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Characterization of myosin isoforms in satellite cell cultures from adult rat diaphragm, soleus and tibialis anterior muscles

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Abstract

Satellite cells were isolated by enzymatic dissociation and Percoll gradient centrifugation from adult rat diaphragm, soleus, and tibialis anterior muscles with fairly reproducible yields. Diaphragm and soleus muscle yielded approximately five times more satellite cells than tibialis anterior muscle. According to light microscopic criteria, no morphological differences existed between the satellite cell cultures of different origin. Contrary to the donor muscles, myotubes from the 10-day-cultured satellite cells contained a uniform myosin heavy chain (MHC) pattern with predominance of an immunochemically identified embryonic heavy chain. The three types of cultures displayed a typical embryonic light chain (LC) pattern with LC1_{emb}, LC1f, LC2f, and traces of LC3f. The isomyosin pattern was characterized by four embryonic isomyosins, eMI–eM4, with similar distributions in the three cultures. In summary, these myosin analyses provide no evidence for the existence of satellite cell diversity among three rat muscles of different fiber-type composition, at least not under the applied in vitro conditions.

Introduction

The ability of skeletal muscle to regenerate relates to its satellite cell population. These quiescent myoblasts, located between sarcolemma and basal lamina [23], are activated after muscle fiber injury to proliferate, fuse and form new fibers [1, 5, 23, 27]. Several laboratories have been successful in establishing satellite cell cultures. These can be derived either from explants [4] or by enzymatic dissociation of skeletal muscle [2, 22, 37].

Circumstantial evidence suggests that satellite cells do not form a homogeneous population but differ depending on the muscle in which they reside. Differences in satellite cell number between slow-oxidative and fast-glycolytic muscle fibers were observed by Schultz in rat muscle [9]. Satellite cell proliferation following deterioration of fast-twitch glycolytic fibers was observed in rabbit fast-twitch muscles undergoing fast-to-slow transformation under the influence of low-frequency stimulation [20]. The satellite cells in these transforming

muscles formed new fibers, some of which expressed slow myosin [21]. We have interpreted these findings as an indication of the existence of more than one satellite cell population [20, 21]. Also, transplantation experiments by Hoh and Hughes [13] provided evidence in favour of satellite cell heterogeneity. These authors observed that cat temporalis muscle regenerating in the bed of the extensor digitorum longus retained the ability to express superfast myosin. The myotubes and muscle fibers originating from satellite cells of the transplanted temporalis muscle obviously expressed the superfast myosin in the foreign environment. Stockdale and Feldman [29, 30] reported on two satellite cell populations in culture derived from slow-tonic chicken anterior latissimus dorsi (ALD) and fast-twitch pectoral muscle. Using monoclonal antibodies directed against specific epitopes of fast and slow myosin heavy chains (MHC), they showed that cultivated ALD satellite cells express, in addition to a fast MHC, minor amounts of slow MHC1. Conversely, satellite cell cultures from pectoralis muscle expressed only a fast MHC [29, 30]. However, evidence exists from the work of Vivarelli et al. [34] that the myogenic programs differ between mammals and birds.

In view of these findings, we were interested to address the question of satellite cell heterogeneity in satellite cell cultures from rat muscles. For this purpose, satellite cells were isolated from selected muscles and kept in culture in order to investigate their phenotype at the level of myosin isoforms. As donor muscles, we chose tibialis anterior, which is predominantly composed of fast-twitch type IIB fibers, and soleus muscle, which mainly contains slow-twitch type I fibers. In addition, satellite cells were obtained from the diaphragm, a specialised, fatigue-resistant muscle with a fiber population mainly composed of the fast fiber subtype IID [3, 32]. The myosin composition of these cultures was analysed electrophoretically for myosin light and heavy chains and isomyosin patterns. Myosin heavy chain isoforms were identified immunochemically with the use of specific antibodies against embryonic, fast and slow MHC isoforms.

Methods

Satellite cell isolation

Satellite cells were obtained from diaphragm, soleus and tibialis anterior muscles of adult (2.5–4 months of age) male Wistar rats. For each preparation, three rats were used. After killing the animals, muscles were removed under sterile conditions, transferred into Dulbecco's modified minimum essential medium (DMEM), and immediately processed for cell isolation. The isolation was performed as previously described [37] with the following modifications: muscle fragments were digested with collagenase type III (Worthington, Freehold, NJ, USA) at a final concentration of 0.04% (mass/vol) for 45 min. The treated tissue was collected by centrifugation at 300 g for 3 min. The supernatant fraction was discarded and the sediment redigested with Dispase (grade II, Boehringer Mannheim, FRG) at a final concentration of 0.24% (mass/vol) for 30 min. Both digestion steps were performed under constant stirring at 37° C. The resulting suspension was forced several times through a 10-ml glass pipet and then filtered through a 50-µm nylon mesh (double layer). Cells were collected by centrifugation at 300 g for 10 min and resuspended in DMEM. This cell suspension was fractionated on a Percoll (LKB Pharmacia GmbH,

Freiburg, FRG) gradient: 7 ml 20% Percoll was layered over 3 ml 60% Percoll in a corex tube. The cell suspension was layered on top and centrifuged for 5 min at 11000 rpm in the SS-34 rotor of a Sorvall centrifuge. Following centrifugation, the cells at the 20/60% Percoll interface were collected, washed free of Percoll in DMEM, counted in a hemocytometer, and used for cultures.

Satellite cell culture

Cells were plated at a density of 2×10^5 cells per 35-mm dish onto gelatin-coated petri dishes in DMEM supplemented with 25% fetal calf serum (FCS), 10% horse serum (HS, HyClone, Greiner, Frikkenhausen, FRG), and 1 % embryo extract. Cells were maintained in a water-saturated atmosphere containing 8% CO₂ in air. Medium was changed 48 h after seeding and then every 2–3 days. After 2 days, the medium was exchanged with a medium containing DMEM with 20% FCS, 5% HS, and 1% embryo extract. After 5 days, the serum concentration was further reduced to 10% FCS, 10% HS, and 1% embryo extract. From day 7 on, cultures were grown in a medium containing 2% FCS, 10% HS, and 1% embryo extract. The cells were kept in culture for up to 14 days. For harvesting, they were washed twice with phosphate-buffered saline (PBS), scraped off the dish, and frozen in liquid N₂.

Muscles

Soleus, tibialis anterior and diaphragm were excised from adult male Wistar rats, frozen, and pulverized in liquid N₂.

Myosin extraction

Myosin was prepared according to Rubinstein and Kelly [26]. The precipitated myosin was dissolved in 40 mM Na₄P₂O₇, 1 mM EDTA, pH 8.8, diluted with an equal volume of glycerol and stored at –20° C. All solutions contained 1 mM phenylmethylsulfonyl fluoride (PMFS). Protein concentrations were determined according to Lowry et al. [19].

Myosin heavy chain electrophoresis

MHC isoforms were separated electrophoretically on Polyacrylamide gradient (PAA) gels in the presence of sodium dodecylsulfate (SDS) as previously described [3] using a 0.75-mm-thick 6%–9% gradient separating gel and a 4% stacking gel, both containing 30% glycerol. Aliquots of the myosin extracts containing 0.1–0.5 µg protein were applied to the gels after 5 min incubation in 10 µl lysis buffer (10% glycerol, 5% mercaptoethanol, 2.3% SDS, 60 mM Tris/HCl, pH 6.8) at 95° C. Electrophoresis at a constant voltage of 120 V lasted 21 h. Gels were silver-stained according to Oakley et al. [24].

Myosin light chain electrophoresis

Myosin light chains (MLC) were separated by two-dimensional electrophoresis according to O'Farrell [25]. For the first dimension, 1.6% (pH 5–8) and 0.4% (pH 3.5–10) ampholines (LKB Pharmacia GmbH, Freiburg, FRG) were used. Electrophoresis in the second dimension was carried out in a 12.5% PAA gel in the presence of SDS according to Laemmli [17].

Isomyosins

Isomyosin separation was performed according to Termin and Pette [31] using the vertical slab gel system DESAPHOR VA 150 (Desaga Heidelberg, FRG). Gels were silver-stained [24].

Immunoblotting

Purified myosin (0.5–1.0 µg) was electrophoretically separated on 6%–9% PAA gradient gels. The proteins were transferred from the gel to nitrocellulose sheets (0.45 µm, Schleicher & Schuell, Dassel, FRG) by the method of Towbin et al. [33]. The blotted proteins were reacted with three different monoclonal antibodies against specific MHC isoforms: anti-embryonic *2B6* [7], anti-slow *96J* [16], and anti-fast *MY32* (Sigma, München, FRG). Production and characterization of these antibodies have been described elsewhere [7, 16]. Antibody 96J was used at 1:1000 dilution, antibody MY32 at 1:500 dilution. Antibody 2B6 as a hybridoma culture supernatant was diluted 1:40. The immunodecoration was performed as previously described [18]. After reaction with the specific antibodies, the nitrocellulose sheet was incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands). The antigen-antibody complex was visualized by staining with α -chloronaphthol and H_2O_2 [11].

Results

Satellite cells from different muscles – yields and morphology

The standardized method of satellite cell isolation via Percoll gradient centrifugation yielded mononucleated cells free from debris and myofibrillar fragments. Based on the number of cells in the 20/60% Percoll interface, we determined satellite cell yields from the different rat muscles under study. Cell counting resulted in fairly reproducible yields from preparation to preparation. A consistent finding was that approximately five times more satellite cells were isolated from both diaphragm and soleus muscle than from tibialis anterior muscle (Table 1). The cell yields from tibialis anterior muscle were in agreement with previously published data on satellite cell yields from chicken pectoralis muscle [37].

Light-microscopic inspection revealed no striking differences in the growth characteristics or morphology of the satellite cell cultures derived from the three muscles (Fig. 1). An intense proliferation could be observed during the first 5 days after seeding. At day 5 in culture, most cells were found to be aligned and first fusion events were observed. After 9 days in culture, fusion was almost complete and well-developed myotubes predominated. Only a few mononucleated cells remained in the culture.

Myosin heavy chains

Myosin heavy chains from 10-day cultured satellite cells and their donor muscles were analysed on PAA gradient gels (Fig. 2). The adult soleus muscle (Fig. 2, lane a) contained MHCI as the major isoform. MHCIIb and MHCIIId were the prominent isoforms of the adult tibialis anterior muscle (Fig. 2, lanes b, d). In the diaphragm, MHCIIId was the major isoform with minor amounts of MHCIIb and MHCI (Fig. 2, lane c). Myotubes in 10-day-old satellite cell cultures from diaphragm, tibialis anterior and soleus muscles (Fig. 2, lanes e, f,

g) uniformly displayed a MHC pattern with one prominent band of low mobility. Two additional faint bands of higher mobility were also seen in these samples. Coelectrophoresis with myosin preparations from the respective adult muscles (Fig. 2, lanes h, i, k) showed that the slowly migrating prominent band had a lower mobility than any of the MHC isoforms in the adult muscles. As judged from the coelectrophoresis experiments, the two faint bands present in the myotube samples, comigrated with MHCIId and MHCIIb.

The electrophoretically separated MHC isoforms were further characterized by immunoblot analyses. After transfer to nitrocellulose sheets, the MHC isoforms of the adult muscles and the respective satellite cell cultures (10- and 14-day-old cultures) were reacted with antibodies against embryonic (2B6, Fig. 3b), adult fast (MY32, Fig. 3c), and slow (96J, Fig. 4b) MHCs. The prominent band in the satellite cell samples reacted strongly with the anti-embryonic MHC antibody (Fig. 3b, lanes B, C, E, G, H) but not with the other two antibodies. Therefore, it was identified as an embryonic MHC. An identification of the additional two faint bands seen in satellite-cell-derived myotubes (Fig. 2, lanes e, f, g) was not possible. The anti-fast MHC antibody stained the MHCIIb and MHCIId bands in tibialis anterior (Fig. 3c, lane D) and the diaphragm (Fig. 3c, lane F), whereas the anti-slow MHC antibody gave a strong reaction with the prominent band in the soleus sample (Fig. 4b, lane A) and a weak reaction with MHCI in the diaphragm (Fig. 4b, lane F). In addition, immunocytochemistry with the anti-slow MHC antibody gave negative results throughout (results not shown).

Myosin light chains

A major result of the MLC analysis by two-dimensional electrophoresis was the detection of appreciable amounts of the embryonic LC isoform, LC1_{emb}, in the satellite-cell-derived myotubes (Fig. 5b, d, f). In addition, all satellite cell cultures expressed, independent of their origin, the two fast light chains LC1f and LC2f, but only trace amounts of LC3f. Although myosin from adult soleus (Fig. 5a) and diaphragm (Fig. 5c) contained appreciable amount of the LC1s_a, LC1s_b, and LC2s, no slow LC isoforms could be detected in the myosin preparations from the corresponding satellite cell cultures.

Isomyosin patterns

Electrophoresis under nondenaturing conditions produced a uniform pattern for all satellite cell cultures. Four bands were separated and designated as embryonic isomyosins eM1, eM2, eM3, and eM4 in the order of decreasing mobilities (Fig. 6). The designation as embryonic isomyosins was justified by their higher mobilities as compared to the mobilities of fast isomyosins from adult muscles (not shown). No significant differences existed between the densitometrically evaluated relative concentrations of the embryonic isomyosins in the three satellite cell cultures. Their relative concentrations (means $\pm$ SEM) were: 20.1 $\pm$ 1.3% for eM4, 46.9 $\pm$ 1.3% for eM3, 25.2 $\pm$ 1.1% for eM2, and 4.1 $\pm$ 2.8% for eM1.

Although no light chain analyses of the individual bands were performed, we speculate on the basis of the above-documented MLC patterns (Fig. 5) that eM4 represents the LC1_{emb} homodimer, eM3 the LC1_{emb}/LC1f heterodimer, eM2 the LC1f homodimer, and eM1 the

LC1f/LC3f heterodimer. Because of the very low amount of LC3f, the corresponding homodimer, which should migrate in front of eM1, was not detectable.

Discussion

The present study addressed possible phenotypic differences between myotube cultures derived from satellite cells of rat skeletal muscles with different fiber type composition. In view of the characteristic myosin phenotypes of the three muscles under study, the analysis of the corresponding satellite cell cultures focused on differences in myosin expression. However, no differences in myosin isoform expression were observed. As judged from the analysis of myosin light and heavy chains and isomyosin patterns, the satellite-cell-derived myotubes remained in an embryonic developmental stage with predominance of an embryonic MHC [36] and the typical myosin light chain pattern of embryonic rat muscle [8, 35]. These findings fit with previous observations by Vivarelli et al. [34] on myotube cultures of late embryonic mouse muscle, which expressed only embryonic but no adult fast myosin isoforms.

Our results do not necessarily exclude the existence of different satellite cell lineages. Hughes and Hoh [14] examined satellite cell cultures derived from cat temporalis, soleus, and EDL muscles. According to a preliminary communication, the “myotubes derived from jaw and limb fast and slow muscles synthesized fetal and slow myosins, but not fast or superfast myosins” [14]. In view of the finding that cat temporalis muscle regenerating in the bed of the EDL muscle expresses superfast myosin [13], these negative results could indicate that satellite cells are unable to express specific intrinsic properties in an in vitro environment due to the lack of permissive factors. In the case of the temporalis muscle, innervation does not seem to be important because the expression of superfast myosin occurs during regeneration in the absence of nerve [13]. It may be speculated that besides unidentified growth factors, interaction with the extracellular matrix may be important for satellite cells to express specific phenotypic properties. It is noteworthy that Matsuda et al. [22] found differences between the myosin LC patterns of satellite cell cultures from fast-twitch pectoralis and slow-tonic ALD muscles. Although these cultures expressed an embryonic LC pattern, the ALD-derived satellite cell cultures differed from the pectoralis muscle derived cultures by their higher synthesis of slow LC1s and LC2s. Using antibodies against MHC isoforms Stockdale and coworkers [29, 30] were able to distinguish immunohistochemically two different satellite cell populations derived from adult chicken fast-twitch pectoralis and slow-tonic ALD muscles. In view of our results, this discrepancy could be due to species-specific differences, but it should also be taken into account that we compared fast- and slow-twitch muscles, whereas the work on avian muscles compared fast-twitch and slow-tonic muscles.

Another possibility which must be considered, is that the isolation and/or culture conditions used in the present study may have favored the isolation and/or proliferation of a unique satellite cell subpopulation. However, the possibility that only a specific subpopulation of satellite cells was isolated seems improbable, in view of the marked predominance of type I fibers in the soleus and type II fibers in the tibialis anterior of the adult male Wistar rat [3, 10, 32]. It seems unlikely that the satellite cells isolated from soleus muscle stem from its

few fast-twitch fibers or that the satellite cells isolated from tibialis anterior muscle stem from its few slow-twitch fibers.

An additional result of the present study relates to the pronounced difference in the yields of satellite cells isolated from muscles of different fiber-type composition. We found an approximately five-fold higher satellite cell content in soleus than in tibialis anterior muscle. This difference is in agreement with data of an electron-microscopic study by Gibson and Schultz [9]. These authors reported a three- to five-fold higher satellite cell content in soleus than in tibialis anterior muscle. However, the absolute satellite cell contents estimated by these authors exceed our values by a factor of three. This discrepancy points to a considerable loss of satellite cells during the isolation procedure. Nevertheless, the loss seems to be similar for both muscles because the relative satellite cell yield varies by the same factor as described for soleus and tibialis anterior by Gibson and Schultz [9].

Evidence exists that the satellite cell content of fast-and slow-twitch limb muscles is under neural control [15]. This may also relate to differences in fiber recruitment. It follows from long-term electromyographic recordings from single motor units of rat limb muscles that slow fibers are much more frequently recruited than fast-twitch fibers during spontaneous locomotor activity [12]. Similarly, physiological or pathological conditions which increase the frequency of muscle fiber recruitment have been shown to evoke increments in satellite cell content. This was observed after an exercise training protocol [6], as well as in the myotonic adr mouse with its typical after-contractions [28]. Also, increased contractile activity as induced by chronic low-frequency stimulation seems to induce satellite cell proliferation [20]. Preliminary studies on satellite cell yields with the methods described in this study, showed threefold elevations in 15-day-stimulated rat tibialis anterior muscle.

Taken together, our results do not provide evidence that satellite cells differentiating in tissue culture differ with regard to their myosin expression patterns. Our negative results do not exclude the possibility that satellite cells residing in different muscles may be capable of developing specific phenotypic properties when they proliferate and fuse to form myotubes in their natural environment. Under the presently applied in vitro conditions, differentiation of satellite does not proceed beyond the embryonic stage. It may be speculated that myofibrillar proteins other than myosin, especially those which do not exist as embryonic isoforms, may be more suitable to study the existence of satellite cell diversity.

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Fig. 1.
a–i. Development of muscle cell cultures derived from satellite cells of adult rat diaphragm, soleus and tibialis anterior muscles. **a–c** Two-day-old cultures; **d–f** Five-day-old structures. **g–i** Nine-day-old cultures. $\times 240$

Fig. 2.

Gradient gel electrophoresis of myosin heavy chain isoforms in adult rat diaphragm, soleus and tibialis anterior muscles and in 10-day-old satellite cell cultures derived from the same muscles: *a*, soleus muscle; *b*, tibialis anterior muscle; *c*, diaphragm; *d*, tibialis anterior muscle; *e–g*, cultured satellite cells derived from diaphragm (*e*), tibialis anterior muscle (*f*), soleus muscle (*g*); *h*, coelectrophoresis of myosins from adult rat diaphragm and 10-day-cultured diaphragm-derived satellite cells; *i*, coelectrophoresis of myosins from adult rat tibialis anterior muscle and 10-day-cultured TA-derived satellite cells; *k*, coelectrophoresis of myosins from adult rat soleus muscle and 10-day-cultured soleus-derived satellite cells. Abbreviations: *SO*, soleus; *TA*, tibialis anterior; *DI*, diaphragm; *HC_{emb}*, *HCIIb*, *HCIIId*, *HCI*, myosin heavy chain isoforms

Fig. 3.

a–c. Immunoblot analyses of myosin heavy chains (MHC) from adult rat muscles and satellite cell cultures derived from the same muscles. **a** Staining for protein after transfer to the nitrocellulose sheet. **b** Immunoblots with the anti-embryonic antibody 2B6. **c** Immunoblots with the anti-adult fast antibody MY 32. Lanes B, E, and G show myosins from 10-day-cultured satellite cells, lanes C and H show myosins from 14-day-old cultures. Abbreviations: SO, soleus; TA, tibialis anterior; DIA, diaphragm; SC, satellite cell culture; HC_{emb}, HCIIId, HCIIb, myosin heavy chain (MHC) isoforms

Fig. 4.

a, b. Immunoblot analyses of myosin heavy chains from adult rat muscles and satellite cell cultures derived from the same muscles. **a** Staining for protein after transfer to the nitrocellulose sheet. **b** Immunoblots with the anti-slow antibody 96J. Lanes B, E, and G show myosins from 10-day-cultured satellite cells, lanes C and H show myosins from 14-day-old cultures. SO, soleus; TA, tibialis anterior; DIA, diaphragm; SC, satellite cell culture; HCl, slow myosin heavy chain isoform

Fig. 5.

Two-dimensional electrophoreses of myosin light chains in adult rat diaphragm, soleus and tibialis anterior muscles and from 10-day-old satellite cell cultures derived from the same muscles. *A*, actin ; *LC1_{emb}*, embryonic myosin light chain ; *LC1f*, *LC2f*, *LC3f*, fast myosin light chains; *LC1s_a*, *LC1s_b*, *LC2s*, slow myosin light chains; *TM*, tropomyosin

Fig. 6.
Electrophoretic separation of native isomyosins from 10-day-cultured satellite cells derived from adult rat diaphragm, soleus and tibialis anterior muscles. *DIA*, diaphragm; *SO*, soleus; *TA*, tibialis anterior; *eM1–eM4*, embryonic isomyosins

Table 1

Yields of mononucleated cells from skeletal muscles of the adult rat

Expt.	Cells/g muscle $\times 10^5$		
	Soleus	Diaphragm	Tibialis anterior
1	8.7	–	0.9
2	9.2	–	1.1
3	6.7	–	1.9
4	15.0	–	2.5
5	5.2	5.8	3.2
6	11.5	12.3	–
7	13.0	12.2	–
8	10.0	13.0	–
9	14.9	14.6	–
10	7.2	9.6	2.2
11	8.2	5.7	2.8
Means $\pm$ SD	9.9 $\pm$ 3.3	10.4 $\pm$ 3.5	2.1 $\pm$ 0.8