Endothelial stress by gravitational unloading: effects on cell growth and cytoskeletal organization

Sofia I.M. Carlsson¹, Maria T.S. Bertilaccio¹, Erica Ballabio, Jeanette A.M. Maier*¹

Laboratory of General Pathology, Department of Preclinical Sciences, LITA Vialba, Università di Milano, Via GB Grassi 74, Milan, Italy

Received 11 April 2003; received in revised form 25 July 2003; accepted 12 August 2003

Abstract

All organisms on Earth have evolved to survive within the pull of gravity. Orbital space flights have clearly demonstrated that the absence or the reduction of gravity profoundly affects eukaryotic organisms, including man. Because (i) endothelial cells are crucial in the maintenance of the functional integrity of the vascular wall, and (ii) cardiovascular deconditioning has been described in astronauts, we evaluated whether microgravity affected endothelial functions. We show that microgravity reversibly stimulated endothelial cell growth. This effect correlated with an overexpression of heat shock protein 70 (hsp70) and a down-regulation of interleukin 1 alpha (IL-1α), a potent inhibitor of endothelial cell growth, also implicated in promoting senescence. In addition, gravitationally unloaded endothelial cells rapidly remodelled their cytoskeleton and, after a few days, markedly down-regulated actin through a transcriptional mechanism. We hypothesize that the reduction in the amounts of actin in response to microgravity represents an adaptative mechanism to avoid the accumulation of redundant actin fibers.

© 2003 Elsevier B.V. All rights reserved.

Keywords: Endothelial cell; Interleukin 1 alpha; Hsp70; Cytoskeleton; Aging

1. Introduction

Endothelial cells cover the entire inner surface of the blood vessels and play a crucial role in maintaining the functional integrity of the vascular wall. Functional properties of the endothelium include an active control of the various components of homeostasis, vascular tone and permeability and medial smooth muscle cell growth [1]. Indeed, endothelial dysfunctions promote several diseases, among which atherosclerosis [2], hypertension [3], diabetes and thrombosis [1]. Moreover, major alterations of endothelial behaviour have been detected in senescent cells and could explain the high frequency of cardiovascular disease in the elderly [4]. In addition, endothelial cells are protagonists in angiogenesis, i.e. the branching and sprouting of capillaries from pre-existing blood vessels, which is a tightly controlled event crucial in development, in reproduction and wound healing [5].

In normal physiological conditions, the endothelium contributes to vascular homeostasis by adaptively altering its functional state [1]. Various pathophysiological factors, such as cytokines, growth factors, hormones and metabolic products can modulate its functional phenotype in health and disease. In addition to these humoral modulators, endothelial cells respond to biomechanical stimulation. The pulsatile nature of blood flow generates a complex interplay of distinct types of fluid mechanical forces, such as wall shear stress and hydrostatic pressure, which play a role in the maintenance of vascular integrity as well as in the development of vascular diseases. Recently, it has been demonstrated that hemodynamically derived stimuli are strong modulators of endothelial gene expression [6,7] and these findings may have relevant implications in the understanding of the mechanisms of vascular homeostasis and atherogenesis.

Another physical force is now clear to be crucial: gravity [8]. Indeed, a number of studies show gravity-dependent modulations of mammalian cell proliferation and differentiation [9,10]. Microgravity seems to affect cellular organization of the cytoskeleton [11], intracellular signalling mechanisms [12], and gene expression [13] and has controversial effects on apoptosis.

* Corresponding author. Tel.: +39-2-5031-9659; fax: +39-2-7004-8045.
E-mail address: maier.jeanette@unimi.it (J.A.M. Maier).
¹ Equally contributed to this work.

0167-4889/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2003.08.003
We therefore evaluated whether microgravity could affect endothelial behaviour. In this report, we show that microgravity modulates the growth, gene expression and phenotype of human umbilical vein endothelial cells (HUVEC).

2. Materials and methods

2.1. Cell culture

HUVEC-C were obtained from the American Type Culture Collection (ATCC) and cultured in M199 containing 10% fetal calf serum, ECGF (150 μg/ml) and heparin (5 U/ml) on 2% gelatin-coated dishes. Cells were subcultured using 0.05% trypsin 0.02% EDTA solution. All culture reagents were from Gibco. For proliferation assays, we cultured the cells for various times, trypsinized, stained with trypan blue solution (0.4%) and counted the viable cells using a Burker chamber. In some experiments, HUVEC were incubated in the presence of an interleukin 1 alpha (IL-1α) antisense oligomer (50 μM) designed to recognize nine nucleotides upstream and nine downstream from the translation initiation codon [14]. Since this oligonucleotide proved to efficiently inhibit IL-1α translation, this is the only antisense oligomer we utilized in our study. As controls, we used the same concentration of the corresponding sense oligomer. After 3 days, cells were trypsinized and counted as described. In other experiments, HUVEC were cultured in the RWV for 96 or 144 h before returning to normal growth conditions. After 2 days, the cells were counted as described.

2.2. Cell culture in microgravity

To generate microgravity, we utilized the Rotating Wall Vessels (RWV) bioreactor (Cellon). Briefly, the RWV are horizontally rotated, fluid-filled culture vessels, equipped with membrane diffusion gas exchange to optimize gas/oxygen supply. The time-averaged gravitational vector acting on these cellular assemblies is reduced to about 10^{-2} g [15]. However, for simplicity, we refer to these conditions as microgravity. To be used in the RWV, cells were seeded on beads (Cytodex 3, Sigma) [16]. As controls, HUVEC grown on beads were cultured in petri dishes or in the vessels not undergoing rotation [16]. Since the results were comparable, the figures shown in the manuscript refer to petri dish controls.

2.3. Fluorescence microscopy

HUVEC were seeded on beads for various times, washed and fixed in phosphate-buffered saline containing 3% paraformaldehyde and 2% sucrose. After extensive washing, cells were permeabilized with Hepes-Triton, and stained with fluorescein isothiocyanate (FITC)-labelled phalloidin (Sigma) to visualize F actin [17].

2.4. Western blot

HUVEC were lysed in 10 mM Tris–HCl (pH 7.4) containing 3 mM MgCl₂, 10 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.5 mM EDTA and protein inhibitors, separated on 10% SDS-PAGE and transferred to nitrocellulose sheets. Western analysis was performed using polyclonal anti-heat shock protein 70 (hsp70) (Santa Cruz) or polyclonal pan-anti-actin antibodies (Santa Cruz). Secondary antibodies were labelled with horseradish peroxidase (Amersham Pharmacia Biotech). The SuperSignal chemilu...
minescence kit (Pierce) was used to detect immunoreactive proteins [18]. The blots were stripped and incubated with an anti-GAPDH antibody (Santa Cruz) to show that comparable amounts of protein were loaded per lane.

2.5. IL-1α synthesis

The IL-1α concentration in cell extracts (50 µg) was detected using Quantikine human IL-1α immunoassay according to the manufacturers’ instructions (R&D Systems) [14]. All experiments were performed in triplicate.

2.6. Northern blot

HUVEC cultured in the RWV for 96 h and their control were rinsed with phosphate-buffered saline and lysed in 4 M guanidinium isothiocyanate. RNA was purified as described [14]. RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, capillary blotted onto nylon membranes and UV cross-linked. β-Actin and GAPDH were labelled with a random primer labelling kit (Ambion). Filters were hybridized in 0.5 M sodium phosphate (pH 7.2) containing 7% SDS, 1 mM EDTA and 20% formamide at 65 °C for 20 h and extensively washed at high stringency before autoradiography.

3. Results

3.1. Modification of the actin cytoskeleton in microgravity

Different cell types cultured in microgravity show cytoskeletal reorganization [19,20]. We therefore stained HUVEC grown in simulated microgravity and their con-
Controls with fluorescent phalloidin to visualize the cytoskeleton. Control cells show a well-organized cytoskeleton, with abundant stress fibers organized into bundles. When HUVEC were grown in the RWV, major modifications of cell shape were evident within 4 h from the beginning of the experiment and maintained for several days (Fig. 1). The cells showed elongated extended podia, the actin fibers were disorganized and formed clusters especially in perinuclear position. After 144 h in the RWV, these clusters disappeared and a marked decrease in stress fibers was observed. These cytoskeletal modifications were reversible upon return to normal growth conditions (not shown).

Fig. 3. Microgravity-stimulated endothelial growth. HUVEC were cultured for different times in the RWV. At 96 and 144 h, some samples of HUVEC were removed from the RWV and returned to normal culture conditions for 48 h. Every 2 days, the cells were harvested by digestion with trypsin and viable cells counted using a Burker chamber. All the experiments were performed in triplicate. Data are expressed as the means ± standard error.

3.2. Stimulation of endothelial proliferation by microgravity

Fig. 2a shows that the steady state levels of β-actin RNA were down-regulated after 96 h culture in the RWV, as detected by Northern blot. Accordingly, by Western blot, we show that the total amounts of actin declined after 96 h in microgravity and were markedly reduced after 144 h (Fig. 2b).

Controversial results have been obtained about the role of microgravity in apoptosis [19,21,22]. We could not detect any difference between RWV-grown HUVEC and controls, since in both conditions the percentage of apoptotic cells was between 5% and 7%, as detected by internucleosomal cleavage and nuclear staining (data not shown). Cell growth is also affected in microgravity [22,23], although the results

Fig. 4. Microgravity up-regulates hsp70. HUVEC were grown in the RWV for different times and compared to controls. Cell extracts (50 μg/lane) were loaded on a 10% SDS-PAGE, blotted onto nitrocellulose filter, incubated with anti-hsp70 antibodies and visualized by chemiluminescence as described. After stripping, the blot was incubated with an anti-GAPDH antibody to show that comparable amounts of proteins were loaded per lane.

Fig. 5. Microgravity inhibits the synthesis of IL-1α. HUVEC were cultured for different times in the RWV. After 96 h in the RWV, some samples of HUVEC were returned to normal culture conditions for 48 additional hours. Cell extracts (50 μg) were utilized to measure the levels of IL-1α as described in Materials and methods. Standard deviation never exceeded ±10%.

176
vary according to the cell type used. We therefore evaluated the proliferation rate of cells grown in the RWV for various times and found that microgravity conferred a time-dependent growth advantage to HUVEC (Fig. 3). When grown in the RWV, the doubling time of HUVEC was about 48 vs. 88 h for the controls.

To determine whether the stimulation of endothelial proliferation was reversible upon return to normal conditions, we cultured HUVEC in the RWV for 96 h and then returned them to normal growth conditions. Every 48 h, the cells were counted and compared both to controls and HUVEC still growing in the RWV. Fig. 3 shows that, upon release from gravitational unloading, HUVEC proliferation rate was reduced and comparable to controls. After 144 h in the RWV, the cells reached the confluency and, therefore, return to normal gravity for 48 additional hours did not exert any effect.

3.3. Up-regulation of hsp70 and down-regulation of IL-1α in microgravity

We investigated whether HUVEC sensed microgravity as a stressful condition and adapted to it by modulating the levels of stress proteins. Fig. 4 shows that RWV-cultured cells up-regulated hsp70 within 24 h and this was maintained up to 96 h. Hsp70 serves multiple functions, including the capability to inhibit the synthesis of proinflammatory cytokines such as IL-1α, an antagonist of endothelial proliferation [24]. We therefore measured the synthesis of IL-1α and found it decreased in HUVEC grown in the RWV (Fig. 5). To demonstrate that IL-1α was responsible for the slower proliferation rate of control cells, we incubated the cells in the presence of an antisense oligomer against IL-1α, which successfully represses IL-1α translation [14]. Indeed, in the presence of the antisense oligomer, control cells grew as fast as HUVEC in the RWV (Fig. 6). The role of IL-1α in modulating endothelial growth in microgravity is further confirmed in the experiments showing that the levels of the cytokine rapidly increased after removal of the cells from the RWV and return to normal conditions (Fig. 5), thus showing that the decreased proliferation rate was paralleled by the up-regulation of IL-1α.

4. Discussion

One environmental factor that has remained constant through evolution is the force of Earth’s gravitational field. All biological mechanisms have developed to allow life within the pull of gravity. These well-known adaptations to gravity became clearly evident during manned orbital space flight. In microgravity, astronauts experience space motion sickness, muscle atrophy, bone demineralization, as well as cardiovascular deconditioning. At the cellular level, it has been shown that microgravity influences proliferation, signal transduction and gene expression [11, 25–28]. Some of the in vitro results explain the biochemical data obtained in the astronauts. For instance, it is now clear that the change in bone mass is the result of decreased bone formation in association with normal or increased bone resorption. The change in bone formation appears to be due, at least in part, to decreased osteoblast differentiation and to alterations in the expression of growth factors and matrix proteins, among which collagen type I and osteocalcin [28]. Because endothelial cells are protagonists in maintaining the functional integrity of the vascular wall [1], we studied their behaviour in simulated microgravity. We found a reversible stimulation of endothelial cell growth in gravitationally unloaded HUVEC. Interestingly, within a few hours from the exposure to microgravity, endothelial cells up-regulate hsp70, showing that the adaptation to microgravity activates the same pathways required in any other stressful condition. A part from its well-characterized function as a chaperonin, hsp70 blocks apoptosis by preventing the constitution of the apoptosome and by protecting against the apoptogenic effects of apoptosis inducing factors (AIF) targeted to the extramitochondrial compartment [29]. While microgravity promotes apoptosis in lymphocytes, osteoblasts and muscle cells, we propose that the absence of apoptosis in HUVEC grown in microgravity is due, at least in part, to the increased amounts of hsp70. Indeed, hsp70 has been shown to increase endothelial survival [30] and to protect endothelial cells from apoptosis by oxidized LDL [31]. Hsp70 also inhibits genetic expression of proinflammatory cytokines, including IL-1α [32]. IL-1α has multiple functions on endothelial cells, among which it inhibits cell growth [24] and promotes senescence [14]. Microgravity markedly down-regulated IL-1α and an antisense against IL-1α-stimulated control cells to proliferate as fast as HUVEC grown in the RWV. In addition, IL-1α levels were rapidly restored when HUVEC were returned to normal growth conditions, an event which correlated with a decrease in the prolifera-
tion rate. Taken together, these results strongly suggest that the down-regulation of IL-1α is responsible for the stimulation of cell growth in simulated microgravity.

In contrast with our results, it has been reported that bone marrow macrophages activated in space secrete more IL-1α and TNF alpha than controls on earth, suggesting that space flight has a significant enhancing effect on immune cell release of cytokines in vitro [33]. Recently, the overexpression of IL-1 receptor antagonist (ILRA), a member of the IL-1 superfamily, was described in space-flown WI38 human fibroblasts [34]. ILRA is a receptor antagonist functioning to neutralize the biological activity of IL-1 alpha and beta. We propose that the down-regulation of IL-1α described in gravitational unloaded endothelial cells and the up-regulation of ILRA in space-flown fibroblasts, both represent a protective mechanism aimed at counterbalancing the detrimental effects of IL-1’s on the vasculature, the muscles and the bones.

It has been suggested that some space flight-dependent physiological states—such as bone demineralization and muscular weakness—resemble those observed as a consequence of aging, and space travel has been proposed as a model for senescence [35]. To this purpose, it is noteworthy that ILRA is overexpressed in vitro senescent WI38 fibroblasts [36], as well as in microgravity [34]. However, in the case of the endothelial cells, gravitational unloading down-regulates IL-1α, an early player in the onset of endothelial senescence [14]. Since treatment of human endothelial cells with an antisense oligomer against IL-1α extends the in vitro lifespan but does not result in the formation of an immortal phenotype, it is conceivable to propose that the program for endothelial senescence contains more end points orchestrated by different molecules [14]. Since, like most normal diploid cells, endothelial cells have a finite replicative potential, we anticipate that the increased proliferation rate detected in microgravity may accelerate the onset of endothelial senescence due to the interventions of factors other than IL-1α.

There is strong evidence suggesting that the cytoskeleton may function in sensing gravity at the single cell level. The cytoskeleton is involved in signal transduction and transition of cells from resting to active proliferation and is known to be sensitive to alterations in gravity [37]. As described in other cell types [11], we found that endothelial cells in microgravity disorganize their cytoskeleton. After a few hours of culture in the RWV, clusters of actin accumulated in the cytoplasm, in particular in the perinuclear region. After 144 h, no cluster of actin were detectable any longer. Interestingly, we describe a downmodulation of β-actin steady state RNA after 96-h culture in the RWV, which correlated with decreased amounts of actin as detected by Western blot. On these bases, we hypothesize a transcriptional regulation of actin in gravitational unloaded HUVEC. Accordingly, it is noteworthy that space flight reduced actin mRNA in the muscle [38]. We anticipate that actin down-regulation represents an adaptable mechanism aimed at avoiding actin accumulation. HUVEC initially redistribute actin fibers; after a few days, a transcriptionally regulated downmodulation of actin leads to a reduced synthesis of the protein and therefore prevents the accumulation of redundant actin fibers in the cytosol. More studies are necessary to understand the molecular mechanisms involved and their relevance in the process of endothelial adaptation to gravitational unloading.

Our studies may also be of interest to afford another challenging issue in biomedicine: the generation of macroscopic tissue equivalents for a variety of basic and applied medical purposes. Indeed, microgravity either in space or on the ground may become an unconventional and attractive venue for the generation of replacement organs for transplantation [16]. RWV bioreactors have been used to promote the differentiation of preassembled bioengineered tissue equivalents, such as skin, cartilage and fetal neuronal cells [16]. However, any attempt to neovascularize these prototissues by inoculating endothelial cells has failed. The study of endothelial behaviour and functions in the RWV may offer important insights to overcome crucial issues in generating artificial tissues.

Acknowledgements

This work was supported by grants from ASI, ESA and AIRC to JAMM. We thank Dr. Giovanna Mazzoleni, University of Brescia, for her suggestions and advice in the use of the RWV. We are also indebted to Dr. Laura Beguinot for helpful discussions.

References
