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Differentiation of mammalian skeletal muscle cells cultured on microcarrier beads in a rotating cell culture system

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Abstract—*The growth and repair of adult skeletal muscle are due in part to activation of muscle precursor cells, commonly known as satellite cells or myoblasts. These cells are responsive to a variety of environmental cues, including mechanical stimuli. The overall goal of the research is to examine the role of mechanical signalling mechanisms in muscle growth and plasticity through utilisation of cell culture systems where other potential signalling pathways (i.e. chemical and electrical stimuli) are controlled. To explore the effects of decreased mechanical loading on muscle differentiation, mammalian myoblasts are cultured in a bioreactor (rotating cell culture system), a model that has been utilised to simulate microgravity. C_2C_{12} murine myoblasts are cultured on microcarrier beads in a bioreactor and followed throughout differentiation as they form a network of multinucleated myotubes. In comparison with three-dimensional control cultures that consist of myoblasts cultured on microcarrier beads in teflon bags, myoblasts cultured in the bioreactor exhibit an attenuation in differentiation. This is demonstrated by reduced immunohistochemical staining for myogenin and α-actinin. Western analysis shows a decrease, in bioreactor cultures compared with control cultures, in levels of the contractile proteins myosin (47% decrease, $p<0.01$) and tropomyosin (63% decrease, $p<0.01$). Hydrodynamic measurements indicate that the decrease in differentiation may be due, at least in part, to fluid stresses acting on the myotubes. In addition, constraints on aggregate size imposed by the action of fluid forces in the bioreactor affect differentiation. These results may have implications for muscle growth and repair during spaceflight.*

Keywords—*Skeletal muscle, Myoblasts, Differentiation, Myogenin, Bioreactor, Simulated microgravity*

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1 Introduction

SKELETAL MUSCLE of adults is composed of multinucleated myofibres. Although these myofibres themselves have no regenerative capacity, a population of mononucleated muscle precursor cells, termed myoblasts or satellite cells, resides between the basal lamina and the sarcolemma of each fibre. These myoblasts, which represent reserve stem cells remaining following normal development of muscle during organ development, are normally quiescent. However, in response to injury or overload, they can migrate, proliferate and differentiate. They will align and fuse into multinucleated myotubes that express muscle specific proteins, such as those that comprise the sarcomeric contractile apparatus. They can fuse with existing fibres and thus contribute to muscle growth and repair.

Satellite cells are responsive to a number of environmental cues, including a variety of mitogens, the extracellular matrix and neural input (BISCHOFF, 1990; FLORINI *et al.*, 1991). In addition to these more prototypical signals, myoblasts are also sensitive to mechanical signals (ILIZAROV, 1989; WINCHESTER and GONYEA, 1992; KULESH *et al.*, 1994). The mechanical signals can originate from a variety of sources, as skeletal muscle tissue is exposed to internal loading from the contractile apparatus, external loading from the myotendinous attachments that is transmitted to the cytoskeleton, and loading that is the result of gravity. Alterations in loading from any of these sources can potentially result in alterations in satellite cell activation, proliferation and differentiation.

The goal of our research is to examine the role of loading in muscle growth and plasticity through utilisation of cell culture systems where other potential signalling pathways (i.e. chemical

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and electrical stimuli) are controlled. In the present study, we explored the effects of loading on muscle differentiation by culturing mammalian myoblasts in a culture model of simulated microgravity, the NASA bioreactor (WOLF and SCHWARZ, 1991; SCHWARZ *et al.*, 1992; WOLF and SCHWARZ, 1992).

We hypothesised that, owing to the microgravity and hydrodynamic environment in the bioreactor, myoblasts grown in the bioreactor would exhibit an alteration in differentiation compared with cells grown under either static or mixing conditions. Results showed that murine myoblasts cultured in the bioreactor on microcarrier beads exhibited reduced differentiation compared with those grown under control conditions. This was demonstrated qualitatively by reduced immunohistochemical staining for proteins specific to muscle differentiation, such as myogenin and α-actinin, and quantitatively by decreased immunoblot levels of the contractile proteins myosin and tropomyosin. Hydrodynamic measurements indicated that the decrease in differentiation may have been, at least in part, due to fluid stresses acting on the myotubes.

2 Materials and methods

2.1 *Methods overview*

Our interest was in investigating the effects of gravity loading, or lack thereof, on differentiation of skeletal muscle cells. We hypothesised that the environment provided by the NASA bioreactor would have effects on skeletal myocyte differentiation as determined by well-accepted protein markers. The advantages of this system include the fact that, if the rotational velocity is adjusted to balance the buoyant forces of the cells or the microcarrier beads upon which they are grown, then the cells remain suspended in a point in space in the laboratory frame of reference, thus negating the forces of gravity and approximating a microgravity environment. In such a system, cultures are three-dimensional and thus provide an environment for the study of tissue-relevant conditions.

There are disadvantages to this system for those studying cells that are dependent upon attachment to substrate for normally regulated growth, such as skeletal muscle cells and mature myofibres. Such cells must be grown on microcarrier beads or an equivalent substitute for study in the bioreactor. A suitable control condition must be designed to approximate all but the lack of gravity loading on the cultures. We chose to grow equivalent cultures on microcarrier beads in teflon bags under normal gravity conditions. These bags provide gas exchange equivalent to that provided in the bioreactor.

2.2 *Culture vessels and microcarriers*

The bioreactor, or rotating cell culture system (RCCS), was developed by NASA (WOLF and SCHWARZ, 1991; 1992; SCHWARZ *et al.*, 1992). It consists of a horizontally rotated cylinder designed to provide efficient mixing under conditions of low shear stress. The bioreactor utilised in the present study was a cylindrical culture vessel (STLV), with a 55 ml capacity*. Sterilisation and handling were similar to previously published methods (SCHWARZ *et al.*, 1992).

A central core in the vessel, of approximately 2 cm diameter, contains a filter for gas exchange. The radius of the outer cylinder is 3.2 cm and was rotated at speeds between 7–25 rpm to maintain the beads and attached cells in suspension. The Reynolds numbers under these conditions ($\mathrm{Re} = \Omega R^2 \rho / \mu$, where Ω is the rotational speed, R is the radius, ρ is the fluid density, and μ is the fluid viscosity) is between 882 and 3153. For a rotating outer cylinder, these conditions yield laminar flow (BIRD *et al.*, 1960). Teflon fluoropolymer 50 ml cell culture bags† were utilised as three-dimensional controls. These inert, transparent bags are gas permeable and temperature resistant. Like the bioreactor, they contain a pair of luer lock access ports to allow medium addition and removal.

Cells were grown on microcarrier beads‡. The beads are about 141–211 μm in diameter, with a density of 1.04 g ml^{-1}, and are coated with a thin layer of denatured collagen. Beads were hydrated and sterilised according to the manufacturer's guidelines and stored in sterile PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) at 4°C until use.

2.3 *Cell culture*

C_2C_{12} cells were obtained from the American Type Culture Collection (Rockville, Maryland). These myoblasts originated from normal adult C3H mouse leg muscle (YAFFE and SAXEL, 1977; BLAU *et al.*, 1985). Under low serum conditions, they readily differentiate into an extensive network of myotubes that express characteristic muscle proteins and spontaneously contract. Cells were grown in DMEM** supplemented with 0.5% chick embryo extract, 0.5% gentamicin** and 10% calf serum††.

2.4 *Experiments*

On the first day of an experiment, the vessels were filled with DMEM and equilibrated in an incubator (5% CO_2, 37°C, 100% humidity) for 1 h. Plates of proliferating myoblasts were trypsinised (0.05% trypsin, 0.53 mM EDTA**) and suspended in growth medium. The beads were also suspended in growth medium. Following equilibration, the bioreactor and bag were filled halfway with medium. The cells and beads were mixed together and then immediately divided between the bioreactor and teflon bag. The vessels were topped up with medium, and all bubbles were carefully removed. The final concentration was 4×10^5 beads and 4×10^6 cells per 50 ml medium. In initial experiments, a second teflon bag was maintained on a rocking platform in the incubator as a control for the mixing of the components. Preliminary results demonstrated no difference between the static and mixed conditions of the teflon bag cultures for any of the measured variables (data not shown), and thus all subsequent experiments were performed with the bag under static conditions. After the mixing of the cells and microcarrier beads in the appropriate proportions in either the bioreactor or culture bags, the cells were allowed passively to attach to the beads, and cultures continued.

Within 24 h, cells attached to the beads. Over subsequent days, aggregates of beads formed in both the bioreactor and the teflon bags. As the size of the aggregates grew, the rotational speed of the bioreactor was adjusted to maintain the velocity of the aggregate close to the velocity of fluid, to maintain the aggregates in suspension. Consequently, aggregate trajectories deviated slightly from those of the fluid, and the net force on the cells was minimised (TSAO *et al.*, 1992). The myoblasts were refed daily, and, when they were 80–90% confluent on the beads (approximately 72 h after seeding), the medium was shifted to DMEM supplemented with 10% horse serum‡‡ and 0.5% gentimicin to promote differentiation. Cultures in the teflon

*Synthecon, Inc., Houston, Texas, USA

†American Fluoroseal Corp., Columbia, Maryland, USA

‡Pharmacia Biotech Inc., Piscataway, New Jersey, USA

**Gibco BRL, Grand Island, New York, USA

††HyClone, Logan, Utah, USA

‡‡Intergen, Purchase, New York, USA

bags were treated equivalently. Cultures were maintained for an additional five ($n = 1$), seven ($n = 2$) or eight ($n = 1$) days so that myotube development could be followed.

2.5 *Cell harvest*

Cell aggregate samples were harvested at a number of time points throughout each experiment to verify cell viability and for immunohistochemistry. Aggregates from the bioreactor were sampled while the vessel was rotating, to ensure a representative sample. Aggregates were also taken from the teflon bag. Samples for staining were prepared according to previously detailed methods (TORGAN *et al.*, 1996). Briefly, samples were washed in ice-cold PBS, fixed in 2% formaldehyde and then permeabilised with 0.25% nonidet P-40 (NP-40). Cells were then stored in rinse solution (PBS with 2% horse serum and 0.1% NP-40) at 4 °C, until samples from all timepoints had been harvested, at which time they were simultaneously immunostained according to the procedures below.

At the termination of an experiment, an aliquot of cells was fixed for staining, and the remaining cells were processed for Western analysis. Cell aggregates were washed twice in cold PBS and then trypsinised for 5–10 min. Samples were vortexed briefly (5 s) to facilitate cell detachment from the beads, the beads were allowed to settle, and the supernatant was collected. This procedure was repeated twice, and the supernatants were pooled. Growth medium was added to inactivate the trypsin, and the sample was spun at 130 × g. The pellet was washed in PBS, spun and then resuspended. This was repeated, and the final cell pellet was stored at −80 °C until analysis. Preliminary studies demonstrated that cells treated in this manner would attach and proliferate if plated, confirming that they remained intact and viable (data not shown).

2.6 *Immunohistochemistry*

Samples were stained as previously described (TORGAN *et al.*, 1996). Cells were incubated with primary monoclonal antibodies against either the Z band protein α-actinin (Sigma; dilution 1 : 800), or the myogenic determination factor, myogenin (F5D*; used as the supernatant). Cells were then sequentially incubated with biotinylated anti-mouse IgG secondary antibody, reagent avidin-biotin complex (ABC), and VIP peroxidase substrate†.

2.7 *Westerns*

Frozen cell pellets were resuspended in lysis buffer (0.6% NP-40, 0.15 M NaCl, 10 mM Tris pH 7.9, 1 mM EDTA, 0.4 mM PMSF, and 0.1 mg ml^{-1} of pepstatin, antipain and leupeptin), incubated for 15 min on ice and then spun at ≈ 20 × g to pellet cellular debris. Protein concentration was determined on the supernatants by a commercially available kit‡ that is based on the Lowry method (LOWRY *et al.*, 1951) and is detergent compatible. Ten μg of protein from each sample were solubilised in Laemmli buffer (LAEMMLI, 1970) and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide). All samples were run in duplicate, and molecular weight standards were included to verify the size of the bands. Samples were transferred to polyvinylidene difluoride (PVDF) membranes**, using a semi-dry transfer unit†† with a discontinuous buffer system, according to the manufacturer's directions.

Following transfer, the membranes were blocked overnight in 5% non-fat dry milk‡‡ in TTBS (0.1% Tween-20, 100 mM Tris, 0.9% NaCl). The PVDF sheets were then cut in half, and the top portion (MW 80–200 kDa) was incubated with an antibody against sarcomeric myosin (MF-20, diluted 1 : 50), and the lower portion (MW 0–80 kDa) was incubated with an antibody against striated muscle tropomyosin (CH1, used as the supernatant; both antibodies from the DSHB), in TTBS with 0.1% BSA for 1 h. The blots were then washed, incubated with biotinylated anti-mouse IgG secondary antibody, diluted 1 : 10 in TTBS with 0.1% BSA for 1 h at 37 °C, washed and then incubated with ABC reagent for 30 min. Following extensive washing, the blots were incubated in ECL substrate*, and exposed to film (for myosin, X-OMAT AR†; for tropomyosin, Hyperfilm MP*) so that bands could be visualised. Exposures were timed to permit sufficient signals for analysis but not to be long enough to saturate the film. A series of protein concentrations, ranging from 2–20 μg, were additionally measured to ensure that all resultant bands were within the linear range of detection (data not shown). Bands were analysed with a laser densitometer and quantification software‡.

2.8 *Statistical analysis*

As indicated cultures were maintained for five ($n = 1$), seven ($n = 2$) or eight ($n = 1$) days of differentiation so that myotube development could be followed. The effect of growth upon the various conditions (bioreactor and teflon bag) was analysed by pooling of the four time points ($n = 4$). For the quantitative data obtained from the Western analyses, unpaired t-tests were utilised to detect differences in tropomyosin and myosin protein levels between the control bag and bioreactor. A level of $p < 0.01$ was set for significance, and reported values are expressed as mean ± S.E.

2.9 *Determination of aggregate dimensions and settling velocity*

To characterise the fluid dynamics and shear forces acting on aggregates, we measured aggregate size and settling velocity. Samples were removed from the bioreactor, and digital images were captured at 40 fold magnification. The area of the aggregate was determined by tracing**. The aggregates were elliptical, with aspect ratios between 0.60 and 0.80. An equivalent radius R_{eq} was determined for an ellipsoid of revolution moving parallel to the axis of symmetry (HAPPEL and BRENNER, 1986). Settling velocity was determined by measuring the time required for aggregates to travel 2.54 cm in the tissue culture medium. From the aggregate radius and settling velocity v_s, the relative aggregate density $\rho_a - \rho$ was determined by performing a force balance on the cell (BIRD *et al.*, 1960):

$$\rho_a - \rho = \frac{9\mu v_s K}{2R_a^2 g} \tag{1a}$$

where K accounts for deviations from Stokes law, μ is the fluid viscosity, and g is the gravitational constant. Reynolds numbers

*Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA

†All from Vector Laboratories, Burlingame, California, USA

‡BioRad, Hercules, California, USA

**Millipore, Bedford, Massachusetts, USA

††LKB, Pharmacia

‡‡Nestlé, Glendale, California, USA

*Amersham, Arlington Heights, Illinois, USA

†Kodak, Rochester, New York, USA

‡Hoeffer Scientific, San Francisco, California, USA

**NIH Image software version 1.51

were in the order of 1 or smaller, and the following expression was used for K (DEEN, 1998):

$$K = 1 + \tfrac{3}{8}\mathrm{Re} + \tfrac{9}{40}\mathrm{Re}^2(\ln \mathrm{Re} + 0.8352) + \tfrac{27}{40}\mathrm{Re}^3 \ln \mathrm{Re}) \quad (1b)$$

3 Results and discussion

3.1 *Qualitative measures of differentiation*

Almost all the myoblasts (>95%) adhered to the microcarrier beads within 24 h of seeding in both the bioreactor and bag, based upon examination of the medium. Over the next 48 h the myoblasts proliferated, so that, by 72 h, the beads were almost completely covered. At this point, the medium was shifted to one that would promote differentiation, and the cells were followed for an additional 5–8 days. Samples were harvested at a number of time points throughout differentiation and were immunostained for myogenin and α-actinin.

Myogenin is a member of the family of myogenic determination factors that can convert non-muscle cells to a myogenic phenotype (FUNK *et al.*, 1991). This muscle-specific transcription factor is not expressed in quiescent satellite cells, but is observed with the onset of differentiation, prior to myoblast fusion into myotubes (SMITH II *et al.*, 1994). In the present study, nuclei of myoblasts stained positive for myogenin following two days of exposure to differentiation medium (Fig. 1) and prior to myotube formation. Although samples from both the bag and bioreactor contained aggregates of beads that were completely covered with myoblasts, aggregates from the bioreactor exhibited fewer nuclei that stained positive for myogenin. This qualitative assessment implies that fewer cells in the bioreactor had initiated the differentiation program.

To detect myotube formation, cultures were immunostained for α-actinin, a major component of the Z band of the sarcomere. The Z band anchors and organises the thin filaments and mechanically transmits the contractile forces from one sarcomere to the next along the myotube (VIGOREAUX, 1994). Alpha-actinin is not present in mononucleated myoblasts, but appears at the onset of myoblast fusion (ENDO and MASAKI, 1984).

As expected, staining for α-actinin was negative in all samples after two days of exposure to differentiation medium (when myogenin was present, but before myotubes had formed; data not shown). Following five days of differentiation, a network of myotubes was evident in both the bioreactor and bag samples (see Fig. 2). In many cases, the myotubes spanned more than one bead. Aggregates from the teflon bag tended to exhibit an extensive network of myotubes, whereas the samples from the bioreactor typically had most of the myotubes in the centre of the aggregate, as visualised by the staining.

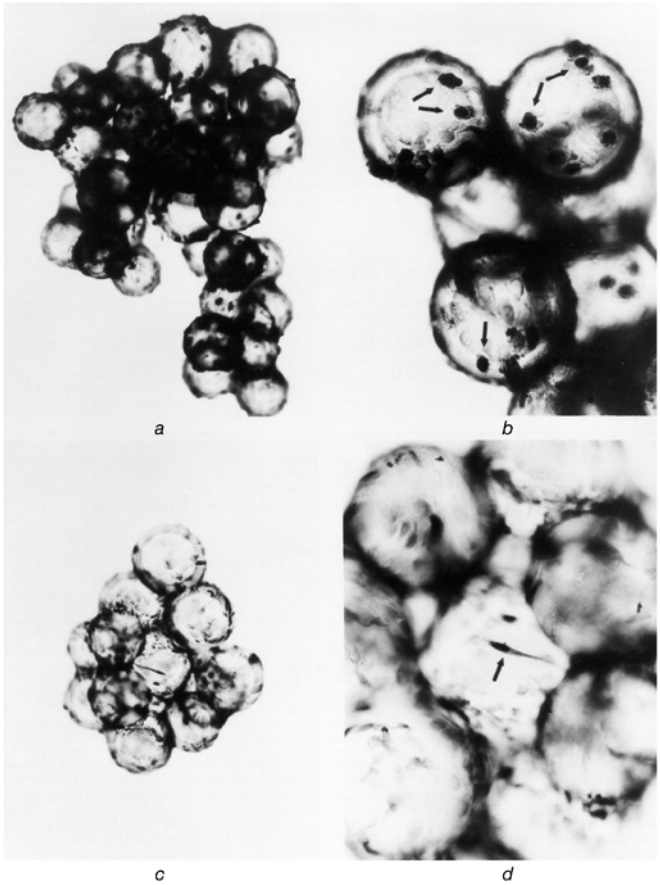

Fig. 1 *Immunohistochemical staining for myogenin following 2 days of differentiation. (a, b) Bead cluster harvested from teflon control bag. Myoblasts are almost completely confluent on beads. Staining for myogenic regulatory protein, myogenin, is detected in nuclei of random cells (arrows). (c, d) Bead cluster harvested from bioreactor. Like sample from bag, myoblasts are almost completely confluent on beads. Myogenin staining is only detected in a few cell nuclei. Higher magnification reveals cell that is positive for myogenin, which has elongated (arrow). Magnification: (a, c): ×100; (b, d): ×250*

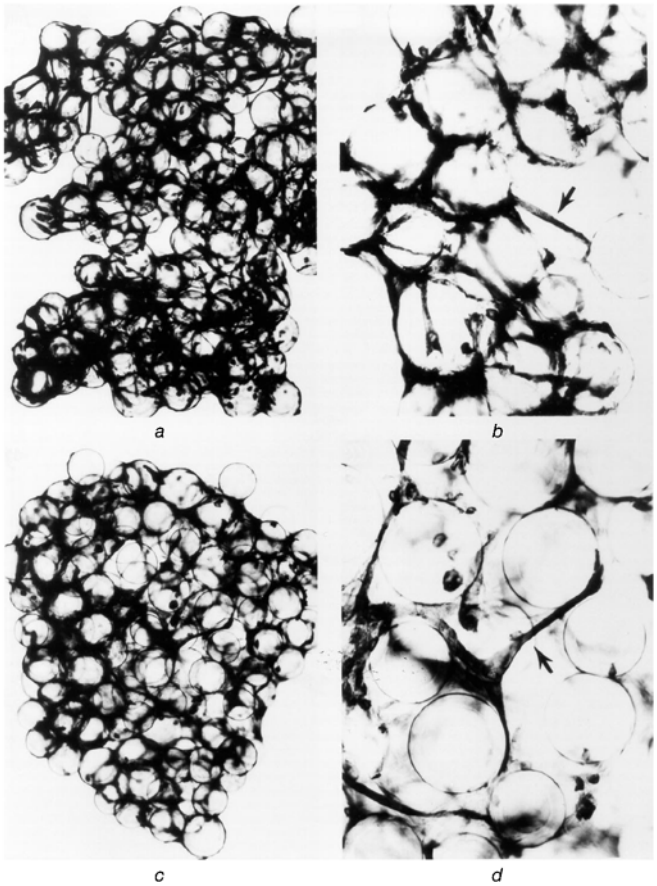

Fig. 2 *Immunohistochemical staining for α-actinin following 5 days of exposure to differentiation medium. (a, b) Portion of bead cluster harvested from teflon control bag. Staining reveals myotube development, as this protein is located at Z band of sarcomeres. Myotubes often span more than one bead. Higher magnification illustrates myotube branched across space between two beads (arrow). (c, d) Bead cluster harvested from bioreactor. Higher magnification shows that, like culture from bag, stained myotubes often span more than one bead (arrow). Magnification: (a, c): ×68; (b, d): ×170*

3.2 *Quantitative measures of differentiation*

To assess whether quantitative differences existed in the extent of differentiation between cells cultured in the bioreactor and in the control bag, protein samples obtained following 5–8 days of differentiation were immunoblotted for the contractile proteins tropomyosin and myosin. Tropomyosin is a thin filament protein that plays a role in calcium regulation of contraction. It was detected by an antibody that recognises all striated muscle forms, including both the skeletal α and β chains, which are detected as separate bands (LIN *et al.*, 1985). Myosin makes up the thick filament of the contractile apparatus and is composed of a hexamer that consists of two heavy chains and four light chains. It was detected by an antibody that recognises all sarcomeric heavy chain isoforms (BADER *et al.*, 1982).

Ten μg of total protein from each sample were assayed in duplicate. The data from the various time points were analysed as one (see Section 2.8). There were no apparent differences in protein yield from the control or experimental samples. Following densitometric assessment, the values for the experimental samples were expressed relative to the parallel control samples.

As shown in Fig. 3, protein levels of both myosin and tropomyosin were significantly reduced in cells cultured in the bioreactor, compared with in the bag. In particular, levels of myosin decreased by 47%, whereas levels of tropomyosin were reduced by 63%. Analysis of the relative amounts of the α and β subunits of tropomyosin revealed that, on average, the α subunit formed 80% of the total protein (see insert in Fig. 3). This is in good agreement with previous work that found that, in differentiating myoblasts, the α subunit is synthesised prior to the β subunit (MONTARRAS *et al.*, 1982).

The attenuation in differentiation in the bioreactor cultures, as demonstrated by decreased myosin and tropomyosin immunoblot levels and reduced immunostaining for α-actinin and myogenin, may be due to a number of factors. It has previously been shown that rat satellite cells cultured in a bioreactor exhibit a decrease in proliferation compared with cells grown in two-dimensional culture (MOLNAR *et al.*, 1997). Thus, in the present study, cells in the bioreactor may have been less confluent at the time that differentiation was initiated, which may in turn have led to a lag in myotube development.

This implies that, over time, myotube development in the bioreactor should have reached levels seen in the control bag. Typically, in our hands, C_2C_{12} myoblasts grown on standard plates differentiate into vast networks of contracting myotubes after only 3–5 days of exposure to differentiation medium, with very little fusion occurring subsequently (data not shown). After 8 days of differentiation, however, protein levels of tropomyosin and myosin from the bioreactor samples remained below those from the teflon bag.

The decrease in myosin and tropomyosin expression may have been due to a decrease in the total number of myotubes, or to a decrease in the amount of each specific protein per myotube. Immunostaining data for α-actinin suggest the former, as a decrease in the total number of myotubes was visually evident. To determine whether differences in the extent of myotube formation between the bioreactor and bag cultures could be due to hydrodynamic influences, aggregate size and settling velocity were monitored.

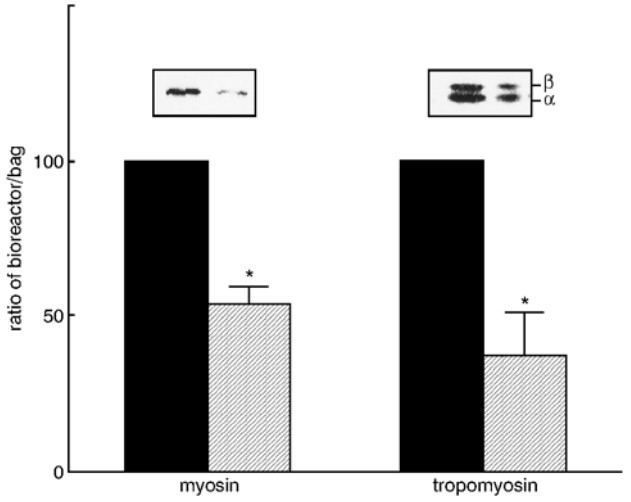

Fig. 3 *Myosin and tropomyosin protein levels of myotubes determined using Western blotting. Ten μg of total protein were analysed in duplicate. Protein levels detected in (▨) bioreactor cultures were expressed relative to those of (■) parallel control teflon bag cultures. Values are mean ± S.E.* * $p < 0.01$. *Inset: Representative autoradiographs for myosin and tropomyosin (control bag sample on left, and bioreactor sample on right of each panel). Both α and β subunit bands of tropomyosin are evident*

3.3 *Aggregate size and sedimentation velocity*

Measurements made on aggregates sampled from the bioreactor showed that the average radius of the aggregate increased with time of culture (Fig. 4*a*). Prior to the shifting to differentiation medium, the aggregates were small and involved only a few beads (Fig. 5). Aggregate size increased during the first 72 h after the shift to differentiation medium and then stabilised. Consistent with the increase in size of the aggregates, the aggregate sedimentation velocity increased with time in the bioreactor (Fig. 4*a*). Interestingly, the effective density of the aggregates decreased with time (Fig. 4*b*). This decrease in density could represent a looser structure that formed in the growing aggregate.

The change in size of the aggregates was accompanied by changes in the distribution of cells throughout the aggregate. Initially, all individual beads and the small aggregates were covered with cells (see Fig. 1). As the aggregates increased in size, the number of cells spanning beads appeared to increase. Beyond 72 h of exposure to differentiation medium, fewer cells were observed on the outer beads of the aggregates, and the myotubes appeared to concentrate in the inner region of fibres spanning the beads (see Figs 2*c* and 5*c*). These changes in the structure of the aggregates are consistent with the decreased effective density during continued culture in the bioreactor. In contrast, cells growing in the cell culture bags rapidly formed large aggregates that coalesced into sheets of cells and beads after only a few days in culture (see Fig. 2*a*).

3.4 *Estimation of fluid shear stresses acting upon myoblasts and myotubes in the bioreactor*

The difference between the behaviours of cells in the bioreactor and in the teflon cell culture bags may be due to fluid forces present in the bioreactor. These forces may prevent aggregates from growing and/or directly affect the function of the myoblasts. To address these questions, we analysed the stresses and forces acting on the aggregates, assuming that aggregate motion was in a quasi-steady state. To evaluate this assumption, we calculated the time constant t_c as v_s/g. The time constant ranged from 0.24 ms to 0.90 ms. For the rotational speed studied (7 rpm), these time constants corresponded to angular movements ranging from 0.012° to 0.047°. These small motions indicate that the aggregates rapidly reached a quasi-steady state.

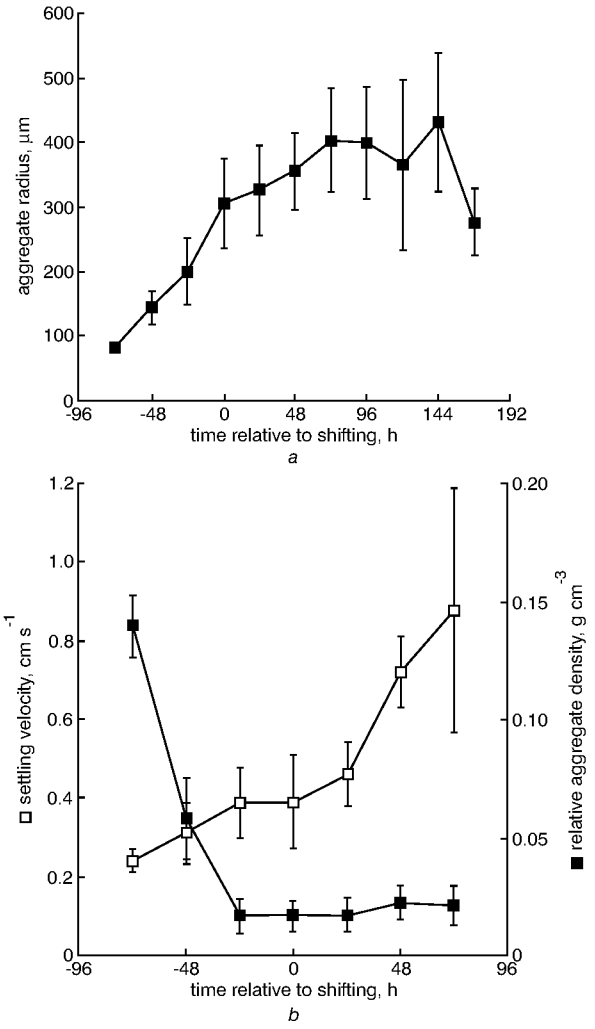

Fig. 4 *(a) Aggregate radius against time of culture, relative to point of shifting to differentiation medium. (b) Settling velocity (left axis) and relative aggregate density (right axis), expressed relative to point of shifting to differentiation medium. Settling velocity and aggregate radius were directly measured, and aggregate density was calculated (see text for details)*

A schematic diagram of the bioreactor is shown in Fig. 6. Steady-state force balances are in the r and θ directions. Forces acting on the aggregates include Stokes drag, gravity, buoyancy and centrifugal forces. Assuming a quasi-steady state, these force balances yield

r direction :

$$0 = -6\pi\mu R_a(v_{ra} - u) - (\rho_a - \rho)g\frac{4\pi R_a^3}{3}\cos\theta + \frac{(\rho_a - R)}{r}\frac{4\pi R_a^3}{3}v_{\theta a}^2 \quad (2)$$

θ direction :

$$0 = -6\pi\mu r_a(v_{\theta a} - v) - (\rho_a - \rho)g\frac{4\pi R_a^3}{3}\sin\theta \quad (3)$$

where v_{ra} and $v_{\theta a}$ are the aggregate velocities in the r and θ directions; u and v are the aggregate velocities in the r and θ directions; R_a is the aggregate radius; μ is the fluid viscosity; and ρ_a and ρ are the densities of the aggregate and fluid, respectively. There was no perfusion of the fluid, and fluid motion was treated as a solid body rotation with a component in the θ direction only.

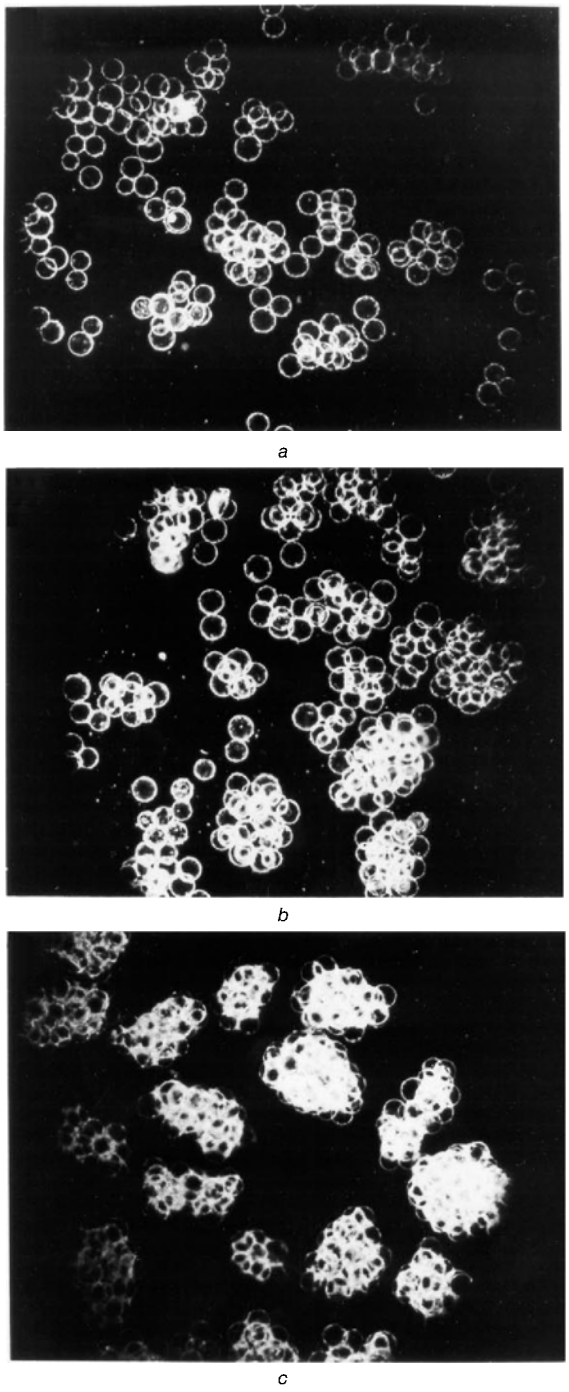

Fig. 5 *Microcarrier bead aggregates harvested from bioreactor. Samples drawn from rotating bioreactor (a) 48 h after seeding, (b) 72 h after seeding, or (c) following 3 days of exposure to differentiation medium. Increase in aggregate size over time is observable. Magnification ×26*

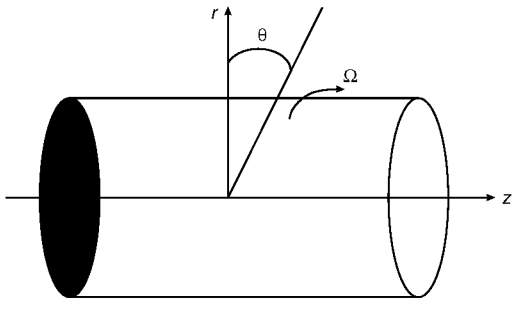

Fig. 6 *Schematic diagram of bioreactor with radial (r), angular (θ) and axial (z) co-ordinates. Bioreactor rotates in angular direction with speed Ω*

The resulting aggregate velocity components relative to the fluid velocity are

$$r \text{ direction}: \quad v_{ra} = -v_s \cos(\omega t) + \frac{v_s}{gr}(v - v_s \sin(\omega t))^2 \quad (4)$$

$$\theta \text{ direction}: \quad v_{\theta a} = -v_s \sin(\omega t) \quad (5)$$

where v_s is the settling velocity

$$v_s = \frac{2g(\rho_a - \rho)R_a^2}{9\mu} \quad (6)$$

The angular velocity component is (BIRD *et al.*, 1960)

$$v = \Omega R\left(\frac{\kappa R/r - r/\kappa R}{\kappa - 1/\kappa}\right) \quad (7)$$

where κ is the ratio of the inner to the outer radius.

The term $v_s/gr(v - v_s \sin(\omega t))^2$ is generally much smaller than $v_s \cos(\omega t)$ and is neglected. The root mean square velocities in the radial and angular directions are each $v_s/\sqrt{2}$, and the net velocity is v_s. The root mean square shear stress acting on an aggregate equals the ratio of the force divided by the surface area (BIRD *et al.*, 1960). Using the equivalent radius for the ellipsoid, the surface area is that of an equivalent sphere, and the root mean shear stress is

$$\tau_{rms} = \frac{3\mu v_s}{2\sqrt{2}R} \quad (8)$$

Using values for the sedimentation velocity and aggregate radius, the shear stress acting on the aggregates ranges from 0.199 to 0.311 dyne cm^{-2}.

At a given rotational speed, some aggregates will be smaller and some larger than the mean size. Nevertheless, their velocities relative to the local fluid velocity will be proportional to the settling velocities, and the shear stresses will be given by eqn 8. The reason for this is that only the net motion of the aggregates relative to the fluid will generate a shear stress. The largest aggregates (550 μm) will experience a shear stress of 0.23 dyne cm^{-2}, and the smallest aggregates will experience a shear stress of 0.50 dyne cm^{-2}. These are less than the suggested upper limits of 0.51 dyne cm^{-2}, which may affect cell function in the bioreactor (TSAO *et al.*, 1992). These values are also much smaller than the value of 8 dyne cm^{-2} known to alter endothelial cell morphology (DAVIES, 1995). Nevertheless, these shear stresses may produce changes in second messenger production that may affect cell function (DAVIES, 1995; SMALT *et al.*, 1997).

This hydrodynamic analysis was extended to examine the stresses acting on a myotube bridging two microcarrier beads. For this case, the radius was approximated as the fibre radius, 20 μm. Under these conditions, the shear stress acting on the fibre is about 1.0 dyne cm^{-2}. Asymmetric rotation of the aggregate with the new attachment may place additional stresses on the fibre. These higher stresses may prevent the fibre from adhering to another microcarrier bead and thus limit the growth of the aggregate. Growth of the fibres within the aggregate would shield the cells.

Based upon this hydrodynamic analysis, we propose that the primary effect of fluid stresses is to prevent the growth of the aggregates. The large stresses acting on individual fibres spanning microcarrier beads prevent the growth of the aggregate such that the size remains small. This geometric constraint causes the fibres to grow in a tight network away from the aggregate surface. In contrast, the low shear stresses acting on cells or fibres on the aggregate surface probably are not high enough to alter cell function.

In conclusion, we observed an attenuation in differentiation in skeletal myoblasts cultured in a bioreactor. In line with this, it has been shown that subconfluent myoblast cultures, which were flown on a space shuttle mission for 7–10 days, were unable to fuse and differentiate into myotubes in follow-up cultures on earth (KULESH *et al.*, 1994). Clearly, further studies are needed to elucidate the effects of mechanical forces, including microgravity, that influence skeletal muscle differentiation and adaptation and repair.

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