Impact of modeled microgravity on microvascular endothelial cells

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Abstract

Microvascular endothelial cells are protagonists in inflammation and angiogenesis. They contribute to the integrity of microvasculature by synthesizing a large array of cytokines, growth factors and mediators active on the endothelium itself, on smooth muscle cells and circulating leukocytes. Because space flight (i) associates with vascular impairment and (ii) modulates the cytokine network, we evaluated the effect of modeled microgravity on microvascular 1G11 cells. We found that modeled microgravity reversibly inhibits endothelial growth and this correlates with an upregulation of p21, a cyclin-dependent kinases inhibitor. By protein array, we found that microgravity inhibits the synthesis of interleukin 6, an event that may contribute to growth retardation. We also detected increased amounts of nitric oxide, a mediator of inflammatory responses, a potent vasodilator and a player in angiogenesis. The increased synthesis of nitric oxide is due, at least in part, to an upregulation of endothelial nitric oxide synthase. Because low levels of IL-6 might contribute to endothelial growth retardation as well as to the enhancement of nitric oxide synthesis, we hypothesize a central role of IL-6 in modulating microvascular endothelial cell behaviour in modeled microgravity.

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1. Introduction

The endothelium is a dynamic, highly heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic and immunologic functions. Functional properties of the endothelium include an active control of the various components of homeostasis, vascular tone and permeability and mural smooth muscle cell growth [1]. The endothelium is heterogeneous [2]. Several factors influence this heterogeneity including: (i) morphological and functional differences between large and small vessels and between cells derived from various microvascular beds; (ii) different response to growth factors; (iii) organ specificity reflecting the cumulative expression of post-translation modifications and also the expression of unique genes under the control of organ-specific regulatory elements.

Endothelial dysfunction promotes several common diseases. In particular, impairment of macrovascular endothelial cell function contributes to the pathogenesis of atherosclerosis [3], hypertension [4], diabetes and thrombosis [1]. On the other hand, microvascular endothelial cells are the protagonists in angiogenesis, i.e., the outgrowth of new capillaries from the pre-existing primary plexus, a crucial event to respond to tissue demands both in physiological and pathological conditions [5]. Dysregulated angiogenesis is considered a common denominator in most frequent diseases, from cancer to ischemic heart disease, from blindness to psoriasis and arthritis [6]. Microvascular endothelial cells are also crucial players in inflammation. Indeed, their activation leads to increase in vascular permeability, cell swelling and loss of barrier function, leukocyte adherence with cell clumping, and microthrombi formation.

In addition to different humoral modulators, endothelial cells respond to biomechanical stimulation. Recently, it has been demonstrated that hemodynamically derived stimuli are strong modulators of endothelial gene expression [7,8] and these findings may have relevant implications in the understanding of the mechanisms of vascular homeostasis. Another physical force that influences cell function is gravity [9] and this became clearly evident during manned orbital space flight. We have shown that human umbilical vein endothelial cells (HUVEC) are very sensitive to gravitational unloading [10], thus providing some explanation at the cellular level to an adaptive cardiovascular response experienced by astronauts
known as cardiovascular deconditioning. Because of the aforementioned endothelial heterogeneity, we studied the effects of modeled microgravity (from now microgravity) on microvascular endothelial cells, and found that it profoundly affects microvascular endothelial cell functions.

2. Material and methods

2.1. Cell culture and simulation of microgravity

Murine microvascular endothelial 1G11 cells were a gift from Drs. A. Mantovani and A. Vecchi (Istituto Mario Negri, Milano, Italy) [11]. Briefly, 1G11 cells were isolated from implanted subcutaneous sponges by collagenase digestion and selected with anti-CD31 monoclonal antibody-coated magnetic beads. A second cycle of immunomagnetic selection was performed to obtain a pure endothelial population. These cells expressed CD31, VE-cadherin, CD34, ICAM, VCAM and P-selectin as detected by flow cytometry and ELISA [11]. We routinely evaluated the expression of endothelial markers, i.e., VE-cadherin and CD34. By RT-PCR the cells do not express the VEGF-C/D receptor (Flt4) mRNA, which suggests no contamination by lymphatic endothelial cells occurs. The cells were utilized between passages 5 and 10 and serially passaged in DMEM containing 10% FCS, 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.

No device on earth can actually model the conditions in space. However, some bioreactors have been developed to simulate some aspects of 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 [12]. However, for simplicity, we refer to these conditions as microgravity. Subconfluent cells were seeded on beads (Cytodex 3, Sigma) [13]. As controls, subconfluent 1G11 cells grown on beads were cultured in Petri dish or in the vessels not undergoing rotation [13]. These two controls yielded comparable results. For proliferation assays, we trypsinized, stained with trypan blue solution (0.4%) and counted the viable cells using a Burker chamber. In other experiments, 1G11 cells were cultured in the RWV and, after 72 h, some samples were removed from the RWV and left in the incubator without undergoing rotation. After 3 additional days, the cells were counted as described. All the experiments were performed in triplicate at least three times.

2.2. Western blot

1G11 cells 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 SDS-PAGE and transferred to nitrocellulose sheets. Western analysis was performed using antibodies against p21, p53, endothelial nitric oxide synthase (eNOS), hsp70 and GAPDH (Tebu Bio-Santa Cruz). The antibody against murine IL-6 was kindly provided by Drs. Mantovani and Vecchi. Secondary antibodies were labelled with horseradish peroxidase (Amersham Pharmacia Biotech). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins [14].

2.3. Interleukin (IL)-1α synthesis

IL-1α concentration in cell extracts (50 μg) was detected using Quantikine mouse IL-1α immunoassay according to the manufacturer’s instructions (R&D Systems). The experiments were performed in triplicate for three times with similar results.

2.4. Protein array

1G11 cells were cultured in the RWV as described for 3 days. 100 μg of cell extracts were utilized to incubate the membranes on which 22 cytokines were spotted in duplicate (RayBiotech-Tebu Bio). The assay was performed according to the manufacturer’s instructions, as described [14].

2.5. Nitric oxide synthase (NOS) activity

NOS activity was measured by monitoring the conversion of 3H-arginine to 3H-citrulline. The assay was performed on 40 μg of cell extracts. Briefly, cell extracts were incubated in a reaction buffer (10 mM NADPH, 1 μM 3H-arginine, 25 mM Tris–HCl, 3 mM MgCl2, 1 mM FAD, 1 mM FMN, 100 mM CaCl2, and 60 μM CaC12) for 1 h at room temperature. The assay was terminated by the addition of a stop buffer (50 mM HEPES pH 5.5, 5 mM EDTA). 100 μl of pre-equilibrated resin (DOWEX 50WX8 200—400 mesh, H form, Sigma) were added to each sample, shaked, and centrifuged. The flow-through was collected and the radioactivity was quantified in a scintillation counter [15].

3. Results

3.1. Modulation of endothelial proliferation by microgravity

No experiment conducted on earth can model the conditions in space. However, different tools are available to isolate specific individual components of the collective effects induced by gravity. We have utilized the RWV [10,13].

Murine microvascular endothelial 1G11 cells were grown in the RWV for different times. As shown in Fig. 1A, culture in microgravity inhibited 1G11 cell proliferation at all the time points tested. Accordingly, culture in the RWV inhibited thymidine incorporation in 1G11 cells (data not shown). To determine whether the inhibition of endothelial proliferation was reversible upon return to normal conditions, we cultured 1G11 cells in the RWV for 72 h and then returned them to normal growth conditions for 72 additional h. Fig. 1B shows that the inhibitory effect of microgravity was reversible upon return to normal culture conditions.

Interestingly, we found that the growth inhibition in microgravity correlated with the upregulation of p21 (WAF1), an inhibitor of cyclin-dependent kinases, as detected by Western blot, and this event seems to be p53-independent since no modulation of p53 was observed in 1G11 cells in microgravity (Fig. 2). p21 levels were similar to the controls after return to normal culture conditions (data not shown).

HUVEC sensed microgravity as a stress and upregulated hsp70 [10]. On the contrary, 1G11 cells showed a slight (1.8 fold), but reproducible, downmodulation of hsp70 by Western analysis (Fig. 2).

3.2. Modulation of the synthesis of cytokines by microgravity

Since we have shown a downregulation of IL-1α in HUVEC cultured in microgravity [10], we evaluated the amounts of IL-1α by ELISA in 1G11 after 48 and 96 h in microgravity and found no modulation (Fig. 3). To obtain a broader profile of cytokine synthesis in 1G11 cells cultured in microgravity, we performed a protein array. 1G11 cells were cultured for 72 h in the RWV. Cell extracts were then utilized for the proteomic analysis. Table 1 shows an overview of the cytokines synthesized by 1G11 cells. Out of 22 cytokines evaluated, we found a significant decrease
of the amounts of IL-6 and a modest increase of the levels of MCP-1 in RWV cultured 1G11 cells (Table 1). Interestingly, no modulation of TNF\(\alpha\) was detected. We confirmed the decreased levels of IL-6 by Western blot using anti-murine IL-6 antibodies (Fig. 4).

3.3. Modulation of nitric oxide synthase expression and activity in microgravity

We evaluated NOS expression and activity in cells cultured for 72 h in microgravity. Fig. 5A shows an upregulation of eNOS (2.8 fold). This result may explain, at least in part, the higher NOS activity detected in cells cultured in the RWV in respect to controls, as measured by the conversion of arginine to citrulline (Fig. 5B). Because NO regulates endothelial proliferation, it is noteworthy that inhibition of NO synthesis by N(G)-nitro-L-arginine methyl ester (L-NAME) did not alter 1G11 proliferation in response to microgravity (not shown).

4. Discussion

Influences of microgravity on cell behavior have been suggested by a number of reports, describing alterations of proliferation, signal transduction and gene expression [16–19]. Some of the results obtained in vitro offer an explanation to altered clinical and biochemical parameters during manned orbital space flight. 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 [19].
We have recently shown that microgravity affects HUVEC behaviour [10]. When exposed to microgravity, HUVEC activate a stress response and downregulate IL-1\(\alpha\). Since IL-1\(\alpha\) is an inhibitor of endothelial growth, the decreased levels of IL-1\(\alpha\) contribute to a stimulation of cell proliferation. Also, bovine aortic endothelial cells grow faster than controls in microgravity, although the molecular mechanisms involved have not been elucidated [20]. Because endothelial cells are highly heterogeneous [2] and most of the studies are performed on macrovascular endothelial cells, we have investigated the response of microvascular endothelial cells to simulated microgravity. We dissected some molecular pathways and found that 1G11 cells behave very differently from other endothelial cells. It is reasonable to propose that some of the differences observed could be dependent on the species from which the endothelial cells originate. However, it is worth to report that preliminary results indicate that human microvascular endothelial and 1G11 cultured in the RWV cells show a similar behavior. Since microvascular cells are protagonists in angiogenesis, inflammation and repair, we pointed our attention on events that are critical in the aforementioned processes, i.e. proliferation and cytokine synthesis. We found that microgravity inhibits 1G11 cell growth and this correlates with the upregulation of p21. p21 negatively regulates cell cycle progression by binding and inhibiting the activity of cyclin/CDK2 complexes necessary for the transition from the G1 to the S phase. In addition, p21 can block DNA synthesis by DNA polymerase \(\delta\) through its direct interaction with PCNA [21]. It has been demonstrated that p21 is regulated at least by two alternative mechanisms, p53 dependent and p53 independent [22]. p53 is induced in response not only to DNA damage, but also to a wide variety of stimuli, including hypoxia and redox stress [22]. We did not detect any increase of p53, suggesting that it is not involved in p21 induction by microgravity. It is noteworthy that laminar shear stress causes a sustained p53 activation, which is responsible for the upregulation of p21, thus arresting endothelial growth [23]. We conclude that different mechanical stimuli, such as microgravity and shear stress, utilize different molecular pathways to inhibit endothelial proliferation. It is noteworthy that a significant decrease of the density of small vessels was reported in the quail chorioallantoic membranes after space flight [24]. In addition, the development of vascular channels in a rat fibular osteotomy model was inhibited after a shuttle flight, suggesting that bone healing may be impaired during space travel [25]. Accordingly, orbital spaceflight retards the capacity of wounds to

**Table 1**

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<th>Protein Array</th>
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Cell extracts were utilized to evaluate the levels of 22 cytokines spotted in duplicate on the filters. The spots were quantitated by densitometry. The results are shown as arbitrary units ranging from (–) negative to (++++) highly expressed.
heal in the rat [26]. Because angiogenesis and, therefore, endothelial proliferation are crucial in wound healing, our results on microvascular endothelial cells may shed some light on the molecular mechanisms contributing to the impairment of wound healing in reduced gravity.

Interestingly, the inhibition of cell growth and p21 upregulation were reversible upon return to normal gravity. Analogously, also in HUVEC microgravity-induced dysfunctions are rapidly reversible upon return to normal culture conditions [10].

While HUVEC adapted to microgravity through the upregulation of hsp70 [10], stress proteins seem not to be involved in 1G11 response to microgravity, underscoring that endothelial cells of different origin activate different mechanisms to adapt to microgravity. While some cell types undergo apoptosis in microgravity, this is not happening in HUVEC and 1G11 cells. We proposed that the upregulation of hsp 70 could protect HUVEC from apoptosis [10]. In the case of 1G11 cells, we hypothesize a role of p21, since high amounts of p21 prevent endothelial apoptosis [27].

Microgravity profoundly impacts on cytokine expression. It has been reported that bone marrow macrophages activated in space secrete more IL-1α and TNFα than controls on earth [28]. Recently, the overexpression of IL-1 receptor antagonist (ILRA), a member of the IL-1 superfamily, was described in space-flown WI38 human fibroblasts [29]. Also endothelial cells participate to the immune response because they are a good source and a target of cytokines, some of which are fundamental both in angiogenesis and inflammation [1]. In contrast with results obtained in HUVEC, 1G11 microvascular endothelial cells do not modulate IL-1α synthesis. To have a profile of cytokine synthesis in 1G11 cultured in microgravity, we used a proteomic approach—i.e. protein array. We detected a marked reduction of IL-6, which was confirmed by Western blot. IL-6 is a pleiotropic cytokine implicated in acute phase response and inflammation [30]. Since IL-6 is an autocrine growth factor for endothelial cells [31], it is feasible that its downregulation in microgravity may have a role in inhibiting 1G11 cell proliferation.

We also show that nitric oxide synthesis is increased by microgravity. These data are in agreement with the results obtained in bovine aortic endothelial cells cultured in the RWV [20] as well as with the increased NO synthesis observed in rodents subjected to hindlimb unloading (HU), a model which simulates cardiovascular deconditioning in humans [32]. In particular, differential alterations of NOS expression and nitric oxide synthesis of different arteries after hindlimb unweighting were reported, and this represents, at least in part, a localized adaptation to body fluid redistribution [33].

NO acts in a paracrine fashion to stimulate smooth muscle cells guanylyl cyclase to produce 3’,5’-cyclic monophosphate which causes relaxation of the blood vessels [34]. NO also contributes to the angiogenic program by triggering endothelial cell growth and differentiation [35]. Interestingly, an inverse relation has been described between NO and IL-6 [36]. We hypothesize that the downmodulation of IL-6 may have a central role in microvascular endothelial cell response to microgravity, since low amounts of IL-6 might contribute to growth retardation as well as to the enhancement of NO synthesis. Because increased NO may have a role in post-flight orthostatic intolerance on return to earth, our results may offer new insights to counteract cardiovascular deconditioning.

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References


