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SKELETAL MUSCLE SATELLITE CELLS CULTURED IN SIMULATED MICROGRAVITY

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SUMMARY

Satellite cells are postnatal myoblasts responsible for providing additional nuclei to growing or regenerating muscle cells. Satellite cells retain the capacity to proliferate and differentiate *in vitro* and, therefore, provide a useful model to study postnatal muscle development. Most culture systems used to study postnatal muscle development are limited by the two-dimensional (2-D) confines of the culture dish. Limiting proliferation and differentiation of satellite cells in 2-D could potentially limit cell-cell contacts important for developing the level of organization in skeletal muscle obtained *in vivo*. Culturing satellite cells on microcarrier beads suspended in the High-Aspect-Ratio-Vessel (HARV) designed by NASA provides a low shear, three-dimensional (3-D) environment to study muscle development. Primary cultures established from anterior tibialis muscles of growing rats (~ 200 gm) were used for all studies and were composed of greater than 75% satellite cells. Different inoculation densities did not affect the proliferative potential of satellite cells in the HARV. Plating efficiency, proliferation, and glucose utilization were compared between 2-D culture and 3-D HARV culture. Plating efficiency (cells attached ÷ cells plated × 100) was similar between the two culture systems. Proliferation was reduced in HARV cultures and this reduction was apparent for both satellite cells and nonsatellite cells. Furthermore, reduction in proliferation within the HARV could not be attributed to reduced substrate availability because glucose levels in medium from HARV and 2-D cell culture were similar. Morphologically, microcarrier beads within the HARV were joined together by cells into 3-D aggregates composed of greater than 10 beads/aggregate. Aggregation of beads did not occur in the absence of cells. Myotubes were often seen on individual beads or spanning the surface of two beads. In summary, proliferation and differentiation of satellite cells on microcarrier beads within the HARV bioreactor results in a 3-D level of organization that could provide a more suitable model to study postnatal muscle development than is currently available with standard culture methods.

Key words: satellite cells; postnatal myogenesis; microcarrier beads; HARV bioreactor.

INTRODUCTION

Satellite cells are postnatal myogenic stem cells that provide additional nuclei to growing and regenerating muscle cells/myofibers (5). Satellite cell postnatal myogenesis involves proliferation, differentiation, and maturation (2,10,17). Satellite cell proliferation results in daughter nuclei that can be incorporated into the myofiber or used to increase the population of satellite cells (11). Differentiation results in the satellite cell fusing with the myofiber and synthesizing muscle-specific proteins that provide for growth of the myofiber (18) and biochemically defined properties associated with muscle contraction (13). Maturation involves a progressive change in the pattern of contractile protein isoforms synthesized by satellite cell-derived myonuclei to a pattern more characteristic of the adult myofiber.

Although the blueprint for satellite cells to proliferate, differentiate, and form contraction-competent myotubes is retained when cultured *in vitro*, the expression of contractile protein isoforms is restricted to an embryonic/fetal pattern (4). Also, myofibers often contract themselves off the dish or degenerate within 20 d of culture (9) and myofibers generated in two-dimensions (2-D) do not morphologically resemble the parallel alignment of myofibers and the placement of connective tissue characteristic of *in vivo* muscle. Advanced stages of maturation have been obtained using modified culture dishes that allowed embryonic muscle cells and fibroblasts to remain in culture for up to 40 d (14). Long-term (2 mo.) cultivation of embryonic muscle cells in three dimensions (3-D) has been demonstrated using microcarrier beads (12). These studies suggest that 3-D organization of skeletal muscle can be achieved *in vitro* and may provide a more realistic model to represent the *in vivo* state.

Spaceflights of short duration and limb immobilization both produce atrophy of weight-bearing muscles (15). We have developed a comprehensive research program to study muscle atrophy and regeneration. One aspect of this program is using the High-Aspect-Ratio-Vessel (HARV) to facilitate growth and differentiation of satellite cells in 3-D. Removing the restriction of satellite cell growth and differentiation in 2-D could result in muscle tissue that more closely resembles their *in vivo* counterpart and thus provide a better model to study muscle atrophy and regeneration. A description of the

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TABLE 1

COMPARISON OF MICROCARRIERS

Specifications	CYTODEX-3	BIOSILON
Bead composition	Cross-linked dextran	Polystyrene
Density	1.04 g/cc	1.05 g/cc
Diameter	133–215 microns	160–300 microns
Surface area	4600 cm^2/gm dry weight	255 cm^2/gm
# beads/g dry weight	4.0×10^6	2.41×10^5
Surface layer	Gelatin (type I collagen)	Nunclon △

HARV bioreactor and the conditions provided by it are detailed in a companion manuscript (1). In this study, we use the HARV bioreactor to facilitate growth and differentiation of satellite cells in 3-D and to characterize satellite cell proliferation.

Materials and Methods

Matrices for cell culture. A number of microcarrier systems are commercially available for growth of anchorage-dependent cells within a suspension system. Table 1 provides information concerning two microcarrier bead types (Cytodex 3, Sigma Chemical Co., St. Louis, MO and Biosilon, Nunc, Roskilde, Denmark) that we have used successfully to grow primary cultures of satellite cells. Our previous studies with 2D cultures have made use of Nunc tissue culture dishes; therefore, studies presented in this paper used Nunclon Biosilon beads. Both culture plates and beads were coated with a 1:100 dilution (in basal medium) of the membrane extract Matrigel™ (Collaborative Research, Bedford, MA) by incubating the plastic surfaces with 2.5 ml/55 cm^2 of surface area for 30 min at 37°C. Beads were gently mixed to allow uniform contact with Matrigel. Prior to addition of cells, culture plates and beads were washed twice in Dulbecco's Modified Eagles' medium (DMEM) containing 20% fetal bovine serum (FBS). Treatment of microcarrier beads with lower dilutions of Matrigel (1:50 or lower) resulted in serious clumping of beads within the HARV.

Methodology for culturing in HARV. Culture of satellite cells on microcarrier beads in the HARV bioreactor provides a low shear environment not obtained when using conventional spinner flasks to suspend microcarriers (8). By design, loading of cells, feeding, and sampling is carried out through two syringe ports built into the front face of the HARV. During feeding or sampling, it is important that both syringe ports (with syringes attached) are open so that positive or negative pressure within the HARV does not develop and damage the internal oxygenation membrane. For example, loading of a 10-ml HARV is done using two 15-cc syringes. The plunger is removed from one syringe and this syringe is attached to one sampling port and another syringe with plunger is attached to the other sampling port. Beads, cells, and medium (added in this order to insure that beads and cells are rinsed through by media and not left in the sampling port) are individually added to the plungerless syringe and each component is drawn through by pulling up on the plunger of the adjacent syringe. After the vessel is filled, it is critical to remove all entrapped air. Following attachment to the base, movement of microcarrier beads relative to medium is of paramount importance. To minimize shear, rotation of the HARV must be adjusted such that beads and fluid rotate as a solid body. For 10 ml HARVs, we have determined that about 30 rpm approximates solid body rotation.

Criteria for evaluating satellite cell proliferation in 3-D culture. For this study, criteria used to compare growth of satellite cells in the HARV bioreactor versus flat culture plates included: (a) plating efficiency; (b) proliferation; (c) characterization of proliferating cell types; (d) glucose utilization rates; and (e) morphological examination using scanning electron microscopy. For all experiments, satellite cells were isolated from anterior tibialis muscle of male Sprague Dawley rats (200 gm) exactly as described (6). All experiments used DMEM containing 20% FBS. The percentage of satellite cells was determined for each isolation by desmin staining using monoclonal antibody D3 reported to be specific for satellite cells (3). The D3 hybridoma was obtained from the Developmental Studies Hybridoma Bank maintained by the department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD), and the Department of Biological Sciences, University of Iowa (Iowa City, IA), under contract N01-HD-6-2915 from the NICHD. The percentage of satellite cells was determined microscopically, as the ratio of desmin positive cells to the total number of cells. Primary isolates consisted of greater than 75% satellite cells. For plating efficiency (cells attached ÷ cells plated × 100), 18 000 cells/cm^2/0.18 ml medium were plated in Nunc tissue culture dishes and Nunclon Biosilon microcarrier beads within the HARV. At 16 h a sample of beads was removed from each HARV, placed into an 0.75 ml Eppendorff tube, washed with two to three changes of phosphate-buffered saline (PBS) to remove nonadherent cells, and suspended in 0.5 ml of 0.25% trypsin. Culture plates were rinsed twice with PBS, and 1 ml of 0.25% trypsin was added to detach cells. Following detachment, cells were counted using a hemacytometer. For HARV samples, it was also necessary to calculate total number of beads so that the amount of surface area could be calculated. This was accomplished by removing 2–15 μl aliquots from each 0.5 ml HARV sample and microscopic counting of beads in each aliquot using a 2.5× objective. For proliferation studies, cells were plated at 18 000 cells/cm^2 in DMEM containing 20% FBS. Cells were enumerated (as described above) from three 10-ml HARVs and three 8-cm^2 culture plates at 16, 48, 72, 96, and 120 h. Desmin staining was used to distinguish between cell types during proliferation. For each time period, cells were detached from the surface of cell culture plates and microcarrier beads using 0.25% trypsin. Cells were enumerated using a hemacytometer and plated at equivalent densities onto Matrigel-coated culture plates. Cultures were fixed 24 h later and the percentage of desmin positive cells determined by cell counting. Glucose concentration was determined using a Trinder assay (Sigma Chemical Co., St. Louis, MO) kit on medium samples (48, 72, 96, and 120 h) 24 h after feeding fresh medium.

Results of Culturing Satellite Cells in HARV Bioreactor

A characteristic of microcarrier cell culture is the ability to vary the ratio of surface area and total number of cells per volume of medium. Because proliferation of satellite cells is known to be affected by medium conditioning, and the bioavailability of growth factors to cells within the HARV was unknown, it was essential to determine if a difference in satellite cell proliferation occurred when HARVs were inoculated with three different levels (0.5×10^6, 1.0×10^6, and 2.0×10^6) of cells (Fig. 1). These levels were chosen to approximate previously reported inoculation densities (7). These data indicate that neither changing the total number of cells within the HARV nor increasing the total surface area and associated Matrigel substratum, which is known to contain low levels of growth factors, yielded statistically different rates of cell proliferation.

Plating efficiency is a function of a cell's ability to properly orient relative to the culture substratum and interact with the substratum through focal contacts. Using equivalent cell number/surface area/volume ratios, the efficiency by which satellite cells attached to microcarrier beads in the HARV bioreactor relative to flat cultures was determined (Fig. 2). In addition, the fraction of myogenic (satellite cells) versus nonmyogenic cells was determined by desmin staining. The data indicate that total cells and satellite cells attach to Matrigel-coated microcarrier beads suspended in the HARV with the same efficiency as cells plated onto flat culture dishes. Cells remaining unattached after 18 h failed to attach when replated for an additional 24 h on culture dishes (data not shown).

The proliferative potential of satellite cells was compared between flat cultures and HARV cultures. Satellite cells (1.0×10^6 per HARV) were inoculated at a ratio of 20 cells/microcarrier bead. A similar number of cells/cm^2 surface area/medium volume was established in 2-D cultures. The number of cells/mm^2 was determined at 16, 48, 72, 96, and 120 h for HARV and flat cultures (Fig. 3*A*). Fig. 3*B* illustrates the proliferation data generated in Fig. 3*A* when myogenic and nonmyogenic cell types were individually analyzed. These data suggest that cell proliferation was reduced for myogenic and nonmyogenic cell types cultured in the HARV relative to flat

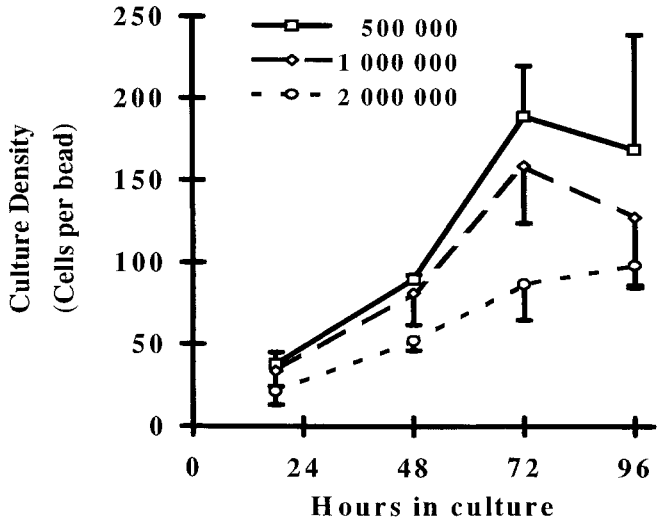

FIG. 1. Effect of cell number and surface area on cell proliferation. Three 10-ml High-Aspect-Ratio Vessels were inoculated with different levels of cells (0.5×10^6, 1.0×10^6, and 2.0×10^6 per 10 ml HARV) and proportionate numbers of beads (total surface area of 27.5 cm^2, 55 cm^2, and 110 cm^2) such that the ratio of cells/bead was maintained at 20:1. The number of cells/bead was determined at the indicated times by microscopic counting. Each data point represents the mean ± the standard error for three separate experiments (one replicate/experiment). No statistical differences between inoculation densities were found at any time point.

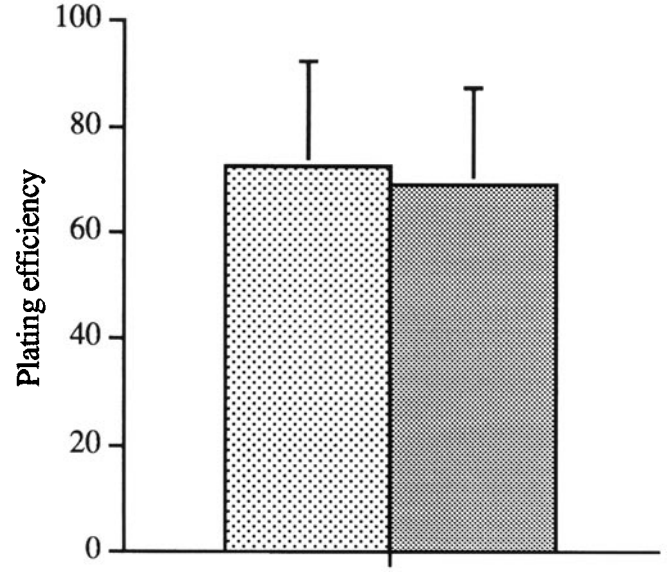

FIG. 2. Plating efficiency (cells attached ÷ cells plated × 100) of satellite cells in two-dimensional (2-D) culture dishes and microcarrier beads suspended in the High-Aspect-Ratio- Vessel (HARV) bioreactor was determined 18 h after initial plating. Data is presented as the mean ± the standard error for three experiments (three replicates/experiment).

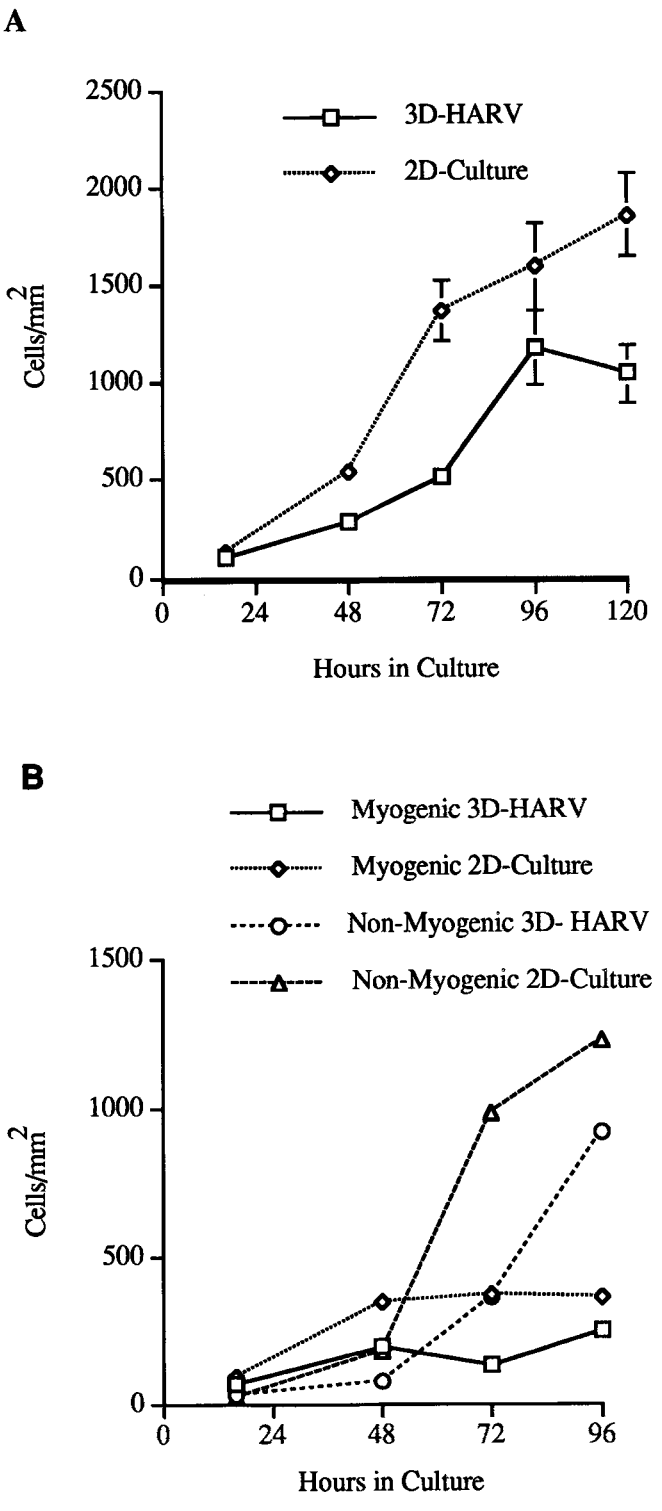

FIG. 3. *A*, Proliferation of cells was compared in two-dimensional (2-D) culture dishes and microcarrier beads suspended in the High-Aspect-Ratio-Vessel (HARV) bioreactor. *B*, Desmin staining was used to distinguish between myogenic and nonmyogenic cell types during proliferation. For each time period, cells were liberated from the surface of cell culture plates or microcarrier beads. Cells were counted and plated at equivalent densities onto Matrigel-coated culture plates. Cultures were fixed 24 h later and the percentage of desmin- positive cells determined by cell counting.

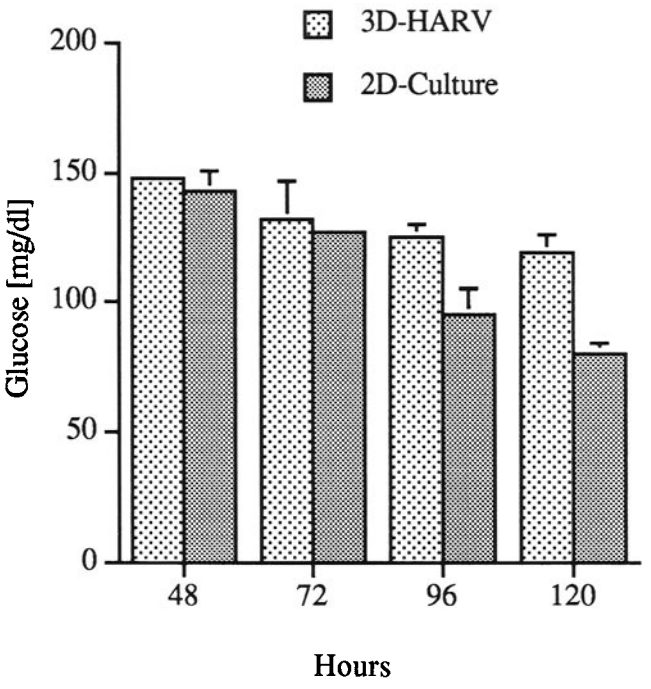

FIG. 4. Glucose levels (mg/dl) in 10 μl of media from High- Aspect-Ratio-Vessel (HARV) and static cell cultures were determined at times indicated. Mean values ± standard error from two experiments (two replicates/experiment) are presented.

culture. Reduced proliferation of cells within the HARV bioreactor did not result from reduced nutrient (glucose) levels compared to standard culture (Fig. 4). Finally, reduction in proliferation of satellite cells between 48 and 72 h coincides with the onset of myotube formation in flat cultures (Fig. 3*B* and 5).

DISCUSSION AND FUTURE PROSPECTS

In this study, satellite cells were collected from growing rats, and cultured within the HARV bioreactor. Unlike conventional spinner flasks, the HARV bioreactor provides a low shear, 3-D environment for the suspension of cells attached to microcarrier beads. Our goal is to utilize the advantage of the quiescent environment provided by the HARV bioreactor to study postnatal myogenesis in the absence of constraints put forth by 2-D culture dishes. Towards this end, we have demonstrated that primary cultures of rat satellite cells can proliferate, differentiate into myotubes, and form 3-D aggregates within the HARV bioreactor.

The majority of data presented here have dealt with the characterization of satellite cell proliferation within the HARV bioreactor. Proliferation was compared between satellite cells cultured in the HARV and satellite cells cultured on 2-D culture dishes. Proliferation of satellite cells within the HARV was not statistically affected by increasing either the number of cells and the total surface area within the HARV. These results suggest that the microenvironment surrounding individual cells attached to a microcarrier bead, which includes nutrient exchange, nutrient availability, waste material buildup, cell-to-cell contact, and media conditioning, was not affected by changing the total number of cells and the available surface area (microcarriers) within a 10-ml HARV. However, a trend toward lower proliferation rates at higher inoculation densities was noted and could be a result of the decreased surface area available due to aggregation of the beads (10–30 beads per aggregate, Fig. 5). We are continuing to evaluate satellite cell proliferation in the HARV. Our future experimental goal is to characterize the role of insulin-like growth factor I, fibroblast growth factor, and transforming growth factor β on satellite cell proliferation in 3-D.

Satellite cells require attachment to substratum prior to proliferation. For 2-D cell culture, cells settle to the bottom of the culture dish under the force of gravity and must contact the substratum. This is not true in the HARV. In addition, microscopic observation of stained preparations of cells attached to microcarrier beads after 18 h of culture showed that the number of cells per bead was relatively uniform. Using desmin immunostaining to distinguish satellite cells from nonsatellite cells, no difference in the efficiency of attachment for either cell type was evident in 2-D culture versus the HARV bioreactor. With demonstrated equivalent plating efficiencies, equivalent inoculation densities can be used in subsequent studies when comparing proliferation of satellite cells between 2-D cell culture and the HARV.

Proliferation of satellite cells and nonsatellite cells was delayed in the HARV bioreactor relative to 2-D culture plates. Cell proliferation is primarily controlled by growth factor levels in the medium (16). Because we demonstrated that proliferation is not statistically affected by different inoculation densities within the HARV, it seems unlikely that levels of growth factors or substrates in the culture medium were limiting, or could account for decreased proliferation of cells in the HARV. This contention is supported by the finding that similar glucose levels were found for both HARV and flat cultures.

Although the levels of factors/nutrients may not differ between HARV and 2D culture, the ability to respond to, or be presented with, the growth factors may differ between cells in flat culture versus cells within the HARV. Alternatively, differences in proliferation may reflect that cells suspended on microcarrier beads within the HARV would be exposed to more shear, relative to cells in flat cultures. Decreased proliferation for some cell types in the presence of measured shear has been reported (8).

Formation of cellular aggregates on microcarrier beads adds a layer of complexity to cells cultured within the HARV. As early as 48 h, aggregates of beads bridged together by cells were forming. Thus, a logical question is whether cells actively involved in the formation of 3-D aggregates are able to proliferate at the same rate as cells on the surface of microcarriers not involved in aggregate formation. If not, the population of cells capable of proliferating would be decreased in the HARV due to their participation in aggregate formation.

In summary, we have demonstrated that satellite cells can proliferate and differentiate on microcarrier beads suspended in the HARV bioreactor. It is our desire to use the HARV to facilitate the formation of 3-D skeletal muscle from mononucleated satellite cells. The criteria for success using the HARV bioreactor will be how closely muscle tissue derived from satellite cells in the HARV mimics the situation *in vivo*.

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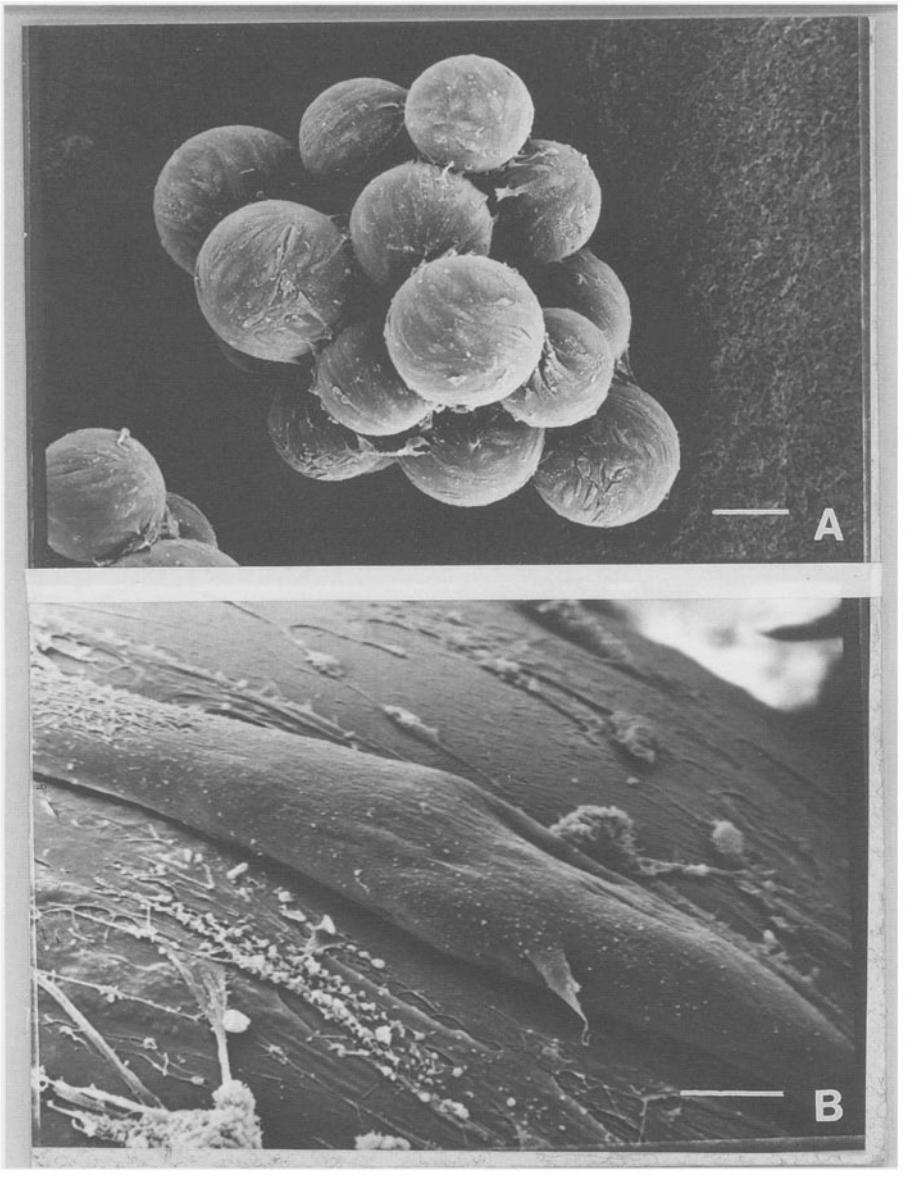

FIG. 5. Scanning electron micrographs showing 3-dimensional (3-D) aggregation of microcarrier beads with cells on surface (*A*, *bar* = 100 μm) and satellite cell-derived myotube spanning the surface of a single bead (*B*, *bar* = 10 μm).

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