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Effects of Mechanical Stretching on Caspase and IGF-1 Expression During the Proliferation Process of Myoblasts

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It has been reported that the synthesis, degradation, and metabolism of muscle proteins in myoblasts, as well as the proliferation and differentiation of cells, are influenced by various related to extracellular signaling molecules, such as neural transmitters, growth factors, and hormones, when muscle tissue has been exposed to mechanical stimulation. However, reports regarding the expression of growth factors during mechanical stimulation of myoblasts are few, and many questions remain unanswered. We examined the mRNA expression of insulin-like growth factor 1 (IGF-1) in myoblasts subjected to mechanical stretching *in vitro*. In addition, apoptosis caused by intracellular stress has been reported to occur during muscle development at the embryonic stage. To clarify the expression of intracellular stress factors, we here investigated related gene expression. Expression of IGF-1 increased in the early stage of cell stretching, followed by a decrease in the late stage. This suggests that mechanical stimulation resulted in an immediate increase in IGF-1 expression, followed by a decrease as cells acclimated to the inducing environment. Caspase was significantly expressed in a stretch group at 12 hours after the beginning of mechanical stimulation, compared with a control group. This suggests that cellular proliferation is also regulated by intracellular stress factors involving the endoplasmic reticulum, mitochondria, and other organelles during the process of muscle proliferation and differentiation

Key words: myoblast, mechanical stress, IGF-1, caspase, muscle protein

INTRODUCTION

The proliferation and differentiation of myocytes are influenced by various extracellular signaling molecules when muscle tissue has been exposed to mechanical stimulation (Towler et al., 2004). Mediators of extracellular signaling include endocrine-system factors (Brunetti et al., 1989; Muscat et al., 1994; Sotiropoulos et al., 2006) and growth factors (Cheema et al., 2005; Tortorella et al., 2001). Moreover, muscle tissue has been reported to enlarge in experimental animals with the elimination of endocrine influence by removal of endocrine glands when cells are subjected to mechanical loading (Spencer et al., 1991). This suggests that either myocytes or surrounding cells secrete growth factors that are responsible for muscle enhancement upon exposure to mechanical loading (Cheema et al., 2005).

Although insulin-like growth factor (IGF) (Florini et al., 1991) and fibroblast growth factor (Tortorella et al., 2001) have been reported to be important factors within the growth-factor family, IGF seems to have a greater influence on myocytes. IGF is a polypeptide with a molecular weight of about 7 kDa. There are two forms of the IGF gene, IGF1 and IGF2, which are similar in structure to proinsulin. Both IGF genes promote the proliferation of various cell types (Florini et al., 1996).

Recent studies have shown that mechanical stretching of myocytes induces cell proliferation and influences the heavy chains of the muscle structural protein myosin (Kumar et al., 2004; Sakiyama et al., 2005; Kurokawa et al., 2007). Mechanical stress on myocyte not only promotes cell proliferation but also changes the characteristics of the cells. However, how these alterations occur and which molecular expression effects these changes remain largely unknown. Moreover there have been few reports regarding the time course of growth-factor expression after cells have been subjected to mechanical stretching.

Apoptosis during muscle development has recently

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drawn attention. Nakanishi et al. (2005) reported that myoblasts proliferate temporarily in large numbers in the embryonic stage, but that most of them die by apoptosis due to intracellular stresses involving the endoplasmic reticulum and mitochondria. Only those myoblasts strong enough to resist intracellular stresses and escape from apoptosis continue to the process of differentiation and maturation. Thus intracellular stresses are an important factor in the processes of muscle-cell proliferation and differentiation.

Our study focused investigated in detail of IGF-1 growth factor and intracellular stress-related caspase family expression in response to a mechanical stretching stimulus, using the C2C12 cell line derived from mouse myoblasts. For intracellular stress signaling, we studied caspase-12 for the role of the endoplasmic reticulum stress pathway, and two other caspases, caspase-9 and caspase-3, for involvement of the mitochondrial pathway.

MATERIALS AND METHODS

Cell culture

C2C12 cells, an established cell line originating from mouse skeletal muscle, were used in this study. Sub-culturing and proliferation of the cells were stable. Differentiation of C2C12 cells was induced using media with relatively low serum concentrations, such as serum-free media or media containing 2% horse serum. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Co., St. Louis, MO) containing penicillin-streptomycin solution (Sigma Aldrich Co.) and 10% fetal bovine serum (FBS) (ICN Biomedicals Inc., Aurora, OH). We used stretching medium containing 10% FBS. Myoblasts were incubated in a humidified atmosphere of 5% carbon dioxide at 37°C. C2C12 cells (1.0×10^5 cells/well) were cultured using type-1 collagen-coated 6-well BioFlex® culture plates (well diameter: 25 mm) (Flexcell international Corp., Mckeesport, PA). To attach these cells to the plates, cells were cultured in 2.0 ml of DMEM containing 10% FBS for a day. After 24h, the culture solution was exchanged, and the cultured cells were subjected to mechanical stretching. Four observation stages were established: Stage 1 (36 h after the start of culturing, or 12 h after the start of stretching), Stage 2 (12 h after Stage 1), Stage 3 (12 h after Stage 2), and Stage 4 (12 h after Stage 3). The culture solution was exchanged daily.

Mechanical stretching

Mechanical stretching was conducted using a Flexercell® strain unit (Flexcell International Corp.). C2C12 cells were subjected to stretching by the vacuum created under the membrane by a pump. The intensity of stretching was 15% in the maximally stretched area, and in this stretching stimulation, the membrane was stretched for 1 s and then relaxed for 1 s. This intermittent stimulation was repeated.

Counting cells

At each stage, cultured cells were immersed in trypsin-EDTA (Gibco BRL, Gland Island, NY) for 5 min. After the cells were detached, they were thoroughly soaked in culture solution and counted by using a hemocytometer. Fused cells were counted as one. Cells counts were performed eight times.

RT-PCR analysis

At each stage of culture, cells were harvested, mRNA was extracted by using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), and cDNA was prepared by using a Ready-To-Go Kit (Amersham Pharmacia Biotech UK Ltd.). After determination of the optimal PCR conditions for all primers, experiments were performed using a

LightCycler™ (Roche Diagnostics, Mannheim, Germany), which allows RNA quantification. Four primer sets designed from the sequences of the IGF-1 and caspase-12, -9, and -3 genes were used. Experiments were performed according to the standard protocol for the LightCycler™. Ready-to-use LC FastStart DNA Master SYBR Green I (Roche) was used as a hot-start PCR reaction mixture for the LightCycler™. A series of cDNA dilutions (4.0 ng/μl) including $1/10^5$, $1/10^6$, $1/10^7$, $1/10^8$, and $1/10^9$ were prepared. PCR reactions for the diluted standards contained 10.2 μl of sterile water, 5 μl of diluted control cDNA product, 1.6 μl of 25 mM MgCl₂, and 2 μl of LC FastStart DNA Master SYBR Green I containing SYBR Green I (1/60,000 dilution). In addition, 0.6 μl of each of forward and reverse primers designed by the software (Biogene Ltd.) were added to reach a final reaction volume of 20 μl for each tube. Primers based on sequences of the IGF-1 and caspase-12, -9, and -3 genes were designed from specific segments of the entire DNA sequence, and are shown in Table 1. Each PCR mixture (final reaction volume, 20 μl) contained 14.2 μl of sterile water, 1.6 μl of 25 mM MgCl₂, SYBR Green I (1/60,000 dilution), 2 μl of LC FastStart DNA Master SYBR Green I, 0.6 μl of forward primer (10 pmol/μl), 0.6 μl of reverse primer (10 pmol/μl), and 1 μl of target DNA. The PCR mixtures (20 μl each) prepared for caspase-12, -9, or -3 were added to the glass portion of capillaries. PCR conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 80 s. Gene amplification was performed according to a melting program of 70°C for 15 s, and fluorescence was continuously monitored at a rate of 0.1°C/s.

The amount of final expression of each caspase gene was obtained by dividing the caspase expression level by that of a housekeeping gene, GAPDH. The primer sequences for GAPDH are given in Table 1.

Table 1. Base sequences of the primers used in thts study.

IGF-1	Forward: 5'-TCGTCTTCACACCTCTTCTA-3'	
	Reverse: 5'-AAGCAACTCATCCACAAT-3'	Accession: NM_010512
caspase-3	Forward: 5'-GACCATACATGGGAGCAAGT 3'	
	Reverse: 5'-ATCCGTACCAGAGCGAGA-3'	Accession: AK014231
caspase-9	Forward: 5'-ATGGTCACGGCTTTGATGGA-3'	
	Reverse: 5'-TTCTCAATGGACACGGAGCA-3'	Accession: NM_015733
caspase-12	Forward: 5'-AGGATGATGGACCTCAGA-3'	
	Reverse: 5'-GCTGTCAGCATTAGATGTGA-3'	Accession: NM_009808
GAPDH	Forward: 5'-TGAACGGGAAGCTCACTGG-3'	
	Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'	Accession: NM_008084

Western blotting

Cells were collected, washed with ice-cold phosphate-buffered saline, and sonicated in the buffer (10 mM Tris-HCl buffer, pH7.4, containing 10 mM NaCl, 3 mM MgCl, 0.5% Nonidet P-40, and complete protease inhibitor cocktail; Roche Diagnostics, Mannheim, Germany) at 20 W for 1 min. The mixture was then incubated at °C for 30 min. of Proteins (10 μg per lane) were separated on a 12%

SDS-polyacrylamide gel and transferred onto an Immobilon P membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skimmed milk and incubated with anti-IGF-1 (1:1000), anti-caspase-12 (1:1000), anti-caspase-9 (1:1000), or anti-caspase-3 (1:1000) antibody. Antibody binding was detected with a horseradish peroxidase-conjugated anti-rabbit/mouse IgG antibody by using the ECL Plus System (GE Healthcare, Buckinghamshire, UK).

Statistical analysis

Student's t-test was used for statistical analysis, and a p value of <0.05 was designated as significant.

RESULTS

Effects of stretching on cell number

At 12 hours after the beginning of stretching, no significant difference was detected between the stretch and control groups. However, the number of the cells was significantly higher in the stretch group compared to the control

group at 24 hours and 36 hours after the onset of stretching (Fig. 1).

mRNA quantification

IGF-1 mRNA expression was significantly higher in the stretch group compared to the control group at 12 hours and 24 hours after the beginning of stretching. However, the expression level thereafter (at 36 hours and 48 hours after the onset of stretching) was similar in the stretch and control groups, without a significant difference (Fig. 2).

At 12 hours after the beginning of stretching, all mRNA expression of the caspases investigated was significantly higher in the stretch group compared to the control group. However, expression levels thereafter (at 24 hours, 36 hours, and 48 hours after the onset of stretching) were similar in the two groups, without significant differences (Figs. 3, 4, 5).

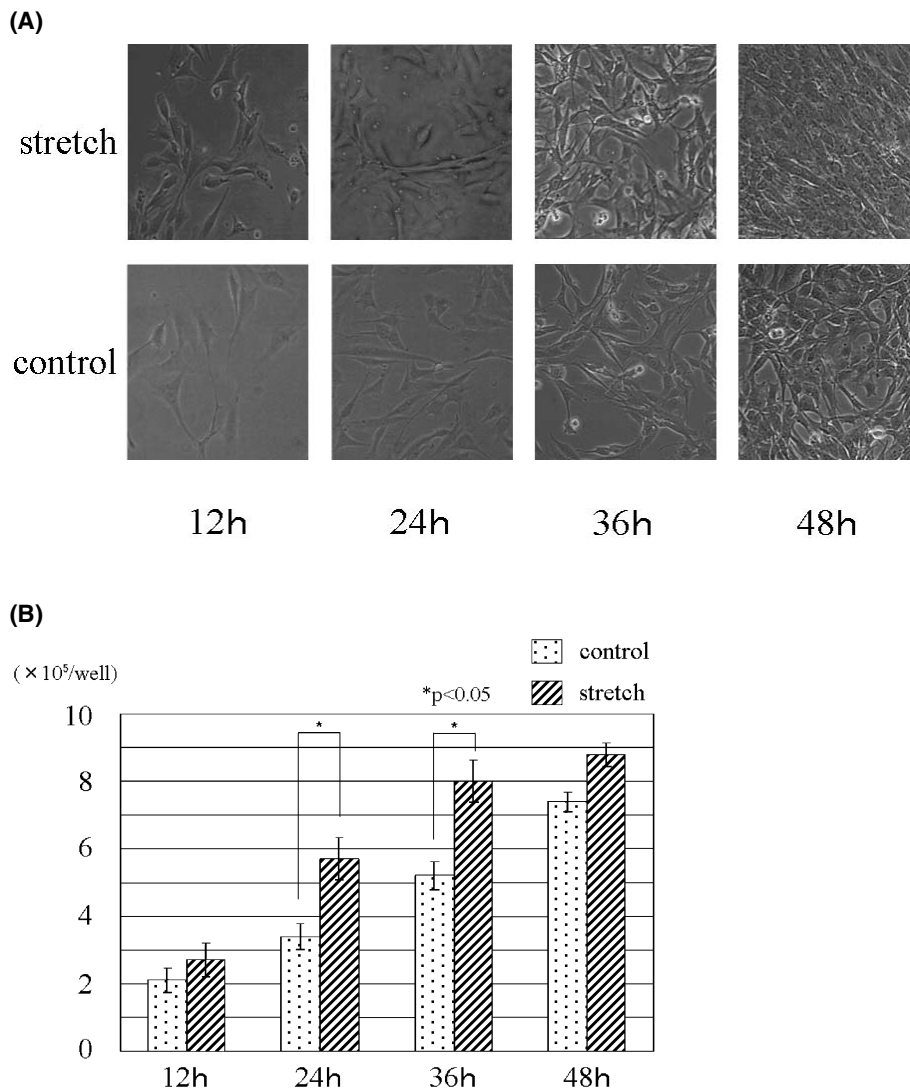


Fig. 1. (A) Phase-contrast microscopic image of myoblasts. (B) Changes in cell number in the stretch and control groups. Both the image and the graph indicate a sequentially higher cell number in the stretch group than in the control group. A significantly higher cell number in the stretch group than in the control group was detected at 24 hours and 36 hours after the onset of the experiment ($p < 0.05$).

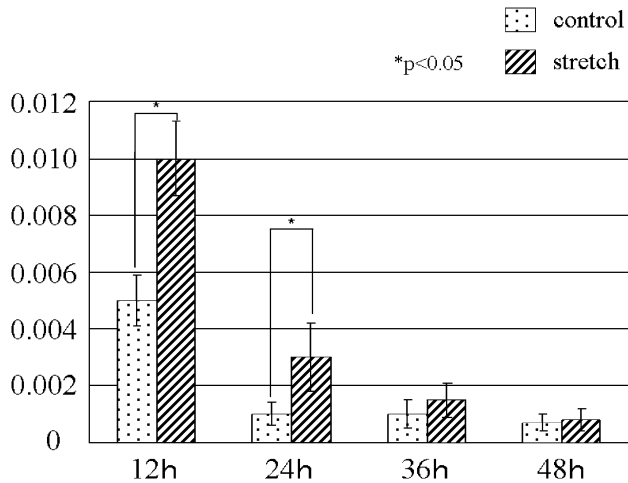


Fig. 2. Comparison of IGF-1 expression levels by LightCycler™ Real-Time RT-PCR. IGF-1 expression was significantly higher in the stretch group than in the control group at 12 hours and 24 hours after the onset of the experiment ($p < 0.05$); thereafter, no significant difference was detected between the groups.

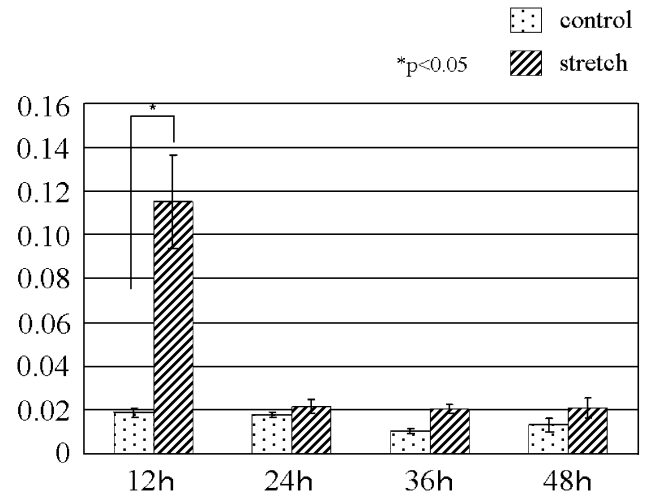


Fig. 4. Comparison of caspase-9 expression levels by LightCycler™ Real-Time RT-PCR. Caspase-9 expression was significantly higher in the stretch group than in the control group at 12 hours after the beginning of stretching ($p < 0.05$); thereafter, no significant difference was detected between the groups.

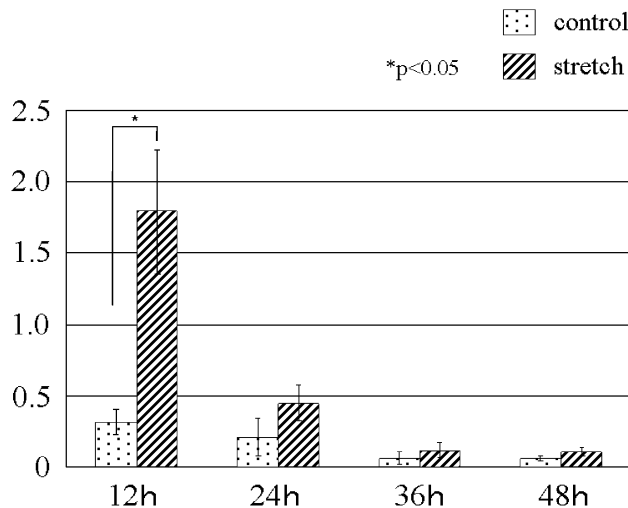


Fig. 3. Comparison of caspase-12 expression levels by LightCycler™ Real-Time RT-PCR. Caspase-12 expression was significantly higher in the stretch group than in the control group at 12 hours after the beginning of stretching ($p < 0.05$); thereafter, no significant difference was detected between the groups.

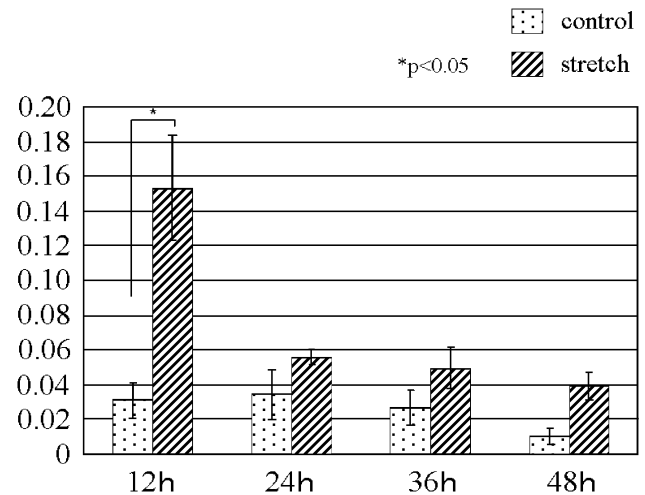


Fig. 5. Comparison of caspase-3 expression levels by LightCycler™ Real-Time RT-PCR. Caspase-3 expression was significantly higher in the stretch group than in the control group at 12 hours after the beginning of stretching ($p < 0.05$); thereafter, no significant difference was detected between the groups.

Western Blotting

Results of polyacrylamide gel electrophoresis of the stretch and control groups of myoblasts are shown in Fig. 6. The protein analysis revealed that IGF-1 was strongly expressed in the stretch group at 12 hours and 24 hours after the beginning of the stretching. However, no expression was noted after this time. In the control group, weak expression was detected at 12 hours after the beginning of the experiment, but no expression was detected thereafter. Strong expression of all caspases was revealed in the stretch group at 12 hours after the beginning of stretching (Fig. 6).

DISCUSSION

Muscle proteins, which contribute to the structure of myoblasts, normally show turnover through new synthesis and degradation (Okubo et al., 2006; Lee et al., 2006; Suzuki et al., 2007). Any imbalance between synthesis and degradation results in an increase or decrease in muscle volume; for example, promotion of the synthesis pathway or inhibition of the degradation pathway in myoblasts will cause an increase in muscle proteins. A muscle volume increase accompanied by loading enhancement could occur through either hypertrophy and proliferation of myoblasts, or acceleration of muscle protein synthesis. In fact, it has already been reported that a loading increase induces signalings pathway for protein syn-

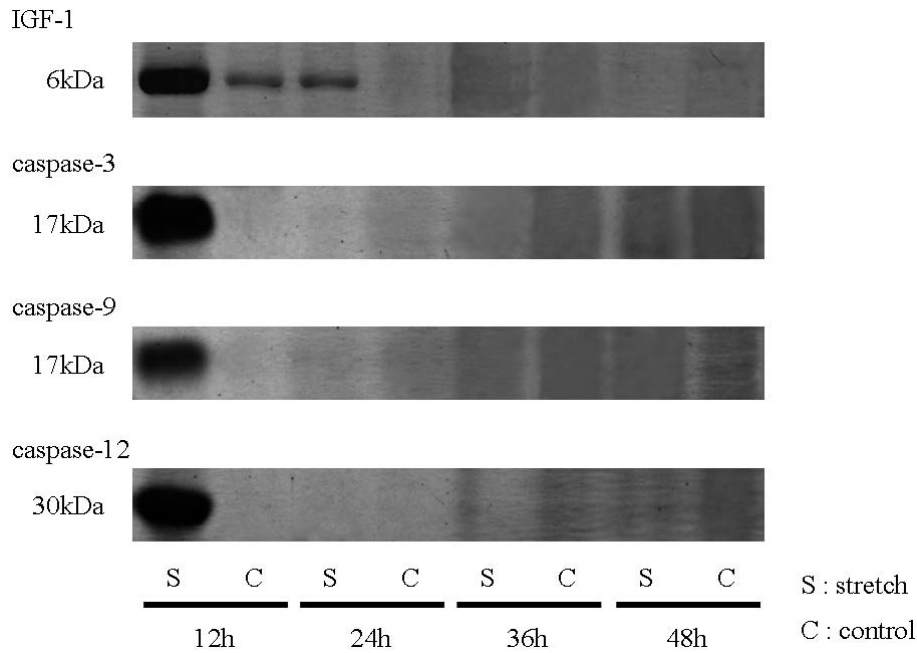


Fig. 6. Results of Western blotting. Strong IGF-1 expression was observed in the stretch group at 12 hours and 24 hours after the beginning of stretching; however, the expression was not remarkable after this time. In the control group, weak expression was detected at 12 hours after the beginning of the experiment, but no expression was detected thereafter. Strong expression of all the caspases was observed in the stretch group at 12 hours after the beginning of stretching, compared to the control group.

thesis (Kumar et al., 2004). Various factors that promote an increase in muscle volume upon mechanical loading have now been identified (Muscat et al., 1994; Sotiropoulos et al., 2006; Tortorella et al., 2001). One of these effectors, growth factor, has been found to influence myoblast hypertrophy (Cheema et al., 2005). From this study, induction of possibly another factor, which accelerates gene expression, occurred when myocytes were subjected to loading. Hsu et al. (1997) suggested that IGF-1 has a great influence on myocytes and plays an important role in their development and differentiation through induction of expression of muscle-specific transcriptional regulatory factors. However, no study has yet been published regarding the expression of either growth factors or muscle-specific transcriptional regulatory factors influencing differentiation and proliferation during mechanical stretching. Many relevant questions remain unanswered.

The caspases, an important family of proteases involved in multiple cascades, are the central executioner molecules of apoptosis (Alnemri et al., 1996; Friedlander et al., 2003). Activation of caspases occurs through death receptors on the cell membrane (Yonehara, 2002; Nagata, 1999; Sakamaki et al., 2002) and involves the endoplasmic-reticulum and mitochondria-mediated pathways. Caspase-12 is involved in apoptosis by the endoplasmic-reticulum stress pathway (Nakagawa et al., 2000; Nakanishi et al., 2005). Caspase-9 has been reported to play a role in apoptosis due to mitochondria-mediated stress (Zamzami et al., 1996). Moreover, activation of caspase-9 induces activity of a downstream molecule, caspase-3, which then executes apoptosis (Earnshaw et al., 1999).

Our study revealed that cell number in the stretch group at 24 hours and 36 hours after the beginning of the mechanical stimulus was significantly higher than in the control

group. This suggests that mechanical stretching enhances cell number. In the fused cells, expression of both IGF and caspase was similar to that in single cells. Although we did not observe the site of fusion of the cells, we suppose that any cell contains receptors for mechanical stimuli and internal organelles for the signaling reaction.

Our study also revealed higher expression of IGF-1 at 12 hours and 24 hours after mechanical stretching. However, no significant difference in expression was detected thereafter between the stretch and control groups. These results suggest that IGF-1 expression was induced just after mechanical stretching of the myoblasts, but later decreased to the previous level as cells became acclimated to the new environment.

Degeneration of myocytes was not observed in the early proliferation stage, possibly due to the strength of the stretching stimulus. Although caspase is an apoptosis-related protein, its expression does not mean execution of the apoptotic reaction. The amount of expression apoptotic molecules is directly related to the strength of the stimulus. Thus, any stimulus that is over a threshold leads to apoptosis. However, the threshold for the apoptotic reaction is not expected to be same for all cells. The apoptotic signal upon mechanical stress likely did not reach the threshold level for execution of apoptosis, which thus explains why apoptotic cells are rarely observed of in the early stages of the stimulus, even though the induction of caspase expression was observed.

All of the caspases we examined demonstrated higher expression in the stretch group than in the control group at 12 hours after the onset of the experiment. Interestingly the expression patterns of IGF-1 and the caspases were clearly similar. This suggests that while mechanical stimulus induces signals, such as IGF-1, for cellular proliferation, stretching simultaneously involves intracellular stress that leads to the

enhancement of caspase expression, which normally inhibits cell proliferation. Thus, regarding the processes of muscle differentiation and proliferation, we consider that mechanical stretching causes intracellular stress in the endoplasmic reticulum and mitochondria and results in the inhibition of cell proliferation. However, whether IGF and caspases co-express simultaneously in the same cell remains unknown, though we suppose that they have a simultaneous, parallel coordinated expression in the same cell, based on the simultaneous appearance and disappearance of their expressions upon mechanical stimulation. Further experiments such as confocal microscopic analysis with different labeling dyes or double immunohistochemistry, which were outside the purpose of the current research, should be performed.

In conclusion, our results suggest that application of a mechanical stimulus promotes the differentiation and proliferation of myoblasts in the early phase through increased expression of the growth factor IGF-1, while the same force also suppresses cell proliferation through intracellular stress signals.

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