Expression of Myosin Heavy-chain mRNA in Cultured Myoblasts Induced by Centrifugal Force

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Abstract

Ballistic muscle training leads to hypertrophy of fast type fibers and training for endurance induces that of slow type fibers. Numerous studies have been conducted on electrical, extending and magnetic stimulation of cells, but the effect of centrifugal force on cells remains to be investigated. In this study, we investigated the effect of stimulating cultured myoblasts with centrifugal force at different speeds on cell proliferation and myosin heavy-chain (MyHC) mRNA expression in muscle fiber. Stimulation of myoblasts was carried out at 2 different speeds for 20 min using the Himac CT6D, a desk centrifuge, and cells were observed at 1, 3 and 5 days later. Number of cells 1 and 5 days after centrifugal stimulation was significantly larger in the 62.5 g and 4,170 g stimulation groups than in the control group. Expression of MyHC-2b mRNA 1 day after centrifugal stimulation was significantly higher in the 2 stimulation groups than in the control group. Almost no expression of MyHC-2a was observed in any group at 1 and 3 days after centrifugal stimulation. However, 5 days after stimulation, MyHC-2a was strongly expressed in the 2 stimulation groups in comparison to the control group. Three days after centrifugal stimulation, expression of MyHC-1 was significantly higher in the 2 stimulation groups than in the control group. The results of this study clarified the effect of different centrifugal stimulation speeds on muscle fiber characteristics, and suggest that centrifugal stimulation of myoblasts enhances cell proliferation.

Key words: Myosin heavy chain—Myoblasts—Centrifugal force

Introduction

Recently, a number of studies have been conducted on the characteristics of skeletal muscle in athletes, and the structural and functional characteristics of muscle fibers
have been clarified in almost all athletic activities. Such studies have indicated that the top athletes exhibit muscle fiber structures suited to their particular athletic activities. For example, in athletes that participate in endurance sports, such as long distance running, cross-country skiing and swimming, the percentage of slow type fibers in the agonist muscles is markedly high, while the percentage of fast type fibers is high in short distance runners\(^8,11\). In athletes that participate in sports requiring rapid exertions of force or speed, such as throwing, jumping and powerlifting, the muscle fiber types are widely distributed, suggesting that other factors, such as technical and muscle strength factors, rather than muscle fiber structures, greatly affect those activities\(^17\). However, it is unclear whether such muscle fiber structures are acquired by exercise or are congenitally determined. Generally, it is a widely held view that ballistic muscle training leads to hypertrophy of the fast type fibers and training for endurance induces that of the slow type fibers.

There have been several in vitro studies on the effects of mechanical stimulation on myoblasts. Naumann and Pette administered electrical stimulation to cells obtained from rat hind leg and soleus muscles via culture medium and reported its effects on expression of myosin heavy-chain (MyHC) isoforms\(^10\). Sakiyama \textit{et al.} reported differences in myoblast proliferation and muscle fiber characteristics induced by extending stimulation\(^13\). Sakuraba \textit{et al.} examined the effects of magnetic stimulation of rat hind leg muscles on expression of mRNA by comparing stimulation and control groups\(^14\).

Methods of applying mechanical stress to cells include electric stimulus, distension stimulus, magnetic stimulus, hydrostatic stimulus and centrifugal stimulus. Centrifugal stimulus involves indirect application of mechanical stress by spinning of cultured cells in a centrifuge. It offers the advantage of being able to easily reproduce a state of constant centrifugal stress. In this study, we investigated the effect of stimulating cultured myoblasts with centrifugal force at different speeds on cell proliferation and expression of MyHC mRNA.

MyHC isoforms are closely related to muscle function\(^12\) and may determine the characteristics of muscles\(^2,3,5,7,15,16,18\). To determine muscle fiber characteristics, we selected expression of MyHC-2b, which is associated with the highest contraction rate, and MyHC-2a, which is associated with the lowest contraction rate.

**Materials and Methods**

1. **Culture methods**

Mouse skeletal myoblast line C2C12 was used. C2C12 is an established cell line derived from mouse skeletal muscle which can be subcultured. The proliferative ability and properties of these cells are relatively stable, even in a mass culture\(^19\). Furthermore, C2C12 cells can be induced to differentiate in culture media containing a low concentration of serum such as media containing 2% equine serum and serum free media\(^6\). The culture medium used in this study was Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich Co., St. Louis, MO), containing 10% fetal bovine serum (ICN Biomedicals Inc., Aurora, OH) supplemented with penicillin (1,000 units). Approximately \(3.0 \times 10^4\) C2C12 cells were seeded on 35 mm laboratory dishes with 2 ml culture medium and cultured with 5% carbon dioxide at 37°C for 2 days.

2. **Stimulation by centrifugal force**

Stimulation by centrifugal force was performed using the Himac CT6D (Hitachi, Tokyo, Japan) with an RT5SA rotor. On Day 2 of cultivation, the culture dish was centrifuged under two conditions of 62.5 \(\times\) g or 4,170 \(\times\) g for 20 min each without removing the 2 ml culture solution. Temperature was set at room temperature. Although these values do not occur within the living body, we believed it was unlikely that they would cause destructive cell damage, as these are the values commonly applied in the centrifugation of subcultures. A control group without centrifugal stimulation was also prepared. After centrifugal stimulation, the cells were cultured under
the same conditions. These experiments were performed 5 times. Cells were observed at immediately and 1, 3 and 5 days after centrifugal stimulation.

3. mRNA expression analyses

In order to visualize the effect of centrifugal stimulation on gene level, the LightCycler™ Instrument (Roche Molecular Biochemicals, Mannheim, Germany) was used to perform quantitative measurement of mRNA expression of MyHC-2b, MyHC-2a and MyHC-1 at each stage. Total RNA was extracted using the Quick Prep micro mRNA Purification Kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) before cDNA was created using the Ready-To-Go (Amersham Pharmacia Biotech UK Ltd.).

The primers for MyHC-2b, 2a and 1 were designed by selecting a unique sequence from the gene sequence of each isoform. The base sequences of the primers for each isoform were: MyHC-2b (Accession: XM_126119, Forward: 5’-ACAGACTAAAGTGAAGGAGG-3’, Reverse: 5’-CTCTCAACAGAAAGATGGGAGGAT-3’); MyHC-2a (Accession: NM-144961, Forward: 5’-CGATGATCTTGCCAGTAATG-3’, Reverse: 5’-ATAACTGAGATACCAGCG-3’); and MyHC-1 (Accession: AY056464, Forward: 5’-GTCGAGGGTGCCAGAAGGTT-3’, Reverse: 5’-CCACCTAAAGGGCTGTGTTG-3’). PCR was performed at 95°C for 10 min, 95°C for 10 sec, 62°C for 10 sec and 72°C for 7 sec for a total of 50 cycles. The amount of each MyHC isoform determined by the method described above was divided by amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the house-keeping gene, to determine the mRNA expression of each isoform. The base sequence of GAPDH was as follows: (Accession: NM_008084, Forward: 5’-TGAACGGGAAGCTCTCAGTGAGTG-3’, Reverse: 5’-TCCACCACCTGTTGCTGTA-3’). Each PCR fragment was verified to be a MyHC isoform using the ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Japan Applied Biosystem, Tokyo, Japan).

4. Statistical analysis

Statistical comparisons were made using a one-way analysis of variance (ANOVA). Tukey’s multiple comparison tests was used for further comparisons between occlusal areas (p<0.05), using SPSS® (SPSS Japan, INC., Tokyo, Japan).

Results

The number of cells 1 and 5 days after centrifugal stimulation was significantly larger in the 2 stimulation groups than in the control group (Fig. 1). In the 2 stimulation groups, the number of cells increased slightly with time.

The MyHC-2b mRNA level 1 day after centrifugal stimulation was significantly higher than the control group (Fig. 2).
the 2 stimulation groups than in the control group (Fig. 2). Three and 5 days after centrifugal stimulation, it was also significantly higher in the 4,170×g stimulation group than in the 62.5×g stimulation group. In all groups, the MyHC-2b mRNA level slightly decreased with time.

Subsequently, the MyHC-2a expression was lower on Day 1 and Day 3 than on Day 5 in all groups (Fig. 3). Five days after stimulation, the MyHC-2a expression was high in the 2 stimulation groups and significantly higher than in the control group. There was no significant difference in the MyHC-2a expression 5 days after centrifugal stimulation between the 62.5×g and 4,170×g stimulation groups, but it was slightly higher in the former.

The MyHC-1 levels became significantly higher in comparison to the control group on Day 1 for the 4,170×g group and on Day 3 for both centrifuged groups (Fig. 4). However, there were no significant differences in MyHC-1 expression 1, 3 and 5 days after centrifugal stimulation between the 62.5×g and 4,170×g stimulation groups.

**Discussion**

The fast type MyHC isoform includes MyHC-2b and MyHC-2a, and the slow type includes fiber MyHC-1. The muscle fibers that express MyHC-2b, MyHC-2a and MyHC-1 are respectively referred to as type II-B, II-A and I fibers.

In this study, the number of cells at 1 and 5 days after was significantly larger in the 2 stimulation groups after centrifugal stimulation than in the control group, suggesting that the differentiation of myoblasts was enhanced by centrifugal stimulation. This result is consistent with the findings of a study by Sakiyama et al. demonstrating the enhancement of myoblast differentiation induced by extending stimulation as observed by electron microscopy.

In addition, the level of MyHC-2b, which had the fastest contraction rate among the Fast Type isoforms, was retrieved in order to observe any differences in muscle fiber characteristics. It substantially increased immediately after centrifugation with a larger load, but decreased thereafter. We believe that, even though the cultured myoblasts temporarily expressed MyHC-2b, which was the isoform with the strongest contraction due to centrifugal stimulation, the MyHC-2b levels decreased thereafter due to continuous stimulation, because the isoform was not suited to undergoing continuous stimulation. MyHC-2a, which had the slowest contraction rate among the Fast Type isoforms, had the high levels of expression when lower loads were applied during centrifugal stimulation.

It has been reported that differing training methods, such as endurance, sprint and muscle exercises, have different effects on muscles in experiments using animals and humans. The effects of endurance training have been
studied most frequently and the following muscle changes have been reported: Histologically, the percentage of muscle fibers with a high level of oxidizing ability is elevated by relative increases in the ratio of type IIA fibers to type IIB fibers. Furthermore, selective hypertrophy of type I or type IIA fibers is observed. Gollnick \textit{et al.} reported that the area of slow type fibers was increased in healthy males by 23\% following 5 months training using a bicycle ergometer at a relatively low load\cite{4}. Andersen \textit{et al.} reported that an 8 week cycling program using a bicycle ergometer at a relatively low load did not lead to changes in the percentage of type I fibers, but significantly increased that of type IIA fibers and decreased that of type IIB fibers in 20 to 23 years old adults. They also reported that the area of type I fibers was not changed, but the areas of types IIA and IIB fibers were increased\cite{1}. In addition, in research concerning cells, Müller reported that slow type fibers were significantly increased in the soleus muscles by endurance training in rats\cite{9}. These 3 reports closely correlate with the results of this experiment, in which the MyHC-2a and MyHC-2b expression increased with the lower load of 62.5\texttimes g and 4,170\texttimes g.

Thus, earlier reports have revealed that training changes the percentage of muscle fibers, \textit{i.e.}, converts the fast type fibers to slow type, and vice versa. The results of this experiment suggest that the application of stress on cells at the level of the myosin heavy chain, in which these muscle fibers are expressed, thus potentially has some effect on the future percentage of muscle fibers.

\textbf{Conclusion}

In this study, cell proliferation was enhanced by centrifugal loading on myoblasts.

Furthermore, the results suggest that such enhanced cell proliferation potentially has some effect on the percentage of muscle fibers.

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