

"Myo Lab": A JAXA Cell Biology Experiment in "Kibo (JEM)" of the International Space Station

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Abstract

Skeletal muscle atrophy is an often-unavoidable response to a prolonged lack of muscle use or spaceflight (referred to as "unloading"), and presents a particular challenge for astronauts during spaceflight. An increased understanding of the molecular mechanisms of muscle atrophy may lead to the development of effective therapies to combat this widespread condition. In this project, we aim to elucidate the molecular mechanisms of microgravity-induced skeletal muscle atrophy, especially physiological relevance of Cbl-b ubiquitin ligase. Japanese experimental module (JEM), "Kibo", in International Space Station has been constructed until June, 2009. This enables us to perform cell culture experiment, which is an easy system to observe direct effects of microgravity on the cell, in space for a long term. This project is called "Myo Lab", which means "Laboratory for Skeletal Muscle". This decal images our dream (space shuttle) that flight beyond Earth forward to "Kibo". In addition, the flame from shuttle means skeletal muscle. ©2009 Jpn. Soc. Biol. Sci. Space; Article ID: 092304020

Key words; Cbl-b ubiquitin ligase, DCC (disposal cell culture) plate, microgravity, rat L6 myotubes

Introduction

Skeletal muscles are vulnerable to rapid and marked atrophy under microgravity conditions (Ikemoto *et al.*,

2001). Unfortunately, despite the fact that almost of all astronauts are afflicted by debilitating atrophy, no treatment besides training exists to halt or reverse its progression, besides training. An increased understanding of the molecular mechanisms of muscle atrophy may lead to the development of effective therapies to combat this condition.

The muscle atrophy caused by microgravity is characterized by both decreased responsiveness to myogenic growth factors (e.g., insulin-like growth factor 1 [IGF-1] and insulin) and increased proteolysis (Ikemoto *et al.*, 2001; Ogawa *et al.*, 2006; Nakao *et al.*, 2009). Previously we showed that simulated microgravity conditions, such as tail-suspension and three dimensional (3D)-clinorotation, resulted in skeletal muscle atrophy through the induction and activation of the ubiquitin ligase Cbl-b (Nikawa *et al.*, 2004; Nakao *et al.*, 2009). Upon induction, Cbl-b interacted with and degraded the IGF-1 signaling intermediate IRS-1. In turn, the loss of IRS-1 activated the FOXO3-dependent induction of atrogenin-1/MAFbx, a dominant mediator of proteolysis in atrophic muscle. Furthermore, Cbl-b-deficient mice were resistant to tail-suspension-induced muscle atrophy and the loss of muscle function. Thus, the Cbl-b-dependent destruction of IRS-1 is a potent dual mediator of both increased protein degradation and reduced protein synthesis observed in microgravity conditions.

In this space experiment, named "Myo Lab", we aim to elucidate this hypothesis on Cbl-b-mediated skeletal muscle atrophy to develop a new therapeutic strategy (drug discovery in space) for muscle atrophy.

Outline of Myo Lab

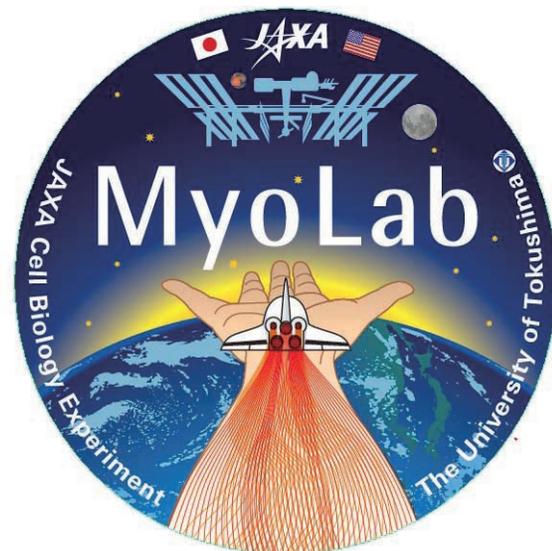


Fig. 1. Decal for the "Myo Lab" space experiment. "Myo Lab" means "Laboratory for Skeletal Muscle". This decal images our dream (space shuttle) that is flying beyond Earth forward to "Kibo", Japanese Experimental Module (JEM), Moon and Mars. In addition, the flame from shuttle means skeletal muscle.

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"Myo Lab" space experiment

"Myo Lab" is a JAXA cell biology space experiment, which was accepted as a candidate of the 4th International Competition of Space Experiment in International Space Station (ISS) in 2002. The decal for this "Myo Lab" is shown in Fig. 1. Although this project was postponed due to the Columbia tragedy in 2003, it will be performed on March, 2010, at last. Astronaut Yamazaki will send our samples to ISS with Space Shuttle "Discovery" (STS-131/19A) on March 18, 2010. Cosmonaut Noguchi will perform our space experiment in ISS.

The outline of "Myo Lab" experiment is schematically illustrated in Fig. 2. To elucidate the molecular mechanism of microgravity-induced muscle atrophy, we will culture rat L6 myotubes in ISS about 10 days. About 7 days before shuttle launch we will seed rat L6 myoblasts (Somers *et al.*, 1975) with Dulbecco's modified Eagle medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS) (Sigma) on 32 disposable culture chambers (DCCs) (Chiyoda Co.) coated with 0.1% gelatin (Sigma). After confluence, we will exchange the media to DMEM containing 0.5% FBS to induce the differentiation to myotubes (Fig. 3A). Medium in DCC will be exchanged with fresh one 2 days before shuttle launch, and 4 DCCs will be packed with an Anaero Pack™ system (Mitsubishi Gas Chemical Co.), which is an easy anaerobic cultivation method using neither water nor catalyst (Deakin *et al.*, 1998). Then, they will be turned over to be launched into space.

L6 myotubes cultured in 32 DCCs will be divided to 4 groups; ① microgravity + vehicle treatment,

② microgravity + IGF-1 treatment, ③ 1G + vehicle treatment, and ④ 1G + IGF-1 treatment. DCCs in Groups ① and ② were cultured at stationary conditions in a cell biology experiment facility (CBEF). Other DCCs in Groups ③ and ④ were cultured in centrifuge producing 1G force in CBEF. About 1 week later, they will be treated with vehicle or IGF-1 with a solution exchanger for 24 hr. A part of DCCs will be subjected to microscopy. Then, they will be fixed with RNeasy Lysis Buffer™ (Ambion) and stocked at -90°C until return to Earth.

Excellent cell culture system in "Myo Lab" experiment

Japanese experimental module (JEM), "Kibo", in International Space Station has been constructed until June, 2009. This enables us to perform cell culture experiment in space for a long time. To observe the response of skeletal muscle cells to microgravity conditions, we will use rat L6 myotubes due to the following reasons. Since astronauts have no time to care cells about 1 week after docking of shuttle to ISS, cells should be alive for a long time without exchanging media. We can keep L6 myotubes vivid for three weeks without exchanging media. In addition, hypergravity during launch is an unavoidable side effect on the cell. However, short-term hypergravity does not affect protein-ubiquitination and proliferation in rat L6 cells (Hirasaka *et al.*, 2005).

Contamination is a severe and unavoidable problem in cell culturing in space. To succeed our space experiment,

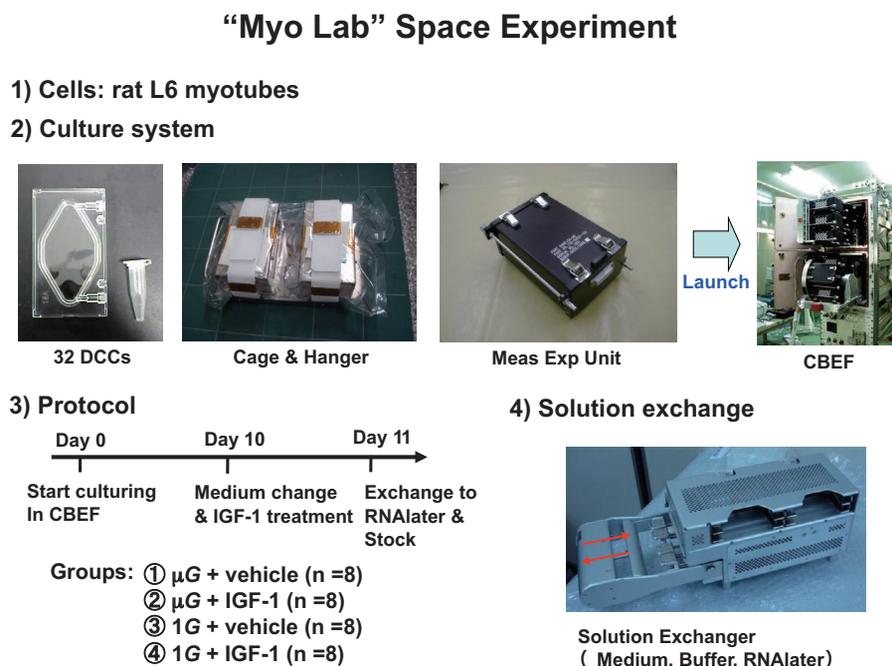


Fig. 2. "Myo Lab" space experiment. Outline of "Myo Lab" space experiment is schematically illustrated. Details are described in the text.

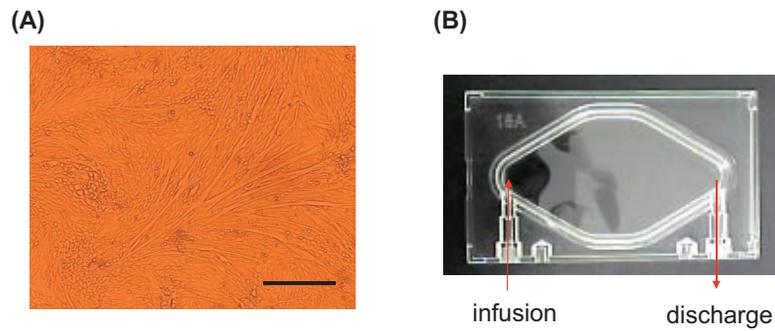


Fig. 3. Rat L6 myotubes (A) and DCC (B)
L6 myoblasts was seed on a DCC (B) coated with 0.1% gelatin. After confluence, we will exchange the media to DMEM containing 0.5% FBS to induce the differentiation to myotubes (A). Then, they will be launched. Bar = 100 μ m.

Chiyoda Co. developed a new culture chamber, named DCC, as shown in Fig. 2 and 3B. DCC significantly decreased contamination rate during culturing L6 cells. DCC is a cheap chamber to easily exchange media or buffer without air babbles by solution exchanger (Fig. 2). If cells were difficult to attach plastic surface of DCC, we can easily coat matrix, such as gelatin and collagen, on the surface. We hope that DCC will become standard cell culture chamber for space cell experiment.

Impacts and challenges in cell biology space experiment

Cell culture experiment in space is an easy system to detect direct effects of microgravity on the cell, compared with animal experiment. Microgravity cause skeletal muscle atrophy in animals via hormonal and neuronal effects as well as direct effects. In addition, we can not distinguish these effects of skeletal muscle in animal experiment. In “Myo Lab” experiment, we will demonstrate that microgravity can induce muscle atrophy only via direct effects, and that skeletal muscle itself has mechanosensor for gravity. On the basis of these findings, we propose that cell biology space experiment is very important and should be continued. Therefore the second generation of CBEF should be developed.

We do not have enough time and equipments to perform biochemical and molecular biological analyses in ISS. Therefore, we should return cells cultured in ISS to the earth. Unfortunately, space shuttle will be retired until September, 2010. That is the biggest problem for cell biology space experiments. Soyuz does not have enough space to return the cells to the earth. Agencies for space development and scientists for space biology must develop a new transporter from ISS. We would like to ask JAXA to modify HII-transfer vehicle (HTV) to a returnable transporter.

Expected results

Our results from simulated microgravity conditions, such as 3D-clinorotation and tail-suspension, indicate that the ubiquitin ligase Cbl-b plays a major role in skeletal muscle atrophy induced by unloading (Nakao

et al., 2009). The mechanism of Cbl-b-induced muscle atrophy is unique in that it does not appear to involve the degradation of structural components of the muscle, but rather it impairs muscular trophic signals in response to unloading conditions (Fig. 4). Recent studies on the molecular mechanisms of muscle atrophy have focused on the role of IGF-1/PI3K/Akt-1 signaling cascade as a vital pathway in the regulation of the balance between hypertrophy and atrophy (Sandri *et al.*, 2004; Stitt *et al.*, 2004). These studies indicate that under muscle wasting conditions, such as disuse, diabetes and fasting,

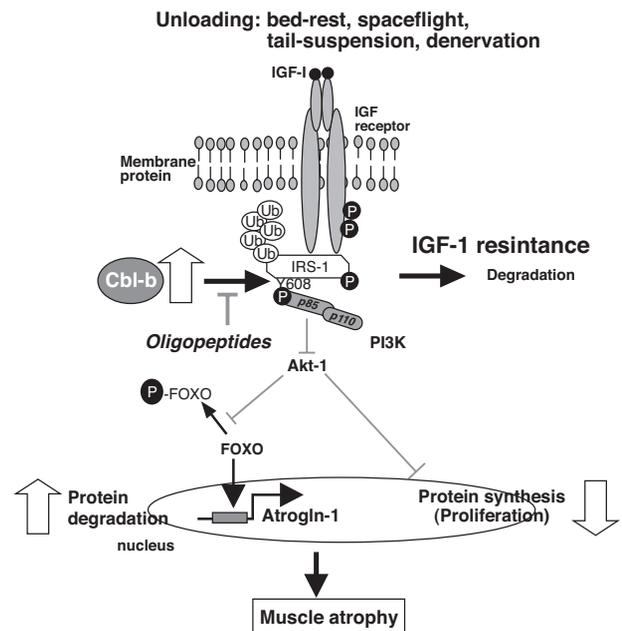


Fig. 4. Mechanistic model of unloading-mediated muscle atrophy.
Unloading induces ubiquitin ligase Cbl-b in myocytes. Cbl-b stimulates ubiquitination and degradation of IRS-1, an important intermediate in IGF-1 signaling pathway, resulting in IGF-1 resistance in myocytes during unloading. IGF-1 resistance induces impaired protein synthesis and enhances protein degradation in muscle, leading to muscle atrophy. GSK3, glycogen synthase kinase 3; mTOR, mammalian target of rapamycin; S6K, p70 S6 kinase; Ub, ubiquitin.



Fig. 5. Future purpose of "Myo Lab"

To prevent muscle atrophy caused by bed-rest as well as spaceflight, we are developing low molecular weight chemical mimetics to Cblin for their efficacy in the inhibition of Cbl-b (See text).

decreased IGF-1/PI3K/Akt-1 signaling augments the expression of atrogin-1, resulting in muscle atrophy. However, these studies did not address the mechanisms of unloading-induced impairment of growth factor signaling. In the present study, we found that under both *in vitro* and *in vivo* experimental conditions, Cbl-b ubiquitinated and induced specific degradation of IRS-1, a key intermediate of skeletal muscle growth regulated by IGF-1/insulin and growth hormone, resulting in inactivation of Akt-1. Inactivation of Akt-1 led to upregulation of atrogin-1 through dephosphorylation (activation) of FOXO3, as well as reduced mitogen response, in skeletal muscle. Thus, activation of Cbl-b may be an important mechanism underlying the failure of atrophic muscle to respond to growth factor-based treatments such as IGF-1 (Fig. 4). To confirm this hypothesis, we will subject L6 myotubes cultured under microgravity conditions to biochemical and molecular biological analyses, such as Western blotting, immunoprecipitation, microarray analysis and real time reverse transcription-polymerase chain reaction, after return of samples to Earth.

Future plan and conclusion

Cbl-b may be a gene highly responsive to mechanical stress (unloading). Since we previously reported that mild oxidative stress occurred in skeletal muscle of suspended or spaceflight rats and low concentration of H₂O₂ induced expression of Cbl-b transcripts (Nikawa *et al.*, 2004), we are examining to identify the transcriptional factor for Cbl-b expression. Elucidating the mechanism of regulation of Cbl-b expression will lead to clarify mechanical sensory system as well as the development of therapeutic strategies for muscle atrophy.

A near universal property of diverse muscle wasting diseases is the presence of IGF-1 resistance. Attempts

to overcome this barrier clinically have led to the use of high doses of IGF-1 sufficient to produce numerous and in many cases prohibitively undesirable side effects (2). "Myo Lab" will reveal the molecular basis of this treatment barrier. Our data from simulated microgravity conditions indicate that Cbl-b is required for microgravity-induced IRS-1 degradation and the resultant loss of muscle mass and function; in its absence, muscle IGF-1 responsiveness was restored (Nakao *et al.*, 2009). Furthermore, we found that a synthetic peptide, named "Cblin (Cbl-b inhibitor)", blocked IRS-1 ubiquitination both *in vitro* and *in vivo*, resulting in the restoration, at least in part, of denervation-induced muscle atrophy. Since peptides are rapidly degraded by aminopeptidase in muscle, our studies indicate that a high dose of Cblin and frequent intramuscular injection were required to prevent the IRS-1 ubiquitination. To prevent muscle atrophy caused by bed-rest as well as spaceflight (Fig. 5), we are synthesizing and testing low molecular weight chemical mimetics to Cblin for their efficacy in the inhibition of Cbl-b.

In summary, we propose a role for Cbl-b in the downregulation of IGF-1 signaling in skeletal muscle under unloading conditions. Our data also suggest that selective inhibition of Cbl-b-mediated IRS-1 ubiquitination may represent a novel therapeutic strategy for wasting diseases in the musculoskeletal locomotor system.

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Abbreviations

CBEF, cell biology experiment module; Cblin, Cbl-b inhibitor; DCCs, disposable culture chambers; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; HTV, HII-transfer vehicle; ISS, international space station; JAXA, Japan Aerospace Exploration Agency; JEM, Japanese experimental module; 3D-clinorotation, three dimensional-clinorotation.

