Type I muscle atrophy caused by microgravity-induced decrease of myocyte enhancer factor 2C (MEF2C) protein expression

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Abstract To investigate the molecular mechanisms of muscle atrophy under microgravity, the paraspinal muscles of rats after 14 days spaceflight and those of ground-based controls were examined. In the microgravitational environment, expressions of 42 genes changed, and the expressions of heat shock protein 70 and t complex polypeptide 1 increased. In Northern blotting, myocyte-specific enhancer binding factor 2C (MEF2C) and MEF2C-related genes including aldolase A and muscle ankyrin decreased. After 9 days ground recovery, expression of MEF2C increased and it was located mainly on the satellite cells in the muscle regeneration state. MEF2C could be a key transcriptional factor for skeletal muscle atrophy and regeneration under microgravity.

Key words: Microgravity; Muscle atrophy; Differential display; Myocyte-specific enhancer binding factor 2C

1. Introduction

Exposure to a microgravitational environment is thought to induce morphological, biochemical, and molecular changes to the human body. The United States and the Soviet Union have conducted several trials to study animal and plant biology in space. As a result, skeletal muscles showed remarkable changes during spaceflight [1]. For example, the greatest level of atrophy was observed in the soleus muscle, a kind of slow-twitch anti-gravity skeletal muscle [2], and many slow-twitch muscle fibers presented certain degrees of phenotypic changes associated with conversion from slow to fast muscle fibers [3]. In contrast, the fast-twitch muscle, e.g. extensor digitorum longus, received less effects [4]. Biochemically, there was a reduction of mitochondrial enzyme activities measured in whole muscle homogenate, though the activities of oxidative enzymes measured in single muscle fibers were usually maintained or even elevated [3,5]. In rabbits, immobilization of anti-gravity muscle for more than 3 days induced muscle atrophy, accompanied by the appearance of abnormal mitochondria and sarcoplasmic changes [6].

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We found that the paraspinal muscle of rats after 14 days spaceflight remarkably decreased the slow Type I myosin heavy chain (MHC), muscle fiber number and muscle volume in comparison to those of synchronous ground control rats. As there have been little genetic studies on the effects of microgravity, we examined the alteration of the muscle gene expression under microgravity using the differential display RT-PCR (DDRT-PCR) method. The aim of this study is clarified to molecular changes in skeletal muscles after spaceflight.

2. Materials and methods

2.1. Animal preparation

This study was a part of the Spacelab Life Sciences 2 (SLS-2) program, the second dedicated life science research mission. The study's animal care and experimental methods were approved by the National Aeronautics and Space Administration of Japan. All experimental procedures were in compliance with the guideline for the Care and Use of Animals described in the American Journal of Physiology [7]. Twenty male Sprague-Dawley rats were used in this study, and they were randomly divided into four groups as described elsewhere [8,9], i.e. R0F, R0C, R9F, and R9C. The two groups, R0F and R9F, were aboard the space shuttle 'Columbia' (Space Transportation System-58; October 18 to November 1, 1993), and returned to Dryden Airforce Base after 14 days of spaceflight. The other two groups, R0C and R9C, were on earth for 14 days. The five R0C rats were used as the synchronous control for the R0F rats. In the same manner, five R9F rats were killed 9 days after the landing, and five R9C rats were synchronous controls for the R9F rats.

2.2. Immunohistochemical analyses and confocal immunofluorescence microscopy

Each paraspinal muscle sample was sectioned into 10 μm thick slices using a cryostat. Anti-MHC (slow) mAb (Medac, Hamburg, Germany) and anti-MHC (fast) mAb (Medac) were used to identify the cells containing the slow Type I and fast MHC protein isoforms, respectively. We prepared anti-MEF2 family rabbit serum against MEF2A, MEF2B, MEF2C and MEF2D. Each antibody recognized the respective carboxy terminus (amino acids 471–498 of mouse MEF2A, amino acids 340–365 of mouse MEF2B, amino acids 439–466 of mouse MEF2C and amino acids 478–506 of mouse MEF2D). After incubation with the primary mAb at 1:100 dilution for 60 min, the tissue sections were rinsed in TBS solution, incubated with biotinylated second anti-mouse IgG (Cappel Research Products, Durham, NC, USA) at 1:300 for 30 min, rinsed with the TBS solution, and stained using an ABC kit (Vector Laboratories, Inc. Burlingame, CA, USA) and diaminobenzidine. Confocal immunofluorescence microscopy examinations were performed according to our methods [10,11]. In short, each paraffin section was coincubated for 1 h with anti-MHC (slow) mAb (diluted 1:200) and with anti-MEF2 rabbit

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serum (1:100) at room temperature. The immunoprecipitation method and peptide tests were used to show that the anti-MEF2C antibody was specific for MEF2C and did not recognize MEF2A, 2B or 2D. The section was rinsed well in phosphate-buffered saline (PBS) and then fluorescein isothiocyanate (FITC)-conjugated Ab, i.e. goat IgG fraction to mouse IgG (dilution 1:250, Cappel) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated Ab, i.e. horse IgG fraction to rabbit IgG (diluted 1:250, Cappel), were added and incubated for 30 min at room temperature. Negative control samples were treated similarly but were not exposed to the first antibody. Confocal laser scanning microscopy (Fluoview FV500; OLYMPUS, Tokyo, Japan) was used to determine the intracellular localization, and protein expression using a two-color scanning program and a three-dimensional image reconstruction program (MicroVoxel, version 2.2, Indec System, Capitola, CA, USA).

2.3. RNA preparation

Some pieces of rat paraspinal muscles were homogenized with 1 ml of Trizol (Gibco, Grand Island, NY, USA) per 100 mg of the sample. It was rigorously shaken after adding 0.2 volume of chloroform. The phase separation was completed, then the top aqueous phase was replaced into the new Eppendorf tube, and it was mixed with an equal volume of isopropanol to precipitate RNA. After washing with 75% ethanol, the RNA pellet was suspended in DEPC-treated water, and it was treated with DNase I for 30 min at 37°C to remove DNA contamination.

2.4. Differential display analysis (DDRT-PCR)

For the differential display analysis, we used the Differential Display Kit™ (Display Systems, LA, USA) with modifications. Briefly, 300 ng DNA-free total RNA was used for the reverse transcription reaction (final volume, 20 μl) with 7.5 units of AMV reverse transcriptase XL, 20 μM dNTP, and 2.5 μM downstream primer which is oligo(dT)primer 5′-T11VV-3′ (where V represented A, C, or G) for 10 min at 30°C, 30 min at 55°C, 5 min at 95°C, and 5 min at 5°C. 1 μl of these first cDNA was added with 10 units of Taq DNA polymerase in the presence of 2 μM dNTP, 2.5 μM downstream primer, and 0.5 μM upstream primer which is 10 bp random primer, then PCR reaction was performed (final volume, 20 μl) at 94°C, 30 s at 40°C, 2 min at 72°C, 30 s for 40 cycles at 72°C, and 5 min at 4°C. Samples were run on 12% native acrylamide gels and the gels were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA).

2.5. Sequencing of differentially expressed bands

Differentially expressed bands were cut and each fragment was eluted by boiling it in 100 μl of double distilled water, and re-amplified in the PCR reaction using the same set of primers. Re-amplified DNA fragments were cloned into the T-Easy vector (Takara, Tokyo, Japan). In short, the DNA fragment was ligated to the T-Easy vector with ligase for 15 h at 4°C. After the transformation was done to the competent cells, it was plated on an LB plate containing X-gal and IPTG. The whitish colonies were picked up and cultured with LB liquid. Then miniprep was conducted, and the obtained plasmids were sequenced using M13 reverse and M13 forward primers with a DNA sequencer model 373A (Perkin Elmer, Foster City, CA, USA). Comparison of DNA homology with the GenBank databases was performed using BLAST.

2.6. Northern blot analysis

20 μg of total RNA was resolved on 1% formaldehyde agarose gel, and the RNA was transferred onto nitrocellulose membrane using a capillary transfer technique. The blots were prehybridized in a formamide-based buffer at 65°C for 2 h. The random primed labeled denatured probe that was generated from the DNA fragment was added at a concentration of 1×10^6 cpm/ml, then the blots were incubated overnight at 65°C, and washed at increasing stringency up to 0.1×SSC/0.1% SDS at 60°C. Membranes were then washed twice with 1×SSC/0.1% SDS, and with 0.1×SSC/0.1% SDS before autoradiography.

2.7. Electrophoretic mobility shift assay (EMSA)

The MEF2C sequences of the sense strands of the oligonucleotide probes were as follows: muscle creatine kinase MEF2 site probe, GATCGCTCTAAAAATAACCCTGTCG; mutant mutant probe, GATCGCTGTAAACATAACCCCTGTCG [12]. MEF2C and its mutant oligonucleotides were annealed and labeled using polynucleotide kinase (Boehringer Mannheim, Germany) and [^{32}P-γ]ATP. 1 μg of labeled MEF2C nucleotide was incubated with 2 ml of in vitro translated MEF2C protein, for 30 min at room temperature in a 20 μl reaction mixture containing 20 mM HEPES (pH 7.9), 0.5 mM EDTA, 2 mM DTT, 10% glycerol, 50 mM KCl and 2 mg of poly dI:dC. Samples were loaded onto a 5% non-denaturing polyacrylamide gel and run in a 0.5×TBE buffer at room temperature. Gels were dried and exposed to Kodak XAR film overnight, with an intensifying screen at −80°C.

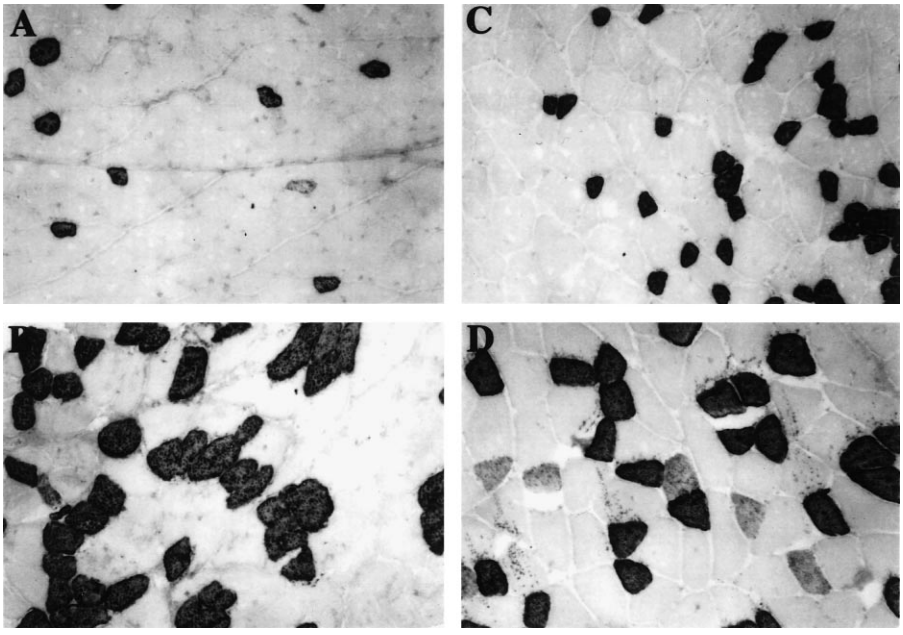

Fig. 1. Immunostaining of myosin heavy chain Type I (slow). Serial tissue sections of rat paraspinal muscle were stained with monoclonal antibodies specific to the slow Type I myosin heavy chain (MHC) protein isoform. A: A rat in the R0F group (14 days spaceflight). B: A rat in the R0C group (ground control). C: A rat in the R9F group (14 days spaceflight and 9 days recovery). D: A rat in the R9C group (14 days plus 9 days ground control).

3. Results

3.1. Changes in the slow Type I myosin heavy chain expressions

The present study examined the paraspinal muscles of 20 rats. The paraspinal muscle is one of the anti-gravity muscles and is composed of both slow-twitch and fast-twitch muscle fibers. The paraspinal muscles (Th5–10) of four rat groups, i.e. R0F, R0C, R9F and R9C, were examined with myosin heavy chain (MHC) monoclonal antibodies of Type I (slow) and Type II (fast). In the 14 days spaceflight rats (R0F, Fig. 1A), remarkable decreases were found in the slow Type I MHC, muscle fiber number, and muscle volume in comparison to those of ground control rats (R0C, Fig. 1B). After placing the spaceflight rats under the one gravity environment for 9 days (R9F group), the number of the slow Type I MHC recovered (Fig. 1C) to an equivalent level in the R0C rats (ground controls). Type I muscle fibers, which did not show any marked differences in the ratios of Type I and Type II in muscle tissue when compared with the R9C ground control rats (Fig. 1D), remained small in size. These observations showed that microgravity induced slow-twitch muscle atrophy in the rat's paraspinal muscle. In contrast, the fast Type II MHC increased after the 14 days spaceflight, indicating that there was a phenotypic change of slow to fast muscle fibers (data not shown).

3.2. Genetic effects of microgravity

Previous morphological studies reported similar findings on the other skeletal muscles [1,3], but there have been no genetic studies on the effects of microgravity. The present study examined the alteration of the muscle gene expression under microgravity using the differential display RT-PCR (DDRT-PCR) method [13], i.e. a proper method for the identification of the actual differences between two well-defined biological situations. We performed DDRT-PCR for the paraspinal muscles of R0C (ground controls) and R0F (spaceflight rats), and obtained 42 PCR fragments which lengths were 200–500 bp as expected. We then sequenced all of these PCR fragments and compared their DNA homology with the GenBank and the EMBL databases using BLAST. Among these samples, some fragments had a high homology score (Table 1).

To clarify the difference of mRNA expressions, we conducted Northern blot analysis using each PCR fragment as a probe. The mRNAs were obtained from the samples of eight rats (two from each of the four groups). The genes of 70 kDa heat shock protein (HSP70), Tcp-1 (t complex polypeptide 1, Fig. 2B), and some kinds of mitochondria-related proteins, e.g. mitochondrial adenine nuclease translocator, and mitochondrial cytochrome oxidase subunit, were increased in the expressions after the 14 days spaceflight. In contrast, myocyte enhancer factor 2C (MEF2C) (Fig. 2A), muscle ankyrin, and aldolase A (Fig. 2B) were decreased in the expressions. Tcp-1

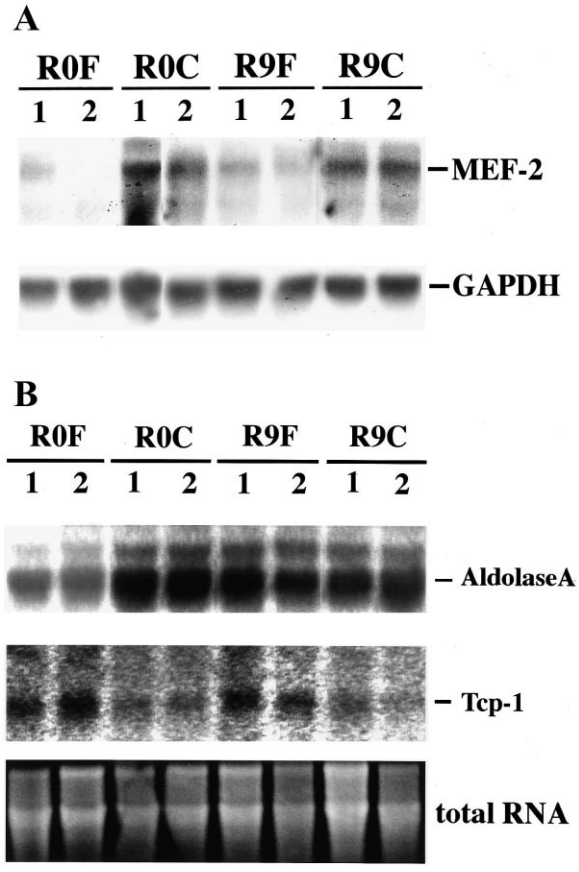

Fig. 2. Northern blotting of MEF2C (A), Tcp-1 and aldolase A (B). Two rats from each of the four groups were used. Expression of MEF2C mRNA in the R0F and R9F rats decreased in comparison to R0C and R9C rats. Expression of aldolase A mRNA in the R0F rats decreased in the same manner as MEF2C, but that in the R9F rats was recovered. Expression of Tcp-1 mRNA in the R0F and R9F rats increased.

Table 1
Differentially expressed genes between spaceflight rats and control rats

Downstream primer	Upstream primer	Effect of spaceflight	Corresponding rat genes	Homology (%)
T11GG	GTTTTCGCAG	up	mitochondrial adenine nucleotide translocator	95
T11GG	TACAACGAGG	up	mitochondrial cytochrome oxidase subunit	98
T11GG	CTTTCTACCC	up	mitochondrial cytochrome oxidase subunits I, II, III	100
T11GG	CTTTCTACCC	up	Na/K ATPase B2 subunit	95
T11GC	GATCATGGTC	up	70 kDa heat shock protein (HSP70)	99
T11AC	CTGCTTGATG	up	complex polypeptide 1 (Tcp-1) mRNA	100
T11AC	TTTTGGCTCC	up	muscle LIM protein	96
T11GG	TACAACGAGG	up	myosin light chain	100
T11GC	GATCCAGTAC	up	mouse mRNA for dexamethasome induce products	98
T11AG	GATCATGGTC	down	mouse muscle mRNA for cyclin B2	86
T11GC	CTGCTTGATG	down	muscle Ank-1 mRNA	100
T11GC	CTGCTTGATG	down	MEF2C (myocyte-specific enhancer binding factor)	94
T11AC	GATCGCATTG	down	aldolase A	98
T11GC	CTGCTTGATG	down	placental pre-GHRH	77

These genes were searched in the GenBank and EMBL databases using BLAST.

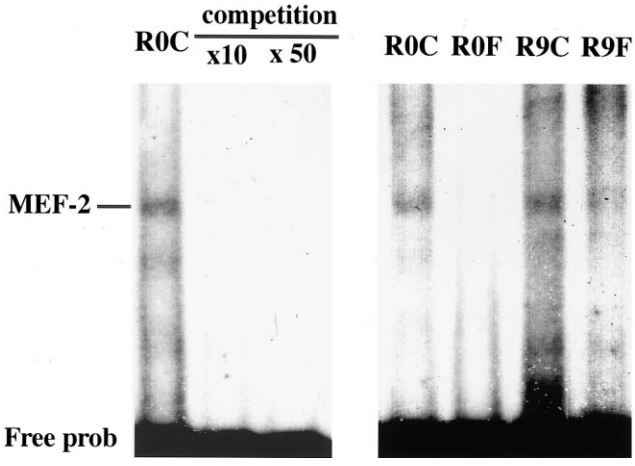

Fig. 3. Electrophoretic mobility shift assay for MEF2C. 5 μg of nuclear protein from the muscle of one rat in each of the four groups was used. MEF2C protein in nuclei decreased after the 14 days spaceflight, but the nuclear MEF2C protein increased during the following 9 days on earth.

and HSP70 are thought to be related to cytoplasmic chaperonin under some stress conditions [14], and their mRNA expressions increased more in the R0F rats than in the R0C (control) rats. This shows that spaceflight, i.e. microgravity itself or the dynamic changes of gravitation, had a great biological stress on the body.

Among these genes which expressions decreased by the spaceflight, MEF2C is an essential element of muscle enhancers and promoters, and it is directly involved with nuclear activity in the muscles. We presumed that MEF2C has effects on atrophy and the regeneration of muscles. To examine nuclear import and activation of MEF2C protein in the muscles of R0F/R9F (spaceflight rats) in comparison to R0C/R9C (ground controls), the electrophoretic mobility shift assay (EMSA) was performed. The nuclear protein in the paraspinal muscles of R0F rats decreased in comparison to R0C rats (both R0F and R0C were killed 5 h after landing), but that of the R9F rats (spaceflight with 9 days recovery) increased (Fig. 3). In order to confirm these results, we examined whether the expression and distribution of MEF2C protein in rat paraspinal muscles was related to the alteration of MHC under microgravity by using a laser confocal immunofluorescence microscopy. MEF2C localized mainly around muscle fibers, and this place was consistent with the location of satellite cells which are presumed to be the sole source of new myonuclei, and a decrease of satellite cell proliferation could reduce myonuclei formation and result in an overall decrease of myonuclei number. In the R0F rats (Fig. 4A), MEF2C (red staining in nuclei) was weakly expressed, and Type I MHC muscle fibers (green stained muscle fibers) were decreased and atrophied in comparison to those of the R0C rats (Fig. 4B). However, an increase of MEF2C protein in myonuclei was found around the increased Type I MHC muscle fibers in R9F rats (Fig. 4C). The R9C ground control rats showed thick Type I MHC muscle fibers and strongly expressed MEF2C in the myonuclei (Fig. 4D). These results indicated that microgravity suppressed MEF2C production and induced the decrease of nuclear import, and recovery of gravity resulted in the increase of MEF2C protein level.

4. Discussion

Many studies using spaceflight rats have been conducted on enzymatic and morphological aspects. For example, in the skeletal muscles under microgravity, α-glycerophosphate dehydrogenase and succinate dehydrogenase activities in each

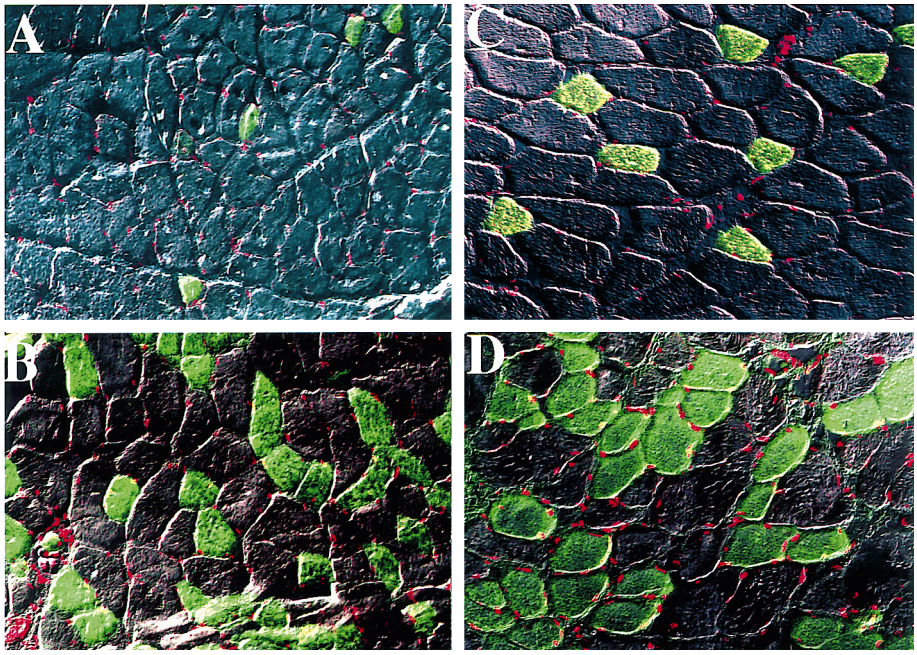

Fig. 4. Confocal immunofluorescence microscopy for rat muscle cross-sections. Representative sections of paraspinal muscle from each rat group were doubly stained with anti-Type I myosin heavy chain (MHC) antibody and anti-MEF2C antibody. In the R0F rats, MEF2C clearly decreased and Type I MHC muscle fibers also decreased and markedly atrophied, in comparison with those of the R0C rats. MEF2C was stained red by TRITC and MHC Type I green by FITC. A: R0F. B: R0C. C: R9F. D: R9C. (Magnification ×300.)

fiber were maintained or elevated, but cytochrome *c* oxidase enzyme activity was significantly reduced [1,2]. Morphologically, previous studies demonstrated that spaceflight resulted in a shift in MHC expression from Type I to Type II in the muscles where slow fiber, the vastus intermedius, is predominant, but not in the vastus medialis and lateralis [3,5,15]. In addition, reduction of MHC Type I and increase of MHC Type II were shown in the rat soleus muscle fibers [16].

Similarly our data of the paraspinal muscles of the spaceflight rats suggested the shift in the fiber distribution from slow (Type I) to fast (Type II). In contrast, Riley et al. reported regeneration of muscle fibers occurred at 14 days after the landing [17]. To clarify the mechanism of these gravity-induced muscle changes, the hindlimb suspension model, i.e. the microgravitational model on the earth, was often utilized, in which the rat soleus muscle is used after 21 days hindlimb suspension. The suspended soleus muscles showed a shift in the MHC isoform distribution with a marked increase in the relative amount of Type II MHC and a corresponding decrease in Type I MHC [4]. Recently, Geary et al. [18] described microgravity increased myogenic tone of the cerebral artery partially through a nitric oxide synthase-dependent mechanism. However, it has not yet been known whether genetic mechanisms of these muscle change during spaceflight.

In the present study, we found 42 genes changed during spaceflight, and demonstrated that the expressions of HSP70 and Tcp-1, which are stress-induced chaperone proteins, were markedly elevated by spaceflight. The Tcp-1 directs the folding of cytoskeletal proteins such as tubulin, actin and centractin, and it is involved in the nucleation of microtubules [19,20]. HSP70 binds to microtubules and protects the centrosome and intermediate filaments during heat shock [21]. Spaceflight brought stronger biological stress on the body than microgravity itself or the dynamic changes of gravitation. The fact that the expressions of HSP70 and Tcp-1 mRNAs in the muscles increased during the spaceflight period could represent a reaction of the organism to the stress that is superfluous for the body.

In general, microgravity in spaceflight produces both general and gene-specific decreases in mRNA levels [22]. In the present study, muscle aldolase A and muscle ankyrin expressions decreased significantly during spaceflight. The muscle-specific promoter of aldolase gene is expressed in fiber type-dependent and muscle location-dependent manner [23]. Ankyrin was also involved in organizing the triad and in immobilizing integral membrane protein in the T-tubes and sarcoplasmic reticulum of rat skeletal muscles [24]. As a mechanism of muscle atrophy due to microgravity, changes in the transcription rate due to the exposure to microgravity were thought to be involved in the reduction of fiber size. However, there must be some specific factors that change under microgravity, because the 'slow to fast' change of the muscle fibers cannot be explained by the general decrease of mRNA. A variety of DNA sequence motifs that are required for muscle-specific gene transcription has been identified in many genes. These include the E-box, N-CAT-box, CarG-box and MEF2 site that serve as binding sites for the myogenic basic helix-loop-helix MyoD1 family proteins, TEF1, serum response factor and MEF2 protein, respectively [25]. It is known that MEF2C itself binds to the promoter of aldolase A or muscle ankyrin, and regulates its expression [26].

In the present study, mRNA expression and protein production of MEF2C decreased dramatically under microgravity, and they returned to normal levels after a 9 days recovery period following spaceflight. These expression and production were located on the satellite cells around the muscle fibers. It was interesting that protein expression tended to recover more rapidly than MEF2C RNA expression after the spaceflight. The reason for this was not clear but two possibilities can be considered. One is that RNA expression appeared rapidly and then decreased within 3 h after the spaceflight, and the other is that MEF2C present in the cytoplasm rapidly migrated into the nuclei in response to gravity and before recovery of RNA expression.

MEF2C is a member of the MEF family of MADS (MCM1, agamous, deficiens, serum response factor)-box transcription factors which bind to an A-T rich DNA sequence associated with muscle-specific genes, and which potentiate the activity of myogenic bHLH factors [27–29]. The MEF family consists of 2A, 2B, 2C and 2D. We prepared specific antibodies for each member of the family, and found that 2A and 2D showed the same staining results as 2C in immunostaining. However, MEF2C showed the most marked decrease in expression of Type I MHC atrophied muscle in the R0F group (data not shown). The band observed in the gel shift assay in Fig. 3 showed a supershift only for the anti-MEF2C antibody (data not shown). These results indicated that 2C exhibits the most potent response to gravity in the MEF family. In mice, MEF2C is an essential regulator for cardiac myogenesis and right ventricular development [30]. We found that expressions of aldolase A and ankyrin mRNAs in the spaceflight rats decreased, and these could occur in consequence to MEF2C decrease. Because the expression of the desmin is controlled by MEF2 [31], we examined the changes in desmin production in Type I muscle atrophy using immunostaining. The results showed decreased desmin expression as well as ankyrin and aldolase A in Type I muscle atrophy during spaceflight and this expression increased with recovery after the spaceflight (data not shown). In the future, a study on adenosine monophosphate deaminase (AMPD), which is expressed predominantly and also controlled by MEF2 family [32] and shows different expressions in Types I and II, will be necessary. A recent study showed MEF2C participates in LPS-induced c-Jun expression through p38-induced phosphorylation [33]. This indicated that MEF2C was not only an inflammation response gene but also a stress response gene, and stress includes the effect of gravitation. Therefore, gravity is thought to control MEF2C expression directly through the degeneration and repair of the muscles. We showed that satellite cells under the muscle fiber repair express MEF2C protein, and this could be the initiation of muscle differentiation.

Because of the recent increase of patients with ischemic cerebral failure, necessity of starting rehabilitation in the early disease stage is emphasized [34]. It is important to protect the occurrence of atrophy in disused muscle in order to maintain or regain the levels of physical capacity and activities of daily living (ADL). MEF2C and its related genes, which we examined in the present study, are important in muscle differentiation, maturity and maintenance of functions. Although there are differences between gravity-free conditions and atrophy in disused muscle control of the expression of these genes should contribute to clarification of the molecular mechanism of atrophy in disused muscle. Spaceflight produces the condition called skeletal unloading which also occurs in bed-ridden pa-

tients and which causes serious bone problems. In the future, an agent that regulates MEF2C expression could be used during spaceflight as well as in the therapy of Type I muscle atrophy, and this would be a new approach to the management of muscle atrophy in bed-ridden patients.

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