.36 myosin (Figure 3B). In addition, a difference in the distribution of the two myosins within colonies became apparent with time: the relative amount of F1.652 myosin was often greater in small myotubes that had probably formed more recently, whereas the larger striated myotubes contained relatively more N3.36 myosin (Figure 3B). Thus, an increase in the total number, as well as the size, of myotubes containing myosin that reacted with the N3.36 antibody occurred with time in culture.

We designed experiments to determine whether the appearance of late myosin in the muscle cultures resulted from coexpression of two different isozymes or reflected a developmental progression in isozyme expression resembling that in the intact organism. It seemed likely from the experiments described above that the early myosin present in day 6 cultures resulted from the formation of new myotubes, since it was present primarily in small, nonstriated myotubes. To test this possibility, we prevented myoblasts from proliferating and forming new myotubes by exposing differentiating muscle cell cultures to an inhibitor of DNA synthesis, cytosine arabinoside (ara-C). As a result, myosin could be monitored in a relatively synchronous population of myotubes as it aged. The outcome of three separate experiments was that within a 1-week period, early myosin declined and was detectable in small, often mononucleated, muscle cells, whereas late myosin was the predominant of the two isozymes and was prevalent in large, striated myotubes (Figure 4). These results were corroborated by gel electrophoresis and Western blotting of protein extracts from parallel cultures (Figure 5). Serial dilutions of proteins extracted from day 1 and day 8 cultures were electrophoresed on polyacrylamide gels and stained either with Coomassie (brilliant) blue or with F1.652 or N3.36 antibodies. The intensity of the band stained by F1.652 in the day 8 samples was equivalent to that in a parallel lane of the day 1 sample diluted 1:3. Thus, the proportion of the total myosin that was early F1.652 myosin dropped from 100% to approxi-
Figure 2. Detection of Early (F1.652) and Late (N3.36) Myosins in Cryostat Sections of Muscle Tissues at Different Stages of Development

Embryonic and adult mouse muscle tissues from C3H mice were frozen in one block and sectioned together. (A and B) Fetal limb muscle (day 17 of gestation) (left) and adult limb muscle (4 month) (right). (C and D) Perinatal limb muscle (1 day before birth, or day 19 of gestation) (left) and adult diaphragm muscle (6 month) (right). Staining of the same field is shown in (A) and (C) with F1.652 and fluorescein-conjugated second antibody and in (B) and (D) with N3.36 and rhodamine-conjugated second antibody. On day 17 of fetal development, fibers appear in clusters or islands well-separated by connective tissue and are ring-like due to the distribution of myosin. Two days later, day 19, the muscle has grown significantly. The staining pattern in the adult diaphragm illustrates that not all fibers in the adult contain N3.36 myosin; the fibers that are not stained are the slow Type I fibers (data not shown). Variation in fiber diameter is frequent in muscle fibers at all stages, including adult muscles; compare the limb (B) and diaphragm (D). Magnification, 310×.

Discussion

Our results contrast with the results of others which suggest that muscle cultures synthesize primarily fetal myosins. The finding of a relatively rapid replacement of the early F1.652 myosin by the late N3.36 myosin may be due to the myoblast type and culture conditions used. The C2C12 cell line was selected in our laboratory for its ability to initiate differentiation rapidly and to form actively contracting myotubes within a few days (Blau et al., 1983a). In addition, the inclusion of an inhibitor of DNA synthesis,
ara-C, enhanced the transition from early to late isozymes in muscle cultures. Ara-C inhibited myoblast proliferation and the continuous production of new myotubes, so that isozyme composition could be monitored in a more synchronous population of myotubes as it aged. Finally, our results may be due to the sensitivity and specificity of our antibodies and assays relative to the peptide mapping, polyclonal sera, and monoclonal antibodies previously employed by others (Whalen et al., 1981; Bandman et al., 1982; Bader et al., 1982).

The pattern of myosin expression during myogenesis is more complex than it first appeared (Whalen et al., 1981). We suggest that the designation of three myosin isozymes specific to fetal, neonatal, and adult stages should be expanded to include multiple myosins that coexist and are characterized by distinct, but overlapping, developmental windows, or time courses of appearance. We have previously determined that there are two distinct fetal myosins in mammalian muscle that differ in their temporal expression (Silberstein and Blau, 1986). Other studies also suggest that more than one myosin isozyme can be present in muscle at a given stage of development (Gauthier and Lowey, 1977; Benfield et al., 1983; Crow and Stockdale, 1984). F1.652 myosin appears to have a time course that is similar to the rat fetal myosin heavy chain isozyme previously described in late fetal and early postnatal stages of limb muscle development (Whalen et al., 1981). On the other hand, N3.36 myosin is unlike other myosins that have been described: it appears at a perinatal stage and persists into adulthood. Possibly the N3.36 antibody recognizes an epitope shared by two distinct myosins present in either perinatal or adult muscle. Alternatively, N3.36 defines a myosin that has not been recognized previously which is present from birth onward. Regardless, it is apparent from analyses of frozen tissue sections and from electrophoresis and Western blotting of protein extracts that N3.36 distinguishes a myosin or myosins typical of more advanced stages of muscle development in the intact organism than the fetal myosin generally observed in cultured muscle. This late isozyme characteristic of stages at or beyond the perinatal stage replaces fetal myosin within a 1 week period in C2C12 muscle cultures.

The developmental progression of muscle gene expression examined here does not require heterogeneous populations of myoblasts. Although myoblasts with different heritable morphological and biochemical properties have been reported by others (Bonner and Hauschka, 1974; Hauschka, 1974; Miller et al., 1986; Miller and Stockdale, 1986), we have detected both the early F1.652 and the late N3.36 myosin isozymes in colonies derived from single myoblasts. Changes in chromatin conformation associated with DNA replication are probably not required.
Developmental Regulation of Myosin

Figure 4: Reduction in Early (F1.652) Myosin and Increase in Late (N3.36) Myosin in ara-C-Treated High Density Cultures

Confluent myoblasts were induced to differentiate by addition of low serum medium and ara-C (10^-5 M) was added after the first 2 days of differentiation. The same cells are shown on day 8 of differentiation with F1.652 (top) and with N3.36 (bottom), using fluorescein-specific and rhodamine-specific optical filters. Magnification, 250x.

for the change in myosin gene expression to occur, since myotubos are generally postmitotic and ara-c was present for the majority of the time course. That sequential expression of myosins occurs in pure muscle cultures within a period of a few days, suggests that these transitions can occur rapidly and do not require innervation, fibroblasts, or complex substrates. Our results raise the possibility that the developmental progression of gene expression in skeletal muscle is primarily regulated by mechanisms that are intrinsic to the muscle cell. Neural and endocrine stimuli such as thyroid hormones (Whalen et al., 1985; Izumo et al., 1986) may subsequently modulate that autonomous expression.

Experimental Procedures

Cell Culture

The C2C12 cell line (Blau et al., 1983a) was a subclone of the C2 line originally isolated by Yaffe and Saxel (1977). Cells were maintained as exponentially growing myoblasts in a high serum medium, Dulbecco's modified Eagle's medium (DMEM; Irvine Scientific) supplemented with 2 g/l glucose, 20% fetal calf serum (JR Scientific, Woodland, CA), and 0.5% chick embryo extract (GIBCO). For studies on differentiated cultures, cells were plated either at high density to form mass cultures (10,000 cells per cm^2) or at low density to form clones (1-2 cells per cm^2) on collagen-coated tissue culture dishes (Falcon) as previously described (Blau and Webster, 1981). Mass cultures reached 70%-80% confluence within 24-48 hr, at which time the medium was changed to low serum medium (DMEM with 2% horse serum; HyClone), and the cultures were fed daily thereafter. Clonal cultures were grown in a conditioned medium, obtained from exponentially growing C2C12 mass cultures, mixed 1:1 with fresh high serum medium and filtered (0.2 µm; Nalgene). After three days, the colonies were fed with fresh high serum medium; after an additional three days, they were changed to low serum medium and fed daily thereafter. Cytosine arabinoside hydrochloride (ara-C, Sigma) was added as indicated at 10^-5 M. A concentration we previously showed reduces the incorporation of [3H]thymidine into TCA-precipitable material to 1% of control levels (Chiu and Blau, 1984).

Isolation of Myosin and Characterization on Western Blots

Myosin was obtained from C3H mouse limbs and trunks of avascularized 17 day old embryos, postnatal back and leg muscles, or differentiated C2C12 mouse muscle cultures. Tissues were homogenized in a Virtis blender (three 10 sec bursts with 1 min rests between), and cultured cells were sonicated for 1 min in low salt buffer (LSB) (40 mM NaCl, 10 mM KPO4, 1 mM MgCl2, and 0.1 mM EDTA (pH 7.5)) with cysteine, (0.1 mg/ml), protease inhibitors PMSF (0.09 mg/ml), aminocaproic acid (1.7 mg/ml), and leupeptin (0.05 mg/ml) at 4°C. The disrupted tissues and cells were centrifuged at 1600 g for 15 min at 4°C and the supernatant was recentrifuged at 16,000 g for 15 min at 4°C and the supernatant was recentrifuged at 16,000 rpm for 15 min at 4°C and resuspended twice in 50 ml of LSB without leupeptin. From the final pellet, which contained myofibrils, myosin was extracted in an equal volume of 0.1 M sodium pyrophosphate (pH 8.3) with 5 mM EGTA, cysteine, and protease inhibitors as described for 1 SR by mixing with a glass rod for 15-30 min at 4°C. The extract
Double-Labeled Immunofluorescent Staining of Cultured Cells

Dishes of muscle colonies were fixed in 1% formalin (Baker) in PBS and stored at −20°C. Dishes were washed as before. Sections were mounted in 16% gelvatol (Monsanto) in PBS/glycerol (2:1) and allowed to harden overnight in the dark at room temperature. The same fields were photographed separately with Kodak Tri-X film using fluorescein-specific (excitation at 470–490 nm) and rhodamine-specific (excitation at 530–560 nm) optical filters and a Leitz epifluorescence Ortholux microscope and 25X/0.25 NA oil immersion objective (Zeiss).

Acknowledgements

We are grateful to Drs. Z. Hall, U. J. McMahan, J. B. Miller, and F. Stockdale for constructive comments on the manuscript. We acknowledge the excellent technical assistance of Dennis Arvanitis and thank Karen Bird for secretarial assistance. This work was supported by grants and a research career development award to H. M. B. from the National Institutes of Health (HD18179 and HD00580) and the Muscular Dystrophy Association (MDA), a postdoctoral fellowship from the MDA to L. S., and an NIH predoctoral training grant (GM07149) to S. G. W.

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Received February 24, 1986; revised June 12, 1986.

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