Title
Effects of mechanical strain on L6J1 rat skeletal myoblasts

Author
Frial, Glenn P.

Publication Date
1996

Peer reviewed|Thesis/dissertation

EFFECTS OF MECHANICAL STRAIN ON L6J1 RAT SKELETAL MYOBLASTS

by

GLENN P. FRIAL

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF ARTS

in

ORAL BIOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

. . . . . .

Date University Librarian

Degree Conferred: . . . . . .

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Arthur Miller, for his dedication and perseverance. He was not only an excellent mentor, but he was also an excellent friend. If not for his dedication, this thesis would not have been possible.

I would also like to thank my committee members, Dr. Sunil Kapila and Dr. Stephen Rosenthal, for their help and input which proved to be invaluable. I also express my gratitude to Dr. Kapila for the use of his laboratory facility.

Thanks also to Ron Joseph for helping me in the initial stages. He was instrumental in getting the project "off the ground".

Finally, I express my love and gratitude to my wife, Carmela. She has always given me love and support.

EFFECTS OF MECHANICAL STRAIN ON L6J1 RAT SKELETAL MYOBLASTS

Glenn P. Frial, D.D.S.

ABSTRACT

Mechanical forces play an important role in modulating the growth of skeletal muscle. Moreover, it has now been well established that muscle development is subject to control by serum growth factors. However, it is not fully understood how physical forces are perceived by cells and how mechanical signals are transduced into the biological alterations associated with mechanically induced cell growth.

The effect of cyclic mechanical strain on subclones from rat thigh skeletal muscle L6J1 cells were assessed. Specifically we examined the effects of cyclic mechanical strain on the proliferation and differentiation of myoblasts as well as the potential role of PDGF-BB in these events. Relatively pure cultures of myoblasts were grown on silicone elastomer plates and were subjected to cyclic strain (6 cycles/min) by application of a vacuum under the plates. 1) We determined if cyclic mechanical stimulation increases the number of cells. Proliferation was determined by visually counting the cells on the microscope using a hemacytometer. 2) We determined the effect of stretch on differentiation. Differentiation was determined by identifying cell morphology after staining with Giemsa. 3) We determined if the conditioned medium supporting stretched cells modifies the cell growth of quiescent, unstretched myoblasts. Strain may induce the production of a growth factor that affects cell proliferation and differentiation. One potential growth factor, platelet-derived growth factor (PDGF), was specifically studied. Western blots of medium conditioned by cells subjected to strain were examined to determine the presence of PDGF-BB. The role of PDGF-BB was examined by blocking its effects with a polyclonal antibody to the BB form of PDGF.

The results demonstrate that cyclic mechanical strain promotes proliferation but not differentiation of skeletal myoblasts. Conditioned medium from stretched myoblasts increased cell proliferation greater than medium from unstretched cells. Western blots indicate that the cells secreted a PDGF-like mitogen. A polyclonal antibody to the BB form of PDGF reduced the mitogenic response to mechanical strain. These studies implicate that the mechanism of strain-induced growth appears to at least partly involve the intermediary action of secreted PDGF-BB. Our results provide the first demonstration that L6J1 rat skeletal myoblasts secrete a PDGF-BB-like protein.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Background

Muscle Development

Throughout embryogenesis of skeletal muscle, progenitor cells proliferate and eventually give rise to fusion-capable myoblasts which are single-nucleated cells. These myoblasts first proliferate then enter the process of myogenesis during fetal development. Myogenesis is the differentiation of proliferating skeletal myoblasts to form contractile, multinucleated myotubes. This process involves withdrawal from the cell cycle and eventual fusion of such cells into multinucleated myotubes that synthesize contractile proteins typical for skeletal muscle. This process occurs mainly at the late fetal stage in the developing rat. Thereafter, muscle growth occurs mainly from enlargement of preexisting myotubes (Jin et al., 1990). Yaffe (1971) showed that myoblasts from newborn rat skeletal muscle tissue must undergo biochemical changes *in vitro* before fusing into multinucleated cells. His experiments also demonstrated that whatever the relation between cell replication and differentiation might be, the number of cell divisions preceding the fusion of a myoblast *in vitro* is not a fixed one and can easily be varied experimentally. Myoblast proliferation and fusion into myofibers can still occur in the adult following injury or other more subtle stresses such as stretch, exercise, denervation, or mild compression (White and Esser, 1989).

An important question related to myogenic cell lineages is how proliferation and differentiation are regulated within a myoblast cell lineage so that there is amplification of the committed cell population. Proliferation of myoblasts is dependent on the ambient nutrient conditions in the cell culture (Yaffe, 1973). The primary components influencing proliferation appear to be serum and embryo extract elements such as insulin-like growth factors (IGF) and/or platelet-derived growth factor (PDGF) and other specific growth-promoting proteins such as fibroblast growth factor (FGF) (Linkhart et al. 1980, 1981). The mechanisms by which cells committed to myogenesis either initiate cell fusion and the

synthesis of muscle-specific cell products or continue to proliferate is dependent upon medium components (Konigsberg, 1971; Nadal-Ginard, 1978).

Implicit in nearly all studies on the mechanisms of differentiation of embryonic skeletal muscle cells (particularly those performed in cell culture) has been the assumption that there is a single lineage of myogenic cells in the developing embryo. However, it is no longer sufficient to view muscle tissue as homogeneous in composition at either the biochemical, structural, or cellular level (Pearson and Epstein, 1982). It is possible that the heterogeneity, in part, is based upon cells committed to different developmental lineages. Not much evidence exists that supports multiple lineages of myoblasts. Most evidence favors a single lineage with diversification being imposed, not at the myoblast level as required by a multiple-lineage model, but most often after the formation of a muscle fiber (Gauthier et al., 1978) . However, there is morphological evidence of diversification at the myoblast level. Evidence that cell interactions can change the diversity of myoblast lineages exists (Bischoff and Holtzer, 1967). Environmental factors, in particular mitogens, playing a role in the growth of such committed myogenic cell populations has not been investigated extensively. It is far from clear that there is any basis for the assumption that the diverse cell types found in skeletal muscle emerge from separate myoblast lineages rather than the modulation of differentiated muscle fibers induced by cell interactions (Pearson and Epstein, 1982).

Hauschka et al. (1977) showed experimental evidence that supports the possibility of commitment of mesenchymal cells to more than a single morphological myogenic cell lineage. These workers have cloned myoblasts from limb buds of both early human and avian fetuses. There appears to be two basic types of myoblasts within the developing limb of these animals. One group, the "early" type of myoblast, requires conditioned medium for differentiation, whereas the "late" type of myoblast does not. Both types of myoblasts differentiate into myotubes (muscle fibers), which differ in morphology. The type that requires conditioned medium for differentiation appears short and has very few nuclei. The

other type that differentiates in unconditioned medium appears large and branched and contains many nuclei (Rutz and Hauschka, 1975; Pearson and Epstein, 1982; Seed and Hauschka, 1984). There is no evidence that these two distinctive myoblast populations differentiate into distinctive muscle fiber types *in vivo*. It also is not known whether the early type is a precursor to the late type.

Studies of avian, rodent, and human myoblasts indicates that developing muscles consist of multiple myogenic populations (Rutz and Hauschka, 1975; Stockdale, 1992; Stockdale and Miller, 1987). In the beginning, this was primarily based on the observations that myoblasts from earlier and later stages of development exhibit different characteristics in culture. For example, the media requirements for their growth and the morphology of myotubes they form are different (Rutz and Hauschka, 1975; Seed and Hauschka, 1984, 1988; White et al., 1975). Based on their developmental stage and embryogenic phase, the early and late myoblasts were named by Stockdale and colleagues as embryonic and fetal myoblasts, respectively. In the chick limb, embryonic myoblasts are most abundant on day 5, while fetal myoblasts are most abundant between days 8 and 12 (Stockdale, 1992; Stockdale and Miller, 1987). It has been suggested that early myoblasts are not likely to be the precursors of late myoblasts (Seed and Hauschka, 1984).

Studies on chick/quail grafts indicate that cells in the satellite cell position in postnatal muscle are derived from the somites similar to myoblasts of the developing muscle (Armand et al., 1983). However, cultured satellite cells isolated from postnatal muscle have different properties than cultured fetal myoblasts that occupy the muscle during development, prior to the emergence of adult myoblasts. Experiments on mammalian myoblasts have shown differences between fetal and adult myoblasts in their sensitivity to a tumor promoter, in expression of acetylcholine receptors, and in the form of acetylcholine esterase expressed (Cossu et al., 1983, 1985, 1987; Senni et al., 1987). Cultured satellite cells express desmin as cycling cells more frequently, fuse into myotubes later, and express more binding sites for PDGF than fetal myoblasts (Yablonka-Reuveni

and Nameroff, 1990; Yablonka-Reuveni et al., 1987, 1990). It is suggested from many of these studies that myogenic precursors with the characteristics of adult myoblasts become dominant in late stages of embryonic development. It is not clear whether adult myoblasts or precursors for adult myoblasts, as in the case of embryonic and fetal myoblasts, are destined as such during early embryogenesis or whether the distinctions of the adult cells are acquired when development progresses. The understanding of the mechanisms governing the regulation of satellite cells in postembryonic life, or the appearance of adult myogenic precursors as a distinct myogenic population in late development, is just in its beginning. Yablonka-Reuveni (1995) suggests that satellite cells represent a unique myogenic population that becomes dominant in late stages of embryogenesis.

The L6 cell line was chosen for this study because it was originally derived from normal skeletal muscle of newborn rats. A cell line was chosen instead of primary cells since primary cultures contain fibroblast-like cells which are capable of binding PDGF and can add to an already complex environment (Yablonka-Reuveni et al., 1988). The rat L6 myoblast line established by Yaffe (Yaffe, 1968) is one of the most widely used muscle cell types. The L6J1 cell line is a subclone of Yaffe's L6 rat myoblasts (Ringertz et al., 1978). These cells grow readily under standard tissue culture conditions, and they are relatively resistant to serum deprivation, yet retain most of the hormone and growth factor responses typical of primary rat myoblasts (Jin et al., 1991; Florini, 1987). The L6 muscle cell line of day-old rat muscle origin expresses many biochemical, morphological, and metabolic characteristics of mature skeletal muscle (Yaffe, 1968, 1969). Nevertheless, the behavior of the L6 cells *in vitro* may not entirely reproduce the way the skeletal muscle cells behave *in vivo*.

Mechanical Stimulation

Mechanical forces play an important role in modulating the growth of a number of different tissues including skeletal muscle, smooth muscle, cardiac muscle, bone,

endothelium, epithelium, and lung (Vandenburgh, 1990). The increasing interest at the molecular level by which mechanical forces are transduced into growth changes has produced strong interest in developing model systems to study these processes in tissue culture.

Skeletal muscle arrangement is regulated by many factors such as nutrition, hormones, electrical activity, and tension development (Vandenburgh et al., 1991). Muscle cells are exposed to both passive and active mechanical forces at all developmental stages. These forces play very important roles but they are poorly understood in regulating muscle organogenesis and growth. For example, the skeleton grows rapidly during embryogenesis and places large passive mechanical forces on the attached muscle tissue.

For the most part, the mechanism by which passive or active mechanical strain are transformed into morphological, biochemical, and molecular alterations in skeletal muscle are unknown. Although active tension induced by muscle contraction shortens the sarcomeres while passive tension induced by muscle stretching lengthens the sarcomeres their effects on muscle metabolism and growth are similar (Vandenburgh, 1987). The mechanical transduction mechanisms involving passive or active mechanical stress on muscle are probably not identical, but a better understanding of the underlying mechanism in one is likely to assist in the understanding of the process in the other.

Cyclic strain has been found to increase cell number and DNA synthesis in cultured bone, epithelial cells, endothelial cells, and smooth muscle cells (Hasegawa et al., 1985; Brunette et al., 1984; Sumpio et al., 1987; Wilson et al., 1993). In fact, the response of bone cells to physical stimulation is dependent on the periodicity or frequency of the applied stimulus. Osteogenic cellular synthesis of bone proteins appears to be activated more by intermittent rather than continuous strain (Carvalho et al., 1994). Moreover, frequency appears to be as important to remodeling as the range of stress applied to the bone.

In the body, cells are constantly subjected to tension and compression. Stressing cells in culture simulates the *in vivo* environment and can cause dramatic morphologic changes and biochemical responses in the cells. Both long- and short-term changes can occur when cells are stressed in culture, such as alterations in the rate and amount of protein synthesis, the rate of cell division and alignment, changes in energy metabolism, changes in rates of macromolecular synthesis or degradation and other changes in biochemistry and bioenergetics.

Platelet-Derived Growth Factor

It has now been well established that myogenesis is subject to control by serum growth factors (Jin et al., 1991). The most extensively studied factors are FGF (Olwin and Hauschka, 1988), IGF (Ewton and Florini, 1981), and transforming growth factor-β (TGF-β) (Massague et al., 1986). FGF increases myoblasts proliferation and inhibits muscle differentiation. TGF-β is a potent inhibitor of muscle differentiation, but shows no mitogenic effects. IGFs stimulate both myoblast proliferation and differentiation (for review see Florini and Magri, 1989).

PDGF is a major mitogen and a potent chemoattractant for many mesenchymal cell types maintained in culture such as fibroblasts, smooth muscle cells, and glial cells (Ross et al., 1986; Westermark and Sorg, 1993). PDGF, a dimeric cationic protein with a molecular weight of about 30 kDa, assembles by disulfide bonds from two distinct but highly homologous polypeptide chains (PDGF-A and PDGF-B). All three possible homo- and heterodimer combinations of PDGF (AA, AB, BB) have been isolated from natural sources (Yablonka-Reuveni, 1995). It is now believed that there are two PDGF receptor subunits, each with a molecular weight of about 170-180 kDa: The α-subunit (PDGFRα), which can bind both PDGF A-chain and B-chain, and the β-subunit (PDGFRβ), which can bind only PDGF B-chain (Seifert et al., 1989; Westermark and Sorg, 1993). These receptor subunits dimerize noncovalently in response to PDGF to form three distinct forms

of high-affinity binding sites for the dimeric PDGF ligand (αα, αβ, ββ). PDGF-AA binds only to the αα receptor, PDGF-AB binds to both the αα and αβ receptors (with much lower affinity to the ββ receptor), and PDGF-BB binds to all three receptor subunit combinations (Seifert et al., 1989, 1993). The binding of PDGF to its receptor activates a cascade of signal transduction events that leads to DNA synthesis (Westermark and Sorg, 1993, Williams, 1989). From these studies, many investigators have proposed roles for PDGF and its receptors in various physiological, pathological, and developmental processes (Ross et al., 1986; Westermark and Sorg, 1993; Wolswijk and Noble, 1992).

PDGF has been shown to be an important factor in wound healing and tissue repair (Ross et al., 1986), but its role during skeletal muscle development and regeneration has not been fully investigated. Although PDGF was initially isolated from platelets and megakaryocytes, it is now well established that other cell types can produce this growth factor (reviewed in Ross et al., 1986). The B chain of PDGF is encoded by the protooncogene c-sis, the precursor of the transforming gene of simian sarcoma virus (SSV), v-sis (Waterfield et al., 1983; Chiu et al., 1984). SSV-infected cells produce a PDGF-like mitogen. Several human tumor cell lines can express either or both of the PDGF-genes, produce PDGF-like proteins, and promote their own growth in an autocrine or paracrine manner (Heldin and Westermark, 1984; Ross et al., 1986). Conceivably, autocrine or paracrine secretion of mitogens may be important also during normal growth and development and in other pathophysiological situations. The c-sis gene has been found to be expressed throughout mouse embryonic development (Slamon et al., 1984). Endothelial cells (DiCorleto et al., 1983) and activated macrophages (Shimokado et al., 1985) express c-sis and produce PDGF-like proteins, however, they lack PDGF receptors. The role of the released factors may in these cases be to stimulate neighboring cells, for example, during wound healing. Smooth muscle cells have also been found to produce PDGF-like mitogens (Wilson et al., 1993; Sejersen et al., 1986).

Sejersen et al. (1986) suggest that rat skeletal myoblasts express the gene for the A chain of PDGF. However, no signs of expression of the B-chain gene were detected. Jin et al. (1990) demonstrated that the genes coding for the PDGF A-chain and the PDGF β-receptor are expressed in rat myoblasts, and that PDGF-BB is a partial mitogen for L6J1 rat myoblasts. Furthermore, Jin et al. (1991) provided the first demonstration that PDGF-BB is potent regulator of myogenesis of L6 rat myoblasts. They found that treatment of L6J1 myoblasts with PDGF-BB increased the rate of DNA synthesis and stimulated cell proliferation. In differentiation medium (Dulbecco's modified Eagle's medium/0.5% FCS), they found PDGF-BB prevented fusion of confluent myoblasts.

Objectives and Specific Aims

Specific Aim 1

Evaluate the effects of mechanical stimulation on cell proliferation and differentiation of skeletal myoblasts. Cell proliferation was determined by visually counting the cells on the microscope using a hemacytometer. Differentiation was determined by identifying cell morphology after staining with Giemsa.

Hypothesis 1

Cyclic mechanical strain increases cell proliferation of myoblasts but decreases differentiation to skeletal myotubes.

Specific Aim 2

Evaluate the effects of conditioned media from mechanically strained myoblasts on quiescent skeletal myoblasts. Quiescent cells were incubated with conditioned media from non-stretched cells and mechanically stimulated cells for 48 h and 96 h.

Hypothesis 2

The conditioned media from stretched cells increases cell proliferation more than media from unstretched cells.

Specific Aim 3

Identify if PDGF is present in conditioned media of mechanically stretched myoblasts and characterize the role of PDGF on cell proliferation and differentiation of mechanically stimulated skeletal muscle cells. A Western blot was used to determine whether the cells subjected to strain secreted PDGF, using a polyclonal antibody to the BB form of PDGF. Cells were subjected to strain and incubated for 48 h and 96 h with a neutralizing antibody to PDGF. Cell proliferation and differentiation were assessed to characterize the role of PDGF.

Hypothesis 3

Cyclic mechanical strain affects cell proliferation and differentiation of rat L6 myoblasts via autocrine and/or paracrine action of PDGF.

Significance

As interest increases in the molecular mechanisms by which mechanical forces are transduced into growth alterations, model systems are being developed to study these processes in tissue culture. These model systems should prove pivotal in the coming decade for understanding how physical forces are perceived by cells and how mechanical signals are transduced into the biochemical and molecular alterations associated with mechanically induced cell growth. A model of autocrine and paracrine growth regulation in L6 rat myoblasts is presented.

MATERIALS AND METHODS

Materials

All materials were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. PDGF antibodies for the Western blots are polyclonal rabbit anti-human PDGF-BB purchased from Genzyme Corp. (Cambridge, MA). Secondary

antibodies are biotinylated anti-rabbit (Amersham Corp., Arlington Hts., IL). Neutralizing antibodies for PDGF were polyclonal rabbit anti-human PDGF-BB obtained from Genzyme Corp. α-Minimal essential medium (α-MEM), fetal bovine serum and horse serum were obtained from Gibco (Life Technologies, Inc. Grand Island, NY). The protein assay kit was obtained from BioRad Laboratories (Hercules, CA).

Experiments

Basically seven experiments were performed (Fig. 1). The first experiments involved changing the serum concentration. It was important to determine the effect of low serum on the cells since all experiments were done in 1% serum to minimize the amount of proteins, growth factors, and hormones in the media. The next set of experiments was to determine the effect of mechanical stimulation. Cell proliferation and cell differentiation were measured. Once an effect had been determined, we wanted to evaluate if mechanical stimulation of myoblasts resulted in altered levels of protein expression so total protein secretion was measured. If a protein was secreted, the next step was to determine if the protein was a growth factor. If a growth factor was found then we wanted to identify which growth factor or growth factors were produced. We specifically studied PDGF-BB. If PDGF-BB was found to be produced, we wanted to characterize its role on cell proliferation and differentiation of mechanically strained myoblasts by blocking its effects with a neutralizing antibody specific to PDGF-BB.

Cell Culture

The L6J1 cell line is a subclone of Yaffe's (1968; Ringertz et al., 1978) L6 rat myoblasts from the thigh muscle. The L6J1 cells were purchased from American Cell Line at passage 5. Cells were grown in α-MEM supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37°C in a water-saturated atmosphere consisting of 95% air, 5% CO_2. Culture medium was changed two or three times per week. Cells were

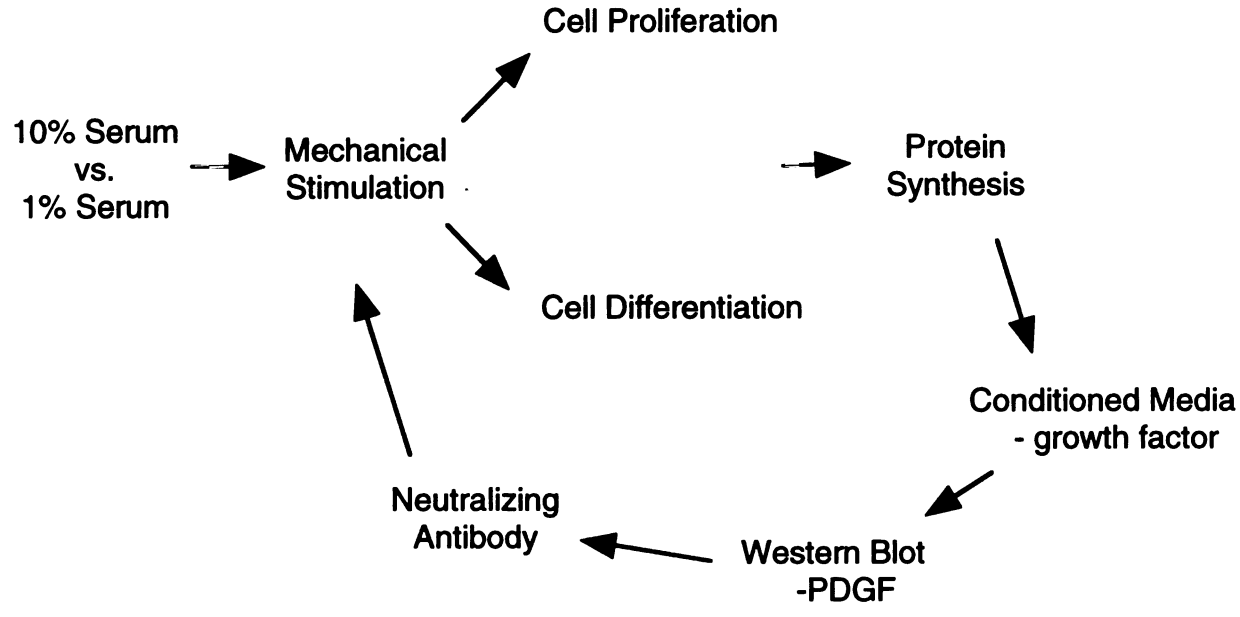

Figure 1. Flow chart demonstrating the experiments performed and the interrelationship between the experiments.

subcultured with trypsin 0.25%-versene 0.02%. Cells from passages 5-10 were used for the current studies. To initiate differentiation, medium was removed and replaced with a differentiation medium (α-MEM containing 1% horse serum with 10 μg/ml bovine insulin, Sigma).

10% Serum vs. 1% Serum

Since the serum content in medium affects myoblast differentiation (Nadal-Ginard, 1978) we first examined the effects of serum concentration on myoblast differentiation by culturing L6J1 cells in 1% and 10% serum. This study was designed to determine optimal media conditions for subsequent experiments. Cells were plated at a density of 2.0×10^4 cells/ml in 35 mm petri dishes. They were grown over 7 days in 10% and 1% serum solutions. Cultures were washed with PBS, fixed with methanol, stained with Giemsa, and mounted with Canada Balsam. The percentage of myoblasts (number of myoblasts/total number of cells) and percentage of myotubes (number of myotubes/total number of cells) were determined with a microscope at 20x. Five areas of the petri dishes were measured (the center and the four corners).

Application of Cyclic Strain to Cultured Cells

We next examined the effects of cyclic mechanical strain on skeletal myoblasts. In this study, the Flexercell Strain Unit (FX-2000) was used. It was developed to provide a mechanically active environment to cells cultured *in vitro*. Cells are grown on silicone elastomer culture plates that are covalently bonded with collagen, elastin, fibronectin, or other attachment proteins. A microprocessor controls negative pressure to the flexible bottoms of the plates resulting in a reproducible deformation of the rubber and the attached cells. A pattern of high intensity (20%) stretch/relaxation at intervals of 6 cycles/min was chosen to simulate functional demands that might be placed on neonatal muscle fibers *in vivo*.

After confluence, myoblasts were trypsinized, collected by centrifugation, washed, and plated in six-well fibronectin-coated silicone elastomer-bottomed culture plates at 3.0 x 10^4 to 5.0 x 10^4 cells/ml (Flex culture plates, Flexcell Corp., McKeesport, PA). The wells are 25mm in diameter. After 24 h incubation in 1% serum to allow for adherence to the plates and to minimize the effects from hormones and growth factors present in the serum, the media was changed to fresh 1% serum. The cells were then subjected to mechanical deformation with the Flexercell Stress Unit (Flexcell Corp.) and placed in a humidified incubator with 5% CO_2 at 37°C. The stress unit is a modification of the unit initially described by Banes et al., (1985, 1989) and consists of a computer-controlled vacuum unit and a baseplate to hold the culture dishes. The stretching regimen consisted of 20% maximal elongation (vacuum at 20 kPa) at 6 cycles per min, with 5 seconds of strain followed by 5 seconds of relaxation. The computer controls the frequency of deformation and the negative pressure applied to the culture plates. Control cells were plated on flexible bottom plates but were not subjected to mechanical stretch.

Cell Proliferation

For analysis of cell proliferation, myoblasts were plated at 3.0 x 10^4 cells per well in 6-well plates. The cells were incubated for 24 h in medium of α-MEM/1% horse serum to allow for adherence to the plates. After 24 h, fresh 1% serum was placed and the myoblasts were stretched as described above. The cells were counted using a hemocytometer. Cell counts were taken at different time points (24, 48, 72, 96 and 120 h). Strained and unstrained wells for both cell populations were washed two or three times in PBS and incubated with trypsin for 10 minutes at 37°C. After the 10 minutes, α-MEM/10% FBS was added to stop the trypsin reaction. Forty-five µl of cell suspension were placed on a hematocytometer and counted. Cell counts included sixteen individual counts at each time point for 6 wells per time point.

Determination of Myoblasts and Myotubes

Cultures to be scored for cell fusion were washed with PBS, fixed with methanol, and stained with Giemsa. The flexcell bases were then mounted on coverslips with Canada Balsam. Myoblasts were defined as round, single-nucleated cells. Myotubes were defined as elongated, multi-nucleated cells. The degree of fusion was determined by the percentage of cells counted containing more than one nucleus by microscopic examination. The percentage of myoblasts and myotubes were determined over 4 days by evaluating four quadrants of each of six wells for all of the time points.

Total Protein

Myoblasts were plated at a density of 3.0×10^4 cells per well and subjected to mechanical stretch for 24, 48, 72, 96, and 120 h as described above. Controls were grown under the same conditions but were not stretched. Media from strained and unstrained cells were removed at each time point and total protein was assayed using the Colorimetric protein assay from Biorad Laboratories (Hercules, CA). The Biorad Protein Assay is used to determine concentration of solubilized protein. It involves the addition of an acidic dye to protein solution, and subsequent measurement at 595 nm with a spectrophotometer or microplate reader. Comparison to a standard curve provides a relative measurement of protein concentration. Fifty samples (25 controls and 25 experimentals) were compared to a standard (Biorad Standard II). Total protein synthesis over 120 h was determined by comparing to a standard curve.

Effects of Myoblast Conditioned Medium on Quiescent Cells

Myoblasts were plated at a density of 5.0×10^4 cells per well and subjected to mechanical strain for 48 h and 96 h. Media from strained and unstrained cells at the two time points were removed and placed on quiescent myoblasts that were plated on six-well Falcon plates at 4×10^4 cells per well. These quiescent cells which were not stretched

were incubated with the media conditioned from strained and unstrained cells to determine the effect of the conditioned media on cell growth. After a 48 h incubation, the quiescent cells were harvested by trypsinization and counted using a hemocytometer. Cell proliferation was only measured since mechanical strain showed no effect on differentiation in the present investigation.

Western Blots

Thirty μl of conditioned culture media from unstrained and strained myoblasts for 24, 48, 72, and 96 h were applied to each of two 10% SDS-polyacrylamide gels. Proteins were electrophoresed under nonreducing conditions, then transferred by electrophoresis to Hybond ECL filters (Amersham Corp., Arlington Hts., IL) in transfer buffer (50 mM Tris, 380 mM Glycine, 20% methanol). Filters were blocked with 10% nonfat dry milk in TBS (20 mM Tris, pH 7.5, 50 mM NaCl, and 0.1% Tween-20), then washed and incubated with polyclonal rabbit anti-human PDGF-BB for 1 h. After washing, blots were incubated with biotinylated anti-rabbit secondary antibody. Then blots were washed and incubated in streptavidin alkaline phosphatase for 1/2 h. The final step involved incubating the blots in 50 μg/ml nitroblue tetrazolium (NBT) and 25 μg/ml of bromochloro-indolyl phosphate (BCIP) and waiting for a color reaction.

Effects of a Neutralizing Antibody on Strain-induced Cell Growth

Myoblasts were plated at a density of 5.0 x 10^4 cells per well. The cells were subjected to mechanical strain and incubated with a neutralizing polyclonal antibody to the BB form of PDGF (3 μg/ml in each well) for 48 h and 96 h. Control myoblasts were also mechanically stimulated; however, they were not incubated with a neutralizing antibody to PDGF. After a 48 h and 96 h incubation, cells were harvested by trypsinization and counted using a hemacytometer. Cell proliferation was only measured since mechanical strain showed no effect on differentiation in the present investigation.

Data Presentation and Statistics

All data have been presented as mean +/- SD for the indicated number of observations (n). Comparisons are made using ANOVA with Bonferroni correction. Values for $P < 0.05$ are considered significant.

RESULTS

Myoblasts and Myotubes

Myoblasts are single-nucleated cells while myotubes are multi-nucleated cells (Fig. 2 and 3). We looked at the effect of mechanical stimulation on myoblasts so it was important to be able to distinguish between the two cell types.

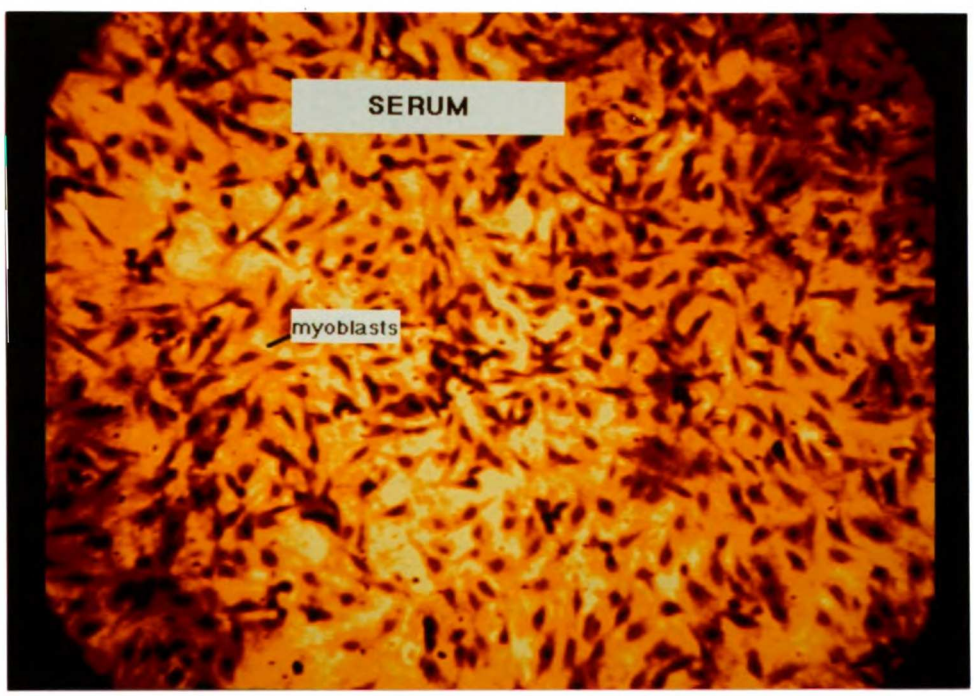

Figure 2. Photomicrograph demonstrating a relatively pure culture of myoblasts taken in 10% serum at 3 days.

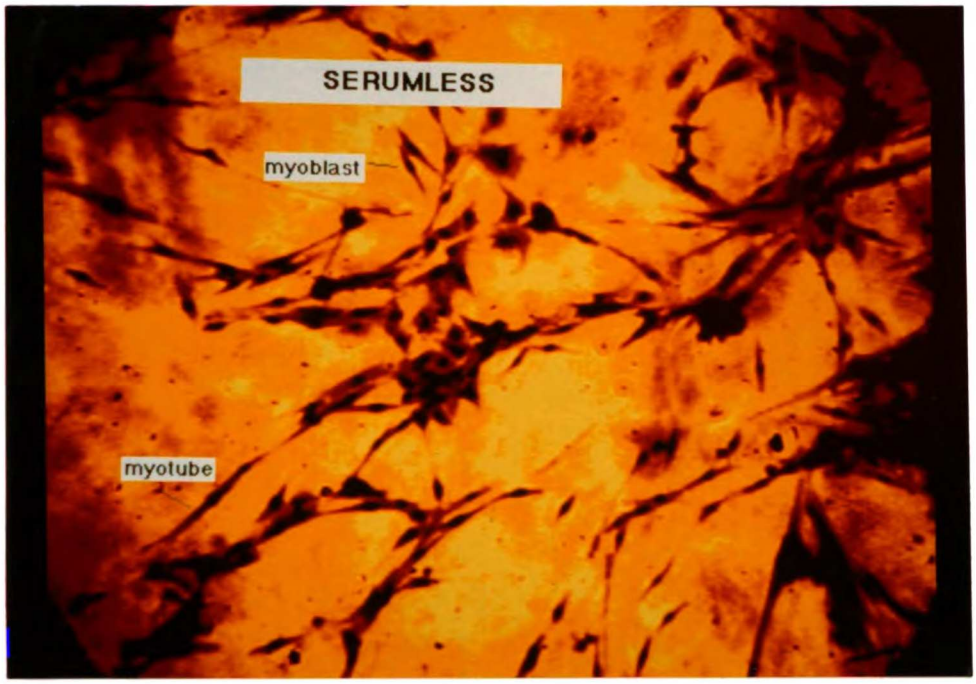

Figure 3. Photomicrograph demonstrating myotubes that was taken at 7 days in 1% serum.

Effect of 10% Serum and 1% Serum Solutions over 7 Days

In 10% serum, single-nucleated cells remained over the seven days with the number rapidly increasing and myotubes were not detected until the seventh day. In contrast, differentiation started in 1% serum at about 4 days and continued throughout the 7 days in nonstretched cells (Fig. 4 and 5). Furthermore, in 1% serum there were major morphological changes in single cells progressing through several stages. Several obvious effects were noted in the stained material. Single cells first congregated in groups. Groups of these cells would extend toward other islands of cells which in turn developed cytoplasmic bridges interlacing networks. The cytoplasmic connections would then develop into multi-nucleated cells.

All of the experiments were done in low serum (1% serum) to minimize the amount of proteins, growth factors, and hormones in the environment. Low or no serum in

medium is necessary in order to evaluate the effect of cyclic mechanical strain on myoblasts. However, since serum deprivation causes myoblast differentiation (Nadal-Ginard, 1978), we first evaluated the effects of low serum (1%) concentration on differentiation of these cells and used this information as baseline data.

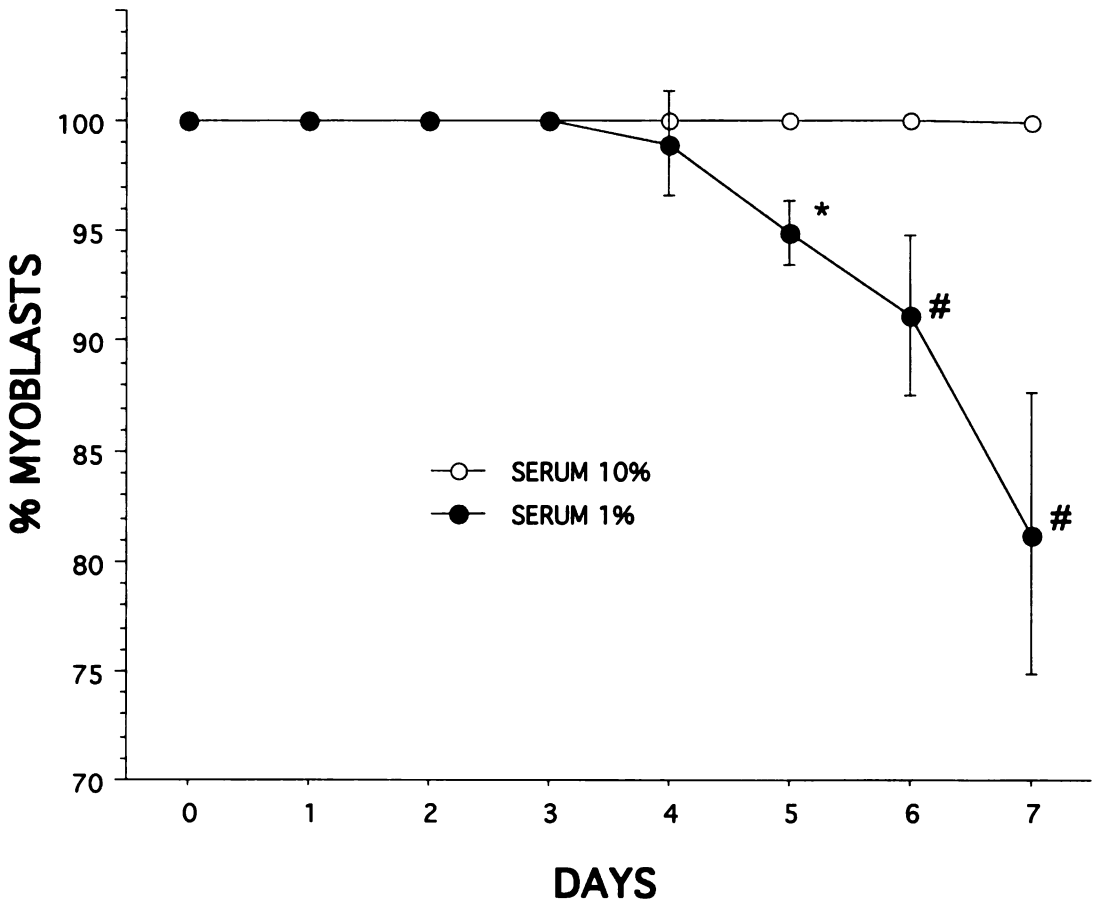

Figure 4. Effect of changing serum concentration on unstretched cells. % myoblasts was measured over 7 days.
Data is mean ± SD
# $p < .001$
* $p < .0001$

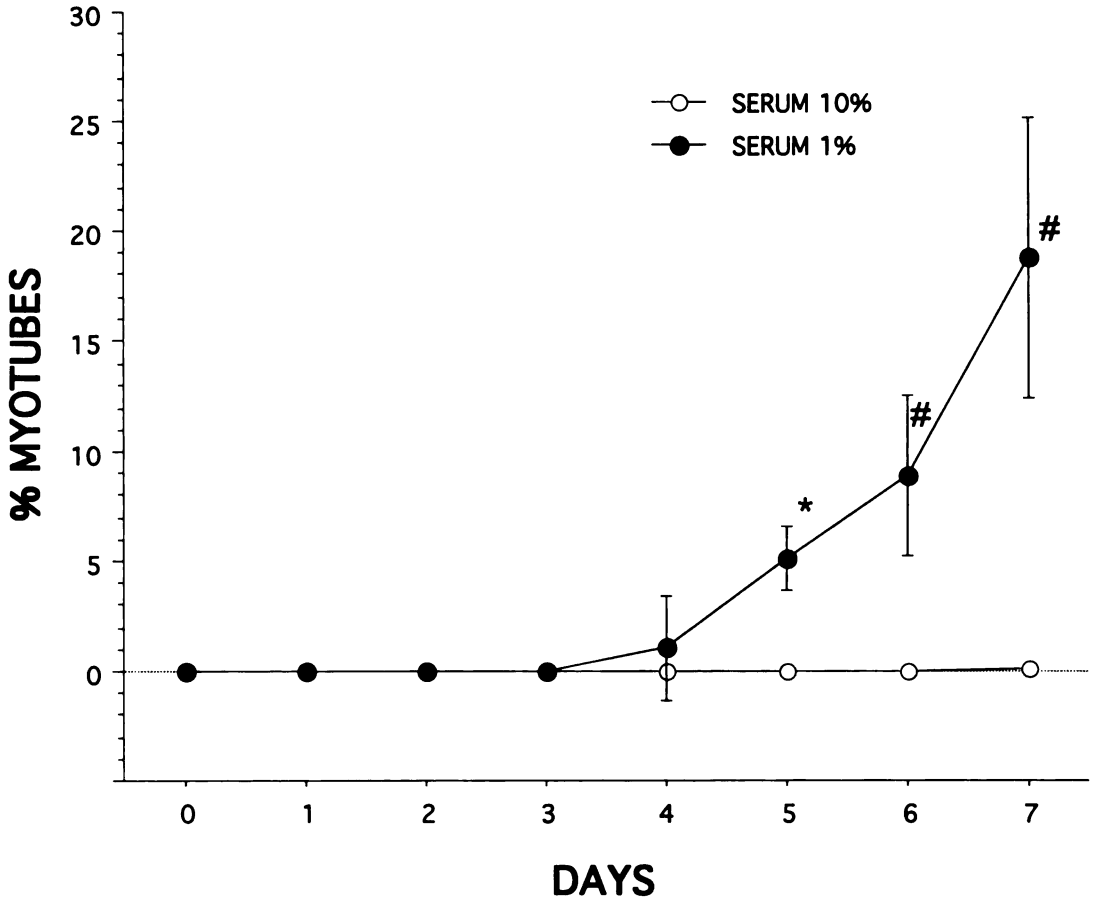

Figure 5. Effect of changing serum concentration on unstretched cells. % myotubes was measured over 7 days.
Data is mean ± SD
# $p < .001$
* $p < .0001$

Effect of Cyclic Strain on Cell Proliferation

To study the effect of cyclic strain on growth of L6J1 skeletal muscle cells, cells were grown in fibronectin-coated silicone elastomer-bottomed plates. In our pilot studies, we originally tried growing the L6J1 myoblasts in amino-coated plates but found that the cells did not adhere to the plates as well. Similar results to the pilot studies using amino-coated plates were found with the elastin- and laminin-coated plates. Controls were grown under identical conditions without application of the strain-producing vacuum. Our pilot studies used strains of 6, 15, and 30 cycles/min. Strain of 15 and 30 cycles/min were eliminated because they allowed minimal relaxation time. Therefore, a pattern of high

intensity (20%) stretch/relaxation at intervals of 6 cycles/min was chosen to simulate functional demands that might be placed on neonatal muscle fibers *in vivo*. The results show that cyclic strain (6 cycles/min) caused a 1.4, 1.5, 3, 3.1, and 3.5 fold increase in cell proliferation after 24, 48, 72, 96, and 120 h, respectively (Fig. 6).

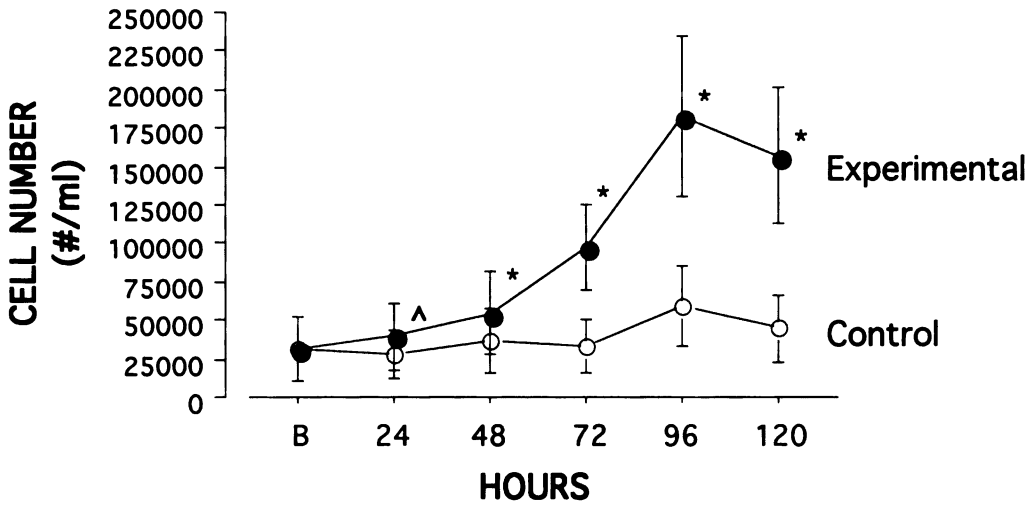

Figure 6. Effect of cyclic mechanical stretch on proliferation of skeletal myoblasts. Cells plated at a density of 3.0 x 10^4 cells per well were subjected to cyclic mechanical strain for 24, 48, 72, 96, and 120 h. Control cells were grown in the same dishes but were not exposed to mechanical strain. After incubation, cells were harvested by trypsinization and counted using a hemocytometer. B = Baseline (cell count at the start of stretching).

Data is mean ± SD

^ $p < .001$

* $p < .0001$

$n = 5$ in all cases

Effect of Cyclic Strain on Cell Differentiation

To determine whether stretching skeletal muscle cells affects cell differentiation, myoblasts were stained with Giemsa and percent myoblasts and percent myotubes were measured for six wells at each time point. Sampling of four sites of each of six wells provided 24 measurements at each time point. The percentage of myotubes increased over a 4 day study for both the control and experimental setups. There was no significant difference with stretching (Fig. 7).

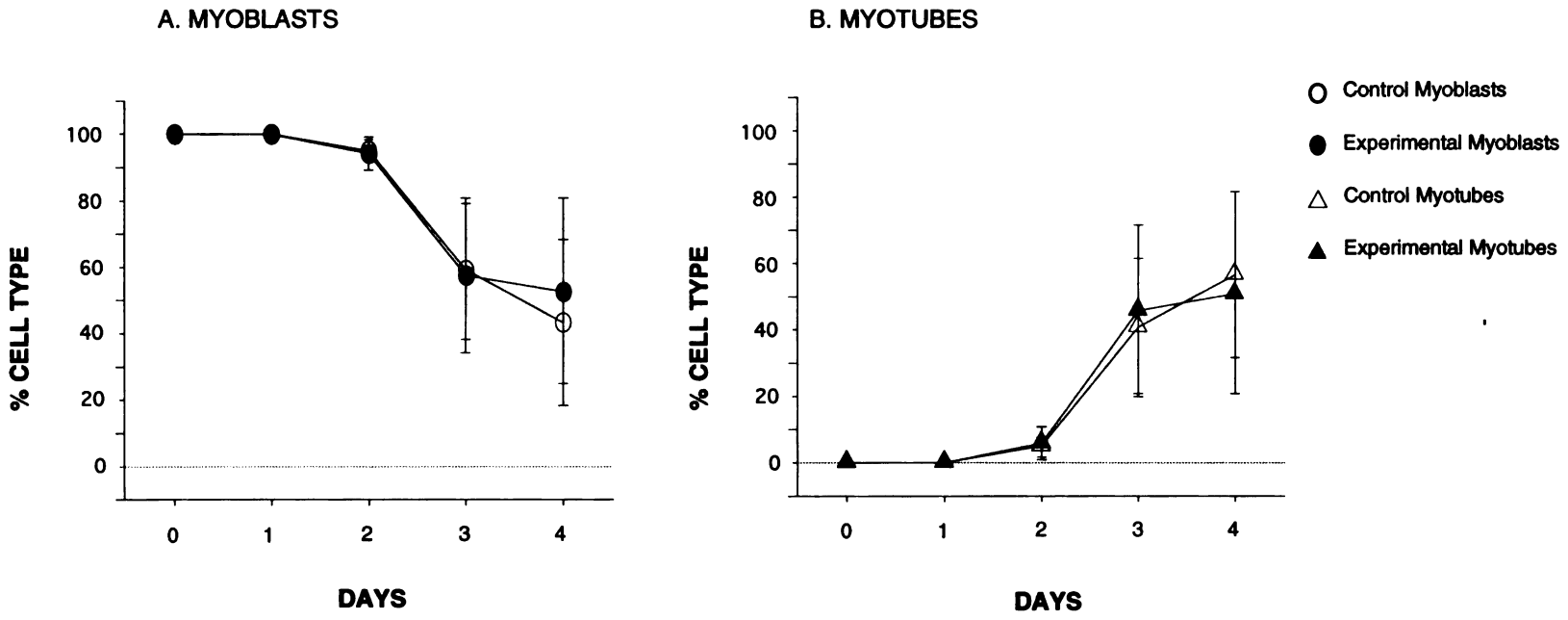

Figure 7. Effect of cyclic mechanical strain on cell differentiation. Cells plated at a density of 5.0 x 10^4 cells per well were unstrained (control) and strained (experimental) over 4 days. After incubation, cells were stained with Giemsa and % cell type was determined under a microscope at 20x: A). % myoblasts was measured for control and experimental conditions. B) % myotubes was measured for control and experimental conditions. $n = 6$ in all cases.

Effect of Cyclic Strain on Secretion of Proteins

To determine whether rat skeletal myoblasts subjected to mechanical stimulation show altered protein expression, we examined total protein synthesis. The results show that medium from cells subjected to strain for periods of 48, 72, and 120 h contain significantly higher concentration of protein than medium from cells not subjected to strain (Fig. 8).

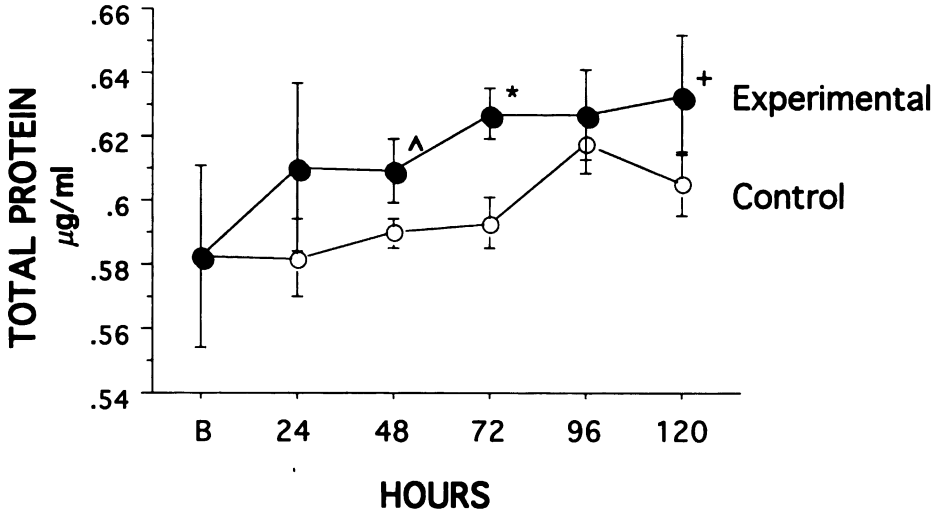

Figure 8. Effect of strain on total protein secretion. Conditioned media from unstrained and strained cells were tested for total protein secretion over 120 h. B = Baseline (cell count at the start of stretching).
Data is mean ± SD
+ $p < .05$
^ $p < .01$
* $p < .0001$
$n = 5$ in all cases

We also examined total protein synthesis per cell. Although there was more total protein produced by strained myoblasts than unstrained myoblasts, the opposite was found when measuring total protein per cell (Fig. 9).

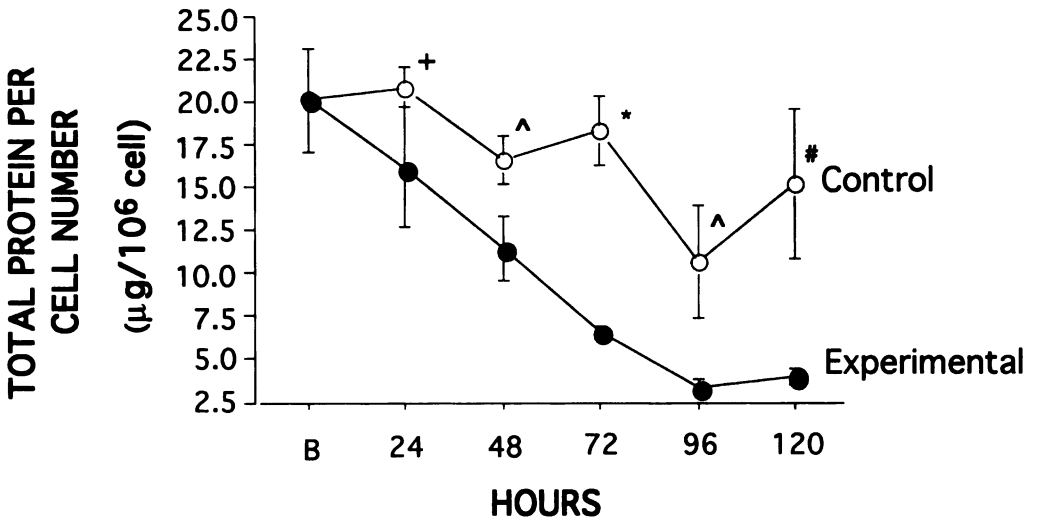

Figure 9. Effect of strain on total protein secretion per cell. The amount of total protein secreted per cell was determined over 120 h. B = Baseline

Data is mean ± SD

+ $p < .05$ ^ $p < .01$

# $p < .001$ * $p < .0001$

$n = 5$ in all cases

Effect of Conditioned Media on Quiescent Cells

In order to determine whether strained skeletal muscle cells produced a growth factor, quiescent skeletal myoblasts were incubated with media that had been conditioned on plates of cells that were static or subjected to strain for 48 h and 96 h. The results show

Table 1. Effect of conditioned media on quiescent myoblasts

Time	Medium from unstrained cells Mean cell count (SD)	Medium from strained cells Mean cell count (SD)	p-value
48 hours	271875 (92613)	297291 (94795)	NS
96 hours	88333 (30689)	111666 (44450)	< .01

To determine whether strained skeletal myoblasts secrete growth factors, quiescent skeletal myoblasts not subjected to strain were incubated with conditioned media. Conditioned media were from cells grown in elastomer-bottomed plates that had been strained or not strained for 48 h and 96 h. The cells were plated at a density of 4.0×10^4 cells per well.

$n = 6$ in all cases.

that media from cells subjected to strain for 48 h and 96 h increased cell growth greater than media from unstrained myoblasts (Table 1). However, this increase was statistically significant only at 96 h (Fig. 10; $p < .01$). Cell numbers were lower for quiescent cells incubated with conditioned medium from unstrained and strained myoblasts for 96 h than for quiescent cells incubated with conditioned media from unstrained and strained myoblasts for 48 h. This probably occurred since media was not changed while the cells were stretched. Therefore, the nutrients in the 1% serum are more depleted in the media after 96 h than after 48 h.

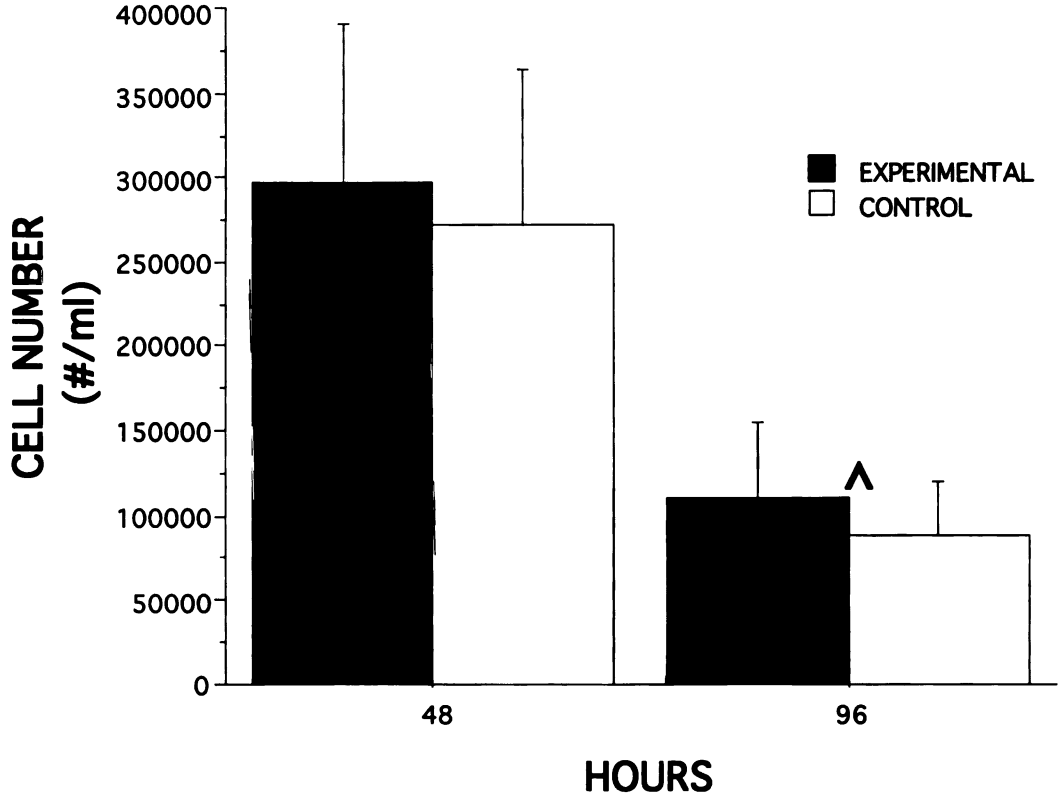

Figure 10. Histogram showing the effect of conditioned media from unstrained (control) and strained (experimental) skeletal myoblasts. (^ $p < .01$).

Western Blots

To determine whether the cells subjected to strain secreted PDGF, 30 μl of medium was removed from such cells, electrophoresed under non-reducing conditions and blots were probed separately with the PDGF-BB antibody. Western immunoblot for PDGF-BB revealed that a PDGF-like protein was secreted at 2, 3, and 4 days of the experiment. The level of the protein appears to be greater in the media from strained cells than media from unstrained cells. Moreover, more of the PDGF-like protein is secreted with each succeeding day (Fig. 11).

Effects of a Neutralizing Antibody on Strain-induced Cell Growth

Experiments with a neutralizing antibody to PDGF-BB added to strained cells reduced cell proliferation at both 48 h and 96 h (Table 2).

Table 2. Effect of a neutralizing polyclonal antibody to the BB form of PDGF on mechanical strain of myoblasts.

Time	Medium without Ab Mean cell count (SD)	Medium with Ab Mean cell count (SD)	p-value
48 hours	93645 (30817)	88750 (35989)	NS
96 hours	111145 (31317)	87604 (27975)	< .0001

To determine whether the secreted PDGF actually contributes to the mitogenic response to strain, cells were subjected to strain and incubated with a neutralizing polyclonal antibody to the BB form of PDGF (3μg/ml in each well) for 48 h and 96 h . The mean cell counts at the start of stretching for the 48 h and 96 h experiments were 55312 and 36250, respectively.
$n = 6$ in all cases.

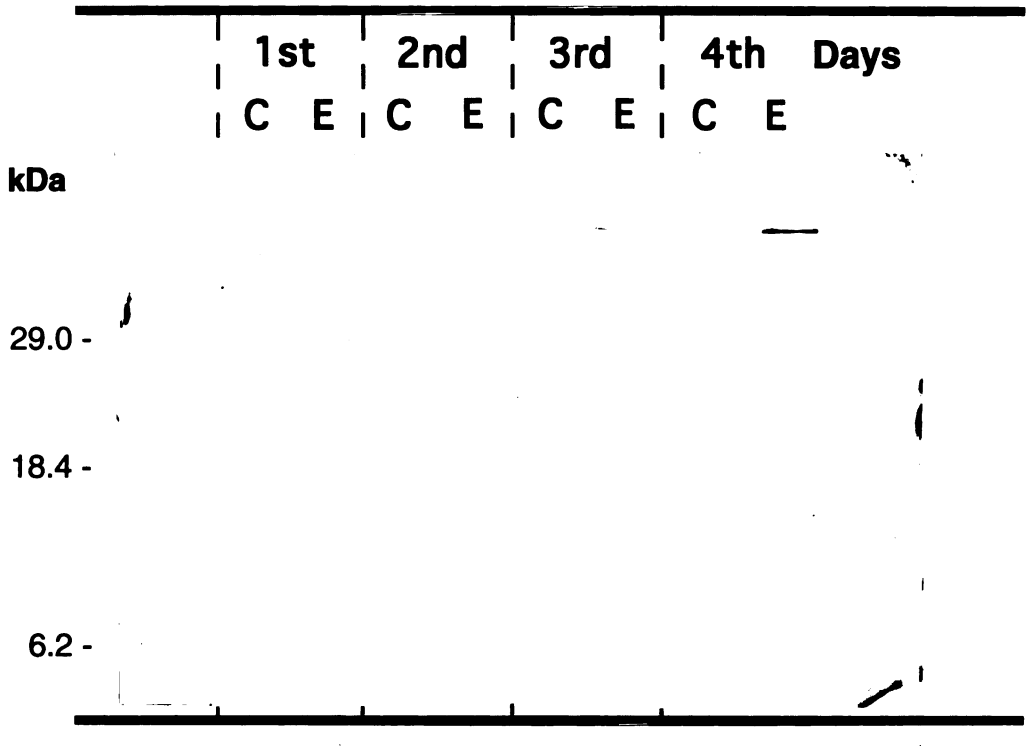

Figure 11. Secretion of a PDGF-like protein by L6J1 myoblasts subjected to strain. Skeletal muscle cells were plated on flex plates in fresh 1% serum medium. 30 µl of media from subconfluent L6J1 cells that were unstrained and strained over 4 days were analyzed. Gels were treated with anti-PDGF-BB. Standard lanes contain prestained protein molecular weight standards.

However, this decrease was statistically significant only at 96 h (Fig. 12; $p < .0001$).

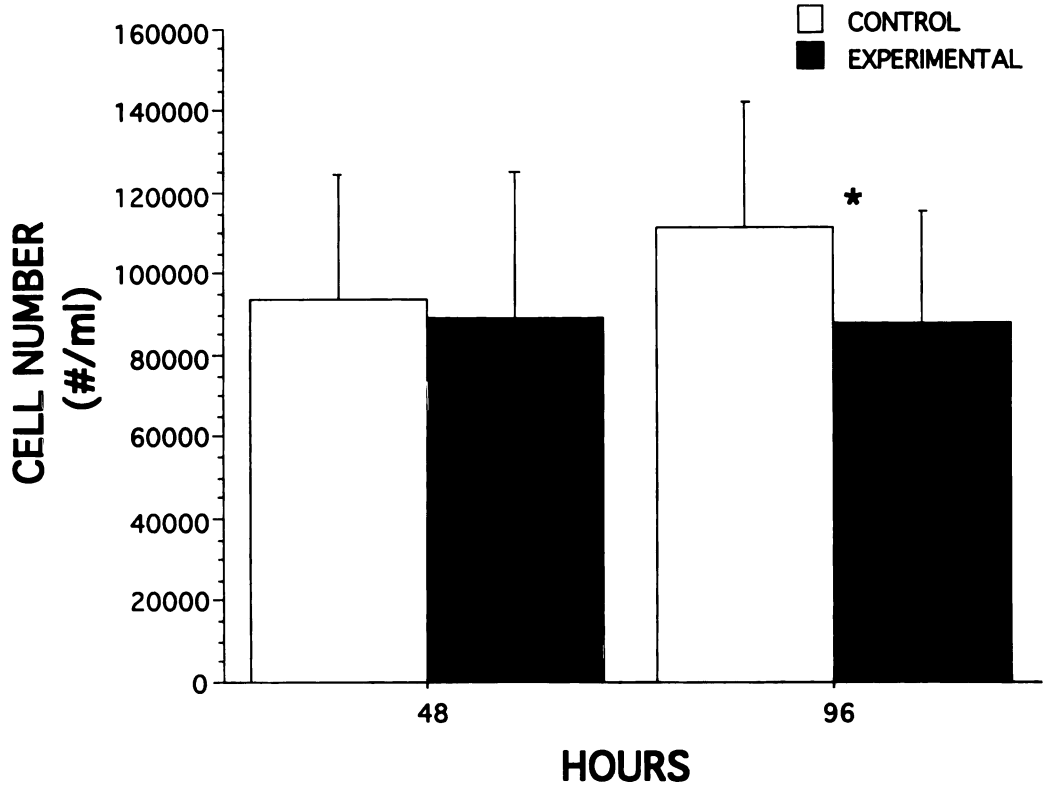

Figure 12. Histogram showing the effect of a neutralizing antibody to PDGF-BB on cell number following cyclic mechanical stimulation of myoblasts. (* $p < .0001$). Control = cells that were subjected to strain only. Experimental = cells that were subjected to strain and incubated with a neutralizing Ab to PDGF-BB.

DISCUSSION

Physical deformation plays an important role in the environment of many cell types. Like all muscle cells, skeletal muscle cells are deformed throughout their development and in the course of performing their biological function. Therefore, it is logical to ask what effect cell deformation, in this case cyclic mechanical strain, has on skeletal muscle cells.

All of the experiments were done in low serum (1% serum) to minimize the amount of exogenous confounding variables. It was important to evaluate the effect of changing the serum concentration since serum deprivation induces myogenic differentiation (Nadal-Ginard, 1978) and the effect of strain on myoblasts was examined. The experiments were

performed up to 5 days and the results showed that the cell type was essentially all myoblasts.

The experiments reported reveal that cyclic strain may play a critical role in skeletal myoblast proliferation. Vandenburgh et al. (1991) found that mechanically stimulating muscle cells induced myoblasts proliferation which agrees with our study. However, our finding that mechanical strain induces growth of cultured skeletal myoblasts differs from their findings in that they found temporary damage to the cells when mechanical stimulation is initiated. The injury-related biochemical responses returned to control levels after 2 to 20 h of continued mechanical stimulation and cell growth began. It was not until 48 to 72 h of stimulation that cell growth began to occur in the Vandenburgh et al. study.

We did not find this initial loss in cell number. Cells started proliferating at 24 h. Potential reasons for the discrepancy between the two studies are possibly the different intensity and rate of cycling used. The previous study used 20% stretch/relaxations at intervals of 2 cycles per hour and studied myofibers as compared to myoblasts studied in the present investigation. This raises the possibility that different skeletal muscle cell types (ie., myoblasts, myotube, myofiber) may respond differently to strain and needs further investigation. In fact, at 120 h (see Fig. 6) the cell numbers decrease for both the experimental and control cells which may have occurred due to the increasing number of myotubes (see Fig. 4, 5 and 7).

Our study shows that mechanical strain has no effect on differentiation. The control and experimental muscle cells show a similar pattern of differentiation. Vandenburgh et al. (1991) found that mechanical strain increased the fusion rates of muscle cells. Again the discrepancy between the two studies could be due to the reasons stated above for cell proliferation. Moreover, the reason we did not find any difference in cell differentiation between strained and unstrained cells could be that our technique was not sensitve enough. We determined differentiation by looking at a change in cell morphology under a microscope. A more sensitive technique would have been to biochemically measure an

early marker for differentiation. Myogenin, a member of the myogenic basic helix-loop-helix transcription factor family, appears to be a good candidate. Myogenin is not detectable until 72 to 96 h, coincidental with the first evidence of differentiation of rat skeletal muscle satellite cells (Smith et al., 1994). Furthermore, Mitsumoto et al. (1994) found that during L6 cell differentiation, the amount of both myogenin mRNA and protein reached a maximal level on day 4 before full myotube formation.

One interesting finding is that myoblast differentiation began at 2 days in the mechanically strained experiments (Fig. 7) while the experiments on changing serum concentration from 10% to 1% show differentiation beginning at 4 days (Fig. 4 and 5). One possible explanation for this observation is that for all of the experiments the cells were normally incubated for 24 h to allow them to adhere to the plates. After 24 h incubation, the environment was changed between the control and experimental. However, in the experiment evaluating the effect of mechanical strain on differentiation the cells were incubated for 48 h. In other words, mechanical stretching did not occur until after the initial 48 h incubation. It is possible that the cells were very close to confluence. When myoblasts become confluent in culture, DNA synthesis is inhibited and myogenic differentiation begins (Yaffe, 1971). Another explanation is that the elastomer-bottomed plates were treated with fibronectin to allow the cells to better adhere to the bottom of the plates while they were stretched. Fibronectin has been known to promote fusion in some instances (Pearson and Epstein, 1982).

Cell deformation may affect cell growth through important biologic agents, such as growth factors. Mechanical forces control skeletal muscle growth or atrophy by regulating the rates of total protein synthesis and degradation (Vandenburgh, 1987). We first measured total protein synthesis to determine if mechanical stimulation of myoblasts resulted in altered levels of protein expression. The results indicate that more total protein was secreted in cultures of strained myoblasts than in unstrained myoblasts. However, significantly less protein per cell was produced in strained cells versus unstrained cells.

We measured total protein so it is possible that even though total protein per cell was less in strained cell, a mitogenic factor or factors produced per cell may be more in strained myoblasts. On the other hand, a small amount of secreted mitogen may have a large effect (ie., cell proliferation). It is also possible that passive stretch sensitizes the L6 muscle cells to respond to macromolecules present in the medium (Misutomoto et al., 1992). Furthermore, mechanical stimulation through passive stretch may trigger other biochemical cascades of events within the cells and may not actually involve exogenous factors.

We evaluated the mitogenicity of the medium by placing conditioned medium from unstrained and strained myoblasts on quiescent muscle cells. It appeared that strain induced the production of one or more growth factors which secondarily caused the cells to divide through autocrine and/or paracrine action. Vandenburgh et al. (1991) showed that mechanically stimulated myofiber growth *in vitro* was dependent on medium growth factors present in serum and/or embryo extract. Moreover, stretched-induced muscle growth *in vivo* is also modulated by circulating hormones and growth factors. We propose that the muscle cells are producing their own growth factor to promote cell growth. One possible growth factor that may interact synergistically with mechanical activity to stimulate muscle cell growth is PDGF.

An interesting finding which was noted in the results section of the conditioned media experiments is that cell numbers at 96 h are lower than at 48 h for both unstrained and strained groups but the decrease in cell numbers are less in cells exposed to medium from strained myoblasts. Possibly the cell numbers at 96 h are influenced not by cell proliferation but by cell survival. As mentioned earlier, the media was not changed while the cells were stretched. Therefore, the nutrients in the 1% serum were more depleted in the media after 96 h than after 48 h.

PDGF-AA can only bind to the αα–receptor, PDGF-AB can bind to the αα- and αβ-receptor, and PDGF-BB can bind to all three receptor combinations: αα, αβ, and ββ (Seifert et al., 1989). Sejersen et al. (1986) demonstrated that L6J1 myoblasts secrete

PDGF, most likely in the the form of PDGF-AA. However, PDGF-AA has been shown to be nonmitogenic (Jin et al., 1990; Yablonka-Reuveni., 1990). PDGF-AB only exerts a slight mitogenic effect (Yablonka-Reuveni, 1990). PDGF-BB is mitogenic for L6 rat myoblasts and C2 mouse myoblasts. Therefore, we studied PDGF-BB in relation to mechanical stretch. Cell proliferation was only measured since mechanical strain showed no effect on differentiation in our study.

As a first step in determining the mitogen or mitogens produced by strained myoblasts Western blots were performed for PDGF-BB. A PDGF-like protein was secreted at 2, 3, and 4 days of culture with increasing amounts being detected with each succeeding day. Based on a comparison of the staining intensity of the bands, strained cells produced more of the factor. Although the bands show that the PDGF-like protein is heavier than PDGF (30 kDa), a neutralizing antibody to the BB form of PDGF reduced the mitogenic effect in cells exposed to mechanical strain. It is possible that PDGF-BB is complexing with another protein, making the bands heavier than 30 kDa. Although the antibody did not completely eliminate the mitogenic response, the data implicates that the mechanism of strain-induced growth appears to at least partly involve the intermediary action of secreted PDGF-BB. The data from the quiescent, Western blot and neutralizing antibody experiments suggests that PDGF is a late-acting autocrine and/or paracrine growth factor in the response to strain. It is, of course, possible that other growth factors are produced by the cells during mechanical strain. Further analysis needs to be done.

In summary, this study shows that cyclic mechanical strain is an important regulatory factor on the growth of skeletal muscle cells. It appears that mechanical strain induces growth of the myoblasts via autocrine and/or paracrine action of a PDGF-like protein. Our results provide the first demonstration that L6J1 rat skeletal myoblasts secrete a PDGF-BB-like protein. As mentioned earlier, Sejersen et al. (1986) showed that L6J1 cells secrete PDGF; however, it most likely was in the form of PDGF-AA. *In vivo*,

production of PDGF by skeletal myoblasts could be of physiological importance during the embryonic and postnatal periods as well as in the repair process in the adult.

REFERENCES

Armand O, Boutineau AM, Mauger A, Pautou MP, Kieny M. Origin of satellite cells in avian skeletal muscle Arch Anat 1983;72:163-181.

Banes AJ, Gilbert J, Taylor D, Monbureau O. A new vacuum-operated stress-providing instrument that applies static or variable duration cycle tension or compression in cells in vitro. J Cell Sci 1985;75:25-42.

Banes JJ, Link GW, Gilbert JW, Tay RTS, Monbureau O. Culturing cells in a mechanically active environment. Am Biotechnol Lab 1989;8:12-22.

Bischoff R, Holtzer H. The effect of mitotic inhibitors on myogenesis. J. Cell Biol 1967;36:111.

Brunette DM. Mechanical stretching increases the number of epithelial cells synthesizing DNA in culture. J. Cell Sci 1984;69:35-45.

Carvalho RS, Scott JE, Suga DM, Yen EHK. Stimulation of signal transduction pathways in osteoblasts by mechanical strain potentiated by parathyroid hormone. J Bone & Mineral Res 1994;9(7):999-1011.

Chiu I-M, Reddy EP, Givol D Robbins KC, Tronick SR, Aaronson SA Nucleotide sequence analysis identifies the human c-sis proto-oncogene as a structural gene for platelet-derived growth factor. Cell 1984;37(1):123-129.

Cossu G, Molinaro M, Pacifici M. Differential response of satellite cells and embryonic myoblasts to a tumor promoter. Dev Biol 1983;98:520-524.

Cossu G, Cicinelli P, Fieri C, Coletta M, Molinaro M. Emergence of TPA-resistant "satellite" cells during muscle histogenesis of human limb. Exp Cell Res 1985;160:403-411.

DiCorleto PE, Bowen-Pope DF Cultured endothelial cells produce a platelet-derived growth factor-like protein. Proc Natl Acad Sci USA 1983;80:1919-1923.

Ewton DZ, Florini JR. Effects of the somatomedins and insulin on myoblast differentiation in vitro. Dev Biol 1981;86:31-39.

Florini JR. Hormonal control of muscle growth. Muscle & Nerve 1987;10:577-598.

Florini JR, Magri KA. Effects of growth factors on myogenic differentiation. Am J Physiol 1989;256 (Cell Physiol 25):C701-C711.

Gauthier GF, Lowey S, Hobbs AW. Fast and slow myosin in developing muscle fibers. Nature 1978;274:25.

Hasegawa S, Sato S, Saito S, Suzuki Y, Brunette DM. Mechanical stretching increases the number of cultured bone cells synthesizing DNA and alters their pattern of protein synthesis. Calcif Tissue Int 1985;37:431-436.

Hauschka SD, Haney C, Angello JC, Linkhart TA, Bonner PH, White NK. Clonal studies of muscle development: Analogies between human and chicken cells in vitro and their possible relation to muscular dystrophies. In Pathogenesis of human muscular dystrophies (ed. L. Rowland), Excerpta Medica, Amsterdam 1977:835.

Heldin C-H, Westermark B. Growth factors: mechanism of action and relation to oncogenes Cell 1984;37:9-20.

Jin P, Rahm M, Claesson-Welsh L, Heldin C-H, Sejersen T. Expression of PDGF A-chain and β-receptor genes during rat myoblast differentiation. J Cell Biol 1990;110:1665-1672.

Jin P, Sejersen T, Ringertz NR. Recombinant platelet-derived growth factor-BB stimulates growth and inhibits differentiation of rat L6 myoblasts. J Biol Chem 1991;266:1245-1249.

Konigsberg IR. Diffusion-mediated control of myoblast fusion. Dev Biol 1971;26:133.

Linkhart TA, Clegg CH, Hauschka SD. Control of mouse myoblast commitment to terminal differentiation by mitogens. J Supramol Struct 1980;14:483.

Linkhart TA, Clegg CH, Hauschka SD. Myogenic differentiation in permanent clonal mouse myoblast cell lines: regulation by macromolecular growth factors in the culture medium. Dev Biol 1981;86(1):19-30.

Massague J, Cheifetz S, Endo T, Nadal-Ginard B. Type β transforming gowth factor is an inhibitor of myogenic differentiation. Proc Natl Acad Sci USA 1986;83:8206-8210.

Mitsumoto Y, Downey GP, Klip AM. Stimulation of glucose transport in L6 muscle cells by long-term intermittent stretch-relaxation. FEBS 1992;301:94-98.

Mitsumoto Y: Liu Z; Klip A. A long-lasting vitamin C derivative, ascorbic acid 2-phosphate, increases myogenin gene expression and promoters differentiation in L6 muscle cells. Biochem and Biophys Res Com, 1994;199(1):394-402.

Nadal-Ginard B. Commitment, fusion, and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. Cell 1978;15:855.

Nilsson J, Sjolund M, Palmberg L, Thyberg J, Heldin CH. Arterial smooth muscle cells in primary culture produce a platelet-derived growth factor-like protein. Proc Natl Acad Sci USA 1985:82(13):4418-4422.

Olwin BB, Hauschka SD. Cell surface fibroblast growth factor and epidermal growth factor receptors are permanently lost during skeletal muscle terminal differentiation in culture. J Cell Biol 1988;107:761-769.

Pearson ML, Epstein HF. Muscle Development: Molecular and Cellular Control. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1982;559-568.

Richler C, Yaffe D. The in vitro cultivation and differentiation capacities of myogenic cell lines. Exp Cell Res 1971;66(1):33-48.

Ringertz NR, Krondahl U, Coleman JR. Reconstitution of cells by fusion of cell fragments. Exp Cell Res 1978;113:233-246.

Ross R, Raines EW, Bowen-Pope DF. The biology of platelet-derived growth factor. Cell 1986;46:155-169.

Rutz R, Hauschka S. Clonal analysis of vertebrate myogenesis. VII. Heritability of muscle colony type through sequential subclonal passages in vitro. Dev Biol 1975;91:103-110.

Sejersen T, Betsholtz C, Sjolund M, Heldin CH, Westermark B, Thyberg J. Rat skeletal myoblasts and arterial smooth muscle cells express the gene for the A chain but not the gene for the B chain (c-sis) of Platelet-derived growth factor (PDGF) and produce a PDGF-like protein. Proc Natl Acad Sci USA 1986;83(18):6844-8.

Seed J, Hauschka SD. Temporal separation of the migration of distinct myogenic precursor populations into the developing chick wing bud. Dev Biol 1984;106:389-393.

Seed J, Hauschka SD. Clonal analysis of vertebrate myogenesis. VIII. Fibroblast growth factor (FGF)-dependent and FGF-independent muscle colony types during chick wing development. Dev Biol 1988;128:40-49.

Seifert RA, Hart CE, Phillips PE, Forstrom JW, Ross R, Murray MJ, Bowen-Pope DF. Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. J Biol Chem 1989;264:8771-8778.

Seifert RA, van Koppen A, Bowen-Pope DF. PDGF-AB requires PDGF receptor α-subunits for high-affinity, but not for low-affinity, binding and signal transduction. J Biol Chem 1993;268:4473-4480.

Senni MI, Castrignano F, Poiana G, Cossu G, Sacarcella G, Biagioni S. Expression of adult fast pattern of acetylcholinesterase molecular forms by mouse satellite cells in culture. Differentiation 1987:36:194-198.

Shimokado K, Raines EW, Madtes DK, Barrett TB, Benditt EP, Ross R. A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. Cell 1985;43:277-286.

Slamon DJ, Cline MJ. Expression of cellular oncogenes during embryonic and fetal development of the mouse. Proc Natl Acad Sci USA 1984;81:7141-7145.

Smith CK 2nd, Janney MJ, Allen RE. Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. J Cell Phys, 1994;159(2):379-85.

Sjolund M, Hedin U, Sejersen T, Heldin CH, Thyberg J. Arterial smooth muscle cells express platelet-derived growth factor (PDGF) A chain mRNA, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype- and growth state-dependent manner. J Cell Biol 1988;106(2):403-413.

Stockdale FE. Myogenic cell lineages. Dev Biol 1992;154:284-298.

Stockdale FE, Miller JB. The cellular basis of myosin heavy chain isoform expression during developmental avian skeletal muscles. Dev Biol 1987;123:1-9.

Sumpio BE, Banes AJ, Levin LG, Johnson Jr. G. Mechanical stress stimulates aortic endothelial cells to proliferate. J Vasc Surg 1987;6:252-256.

Vandenburgh HH. Motion into mass: How does tension stimulate muscle growth? Med Sci Sports Exer 1987;19:S142-S149.

Vandenburgh HH, Hatfaludy S, Karlisch P, Shansky J. Mechanically induced alterations in cultured skeletal muscle growth. J Biomechancs 1991;24(1):91-99.

Vandenburgh HH. Mechanical forces and their second messengers in stimulating cell growth in vitro Am J Physiol 1992;R350-355.

Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermark B, Heldin CH, Huang JS, Deuel TF Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature (London) 1983;304:35-39.

Westermark B, Sorg C. Biology of Platelet-derived growth factor. Cytokines 1993 Vol. 5. Karger, Basel.

White TP, Esser KA. Satellite cell growth and growth factor involvement in skeletal muscle growth. Med Sci Sports Exerc 1989;21:S158-S163.

White NK, Bonner PH, Nelson DR, Hauschka SD. Clonal analysis of vertebrate myogenesis. IV. Medium dependent classification of colony-forming cells. Dev Biol 1975;44:346-361.

Williams LT. Signal transduction by the platelet-derived growth factor receptor. Science 1989;243:1564-1570.

Wilson E, Qing M, Sudhir K, Weiss RH, Ives HE. Mechanical strain induces growth of vascular smooth muscle cells via autocrine action of PDGF. J Cell Biol 1993;123:741-747.

Wolswijk G, Noble M. Cooperation between PDGF and FGF converts slowly dividing O-2A adult progenitor cells to rapidly dividing cells with characteristics of $^{O\text{-}2A}$perinatal progenitor cells. J Cell Biol 1992;118:889-900.

Wright WE, Sassoon DA, Lin VK. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoK. Cell 1989;56(4):607-617.

Yablonka-Reuveni Z, Balestreri TM, Bowen-Pope DF. Regulation of proliferation and differentiation of myoblasts derived from adult mouse skeletal muscle by specific isoforms of PDGF. J Cell Biol 1990;111(4):1623-1629.

Yablonka-Reuveni Z. Development and postnal regulation of adult myoblasts. Microscopy Research and Technique 1995;39(5):366-380.

Yaffe D. Retention and differentiation potentialities during prolonged cultivation of myogenic cells. Proc Natl Acad Sci USA 1968;61:477-483.

Yaffe D. Cellular aspects of muscle differentiation in vitro. Current Topics in Developmental Biology 1969;4:37-77.
Yaffe D. Developmental changes preceding cell fusion during muscle differentiation in vitro. Exp Cell Res 1971;66(1):33-48.

Yaffe D. Rat skeletal muscle cells. In Tissue culture: Methods and applications (ed. Krus PF, Patterson MK, Jr) Academic Press, New York 1973:106.