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Actin dynamics in mouse fibroblasts in microgravity

After stimulating with the growth factor PDGF, cells exhibit abundant membrane ruffling and other morphological changes under normal gravity conditions. These morphological changes are largely determined by the actin microfilament system. Now these actin dynamics were studied under microgravity conditions in mouse fibroblasts during the DELTA mission. The aim of the present study was to describe the actin morphology in detail, to establish the effect of PDGF on actin morphology and to study the role of several actin-interacting proteins involved in introduced actin dynamics in microgravity. Identical experiments were conducted at 1G on earth as a reference. No results in microgravity were obtained due to a combination of malfunctioning hardware and unfulfilled temperature requirements.

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Introduction

Humans exposed to microgravity conditions experience various physiological changes like reduced immunodeficiency, loss of bone mass and muscle deterioration. The underlying mechanism for these phenomena might be within cells on the molecular level. This is supported by a large number of observations in mammalian cells. Gravity effects mammalian cell growth and differentiation and cells were shown to have altered gene expression in microgravity [1, 2, 3, 4, 5]. The presented research fits in an ongoing project that studies the effect of microgravity on mammalian cells.

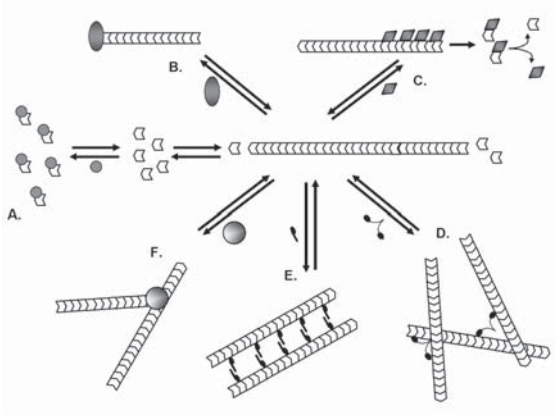

Fig. 1: Actin-binding proteins affect the behavior of actin in various ways. Some examples are illustrated in this figure: (A) Monomer-binding proteins keep the amount of monomeric actin subunits available for polymerization low, to prevent the formation of F-actin filaments. (B) When a fiber of actin is formed it can be capped by a capping protein that prevents further elongation resulting in short capped filaments. (C) Severing proteins increase the depolymerization of filaments. Cross-linking proteins can organize the filaments into gel-like networks (D) or bundles (E). Branching proteins (F) initiate the growth of daughter filaments by serving as nucleation sites on existing filaments resulting in branched actin networks.

During previous studies in microgravity it was found that growth factor-induced signal transduction is sensitive to microgravity. Epidermal growth factor (EGF) induced early gene expression of *c-fos* and *c-jun* was decreased under microgravity conditions [1, 6]. Upstream of this early gene expression, EGF signaling was investigated starting at the receptor by investigating receptor clustering, ligand binding and subsequently by investigating EGF induced signaling cascades from membrane to the nucleus. Selective transduction pathways were identified to be susceptible to changes in gravity conditions whereas other pathways originating from the EGF receptor were not [7]. This was shown in several studies in sounding rocket experiments in adherent A431 epithelial cells. Moreover, cells showed increased cell rounding under microgravity conditions [8]. Cell rounding is largely determined by the actin filament system. Interestingly, the signaling cascade that was susceptible to changes in gravity, the PKC-mediated signal transduction, was demonstrated to be linked to actin. That is why the actin cytoskeleton might well be the microgravity sensitive component in cells [9]. Microgravity might affect the behavior of actin directly or indirectly via actin binding proteins. Actin on its turn could affect the PKC-mediated signaling cascades. The experiments on the DELTA mission were part of a series of experiments that aim at determining whether actin is the microgravity sensitive component in mammalian cells.

Actin is an abundant protein in cells with important functions like migration, cell cycle progression, intracellular transport, signal transduction, transcription, membrane ruffling and cell attachment. It also largely determines the morphology of cells and the shape and position of organelles within cells (for review see [10]). Actin is present both in a polymerized form (F-actin) and a non-polymerized form (G-actin) and cycles between those states in cells thereby forming a highly dynamic network. Many proteins interact with actin and locally determine the behavior of actin. One can characterize these proteins by their function, including cross-linking proteins like α-actinin and filamin, severing proteins like actin-depolymerization factor (ADF) and cofilin, capping proteins like gelsolin, monomer-binding proteins like profilin and membrane-associated proteins like the EGF-receptor. Together these actin binding proteins (ABPs) regulate the dynamics of the actin cytoskeleton (Fig 1.). So actin and actin-binding proteins form a complicated network and are important for many cellular processes.

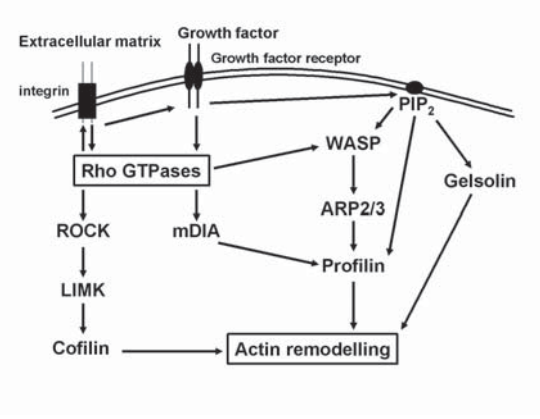

Fig. 2: Overview of the interaction between signal transduction and actin remodeling. Activation of growth factor receptors or integrins by binding to their respective ligands results in actin remodeling through Rho GTPases. Rho GTPases subsequently activate a kinase cascade including ROCK and LIMK to activate the actin-binding protein cofilin. Alternatively, the profilin is modulated through mDIA or the WASP pathway.

Actin-binding proteins on their turn are subject of regulation as well (Fig 2.). Various signaling cascades originating from both integrins and growth factor receptors stimulate or repress ABPs resulting in remodeling of the actin cytoskeleton. Integrins link the actin cytoskeleton to the extracellular matrix and by doing so play a role in cell attachment. Growth factor receptors sense the environment of a cell by binding growth factors from the outer world. Both integrins and growth factor receptors transport these extracellular signals over the membrane into the cell where cells respond by reshaping its cytoskeleton and ultimately altering gene expression.

Central players in these signaling cascades in relation to alterations of the cytoskeleton are the Rho GTPases such as RhoA, Rac1 and cdc42 (for review see [11]). Rho induces stress fiber formation and Rac1 and cdc42 play a role in the assembly of actin filaments.

Starting from the binding of a growth factor to its receptor, one can recognize the following events. The activated receptor generates intracellular signaling peptides like PIP2 and activates Rho GTPases via other peptides. Rho GTPases on their turn start signaling cascades involving proteins such as ROCK, LIMK, mDIA and WASP. Subsequently the activities of actin-binding proteins like cofilin, gelsolin and profilin are modified reshaping the actin cytoskeleton.

As described above, studies in microgravity gave rise to the hypothesis that actin might be the microgravity sensitive component in cells. Actin was indeed shown to behave differently in microgravity [4, 9, 13]. The relative F-actin content was shown to increase during microgravity conditions [14]. So, microgravity seems to either induce actin polymerization or to reduce its depolymerization. Moreover, several studies describe morphological changes of cells in simulated and real microgravity. This indicates a role for actin, since actin largely determines the cell morphology. However, only a few studies did partly investigate the role of actin in simulated or real microgravity [2, 4, 12, 15]. The present study aimed at investigating the behavior of actin in cells in real microgravity, and to investigate possible local differences in behavior of actin in cells. These differences might either be directly induced by the physical environment of a cell or indirectly by modifying the interactions with actin-binding proteins.

To study the behavior of actin in detail we developed a model allowing the study of actin dynamics in cells exposed to micro-

gravity. Actin dynamics is triggered by stimulating growth-factor starved cells with a growth factor called Platelet Derived Growth Factor (PDGF) (Fig 3.). This growth factor binds to the PDGF receptor that on its turn stimulates intracellular signaling cascades. Via RhoGTPases these signaling cascades regulate a number of actin binding proteins resulting in a response of the actin cytoskeleton. After stimulation with PDGF, the number of stress fibers and focal adhesions are reduced in cells. Moreover, circular ruffles are formed on the upper side of these cells. These circular ruffles are dynamic structures formed by actin starting from the periphery of cells and moving towards the middle of cells [16, 17]. Actin pushes the membrane outwards thereby changing the cell morphology.

Using automated flight hardware we planned to stimulate mouse fibroblasts with PDGF and subsequently chemically fix the cells in microgravity during the DELTA mission.

Material and Methods

Materials
Tissue culture nutrients, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Paisley, UK). PDGF-BB was purchased from Upstate (Hampshire, UK). All other chemicals used were obtained from Sigma or Merck (Darmstadt, Germany) and were of the highest purity available. PBUs were manufactured by CCM (Nuenen, the Netherlands). Coverslips sized 9x25 mm or 9x12 mm were purchased from Menzel (Braunschweig, Germany).

Cell culture
C3H10T1/2 mouse fibroblasts were grown at 37 °C in HEPES (25 mM) buffered DMEM supplemented with 7.5% FBS and 5 mM L-glutamine (humidified atmosphere, 5% CO_2). Cells were plated at 12,000 cells/cm^2 on coverslips and allowed to grow for 24 hours after seeding. Cells were growth factor-starved for 24 hours at 37 °C and subsequently growth factor starved for another 8 days at 20 °C during transport from the laboratory to the launch area in a temperature regulated box.

Flight hardware
Plunger Box Units (PBUs) were filled four days before launch except from cells that transported separately to keep them in good condition. Cells on coverslips were inserted one day before launch into the PBUs and kept in fresh serum-free medium.

Flight experiment
Cells were to be kept at 20 °C for the duration of 2 days during flight. 6 hours before stimulation, experimental units were to be transferred to 36.5± 0.5 °C. Plungers were to be activated to stimulate cells with PDGF and to fix them afterwards. Experimental units were to be cooled down to 4 °C and to be transported at 4 °C back to the laboratory.

Immunofluorescent labeling
Cells were fixed by adding 5.3% formaldehyde directly to the culture medium. The refreshment rate of the PBUs is ~70%, resulting in a final concentration of fixative of 3.7%. After fixation, cells were kept at 4 °C during transport to the laboratory. Here coverslips were taken out of the PBUs and washed twice with PBS, permeabilized for 5 min in PBS containing 0.2% Triton X-100, followed by two washes in PBS, and incubated for 10 min with 50 mM glycine in PBS. After washing twice with PBS containing 0.2% gelatin, cells were incubated for 60 min at room temperature with tetramethylrhodamine-5- (and-6) isothiocyanate (TRITC)-conjugated phalloidin and Alexa 488 conjugated DNase I. Subsequently they were washed six times with PBS containing 0.2% gelatin. Finally, cells were mounted in Mowiol-DABCO.

Acquisition of images
Pictures were taken with a Leitz microscope (model Orthoplan Flu 043944) equipped with Leitz objectives 40x/1.3 oil and 63x/1.4 oil, using immersion oil Immersol 518F (Carl Zeiss MicroImaging Inc.). Images were acquired using a Leica CCD camera (model DC350F; Leica Microsystems, Inc.) using Leica Image Manager 50 software, subsequently pictures were processed with Adobe Photoshop 8.0.

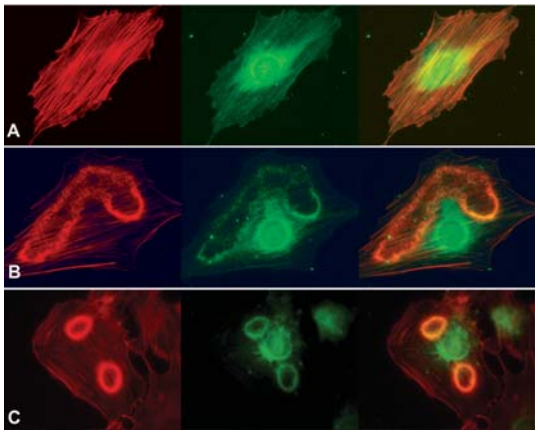

Fig 3: Effect of PDGF on F- and G-actin localization in C3H/10T1/2 mouse fibroblasts. Cells were serum deprived for 24 hours and subsequently incubated in the presence or absence of 20 ng/mL PDGF-BB for 0, 10 and 30 minutes at 37 °C, respectively (A), (B) and (C). Subsequently cells were fixed using formaldehyde and incubated with Phalloidin-Tritc to label F-actin (red) or with DNase I-Alexa488 to label G-actin (green). In non-stimulated cells F-actin is visible in large stress fibers, whereas G-actin is localized mainly around and in the nucleus. Incubation in the presence of PDGF-BB for 10 (B) and 30 minutes (C) results in the formation of membrane ruffles and the partial disappearance of stress fibers. (photo: Critical Reviews™ in Eukaryotic Gene Expression, 15(3):255–275 (2005))

Results

Ground controls were carried out simultaneously with the flight experiments using the same batch of cells. They were successful and gave similar results as normally obtained (Fig 3.)[16, 17]. Cells showed normal actin morphology and number of ruffles indicating that the cells survived the long period of starvation and exposure to room temperature during the experiment. Cells that were not stimulated showed abundant stress fibers and a pool of G-actin mainly localized around the nucleus. After stimulation with PDGF the number of stress fibers decreases and both F-actin and G-actin were found in circular ruffles indicating an extensive reorganization of actin.

No flight results were obtained due to a combination of malfunctioning hardware and unfulfilled temperature requirements.

Conclusion/ Discussion

Growth factor induced actin dynamics in serum starved cells is an interesting model for studying actin dynamics in microgravity. Because of the highly dynamic behavior of actin small differences in actin metabolism in microgravity will be enlarged compared to the situation in a more static cell. Moreover, the mouse fibroblasts were shown to stand the harsh conditions unavoidable when doing experiments in real microgravity. Because there were no results obtained in microgravity for this experiment during the DELTA mission, new flight opportunities will have to provide answers to the questions regarding the dynamics of actin in microgravity listed in this paper.

Acknowledgements

Because of space constraints we have been unable to cite all original papers. We, therefore, apologize to authors whose work in this subject area may have been omitted. This work in our laboratory is subsidized by the Dutch Space Organization (SRON, grant MG-059).

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Actin dynamics in mouse fibroblasts in microgravity: MASER-10 mission

Maarten J. A. Moes • Jose J. Bijvelt • Johannes Boonstra

Abstract

Under normal gravity conditions, cells exhibit distinct morphological changes upon stimulation with the growth factor PDGF (Platelet-Derived Growth Factor), such as the formation of linear and circular membrane ruffles and the formation of lamellae. These morphological changes are largely determined by the actin microfilament system. During the MASER-10 experiment, PDGF-induced actin dynamics were studied under microgravity conditions in mouse fibroblasts. The aim of the experiment was to describe the actin morphology in detail, to establish the effect of PDGF on the actin morphology and to study the role of several actin-interacting proteins in microgravity. Identical experiments were conducted at 1g in space and at 1g on earth as a reference.

Introduction

The ACTIN experiment on the MASER-10 was part of an ongoing program that aims at identifying the microgravity sensitive component of the actin metabolism in mammalian cells. Previous observations gave rise to the hypothesis that the target of gravity in a cell is focused on the regulation of actin polymerisation, being an increased polymerisation rate or a decreased depolymerisation rate. A detailed description is provided in Boonstra, 1999 and Moes et al., 2007.

The experiments on the MASER-10 sounding rocket aimed at studying the actin dynamics that are induced in mouse fibroblasts upon stimulation with the growth factor PDGF. Stimulation with PDGF results in the formation of spectacular actin rearrangements within minutes. During the MASER-10 mission, cells are exposed to microgravity conditions for six minutes. Therefore this model is suited to study induced actin dynamics in this relatively short time frame. The actin dynamics that are induced by PDGF require a change in the activities of actin regulating proteins, such as actin binding proteins. The examination of the role of actin regulating proteins in microgravity was another aim of the present investigation.

The model of mouse fibroblasts stimulated with PDGF was studied previously in real microgravity during a long duration space mission in the International Space Station (ISS)

(Moes et al., 2007). During this experiment cells were subjected to microgravity for several days before stimulating the cells with PDGF. The experiment that was conducted during the MASER-10 mission made use of the same experimental model but the exposure to microgravity was different. The MASER-10 experiment was conducted with a sounding rocket and cells were exposed to microgravity for 6 minutes. This implies there is little time for cells to adapt to their changed environment, i.e. microgravity. This in contrast to long term space missions, where cells may have ample time to adapt to conditions of microgravity. In other words, during short duration space missions cells are investigated that are adapting to conditions of microgravity and during long duration space missions cells are investigated that may have adapted to microgravity. Ground based simulation experiments revealed that cells indeed adapt to conditions of simulated microgravity (Moes et al., 2010). Upon exposure to conditions of simulated microgravity cells round up within minutes (Rijken et al., 1991, Moes et al., 2010) similar as was demonstrated during experiments in real microgravity (Boonstra et al., 1997). However, after exposure to simulated microgravity for more than 1 hour, cells start to spread again, become flattened and exhibit a morphology that is comparably to cells that were not exposed to simulated microgravity (Moes et al., 2010). Because cells exhibit a transient response, one must be rather careful in comparing the results of experiments that expose cells to microgravity conditions for different durations.

During the MASER-10 experiment PDGF induced actin dynamics were studied in mouse fibroblasts that were adapting to conditions of microgravity. The aim of the experiment was to describe the actin morphology in detail, to establish the effect of PDGF on the actin morphology and to study the role of several actin-interacting proteins in microgravity. Identical experiments were conducted at 1g in space and 1g on earth as a reference.

Materials and Methods

Materials

Tissue culture nutrients, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Paisley, UK). PDGF-BB was purchased from Upstate (Hampshire, UK). The MAL antibody was kindly provided by dr. Treisman (Cancer Research UK London Research Institute, UK), the monoclonal antibody directed against β-actin (A1978, clone AC-15) was purchased from Sigma. The goat-anti-mouse-Alexa488 secondary antibody, DNaseI-Alexa594 and Palloidin-Alexa633 were purchased

from Molecular Probes. All other chemicals used were obtained from Sigma or Merck (Darmstadt, Germany). Plungerbox units (PBUs) were manufactured by CCM (Nuenen, the Netherlands). Coverslips sized 9x25 mm or 9x12 mm were purchased from Menzel (Braunschweig, Germany). The experiment system BIM (Biology In Microgravity) was developed by Swedish Space Corporation (Solna, Sweden), Dutch Space (Leiden, the Netherlands) and CCM (Nuenen, the Netherlands).

Cell culture

C3H10T1/2 mouse fibroblasts were grown at 37 °C in HEPES (25 mM) buffered DMEM supplemented with 7.5% FBS and 5 mM L-glutamine (humidified atmosphere). Cells were plated at 12,000 cells/cm^2 on coverslips and allow to grow for 24 hours after seeding. Subsequently, cells were growth factor-starved for 24 hours at 37 °C.

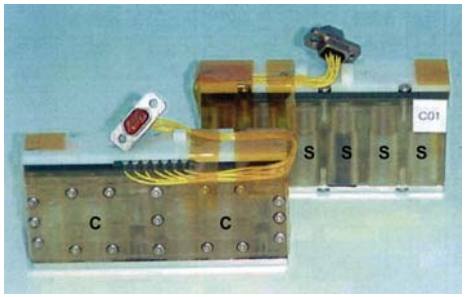

Figure 1. Plungerbox units were used as experiment containers. A plungerbox unit (80x40x20 mm^3) contains two culture compartments (C; 1 ml each) that can be processed independently. The other side of the PBU contains six cylindrical storage compartments (S; 1 ml each) that can be flushed into the culture compartment when a spring-loaded plunger is released. An electrically operated thermal cutter releases the plungers.

Flight hardware

Experiment containers (PBUs) contained two culture chambers (1 ml each) that can be processed independently (Figure 1). Each culture chamber was connected to three liquid storage reservoirs (1 ml each) that can be operated automatically. PBUs were filled one day before launch at 37 °C. Cells on coverslips were inserted into the PBUs 8 up to 18 hours before launch and kept in fresh serum-free medium. Subsequently, experimental containers for the flight experiment were transferred to the BIM (Biology In Microgravity) experiment module (Holm et al., 2005). The BIM facilitated the operation of the PBUs and consisted of two parts, namely the Late Access Insert (LAI; Figure 2) that contained the flight experiments and the system for the ground reference experiments. The LAI facilitated the flight experiments that comprehended both the microgravity set of experiments and the 1g reference set of experiments on the centrifuge. The LAI thermal

control was set to operate at 36.5± 0.5 °C with a tolerance of ± 0.2 °C between PBUs. Four hours before launch, the LAI was placed in the MASER-10 sounding rocket to ensure that cells were recovered from any stress resulting from human handling. The experiment containers for the reference on-ground were placed in an incubator at 36.5± 0.5 °C. During the experiment the temperature was constantly monitored with several temperature sensors (Holm et al., 2005).

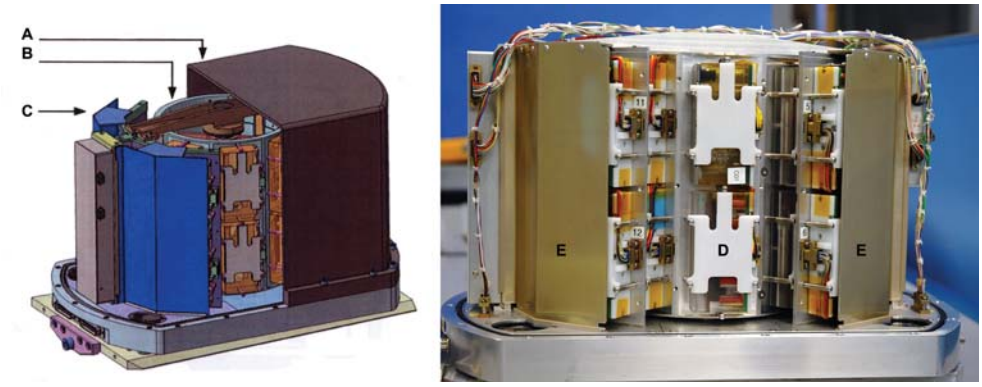

Figure 2. The Late Access Insert of the BIM that facilitates operation of the experiments in space. The drawing of the LAI (Dutch Space) with the cover (A) half-open indicates the position of the centrifuge (B) and the static racks (C). The photo on the right displays the LAI with the PBUs mounted on the centrifuge (D) and the static racks (E). The static racks facilitate the microgravity experiments and the in-flight reference experiments are mounted on the centrifuge.

Flight experiment

Cells were kept at 36.5± 0.5 °C. The launch of MASER 10 was on May 2, 2005 at the launching facility at Esrange outside Kiruna. The experiment was performed simultaneously under microgravity conditions, on a 1g reference centrifuge on board and a 1g reference set-up on-ground. At the onset of microgravity, cells were either directly stimulated and fixed, or stimulated with serum-free medium with PDGF and fixed after 6 minutes, or serum-free medium was added and cells were fixed after 6 minutes. Stimulations and fixations were performed by activating the plungers of the PBUs. All experiments were executed in triplicate. Cells were fixed by adding either 5.3% formaldehyde or 2.1% glutaraldehyde directly to the culture medium. The refreshment rate of the PBUs is approximately 70%, resulting in a final concentration of fixative of

approximately 3.7% formaldehyde and 1.5% glutaraldehyde. Subsequently experimental units were passively cooled down.

Immunofluorescent microscopy

Coverslips were taken out of the PBUs and washed twice with PBS, permeabilized for 5 min in PBS containing 0.2% Triton X-100, followed by two washes in PBS, and incubated for 10 min with 50 mM glycine in PBS. After washing twice with PBS containing 0.2% gelatin, cells were incubated for 60 min at room temperature with the primary antibody. Subsequently cells were washed six times PBS containing 0.2% gelatine. Then, cells were incubated with the secondary antibody and/or Alexa633-conjugated phalloidin and/or DNaseI-Alexa594 and washed six times PBS containing 0.2% gelatin. Finally, cells were mounted in Mowiol-DABCO. Images were acquired with a Zeiss CSLM (Pascal 510) fitted with Zeiss objective lenses (40x N.A. 1.3 and 63x N.A. 1.4). All immunofluorescence studies were performed using single labelings as well.

Environmental Scanning Electron Microscopy (*ESEM)*

Coverslips were taken out of the PBUs and cells were stained in 1% uranyl acetate. After washing in PBS, cells were kept in PBS overnight before analysis in ESEM mode in a Quanta FEG (FEI Company, Eindhoven, The Netherlands). This microscope enables to study cells in a fully hydrated state, avoiding all kind of drying artifacts.

Results

The aim of the Actin experiment was to investigate the behaviour of actin in detail. For this purpose, the intracellular distribution of both G-actin and F-actin can be determined. However, to gain insight in the degree of actin polymerization additional information is required. Investigations of cell lysates can provide such information but the limited number and small sizes of samples that were available for this experiment did not allow both microscopy and biochemistry studies. It was decided to focus on investigating the actin morphology by various methods of microscopy. For studying the degree of actin polymerization in cells exclusively using microscopy techniques, the localization of actin-interacting proteins may provide additional information. In this respect the subcellular localization of the actin binding protein MAL is of particular interest. In serum-starved cells, MAL was described to be mainly present in the cytoplasm and in addition a fraction is present in the nucleus. Upon stimulation with serum, MAL was described to translocate

from the cytoplasm to the nucleus (Miralles et al. 2003). Stimulation of cells with serum results in an increase of actin polymerization and a depletion of the cytoplasmic pool of G-actin. MAL binds to G-actin and upon depletion of the cytoplasmic pool of G-actin, MAL translocates from the cytoplasm to the nucleus (Miralles et al., 2003). Therefore, the localisation of MAL provides information of the degree of actin polymerization in cells. The ratio of cyoplasmic MAL and nuclear MAL within single cells can be measured and subsequently the average ratio of a cell population can be determined. It was investigated if this model could be applied with the experimental set up of the Actin experiment in the sounding rocket MASER-10. For this experiment, fibroblasts were stimulated for only 6 minutes with PDGF-BB before chemical fixation. Control experiments on ground revealed that this is ample time for observing a clear translocation of MAL in mouse fibroblasts (Figure 3).

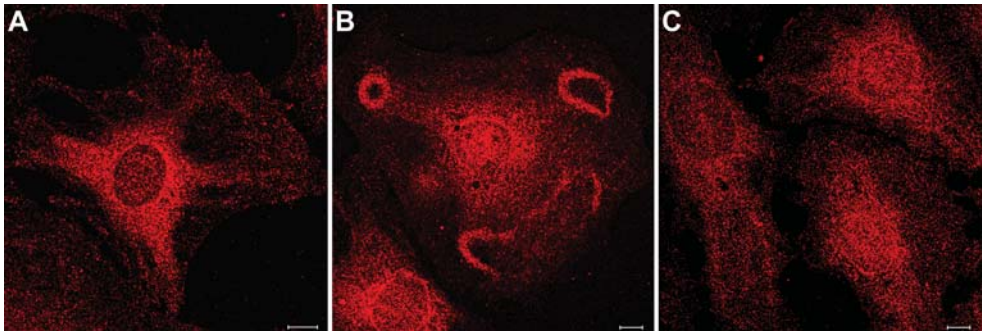

Figure 3. A: In serum-starved mouse C3H10T1/2 fibroblasts, MAL is mainly present in the cytoplasmic pool around the nucleus. B: Upon stimulation with PDGF-BB for 6 minutes, MAL translocates partly to the nucleus. In addition, MAL accumulates in newly formed circular ruffles.
C: 30 minutes after addition of PDGF-BB, MAL is less enriched in the nucleus of cells and the cytoplasmic pool of MAL becomes more abundant.

As mentioned before, previous observations in the lab gave rise to the hypothesis that the target of gravity in a cell is focused on the regulation of actin polymerisation. It was hypothesized that cells exhibit either an increased polymerisation rate or a decreased depolymerisation rate in microgravity (Boonstra, 1999). In view of this, the subcellular localisation of MAL of cells exposed to microgravity might provide interesting information about the degree of actin polymerization in cells. Similar to the translocation upon stimulation with growth factors, the localisation of MAL might change upon

exposure to microgravity conditions as a consequence of a changed polymerization rate or depolymerisation rate of actin.

During flight the Actin experiment in the BIM module was successfully performed. All temperature requirements were fulfilled before flight and during flight and all but one plunger were activated successfully (Figure 4). The recorded speed of the centrifuge during the experiment as well as the start and stop of rotation of the centrifuge were as programmed (Figure 4). After the experiment the MASER 10 sounding rocket experienced a hard landing because of failure of the landing parachute. Due to this hard landing a few glass coverslips were broken and these samples were lost. All samples were examined using phase contrast microscopy and all samples were documented by taking representative pictures of each sample. The quality of the chemical fixation was determined based on the observed morphology and in addition the number of cells that was present on the coverslips and the number of traces left by detached cells were investigated. In the samples of the flight experiments, an increase was found in the number of cells that detached during the experiment. Therefore it was concluded that part of the cells were detached from the substratum due to the hard landing. In previous microgravity studies it was demonstrated that cells round up in microgravity. In addition, cells round up upon stimulation with PDGF-BB. Hence, we assume that especially the cells that responded most to PDGF-BB and cells that responded most to conditions of microgravity did round up during the experiment. Cells that round up are less strongly attached to the substratum. Therefore it can be expected that cells that responded most to the stimulation with PDGF-BB and cells that responded most to microgravity have detached more frequently than other cells due to the impact of the uncontrolled landing. In conclusion, there are strong indications that the cells that were still attached on the glass coverslips might not be representative for the experiment and therefore we cannot draw any conclusion from the experiment with respect to possible effects of microgravity on actin.

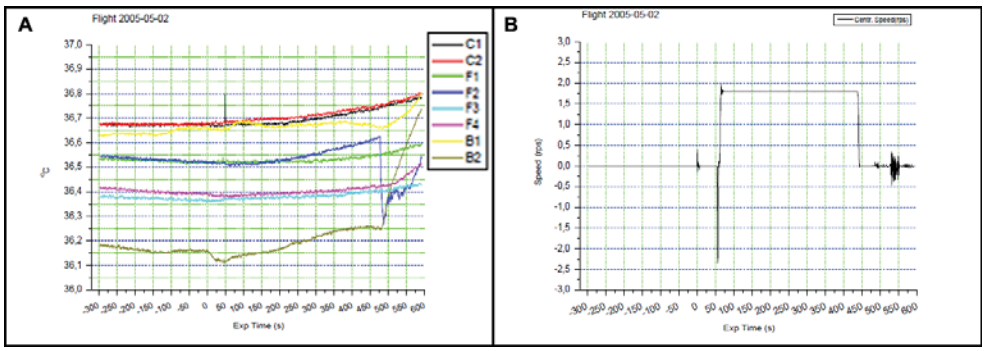

Figure 4. Recorded temperature profile (A) and recorded speed of the centrifuge (B). The onset of microgravity is at t=0 and lasts till t=360, the recording of data ends after the hard landing of the rocket. The temperature sensors on the centrifuge (C) and the sensors on the static racks (F) indicate that temperatures were within 36.5± 0.5 °C with a tolerance of ± 0.2 °C between PBUs during the period of microgravity (B1 is the regulating sensor and B2 is located at the heat exchanger of the LAI). Due to the impact of the rough landing, the thermal control and data logging were not working as planned after the landing. The recorded increase in temperature at the end is probably due to the impact of the landing. The centrifuge moved at spin-up directly after the lift-off of the rocket and at de-spin at about +60 seconds (B). The recorded speed during the experiment as well as the start and stop of rotation of the centrifuge are as programmed.

In addition, unexpected variations in cell morphology were observed in all samples, including the ground samples. These variations could not be explained by a different treatment, the use of different chemicals or differences in cell population. We have not been able to identify the precise cause for the differences that were observed. However, the experiments performed during MASER 10 have been performed many times in hand-operated PBUs. Hand-operated PBUs can be used repeatedly and during the use of hand-operated PBUs the differences in results as indicated above were never observed. The automatic operated flight PBUs can only be used once.

Although no conclusions can be drawn from the Actin experiment of MASER 10, all samples were studied extensively. Except for the observed variation between identical samples, the results from these studies again indicate that the experiment was conducted successfully (Figure 5).

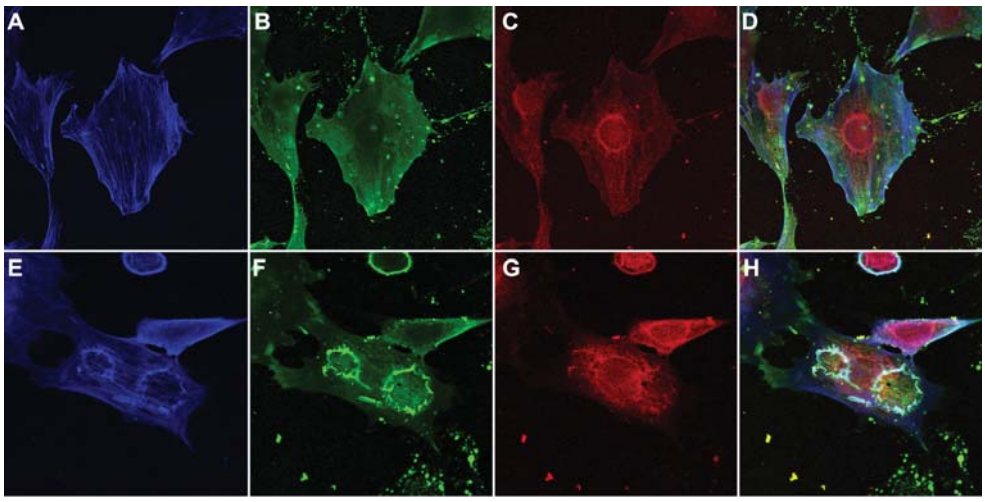

Figure 5. Mouse C3H10T1/2 fibroblasts that are exposed to 1g in the centrifuge of the BIM experiment system in the MASER-10 sounding rocket. Cells are labelled for F-actin (blue), β-actin (green) and G-actin (red). A-D: Serum-starved mouse C3H10T1/2 fibroblasts that are fixed at the onset of microgravity. E-H: Upon stimulation with PDGF-BB for 6 minutes, dorsal circular ruffles are induced by newly formed F-actin (E). These ruffles contain β-actin (F) and are enriched with G-actin (G). The induced ruffle formation in the cells of the in-flight 1g reference experiment seems comparable with the ruffle formation that is observed on earth.

Some single observations suggests some interesting starting points for future research. For example, it was observed that in the remaining cell populations that were still present on the coverslips, dorsal circular ruffles are formed under microgravity conditions. Circular ruffles were observed in various samples exposed to both PDGF-BB and microgravity using phase contrast microscopy (Figure 6), ESEM (Figure 7) and (immuno)fluorescent microscopy using Confocal Scanning Fluorescence Microscopy (CLSM; data not shown). However, since cells that were most influenced by microgravity were lost at the landing, it is not known whether this observation is representative. It can not be excluded that cells that are more susceptible to microgravity exhibit a disturbed ruffle formation.

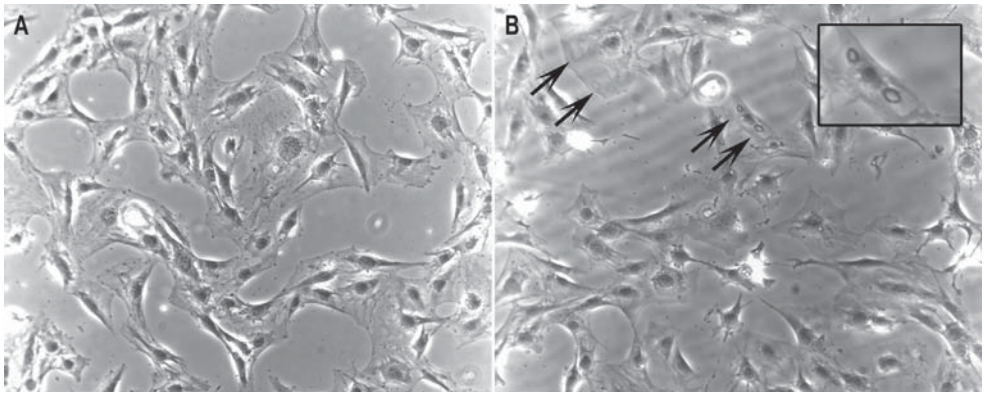

Figure 6. Mouse C3H10T1/2 fibroblasts exposed to microgravity and stimulated with PDGF-BB. Cells are fixed with glutaraldehyde and visualized by phase contrast microscopy. A: Serum-starved cells that are chemically fixed directly upon the start of microgravity. B: Cells that exposed to microgravity and stimulated with PDGF-BB for 6 minutes. Upon stimulation with PDGF-BB, dorsal circular ruffles are formed that are indicated by arrows. Insert is enlarged twice.

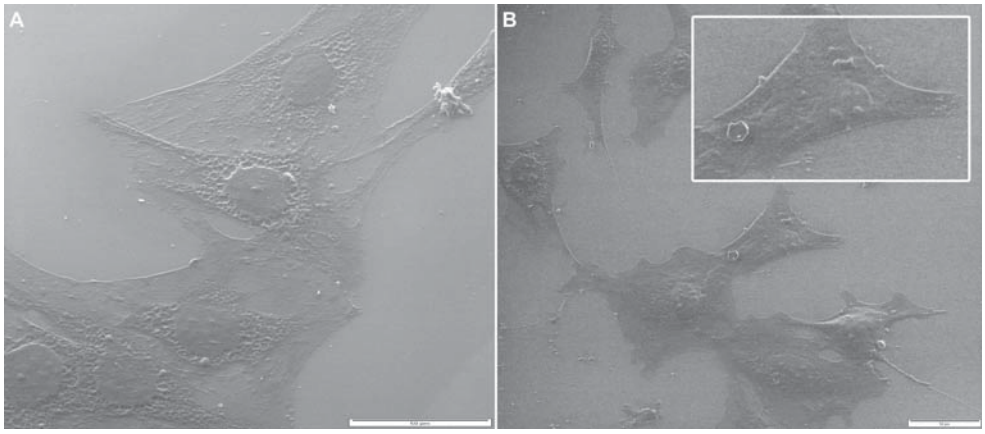

Figure 7. Mouse C3H10T1/2 fibroblasts exposed to microgravity and stimulated with PDGF-BB. Cells are fixed with glutaraldehyde and visualized by ESEM. A: Serum-starved cells that are chemically fixed directly upon the start of microgravity exhibit a flat cell morphology. B: Cells exposed to microgravity and stimulated with PDGF-BB for 6 minutes. Upon stimulation with PDGF-BB, dorsal circular ruffles are formed (insert). Insert is enlarged twice; bar represents 50 μm.

Discussion

No results in microgravity were obtained due to the hard landing of the sounding rocket MASER-10. It was concluded that part of the cells detached from the substrate because of the impact of the uncontrolled landing. We assume that especially the cells that

responded most to microgravity and PDGF-BB have detached. Therefore the samples did no longer represent a representative population of cells and no conclusions can be drawn from the results.

Fibroblasts that are stimulated with a growth factor form an interesting model for studying actin dynamics in microgravity. The actin dynamics that are induced upon exposure to growth factors will enlarge differences in the actin metabolism compared to the situation in more static cells. Moreover, these cells were demonstrated to stand the less ideal conditions unavoidable when doing experiments in real microgravity.

It should be stressed that the Actin experiment is probably the first experiment carried out in a sounding rocket in which all experiments were executed in triplicate. In addition, it is the first experiment in which cell morphology was studied in detail. Our experiment demonstrated the value of doing experiments in triplicate. The variation in results between experimental containers might otherwise not have been detected and results might be interpreted differently. To avoid the problems that were encountered with the variation between samples, re-usable experiment containers should be developed. Re-usable experiment containers would allow to execute the experiment in the lab exactly as it is conducted in the sounding rocket using exactly the same materials, both before and after the space experiment. Any influences of the experimental units can then be excluded.

Sounding rockets provide a unique method for studying cells that are not fully adapted to microgravity conditions. In addition, the facilities for doing biological research are excellent on these unmanned flights. The BIM experiment module did perform excellent and should be rebuilt. The experimental model of mouse fibroblasts that are stimulated with PDGF represents a well described and useful model for future studies under microgravity conditions using sounding rockets. New flight opportunities will provide answers to the questions regarding how cells sense microgravity and the role of the actin cytoskeleton.

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