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Modification of Proteins Secreted by Endothelial Cells During Modeled Low Gravity Exposure

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ABSTRACT

The exposure of the human body to microgravity, conditions that occurs during space flights, causes significant changes in the cardiovascular system. Many cell types have been involved in these changes, and the endothelium seems to play a major role. In endothelial cells (EC), it has been shown that modeled low gravity impairs nitric oxide synthesis, cell adhesion, extracellular matrix composition, cytoskeleton organization, cytokines, and growth factors secretion. Nevertheless, detailed analysis of EC physiological changes induced by microgravity exposure is still lacking. Secretome analysis is one of the most promising approaches for the identification of biomarkers directly related to the physiopathological cellular state. In this study, we analyzed in details the modifications of EC secretome by using umbilical vein endothelial (HUVE) cells exposed to modeled low gravity conditions. By adopting a two-dimensional (2-D) proteomic approach, in conjunction with a technique for the compression of the dynamic range of proteins, we observed that modeled low gravity exposure of HUVE cells affected the secretion of proteins involved in the regulation of cytoskeleton assembly. Moreover, by using Luminex® suspension array systems, we found that the low gravity condition decreased in ECs the secretion of some key pro-inflammatory cytokines, including IL-1α and IL-8, and of the pro-angiogenic factor bFGF. On the contrary, microgravity increase the secretion of two chemokines (Rantes and Eotaxin), involved in leukocytes recruitment. J. Cell. Biochem. 112: 265–272, 2011.

KEY WORDS: MICROGRAVITY; ENDOTHELIUM; CYTOSKELETON REORGANIZATION; INFLAMMATION

Over the 50-year-old history of human space flights it has been demonstrated that the human body suffers from several conditions when it is subject to microgravity: bone mineral density is lost, muscle atrophy and cardiovascular deconditioning occur, pulmonary function and fluid regulating systems are impaired, sensory and balance systems are disturbed. Such negative effects of microgravity have to be reversed when astronauts return to normal gravity especially following long-duration space flights [Aubert et al., 2005]. In particular, space flights affect the cardiovascular system by compromising cardiovascular performances, causing cardiac dysrhythmias, cardiac atrophy, orthostatic intolerance, and inducing a reduced aerobic capacity [Convertino, 2009].

Structural and functional changes in the endothelium have been clearly involved in cardiovascular deconditioning [Carlsson et al., 2003], even if it has to be considered that the direct effect of weightlessness in space on EC is influenced also by physical inactivity and decreased shear stress [Navasiolava et al., 2010]. Since the endothelium plays a pivotal role in the regulation of microvascular homeostasis and of local blood flow, endothelial dysfunctions are likely to be at the basis of weightlessness-induced cardiovascular changes [Coupé et al., 2009]. ECs are key to vascular permeability, inflammation, and angiogenesis and, in fact, these processes seem to be impaired in space. For this reason, several studies have been devoted to the comprehension of how microgravity affects endothelial cell functions, by using different in vitro cell models. ECs are very heterogeneous and, as a consequence, the general mechanisms of ECs adaptation to microgravity are not easy to ascertain. Yet, it is widely recognized that ECs, when cultured

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under low gravity, show increased nitric oxide production and cytoskeletal reorganizations that include a reduction in β-actin expression [Carlsson et al., 2003; Infanger et al., 2007, Versari et al., 2007]. In fact, cytoskeletal alterations and increased nitric oxide synthesis have been observed by using different EC types such as microvascular EC, HUVE cells, and bovine aortic cells [Sanford et al., 2002; Spisni et al., 2006; Mariotti and Maier, 2008]. Nevertheless, the molecular mechanisms responsible of the increased NO secretion seem to vary in different cell types [Spisni et al., 2006; Mariotti and Maier, 2008; Balligand et al., 2009]. With reference to inflammatory responses, a decreased secretion of IL-1α has been observed in HUVE cells cultured in modeled low gravity obtained by using the Rotating Wall Vesel (RWV) bioreactor [Carlsson et al., 2003]. Other studies have shown a slight reduction of IL-8 in microvascular ECs cultured under low gravity, achieved by using the Random Positioning Machine (RPM) [Mariotti and Maier, 2008]. Infranger and collaborators [2007] found that only IL-10 secretion was reduced under low gravity when they tested a panel of six cytokines in EA.hy926 cells, a human ECs line. In consideration of these scattered data, the understanding of ECs inflammatory responses modifications under low gravity is still partial even if it results clear that spaceflight and the ground-based simulation of microgravity affect the production and the activation of various cytokines in vivo [Girten et al., 1995].

The role of microgravity in apoptosis regulation is still debated [Lewis et al., 1998; Jessup et al., 2000; Grimm et al., 2002]. Yet, we have found, together with other labs, that low gravity does not seem to modify the percentage of apoptotic HUVE cells [Carlsson et al., 2003; Spisni et al., 2006]. The effect of microgravity on the angiogenic responses of ECs is of great interest and RWV bioreactors have been also used to obtain preassembled engineered tissues [Unsworth and Lelkes, 1998]. Angiogenesis is a multistep process that includes EC proliferation and migration, basement membrane degradation, and deposition. According to the results obtained to date, different EC types show different behaviors when cultured in modeled low gravity. For example, Carlsson and collaborators [2003] found an increased proliferation rate in HUVE cells exposed to simulated microgravity, while Morbidelli and collaborators [2005] obtained opposite results in porcine aortic ECs. It has also been observed that the expression of many angiogenic molecules are modified by microgravity. In particular, it has been found that the synthesis of NO and tissue inhibitor of metalloproteases TIMP-2, is increased by microgravity exposure, whereas the synthesis of VEGF, Endothelin-1 and IL-8 is decreased [Infanger et al., 2007; Mariotti and Maier, 2008]. While some EC types respond to modeled microgravity by decreasing their angiogenic potential in vitro other types do not [Spisni et al., 2006; Mariotti and Maier, 2008]. Nevertheless, in animal models, several lines of evidence suggest that exposure to space flight strongly affects the angiogenic response [Henry et al., 1998; Davidson et al., 1999].

With the aim of better clarifying EC physiopathological modifications induced by low gravity, we decided to investigate possible pathway biomarkers of microgravity exposure by analyzing HUVE cell secreted proteins. Secretome is one of the most promising biofluids for the detection of markers directly related to a cellular pathological condition. Secretome research is a new and widely adopted strategy to understand disease progression [Bonin-Debs et al., 2004]. For this reason, we have collected the secretome of HUVE cells exposed to low gravity produced by RPM. To broaden our analysis further, Proteominer® technology was adopted for compressing the dynamic range of collected proteins. To this end, we also applied Luminex® technology to identify and quantify the differential secretion of cytokines, chemokines, and growth factors, induced by low gravity exposure.

MATERIALS AND METHODS

REAGENTS

Fetal bovine serum was purchased from BioWhittaker (Cambrex, East Rutherford). All the other reagents for cell culture were purchased from Sigma (St. Louis, MI). Rabbit monoclonal antibody to Gelsolin was from Abcam (Cambridge, UK). Polyclonal antibody to α-tubulin, was from Sigma. Secondary Cy3 and Cy5 conjugated antibodies were from GE (USA). Multiplex cytokine assay kits were from Bio-Rad (USA) and Millipore (USA).

CELL CULTURE AND CONDITIONED MEDIA COLLECTION

HUVE cells were isolated from recently collected unfrozen umbilical cords. Cells were grown in M199 medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, 50 μg/ml endothelial cell growth supplements (ECGS), 5 U/ml heparin, and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml), as previously described [Spisni et al., 2006]. Cells were maintained at 37°C in a 5% CO_2 incubator and used for experiments at third and fourth passages. HUVE cells were assayed for Won Willebrand factor (indirect immunofluorescence) at passage 1 and 3. Batches with positivity below 90% were discarded. Microgravity was generated by a 3D clinostat positioned in a thermostatic room, and particular attention was taken to exclude the possibility of false-positive results. The effects of vibration and gradient accumulation or depletion of nutrients were almost completely eliminated by conducting control experiments. Modeled microgravity conditions were achieved by Random Position Machine (RPM, Dutch-Space). The RPM reproduces gravity acceleration between 10^{-4} and 10^{-3} g [Hoson et al., 1997]. Cell cultures exposed to modeled microgravity were positioned at a maximum distance of 3 cm from the centre of the frame. Rotation of each frame is random, autonomous, and regulated by computer software. The rotation velocity of the frames was 60°/s. Static control cultures (named here as controls) were positioned on the basement of the RPM [Schwarzenberg et al., 1999]. HUVE cells (1.5×10^6) were grown on both sides of OptiCell flasks (Thermo Scientific, USA) without any coating. OptiCells were then entirely filled (to avoid shear stress) with 10 ml of complete medium. OptiCells create a closed cellular environment with high cellular density (100 cm^2/10 ml) in which the O_2 and CO_2 levels into the medium are maintained through the pores of the growth membrane. Using OptiCells, oxygenation of the medium was not required. At confluence, cells were exposed to modeled microgravity for 96 h. Both exposed and control OptiCells were treated identically until the start of clinorotation. The temperature and the pH of the culture medium were measured and kept constant to ascertain that the experiments were conducted under identical conditions. Scrupulous

attention was made to eliminate the formation of bubbles into the OptiCell flasks during clinorotation. Immediately after microgravity exposure, conditioned media were collected and frozen at −80°C before 2-DE and Western blotting analyses.

PROTEOMINER® PROTEIN ENRICHMENT

ProteoMiner beads (BioRad, USA) were used to reduce high-abundance proteins in serum-containing HUVE cells conditioned media. 1 mg/sample of secreted proteins were loaded on ProteoMiner beads, following manufacturer's instructions.

TWO-DIMENSIONAL ELECTROPHORESIS

In 2-DE experiments, the Ettan IPGphor III IEF System (GE) was used for the IEF step. A 150 μg protein sample containing 2% CHAPS and 1% carrier ampholyte mixture, pH 4–7 (GE Healthcare, UK) was loaded onto a 13 cm (pH 4–7) strip (GE Healthcare). Application of the strips and the running procedure were carried out as described by the manufacturer. Rehydratation was performed for 12 h at room temperature and was followed by the IEF run. Strips were kept at 1,000 V or frozen at −80°C until loaded on the second dimension gels. Before starting the second dimension, strips were equilibrated for 20 min in 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris, pH 6.8, and 2% DTT. Afterward, strips were equilibrated in 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris, pH 6.8, and 2.5% iodoacetamide for an additional 20 min. The second dimension was carried out on a SE 600, $14 \times 16\ cm^2$ electrophoresis apparatus (Hoeffer Scientific Instruments, USA), by using 12% SDS–polyacrylamide gels. After runs, gels were stained overnight with Ruthenium II Tris (batho-phenantroline disulfonate; Fluka, USA), as previously described [Lamanda et al., 2004]. Stained gels were scanned by using a Pharos FX (Bio-Rad) scanner at a resolution of 50 μm. Raw images (each sample was analyzed in four different gels) were analyzed by using Proteomweaver 4.0 software (Bio-Rad). Differentially expressed spot, with intensity increased or decreased more than threefold in the average map were collected by using the automated EX Quest spot cutter (Bio-Rad).

WESTERN BLOTTING

Conditioned media proteins were separated by a 12% SDS–PAGE, and then electrophoretically transferred onto PVDF membranes. Membranes were incubated with primary antibodies (rabbit monoclonal antibody to Gelsolin and polyclonal antibody to α-tubulin) and, after washing, with secondary conjugated antibody (anti rabbit Cy3 or Cy5, ECL Plex; GE) following manufacturer's instructions. Immunolabeling was visualized by using a laser scanner system (Pharos FX; Bio-Rad) and bands were quantified by using Quantity One software (Bio-Rad). Normalization was made against the total amount of secreted proteins loaded.

IN-GEL TRYPTIC DIGESTION AND RP-LC-MS/MS ANALYSIS

Gel spots were washed in 25 mM ammonium hydrogencarbonate, 50 mM ammonium hydrogencarbonate/acetonitrile 1:1 and covered with acetonitrile until gel pieces shrunk. Residual acetonitrile was removed and the gel pieces dried by centrifugation under vacuum by SPD SpeedVac. In-gel digestion was performed by adding 12.5 ng/μl of bovine trypsine (Promega Madison, WI) in 25 mM ammonium hydrogencarbonate at 37°C overnight under stirring. The identification of proteins was performed on peptide mixture analyzed by nanoflow reversed-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/MS). LC-MS/MS analysis was performed in positive mode, on an LTQ XL linear ion trap with nanoESI source (Thermo electron, San Josè, CA) coupled on line to a Dionex Ultimate 3000 (DIONEX, Sunnyvale, CA) equipped with an Ultimate 3000 autosampler. Peptides (10 μl) were desalted in a trap column (Acclaim PepMap 100 C18, LC Packings, DIONEX) with 96% of solution A (H_2O with 4% ACN, 0.1% formic acid) and 4% of solution B (ACN with 5% H_2O with 0.1% formic acid) for 5 min then separated in a reverse phase column, a 10 cm long fused silica capillary (Silica Tips FS 360–75–8, New Objective, Woburn, MA), slurry-packed in-house with 5 μm, 200 Å pore size C18 resin (Michrom BioResources, CA). Peptides were eluted using a linear gradient from 4–60% B in 40 min at 300 ml/min flow rate. The column was washed for 5 min with 90% B and re-equilibrated with 4% B for 10 min.

The LTQ operated in data-dependent mode in which each full MS scan event was followed by five MS/MS scans, where the five most abundant parent ions were dynamically selected and fragmented with collision induction dissociation (CID) using a collision energy of 35%. All spectra was acquired with an m/z scan range of 200–2,000. Tandem mass spectra were matched against National Center for Biotechnology Information (NCBI) Human database through SEQUEST [Yates et al., 1995] algorithm incorporated in the Bioworks software (version 3.3, Thermo Scientific) using the following search parameter: static cysteine alkylation by iodoacetamide; partial methionine oxidation, mass tolerance of precursor 2.0 Da and mass tolerance of fragment ions set 1.0 Da. A peptide has been considered legitimately identified when it achieved cross correlation scores of 1.5 for [M + H]1+, 2.5 for [M+2H]2+, 3 for [M+3H]3+, and a peptide probability cut-off for randomized identification of $P < 0.001$.

CYTOKINE DETECTION

The simultaneous detection of 28 extracellular factors released by HUVE cells was performed by using Luminex® technology based multiplexed bead immunoassay, following manufacturer's instructions. The levels of 27 cytokines, chemokines, and growth factors (IL-1β, IL-1 RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, PDGF-B, bFGF, G-CSF, GM-CSF, VEGF, TNFα, IFNγ, IP-10, Eotaxin, MCP-1, MIP-1α, MIP-1β, Rantes) were measured using the human ultrasensitive cytokine 27-plex antibody bead kit (BioRad), while IL-1α level was measured using Milliplex® Map kit (Millipore). The concentrations of analytes into the samples were estimated from the standard curve, using a fifth-order polynomial equation, and expressed as pg/ml (Bio-Plex Manager software 5.0). The intra-assay CV averaged 19%.

STATISTICAL ANALYSIS

Data were expressed as mean ± SEM of three independent experiments. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences. Differences were considered statistically significant at $P < 0.01$.

RESULTS

In order to study the effects of modeled low gravity on HUVE cells secreted proteins, we decided to grow ECs for 96 h under modeled low gravity. During this time, secreted proteins accumulate into the culture media and reach amounts compatible with 2-DE analysis. To maintain a physiological extracellular milieu during this time, we decided to maintain serum in the culture media, even if this may increase the abundance of background proteins detected in the following proteomic analysis. It must also be noted that serum-free conditions significantly modify the ECs proliferation rate after 24 h. As a consequence, EC physiology is strongly affected after 96 h of serum deprivation. ProteoMiner® beads were used to reduce the amount of high-abundance serum proteins present in our conditioned media before 2-DE analyses. This technology is based upon a combinatorial random hexapeptides library, capable of interacting with most, if not all, proteins in any given proteome. The limited binding capacity of the beads strongly reduce the amount

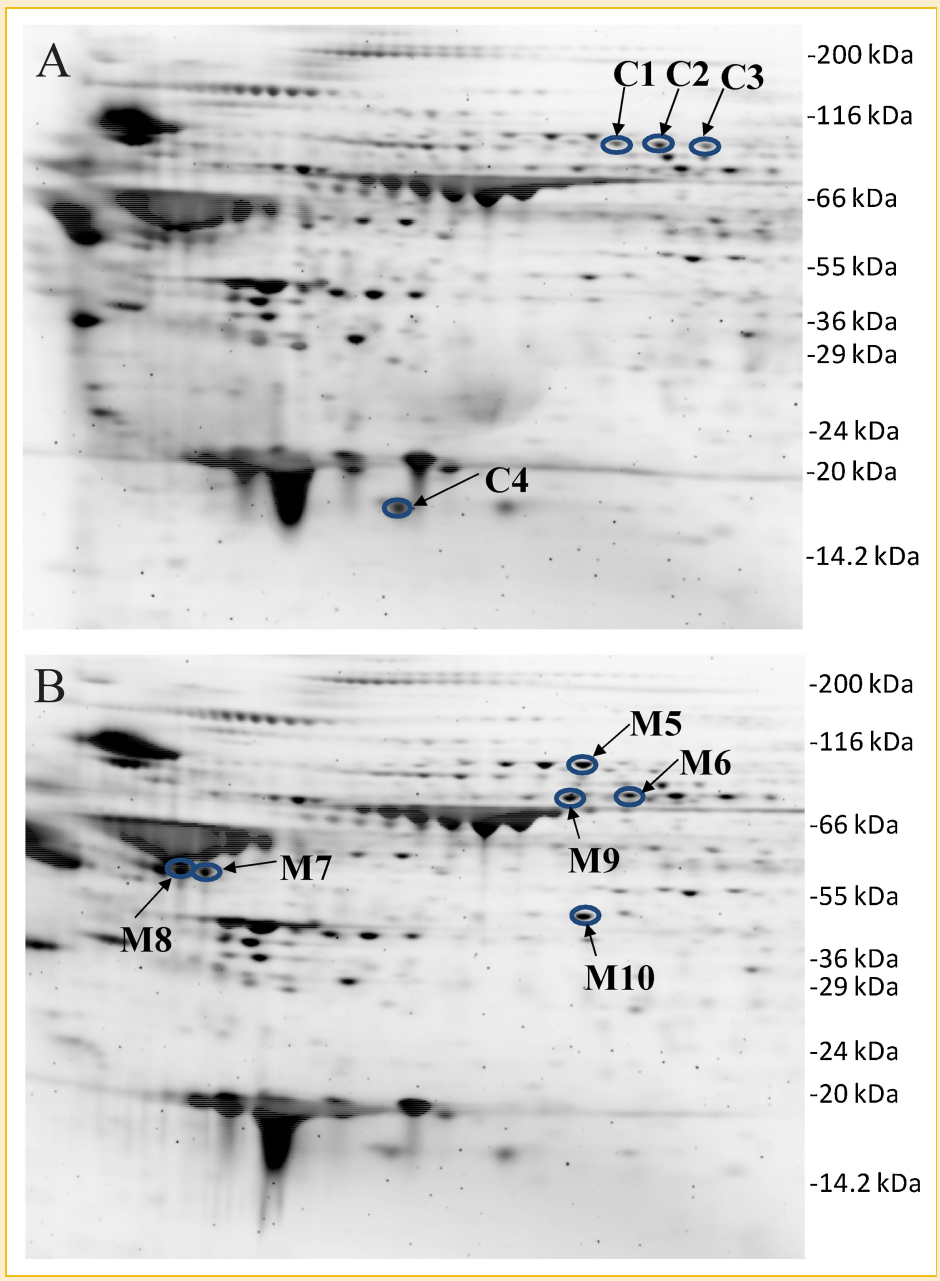

Fig. 1. 2-D proteome analysis of cellular secretome of normal gravity (A) and low gravity (B) cultured HUVE cells. Proteins derived from supernatants were depleted of high-abundant proteins by using Proteominer® technology and then resolved (150 μg for each gel) by 2-DE (n = 4 gels/condition) and stained with Ruthenium fluorescent dye. Circles indicate the differentially expressed spots (C1–4 downregulated, M5–10 up-regulated in modeled microgravity). These spots were then identified by MS/MS analyses (see Table I). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I. Summary of HUVE Cells Secreted Proteins That Were Differentially Expressed Under Normal Gravity Versus Microgravity Conditions

Spot	Reference	P (pro)	Cov	Score	NCBI ID
C1	AF304164_1 keratin 1 [Homo sapiens]	1,45E-11	8	80,26	gi|11935049
C2	AF304164_1 keratin 1 [Homo sapiens]	5,03E-14	6	60,28	gi|11935049
C3	AF304164_1 keratin 1 [Homo sapiens]	1,55E-10	6	60,24	gi|11935049
C4	beta globin [Homo sapiens]	8,54E-07	1	10,20	gi|4504349
M5	gelsolin isoform b [Homo sapiens]	1,00E-30	11	110,42	gi|38044288
M6	coagulation factor II precursor [Homo sapiens]	5,62E-12	5	30,25	gi|4503635
M7	tubulin, alpha 1a [Homo sapiens]	2,10E-09	5	50,26	17986283
M8	tubulin, alpha 1a [Homo sapiens]	6,05E-12	14	140,27	17986283
M9	coagulation factor II precursor [Homo sapiens]	1,39E-09	3	30,19	gi|4503635
M10	S-adenosylhomocysteine hydrolase [Homo sapiens]	1,12E-10	6	60,33	gi|9951915

Major identification parameters such as probability, score and coverage (number of unique peptides identified) are reported.
NCBI, National Center for Biotechnology Information.

of high-abundant proteins and, at the same time, concentrate low-abundant proteins, thereby decreasing the dynamic range of proteins in the sample. ProteoMiner® has been successfully adopted with a wide number of human biological fluids, including serum, urine, bile, cerebrospinal fluid as well as cell lysates [Guerrier et al., 2007; Sennels et al., 2007; Boschetti et al., 2008; Righetti and Boschetti, 2008].

The 2-DE analysis of HUVE cell secretome in normal or in microgravity conditions, after ProteoMiner® enrichment (Fig. 1A,B), revealed that six proteins were upregulated in the conditioned media of ECs exposed to low gravity, while four proteins were down-regulated. MS/MS mass spectrometry identified them as reported in Table I. Among such proteins, we focused our attention on gelsolin and α-tubulin, since both secreted proteins are involved in the reorganization of the cytoskeleton. Western blotting analysis (Fig. 2) confirmed the increased secretion of the two proteins under low gravity conditions. Moreover, in order to expand our findings to cytokines, chemokines, and growth factors, we decided to analyze EC secretome by using multiplex suspension arrays since such proteins are not detectable in 2-DE gels owing to their low abundance or their low molecular weight. We chose a panel of 28 analytes involved in inflammation and angiogenesis. Our results confirm that modeled microgravity reduces the secretion of IL-1α (from 8.70 ± 0.74 to 2.82 ± 0.23 pg/ml) and IL-8 (from 8,152 ± 743 to 4,631 ± 384 pg/ml; Fig. 3) as previously reported by other groups [Carlsson et al., 2003; Mariotti and Maier 2008]. Furthermore, we also found that microgravity significantly reduces the secretion of bFGF (from 7.85 ± 0.69 to 3.76 ± 0.33 pg/ml). This contributes to explain the anti-angiogenic phenotype of ECs observed in gravitational unloading [Mariotti and Maier, 2008]. For what chemokines secretion concerns, we found a significant increase in the secretion of two chemokines: Rantes (from 2.61 ± 0.21–4.68 ± 0,41 pg/ml) and Eotaxin (from 11.81 ± 1.04–18.48 ± 1.65 pg/ml). Both play active roles in leukocytes recruitment (Fig. 5).

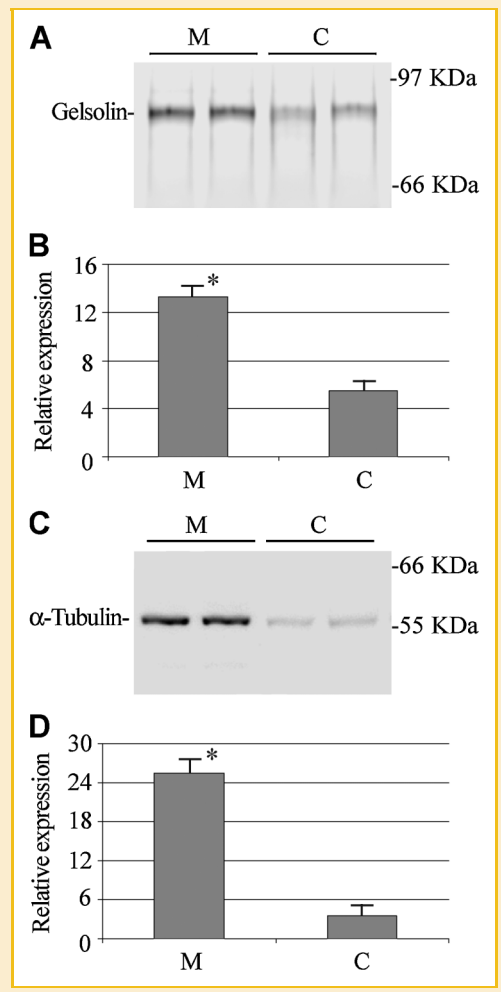

Fig. 2. Validation of the proteomic data. The increased level of secreted gelsolin (A,B) and α-tubulin (C,D) after 96 h of modeled microgravity were confirmed by Western Blotting analyses. Experiments were repeated three times (n = 6) and the increased levels of the secreted proteins were quantified by densitometric analysis (panels B and D, see details in Materials and Methods Section), normalizing against the total amount of secreted proteins loaded.

DISCUSSION

RPM bioreactor has proved to be a valuable device to study simulated microgravity on Earth [Versari et al., 2007]. The exposure

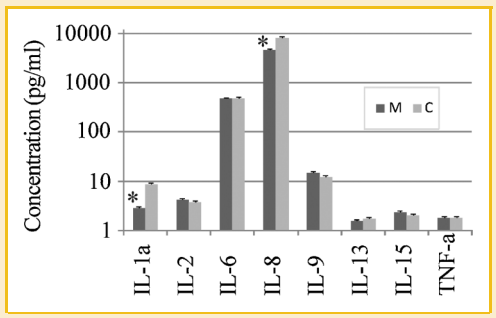

Fig. 3. Cytokines released in the conditioned media by HUVE cells after 96 h of normal gravity or modeled microgravity. Levels of cytokines were quantified by using the Luminex®-based multiplexed bead immunoassay. Concentration values are expressed as log (pg/ml) and represent the mean ± SEM of three independent measurements; $^{*}P < 0.01$.

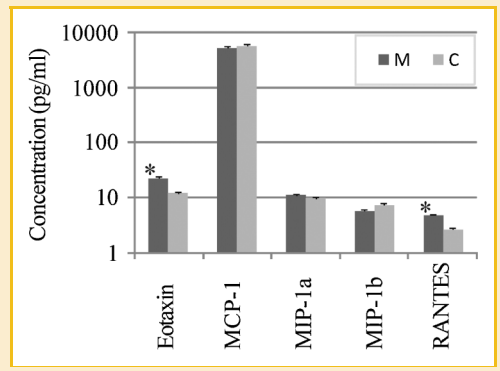

Fig. 5. Chemokines released in the conditioned media by HUVE cells after 96 h of normal gravity or modeled microgravity. Levels of chemokines were quantified by using the Luminex®-based multiplexed bead immunoassay (see details in "Materials and Methods" Section). Concentration values are expressed as log(pg/ml) and represent the mean ± SEM of three independent measurements. $^{*}P < 0.01$.

of HUVE cells to the simulated microgravity obtained by using a RPM produced interesting modifications of the EC secretome. Secreted proteins and peptides include members of signaling pathways, blood coagulation and immune system factors, as well as components of the extracellular matrix. This class of proteins is generated from about one-tenth of the human genome (a secreted protein database is available at: http://spd.cbi.pku.edu.cn).

By using 2-D electrophoresis, associated with a technique for the compression of the dynamic range of proteins, we found that six proteins were differentially secreted under microgravity conditions. Interestingly, among them we found two proteins involved in cytoskeleton rearrangements: gelsolin and α-tubulin. Gelsolin exists both as a cytoplasmic and as a secreted isoform. Secreted gelsolin normally circulates in plasma (pGSN) and is mainly produced by monocytes and endothelium [Lee et al., 2009]. While the cytosolic gelsolin participates in actin dynamics exclusively in the intracellular compartment [Sun et al., 1999], the secreted isoform has been linked to the protection of tissues against different types of insults [Christofidou-Solomidou et al., 2002; Becker et al., 2003; Rothenbach et al., 2004]. pGSN is capable of binding and modulating several bioactive lipids, such as platelet-activating factor [Osborn et al., 2007]. For this reason, it has been suggested that pGSN may play a role in innate immunity. In animal models and humans, pGSN is capable of suppressing the secretion of pro-inflammatory cytokines [Lee et al., 2007]. It is likely that its increased secretion in EC exposed to low gravity is a response, which protects endothelium from the increased secretion of pro-inflammatory cytokines. Also α-tubulin exists both as a cytoplasmic and as a secreted isoform. Cellular secretion of α-tubulin has been recently observed during the differentiation of monocyte to macrophage [Sintiprungrat et al., 2010] and this process has been linked to the cytoskeleton rearrangement that happens in the microtubule during monocyte/macrophage differentiation. Cytoskeleton rearrangement was observed in HUVE cells exposed to modeled microgravity a few years ago [Carlsson et al., 2003; Versari et al., 2007], but only recently [Li et al., 2009] have clearly demonstrated that low gravity rapidly disorganize microtubules in human MCF-7 cells. Therefore, α-tubulin secretion could be considered a good biomarker of the microtubule reorganization that happens in endothelial and other epithelial cells exposed to microgravity. We also identified, with quite high scores, the presence of keratin 1 in microgravity-exposed ECs secretome. The keratin 1 detected in 2-DE may be secreted [Srisomsap et al., 2010], but it can also be the result of contamination. Since the picked spots had a higher molecular weight than that expected for keratin 1, it is very likely that the presence of keratin 1 detected in our gels was due to contamination.

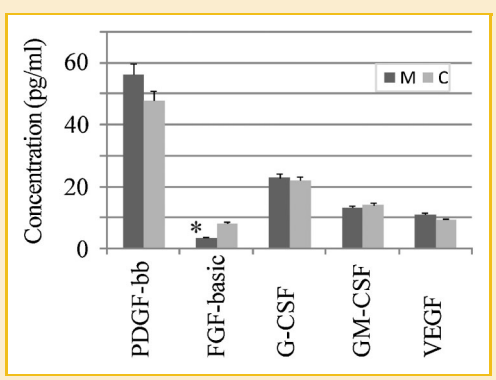

Fig. 4. Growth factors released in the conditioned media by HUVE cells after 96 h of normal gravity or modeled microgravity. Levels of growth factors were quantified by using the Luminex®-based multiplexed bead immunoassay (see details in "Materials and Methods" section). Concentration values are expressed as (pg/ml) and represent the mean ± SEM of three independent measurements; $^{*}P < 0.01$.

A decreased secretion of the pro-inflammatory cytokines IL-1α and IL-8 has been observed in HUVEC and microvascular endothelial cells (HMEC) exposed to modeled microgravity [Carlsson et al., 2003; Mariotti and Maier, 2008]. Nevertheless, contrasting literature data exist in regard to the anti-inflammatory phenotype expressed by ECs exposed to microgravity. For example, Infanger and collaborators [2007] found a decreased secretion of the anti-inflammatory cytokine IL-10 suggesting an increased inflammatory

state in EA.hy926 EC exposed to low gravity. Similar conclusions were drawn by Ulbrich et al. [2008] who observed an increased secretion of the pro-inflammatory IL-6 and IL-8 after culturing EA.hy926 cells for 24 h in a RPM machine. Our results sustain the hypothesis that microgravity is not a pro-inflammatory stimulus in ECs, at least for what the cytokine secretion concerns. In fact, analyzing the secretion of 18 different cytokines after 96 h of clinorotation, we only observed a significant decrease of IL-1α and IL-8. These contrasting results suggest that microgravity elicit different responses in different EC types. In particular, it seems that the cell line EA.hy926 behaves differently in comparison to primary ECs. In further support of this theory, EAhy926 was found to behave very differently from endothelial cells, at least with respect to cell proliferation and apoptosis [Boerma et al., 2006]. We also observed that among the six polypeptide growth factors analyzed, only bFGF secretion was modified by low gravity and showed a significant reduction. This piece of data may explain why HUVE cells angiogenic potential does not increase under modeled microgravity [Spisni et al., 2006], even if such condition promotes HUVE cells proliferation by decreasing IL-1α secretion and by increasing nitric oxide and prostacyclin synthesis.

Analysis of the secreted chemokines revealed that low gravity increased the release of both RANTES and Eotaxin, which are molecules capable of recruiting leukocytes and triggering inflammation. This suggests that microgravity may elicit contrasting effects on ECs. In fact, on the one hand it decreases the secretion of pro-inflammatory cytokines and on the other hand it increases the secretion of chemoattractant chemokines. In conclusion, our results provide novel insight into the modifications of EC physiology in the presence of low gravity and contribute to a better understanding of the physiopathological modifications of the vascular system occurring during space flights. It would be of utmost interest to ascertain if plasma gelsolin levels may be used as a biomarker of microgravity adaptation in animals and humans.

REFERENCES

Aubert AE, Beckers F, Verheyden B. 2005. Cardiovascular function and basics of physiology in microgravity. Acta Cardiol 60:129–151.

Balligand JL, Feron O, Dessy C. 2009. eNOS activation by physical forces: From short-term regulation of contraction to chronic remodelling of cardiovascular tissues. Physiol Rev 89:481–534.

Becker PM, Kazi AA, Wadgaonkar R, Pearse DB, Kwiatkowski D, Garcia JG. 2003. Pulmonary vascular permeability and ischemic injury in gelsolin-deficient mice. Am J Respir Cell Mol Biol 28:478–484.

Boerma M, Burton GR, Wang J, Fink LM, McGehee RE, Jr., Hauer-Jensen M. 2006. Comparative expression profiling in primary and immortalized endothelial cells: Changes in gene expression in response to hydroxy methylglutaryl-coenzyme A reductase inhibition. Blood Coagul Fibrinolysis 17:173–180.

Bonin-Debs AL, Boche I, Gille H, Brinkmann U. 2004. Development of secreted proteins as biotherapeutic agents. Expert Opin Biol Ther 4:551–558.

Boschetti E, Giorgio Righetti P. 2008. Hexapeptide combinatorial ligand libraries: The march for the detection of the low-abundance proteome continues. Biotechniques 44:S663–S665.

Carlsson SIM, Bertilaccio MTS, Ballabio E, Maier JAM. 2003. Endothelial stress by gravitational unloading: Effects on cell growth and cytoskeletal organization. Biochim Biophys Acta 1642:173–179.

Christofidou-Solomidou M, Scherpereel A, Solomides CC, Muzykantov VR, Machtay M, Albelda SM, DiNubile MJ. 2002. Changes in plasma gelsolin concentration during acute oxidant lung injury in mice. Lung 180:91–104.

Convertino VA. 2009. Status of cardiovascular issues related to space flight: Implications for future research directions. Respir Physiol Neurobiol 169: S34–S37.

Coupé M, Fortrat JO, Larina I, Gauquelin-Koch G, Gharib C, Custaud MA. 2009. Cardiovascular deconditioning: From autonomic nervous system to microvascular dysfunctions. Resp Physiol Neurobiol 1695:510–512.

Davidson JM, Aquino AM, Woodward SC, Wilfinger WW. 1999. Sustained microgravity reduces intrinsic wound healing and growth factor responses in the rat. FASEB J 13:325–329.

Girten B, McPherson S, Lee M, Grethe N, McDowell R, McPherson L, Tuttle RR. 1995. Effectiveness of a cytokine restraining agent (CRA (TM)) in attenuating disuse deconditioning induced by hindlimb unloading in rats. J Gravit Physiol 2:121–122.

Grimm G, Bauer J, Kossmehl P, Shakibaei M, Schoberger J, Pickenhahn H, Schulze-Tanzil G, Vetter R, Eilles C, Paul M, Cogoli A. 2002. Simulated microgravity alters differentiation and increases apoptosis in human follicular thyroid carcinoma cells. FASEB J 16:604–606.

Guerrier L, Claverol S, Fortis F, Rinalducci S, Timperio AM, Antonioli P, et al. 2007. Exploring the platelet proteome via combinatorial, hexapeptide ligand libraries. J Proteome Res 6:4290–4303.

Henry MK, Unsworth BR, Sychev V, Guryeva TS, Dadasheva OA, Piert SJ, Lagel KE, Dubrovin LC, Jahns GC, Boda K, Sabo V, Samet MM, Lelkes PI. 1998. Launch conditions might affect the formation of blood vessels in the quail chorioallantoic membrane. Folia Vet 42:S25–S31.

Hoson T, Kamisaka S, Masuda Y, Yamashita M, Buchen B. 1997. Evaluation of the three-dimensional clinostat as a simulator of weightlessness. Planta 203:S187–S197.

Infanger M, Ulbrich C, Baatout S, Wehland M, Kreutz R, Bauer J, Grosse J, Vadrucci S, Cogoli A, Derradji H, Neefs M, Küsters S, Spain M, Paul M, Grimm D. 2007. Modeled gravitational unloading induced downregulation of endothelin-1 in human endothelial cells. J Cell Biochem 101:1439–1455.

Jessup JM, Frantz M, Sonmez-Alpan E, Locker J, Skena K, Waller H, Battle P, Nachman A, Bhatti WME, Thomas DA, Curbeam RL, Jr., Baker TL, Goodwin TJ. 2000. Microgravity culture reduces apoptosis and increases the differentiation of a human colorectal carcinoma cell line. In Vitro Cell Dev Biol Anim 36:367–373.

Lamanda A, Zahn A, Röder D, Langen H. 2004. Improved Ruthenium II tris (bathophenantroline disulfonate) staining and destaining protocol for a better signal-to-background ratio and improved baseline resolution. Proteomics 4:599–608.

Lee PS, Waxman AB, Cotich KL, Chung SW, Perrella MA, Stossel TP. 2007. Plasma gelsolin is a marker and therapeutic agent in animal sepsis. Crit Care Med 35:849–855.

Lee PS, Sampath K, Karumanchi SA, Tamez H, Bhan I, Isakova T, Gutierrez OM, Wolf M, Chang Y, Stossel TP, Thadhani R. 2009. Plasma gelsolin and circulating actin correlate with hemodialysis mortality. J Am Soc Nephrol 20:1140–1148.

Lewis ML, Reynolds JL, Cubano LA, Hatton JP, Lawless BD, Piepmeier EH. 1998. Spaceflight alters microtubules and increases apoptosis in human lymphocytes (Jurkat). FASEB J 12:1007–1018.

Li J, Zhang S, Chen J, Du T, Wang Y, Wang Z. 2009. Modeled microgravity causes changes in the cytoskeleton and focal adhesions, and decreases in migration in malignant human MCF-7 cells. Protoplasma 238:23–33.

Mariotti M, Maier AM. 2008. Gravitational unloading induces an anti-angiogenic phenotype in human microvascular endothelial cells. J Cell Biochem 104:129–135.

Morbidelli L, Monici M, Marziliano N, Cogoli A, Fusi F, Waltenberger J, Ziche M. 2005. Simulated hypogravity impairs the angiogenic response of endothelium by up-regulating apoptotic signals. Biochem Biophys Res Commun 334:491–499.

Navasiolava NM, Dignat-George F, Sabatier F, Larina IM, Demiot C, Fortrat JO, Gauquelin-Koch G, Kozlovskaya IB, Custaud MA. 2010. Enforced physical inactivity increases endothelial microparticle levels in healthy volunteers. Am J Physiol Heart Circ Physiol 299:248–256.

Osborn TM, Dahlgren C, Hartwig JH, Stossel TP. 2007. Modifications of cellular responses to lysophosphatidic acid and platelet-activating factor by plasma gelsolin. Am J Physiol Cell Physiol 292:1323–1330.

Righetti PG, Boschetti E. 2008. The ProteoMiner and the FortyNiners: Searching for gold nuggets in the proteomic arena. Mass Spectrom Rev 27:596–608.

Rothenbach PA, Dahl B, Schwartz JJ, O'Keefe GE, Yamamoto M, Lee WM, et al. 2004. Recombinant plasma gelsolin infusion attenuates burn-induced pulmonary microvascular dysfunction. J Appl Physiol 96:25–31.

Sanford GL, Ellerson D, Melhado-Gardner C, Sroufe AE, Harris-Hooker S. 2002. Three-dimensional growth of endothelial cells in the microgravity-based rotating wall vessel bioreactor. In Vitro Cell Dev Biol Anim 38:493–504.

Schwarzenberg M, Pippia P, Meloni MA, Cossu G, Cogoli-Greuter M, Cogoli A. 1999. Signal transduction in T lymphocytes—A comparison of the data from space, the free fall machine and the random positioning machine. Adv Space Res 24:793–800.

Sennels L, Salek M, Lomas L, Boschetti E, Righetti PG, Rappsilber J. 2007. Proteomic analysis of human blood serum using peptide library beads. J Proteome Res 6:4055–4062.

Sintiprungrat K, Singhto N, Sinchaikul S, Chen ST, Thongboonkerd V. 2010. Alterations in cellular proteome and secretome upon differentiation from monocyte to macrophage by treatment with phorbol myristate acetate: Insights into biological processes. J Proteomics 73:602–618.

Spisni E, Toni M, Strillacci A, Galleri G, Santi S, Griffoni C, Tomasi V. 2006. Caveolae and caveolae constituents in mechanosensing. Cell Biochem Biophys 46:155–164.

Srisomsap C, Sawangareetrakul P, Subhasitanont P, Chokchaichamnankit D, Chiablaem K, Bhudhisawasdi V, Wongkham S, Svasti J. 2010. Proteomic studies of cholangiocarcinoma and hepatocellular carcinoma cell secretomes. J Biomed Biotechnol 2010/437143:1–18.

Sun HQ, Yamamoto M, Mejillano M, Yin HL. 1999. Gelsolin, a multifunctional actin regulatory protein. J Biol Chem 274:33179–33182.

Ulbrich C, Westphal K, Baatout S, Wehland M, Bauer J, Flick B, Infanger M, Kreutz R, Vadrucci S, Egli M, Cogoli A, Derradji H, Pietsch J, Paul M, Grimm D. 2008. Effects of basic fibroblast growth factor on endothelial cells under conditions of simulated microgravity. J Cell Biochem 104: 1324–1341.

Unsworth BR, Lelkes PI. 1998. Growing tissues in microgravity. Nat Med 4:901–907.

Versari S, Villa A, Bradamante S, Maier JA. 2007. Alterations of the actin cytoskeleton and increased nitric oxide synthesis are common features in human primary endothelial cell response to changes in gravity. Biochim Biophys Acta 1773:1645–1652.

Yates JR, Eng JK, McCormack AL, Schieltz D. 1995. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. Anal Chem 67:1426–1436.