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Differentiation of Myoblasts in Serum-Free Media: Effects of Modified Media Are Cell Line-Specific

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Key Words

Myogenesis · Differentiation · Myoblast · Serum-free culture

Abstract

Myoblast cell lines are grown and differentiated readily in cell culture. Two cell lines typically used for investigating the growth and differentiation of muscle are the mouse cell line C2C12 and the rat cell line L6. The differentiation of these cells in vitro requires a switch from a serum-rich medium to a less rich medium after the cells have reached confluence. Since the components present in serum are not well characterized, the use of a better defined medium for these studies was investigated. C2C12 and L6 myoblasts were differentiated in both serum-containing and serum-free media. The differentiation state of these cultures was then tested both microscopically and biochemically. Cultures were checked for myotube formation, the activity of creatine phosphokinase and the presence of sarcomeric actin. In C2C12 cells, the extent of differentiation was greater in the serum-free than in the serum-containing system. In both media types, the C2C12 cells produced sarcomeric actin, showing the presence of sarcomere structure in the myotubes. In L6 cells, however, myotubes were readily formed in medium containing 2% horse serum, but not in the serum-free system. In addition, the ability of C2C12 cells to differentiate on substrates coated with extracellular matrix proteins was shown to be media-dependent. The presence of extracellular matrix proteins did not enable L6 cells to form myotubes when cultured in serum-free media. Primary cultures of chick myoblasts were able to differentiate in both media tested, with Dulbecco's modified Eagle medium containing horse serum being a more efficient medium for cell fusion. This study shows a divergence in muscle cell line responses in three cell lines, two of which are typically used as 'model systems' for understanding muscle growth and development.

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Introduction

Skeletal myoblasts have long provided researchers with a good in vitro tool for studying muscle cell proliferation and differentiation. Understanding of these processes is essential in understanding both muscle development and repair. Myoblasts in culture are able to exhibit

Abbreviations used in this paper

BSA	bovine serum albumin
CPK	creatine phosphokinase
DMEM	Dulbecco's modified Eagle's medium
PBS	phosphate-buffered saline
TBS	Tris-buffered saline

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all of the features of muscle myogenesis, including proliferation, migration, fusion, myotube formation and contraction. Muscle cells grown in a medium containing 10% fetal calf serum continue to proliferate even after the point of confluence, while these same cell lines, when grown in media containing 2% horse serum, readily fuse and form myotubes. Two cell lines that have been used extensively in the study of myoblast proliferation and differentiation are the mouse cell line C2C12 and the rat cell line L6.

There are a number of factors that can play a pivotal role in muscle cell differentiation. These include intracellular factors such as Myo-D, Myf-5 and myogenin [Weintraub et al., 1991; Edmonson and Olsen, 1993; Lasser et al., 1994] and the proteinase urokinase [Miralles et al., 1998], metalloproteinases [Guerin and Holland, 1995], meltrin-α [Yagami-Hiromasa et al., 1995; Gilpin et al., 1998], calpain [Kwak et al., 1993; Dourdin et al., 1997], and cathepsin B [Gogos et al., 1996]. Extracellular elements such as growth factors [Harper et al., 1986] and extracellular matrix proteins [Mayne and Sanderson, 1985; Engvall, 1993; Lasser et al., 1994] have a large effect on myoblast cells in culture. One of these proteins, laminin, which is normally found in the basement membrane of cells, has been shown to play a role in promoting myoblast adhesion, migration, proliferation and myotube formation [Kuhl et al., 1982; Foster et al., 1987; Von der Mark and Ocalon, 1989; Kroll et al., 1994], and may also be required for myotube stability and survival [Vachon et al., 1996]. Fibronectin has been shown to promote myoblast adhesion and proliferation, but appears to inhibit myoblast differentiation [Podleski et al., 1979; Foster et al., 1987; Von der Mark et al., 1989]. Whether the extent of the effects of these proteins varies between cell lines is unknown.

Fetal serums may contain more than 1,000 distinct components, including proteins, electrolytes, lipids, carbohydrates, attachment factors, hormones, enzymes and inhibitory factors. Many of the substances in serum are related to factors that have been shown to have a large effect on the development of muscle cells. The identities and concentrations of these substances in serum are not well characterized, and therefore the use of serum introduces a large number of confounding variables to any experiment. The use of a serum-free medium is, therefore, an important tool for eliminating many of these variables.

For some time, researchers have tested serum-free media for the study of myoblasts in culture [Florini and Roberts, 1979; Dollenmeier et al., 1981; Yoshiko et al., 1996; Minotto et al., 1998; Goto et al., 1999]. In each of these studies, a medium was found that effectively supports proliferation and differentiation in a single myoblast cell line. Not all myoblast cell lines have the same requirements for growth and survival. In this study, we test the effects of a serum-free medium on both C2C12 cells and L6 cells to determine whether one medium could induce differentiation in both of these 'model' systems. We have also tested the ability of a primary culture of chick myoblasts to fuse under these conditions. One serum-free medium that has been used extensively for a large number of cell lines including macrophages [Helsinki et al., 1988], T cells [Causey et al., 1994; Slunt et al., 1997] and fibroblasts [Ellis et al., 1996] is Aim-V medium. Aim-V medium is especially effective for cell lines that are usually grown in Dulbecco's modified Eagle medium (DMEM) containing serum. Since myoblasts grow readily in DMEM, Aim-V was used to study the effects of a serum-free system on C2C12 and L6 cells in this study. The results demonstrate that Aim-V medium could not only efficiently support myotube formation, stimulate creatine phosphatase activity and induce the production of sarcomeric actin in C2C12 cells, but enhance each of these processes. In L6 cells however, Aim-V medium was not only unable to induce myoblast fusion, but it was also unable to support the survival of the rat myoblast cell line.

Materials and Methods

Cell Culture

C2C12 and L6 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, Va., USA) and maintained at 37 °C under a continuous 5% CO_2 stream. The cells were plated at 7,500 cells/cm^2 and grown to confluence in DMEM (Life Technologies, Denmark) containing 10% fetal bovine serum. Cell cultures appeared confluent after approximately 24 h of incubation. The culture medium was then replaced with either fresh DMEM with fetal bovine serum, DMEM containing 2% horse serum, or Aim-V serum-free medium (Life Technologies, Denmark) and incubated for up to 10 days to stimulate myotube formation.

Isolation of Chick Myoblasts

Fertilized chick eggs were purchased from Tybjerg Kontrolhønseri (Tybjerg, Denmark) and incubated at 35 °C until embryonic day 10. The pectoralis muscles were removed from each embryo, minced and incubated in Hanks' balanced salt solution containing 0.25% trypsin for 30 min at 37 °C and 5% CO_2. The trypsinized samples were then filtered through filter paper and centrifuged. This procedure was repeated 3 times to remove muscle debris. The cells were then resuspended in DMEM containing 10% fetal calf serum at a concentration of 3×10^5 cells/ml. Fibroblasts were removed from the cultures by repeated differential adhesion steps whereby the

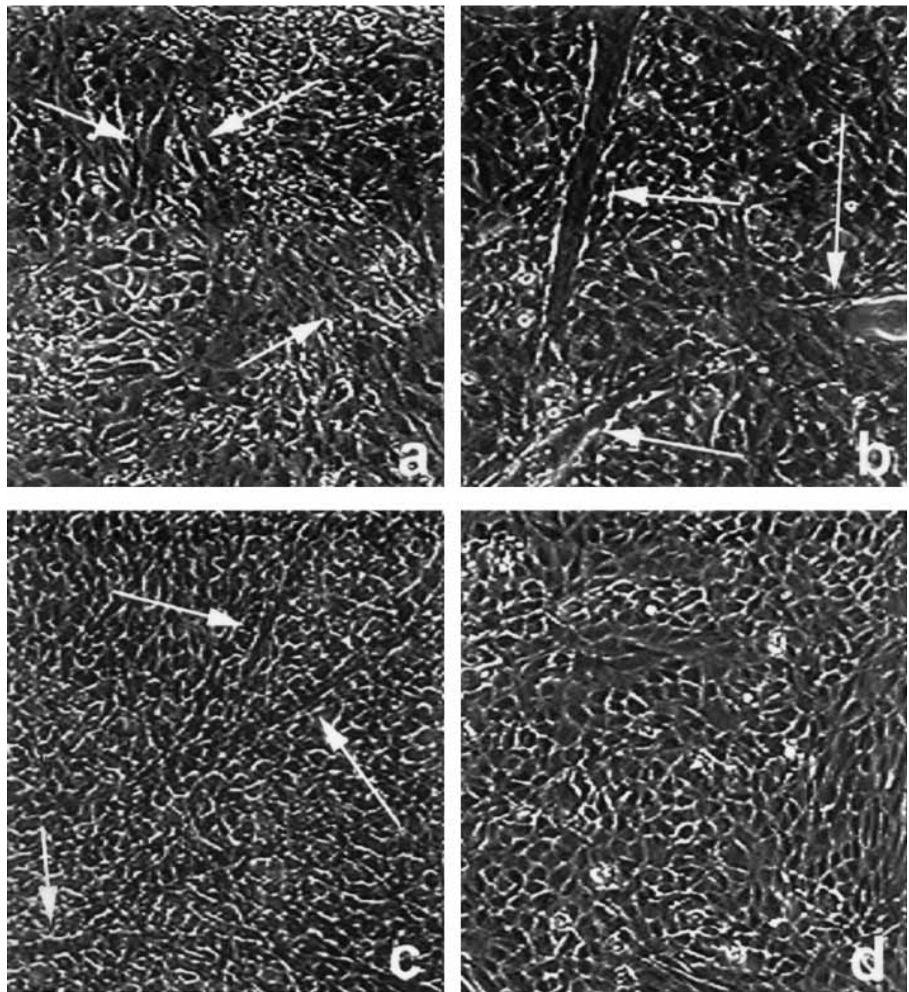

Fig. 1. C2C12 and L6 cells differentiated in serum-containing and serum-free media. C2C12 cells (**a**, **b**) and L6 cells (**c**, **d**) were differentiated in media containing 2% horse serum (**a**, **c**) or in serum-free medium (**b**, **d**). Several of the resultant myotube structures are indicated with arrows. Myotubes are readily seen in all cells grown and differentiated in serum-containing medium, but L6 cells in serum-free medium do not appear to make visible myotube structures.

supernatant containing the slowing adhering myoblasts was removed and transferred to fresh flasks, and the fibroblasts discarded. This isolation step was repeated 3 times, and the representative cultures stained for the presence of desmin. Desmin staining showed that the cell cultures used contained >98% myoblasts (data not shown). All chick cell cultures were used within 5 days after isolation.

Microscopy and Image Acquisition

Cell cultures were viewed using a Leica IRB inverted fluorescent microscope (Leica A/S, Denmark) using phase-contrast optics, and equipped with a Kappa (Kappa Messtechnik, Germany) video camera. Images were obtained using Image Pro Plus image acquisition software (Image House A/S, Denmark) and the final images reproduced using Adobe Photoshop (Adobe Systems, San Jose, Calif., USA).

Cell Proliferation Assay

Cell cultures were fixed with 4% paraformaldehyde for 5 min, and cell nuclei were stained with 4,6-diamidino-2-phenylidone as previously described [Yablonka-Reuveni and Rivera, 1994]. The cells were then rinsed and viewed as described above. For each field, the number of nuclei were counted using the Image Pro plus image analysis system. 10 fields were counted per dish, with 3 dishes used for each condition.

Creatine Phosphokinase Activity Assay

Cells were cultured and differentiated as described above. Where indicated, the flasks were first coated with 0.1 μg/ml of either collagen I, collagen IV, laminin, fibronectin or bovine serum albumin (BSA) for 1 h and then rinsed. Cells were rinsed with phosphate-buffered saline (PBS), and then scraped from the surface of the flask in the presence of 2 ml PBS. The cell suspension was homogenized for 1 min. Protein determination was carried out using the BCA protein determination kit (Pierce, Rockford, Ill., USA). According to the manufacturer's instructions, 0.1 ml of the cell suspension was added to one vial of the creatine phosphokinase (CPK) assay kit (Sigma, St. Louis, Mo., USA) that had been diluted with 3 ml distilled water. The mixture was inverted several times and then transferred to a cuvet. After 5 min a baseline reading at 340 nm was recorded. 5 min later a second 340-nm reading was taken. The difference between the final and initial readings was used to calculate the CPK activity in the samples.

Table 1. Proliferation rates for L6 and C2C12 cells grown and differentiated in different medium types

	L6			C2C12		
	in DMEM + 10% FCS	in DMEM + 2% HS	in serum-free medium	in DMEM + 10% FCS	in DMEM + 2% HS	in serum-free medium
16 h	329±38	306±40	308±39	261±44	268±39	259±46
56 h	375±41	321±39	277±51	359±35	283±42	265±37
96 h	441±43	344±44	291±43	429±37	296±39	270±49

Number of nuclei per field ± SD is given.
HS = Horse serum.

Determination of Sarcomeric Actin Production

Cells were grown to confluence and myotube formation was stimulated as described above. Cells were rinsed with PBS and then scraped from the surface of the flask in the presence of 2 ml PBS. The cell suspension was homogenized for 1 min and 1 ml of the solution was diluted 1:1 with sample buffer. After protein determination (as described above), the protein concentrations of each sample were adjusted to the same value. 4 μl of cell homogenate containing 10 μg protein was applied to nitrocellulose filters by dot blot. The membranes were then blocked for 1 h in 3% milk in Tris-buffered saline (TBS). The blots were incubated in a 1:500 dilution of primary antibody in the 3% milk buffer overnight at 4°C. The blots were then rinsed thoroughly with TBS and incubated in a 1:10,000 dilution of alkaline phosphatase-conjugated secondary antibody in TBS for 1 h at room temperature. After thorough rinsing in TBS the protein spots were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Dot blots were analyzed using NIH Image (Wayne Resband, NIH, Bethesda, Md., USA, program available by ftp from zippy.numh.nih.gov).

Results

Serum-Free Media Enhances Myotube Formation in C2C12 Cells but Not in L6 Cells

After 5 days in serum-free media, C2C12 cells show considerable numbers of fused myotubes (fig. 1b). These structures are also seen in the cultures grown in medium containing 2% horse serum, but a far greater number of undifferentiated myoblasts can be seen in the cultures (fig. 1a). In general, the L6 cell cultures showed a smaller number of myotubes than the C2C12 cells, although the cells grown in 2% horse serum did show a number of these structures (fig. 1c). The L6 myotubes appear smaller than C2C12 myotubes, and this is probably due to the fact that the L6 myotubes contained an average of 40% less nuclei (data not shown). Myotubes were virtually absent in the L6 cell cultures grown in serum-free medium (fig. 1d). In addition, by 6 days of growth in serum-free media, the L6 cell cultures began to lift off the tissue culture dish (data not shown). The C2C12 cultures remained strongly adherent even up to 10 days in the serum-free media. There was therefore an undefined component present in horse serum that was necessary for not only the differentiation of L6 cells, but for their adherence as well. This component was not necessary for either differentiation or adherence of C2C12 cells.

Incubation in Differentiation Medium Effectively Halts Cell Proliferation

An increase in cell proliferation can be measured by an increasing number of cell nuclei in each culture. When C2C12 cells were cultured in DMEM containing 10% horse serum, the number of cell nuclei increased significantly after reaching what looked by eye to be cell confluence (table 1). When cells were grown in myoblast differentiation media, there was no significant increase in the number of nuclei seen in each field of cells. Therefore, the lack of differentiation seen in L6 cells subjected to serum-free media was not due to the cells remaining in the proliferative state. Additionally, the observation that the L6 cells lifted off the surface of the dish after 5–6 days in serum-free media could not be due to the cells becoming hyperconfluent.

CPK Activity Increases in C2C12 Cells but Not L6 Cells Grown in Serum-Free Medium

The activity of CPK increases as myoblasts fuse into elongated myotubes. The activity of this enzyme was assayed 5 days postconfluence in C2C12 and L6 cells grown in serum-free medium, or medium containing 2% horse serum (fig. 2). The increase in CPK activity was approximately twice as high in C2C12 cells grown in serum-free media than those grown in medium containing 2% horse serum. L6 cells showed only a very small

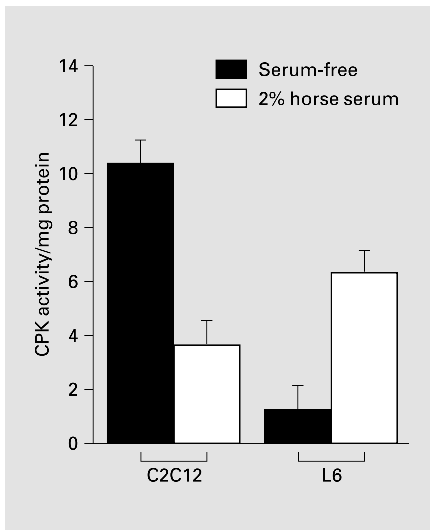

Fig. 2. CPK activity of cells grown in serum-containing and serum-free media. C2C12 cells and L6 cells were differentiated in either serum-containing or serum-free media and assayed for CPK. C2C12 cells showed a higher CPK activity when grown in serum-free medium, while L6 cells showed a higher CPK activity when differentiated in medium containing horse serum. The experiment was repeated on 3 separate days with different cultures, and a representative data set is shown.

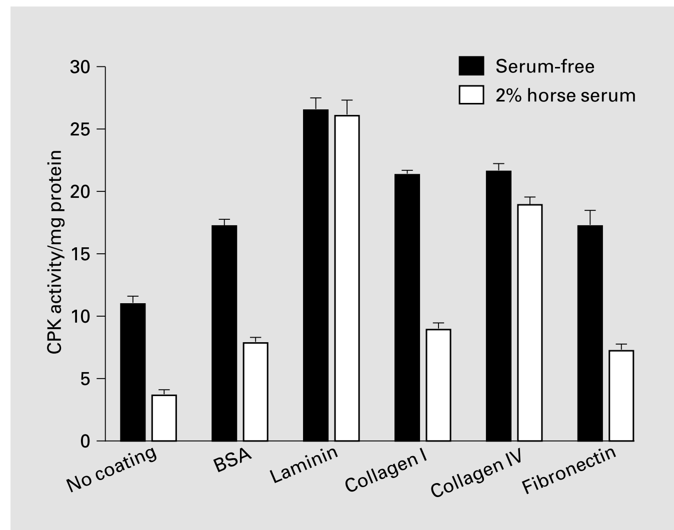

Fig. 3. CPK activity of C2C12 cells grown on different substrates and differentiated in the presence or absence of serum. C2C12 cells were plated on tissue culture flasks that had been coated with components of the extracellular matrix, differentiated in the presence of either serum-free media or DMEM containing 2% horse serum, and assayed for CPK activity. In serum-free medium, CPK activity was elevated in cells grown on all the proteins tested, with cells grown on laminin exhibiting the highest activity. CPK activity for cells grown in serum-containing medium was significantly higher only for cells plated onto laminin or collagen IV. The experiment was repeated on 3 separate days with different cultures, and the data is normalized against the protein concentration on a given day to allow for small differences in the number of cells in each culture.

increase in CPK activity when grown in serum-free medium and is 2–3 times less than in cultures grown in 2% horse serum. This finding corresponds to the microscopic data, where L6 cells formed few myotubes in serum-free media, while this fusion was readily apparent in C2C12 cells grown under the same conditions. The CPK activity did increase in each culture over time (data not shown).

The Presence of Basement Membrane Proteins Allows Increased Myotube Formation in C2C12 Cells Grown in Medium Containing 2% Horse Serum

The C2C12 cells that were grown and differentiated on dishes that had been previously coated with extracellular matrix proteins showed a higher CPK activity than those cultured on BSA-coated surfaces of tissue culture plastic alone. Cells that had been grown on laminin and differentiated in serum-free medium showed the highest rate of myotube formation (fig. 3) as determined using the CPK assay as a marker for myotube formation. Smaller increases in the activity of CPK were observed for cells grown on collagen I, collagen IV and fibronectin. When cells were grown on untreated tissue culture flasks, or those that had been pretreated with BSA or collagen I, and differentiated with medium containing 2% horse serum, the CPK activity was less than half of that seen with cells differentiated in serum-free medium. Cells grown on laminin and collagen IV, however, showed little differences in CPK activity after being differentiated in either serum-free or serum-containing medium. Therefore, adherence to specific basement membrane proteins negates any differences in cell fusion due to the presence or absence of serum proteins.

The Presence of Basement Membrane Proteins Allows Increased Myotube Formation in L6 Cells Grown in Medium Containing 2% Horse Serum, but Has No Effect on These Cells Grown in Serum-Free Media

When L6 cells were grown on flasks coated with extracellular matrix proteins, the presence of the proteins did nothing to recover the ability of L6 cells to fuse and form myotubes in serum-free media as measured by the CPK activity of the cell culture (fig. 4). When grown in media containing 2% horse serum, L6 cells showed the highest level of CPK activity when grown on the proteins normal-

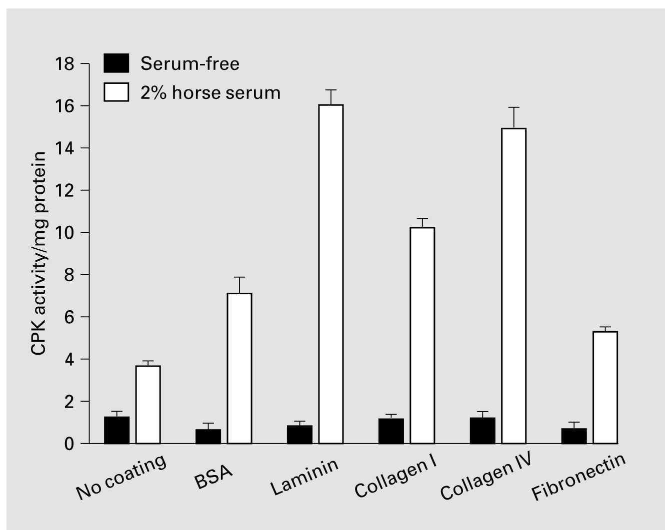

Fig. 4. CPK activity of L6 cells grown on different substrates and differentiated in the presence or absence of serum. L6 cells were plated on tissue culture flasks that had been coated with components of the extracellular matrix, differentiated in the presence of either serum-free media or DMEM containing 2% horse serum, and assayed for CPK activity. Cells grown in the presence of serum-free media did not show significant amounts of CPK activity on any of the substrates tested. In serum-containing media, CPK activity was highest in cells grown on the basement membrane proteins laminin and collagen IV. The experiment was repeated on 3 separate days with different cultures, and the data is normalized against the protein concentration on a given day to allow for small differences in the number of cells in each culture.

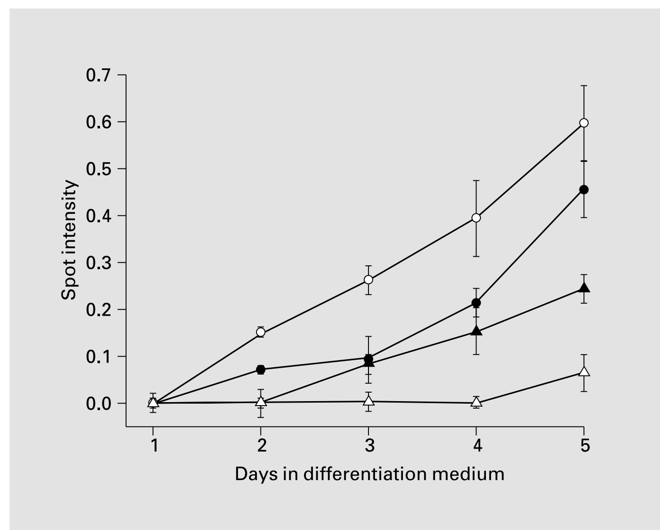

Fig. 5. Production of sarcomeric actin in cells differentiated in serum-free and serum-containing media. C2C12 cells and L6 cells were differentiated in either serum-free or serum-containing media and assayed for the production of sarcomeric actin. C2C12 cells in serum-containing media (●) showed a small amount of sarcomeric actin present at day 2 postconfluence, while significantly more of the protein was seen at this time in cells grown in serum-free medium (○). Cells in both media showed a daily increase in the amount of sarcomeric actin present, with the highest level seen in the serum-free cultures after 5 days. Treated similarly, L6 cells grown in serum-containing media showed the presence of sarcomeric actin on day 3 post-confluence (▲). The concentration of sarcomeric actin on day 5 in this culture was less than seen in the C2C12 cultures. L6 cells grown in serum-free media produce little if any sarcomeric actin (△).

ly found in the basement membrane, laminin and collagen IV. There was also a slight increase seen in cultures grown on either collagen I or fibronectin, with little effect seen on cultures grown on flasks coated with BSA. Therefore, in the presence of medium containing 2% horse serum, L6 cells show little behavioral variation compared to C2C12 cells, while in serum-free medium their ability to differentiate varies greatly.

C2C12 Cells Produce Sarcomeric Actin in Both Serum-Containing and Serum-Free Media, while L6 Cells Produce Little if Any after 5 Days in Culture

While the presence of myotubes was readily visible after 5 days in all C2C12 cultures and in L6 cultures grown in serum containing media, it was not known whether the fused myotubes were assembling sarcomeric structures. Using the dot blot technique described, the cultures were probed for the presence of sarcomeric actin (fig. 5). After 2 days in serum-free medium, C2C12 cells produced a significantly greater amount of sarcomeric actin than was seen in cells grown in medium containing 2% horse serum. Sarcomeric actin was first detected in L6 cells at day 3 in the serum-containing media. Very small but detectable amounts of sarcomeric actin were present at day 5 of L6 cells in serum-free media. Cells that had been cultured for a longer time period became detached from the tissue culture plate and were no longer viable (data not shown).

Chick Myoblast Differentiate Well Both in DMEM Containing 2% Horse Serum and in Serum-Free Medium

Chick myoblasts were able to differentiate in all media tested (fig. 6). Cells cultured in DMEM containing 10% fetal calf serum showed less than half the CPK activity of cells incubated in either of the differentiation media. Cells differentiated in DMEM showed the highest CPK activity after a 5-day incubation period. The cultures continued to be adherent for up to 10 days in all media (data not shown).

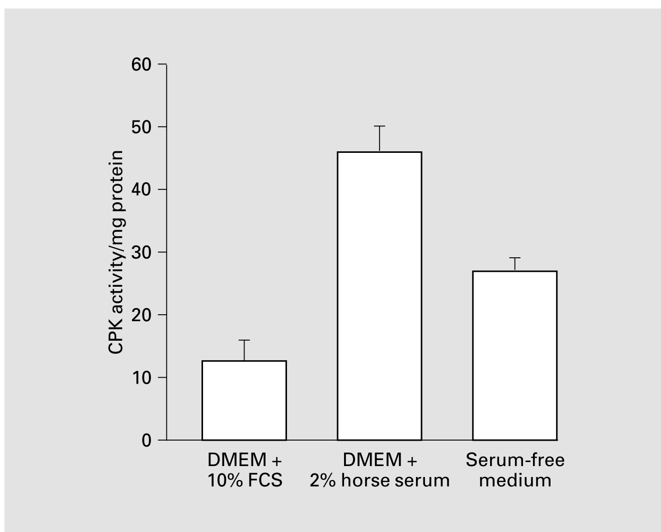

Fig. 6. CPK activity of chick myoblasts differentiated in the presence or absence of serum. Chick myoblasts were plated on tissue culture flasks, differentiated in the presence of either serum-free media or DMEM containing 2% horse serum, and assayed for CPK activity. Cells differentiated in both media showed significant amounts of CPK activity with those grown in 2% horse serum showing greater activity. The experiment was repeated on 3 separate days with different cultures, and the data is normalized against the protein concentration on a given day to allow for small differences in the number of cells in each culture.

Discussion

C2C12 and L6 cells both effectively differentiate when grown in medium containing low levels of horse serum. They form myotubes that successfully produce components of sarcomeric structures, and the myotubes generated are readily visually apparent. When the cells are grown in serum-free medium however, the L6 cells not only are unable to differentiate, but are nonadherent after 6 days in culture. Since many of the proteins in serum are not identified, it is very difficult to pinpoint the protein absent from serum-free media that is essential for L6 function. This diversity of response between two cell lines which are so often used as model systems to investigate muscle cell development is a concern. Transformed cell lines such as C2C12 have been shown to contain a heterogenous population of cells, most of which are deficient in the production of Myf-5 and Myo-D [Yoshida et al., 1998]. Both of these proteins are involved in the myoblast differentiation process. The cells deficient in these proteins do not go on to form myotubes in culture, but when subcloned, a small subset of these cells reverts back to Myf-5 and Myo-D-producing myoblasts. This behavior is worrisome, as this type of mixed population, with such a large portion of nondifferentiating cells, does not necessarily mimic in vivo conditions. The reduced myotube formation in L6 cells in the present study is probably due similarly to a loss of Myf and Myo-D production.

Choosing a model system for the study of myoblast development is a daunting task. Mammalian myoblasts are quite diverse in their reactions to environmental conditions during differentiation. Most mammalian cell lines require a signal that is generated when the cells are switched from a serum-rich environment to a serum-deprived one. This is not true of all myoblasts however. Chicken pectoralis myoblasts are able to differentiate successfully in serum-rich conditions [Yeoh and Holtzer, 1977], and the impetus to switch from a proliferative state to a differentiating state may well lie in a signal generated from cell-cell contact, not a chemical change in the environment. We investigated the effects of Aim-V medium on primary chick myoblast cultures, and found that these cells differentiated in both serum-rich and serum-free conditions, but the rate of myotube formation was significantly higher in both differentiation media tested. In the mouse and rat cell lines investigated in this study, few myotubes were formed when the cells were left in serum-rich media after reaching confluence. Therefore, transformed cell lines, such as those used in this study, would be poor model systems for understanding avian muscle development. There may also be significant variations in myoblast behavior in different muscles types in a single animal, which makes the choice of a model system even more difficult.

It has been previously shown that myoblasts grown on the basement membrane proteins laminin and collagen IV differentiate more readily than cells grown on other extracellular matrix protein or on tissue culture plastic alone [Thyberg and Hultgardh-Nilssen, 1994]. This has been confirmed in this study. What is interesting, however, is that differentiation of C2C12 cells in serum-free media is equally enhanced by growth on all of the extracellular matrix proteins tested. Even fibronectin, which has been previously shown to inhibit myoblast fusion, seems to enhance differentiation of the myoblasts readily in a serum-free environment. This can be due to a number of factors. There may well be a component of serum that in conjunction with cell adhesion to fibronectin, presumably through integrin proteins, signals cells to remain in the proliferative state. This signal could be reduced or eliminated in the absence of certain serum proteins, possibly growth factors. This possibility is currently being investigated. Another possibility is that components of

the serum-free media itself block integrin clustering and plaque formation or integrin-mediated cell signalling. Our preliminary work has shown, however, that integrin-containing adhesion sites on myoblasts grown on fibronectin appear identical in both size and number in the two medium types tested (data not shown). More work is currently underway in the laboratory to better understand this phenomenon.

C2C12 and L6 cells have both been used as model cell lines for the study of muscle development. Both cell lines have been isolated from rodent cell lines and are thought to be closely related in many aspects. As we have shown, however, these two cell lines can respond quite differently to experimental conditions. Chick myoblast cells do not respond in the same way to differentiation media as either of the model systems. It is not clear whether either response more closely resembles the majority of mammalian cells in vivo. As we are, in many cases, restricted to the study of cells in vitro, the careful consideration and testing of any model system is essential.

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