MYOFIBRILLAR PROTEIN SYNTHESIS AND ASSEMBLY IN SATELLITE CELL CULTURES ISOLATED FROM SKELETAL MUSCLE OF MICE

Ronald B. Young, Timothy R. Miller and Robert A. Merkel

Michigan State University, East Lansing 48824

SUMMARY

Several developmental properties of replicating myogenic cells isolated from the hind limb muscles of neonatal, rapidly growing and young adult mice were examined in cell culture. Satellite cell progeny fused to form multinucleated myotubes that elaborated myofibrillar proteins and underwent spontaneous contractile activity. Additionally, the maximum extent of fusion was similar in cultures isolated from the three ages of mice when cultures were treated with fluorodeoxyuridine (FdU) to inhibit replication of non-muscle cells. However, the onset of fusion was delayed in the rapidly-growing and adult cultures compared to neonatal cultures. This differential onset of fusion was attributed to a higher proportion of non-muscle cells in the cell preparations from the older mice rather than to inherent cellular differences in fusion capability. To determine if the rates of myofibrillar protein synthesis and total protein synthesis were influenced by the age of mouse from which cells were isolated, cultures were pulse labeled with [3H]leucine. The distribution of polypeptides synthesized by these cells, the rate of myosin heavy chain synthesis and the rate of total protein synthesis were not appreciably affected by the age of mouse from which cells were isolated. Thus, it is concluded that the intrinsic myofibrillar protein synthetic capacity of satellite cell progeny is unaffected by mouse age.

(Key Words: Satellite Cells, Cell Culture, Mice, Growth, Myofibrillar Protein Synthesis.)

INTRODUCTION

Satellite cells are small, mononucleated cells located between the basement membrane that surrounds each muscle fiber and the sarcolemma or cell membrane of the muscle fiber (Aloisi, 1970; Church, 1969; Kahn and Simpson, 1974; Konigsberg et al., 1975; Mauro, 1961; Muir et al., 1965; Ontell, 1973). These quiescent cells are within the myogenic lineage, are normally responsible for adult muscle regeneration and, after activation to proliferate, are indistinguishable from embryonic presumptive myoblasts (Bischoff, 1975; Konigsberg et al., 1975; Young et al., 1978). Satellite cells are always found in adult skeletal muscle; however, the mechanism ensuring their presence is not known. These dormant satellite cells in adult skeletal muscle are morphologically distinct from the replicating presumptive myoblasts found in embryonic muscle, but if properly activated to proliferate, satellite cells could feasibly contribute additional genetic information to the muscle fibers of growing or mature meat animals. Therefore, the present study was initiated to examine the effect of animal age on the inherent capability of satellite cell progeny to synthesize and assemble the myofibrillar proteins in cell culture.

MATERIALS AND METHODS

Animal Model. White mice, outbred Spartan HA(ICR), were obtained from Spartan Research Animals, Inc. of Haslett, MI. All mice were

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anesthetized with ether immediately prior to excision of their hind limbs.

**Preparation of Cells for Muscle Culture.** The procedure used for isolating myogenic cells was patterned after that of Bischoff (1974). Excised muscle was immediately placed in a 10 cm culture dish containing buffered saline solution (137 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, .14 mM Na₂HPO₄, 1.35 mM Na₂HPO₄, 6 mM NaHCO₃, 5.5 mM glucose, 37 C, pH 7.4). Muscle was trimmed free of excess connective tissue, and approximately 1.5 g were placed in a clean, uncoated culture dish containing 10 ml of fresh buffered saline solution (BSS). The muscle was then minced with a scalpel into approximately 1 mm³ fragments, pronase (Calbiochem, B Grade) was added to a final concentration of 1 mg/ml, and the suspension was incubated at 37 C for 60 min while undergoing agitation in a water bath. This suspension was centrifuged at 1,500 x g for 1 min and the pellet was resuspended in 15 ml of complete medium consisting of 85% Eagle's Minimum Essential Medium, 10% horse serum, 5% chicken embryo extract, 125 units/ml penicillin, 3.4 μg/ml fungizone and 125 μg/ml streptomycin. The tissue suspension was agitated with a vortex mixer for 30 sec and centrifuged at 700 x g for 1 min. The supernatant from this centrifugation was saved, and the pellet was resuspended in an additional 5 ml aliquot of complete culture medium with the aid of a vortex mixer. The suspension was centrifuged two more times exactly as described above, and the supernatants from all three centrifugation steps were combined. These combined supernatants were centrifuged at 1,500 x g for 3 min. The myofibrils were resuspended in 3 ml of 1 mM EDTA, pH 7.5, collected by centrifugation at 500 x g for 3 min, and finally resuspended in .5 ml of .15 M NaCl.

**Measurement of Myosin Heavy Chain Synthesis Rate.** Muscle cell cultures were evaluated for myosin heavy chain (MHC) synthesis rate at each stage of differentiation by pulse labeling with 20 μCi/ml of [3H]leucine (New England Nuclear). Cultures were labeled for 4 hr at 37 C in complete medium. At the completion of the labeling period, dishes were rinsed twice with cold .25 M KCl, .01 M MgCl₂, .01 M Tris-HCl, pH 7.4. The cells were scraped from the surface into .5 ml of the above buffer. Cells were homogenized with 20 strokes of a 7 ml Dounce homogenizer (tightly fitting pestle), and the cell homogenate was centrifuged at 1,600 x g for 20 min at 2 C. The KCl concentration of the supernatant was lowered to .025 M by the addition of cold distilled water, and 20 μg of previously purified carrier MHC was added. Tubes were placed at 2 C for approximately 12 hr and the myosin-containing material was collected by centrifugation at 1,600 x g for 1 hour. Pellets were dissolved by heating at 80 C for 20 min in 100 μl of a solution containing 1.0% SDS, .05 M Tris-HCl, pH 7.1, 20% glycerol, .01% pyronin y. The dissolved samples were quantitatively
loaded onto 8 cm, 10% polyacrylamide gels and were electrophoresed at .5 milliamps per gel in a solution containing .1% SDS, .2 M Tris-glycine, pH 8.8. Gels were then stained with .02% Coomassie Blue and then destained in a H_2O:glacial acetic acid:methanol mixture (87.5:7.5:5 v/v).

Destained gels were frozen in dry ice, and .5 mm slices were taken through the MHC band on the gel. Slices were dissolved in .2 ml of 30% H_2O_2 in scintillation vials by heating at 50 C for 2 hours. Aquasol (New England Nuclear) was added to each vial and samples were counted in a Beckman 3133P Liquid Scintillation Counter. CPM were converted to DPM using the external standard channels ratio method with chloroform as the quenching agent.

**Measurement of Total Protein Synthesis Rate.** The rate of total protein synthesis and the rate of MHC synthesis were measured from the same set of cultures. This was necessary because of the large number of mice needed for each experiment (i.e., approximately 15 neonatal, 50 rapidly growing and 75 adult mice). The pellet from the first centrifugation of the cell homogenate and the supernatant remaining after centrifugation of the myosin-containing material were recombined. These combined fractions were adjusted to a final concentration of 5% trichloroacetic acid (TCA) and left at 2 C for 2 hours. The TCA-insoluble material was collected by centrifugation and rinsed three times in order to remove non-protein radioactivity. The pellet was finally dissolved in 10 ml of 20% NaOH, and aliquots of this solution were removed and the radioactivity analyzed. In order to measure the radioactivity in proteins other than MHC on the polyacrylamide gels described in the previous section, all gel slices prior to the MHC band and the remainder of the gel below the MHC band were placed into a 10 ml volumetric flask. The gel material was dissolved by adding 2 ml of 30% H_2O_2 and heating at 50 C for 3 hours. This solution was brought to a final volume of 10 ml by the addition of water. Aliquots of this solution were removed and radioactivity was analyzed. The total protein synthesis rate was then calculated as the sum of radioactivity in the gel slices making up the MHC band, the radioactivity in all non-MHC fractions of SDS-polyacrylamide gels and the radioactivity incorporated into TCA-insoluble protein. Since the number of cells in each culture was calculated from Giemsa stained plates as described earlier, the rate of total protein synthesis and the rate of MHC synthesis were calculated as the quantity of [³H]leucine incorporated per nucleus. This was necessary in order to alleviate the fact that neither cell density nor percentage fusion was constant throughout the course of the differentiation process.

**RESULTS AND DISCUSSION**

The goal of this study was to evaluate satellite cell progeny from mice at different stages of growth for their ability to synthesize myofibrillar proteins and to assemble them into intact myofibrils. A rapidly growing strain of white mice was selected so that experiments could be carried out within a reasonable time limit. The three ages of mice chosen for the experiments were neonatal (within 24 hours after birth), rapidly growing (3 weeks) and young adult (7 weeks). Neonatal mice are very immature and were considered to be embryonic for the purposes of the present investigation. Mice at 3 weeks of age were selected because they were making the most rapid weight gains, and 7-week mice were examined because they had essentially attained maximum body weight (figure 1).

Satellite cell suspensions prepared from hind limb muscle were placed in collagen-coated petri dishes at approximately equal densities and incubated for various lengths of time. Cell

![Figure 1. Body weight of Spartan White mice versus mouse age. The data for this growth curve were generously provided by Spartan Research Animals, Inc. of Haslett, MI.](image-url)
attachment and proliferation began within a few hours after plating (Young et al., 1978). Myogenic cell proliferation continued for several days until cell density became adequately high to permit initiation of cell fusion. The percentage of nuclei found in multinucleated myotubes was then utilized as an index of the degree of morphological differentiation, and typical fusion curves are shown in figure 2 for the three ages of mice examined. The lower curve in each panel of figure 2 shows the percentage fusion in cultures that were allowed to differentiate normally for the periods of time indicated. While muscle nuclei within multinucleated myotubes were incapable of mitosis, the non-myogenic cells replicated continuously, and the actual percentage of multinucleated cells decreased in these control cultures after the majority of myogenic nuclei had fused. The absolute number of myotube nuclei either remained constant or increased slightly during the period of decreasing percentage fusion illustrated in figure 2. Our goal was to obtain muscle cell cultures with as high a percentage of multinucleated cells as possible; therefore, mitotic inhibitors were added on the day at which fusion was first noted to enhance selection of a post-mitotic myogenic cell population. Either fluorodeoxyuridine (FdU) or cytosine arabinoside (Ara C) was employed with similar results, but FdU was used in the experiments reported here because it seemed to be slightly less toxic to multinucleated myotubes than Ara C. The percentage of multinucleated cells continued to increase for several days subsequent to addition of the antibiotic (figure 2, upper curves).

The amount of time elapsed between culture initiation and onset of multinucleated myotube formation was dependent on mice age. For example, in a typical experiment such as that shown in figure 2, multinucleated myotubes were first observed on day 2, 5 and 7 in neonatal, rapidly growing and adult satellite cell progeny, respectively. At least two factors can be identified which possibly affect the onset of myogenic cell fusion. Firstly, the spatial proximity of fusion competent cells may vary, either as the result of unequal plating densities or unequal proportions of myogenic and non-myogenic cells in a given culture. Secondly, there could be an age-dependent, inherent minimum lag time between the onset of satellite cell proliferation in culture and the time at which these cells develop the ability to fuse with each other. The results of the present experiments cannot be used to unequivocally distinguish between these possibilities. For example, while the initial, absolute cell density was similar in all cultures (Materials and Methods), the percentage of satellite cells found in total cell preparations decreases with age (Young et al., 1978). In addition, onset of fusion in neonatal cultures is predictably rapid, considering that this tissue is essentially embryonic and that it therefore contains a substantial quantity of post-mitotic myoblasts capable of immediate fusion. Two additional observations indirectly suggest that the delay in fusion results from dilution of satellite cells by the age-associated increase in frequency of non-myogenic cells. Cell proliferation in mixed cultures is initiated within 36 hr after plating in all cases (Young et al., 1978), and the time of

![Figure 2. Fusion of satellite cell progeny from neonatal, rapidly growing, and adult mice. Cultures were fixed with methanol, stained with Giemsa stain and evaluated for the percentage of nuclei within multinucleated cells as described in Materials and Methods. Solid lines indicate fusion curves of cultures to which \(10^{-7}\) M FdU had been added on the first day that fusion was observed, and the lower curve indicates the percentage fusion in cultures to which no FdU was added. Each point within an experiment represents the average of duplicate determinations.](image-url)
onset of fusion in clonal cultures of satellite cells is unaffected by donor age (Miller, 1977).

Because the onset of fusion varied between ages of mice, a particular day common to all cultures could not be directly chosen for data comparison. Therefore, cultures were standardized by making all measurements at specific times relative to the first day at which multinucleated myotubes were found. Live cultures were examined daily by phase microscopy, and the first day at which fusion could be observed was designated as day 1 of the experiment. Measurements were then made on this day and at 2-day intervals thereafter. Figure 3 shows the morphological appearance of satellite cell progeny shortly after the onset of fusion but before the FdU had eliminated most of the mononucleated cells between the myotubes. Cultures treated with FdU for 6 to 10 days were identical in appearance to the cells shown in figure 3, except that essentially no mononucleated cells were visible between the myotubes.

The primary criterion for identifying myofibrillar protein gene activation in muscle cells is the appearance of myofibrils. Additionally, myosin composes approximately 55% of the myofibril, and experiments illustrating the presence of substantial quantities of myosin would confirm that the myofibrillar protein genes have been activated. In all of our experiments with satellite cell progeny, extensive morphological differentiation (i.e., figure 3) and frequent spontaneous contraction of myotubes were observed; however, characteristic myofibrillar cross-striations were scarce. To further examine whether myofibrillar myosin was synthesized in satellite cell progeny, additional biochemical experiments were carried out. The molecular weight of myosin heavy chain (MHC) is approximately 200,000 daltons, a molecular weight different from that of most other cellular proteins; therefore, this peptide can be separated from other cellular proteins by SDS-polyacrylamide gel electrophoresis. Satellite cell progeny cultures from neonatal, rapidly growing and young adult mice were pulse labeled with [3H]leucine as described in Materials and Methods, and cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis. The radioactivity was then measured in gel slices throughout the length of the gel. As shown in figure 4, satellite progeny were capable of directing the synthesis of substantial quantities of MHC (i.e., the radioactivity in slices 11 to 15), and the overall pattern of labeled proteins was similar for the myogenic cell progeny from all three ages of mice. In related experiments examining the molecular components of the myofibrils isolated from
Figure 4. Distribution of [3H]leucine in the low-KCl insoluble protein fraction extracted from satellite cell progeny of neonatal, rapidly growing and adult mice. Cultures were pulse labeled and radioactivity was analyzed as detailed in Materials and Methods. The peak of radioactivity corresponding to slices 11 through 15 was identified as myosin heavy chains by simultaneous electrophoresis of purified myosin.

satellite cell progeny cultures, SDS-polyacrylamide gel electrophoresis was carried out on crude myofibrils prepared as described in Materials and Methods. Gel scans of these preparations, when compared to myofibrils isolated from adult mouse skeletal muscle tissue (figure 5), illustrate that several of the predominant bands found in adult muscle tissue myofibrils are present. Particularly noticeable is the similarity in relative quantity of myosin (Mr = 1.2 cm) and actin (Mr = 4.6 cm), which compose 75% of the myofibrillar fraction. Other general similarities are also apparent; however, it is noteworthy that the myosin light chain pattern (Mr = 7 to 8 cm) is qualitatively altered in the culture myofibril preparations. In summary, the data in figures 4 and 5 suggest that satellite cell progeny are capable of elaborating and assembling adequate quantities of myofibrillar contractile proteins to carry out their contractile function.

Figure 5. Gel scans of SDS-polyacrylamide gels loaded with myofibrils isolated from satellite cell cultures. A. Neonatal, B. Rapidly growing, C. Adult and D. Myofibril preparation isolated from adult mouse skeletal muscle tissue was described by Miller (1977).

The studies described earlier have shown that the existence of satellite cells in a dormant
or quiescent state throughout much of muscle growth does not irreversibly prohibit subsequent myofibrillar protein gene activation under the proper in vitro conditions. However, because it was possible that temporary quiescence could have quantitatively affected the eventual ability of satellite cell progeny to direct the synthesis of myofibrillar proteins, experiments were carried out to test the effect of mice age on the rate of total and myofibrillar protein synthesis in satellite cell progeny cultures. Myosin heavy chain synthesis rate was examined in these experiments because myosin is the major component of the myofibril and because factors affecting myofibrillar protein gene activation would be expected to affect the major myofibrillar constituent. Table 1 shows that the maximum rates of total protein synthesis and MHC synthesis were not measurably affected by the age of the donor mouse.

The pattern of MHC synthesis throughout satellite cell progeny development is shown in figure 6 for both control cultures (lower curves) and cultures treated with 10⁻⁷ M FdU (upper curves). Myosin heavy chain synthesis in mixed cultures of non-muscle cells and multinucleated myotubes is contributed primarily by the multinucleated muscle cells. When total MHC synthesis rate in these mixed cultures is divided by the total number of nuclei present, a low MHC synthesis rate/nucleus is obtained because of the overwhelming proportion of non-muscle cells (c.f. figure 2, control cultures). FdU treatment eliminates the majority of mononucleated cells (c.f. figure 2, FdU-treated cultures); therefore the observed MHC synthesis rate per nucleus in FdU-treated cultures more accurately reflects the quantitative rate of myosin synthesis within the multinucleated myotubes. Figure 2 also shows that the rate of MHC synthesis increases several-fold shortly after myoblast fusion and then reaches a level of maximum synthesis rate that is maintained for several days. Eventually the MHC synthesis rate subsides considerably. The most likely explanation of this pattern of MHC synthesis is that MHC synthesis rate is most rapid shortly after muscle-specific gene activation in order for the multinucleated muscle cells to accumulate substantial quantities of myofibrillar myosin (Emerson and Beckner, 1975; Paterson and Strohman, 1972; Young et al., 1975). After this maximum MHC synthesis rate is reached, it is maintained until adequate quantities of myosin have accumulated to support the contractile activities of the muscle cells. The overall MHC synthesis rate then decreases because only enough synthesis is required to maintain a constant quantity of myofibrillar myosin. Figure 6 (also table 1) shows that the maximum MHC synthesis rate is essentially the same for satellite cell progeny of each age of mice examined. Thus, existence of satellite cells for extended periods of time in the quiescent

![Figure 6. Rate of MHC synthesis in satellite cell cultures either in the presence (solid lines) or absence (dashed lines) of 10⁻⁷ M FdU. Cultures were pulse labeled with [³H]leucine, myosin-containing material was extracted, and electrophoresis was carried out as described in Materials and Methods. Each point represents the mean of 4 to 6 experiments. Variation in these experiments was sufficiently high that none of the points shown was different at the .05 probability level. (o--o), neonatal control; (O--O), rapidly growing control; (●--●), adult control; (●--●), neonatal plus FdU; (O--O), rapidly growing plus FdU; (●--●), adult plus FdU.]

### Table 1. Effect of Growth Stage on the Maximum Rate of [³H]Leucine Incorporation into Total Protein and MHC in Satellite Cell Cultures

<table>
<thead>
<tr>
<th>Mouse age</th>
<th>Synthesis rate (pmol [³H] Leu/10⁴ nuclei)</th>
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<tbody>
<tr>
<td></td>
<td>Total protein</td>
</tr>
<tr>
<td>Neonatal</td>
<td>6.40 ± .31</td>
</tr>
<tr>
<td>3 weeks</td>
<td>6.10 ± .25</td>
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<tr>
<td>7 weeks</td>
<td>5.97 ± .36</td>
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*aEach value represents the mean ± SD of five experiments. Cells were pulse labeled with 20 μCi/ml [³H]leucine for 4 hr as described in Materials and Methods. Maximum total protein and MHC synthesis rates were normally observed 4 to 6 days after the onset of myoblast fusion.
stage neither affected the ability of myofibrillar myosin genes to be activated nor suppressed the ultimate maximum level of MHC synthetic activity. It should be noted that the increase in MHC synthesis rate following fusion of adult satellite cell progeny tended to be slower than the rate of increase of the other two cell types. This effect was observed in most experiments, but it is unclear whether this observation results from an intrinsic difference in the genetic capability of the satellite cell, or whether it could somehow be a manifestation of the lower proportion of satellite cells generally found in adult cell preparations. At any rate, satellite cell progeny are able to elaborate the myofibrillar proteins and assemble them into functional myofibrils.

During embryonic differentiation of skeletal muscle cells, presumptive myoblasts undergo a quantal mitosis that results in development of myoblasts, the immediate precursor for myogenic cell fusion (Abbott et al., 1974). The underlying goal of the present research was to determine if satellite cells (which are quiescent, mononucleated cells in adult skeletal muscle) are identical in some of their metabolic options and biochemical properties to embryonic presumptive myoblasts (which are present in large numbers in neonatal mice). As indicated earlier, the mechanisms responsible for ensuring the presence of satellite cells in adult skeletal muscle are not known. One possibility is that satellite cells are in a uniquely differentiated state and possess the capabilities to become activated, proliferate and further differentiate into fusion-competent myoblasts. Another possibility is that replicating presumptive myoblasts during embryonic muscle development somehow become trapped between the basement membrane and the sarcolemma of a muscle fiber. These trapped cells, which are unable to fuse or proliferate further, simply exist until an external stimulus reactivates their proliferative machinery. Neither of these mechanisms can be ruled out by the present experiments; however, it is clear that satellite cells must be irreversibly committed to the myogenic cell lineage. This conclusion is drawn from the experiments of Young et al. (1978) in which satellite cells from adult skeletal muscle were examined in clonal cultures. In the satellite cell colonies derived from adult skeletal muscle, no clones were observed containing cells with both muscle and non-muscle cell morphologies. These results imply that the satellite cell is not only within the myogenic lineage, but that it does not possess the capability to differentiate into any other cell type.

CONCLUSION

Mononucleated myogenic cells in neonatal, rapidly growing and adult skeletal muscle seem identical both quantitatively and qualitatively in their ability to differentiate and to synthesize and assemble the myofibrillar proteins in cell culture. These data suggest that temporary existence of satellite cells in a dormant state does not affect their ultimate myogenic capabilities. Because satellite cell nuclei are potentially capable of increasing the DNA content of skeletal muscle, and because these added nuclei would contribute additional genetic information that could be transcribed and translated into myofibrillar proteins, the present experiments establish a biochemical basis for conducting additional experiments on activation of proliferation of satellite cells during muscle growth.

LITERATURE CITED

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