A modified enrichment protocol for adult caprine skeletal muscle stem cell

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Abstract To establish an adequate model to study the proliferation and differentiation of adult caprine skeletal muscle in response to bioactive compounds, a pool of satellite cells (SC) was derived from the rectus abdominis muscle of adult goat. Skeletal muscle contains a population of adult stem cells, named as satellite cells that reside beneath the basal lamina of skeletal muscle fiber and other populations of cells. These SC are multipotent stem cells, since cells cultured in the presence of specific cell lineage inducing cocktails can differentiate into several types of mesenchymal lineage, such as osteocytes and adipocytes. In the present study, we have developed a modified protocol for isolating satellite cells (>90%) and examined their myogenic and contractile properties in vitro.

Keywords Caprine · Satellite cells · Goat · Multipotent

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Introduction

Cell culture is a common method for the investigation of cells from various tissues. It has been proven to be an invaluable technique for researchers exploring mechanisms of animal growth and development, which allows us to separate these interactions and more clearly examine the cell's nutritional, hormonal and environmental requirements for maintenance, proliferation and differentiation (Mcfarland 1992).

Skeletal muscle contains several types of stem cells, including satellite cells (SC) that reside beneath the basal lamina and plasma membranes of skeletal muscle fibers in vivo (Charge and Rudnicki 2004). Adult skeletal SC have long been considered as myogenic lineage-committed cells with either self-renewing or differentiating into multinucleated myotubes in vitro and are mitotically quiescent. They can be activated during muscular growth and traumatic regeneration. When challenged by mitogens, such as fetal bovine serum after extirpation into culture, they are activated and proliferate to produce progeny (myoblasts). After differentiation, they fuse to form multinucleated cells (myotubes) (Chen et al. 2005). Recent observations in rodents and humans indicate that skeletal muscle stem cells (both SC and the cells in the interstitial spaces of skeletal muscle) possess the capability not only to be myogenic stem cells but also to be multipotent stem cells, since cells cultured in the presence of specific cell lineage inducing cocktails can differentiate into several types of

mesenchymal lineage, such as osteocytes and adipocytes (Asakura et al. 2001; Wada et al. 2002).

In vitro growth of skeletal muscle is controlled by several growth factors like fibroblast growth factors (FGFs), insulin-like growth factor-I (IGF-I) and its alternative splicing variant (Kawada et al. 2001). The skeletal muscle is known to be a complex object containing various mononuclear cells capable of in vitro cultivation. These include fibroblasts (connective tissue), various blood vessel cells, adipocytes, and the proper muscle SC. That is why obtaining a pure SC culture remains a complex problem and requires special procedures. A number of approaches have been proposed to solve this problem: (1) separation of the cells by sedimentation rate or attachment to the plastic surface (Anderson 2000; Springer et al. 1997); (2) getting rid of the present fibroblasts by flow cytometry using a cell sorter (Linge et al. 1989); and (3) using special (selective) media (containing D-valine, growth factors, etc.) (Rando and Blau 1994). However, due to various complications, the real purity of the obtained myoblast cultures can be low (Terekhov et al. 2001).

Despite the accumulated evidence showing the presence of stem cells harboring mesenchymal multipotency in the skeletal muscle of rodents and humans, few studies have been reported regarding the multipotency of small ruminants particularly of caprine muscle cells. There are many different procedures and media for the cultivation of myogenic cells derived from large mammals like sheep or cattle (Dodson et al. 1987; Roe et al. 1989), but there is only a few reports for the establishment and characterization of caprine myogenic cell (Yamanouchi et al. 2007, 2009). There are reports that myoblasts grow better in F10 media than in DMEM or other media as it gives advantages over contaminating fibroblast (Springer et al. 1997). Through the combination and improvement of different protocols given in the above mentioned literature, we developed a protocol that enables to establish a myogenic cell pool highly enriched for myoblasts/satellite cells and to study the proliferation, differentiation and contractile properties of caprine myoblasts in response to a variety of factors under different media conditions.

Materials and methods

Isolation of caprine satellite cells

To establish an in vitro system of caprine myoblasts from muscle SC for various types of investigations, goat rectus abdominis muscle (RA) tissue was collected from slaughter house with all aseptic precautions in Dulbecco's phosphate-buffered saline (D-PBS, calcium- and magnesium free, Sigma) and transported to laboratory. Tissues were digested to release myogenic cells according to modification of a protocol described previously by Springer et al. (1997) and Mau et al. (2008) (Fig. 1). Briefly, connective tissue was removed and muscles were minced to a slurry in a minimal quantity of enriched D-PBS (50 IU/mL penicillin, 100 µg/mL streptomycin and 5 µg/mL gentamycin) and digested twice with 0.10% trypsin (Sigma) for one hr each at 37 °C, with inversion every 15 min. Samples were triturated for 10 min, and enzyme action was stopped by 10% fetal bovine serum (Invitrogen). After filtering the cell suspension through sterile muslin cloth, it was pelleted by centrifugation (1,200 rpm for 10 min) at room temperature and supernatant was discarded. Cell pellet was suspended in F10 medium without serum and plated in a sterile 90 mm Petri dish (Axygen) and incubated for 3 h at 37 °C under 5% CO₂. After 3 h of incubation, without disturbing sediment action, supernatant was collected, centrifuged (1,200 rpm for 10 min) and precursor cells were counted by a hemocytometer before seeding at a concentration of approximately 1×10^6 cells/mL into a 25 cm² culture flask (Corning) in 10 ml growth medium [F10, containing with 200 mM glutamine, 50 IU/mL penicillin, 100 µg/mL streptomycin and 5 µg/mL gentamycin, 5 µg/mL insulin (Sigma) and 10% FBS]. All incubations were performed at 37 °C under a humidified atmosphere of 5% CO₂. The medium collected after 4–5 days of incubation was having mostly unattached myogenic precursor cells. This supernatant medium was centrifuged to collect unattached SC (1,200 rpm for 10 min), and the pellet was suspended in two ml of D-PBS. These unattached SC were again enriched by using a Percoll (Sigma–Aldrich) gradient (Roe et al. 1989). After washing the SC band with D-PBS, it was diluted with growth

Fig. 1 Isolation of satellite cells from the *rectus abdominis* muscle of adult goat in modification to Mau et al. (2008), Springer et al. (1997) and Roe et al. (1989)

medium containing 5 ng/mL bFGF (basic fibroblast growth factor) and seeded in 25 cm² culture flask again. Subsequently, medium was changed approximately 2× per week until growth foci were seen, which were not allowed to over grow and monolayers were harvested with 0.10% trypsin. Aliquots of the cells were frozen in liquid nitrogen until required in 10% dimethyl sulfoxide (DMSO, Sigma) and 90% FBS. Later on, aliquots of the SC were thawed at the beginning of each experiment, excessive DMSO was washed out by a single centrifugation step (1,000 rpm, 10 min, RT), and cells were subsequently seeded in 25 cm² cell culture flasks with F10 + 10% FBS and other growth factors (insulin, bFGF).

For proliferation study, cells were counted with a cell cycle analyzer (CCA) system (Partec) as per manufacturer's instruction. In brief, cells were trypsinized, pelleted, lysed in cell lysis solution and then counterstained by nuclear staining solution, which were then counted with a CCA.

Immunofluorescence

Cells were subjected to indirect immunofluorescence for detection of desmin as it is only present in muscle fiber (SC, myoblast, fused myotubes) and not in any other tissue (Yamanouchi et al. 2007). In brief, after fixation in ice cold methanol, the cells were washed 3 times with PBS and then blocked with 5% normal goat serum (NGS)/PBS (Sigma) for 20 min at RT. After blocking, the cells were washed 3 times with PBS and then reacted with anti-desmin (mouse, clone DE-U-10, Sigma, 1:400 dilution) primary antibodies for 2 h at RT. After 3 washes again with PBS, the cells were incubated with AlexaFluor conjugated-secondary antibodies (Invitrogen) for 1 h at RT followed by counterstaining of nuclei with DAPI (Lonza). Observations were made using a fluorescence microscope (LEICA DMIL) equipped with a digital camera. All primary antibodies and AlexaFluor conjugated-secondary antibodies were diluted with 5% NGS/PBS (1:400).

Results and discussion

Cultivation of proliferating and differentiating caprine myoblasts

Primarily murine and avian myoblast cultures have been widely used to study the proliferation and differentiation of skeletal muscle cells. However, data originating from these systems may not be directly transferable to studies of skeletal muscle growth and differentiation in meat-producing animals (Hembree et al. 1991). So the aims of the present study were to isolate and establish a culture system for goat skeletal muscle stem cells and to examine their myogenic and contractile properties in vitro.

Desmin, one of the intermediate filaments, has shown to be a useful marker for identifying proliferating SC (adult myoblasts) in many species (Dodson et al. 1996). Therefore, an indirect immunofluorescent analysis for the expression of desmin was carried out to find out the proportion of isolated SC. Muscle dissociation and isolation processes were found to release mainly two types of cell population consisting mostly of SC and a smaller proportion of nonmyogenic cells. Since the attachment of SC to the tissue culture plate is slower than that of the accompanying

nonmyogenic cells, preplating was done to select SC, which resulted into 65–70% desmin positive population in comparison to percoll gradient separation of SC (75–80%) at time of muscle tissue trypsinization. Whereas our modified protocol produces populations that consist of >90% SC without addition or any other selecting medium of chemical compound and we found no difference in the subsequent growth and fusion behavior of myoblasts.

Proliferation and differentiation of skeletal muscle stem cells are under the control of several growth factors and is a highly controlled multi-step process. Amongst these growth factors, bFGF is a well-characterized regulatory factor that is indispensable in the control of myogenesis (Charge and Rudnicki 2004). In general, bFGF is known to enhance proliferation of myogenic cells and to strongly suppress their terminal differentiation. This has been shown in several species, including murine (Allen and Boxhorn 1989), equine (Byrne et al. 2000), and avian species (McFarland 1999; Velleman et al. 2004).

In order to evaluate the effect of insulin and bFGF on goat skeletal muscle stem cells, the cells were cultured in 10% FBS/F10, with/or without insulin (5 µg/mL), bFGF (5 ng/mL) for 6 days. For proliferation studies, cells were seeded with a cell number of 1.2×10^4 cells per well into 24 well culture plates (Corning) and incubated with F10 + 10% FBS, F10 + insulin + 10% FBS, F10 + insulin + bFGF + 10% FBS for 6 days. Addition of bFGF strongly enhanced growth of myoblast ($N = 4$, $p < 0.01$, Fig. 2). The effect of insulin was not as much as observed by Yamanouchi et al. (2007), however in combination with bFGF, it was significantly higher. In this regard, however, it should be noted that Dodson and Mathison (1988) had reported insulin promoted proliferation of ovine SC, while it did not promote that of rat SC and suggested species differences in the responsiveness of SC to hormones and/or growth factors. Allen and Boxhorn (1989) also reported that insulin-like growth factor I (IGF-I) stimulate proliferation to a small degree but demonstrated a more pronounced stimulation of differentiation and maximum stimulation of proliferation in the presence of both FGF and IGF-I.

For differentiation study, cells were seeded with a cell number of 1.5×10^5 per well in a 6 well culture plate (Corning) in reduced serum MEM (Hyclone)

Fig. 2 Relationship between myoblast monolayer growth and growth-stimulating admixtures in the culture medium

and 10% FBS. We noticed that there were no fusions when F10 medium was being used (Fig. 3a) as only a monolayer was formed. In MEM medium, once cells reached confluence, they started fusing and formed multi-nucleated myotubes which were formed on top of the monolayer of other contaminating cells (Fig. 3b, c). After 10–12 days, many fused myotubes started exhibiting frequent spontaneous twitching which were independent of other fused myotubes (*Online Resource 1*). During this period, medium was replaced once a week. 10 days after fusion, striation started appearing on myotubes (Fig. 3d). So far not too many reports are available for myoblast fusion and their contraction especially in caprine myoblast culture. Clair et al. (1992) reported similar cross-striation and frequent spontaneous twitching in human myoblast culture when these cells were grown in a serum free medium, MCDB 120, contain insulin, epidermal growth factor (EGF) and bovine serum albumin (BSA). Das et al. (2006) have reported similar fusion and contraction in mouse myotubes in a serum free medium. Mau et al. (2008) have reported fusion events in porcine myoblast culture in DMEM +10% FBS +10% HS.

Proliferating myoblasts and fused myotubes were confirmed with desmin immunostaining as desmin has only been reported in myoblast and myotubes but not contaminating nonmyogenic cells (Fig. 4a, b). Of the myogenic marker examined, species differences in the expression of desmin in adult myoblasts (proliferating SC) have been noted previously. Allen and Boxhorn (1989) showed that desmin is expressed in proliferating rat SC but not in proliferating bovine

Fig. 3 Differentiation of caprine myoblasts into myotubes. **a** Proliferating myoblasts were prepared for differentiation in F10 growth medium with 10% FBS by the addition of 5 $\mu\text{g}/\text{mL}$ of insulin and 5 ng/mL bFGF (H&E stain). **b** Cell culture in

MEM after 48 h, short myotubes are formed (H&E stain). **c** After 72 h in differentiating medium, numerous myotubes are apparent. **d** Striations in fused myotubes (5 days after fusion). Bar = 100 μm

Fig. 4 Combined staining of a caprine myoblast culture differentiated in MEM (reduced serum) medium for **(a)** desmin using an antidesmin antibody and **(b)** DNA by DAPI. Multinucleated myotubes are desmin-positive by green

fluorescence; nuclei exhibit blue fluorescence. This staining can be used to determine the degree of fusion as the percentage of nuclei located within myotubes. Bar = 100 μm

SC. Yablonka-Reuveni and Nameroff (1990) noted that the progeny of adult myoblasts in the chicken exhibit more desmin-expressing cells than embryonic myoblasts do. We have also noticed that the desmin expression level also varies between chicken embryonic myoblast cells (unpublished report).

In conclusion, we have found that SC remain unattached even after four-five days since first plating, as these cells have still capacity to proliferate and

differentiate into contractile myotubes. The proliferating and differentiating cultures grown under the described conditions are eminently suitable to study the effects of various bioactive compounds like nutrients, hormones, and growth factors. Likewise, the hereby provided protocols should be easily adaptable to isolate and cultivate myoblasts derived from various caprine skeletal muscles and from fetal, postnatal and adult stages of development.

Establishment of this culture system will be of value in elucidating the mechanisms that control growth/differentiation of stem cells in ruminant skeletal muscle.

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