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Controlled differentiation of myoblast cells into fast and slow muscle fibers

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要旨

骨格筋は脊椎動物の体の大部分を占める組織であり、動物の運動に必要な器官である。骨格筋は多くの筋線維で構成されている。骨格筋は損傷を受けると、筋線維の形質膜と基底膜との間に存在する骨格筋衛星細胞と呼ばれる休止状態の未分化な細胞が活性化され、増殖を開始し筋芽細胞へと分化する。この筋芽細胞は互いに融合することにより筋管を形成し、さらに、収縮蛋白質を合成して筋線維の再生が行われる。

骨格筋線維は速筋線維及び遅筋線維に分類することができる。それらの収縮速度は速筋線維では速く、遅筋線維は速筋に比べて遅い。一方で、速筋線維では解糖系をエネルギーの供給源にしているのに対し、遅筋線維では酸化的リン酸化を主なエネルギーの供給源としており、遅筋線維は速筋線維に比べ長時間にわたり張力を発揮できる。また、速筋線維と遅筋線維では発現しているミオシン重鎖 (MyHC) のタイプが異なっており、速筋線維では速筋型の、遅筋線維では遅筋型の MyHC が発現している。そのため、MyHC アイソフォームをマーカーとして筋線維の分類をすることが可能である。各骨格筋は、これら速筋線維および遅筋線維の含まれる割合により速筋および遅筋に分類される。発生時において、これら筋線維のサブタイプの形成は位置特異的に起こり、損傷からの再生後においてもサブタイプを維持することが知られている。しかしながら、この速筋線維および遅筋線維間の分化決定がどのように調節されているかは分かっていない。

そこで、本研究では筋芽細胞から筋線維へと分化する際サブタイプ間の分化が筋芽細胞の時点ですでに運命決定されているのか、あるいは筋管形成中に決定されるのかを明らかにし、再生時における筋線維サブタイプ間の維持機構を解明することを目的とした。ここでは、速筋および遅筋自身が筋線維のサブタイプ形成に影響を与えている可能性を検証するために、ニワトリの速筋ならびに遅筋から筋抽出液を作成し、この筋抽出液が筋芽細胞から各サブタイプへの分化にどのような影響を与えるかを与えるかを検討した。

まず、ニワトリ骨格筋を、速筋型 MyHC および遅筋型 MyCH に特異的な抗体を用いて、ウェスタンブロッティングにより分類した。その結果、小胸筋は速筋、前広背筋は遅筋、

縫工筋およびヒラメ筋は中間タイプであることが明らかにされた。次いで、それらより抽出液を作成した。さらに、ニワトリ胚後肢より取り出した筋芽細胞を分化培地中に各骨格筋抽出液を添加した状態で 7 日間培養し、各サブタイプへと分化させた。この際に、上記の各骨格筋抽出液を分化培地に加えたときの効果を、速筋型および遅筋型 MyHC に対する抗体を用いて、免疫二重染色法およびウェスタンブロッティングにより評価した。その結果、対照群では半数以上の筋管が速筋型の MyHC のみを、残りは速筋型と遅筋型の MyHC を同時に発現するのに対し、遅筋である前広背筋の抽出液を添加した群では、遅筋型の MyHC のみを発現する筋管が見られるようになり、また速筋型の MyHC のみを発現する筋管の割合が対照群と比較して減少した。反対に、速筋である小胸筋抽出液を添加した群では対照群に比べ速筋型の MyHC のみを発現する筋管の割合が増加した。中間型である縫工筋およびヒラメ筋の抽出液を添加した群においては、前広背筋の抽出液を添加した群よりも少ない割合ではあるが、遅筋型の MyHC のみを発現する筋管が出現した。この結果から、筋芽細胞は筋管形成時に筋線維からの影響を受けてサブタイプへの分化を制御している可能性が示唆された。

しかしながら、以上の実験で用いたニワトリ筋芽細胞には様々なタイプの筋芽細胞が含まれている可能性があり、あらかじめ遅筋タイプの筋線維に分化することが運命付けられている細胞が特異的に増殖および分化した結果なのか、それとも、遅筋抽出液中に含まれる成分により筋芽細胞の分化の方向性が遅筋タイプへと変えられたのかを判別することはできない。そのために、マウス筋芽細胞由来の株化細胞であるC_2C_{12}細胞のsingle cloneを用いて同様の実験を行った。その結果、前広背筋抽出液を添加した群では、遅筋型のMyHCを発現する筋管の割合が対照群と比較して有意に増加した。このことから、一つの筋芽細胞が速筋型および遅筋型の両方へと分化可能であり、遅筋への分化誘導が遅筋抽出液により起こることが示された。筋芽細胞を遅筋へと誘導する因子は分子量が約５０Ｋの蛋白質で尿素変性後緩衝液に透析することにより活性を回復することから分泌性の蛋白質であると示唆された。

これらの結果から、筋細胞のサブタイプの分化はその環境により制御されることこと

が明らかになった。発生時においても、再生時と同じような制御機構が存在するものと考えられる。

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General Introduction

The origin of myogenic cell

The skeletal muscle is an important tissue, which comprises a large part of body of animal. The skeletal muscles of vertebrates are divided into a lot of parts, and their roles in physiological motion are highly specified, and these skeletal muscles consist of many multinucleated cells called muscle fibers.

In the developmental stage, myogenic cells of the limb rise from somites, which are initially specified into sclerotomes and dermomyotomes (Francis-West et al., 2003). The dermomyotome give rise to myogenic precursors and the dermis. The dorsal-medial edge of the dermomyotome involutes to give rise to the myotome, consisting of myogenic precursor cells. In this stage, myogenic precursors express transcription factors *Pax3*, *Lbx1* and *c-met*, tyrosine kinase receptor. In the limb formation stage, myogenic precursor cells are delaminated by scatter factor and fibroblast growth factor (FGF) librated from lateral plate mesoderm, and myogenic precursor cells migrate from myotome into limb bud and begin to express the myogenic commitment inducer *MyoD* and *Myf5* (Langley et al., 2002; Buckingham et al., 2003). Myogenic precursor in this stage is called “myoblast”, which commit to form skeletal muscle. Then, the myoblast expresses *Myf5*, followed by *MyoD*. *MRF4* is then transiently expressed and this is followed by *myogenin* expression (Francis-West et al., 2003). These factors are essential for skeletal muscle development, and they are called “myogenic regulatory genes (MRFs)”. Next, myoblast cells up regulate the expression of cyclin-dependent kinase inhibitor p21 (Palacios and Puri, 2006), and they induce the phosphorylation of p38 (Lluis et al., 2005), to escape from cell cycle, and then, they enter the terminal differentiation. Differentiating myoblast cells fuse and differentiate into multinucleated myotubes. During terminal differentiation of myogenic cells, myotubes induce the expression of myosin heavy chain (MyHC), and form myofibrils.

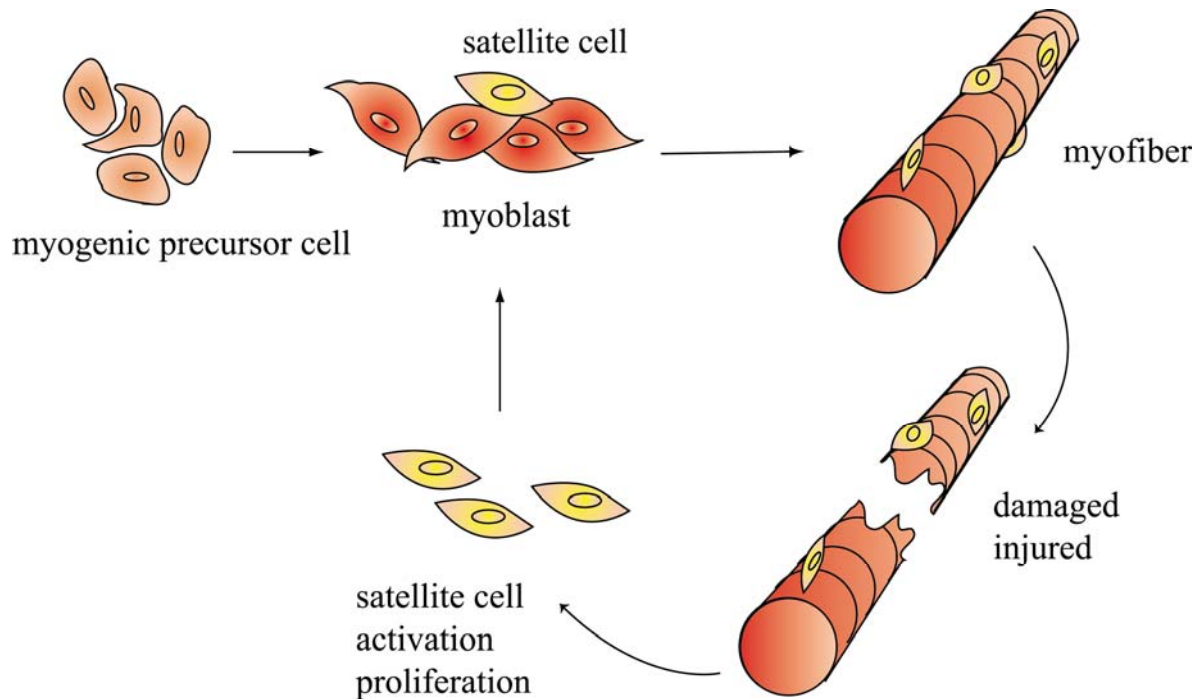

Fig. 1 The life cycle of myogenic cells. Myogenic precursor cells in the myotome migrate into limb bud and differentiate into committed "myoblast" cells or quiescent satellite cells. Myoblast cells fuse and differentiate into multinucleated myofibers, and quiescent satellite cells remain adjacent to muscle fibers. When muscle is damaged or injured, quiescent satellite cells are activated and they initiate proliferation and differentiation into myobalst cells. The activated satellite cells repair or regenerate muscle fibers, and some of satellite cells reenter into quiescent state after proliferation, and the number of satellite cells is maintained by a self-renewal manner.

Muscle regeneration and muscle satellite cell

During muscle development, there remain undifferentiated cells called as “satellite cells”, which located between the matrix of basal lamina and plasma membrane of muscle fiber. The satellite cell is a progenitor cell, which possesses differentiation capability for myogenic cell. The satellite cell is usually in the quiescent state, but when muscle fiber is damaged or injured, the satellite cell is activated and proliferates and differentiates into myoblast, and then, regenerates and repairs the muscle fiber (Montarras et al., 2005). The origin of satellite cells in the development stage has been discussed for long time. Recent studies suggest that the transcriptional factors Pax3 and Pax7 positive population in the myotome are essential for myogenesis over the embryonic stage and in fetal development, these cells adopt a satellite cell position (Relaix et al., 2005). The development of muscle fiber from myogenic precursor cell and life cycle of satellite cell are summarized in Figure 1. Myogenic precursor cells in the myotome migrate into limb bud and differentiate into committed myoblast cells or quiescent satellite cells. Myoblasts fuse and differentiate into multinucleated myofibers, but quiescent satellite cells remain adjacent to muscle fiber. When muscle was damaged or injured, quiescent satellite cells are activated, and they initiate proliferation and differentiation into myoblasts. The activated satellite cells repair or regenerate muscle fibers, and some of satellite cells reenter into quiescent state after proliferation, and maintain the number of satellite cells by self-renewal manner (Zammit et al., 2004). In addition, cloned cell line of myogenic cell, C_2C_{12} cell, is established from mouse myoblast (Blau et al., 1983). The C_2C_{12} cell is useful for studies about muscle differentiation mechanism using culture system (Amack and Mahadevan, 2004).

Table 1. The differences between fast and slow muscle fibers

	Fast-twitch fiber	Slow-twitch fiber
Contraction speed	Fast	Slow
Myosin heavy chain	Fast-type	Slow-type
Myoglobin content	Low	High
Motoneuron	Fast-type	Slow-type
Creatine phosphokinase activity	High	Low
Mitochondrial density	Low	High

Specification of slow and fast muscle

Skeletal muscle is separated into slow and fast types. The slow muscle is also called “Red muscle” and the fast muscle is also called “White muscle”. Their differences on their name are derived from its physical appearance because “Red muscle” contains large amount of myoglobin, on the other hand, “White muscle” contains small amount of myoglobin. Therefore, the color of “Red muscle” looks red and “White muscle” looks white, respectively (Oh et al., 2005). The differences of them on the molecular and physiological aspects are well studied. The characteristic differences between fast muscle fiber and slow muscle fiber were summarized in Table 1. Fast and slow muscles are defined from their contraction properties, and expression of myosin heavy chain isoforms (Pette and Staron, 2000; Schiaffino and Reggiani, 1994). In addition, these muscles are highly specified in motoneuron projecting manner that slow muscles are projected by slow type motoneurons and fast muscles are projected fast type motoneurons (Buller et al., 1960). However the origin of slow and fast muscle in the development and regeneration stage is not yet known. Many studies has addressed this question and there have been two major theories (1) myogenic progenitor is committed to differentiate into slow or fast type myoblast and this myoblast form slow or fast muscle fiber (DiMario et al., 1993) and (2) myoblast is uncommitted to their fate to terminal differentiation type, and surrounding tissues define the terminal differentiation of muscle fiber type (Nikovits et al., 2001; Van Swearingen and Lance-Jones, 1995). Therefore, the mechanism of patterning of slow and fast muscle fiber formation in the development and muscle regeneration stages is an issue needing more studies.

In this paper, the author studied about the differentiation of muscle fibers into fast and slow fibers during muscle regeneration. The paper is divided into two parts. In part A, the author studied about the mechanism of muscle fiber subtype differentiation by using chick myoblast cell and C_2C_{12} cells. The author found that muscle fiber subtype differentiation is controlled by extracts of fast and slow muscles. This phenomenon clearly indicates that myoblast is not committed to subtype differentiation pathway, but that subtype differentiation is controlled by their surroundings.

In part B, the author studied about the relation between muscle differentiation and muscle cell metabolic state by using creatine and its analog β-guanidinopropionic acid. Muscle differentiation is affected by the metabolic state of myoblast cells associated with morphological changes of nucleoli. These findings give new knowledge on the mechanism of muscle differentiation.

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Part A

Controlled Differentiation of Myoblast Cells into Fast and Slow Muscle Fibers

Abstract

Skeletal muscles are classified into fast and slow muscles, which are characterized by the expression of fast-type myosin heavy chains (fMyHCs) or slow-type myosin heavy chains (sMyHCs), respectively. However, the mechanism of subtype determination during muscle fiber regeneration is unclear. It has been analyzed whether the type of muscle is determined in the myoblast cells or is controlled by the environment in which the muscle fibers are formed from myoblast cells. When myoblast cells from 7-day-old chick embryo were cultured and formed into muscle fibers, more than half of the fibers produced only fMyHCs, and the remaining fibers produced both fMyHCs and sMyHCs. However, when myoblast cells were cultured in medium supplemented with a small amount of slow muscle extract, the expression of sMyHCs in muscle fibers increased, whereas the expression of fMyHCs increased in the group supplemented with fast muscle extract compared with the control group. The same results were obtained when cloned mouse myoblast cells (C_2C_{12} cells) were cultured and formed into muscle fibers. The data presented here thus show that the subtype differentiation of muscle fiber is controlled by the environment in which the muscle fiber forms.

Introduction

The skeletal muscle in vertebrate is made from multi-nuclear cells called muscle fibers; they are divided into fast and slow fibers (Stockdale 1997). For living vertebrate, the existence of two types of fibers is important, since muscle should undertake multiple tasks depending on its location in the body. These two fibers have differences in contraction speed and mechanism of energy supplement. Furthermore, the expression of myosin heavy chains (MyHCs) is modulated in these two fibers (Volosky and Keller 1991), i.e. fast fiber expresses fast-type MyHCs, and slow fiber expresses slow-type MyHCs, respectively (Billeter et al. 1981; Mahdavi et al. 1987; Dix and Eisenberg 1988; Sweeney et al. 1989; Fladby and Jansen 1990; Feghali et al. 1992; Ohira et al. 1992; Page et al. 1992; Nikovits et al. 1996; Wright et al. 1997; Staron et al. 1999; Francis-West et al. 2003).

The mechanisms of the subtype determination of muscle fibers during development and the regeneration of skeletal muscle fibers have been unclear. It was suggested that myoblasts had already determined their differentiation pathways into fast- or slow-type muscle in their early developmental stages (DiMario et al. 1993). Furthermore, it was suggested that myoblasts form two different types of muscle fibers, which are called first and secondary fibers (Miller and Stockdale 1986a). In chick muscle, the first fibers are formed at the E3-E6 stage, and they are thought to become scaffold fiber for the formation of secondary fibers. When myoblast cells were dissected from this stage, three types of MyHCs expression appeared: only fMyHCs, only sMyHCs, or both fMyHCs and sMyHCs, in vitro. However, when myoblast cells from E7-E18 chick muscle, which form secondary fibers, are cultured, most myotubes express only fMyHCs or both fMyHCs and sMyHCs, and this trend is prominent in the later stage (Kocamis and Killefer 2003). Moreover, a myoblast transplantation study revealed that when myoblasts from quail were transplanted into chick thigh muscle, donor myoblasts expressed their original phenotypes in host muscle (Van Swearingen and Lance-Jones 1995; Nikovits et al. 2001). On the contrary, it was reported that when cloned myoblasts were injected into injured muscle, they could enter various types of myofibers (Hughes and Blau 1992).

In this part, it was examined whether the differentiation of myotube subtype was intrinsically determined or was controlled by their environment where myotube was formed. Chick myoblast cells and a murine C_2C_{12} cell line have been used. It was found that the type of MyHC isoforms expressed in myotubes is regulated by the addition of fast or slow muscle extract to the culture medium. The results thus suggest that the differentiation of muscle fiber type is modulated by its environment.

Materials and Methods

Animals

Fertilized eggs of white-leghorn chicken were purchased from Takeuti-Huranjo Co., Nara, Japan, and incubated at 37.5ºC in a humidified forced-draft incubator. The stage of the embryo at the time of surgery was scored according to Hamburger and Hamilton (1951). Female chickens (6 months old) were purchased from Okada-Youkeijo Co., Osaka, Japan.

Muscle Myosin Quantitation

The amounts of sMyHC and fMyHCs in the pectoralis minor (PM), anterior latissimus dorsi (ALD), sartorius, or soleus muscles of 6-month-old female chickens were determined as follows. Each muscle (0.1 g) was homogenized in 400 μl of lysis buffer (0.5% SDS, 1% Triton X-100, 50 mM NaCl, 30 mM NaPPi, 50 mM NaF, 2 mM EDTA, 0.1 mM Na_3VO_4, 1 mM PMSF, 10 mM Tris-HCl pH 7.5) by using a glass homogenizer, and total proteins were extracted for 24 hours at 4ºC. The mixture was centrifuged at 16,000 g for 15 minutes at 4ºC, and debris were removed. The concentration of muscle protein in each sample (measured by BCA Protein Assay Reagent; Pierce, USA) was approximately 10 mg/ml. The samples were diluted 10 times with lysis buffer, mixed with an equal volume of the sample buffer (40% glycerol, 5% β-mercaptoethanol, 10% SDS, 0.5 M Tris-HCl at pH 7.8), and each sample (10 μl) was separated by SDS-polacrylamide gel electrophoresis (SDS-PAGE) with 7.5% (upper) and 15% (lower) acrylamide bilayer gels. After SDS-PAGE, the proteins were stained with Coomassie Brilliant Blue (CBB) or they were analyzed by immunoblotting. The proteins were transferred to PROTRAN Nitrocellulose Transfer Membrane (Schleicher & Schuell, Germany), and the transfers were incubated with monoclonal antibody (mAb) S46 (Miller and Stockdale 1989), which is specific for chicken sMyHCs or mAb F59 (Miller and Stockdale 1989; Miller et al. 1989), which is specific for chicken fMyHCs. These antibodies

were kindly gifted by Dr. F. E. Stockdale (Stanford University, USA). Each antibody was diluted at 1:200 with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20, respectively. Antibody binding was visualized by 1:5000 anti-mouse IgG antibody conjugating with horseradish peroxidase (Bio-Rad, USA). The enzyme reactions were performed using the chemifluorescence method. The protein bands were detected by using cooled charge-coupled device (CCD) camera, (LAS-1000; Fuji-Film, Japan), and the images were processed by using Adobe Photoshop Software. The band intensity was measured by an NIH image program. The amounts of MyHCs in each chicken muscle were calculated from the band intensity after CBB staining by using purified rabbit white skeletal muscle myosin (0.1 μg) as a control. The amounts of fMyHC or sMyHCs were calculated from the band intensity after immunoblotting, based on the band intensity of PM or ALD muscle myosins that contain fMyHCs or sMyHCs, respectively, with 95% purity.

Preparation of Muscle Extracts

Muscle extracts were prepared from PM, ALD, sartorius, and soleus muscles of 6-month-old chicken. After tendon and connective tissue were removed, muscles were minced by using a surgical knife, on ice. Each muscle (2.0 g) was then homogenized in 8 ml of Ca^{2+} and Mg^{2+}-free (CMF)-Phosphate-buffered Saline (PBS), using glass homogenizer. The homogenate was centrifuged at 1,100 g for 15 minutes at 4ºC. The supernatant was collected and filtered through 0.22 μm pore size PVDF filter (Millipore, USA) to remove contaminated cells and bacteria. The concentration of proteins in muscle extract was measured by a BCA Protein Assay Reagent (Pierce, USA). The protein concentrations of PM, ALD, sartorius, and soleus muscle extract were 13.9 ± 1.6, 6.4 ± 1.8, 8.2 ± 2.3 and 8.2 ± 2.1 mg/ml, respectively. Each extract was then adjusted to 5.0 mg/ml protein with PBS. The extract was quickly frozen using liquid nitrogen and stored at –80ºC until use. To avoid the change in the Ca^{2+} concentration of culture medium, extract was added maximum 1% to the culture medium.

Cell culture

Chick myoblasts were prepared from whole thigh muscles of 7-day-old chick embryo (stage 26-27, Hamburger and Hamilton, 1951). Primary culture was performed according to previously described methods (O'Neill and Stockdale, 1972), with some modification. The thighs were dissected, minced, and digested with 0.25% trypsin-EDTA (Gibco, UK). The digest was transferred into Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal-bovine serum (FBS) and triturated vigorously to release single cells. The cell suspension was then filtered through double-layered lens papers to remove debris. The cells were collected by centrifugation at 290 g for 5 minutes and resuspended in growth medium (GM; DMEM with 10% FBS). To remove fibroblast cells, cells were placed on a non-coated plastic culture dish and incubated at 37ºC in 5% CO_2 for 3 hours. The cells in culture medium were then collected and centrifuged at 290 g for 5 minutes and used for experiments. The C_2C_{12} cell line was provided to us from Dr. I. Suetake (Osaka University, Japan), and a single clone of C_2C_{12} cell was cultured in GM until a cloned colony was formed. The colony was removed to a 90 mm culture plate by trypsination and cultured continuously until a sufficient number of cells were obtained.

Chick primary myoblasts or C_2C_{12} cells were plated on gelatin-coated plates at 1.0×10^4 cells/cm^2 and precultured in GM. After 24 hours, GM was replaced by the differentiation medium (DM; DMEM with 2% horse serum), and 0.1% (5 μg/ml in the final protein concentration) of PM, ALD, sartorius, or soleus muscle extract or 0.1% (5 μg/ml in the final protein concentration) of BSA solution (control) was supplemented. They were cultured for another 7 days, and the culture medium was replaced with fresh medium every 3 days. To titrate the dose of the muscle extract activity, various concentrations (0, 0.1, 0.5, 1.0%) of PM or ALD extract, BSA solution, or the same volume of PBS (0%) were added into the culture medium.

Immunostaining

The immunostaining of muscle fibers in culture was performed by using monoclonal antibodies (mAbs) against sMyHCs and fMyHCs, as described by Miller and Stockdale (1986b). On the 7th day of the culture, myotubes were fixed and processed for immunohistochemistry. The expression of MyHC isoforms in cultured chick myotubes were detected by mAbs S58, which is specific for chicken embryonic and adult sMyHCs, and F59, which is specific for fMyHCs, respectively (Miller and Stockdale 1989). The expression of MyHCs in mouse C_2C_{12} cells was also detected by mAbs S58 and F59. For mouse MyHCs, mAb S58 is specific for sMyHCs, but F59 stains both slow and fast MyHCs (Miller 1990). The mAbs S58 or F59 was visualized by anti mouse IgA conjugated to rhodamine or anti mouse IgG conjugated to Alexa Fluor 488, respectively. Each of the secondary antibody was diluted 1:100 in PBS containing 10% goat serum, and incubation was performed for 1 hour at room temperature. The nuclei were stained with Hoechst 33342 (Calbiochem Co., USA). Four randomly chosen 0.64-mm^2 fields of each well under the fluorescence microscope (Olympus) were recorded with a cooled CCD camera (Photometrics, Tucson, AZ) and the software package IP lab Spectrum version 3.0.1 (Signal Analytics Co.). The images were processed using Adobe Photoshop Software. The number of sMyCH-, fMyHC-, or both sMyHC- and fMyHC-positive myotubes containing more than two nuclei was counted. The data represents the mean ± s.e.m. Significance was tested by Student's *t*-test. All comparisons of statistical significance have *P* values of $P < 0.05$.

Immunoblotting of MyHC expression by cultured cells

The expression of MyHC isoforms in myotubes formed by cultured chick myoblasts or cloned C_2C_{12} cells was detected by Western immunoblotting. On the 7th day in culture, cells in the 90-mm culture dish were collected and centrifuged at 290 g for 5 minutes at 4ºC. They were then homogenized in 50 µl of lysis buffer and mixed with an equal volume of the sample buffer. The concentration of protein in the sample was measured by a BCA Protein Assay Reagent. The samples were then adjusted to 2.5 mg/ml in sample buffer. Each 10-µl of sample (25 µg protein) was

electrophoresed, and the amount of fMyHCs or sMyHCs was analyzed by Western immunoblotting using mAbs S46 and F59 for chick myoblast, or NCL-MHCs (Novocastra, UK) and NCL-MHCf (Novocastra, UK) for C_2C_{12} cell, respectively (Messi and Delbono 2003).

Results

Subtype of chicken skeletal muscles

It has been addressed whether myoblast cells differentiate into either fast-type or slow-type muscle fibers, depending on the surroundings in which the cells differentiate. First, it was examined the contents of fMyHC and sMyHC isoforms in adult chicken muscles from which the author prepared the extract. The total proteins were extracted from pectoralis minor (PM), anterior latissimus dorsi (ALD), sartorius, and soleus of 6-month-old female chicken by using the lysis buffer (see Materials and Methods). The amount of MyHCs in muscle was estimated from the band intensity after Coomassie Brilliant Blue (CBB) stain by comparison with that of 0.1 μg of rabbit white skeletal muscle myosin (Fig. 1a). The total content of sMyHCs and fMyHCs in PM, ALD, sartorius, and soleus muscles were 9.4, 5.1, 11.1, and 5.3 mg/g muscle, respectively.

The author next determined the muscle contents of MyHC isoforms by Western immunoblotting with MyHC monoclonal antibodies (mAbs) F59 and S46 recognizing chicken fMyHCs and sMyHCs, respectively (Fig. 1b). PM muscle contained almost 95% fMyHCs (9.0 mg/g muscle). Therefore, the author defined chicken PM as fast muscle. Chicken ALD muscle contained almost 95% sMyHCs, (4.9 mg/g muscle; Fig. 1b,c), and sartorius and soleus muscle contained either fMyHCs or sMyHCs (Fig. 1b). Therefore, the author defined chicken PM as fast muscle. Chicken ALD muscle contained almost 95% sMyHCs, (4.9 mg/g muscle; Fig. 1b,c), and sartorius and soleus muscle contained either fMyHCs or sMyHCs (Fig. 1b). Two individual bands were detected with mAb S46 in ALD, sartorius, and soleus muscle (Fig. 1b, bottom). From the electrophoretic mobility, these bands were assumed to be sMyHC1 (Fig. 1b, bottom) and sMyHC2 (Fig. 1b, top; Miller and Stockdale 1989). Based on the band-intensity calibration curve of fMyHCs and sMyHCs, the contents of fMyHCs and sMyHCs in sartorius muscle were estimated to be 8.1 and 2.9 mg/g, respectively, and those in soleus muscle were 1.5 and 3.7 mg/g, respectively (Fig. 1c). Generally, soleus muscle has been defined as a slow-twitch muscle in rodents and humans (Baldwin

and Haddad 2001). However, chicken soleus muscle contains both fMyHCs and sMyHCs. Thus, in this study, the author used ALD muscle as slow muscle.

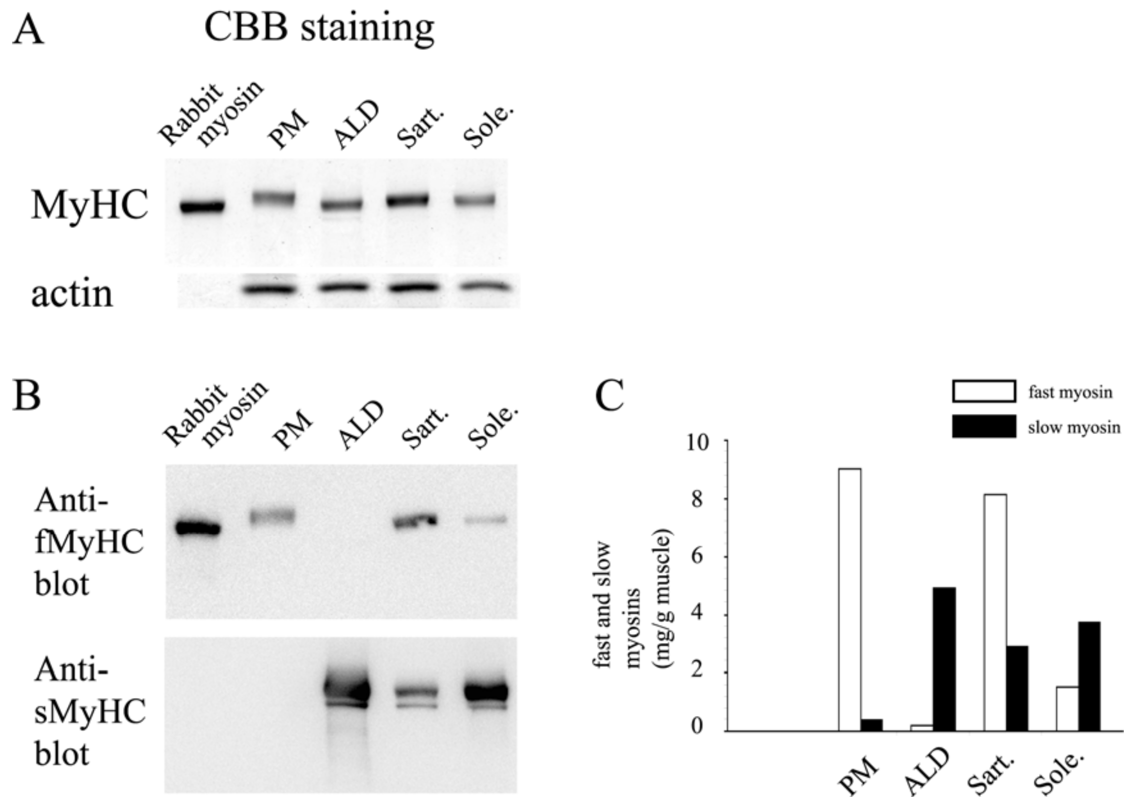
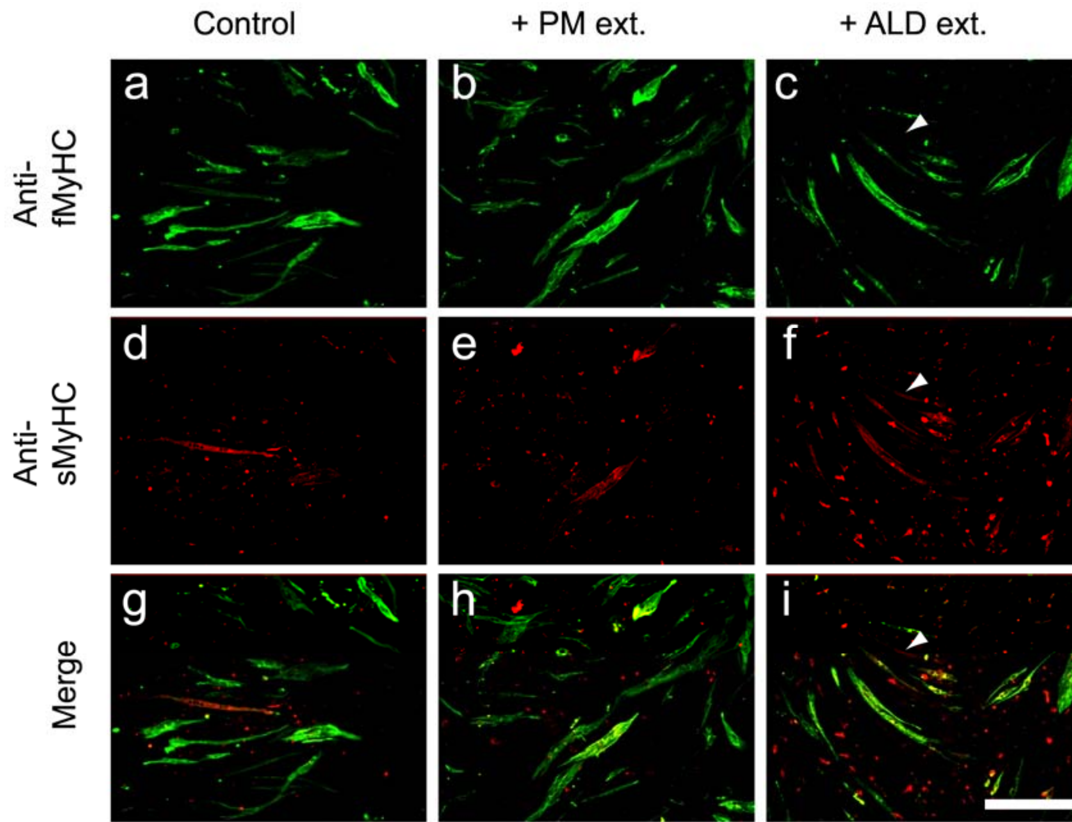


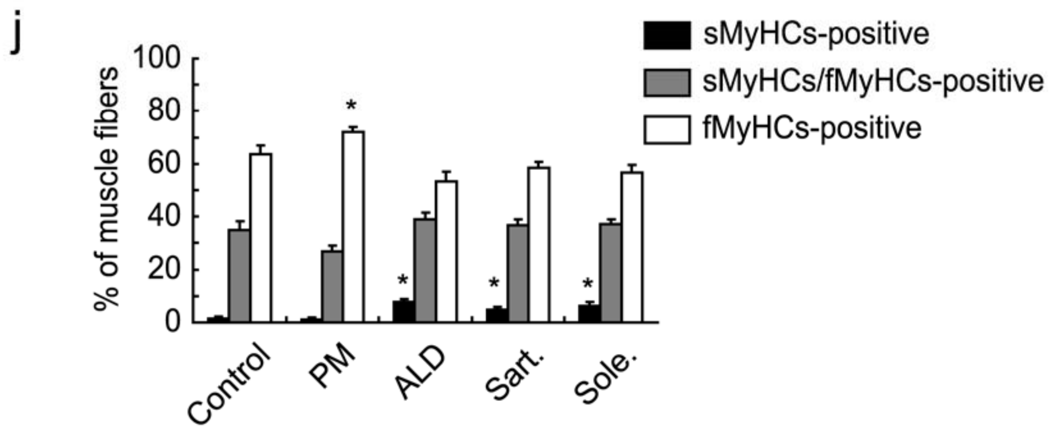
Fig. 1 Expression of slow and fast myosin heavy chain (MyHCs) isoforms in adult chicken skeletal muscles. A total protein sample was prepared from pectoralis minor (PM), anterior latissimus dorsi (ALD), sartorius (Sart.), or soleus (Sole.) of 6-month-old female chicken. Proteins (5 µg) were applied to 7.5% acrylamide gel, and SDS-PAGE was performed. Purified rabbit myosin (0.1 µg), which was extracted from rabbit white skeletal muscle, was applied as a control. **a:** Protein bands visualized by Coomassie brilliant blue (CBB) staining. Note the bands of MyHCs and actin at about 200 kDa and 42 kDa, respectively. **b:** Immunoblotting was performed after SDS-PAGE by using monoclonal antibodies (mAb) specific to fast MyHCs (Anti-fMyHCs) or slow MyHCs (Anti-sMyHCs). **c:** Total amount of MyHCs estimated from the band intensity of MyHCs after CBB staining of the gel. The contents of fMyHCs (open bars) and of sMyHCs (filled bars) were calculated from the band intensity after immunoblotting

Effects of chicken fast- and slow-muscle extracts on expression of MyHCs isoforms in myotubes formed by fusion of chick myoblast cells

The author investigated the types of muscle fibers formed by cultured myoblast cells when an extract of fast or slow muscle was added to the medium. Muscle extracts were prepared as in Materials and methods. Myoblasts were isolated from E7 chick thigh muscle and cultured at 1.0×10^4 cells/cm^2 in GM for 24 h. The culture medium was then replaced with DM supplemented with 0.1% of PM, ALD, sartorius, or soleus muscle extract (n=8 wells, each group). BSA solution was added to DM as a control (n=8 wells). By 7 days in culture, most of the myoblast cells had fused into multinucleated myotubes. Cultured myotubes were then fixed, and a double-immunofluorescence analysis was performed by using mAbs F59 and S58 recognizing chicken fMyHCs and sMyHCs, respectively (Fig. 2a-i). The nuclei were visualized by staining with Hoechst 33342. When the BSA solution or the PM muscle extract was added to the DM, most myotubes synthesized only fMyHCs or both fMyHCs and sMyHCs (Fig. 2a-i, left and middle). However, when the ALD muscle extract was added to the DM, the number of myotubes that synthesized only sMyHCs increased dramatically (Fig. 2a-i, right). The number of multinuclear myotubes expressing only sMyHCs, only fMyHCs, or both sMyHCs and fMyHCs, were counted, and the average percentages of them in each field presented graphically (Fig. 2j). In the control group, 1.5±0.6%, 35.1±3.4%, and 63.4±3.3% of the myotubes synthesized only sMyHCs, both fMyHCs and sMyHCs, and only fMyHCs, respectively (Fig. 2j). When the culture medium was supplemented by the PM (fast) muscle extract, the percentages of myotubes expressing only fMyHCs increased slightly to 72.0±1.9%, compared with the control group (Fig. 2j). On the other hand, when the ALD (slow) muscle extract was added as a supplement, the percentages of myotubes expressing only sMyHCs increased significantly to 7.6±1.3%, from 1.4±0.6% (Fig. 2j). The sartorius and soleus muscle extracts also increased the percentages of myotube expressing only sMyHCs to 4.9±1.2% and 6.4± 1.3%, respectively.



Control
+ PM ext.
+ ALD ext.
a
b
c
Anti-
fMyHC
d
e
f
Anti-
sMyHC
g
h
i
Merge



j
sMyHCs-positive
sMyHCs/fMyHCs-positive
fMyHCs-positive
% of muscle fibers
100
80
60
40
20
0
Control
PM
ALD
Sart.
Sole.

Fig. 2 Effect of muscle extracts on the expression of fMyHCs and sMyHCs in myotubes formed by cultured E7 chick myoblast cells. Myoblasts were prepared from thigh muscles of E7 chick embryo and were pre-cultured in growth medium for 24 h. Then, the culture medium was replaced with differentiation medium and supplemented with 0.1% PM (b, e, h) or ALD (c, f, i) extract (ext.). The control group was supplemented with 0.1% bovine serum albumin (BSA, 5 mg/ml) solution (a, d, g). The myoblasts were cultured for 7 days to allow the formation of myotubes and were then fixed and processed for double-immunofluorescence analysis. **a–c**: Alexa Fluor 488 fluorescence, representing fMyHCs. **d–f:** Rhodamine fluorescence of the same fields, representing sMyHCs. **g–i:** merged images of Alexa Fluor 488 and rhodamine fluorescence (arrowheads in c, f, i myotubes expressing only sMyHCs. Bar 250 μm. **j:** Chick myoblasts were cultured for 7 days in DM with BSA or extract of chicken PM, ALD, sartorius (Sart.), or soleus (Sole.). The number of multinucleated myotubes (nuclei ≥ 2), which expressed sMyHCs, both sMyHCs and fMyHCs, or fMyHCs, in four randomly chosen 0.64-mm^2 fields of each well, were separately counted. Their average percentages are shown by black, gray, and white bars, respectively. Data are means ± s.e.m. *$P<0.05$ vs. control group.

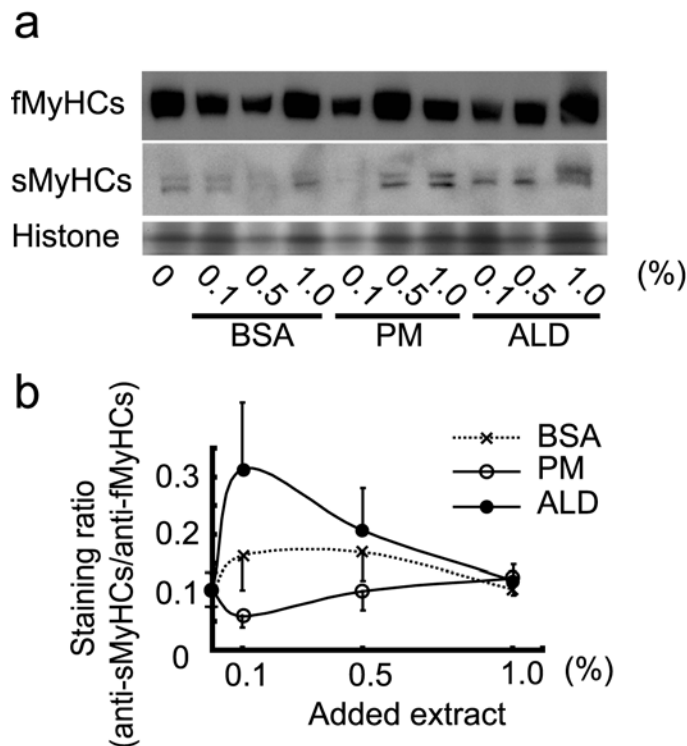
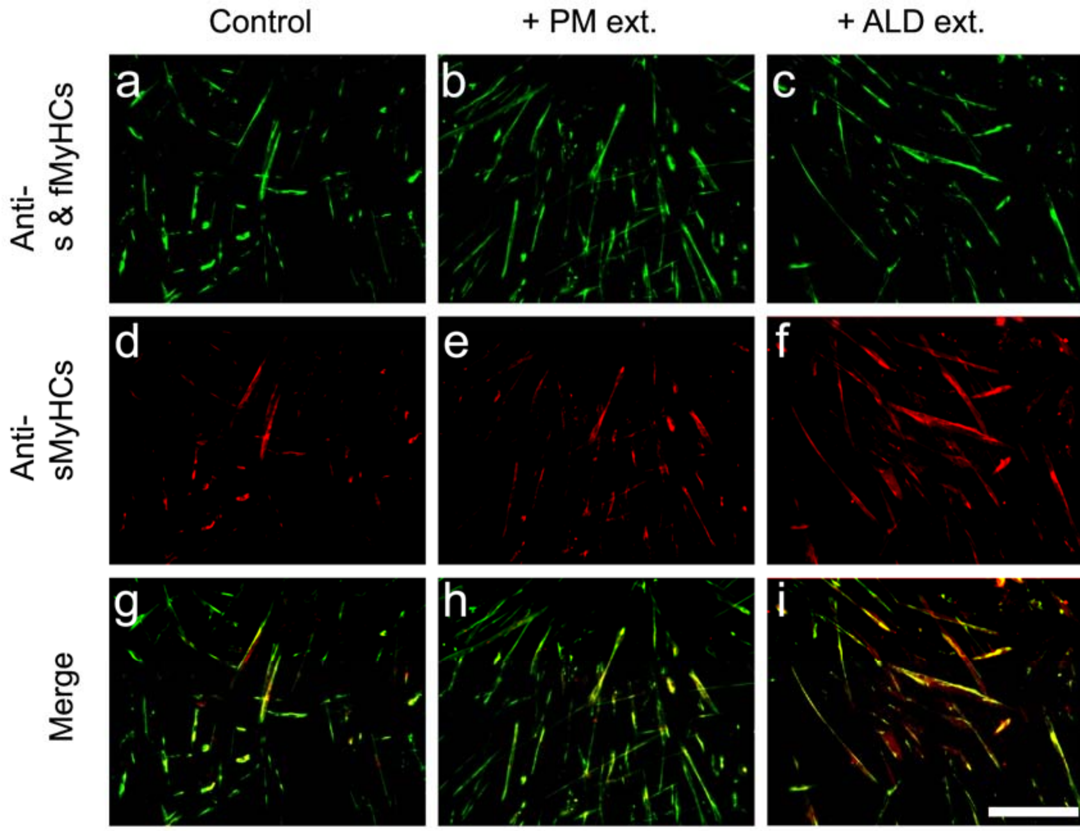


Fig. 3 Amount of sMyHCs and fMyHCs expressed in myotubes formed by cultured myoblast cells following the addition of muscle extracts. Various concentrations (0, 0.1, 0.5, or 1.0%) of PM or ALD muscle extracts were added as supplements to the myoblast culture medium for 7 days. **a:** Total protein was extracted from cultured cells by lysis buffer, 25 μg protein sample was loaded onto the gel, and the expressions of sMyHCs and fMyHCs were determined by Western immunoblotting with mAbs S46 and mAb F59, respectively. Histone was used as a control. **b:** Staining ratio of sMyHCs/fMyHCs in the groups supplemented with BSA solution (crosses), PM extract (open circles), or ALD extract (filled circles) plotted against extract concentration. The average values of three independent experiments are shown. Data are means ± s.e.m.

To confirm the effects of various concentrations of muscle extracts on myoblast subtype differentiation, DM was supplemented with increasing doses of 0, 0.1, 0.5, or 1.0% of PM or ALD muscle extract. BSA solutions were added to DM as a control. On the 7th day, cells were collected, and the expression of fMyHCs and sMyHCs was examined by Western immunoblotting. Figure 3 shows the relationship between a dose of PM or ALD muscle extract and the subtype differentiation of myotubes. The band images of fMyHCs and sMyHCs were detected by Western blotting using mAb F59 and S46, respectively (Fig. 3a). When 0.1% of PM muscle extract was added to the medium, the ratio of sMyHCs/fMyHCs in cultured myotubes decreased from 0.10 ± 0.03 to 0.06 ± 0.03, whereas the addition of more PM muscle extract induced an increase in the value of the expression ratio to the control level. On the other hand, the ALD muscle extract at 0.1% induced an increase in sMyHCs/fMyHCs to 0.32 ± 0.12. However, the addition of more ALD muscle extract induced a decrease in the sMyHCs/fMyHCs ratio to control levels (Fig. 3b). BSA solution did not affect the expression ratio. Because the value of sMyHCs/fMyHCs was constant when excess amounts of the extract were added to the medium, it was used 0.1% of the muscle extract for further study.


Control
+ PM ext.
+ ALD ext.
Anti-
s & fMyHCs
Anti-
sMyHCs
Merge
a
b
c
d
e
f
g
h
i

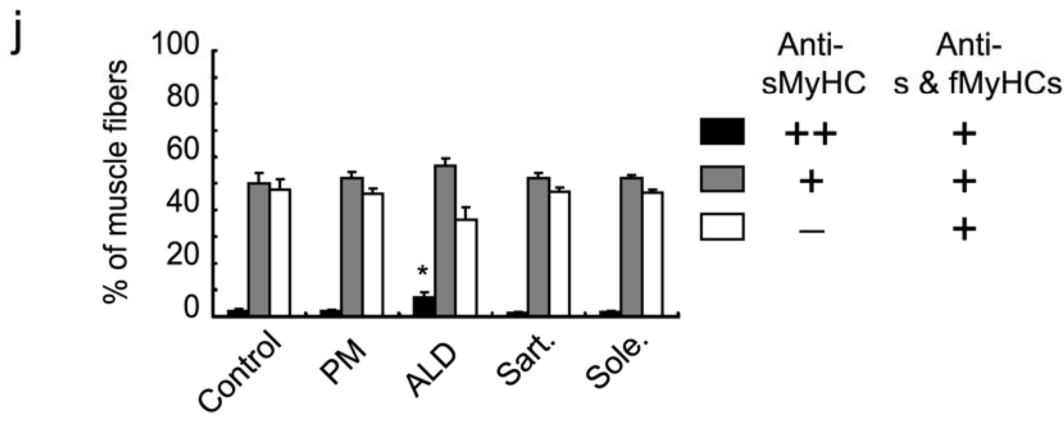

j
% of muscle fibers
100
80
60
40
20
0
*
Control
PM
ALD
Sart.
Sole.
Anti-
sMyHC
Anti-
s & fMyHCs
++
+
+
+
–
+

Fig. 4 Effects of muscle extracts on subtype differentiation of myotubes formed by C_2C_{12} cells. **a–f:** C_2C_{12} cells were cultured for 7 days with 0.1% BSA (a, d, g), PM extract (b, e, h), or ALD extract (c, f, i). The expression pattern of MyHCs was visualized with mAb F59, which recognizes either mouse sMyHC and fMyHCs isoforms, by using antibody conjugated to Alexa Fluor 488 (a–c), or mAb S58, which recognizes only mouse sMyHC, by using rhodamine-conjugated antibody (d–f). **g–i:** Merged images. Bar 250 μm. **j:** C_2C_{12} cells were cultured for 7 days in DM with BSA or with extract of chicken PM, ALD, sartorius (Sart.), or soleus (Sole.). The numbers of multinuclear myotubes (nuclei ≥ 2) that strongly reacted with mAb S58, either mAb S58 or F59, and only mAb F59 were counted after double immunofluorescence staining (black, gray, and white bars percentages of myotubes expressing sMyHC strongly [antisMyHC ++, anti-sfMyHCs +], both sMyHC and fMyHCs [antisMyHC +, anti-sfMyHCs +], and only fMyHCs [anti-sMyHC -, and anti-sfMyHCs +], respectively). Data are means ± s.e.m. *P<0.05 vs. control group.

Effect of chicken fast- and slow-muscle extracts on subtype differentiation of C_2C_{12} cells

The expression of fMyHCs or sMyHCs was increased respectively by the addition of extracts of fast or slow muscle in chick myoblast culture. Two explanations were possible: (1) the number of myoblasts determined as fast- or slow-type muscle fibers was increased, or (2) the differentiation pathway of myoblast was affected by muscle extracts. The author therefore further studied the effect of muscle extract by using the murine C_2C_{12} cell line. A single clone of C_2C_{12} cells were cultured, plated at 1.0×10^4 cells/cm^2, and then cultured for a further 24 h in GM. The medium was subsequently replaced with DMs supplemented with 0.1% of PM, ALD, sartorius, or soleus muscle extract or 0.1% of BSA solution (n=8 wells, each group), and the cells were cultured for another 7 days. The myotubes were then fixed and processed for double immunofluorescence (Fig. 4a-i). The nuclei were also visualized by staining with Hoechst 33342. The expression pattern of total MyHCs was detected by mAb F59, which recognizes either mouse sMyHC or fMyHC isoforms (Miller 1990; Fig. 4a-c). The expression of sMyHC was detected by mAb S58, which recognizes only mouse sMyHC (Fig. 4d-f). The author found three types of myotubes in the culture: (1) the myotubes reacted with mAb S58 more strongly than mAb F59 (anti-sMyHC ++, anti-s and antifMyHCs +); (2) the myotubes reacted with both mAb S58 and mAb F59 (anti-sMyHC +, anti-s and antifMyHCs +); (3) the myotubes did not react with mAb S58, but reacted only with mAb F59 (anti-sMyHC -, anti-s and anti-fMyHCs +). The author thus considered that type (1) myotubes expressed sMyHC to a greater extent than fMyHCs, and the author counted them separately from the type (2) myotubes (Fig. 4j). In the control group, the percentages of type (1), type (2), and type (3) myotubes were 2.1 ± 0.5%, 50.2 ± 3.6%, and 47.7 ± 4.0%, respectively. The percentages of these myotubes were not significantly altered by PM, sartorius, or soleus muscle extract. However, in the group supplemented with ALD (slow) muscle extract, the percentage of type (1) myotubes increased significantly to 7.1 ± 1.9%, from 2.1 ± 0.5%.

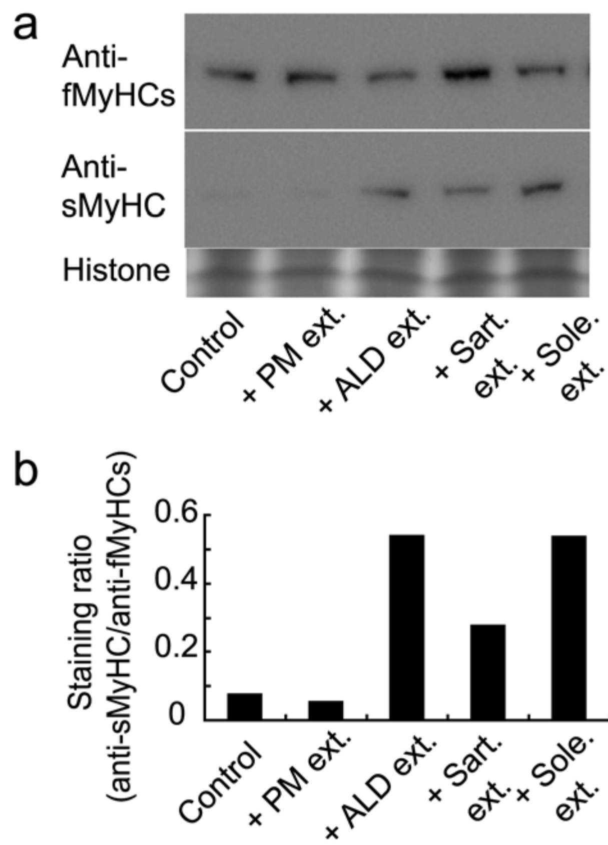


Fig. 5 Immunoblot analysis of MyHCs isoforms expressed in myotubes formed by cultured C_2C_{12} cells. C_2C_{12} cells were cultured for 7 days with 0.1% BSA (control) or 0.1% chicken muscle extracts (Sart. sartorius, Sole. soleus, ext. extract). Total protein was then extracted from cultured C_2C_{12} cells by the lysis buffer. **a:** Immunoblotting was performed by anti-mouse fast-type MyHCs (fMyHCs) mAb (NCL-MHCf) or anti mouse slow-type MyHC (sMyHC) mAb (NCL-MHCs). **b:** Staining ratio of the band intensity of sMyHC/fMyHCs.

To confirm the increase of sMyHC expression in C_2C_{12} myotubes by slow muscle (ALD) extract, the author performed Western immunoblotting with sMyHC and fMyHCs specific mAbs. C_2C_{12} cells were cultured in 90-mm plastic dishes with 0.1% BSA solution or 0.1% of each muscle extract. After 7 days, C_2C_{12} cells were collected, and Western immunoblotting was performed by using an anti-sMyHC mAb, viz., NCL-MHCs (specific for a slow class of mouse MyHC isoforms), or an anti-fMyHCs mAb, viz., NCL-MHCf (specific for a fast class of mouse MyHCs isoforms; Miller 1990) (Fig. 5a). The expression ratio of sMyHCs versus fMyHCs in the C_2C_{12} myotubes was increased by the ALD muscle extract to 0.18, from 0.05, and the expression ratio of sMyHCs versus fMyHCs in C_2C_{12} myotubes was decreased by the PM muscle extract to 0.04 (Fig. 5b). Furthermore, the expression ratio of sMyHCs versus fMyHCs in C_2C_{12} myotubes slightly increased after supplementation with the sartorius and soleus muscle extract (0.09 and 0.09). Thus, these results confirmed that the expression of sMyHC was increased by ALD (slow) muscle extract, as observed previously (Figs. 1, 2) by immunohistochemistry.

The factor(s) for inducing slow-type muscle fiber in the ALD muscle extract is considered to be protein, since its activity disappeared by standing at 95°C for 20 min. Furthermore, the active component did not pass through the dialysis membrane, and when the ALD muscle extract was applied to a sepharose gel (S-300) column, the greatest activity appeared in the void fraction.

Discussion

Muscle fiber can be regenerated from myoblast cells. However, the way in which the type of muscle fiber is determined during fiber formation is as yet unknown. The two possibilities are that: (1) the differentiation pathways of myoblasts have previously been determined, and that predetermined myoblasts form each type of muscle fiber, or (2) the subtype of myoblast differentiation is controlled by the environment. To answer this question, the author has investigated whether the determination of fiber type differentiation in vitro can be regulated by various muscle extracts. The results in this paper indicate that the number of slow-type myotubes increase in proportion to the content of slow muscle fibers in the muscle from which the extract is prepared (Figs. 1c, 2j). Of note, avian skeletal muscle expresses three types of sMyHC isoforms, sMyHC1, sMyHC2, and sMyHC3 (for a review, see Bandman and Rosser 2000). sMyHC1 is expressed predominantly in the developmental slow muscle, sMyHC2 is expressed in the adult slow muscle (Stockdale et al. 2000), and sMyHC3 is expressed in atria of adult heart or early developmental skeletal muscles committed to be slow muscle in the adult (Page et al. 1992). In this study, the author has used mAbs S46 and S58 to detect the expression of sMyHC isoforms in myotubes. As mAb S46 identifies three sMyHC isoforms and mAb S58 identifies both sMyHC2 and sMyHC3 (Page et al. 1992), the author has not examined the expression of each sMyHC isoform. Conversely, the extract of PM (fast) muscle increases the expression of fMyHCs in myotubes. Therefore, the subtype of myotube is controlled by its environment. In recent studies, many investigators have reported that the type of muscle fiber can be changed from the fast- to the slow-type or from the slow- to the fast-type (Hamalainen and Pette 1996; Pette and Staron 2001; Chin et al. 2003; Ustunel et al. 2003; Agbulut et al. 2004; Ishihara et al. 2004; Mousavi et al. 2004). These fiber type conversions are modulated by the innervation of the muscle fibers. Cross-reinnervation studies have shown that the type of muscle fibers depends on the type of motoneuron projecting muscle fibers (Buller et al. 1960). Furthermore, the expression of the slow-type genes has been suggested to be stimulated by the Ca^{2+}/calcineurin pathway, which is activated by the neural stimulation of muscle contraction (Chin et al. 1998; Wu

et al. 2000; Dunn et al. 2001; Chakkalakal et al. 2003; Kubis et al. 2003; Ryder et al. 2003; McCullagh et al. 2004). However, innervation is insufficient to cause a change in the fiber type (Laing and Lamb 1983; Cho et al. 1993; Lefeuvre et al. 1996; DiMario and Stockdale 1997; Jiang et al. 2004), and the ratio of slow and fast muscle fibers is different between regenerating fast and slow muscles when they are denervated and electrically stimulated (Kalhovde et al. 2005).

The default type of MyHC in regenerating fiber is thought to be the fast-type as revealed by results from chicken or mouse muscles (Jerkovic et al. 1997; Francis- West et al. 2003). Our results also indicate that fast-type myotube and mixed-type myotube are predominantly represented in the control group. However, human satellite cells isolated from fast and slow muscle form myotubes that co-express both fast and slow MyHCs independently of the fiber type from which they are prepared (Bonavaud et al. 2001). These observations suggest that a part of the mechanism of the determination of muscle fiber type is different between human and chick during myotube formation. Nevertheless, our results have revealed the similarity of mechanisms of fiber type determination between human and chick or mouse. In the present study, the author has used chicken muscle or mouse cloned myoblast cells and have shown that, when fast muscle extract is added to the culture medium, the number of fast-type myotubes increases (Fig. 2j). On the other hand, when slow muscle extract is added to the culture medium, the number of slow-type myotubes increases dramatically (Fig. 2). When higher concentrations (more than 0.5%) of PM or ALD extract are supplemented, the expression ratio of sMyHCs versus fMyHCs returns to control levels (Fig. 3). This may be because skeletal muscle of chick contains both fast and slow fibers, even in PM or ALD. Thus, the myoblast differentiation pathway of chick or mouse is not completely determined and might be regulated by muscle extract.

With regard to the experiment on chicken myoblast, the possibility remains that myoblasts from chick whole thigh muscle contain both fast and slow myoblast lineages, and that only the proliferation of slow myoblast is promoted by slow muscle extract. To eliminate this possibility, the author has investigated the effects of muscle extracts on cultures of the C_2C_{12} cell line, which is subcloned from mouse myoblasts (Blau et al. 1983). ALD (slow) muscle extract also significantly

increases the expression of sMyHC in the single clone that the author has raised from the C_2C_{12} cells (Fig. 4). This result suggests that myoblasts have a variability of their subtype differentiation that is controlled by their surroundings. Moreover, neither the number of myotubes nor the number of nuclei in each of the groups supplemented with muscle extract significantly changes in comparison with the control groups of both the chick myoblasts and the C_2C_{12} cells. This observation suggests that the differentiation of the muscle fiber subtype is not controlled by the proliferation of myoblasts that are fated to be fast or slow muscle fibers, but that it is controlled by the regulation of the differentiation pathways.

In conclusion, myoblast subtype differentiation is not completely pre-committed to the slow- or fast-type, and myoblasts have some variability in their fiber-type determination. Their environment regulates the differentiation of muscle fiber type. The author suggest that, following muscle fiber damage or injury, this variability is essential for maintaining muscle fiber types, when muscle satellite cells differentiate into myoblasts during muscle fiber regeneration.

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Part B

Effect of β-guanidinopropionic acid on the growth and nucleoli in cultured muscle cells

Abstract

Effects of supplementation of creatine (Cr) or its analogue, β-guanidinopropionic acid (β-GPA), on the differentiation of mouse myoblast cell line C_2C_{12} cells to myotubes and/or number of nucleoli within a single nucleus were studied. Here the author reports that the differentiation of C_2C_{12} cells was stimulated by treatment with β-GPA. Growth-related increase of nuclear number in myotubes was enhanced by supplementation of β-GPA to culture medium. The mean number of nucleoli in a single nucleus was decreased from 5.3 to 1.7 following the 6-day culture with normal differentiation medium. And all of the nuclei in myotubes were p21-positive. It was also suggested that β-GPA supplementation stimulated the differentiation of myoblast cells through the induction of p21 expression, which is closely associated with cell cycle. Supplementation of Cr, on the contrary, inhibited these phenomena slightly, and increased myotube diameter, and inhibited the differentiation-related decrease of nucleoli in the myotubes.

Introduction

Creatine (Cr) is essential for synthesis of adenosine triphosphate (ATP) in the animal cells (Ohira *et al*. 1994; Jensson *et al*. 1996) and is mostly comprised as phosphocreatine in muscles. The supplementation of Cr and its analogue, β-guanidinopropionic acid (β-GPA), increases and decreases phosphocreatine and ATP contents in animal tissues *in vivo*, respectively (Boehm *et al*. 1995; Bergeron *et a*l. 2001; Ohira *et al.* 2003). Addition of β-GPA also decreases the level of intracellular ATP in cultured neutrophil (Hirayama *et al.* 2000). Moreover, it was reported that β-GPA suppressed the proliferation of Ehrlich ascites tumor cells *in vivo* and *in vitro* (Ohira *et al.* 1991; Ohira *et al.* 1995). These results suggest that supplementation of Cr or β-GPA affects the cell metabolism. In addition, Cr supplementation increases protein concentration of cultured C_2C_{12} mouse myoblast cells, associate with hypertrophy (Louis *et al.*2004).

In the nucleolus, chromosome is highly condensed and is surrounded by fibrillar and granular components, and forms nucleolar organization regions (Maggi and Weber 2005). It is reported that the conformation and number of nucleoli are associated with the cell cycle progression and malignant transformation (Sulic *et al*. 2005). The nucleolus disappears during the prometaphase and is reconstituted as the cell cycle progressed (Leblond and El-Alfy 1998). Nucleoli of cancer cells are generally larger than those of normal cells. Therefore, morphological change in the nucleolus has been used as the marker of malignant transformation (Rosenwald 2004). However, it is not known yet whether the nucleolar number can be changed independently from the cell cycle progression and malignant transformation.

Recently, we reported that the number of nucleoli in a single myonucleus of rat soleus muscle fibers was increased in response to functional overload by ablation of synergists (Kawano *et al.* 2007). These observations suggest a possibility that the number of nucleoli in the differentiated muscle fibers responds to mechanical load, applied to the muscle fibers, and the protein synthesis activity of myonucleus is stimulated. It is also reported that the regulation of muscle proteins in adult rats is related to both mechanical and neural stimuli (Ohira *et al.*2006). In response to feeding

of β-GPA, which caused a drastic depletion of phosphocreatine especially, the levels of mitochondrial enzyme levels in skeletal muscles were increased (Ohira *et al*. 1992; Ren *et al*. 1993), as was seen after a strenuous exercise training (Holloszy *et al.* 1967). However, the levels of daily voluntary activity and electromyogram of hindlimb muscle in these rats were significantly less than those of normal controls (Ohira *et al.* 2003). Further, the muscle mass was less than controls (Ohira *et al.* 2003). These results clearly suggest that the key factor for regulation of morphological and metabolic properties of skeletal muscles of matured animals is different. But, it is still unclear how the development of muscle fibers is regulated. Therefore, the author investigated how the differentiation of muscle cells and the number of nucleoli in single nucleus, which may influence the level of protein synthesis (Maggi and Weber 2005), respond to the changes of metabolic conditions, caused by supplementation of Cr or β-GPA in C_2C_{12} cells.

Materials and Methods

Cell culture

Mouse myoblast cell line C_2C_{12} cells were plated on a coverslip, coated with 0.2% gelatin, at 2 x 10^4 cells per well and pre-cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/l) containing 10% fetal bovine serum (growth medium) for 24 hours, and then, the culture medium was changed to DMEM with low glucose (1 g/l) containing 2% horse serum (differentiation medium) for 4 days under the stable 5% CO_2 at 37 ºC in an incubator. At the 4th day, the cells were separated into 3 groups; Cr-supplemented, β-GPA-supplemented, and control groups. The Cr or β-GPA was added into the differentiation medium of each group at the final concentration of 1 mM, respectively. No supplementation was performed for the control cells. The cells were subsequently cultured for additional 2 days.

Immunostaining

The cells were fixed using 10% formalin at the end of 6-day culture. Then, the cells were incubated overnight at 4°C with the primary antibodies specific for both fibrillarin (rabbit IgG, Santa Cruz Biotechnology, Inc., CA, USA) and p21 (mouse IgG, Santa Cruz Biotechnology, Inc., CA, USA), diluted at 1:200 with phosphate-buffered saline (PBS) containing 0.5% Tween 20 and 0.5% bovine serum albumin (BSA). The primary antibody-reacted regions for the fibrillarin and p21 were then detected by incubation with the secondary antibody conjugated with rhodamine and fluorescein, diluted at 1:200 with PBS containing 0.5% Tween 20 and 0.5% BSA for 2 hours, respectively. Staining of fibrillarin, which locates in nucleolus (Ochs *et al.* 1985), was performed as the marker for counting the number of nucleoli. The nuclei of the cultured cells were visualized by staining using Hoechst 33342. The data obtained from the cells immediately before the change from growth

to differentiation medium were used as the pre-control.

Immunofluoresence microscopy and analysis of muscle cells

Images of 4 randomly chosen fields (0.64 mm^2) of each well were stored using a cooled CCD camera under the fluorescence microscope (Olympus, Tokyo, Japan). The number of myotubes in each field was counted using Adobe Photoshop Software. To avoid miss counting of the proliferating myoblasts and binucleated myotubes, fused myotubes containing more than 3 nuclei were assessed. Myotube diameter and cross-sectional area of nucleus (n=40 nuclei per each group) and nucleolus (n=16 nucleoli per each group) were determined by using Image J program. Nuclear number per myotube was also analyzed.

The number of nucleoli in a single nucleus of 50 randomly chosen mononucleated myoblasts in each well (pre: n=16, control: n = 21, Cr: n = 12, and β-GPA: n = 24) and 10-50 multinucleated (with more than 3 nuclei) myotubes in each well (control: n = 17, Cr: n = 8, and β-GPA: n = 18) was counted. Then, the myoblasts and myotubes were separated into 2 groups with p21-positive and -negative nuclei, and the percentage of p-21-positive nuclei per total nuclei was calculated. The numbers of nucleoli in p21-positive and/or –negative nuclei were also counted by using a FV-300 confocal microscope (Olympus, Tokyo, Japan) and the images were recorded by using Fluoview Software. The images were processed using Adobe Photoshop Software.

Statistical analysis

Data were presented as means ± SEM. Statistical significance was examined by analysis of variance followed by Scheffé's post hoc test. Differences were considered significant at the 0.05 level of confidence.

Results

Culture study was performed using mouse myoblast C_2C_{12} cells to investigate the effects of Cr or β-GPA supplementation on myobalst differentiation and the number of nucleoli within a single nucleus. The numbers of myotubes in each field was not significantly influenced by supplementation of Cr or β-GPA to differentiation medium for 2 days, although the number of myotubes in the Cr-supplied group tended to be less than controls ($p>0.05$, Fig. 1A). The average diameter of myotubes in Cr-supplied group was greater than control group (31%, $p<0.05$), but was not significantly different from that in β-GPA-treated group (Fig. 1B). The number of nuclei per myotube in β-GPA-supplied group was significantly greater than the control (59%, $p<0.05$) and Cr-supplied group (71%, $p<0.05$, Fig. 1C). The levels of control and Cr-supplied groups were similar.

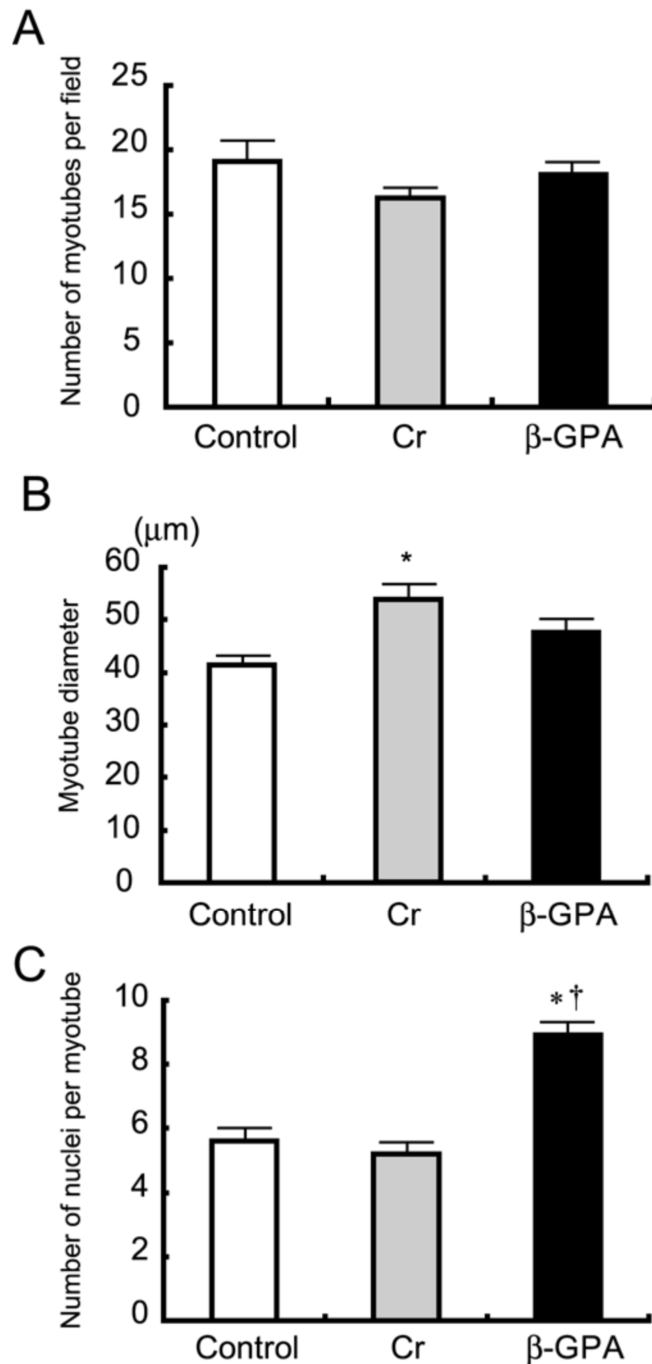


Fig. 1 Effects of the supplementation of creatine (Cr) or β-guanidinopropionic acid (β-GPA) on the myotube formation during the last 2 days of 6-day culture in differentiation medium. (A): The average numbers of myotubes in each field (n = 8 wells in each group. (B): The average diameter of 10 largest myotubes in each field (n = 8 wells in each group). (C) The number of nuclei in randomly chosen myotubes (10-50 myotubes in each well), which was counted under the fluorescence microscope (control: n = 17 wells, Cr: n = 10 wells, and β-GPA: n = 18 wells). Mean ± SEM. * and †: $p<0.05$ vs. control and Cr, respectively.

The distributions of nucleoli, which are stained positively with antibody specific for fibrillarin and/or p21, are shown in Figure 2A-C. Figure 2C indicates the typical morphology and distribution of nucleoli in a single nucleus of myobalst and myotube. The cross-sectional areas of nuclei in myoblasts decreased significantly after 6-day culture relative to those obtained immediately before changing from growth to differentiation medium ($p<0.05$, Fig. 3A). Although these levels in myotubes also tended to be less than the pre-control myoblasts, the mean size was slightly greater than the myoblasts treated in the same way ($p>0.05$).

The number of nucleoli in the nucleus of control myoblast cell (5.4 per nucleus) was not different from the pre-control level (5.3, Fig. 3B). The nucleolar number of myoblasts was not influenced by supplementation of Cr, but that in β-GPA-treated cells (4.9 per nucleus) was significantly less than the control and Cr-treated groups (~10%, $p<0.05$). The number of nucleoli within a single nucleus in all groups decreased drastically following myotube formation. The mean number in myotubes of control group was 1.7 per nucleus. Although nucleolar number in β-GPA group (1.6) was identical, that in Cr group (2.4 per nucleus) was greater than in control myotube (38%, $p<0.05$). The size of nucleoli in myotubes tended to be greater than in myoblasts (Fig. 3C). The mean size in myotubes of β-GPA-treated group was significantly greater than that in pre-control myoblasts, control myoblasts, myoblasts treated in the same way, and Cr-treated myoblasts or myotubes ($p<0.05$).

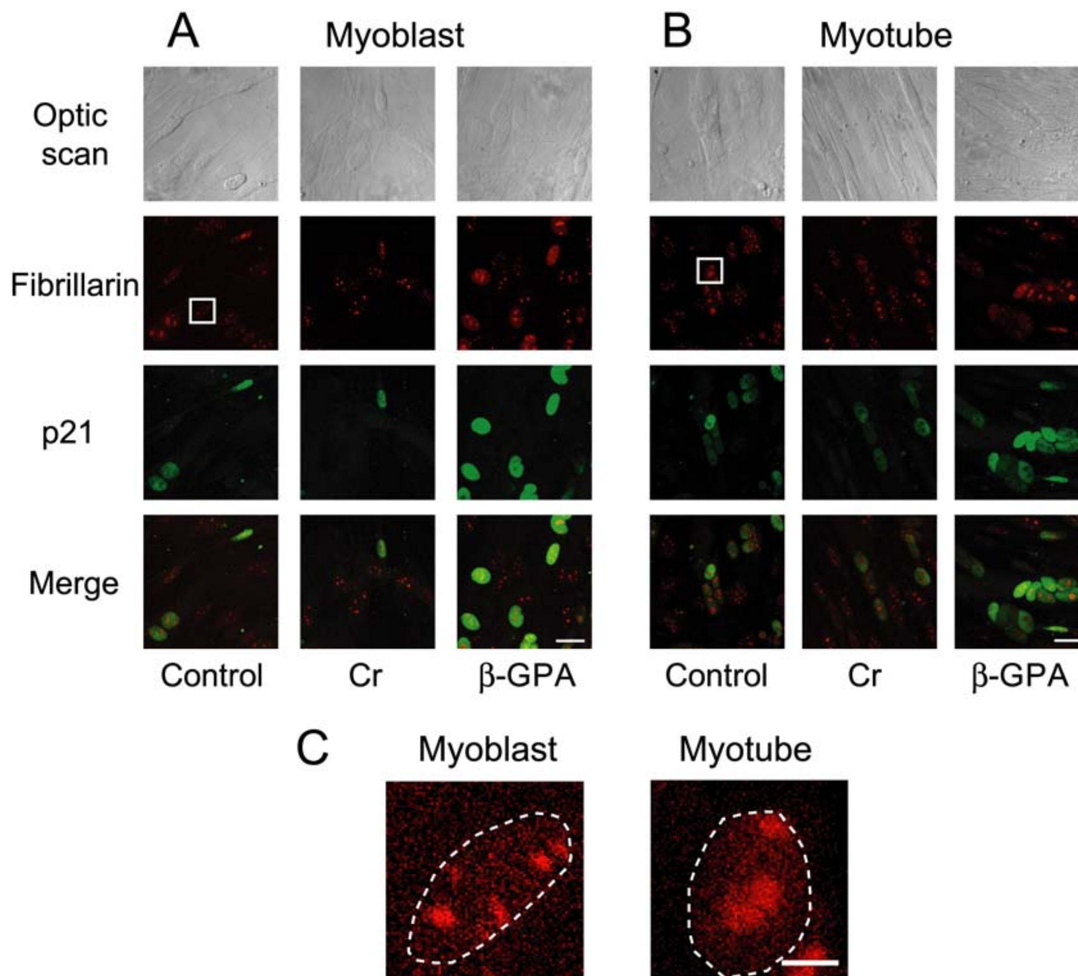
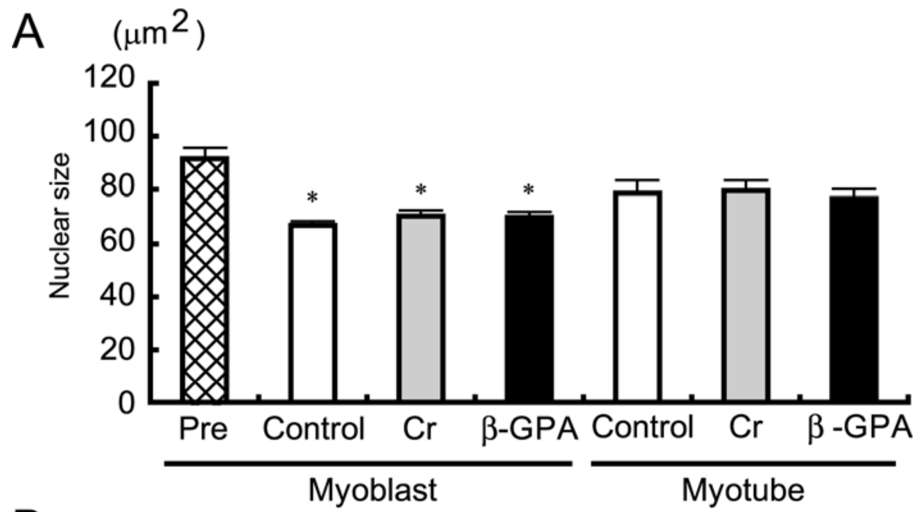


Fig. 2 Effects of the supplementation of Cr or β-GPA on the characteristics of nuclei and nucleoli of the cultured myoblast and myotube. The pictures show the optic scan images and immunohistochemical visualizations of fibrillarin (red) and p21 (green) in the myoblast (A) and myotube (B) after 2 days of Cr or β-GPA supplementation during 6-day culture in differentiation medium. Scale bar = 25 μm. C: High magnification images of fibrillarin in the framed field of A (left) and B (right), respectively. Single nucleus was framed by dotted line. Scale bar = 5 μm. See Figure 1 for the abbreviations.


A
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120
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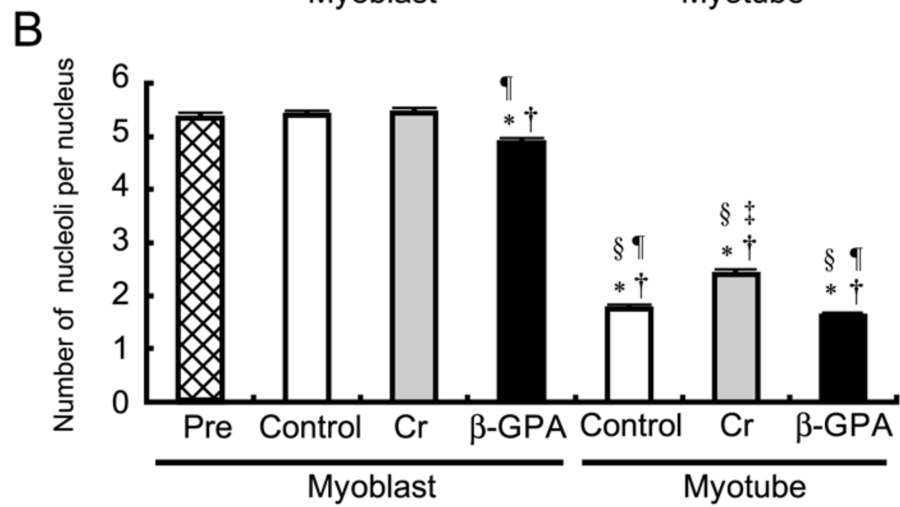
B
Number of nucleoli per nucleus
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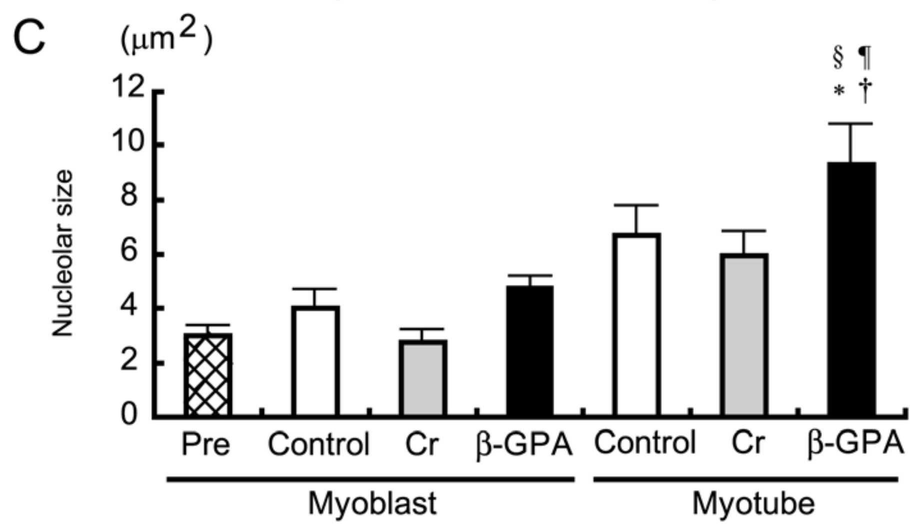
Myoblast
Myotube


C
(μm2)
Nucleolar size
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Control
Cr
β-GPA
Control
Cr
β-GPA
Myoblast
Myotube

Fig. 3 The mean cross-sectional area of nucleus (A), number of nucleoli per nucleus (B), and the cross-sectional area of nucleolus (C) in myoblast and myotube. The data obtained immediately before the change from growth to differentiation medium were used as the pre-control (pre). Mean ± SEM. *, †, §, ‡, and ¶: $p<0.05$ vs. pre-control myoblasts, control myoblasts, myoblasts treated in the same way, control myotubes, and Cr-treated myoblasts or myotubes, respectively. See Figure 1 for the abbreviations.

In both myoblasts and myotubes, p21 was expressed in the nuclei (Figs. 2A and B, 4A and B). All nuclei in the myotubes of all groups were p21-positive (Fig. 4A), indicating that the differentiating myotubes escaped from the cell cycle. Further, the percentage of p21-positive myoblast cells in the β-GPA-supplied group was greater than that of controls (96%, $p<0.05$, Fig. 4A). That of the Cr-supplied group tended to be less than controls (54%, $p>0.05$). The number of nucleoli per nucleus in myoblast of all groups was approximately 25% less in the p21-positive than -negative nucleus ($p<0.05$, Fig. 4B). No effect of either Cr or β-GPA was observed.

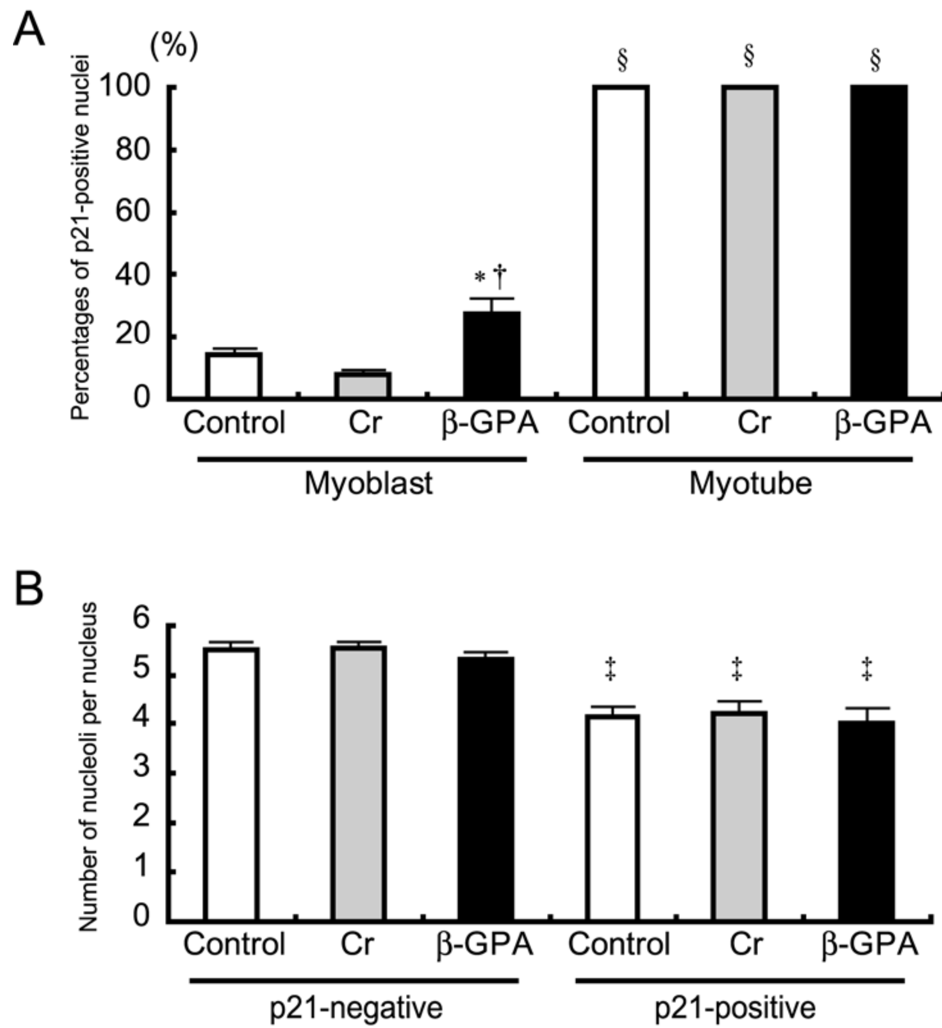

Fig. 4 A: The mean percentage of p21-positive nuclei per total nuclei in a myoblast (left) and a myotube (right). **B:** The mean number of nucleoli in p21-negative (left) or -positive (right) nucleus in a myoblast cell (control: n = 29 wells, Cr: n = 12 wells, and β-GPA: n = 32 wells). Mean ± SEM. *, †, §, and ‡: $p<0.05$ vs. control myoblasts, Cr-treated myoblasts, myoblasts treated in the same way, and p21-negative myoblasts treated in the same way, respectively. See Figure 1 for the abbreviations.

Discussion

Here it is reported that the differentiation of mouse myoblast cell line C_2C_{12} cells to myotubes was stimulated by treatment with β-GPA. Growth-related increase of nuclear number in myotubes was enhanced by supplementation of β-GPA. The mean number of nucleoli in a single nucleus was decreased from 5.3 to 1.7 following the 6-day culture with normal differentiation medium. And all of the nuclei in myotubes were p21-positive. Such phenomena were further stimulated by β-GPA treatment. Supplementation of Cr, on the contrary, inhibited these phenomena slightly.

The cyclin-dependent-kinase inhibitor, p21, is highly expressed in developmental embryonic muscle and is increased during myoblast differentiation (Guo *et al*. 1995; Halevy *et al.* 1995). It is reported that p21 plays a role in induction of G1 cell cycle arrest (Myers *et al*. 1995) and cell cycle withdrawal in differentiating muscle cells (Guo *et al.* 1995; Halevy *et al.* 1995). It was also reported that the p21 was expressed by myogenic cell specific basic helix-loop-helix protein MyoD (Halevy *et al.* 1995). All of the myonuclei in myotube was p21-positive (Fig. 4A). This result agrees with the recent reports (Odelberg *et al.* 2000), although the expression of p21 is down-regulated during the late myotube differentiation *in vitro* (Cabane *et al*. 2003) and myonuclei in the matured myofiber do not express p21 *in vivo* (Ishido *et al.* 2004).

It was reported that the number of nucleoli per nucleus decreased from 1.7, observed 24-hour after initiation of cell differentiation, to 1.2 after 216 hours of differentiation in the chicken myoblast primary culture (Bachmann *et al*. 1980). Our data showed that the number of nucleoli was more drastically reduced in response to differentiation from myoblast to myotube in mouse myoblast cell line. The number of nucleoli in myoblast cell (5.3) was greater than that in myotube (1.7), which was similar to that in normal rat soleus fibers (1.6-1.7) (Kawano *et al.* 2007), suggesting that the number of nucleoli in differentiated cell is identical between myotubes and matured myofibers. Moreover, the number of nucleoli per nucleus in p21-positive myoblast was less than that of p21-negative myoblast cells. These results suggest that the decrease of nucleolar number is initiated even during the early myogenic differentiation stage.

On the contrary, the size of nucleoli in myotube (6.0-9.3 μm^2) tended to be greater than that of myoblast treated in the same way (2.8-4.4 μm^2). Treatment with β-GPA significantly enhanced the nucleolar size further. Although the precise cause is still unclear, this phenomenon may have some relationship with the increase of nuclear number caused by β-GPA supplementation (Fig. 1C). It was indicated, for the first time, that the number of nucleoli decreased but the nucleolar size was increased during muscle differentiation, which caused a reduction of nucleolar number (Bachmann *et al*. 1980). The direct cause of these modulations is not clear, but a similar morphological change of nucleoli in cancer cells was observed by Espada *et al*. (2007). They reported that wild type cancer cells had large spherical nucleoli, but cancer cells, which lack DNA methyltransferase 1, had smaller mass. They speculated that these conformational changes of nucleoli may be associated with a change in chromatin.

The sizes of nuclei in myoblasts ($p<0.05$) and myotubes ($p>0.05$) decreased after 6 days of culture. It is also reported that the size of nuclei was decreased after 216 hours, when chick myoblast was cultured in differentiation medium (Bachmann *et al*. 1980). These results suggest that long-term culture in differentiation medium decreases the nuclear size of muscle cells, although the mechanism of these phenomena is unknown. It was recently reported that the myonuclear cross-sectional area in the normal rat soleus muscle fibers increased following the gravitational unloading and it was also suggested that the protein synthetic capability of these nuclei are inhibited (Wang *et al.* 2006; Kawano *et al*. 2007). In this study, the mean nuclear size of in the C_2C_{12} cells was approximately 70 μm^2. However, neither Cr nor β-GPA affected the size of muscle nuclei. These observations suggest that the mechanical stimuli and Cr or β-GPA affects the cell growth via different pathways.

It was reported that the treatment with β-GPA suppressed the cell proliferation activity of Ehrlich ascites tumor cells (Ohira *et al.* 1991; Ohira *et al.* 1995). However, the results obtained from the present study suggested that the differentiation to myotubes was stimulated by treatment with β-GPA. Enhanced myonuclear incorporation (Fig. 1C), observed in newly formed myotubes, might suggest that the longitudinal growth of myotubes may be stimulated by β-GPA treatment. In a

myoblast, the number of nucleoli in a single p21-positive nucleus was decreased to ~4, when the nucleus started to express p21 (vs. ~5 in p21-negative nucleus) (Fig. 4B). Further, the percentage of p21-positive nuclei was increased by β-GPA treatment (Fig. 4A). As the results, the mean number of nucleoli per nucleus in the β-GPA group was less than control, when both p21-negaive and –positive nuclei were combined (Fig. 3B). It was also reported elsewhere that the number of nucleoli per nucleus decreased during myoblast differentiation in the chicken myoblast primary culture (Bachmann *et al*. 1980). These data also suggested that β-GPA supplementation stimulated the differentiation of myoblast cells through the induction of p21 expression.

The increase of myotube diameter in the group cultured with β-GPA was not statistically significant, although the mean value (47.5 μm) tended to be greater than the control group (41.5 μm, $p>0.05$, Fig. 1B). It was reported that the cross-sectional area of hindlimb muscle fibers of rats fed β-GPA was less than that of rats fed normal control diet (Ohira *et al.* 2003), but the mitochondrial enzyme levels were increased (Ohira *et al*. 1992; Ren *et al*. 1993). These results clearly suggest that the treatment with β-GPA stimulated the synthesis of metabolic enzymes but not the structural proteins.

The myotube formation (Fig. 1A) and the percentage of p21-positive nuclei in myoblast (Fig. 4A) tended to be inhibited by Cr supplementation ($p>0.05$). Myotubes with large diameter (Fig. 1B) and greater number of nucleoli (Fig. 3B), seen in Cr-treated group, agrees with our recent report (Kawano *et al.* 2007), suggesting that the nucleolar number in a nucleus of muscle cells may be closely related to the regulation of muscle fiber size. However, the greater number of nucleoli seen in Cr-treated group was not due to an increase of the number. It was just caused by the inhibited decrease of nucleoli, when myoblasts differentiate to myotubes. Since the number of nuclei per cell was identical to the controls and significantly less than the β-GPA group, the length of the myotubes may be similar to controls. However, it is not clear why Cr treatment caused the formation of myotubes with large diameter.

In conclusion, the mean number of nucleoli in a single nucleus was decreased from 5.3 to 1.7 following the progression of cell cycle during 6-day culture with normal differentiation medium. All

of the nuclei in myotubes became p21-positive. And the number of nucleoli in p21-positive nucleus was less than that in p21-negative one in a myoblast. The growth-related increase of nuclear number in myotubes was enhanced by supplementation of β-GPA to culture medium. Further, the number of nucleoli in a nucleus was decreased and that of p21-positive nuclei was increased by β-GPA treatment even in myoblasts, suggesting that β-GPA supplementation stimulated the differentiation of myoblast cells through the induction of p21 expression. Supplementation of Cr, on the contrary, inhibited these phenomena slightly.

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2. Lan YB, Terada M, Higo Y, Kawano F, Matsuoka Y, Wang XD, Nakai N, Ohira Y. Effects of mechanical and/or neural stimulation on the propertiesof soleus muscle fibers in mdx mice. Jpn. J. Aerospace Environ. Med. 44:19-29, 2007. (Technical support)
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1. Y. Matsuoka, F. Kawano, X.D. Wang, M. Terada, Y. Higo, Y.B. Lan, A. Ogura, N. Nishimoto, Y. Adachi, and Y. Ohira. Blockade of interleukin-6 signaling pathway promotes muscle cell differentiation. Journal of Gravitational Physiology, volume 13, Number 1, P-83, 2006.
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1. Y. Matsuoka, and A. Inoue. Controlled Differentiation of Myoblast Cells into Fast and Slow Muscle Fibers. 46th Annual Meeting of the American Society for Cell Biology. 2006. (Poster presentation)
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