Characterization of Sheep Semimembranosus Muscle and Associated Satellite Cells: Expression of Fast and Slow Myosin Heavy Chain

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Abstract

The following study characterized sheep semimembranosus muscle on the basis of fiber type and myosin heavy chain (MHC) isoform composition. Histochemistry (ATPase) of 10 µm cryosections indicated that sheep semimembranosus muscle was composed of fast and slow populations of muscle fibers. Western blots using monoclonal antibodies F-59 and S-58, specific for fast and slow MHC isoforms respectively, demonstrated that the major protein fraction of the semimembranosus muscle included fast and slow MHC isoforms. Satellite cells were subsequently collected from the semimembranosus muscle and allowed to form myotubes in vitro. Satellite cell-derived myotubes synthesized both fast and slow MHC isoforms. Further, Western blots indicated that more slow MHC was present in whole muscle extracts relative to samples collected from satellite cell-derived myotubes. Neither longevity in culture (5 to 20 d post-fusion), nor three subpassages, changed the proportion of fast or slow MHC expressed in primary cultures of satellite cell-derived myotubes. Using immunocytochemistry, binding of McAb S-58 occurred in localized regions of 10 d post-fusion myotubes. In contrast, binding of McAb F-59 was dispersed throughout the myotube and in some instances within mononucleated-like cells. Mononucleated cells produced a protein that co-migrated with MHC following electrophoresis, but was not detected with available antibodies. In summary, satellite cells isolated from the semimembranosus muscle formed myotubes in primary culture that synthesized fast and slow myosin heavy chain isoforms. Future studies with clonal cultures will be required to determine if individual satellite cell-derived nuclei co-express fast and slow MHC isoforms, or if individual myonuclei are restricted in their expression of fast and slow MHC isoforms.

Key words: sheep, muscle, primary culture, satellite cells, myosin.

BAM 2 (3): 183- 190, 1992

Muscle fibers (myofibers) maintain a large intracellular volume by combining the activity of hundreds of myonuclei distributed along the longitudinal axis of the myofiber [24]. Myofibers acquire myonuclei during embryonic myogenesis from myoblasts, or during postnatal myogenesis from satellite cells [4, 5]. Regardless of the developmental period of acquisition, myonucleic act in concert to produce muscle-specific proteins that maintain the protein profile characteristic of a specific muscle fiber [13, 36]. The extent to which intrinsic differences between myonuclei, or environmental regulation of myonuclei, account for diversity between postnatal myofibers remains unresolved.

Using immunological probes to fast and slow MHC isoforms, initial differences between myofibers are detected during early embryogenesis, prior to innervation [6, 7]. These data could be interpreted to suggest that initial myofiber diversity results from the fusion of myoblasts that intrinsically differ in their capacity to synthesize specific MHC isoforms [1, 28].

Since the majority of postnatal myonuclei are obtained from satellite cells during postnatal development [3], it seems important to determine the role that satellite cell-

derived nuclei play in regulating the phenotype of adult myofibers. It has been suggested that satellite cell-derived myonuclei differ intrinsically in their capability to express contractile protein isoforms, depending upon the fiber type to which they have been juxtaposed [15, 16]. When jaw-closing muscles from cats are transplanted to an ectopic muscle bed that differs in the pattern of innervation, myofibers regenerating from satellite cells continue to express an isoform of MHC characteristic of jaw muscle [15, 16]. In a similar experiment, transplantation of chicken breast muscle into the leg musculature resulted in the expression of troponin isoforms (following regeneration) characteristic of the pectoralis muscle [37]. These results suggest that satellite cells are programmed to express specific contractile protein isoforms independent of environmental modulation.

The ability to propagate satellite cells in vitro, offers the opportunity to analyze protein expression patterns in myotubes independent of innervation. Satellite cells from avian skeletal muscle differ depending upon the muscle fiber type from which they are isolated [19, 29]. More specifically, satellite cells from a fast muscle fiber appear to form myotubes that express only the fast isoform of MHC. In contrast, cultured satellite cells from a slow muscle fiber, form myotubes that express both fast and slow MHC isoforms [29]. Using colonies of satellite cells, two populations of satellite cells committed to the formation of fast, or mixed (fast/slow) myofibers are detected in avian skeletal muscle [14]. Expression of myosin light chain isoforms by satellite cell-derived myotubes, also depends on the fiber type of the muscle from which the satellite cells are isolated [19]. In contrast to studies with avian satellite cells, satellite cells from rat soleus muscle (slow fiber type) express only the fast isoform of MHC following myotube formation [11], indicating that differences among species may exist with regard to the expression of MHC isoforms by satellite cell-derived myotubes, *in vitro*.

In the current study, satellite cells were collected from sheep skeletal muscle (mixed fiber type) and characterized on the basis of MHC expression (fast and/or slow), following myotube formation in vitro. The hypothesis tested in this study was that satellite cells isolated from a mixed-fiber type muscle will form myotubes in primary cultures composed of fast and slow MHC isoforms. This research represents an initial effort to determine if satellite cells in sheep skeletal muscle are present in multiple populations which differ in the expression of contractile protein isoforms.

Materials and Methods

Antibiotics [penicillin, streptomycin and fungizone (lot # 0020743)], were purchased from Flow Laboratories (Melean, VA.). Culture medium, fetal bovine serum (FBS) and horse serum (HS) were purchased from Gibco (Grand Island, N.Y.). Cultureware was purchased from Costar (Cambridge, MA.). All electrophoresis supplies and Western blot materials were purchased from Bio-Rad (Richmond, CA.). 1,4-Diazabicyclo[2.2.2]octane was purchased from Aldrich (Milwaukee, WI.). Protease inhibitors (phenylmethyl-sulfonyl fluoride, leupeptin, pepstatin-A, 1,10-phenanathroline), and gelatin (swine-skin, type-I) were purchased from Sigma (St. Louis, MO.). Reagents used to detect antibody binding were purchased from Vector Laboratories (Burlingame, CA.).

Monoclonal antibodies

Monoclonal antibodies specific for fast and slow isoforms of chicken myosin heavy chain (MHC) were kindly provided by Dr. Frank Stockdale, Stanford University. Specificity of these antibodies has been well characterized [22, 23]. Monoclonal antibody F-59 is an IgG that recognizes all fast MHC isoforms, and the β-cardiac/slow MHC isoform in rats. Monoclonal antibody S-58 is an IgA that reacts strongly with slow MHC-2 and very weakly to slow MHC-1 in chickens.

Cryosectioning

The fiber-type distribution in a semimembranosus muscle from a six month old wether lamb was determined, using ATPase histochemistry. Strips of semimembranosus muscle (15x5 mm) were removed and submerged in isopentane cooled to -160°C with liquid nitrogen. Transverse sections (10 μm) were cut with a cryostat and placed on a glass coverslip. Sections were air dried and processed for ATPase staining as previously described [17].

Cell culture

The semimembranosus muscle was aseptically collected from a six month old wether lamb (approximately 45 kg) and satellite cells were isolated exactly as previously described [9]. All experiments were performed using cultureware pre-coated with 0.5% (w/v) pig-skin gelatin [8]. Cultures were established using 15 gm of original muscle mass equivalence (wet weight). Cultures were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Fusion of satellite cell nuclei and formation of myotubes occurred spontaneously, approximately 7 d after plating. Following fusion, 10% horse serum was used in place of FBS and antibiotics were omitted from the medium. Cultures were provided fresh media every other day, prior to confluency. Following confluency, medium was exchanged daily by removing approximately 1/2 of the medium per well and adding the same volume of fresh medium back to the culture well.

The effect of culture age on the expression of MHC was studied first, in an attempt to determine the phenotypic stability of MHC expression. Cells were grown on four 10 cm plates and myosin was extracted 5, 10, 15 and 20 d post-fusion. The isoform(s) of MHC present at each time period was determined by Western blotting with McAbs F-59 and S-58. Second, the effects of three subpassages on the expression of MHC isoforms were defined. Duplicate (10 cm) cultures were used for subpassaging experiments.

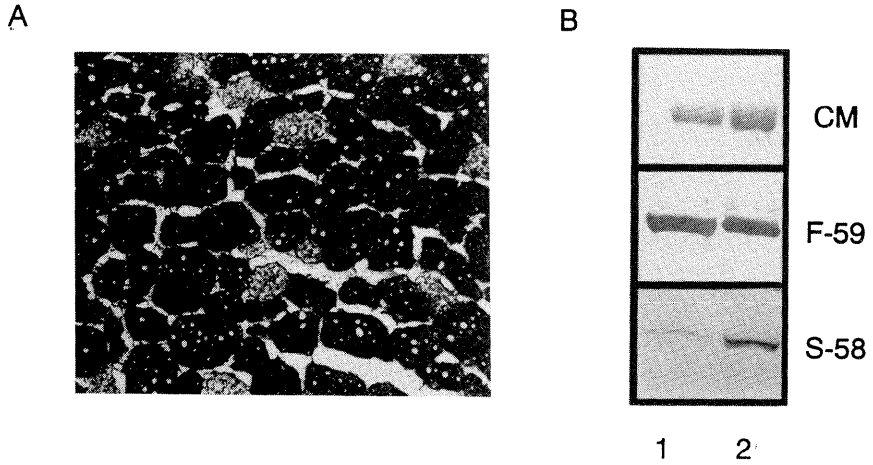

Figure 1. Determination of fiber types in ovine semimembranosus muscle, also MHC isoforms present in whole muscle extracts and satellite cell-derived myotubes. (A) Transverse section (10 μm) of sheep semimembranosus muscle stained for myofibrillar ATPase activity. Darkened regions indicate higher ATPase activity. (B) Equal amounts of MHC were loaded and blotted with McAb F59 and S58 specific for fast and slow MHC, respectively. Concentrations of MHC were determined by scanning Coomassie stained gels (Cm) with a densitometer. Myosin was extracted from satellite cell-derived myotubes (lane 1) or whole muscle (lane 2). Fast and slow MHC isoforms were present in whole muscle and myotubes derived from satellite cells. However, more slow MHC was consistently detected in whole muscle extracts compared to myosin isolated from satellite cell-derived myotubes.

Myosin was collected from one of the duplicate cultures (10 d post-fusion) at each of the three passages for Western blot analysis. Cells in the second culture were liberated using 0.25% (v/v) trypsin/1mM ethylenediamine tetraacetic acid (EDTA), and filtered through 40 μm Nytex to remove myotubes. Mononucleated cells were counted using a hemacytometer, and replated (2.0×10^5 cells) onto two (10 cm) culture plates to represent the next passage. The contribution of mononucleated cells to the expression of MHC was also studied. Mononucleated cells (2.0×10^6) were collected at each subpassage and processed for MHC content.

Myosin extraction

Myosin was extracted from myotubes in culture as described [22]. Cells were rinsed with cold PBS (pH 7.08), scraped from the plate using a rubber policeman and collected by centrifugation for 3 min at 1000 x g. The supernatant was decanted and cold extraction buffer composed of 0.6M sodium chloride, 10mM sodium phosphate (dibasic), 1mM sodium pyrophosphate, 0.5mM magnesium chloride, 0.1mM ethylene glycol bis N, N, N, N-tetraacetic acid, 1mM dithiothreitol and 0.5% (v/v) Triton x-100 (pH to 6.8) was added (1 ml/100 mm culture plate) to the cell pellet. To inhibit proteolysis, leupeptin (10 μg/ml), 1,10-phenanthroline (5 mM), pepstatin (0.1 μg/ml) and phenylmethylsulfonyl fluoride (2 mM) were added. The pellet was dispersed with agitation while being maintained on ice for 15 min. Next, the extract was centrifuged (5 min at 1500 x g) and the supernate containing the solubilized myosin was collected and added directly to Laemmli buffer 1:4 [18]. For some preparations, myosin was further concentrated by low salt precipitation. Five volumes of water were added to the supernatant, mixed, and set on ice for 15 min. Precipitated myosin was collected by centrifugation at 15,000 x g for 10 min. The supernatant was removed and Laemmli buffer was added to the pellet. The pellet was solubilized by heating for 3-5 min at 90°C.

Electrophoresis and immunoblotting

Proteins were separated by SDS-PAGE using a 5% (v/v) bis/acrylamide (29.3 gm acrylamide, 0.7 gm bis) running gel and a 3.5% stacking gel. Gels were run at 100 mV constant current. The gel was stained with Coomassie Brilliant Blue R-250 and MHC was quantitated using an ISCO UA-5 gel scanner. Estimation of MHC concentrations in unknown samples were made by comparison to a standard curve generated with known concentrations of Sigma Rabbit MHC (lot # 13F-9002). For Western blotting, equal amounts of MHC were separated as described above and electrophoretically transferred to nitrocellulose [31]. Following transfer, the membrane was incubated for 1 h at room temperature with PBS (pH 7.6) containing 2% (w/v) powdered milk. The membrane was then incubated for 1 h with antibodies F-59 or S-58 diluted 1:10 in PBS pH 7.6 containing 2% (w/v) powdered milk. Binding of monoclonal antibody S-58 was detected using an amplified (avidin/biotin) alkaline phosphatase system [10]. Antibody F-59 was detected using a non amplified, goat anti-mouse peroxidase system [33].

Immunocytochemistry

Cells were grown on glass coverslips (12 mm dia.) coated with 0.5% (w/v) pig skin gelatin. Coverslips with myotubes on their surface, were rinsed with PBS (pH 7.6)

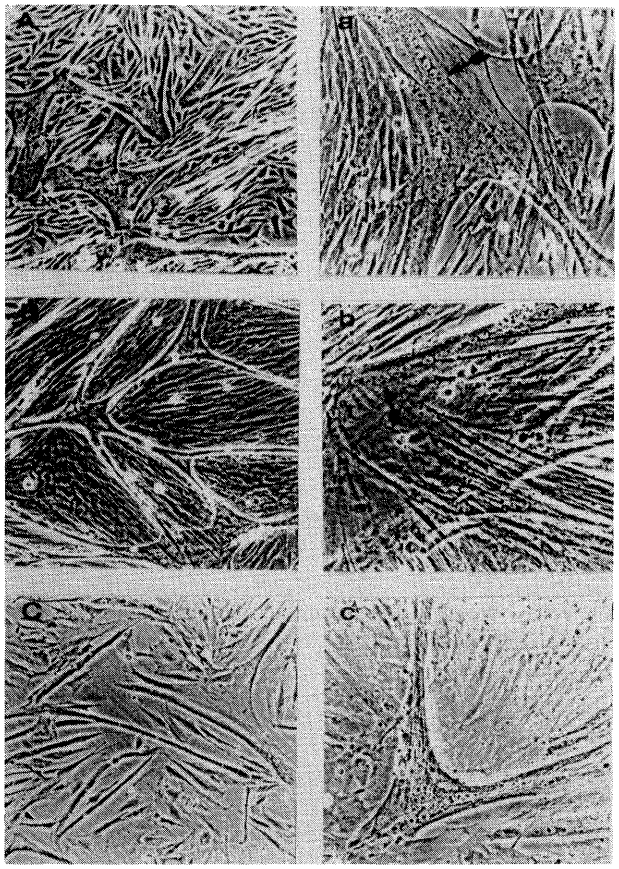

Figure 2. Phase micrographs of satellite cell-derived myotubes in primary culture. A,a and B,b represents 5 and 15 d postfusion myotubes; C,c are myotubes derived from third passage mononucleated cells (29 d old). Large letters indicate magnification of 100x while small letters indicate magnification of 200x. Arrows in a and b show the location of satellite cell-derived myonuclei in myotubes 5 and 15 d following fusion. As myotubes age, nuclei become situated along the edges of the myotube, in contrast to their central location in younger myotubes.

and fixed with 95% ethanol at room temperature for 5 min [22]. Coverslips were then incubated at room-temperature for 1 h with antibodies F-59 or S-58 diluted 1:10 into PBS (pH 7.6) containing 0.4% (v/v) horse serum. Binding of McAb F-59 was detected using a FTIC conjugated second antibody. In contrast, an amplified (avidin/biotin) system was required for the detection of McAb S-58 binding. For the amplified system, coverslips were incubated for 1h at room temperature with biotinylated anti-mouse IgG, diluted 1:200 in PBS (pH 7.6) with 0.4% (v/v) horse serum. Next, coverslips were incubated with FTIC avidin-D diluted 1:160 in PBS (pH 7.6). Between all reagent changes, coverslips were washed with PBS (pH 7.6). After the final incubation, all coverslips were washed and mounted in 50% glycerol/PBS (pH 8.6) containing 2.5% (w/v) 1,4 diazabicyclo[2,2,2]octane to prevent fluorescent bleaching [14]. Fluorescence was detected using a Zeiss microscope modified with epifluorescence.

Results

To determine if McAbs F-59 and S-58 crossreact in sheep skeletal muscle and to ensure that satellite cells were being isolated from a mixed population of muscle fibers, elucidation of the fiber type composition of the sheep semimembranosus muscle was requisite. Using ATPase histochemistry (Fig. 1A) the semimembranosus muscle was found to be composed of mixed (fast and slow) fiber types. Results from immunoblots (Fig. 1B), using McAbs F-59 and S-58 supported the ATPase histochemistry data, that both fast and slow MHC isoforms were present in the semimembranosus muscle. Furthermore, histochemical and immunological data indicated that sheep semimembranosus muscle has principally fast-type fibers.

Expression of fast and slow MHC isoforms in whole muscle could result from environmental modulation of myofiber nuclei [1], or be an intrinsic property of the myofiber nuclei [28]. Determination of the MHC composition of satellite cell-derived myotubes in vitro, offered an opportunity to initially distinguish between these alternatives. Western blot analysis (Fig 1B) indicated that 10 d post-fusion satellite cell-derived myotubes, were composed of fast and slow MHC isoforms and that myosin from whole muscle extracts had more slow MHC compared to myosin extracted from satellite cell-derived myotubes. The Coomassie (CM) stained gel (Fig. 1B), was included to demonstrate that equal amounts of MHC were added for immunoblots.

The effect of culture age on the expression of MHC isoforms (fast vs slow), was determined by Western blotting of myosin collected from primary cultures of satellite cell-derived myotubes 5, 10, 15, and 20 d postfusion. Figure 2 (A-C), shows photomicrographs of satellite cell-derived myotubes. Although the exact age of individual myotubes were unknown, areas of highly branched myotubes were present 15 and 20 d after initial fusion (Fig. 2 B). Western blots indicated that fast and slow MHC isoforms were synthesized by satellite cell-derived myotubes for each time period (Fig. 3A). Also, for each time period the amount of slow MHC was low, relative to fast.

Formation of myotubes from sheep satellite cells in primary culture was an asynchronous process. Myotubes initially formed 5-10 d after plating of mononucleated cells. However, new myotube formation has occurred as late as 40 d following initial plating. Thus, to determine if early and late fusing mononucleated satellite cells differed in their expression of MHC isoforms, myosin was collected from 10 d post fusion myotubes prior to each of three subpassages. Third passage mononucleated cells (26 d old) maintained the capacity for myotube formation (Fig. 2C) reiterating the asynchronous process of satellite cell fusion in primary culture. Fast and slow MHC isoforms were

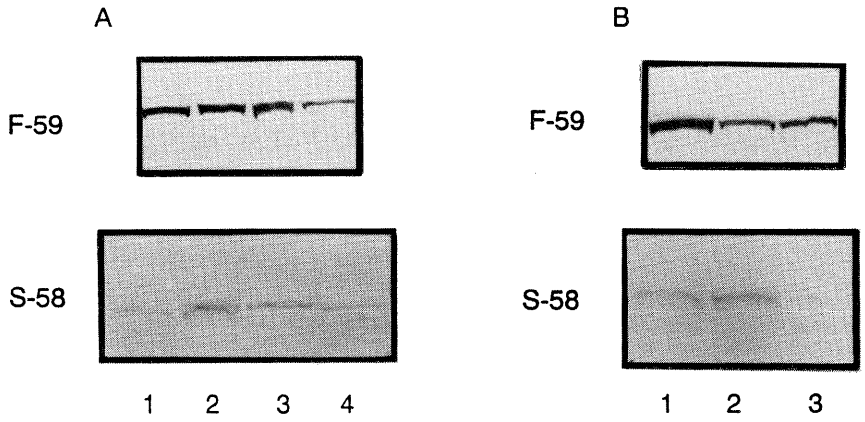

Figure 3. (A) Immunoblots of MHC isolated from primary cultures 5, 10, 15 and 20 d postfusion, lanes 1-4, respectively. (B) Immunoblots of MHC collected from 10 d postfusion myotubes following 1-3 subpassages. Lanes 1-3 represent subpassage 1-3. For (A) and (B), top row was reacted with McAb F59 and bottom row was reacted with McAb S58. Sample wells reacted with McAb S58 contained twofold more myosin.

produced by myotubes at each subpassage (Fig. 3B). Prior to each subpassage (15 d and 28 d) and before initial myotube formation (4 d), myosin was extracted from (2.0 x 10^6) mononucleated cells. A protein that co-migrates with MHC was detected in second and third passage mononucleated cells, following Coomassie staining of the gel (Fig 4A). However, myosin from mononucleated cells was not detected with McAbs specific for fast (Fig 4B) or slow MHC isoforms.

Immunofluorescent staining of 10 d postfusion myotubes using McAb F-59 and S-58 (data not shown) was used to confirm Western blot data and determine the location of McAbs F-59 and S-58 binding. Monoclonal antibodies S-58 and F-59 recognized MHC proteins within satellite cell-derived myotubes. In addition, McAb F-59 occasionally detected MHC in mononucleated-like cells.

Discussion

The majority of myofiber nuclei responsible for the synthesis of myofibrillar proteins are derived during postnatal development from satellite cells. To account for the diversity in protein composition between myofibers [13], satellite cell-derived myonuclei must be flexible in their protein expression patterns, exist as multiple populations that differ in protein expression, or combine properties of both. The isoform(s) of MHC present in myofibers may be used as molecular markers to distinguish between patterns of protein synthesis *in vitro* or *in vivo* [34]. In avian skeletal muscle, satellite cells appear to be predetermined in their expression of fast and/or slow MHC isoforms [14, 1]. In contrast, the majority of data for mammalian satellite cells indicate that satellite cells are not predetermined with regards to the expression of MHC isoforms [2, 25, 35].

Data from the current study suggest that sheep satellite cell-derived myotubes produce both fast and slow MHC isoforms. Although satellite cells from avian skeletal muscles similarly express both fast and slow MHC isoforms in primary culture [29], synthesis of slow MHC by cultured mammalian satellite cells is not well documented. Satellite cells from rat soleus muscle (composed primarily of slow contracting myofibers) form myotubes that synthesize only the fast isoform of MHC in vitro [11]. Differences between sheep and rat satellite cell-derived myotubes in ability to express slow MHC could represent divergency between species, differences in antibody specificity, and/or differences in methods used to detect antibody binding. In the current study, detection of McAb S-58 binding required the addition of at least 4 µg of MHC to the SDS PAGE gel and an amplified (avidin/biotin) antibody detection system. Whereas, the prior study with rat satellite cells utilized a blot system in which only 0.5-1.0 µg of MHC was loaded and an amplified detection system was not employed.

In the present study, all cultured sheep satellite cell-derived myotubes bound to McAb F-59. The pattern of binding was distributed throughout the myotube. In contrast, few myotubes bound McAb S-58 and binding was localized to discrete regions of the myotube. Thus, myotubes were predominantly expressing the fast MHC isoform. However, it remains unclear if individual nuclei were co-expressing both MHC isoforms, or if a distinct population of satellite cell-derived myonuclei were present that expressed only the slow isoform of MHC. Studies using clonal cultures are in progress to determine if specific sheep-derived satellite cells are restricted to an individual pattern of MHC synthesis following myotube formation.

Regardless of whether an individual satellite cell-derived myonucleus is co-expressing fast and slow MHC, or if different populations of satellite cells are responsible for the synthesis of these two MHC isoforms, the current study does suggest that very little slow MHC is synthesized, relative to fast. This decreased detection of the slow MHC isoform may be a result of: 1) the presence of small numbers of satellite cells that express slow MHC, 2) a low basal rate of slow MHC synthesis or 3) inadequate methods

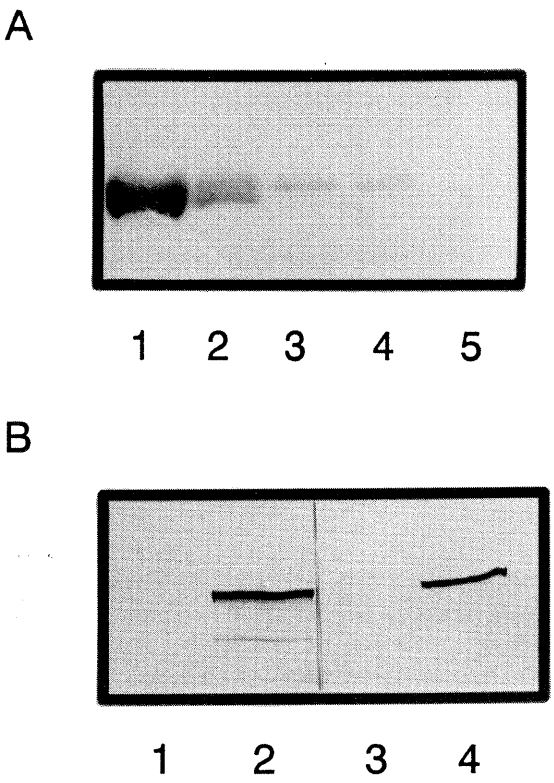

Figure 4. (A) Coomassie stained gel showing protein collected from mononucleated cells (2.0 x 10^6) prior to each subpassage that comigrates with MHC. Lane 1 is myosin molecular weight standard, lane 2 is MHC collected from primary cultures (mononucleated cells and satellite cell-derived myotubes) and lanes 3-5 represent protein collected from mononucleated cells prior to subpassage 1, 2, and 3. (B) Protein collected from mononucleated cells was not reactive with McAb F59 or F27, both specific for fast MHC isoforms. Lanes 1, 3 were loaded with protein extracted from subpassage 2 mononucleated cells and reacted with McAb F59 and F27, respectively. Lanes 2, 4 were loaded with protein extracted from primary cultures and reacted with the same antibodies.

used to detect slow MHC. If the numbers of satellite cells that express slow MHC were low in the original cell isolate, eventual proliferation of this population should result in some clonal colonies that formed myotubes, expressing only the slow MHC isoform [21]. Since binding of McAb S-58 only occurred in sections of the myotube, it is unlikely that myotubes are formed from satellite cells that express only slow MHC isoform. Further, no change in the expression of slow MHC was detectable in myotubes prior to three subpassages. This eliminates the possibility that satellite cells expressing slow MHC are slower to proliferate and are thus being overgrown by other cell populations. However, this interpretation does not exclude the possibility that protocols used during the collection or initial culture of satellite cells, may select against the proliferation and/or differentiation of satellite cells that would normally express slow MHC following myotube formation.

Numerous intracellular mechanisms might also account for the low level of slow MHC in primary cultures of satellite cell-derived myotubes. Synthesis of slow MHC could be regulated at the level of gene transcription, mRNA processing, mRNA translation or degradation of the mRNA and/or protein. The majority of available data indicate that MHC levels are primarily regulated at the transcriptional level [32]. Decreased transcription of the slow MHC gene could result from restricting access to the slow MHC control elements (promoter or enhancer). Further, the current *in vitro* system may lack the positive trans-acting factors required for efficient gene activation [30]. If transcriptional regulation, or any other intracellular mechanism, accounted for the low levels of slow MHC synthesis in our system, then sheep-derived satellite cells would not be predetermined to express slow MHC following differentiation in vitro.

More of the slow MHC isoform is present in whole muscle extracts than in satellite cell-derived myotubes. Since the source of nuclei responsible for the synthesis of slow MHC differs between whole muscle and myotubes collected in vitro, this may not represent a parallel comparison. For example, embryonic and fetal myoblasts (not present in our culture system) are known to form myofibers composed of slow MHC. Since these cells are not present in our culture system, the percentage of the slow MHC isoform may be decreased. Another possible explanation for the low level of slow MHC detected in vitro is that monoclonal antibody S-58 may have a low affinity for the isoform of slow MHC synthesized by myotubes derived from sheep satellite cells. In chicken skeletal muscle, McAb S-58 displays a high affinity for the adult isoform of slow MHC and a lower affinity for the slow isoform of MHC present in the fetus [14]. If sheep satellite cell-derived myotubes synthesize an isoform of slow MHC similar to fetal MHC in chickens, McAb S-58 may underestimate the amount of slow MHC present in sheep satellite cell cultures. In the current study, some mononucleated cells produced a protein that co-migrated with MHC, but which was non-reactive with antibodies S-58 and F59. This protein migrated at a higher molecular weight relative to the protein recognized by McAb F-59. Therefore, it probably does not represent a degradation product of MHC. This protein most likely represents a degradation product from a higher molecular weight protein, even though the abundance of this protein excludes most higher molecular weight muscle proteins [26].

Proteins which co-purify with myosin have molecular weights less than MHC [27]. Fibronectin is synthesized by myoblasts and fibroblasts [20] and upon degradation produces a fragment of a similar molecular to MHC [12]. The

fibronectin fragment which co-migrates with MHC can be removed using Sepharose-4B affinity chromatography [12]. In the current study Sepharose-4B did not remove the protein that co-migrated with MHC (data not shown).

In conclusion, sheep satellite cells derived from a mixed fiber type muscle, formed myotubes in vitro that produced both fast and slow MHC isoforms. The amount of slow MHC present was much less than the amount of fast and the ratio of fast to slow MHC produced was not changed by longevity or subpassaging of the cells. Evaluation of cloned satellite cells will be required to determine if satellite cells are flexible enough to coexpress both isoforms of MHC following myotube maturation in vitro. Alternatively, populations of satellite cells may be present in sheep skeletal muscle that are programmed to express distinct MHC isoforms.

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Acknowledgments

Supported by United States Department of Agriculture competitive grant 86-CRCR-1-1961, regional project NC-131 and Washington State Agricultural Research Center projects 0743 and 0913, paper number 8064.

We thank Dr. Everett Martin for assistance in muscle isolation and Janet Hume and Laura Lee Clark for their assistance in manuscript preparation. In addition, the contribution of monoclonal antibodies S-58 and F-59 by Dr. Frank E. Stockdale (Stanford University) is greatly appreciated.

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