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Modeled Gravitational Unloading Induced Downregulation of Endothelin-1 in Human Endothelial Cells

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Abstract Many space missions have shown that prolonged space flights may increase the risk of cardiovascular problems. Using a three-dimensional clinostat, we investigated human endothelial EA.hy926 cells up to 10 days under conditions of simulated microgravity (μg) to distinguish transient from long-term effects of μg and 1g. Maximum expression of all selected genes occurred after 10 min of clinorotation. Gene expression (osteopontin, Fas, TGF-β_1) declined to slightly upregulated levels or rose again (caspase-3) after the fourth day of clinorotation. Caspase-3, Bax, and Bcl-2 protein content was enhanced for 10 days of microgravity. In addition, long-term accumulation of collagen type I and III and alterations of the cytoskeletal alpha- and beta-tubulins and F-actin were detectable. A significantly reduced release of soluble factors in simulated microgravity was measured for brain-derived neurotrophic factor, tissue factor, vascular endothelial growth factor (VEGF), and interestingly for endothelin-1, which is important in keeping cardiovascular balances. The gene expression of endothelin-1 was suppressed under μg conditions at days 7 and 10. Alterations of the vascular endothelium together with a decreased release of endothelin-1 may entail post-flight health hazards for astronauts. J. Cell. Biochem. 101: 1439–1455, 2007.

Key words: apoptosis; cytokines; endothelial cells; extracellular matrix proteins; cytoskeleton

Prolonged exposure of humans to space radiation and extended weightlessness may seriously affect their health [Baldwin, 1996; Buckey et al., 1996; White and Averner, 2001]. Many of the health problems experienced during space missions are thought to be

Abbreviations used: ET-1, Endothelin-1; PAI-1, plasminogen activator inhibitor-1; TIMP-1, tissue inhibitor of metalloproteinase-1; MIP-1β, macrophage inflammatory protein-1β; MCP-1, monocyte chemoattractant protein-1; TNF-alpha, tumor necrosis factor-alpha; TNF-R2, tumor necrosis factor receptor-2; IFN-gamma, interferon-gamma; RANTES, regulated upon activation, normal T cell expressed and secreted; IGF-I, insulin-like growth factor-I; IL-2, interleukin-2; IL-3, interleukin-3; IL-6, interleukin-6; IL-8, interleukin-8; VEGF, vascular endothelial growth factor; IgE, immunoglobulin E.

Manfred Infanger and Claudia Ulbrich contributed equally.

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attributable to the influence of microgravity on cellular features [Grimm et al., 2002]. The effects of weightlessness on different cell types are therefore a topic of current interest. At a cellular level, alterations of the cytoskeleton [Vassy et al., 2001; Grimm et al., 2002; Uva et al., 2002], loss of T-cell activation [Boonyaratanakornkit et al., 2005] and changes in gene expression patterns [Hammond et al., 2000; Lewis et al., 2001] have already been observed. Furthermore, three-dimensional growth of normal cells [Martin et al., 2000; Clejan et al., 2001; Kossmehl et al., 2003; Pardo et al., 2005] and tumor cells [Chopra et al., 1997; Grimm et al., 2002; Kossmehl et al., 2003] is induced, microtubule and mitochondria organization is altered [Grimm et al., 2002; Uva et al., 2002], and the production of extracellular matrix and cytoskeletal proteins is modified [Grimm et al., 2002; Kossmehl et al., 2003], while apoptosis is initiated in various types of cells [Grimm et al., 2002; Uva et al., 2002; Kossmehl et al., 2003]. Endothelial cells are a type of cells that are affected by weightlessness [Sangha et al., 2001; Buravkova et al., 2005]. In vivo, they form the endothelium, which is located at the interface between the blood and the vessel wall [Li et al., 1994; Michiels, 2003]. Each cell is anchored to an underlying basal lamina and individual cells are anchored together by adhesion junctions, including prominent tight junctions that prevent diffusion at cell–cell contact sites. Endothelial cells may respond to changes in local conditions such as blood pressure, oxygen tension, and blood flow by secreting substances (e.g., endothelin-1 (ET-1)), which have powerful effects on the tone of vascular smooth muscle. Under certain circumstances, especially in response to adverse stimuli such as wounds, infections or tumor challenge, an endothelial cell may become activated and change its function. In tissue regeneration, endothelial cells contribute to the formation of new vessels. This process called angiogenesis may be mediated by proliferation, migration, and remodeling of fully differentiated endothelial cells from pre-existing vessels [Tang and Conti, 2004; Infanger et al., 2006]. In an earlier study, we investigated short-term effects of simulated weightlessness on endothelial cells and found that morphology and differentiation stages changed, apoptosis was initiated and the expression of extracellular matrix and cytoskeletal proteins was enhanced within a few hours of clinorotation [Infanger et al., 2006]. In addition, we demonstrated that vascular endothelial growth factor (VEGF) counterbalanced the effects of altering the physical parameter of gravity [Infanger et al., 2004; Infanger et al., 2006]. In the present study, we investigated endothelial cells under simulated long-term microgravity conditions. The principal aim of this study was to distinguish between transient and long-term effects of simulated microgravity. Gene expression of Fas (CD 95), caspase-3, osteopontin, TGF-β_1, and endothelin-1 and long-term changes in protein levels induced by the influence of simulated microgravity were evaluated. Moreover, the quantity of endothelial cell-specific factors released in the supernatant was addressed and morphological alterations of the cytoskeleton were examined.

MATERIALS AND METHODS

Random Positioning Machine (RPM)

The RPM or three-dimensional clinostat was manufactured by Dutch Space, Leiden, NL. The RPM is a laboratory instrument to randomly change the position of an accommodated (biological) experiment in three-dimensional space, under the control of dedicated software running on a PC.

The layout of the RPM consists of two cardanic frames and experiment platform. The frames are driven by means of belts and two electromotors. Both motors are controlled on the basis of feedback signals generated by encoders, mounted on the motor-axes, and by "null position" sensors on the frames. Rotation rate ω and geometrical distance from the center of rotation (R) yield "g-contours," through $g = \omega^2 R/g_0$ ($g_0 = 9.81\ m/s^2$), that provide guidelines for the design and layout of experiment packages and for the interpretation of the experimental results.

On the RPM, the samples are fixed as close as possible to the center of the inner rotating frame. This frame rotates within another rotating frame. Cell culture containers were fixed in the center of the RPM. The RPM was operated as a random walk 3D clinostat (basic mode). The RPM was located in a room with an ambient temperature of $37 \pm 1^{\circ}C$ held thermostatically.

Cell Culture Procedure

The human endothelial EA.hy926 cells [Van Oost et al., 1986] were grown in

DMEM (Invitrogen, Eggenstein, Germany). The medium was supplemented with 10% fetal calf serum (FCS) (Invitrogen, Eggenstein, Germany), 100 units penicillin/ml, and 100 μg streptomycin/ml. Subconfluent monolayers (10^6 cells/flask) were randomized to the following study groups: 70 ground controls (10 min, 30 min, 1 h, 2 h, 4 days, 7 days, and 10 days; $n = 10$ each group), and 70 samples for the clinorotation experiments (10 min, 30 min, 1 h, 2 h, 4 days, 7 days, and 10 days; $n = 10$ each group). Ground control cultures were kept in the same room as the RPM. In parallel, we investigated 10 incubator control cultures. Incubator control cultures were incubated under normal cell culture conditions (37°C and 5% CO_2) in an incubator. The cells grew in 25 cm^2 culture flasks (Sarstedt, Nümbrecht, Germany). To start a clinorotation culture, the culture flasks were filled with complete medium with a syringe, taking care to avoid air bubbles. The filled culture flasks were fixed onto the clinostat. The system was placed in a room with a temperature of 37°C. Rotation was 60°/s. Complete medium was changed every day. Many endothelial cells grew in form of 3D structures, but also single cell suspensions were obtained. After 10 days, 3D aggregates were collected for microscopy.

Determination of Micromilieu

To measure pH, bicarbonate, sodium, potassium, pO_2, and pCO_2 content in EA.hy926 cell culture supernatants, we used a commercially available radiometer (EML 100, Radiometer Copenhagen, Denmark).

Histological Evaluation and Polarization Microscopy

Ten-day-old 3D aggregates in complete medium were collected after 10 days in simulated microgravity and were cultured in chamber slides. After 4 h adhesion time, the samples were washed in PBS and fixed with 4% PFA/PBS. The samples were subjected to sirius-red staining as a specific dye for connective tissue structures. The spheroids were investigated using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany). Polarization microscopy was performed according to Junqueira et al. [1978]. Collagen type I has a yellow, orange or red color while collagen type III appears in green.

Flow Cytometric Analysis of Cellular Antigens

In each test, monoclonal antibodies were added to 10^5 single cells that had been prepared and fixed in ethanol (70%) as previously described [Grimm et al., 1997, 2002]. The cells were incubated with unconjugated antibodies (caspase-3 (Santa Cruz), Bax, Bcl-2, and Fas (Chemicon), collagen type I (Sigma, Taufkirchen, Germany), collagen type III (Monosan, Beutelsbach, Germany) for 60 min at room temperature in darkness and washed three times with PBS containing 2% FCS. The cells were treated again for 45 min at room temperature with FITC-conjugated anti-mouse-IgG antiserum (DAKO, Hamburg, Germany) and washed. The cell suspensions were analyzed with a Facscan flow cytometer (Becton Dickinson, Heidelberg, Germany) equipped with an argon laser as previously described [Grimm et al., 1997, 2002]. Cells exerting fluorescence intensities above the upper limit of the negative control distribution were considered positive.

Cytoskeletal Evaluations

The morphology of the microtubule cytoskeleton (alpha-tubulin, beta-tubulin) was evaluated by indirect immunofluorescence. Static control cells and cells cultured under simulated microgravity were grown on super cell chamber slides (Becton Dickinson), and were then washed twice in PBS. Subsequently, the cells were fixed with ethanol/methanol solution (2:1) at room temperature for 30 min. For immunofluorescence staining, the cells were washed twice in PBS and then incubated with the first antibody (alpha-tubulin, beta-tubulin (Sigma)) for 24 h at room temperature. They were then rinsed in PBS and incubated for 24 h with anti-mouse FITC-conjugated immunoglobulin antibody (DAKO). Subsequently, they were rinsed again in PBS, mounted with Vectarshield immunofluorescence mounting medium (Vector, Burlingame, CA) and sealed with nail polish. Microtubule filament morphology was visualized by means of fluorescence microscopy.

For F-actin visualization, the cells were fixed with 3.7 % formaldehyde. After washing with PBS, cells were permeabilized for 5 min with 0.1% Triton X-100 in PBS and rinsed three times with PBS. Staining of F-actin filaments was performed with rhodamine-phalloidin (Molecular Probes, Eugene, OR) in

PBS containing 1% BSA (Sigma) as described by the manufacturer. Coverslips were mounted in Moviol and examined by means of a Zeiss Axiovert wide-field microscope. Images were collected using a CCD camera (Hamamatsu, Solothurn, Switzerland) and a standard RITC filter.

RNA Isolation

Total RNA from EA.hy926 cells was isolated with the Qiagen Minikit (Hilden, Germany) according to the manufacturer's instructions. The isolated RNA had an A260/280 ratio of >1.5. Quality control of the RNA by agarose gel electrophoresis showed no degradation. RNA concentrations were determined spectrophotometrically at 260 nm.

Quantitative Real-Time PCR (TaqMan PCR)

We employed the real-time quantitative RT PCR to quantify expression levels of the genes of interest [Kossmehl et al., 2005]. Appropriate primers and fluorogenic probes were designed with the Primer Express® software. The ABI PRISM® 7000 SDS instrument in conjunction with the ABI TaqMan Universal Master Mix and the SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) was employed to perform the assays. The reaction volume was 25 μl with a final concentration of 500 nM for the primers and, where necessary, 200 nM for the probes. The PCR conditions were as recommended by the manufacturer. The primers and fluorogenic probes were synthesized by TIB Molbiol (Berlin, Germany) and Applied Biosystems (Table I).

Relative quantification was effected by means of the standard curve method. A PCR fragment containing the sequence of the TaqMan system was generated for each gene. Seven serial 1:10 dilutions of this fragment served as a standard curve that was assayed together with the corresponding unknown samples on each plate. Every sample was measured in triplicate. To normalize our expression data, we utilized 18S rRNA as a housekeeping gene.

Cytokine Measurement by Multiplex Array Assay

All supernatant samples were stored at −80°C until tested. The samples were thawed at room temperature, vortexed, spun at 13,000*g* for 5 min for clarification and 150 μl were transferred for multianalyte profiling (MAP) antigen analysis into a master microtiter plate. Using automated pipetting, an aliquot of each sample was introduced into one of the capture microsphere multiplexes of the Human Antigen MAP. These mixtures of sample and capture microspheres were thoroughly mixed and incubated at room temperature for 1 h. Multiplexed cocktails of biotinylated reporter antibodies for each multiplex were then added using a robot. After thorough mixing, they were incubated for an additional hour at room temperature. Multiplexes were developed using an excess of streptavidin-phycoerythrin solution which was thoroughly mixed into each multiplex and incubated for 1 h at room temperature. The

TABLE I. Primers and Probes Used for Quantitative Real-Time PCR

Gene	Primer name	Sequence
CD95	CD95-Tq-F	AGGGATTGGAATTGAGGAAGACT
	CD95-Tq-R	AGTCTGGTTCATCCCCATTGAC
	CD95-Pr	ACTCAGAACTTGGAAGGCCTGCATCATG
CD95-Ligand	CD95L-Tq-F	CCCATTTAACAGGCAAGTCCAA
	CD95L-Tq-R	CTTCACTCCAGAAAGCAGGACAAT
	CD95L-Pr	CCATGCCTCTGGAATG
Osteopontin	OSP-Tq-F	CGAGGTGATAGTGTGGTTTATGGA
	OSP-Tq-R	CGTCTGTAGCATCAGGGTACTG
Endothelin 1	EDN1-Tq-F	TGCCACCTGGACATCATTTG
	EDN1-Tq-R	CTCCAAGGCTCTCTTGGACCTA
	EDN1-Pr	ACACTCCCGAGCACGTTGTTCCG
Caspase-3	Casp3-Tq-F	GCAGCAAACCTCAGGGAAAC
	Casp3-Tq-R	AACTGCTCCTTTTGCTGTGATCT
TGF-β_1	TGFb-Tq-F	AAATTGAGGGCTTTCGCCTTA
	TGFb-Tq-R	CCGGTAGTGAACCCGTTGA
18S	18S-Tq-F	GGAGCCTGCGGCTTAATTT
	18S-Tq-R	CAACTAAGAACGGCCATGCA
	18S-Pr	CAATCTGTCAATCCTGTCCGTGTCCG

All probes were labeled with FAM at the 5′-end. The CD95L-probe had an MGB, all other probes were labeled with TAMRA at the 3′-end: If no probe is given, the assay is SYBR Green-based.

volume of each multiplexed reaction was reduced by vacuum filtration and the volume increased by dilution into matrix buffer for analysis. Analysis was performed in a Luminex 100 instrument and the resulting data stream was interpreted using proprietary data analysis software developed at Rules-Based Medicine and licensed to Qiagen Instruments. For each multiplex, both calibrators and controls were included on each microtiter plate. Eight-point calibrators were run in the first and last column of each plate and three-level controls were included in duplicate. Investigation results were determined first for the high, medium, and low controls for each multiplex to ensure that a proper assay had been performed. Unknown values for each of the analytes localized in a specific multiplex were determined by means of four and five parameters, weighted and non-weighted curve fitting algorithms included in the data analysis package. The least detectable dose for each cytokine was the following: endothelin-1 (ET-1; 1.4 pg/ml), brain-derived neurotrophic factor (0.0059 ng/ml), IFN-gamma (0.034 ng/ml), IgE (2.8 ng/ml), IGF-1 (0.8 ng/ml), IL-10 (3.1 pg/ml), IL-12p40 (0.24 ng/ml), IL-12p70 (19 pg/ml), IL-13 (11 pg/ml), IL-15 (0.26 ng/ml), IL-16 (13 pg/ml), IL-18 (11 pg/ml), IL-1 alpha (0.033 ng/ml), IL-2 (12 pg/ml), IL-3 (0.035 ng/ml), IL-6 (2.4 pg/ml), IL-7 (11 pg/ml), IL-8 (0.7 pg/ml), MCP-1 (10 pg/ml), MIP-1β (7.6 pg/ml), MMP-3 (0.04 ng/ml), MMP-9 (7.4 ng/ml), PAI-1 (0.0045 ng/ml), RANTES (0.00024 ng/ml), stem cell factor (11 pg/ml), tissue factor (0.17 ng/ml), TIMP-1 (0.042 ng/ml), TNF-R2 (0.00065 ng/ml), TNF-alpha (0.80 pg/ml), thrombopoietin (0.65 ng/ml), VEGF (1.5 pg/ml), and von Willebrand factor (0.002 μg/ml). For each cytokine, the lowest detectable dose was 3 standard deviations above the mean of the background signal.

Statistics

Statistical analysis was performed using SPSS 12.0 (SPSS, Inc., Chicago, IL). All data are expressed as means ± standard deviation (SD). We tested all parameters for deviations from the Gaussian distribution using the Kolmogorov–Smirnov test and compared the cases with the controls as well as remote areas with the independent–sample t test or the Mann–Whitney test (depending on the results of the normality test). In addition, we compared changes at different times on the same group with a repeated measure test (paired t-test). Differences were considered significant at the level of $P < 0.05$ (ns = not significant). For the cytokine and growth factor measurements, comparisons between various groups were made with the paired Student's t-test. A $P < 0.05$ was considered statistically significant.

RESULTS

In order to distinguish transient from long-term effects of simulated microgravity on human endothelial cells, we studied time dependent changes of selected genes, long-term accumulation of extracellular matrix components, and increase in apoptosis-related factors, as well as cytoskeletal alterations and the release of soluble factors into the culture supernatant.

Micromilieu

Clinorotation of EA.hy926 cells had no effect on the cellular micromilieu in terms of relative pH (P = ns; 10 min, 30 min, 1 h, 2 h, 4 days, 7 days, and 10 days vs. incubator and ground controls), sodium and potassium (P = ns; 10 min, 30 min, 1 h, 2 h, 4 days and 10 days vs. incubator and ground controls), bicarbonate (P = ns; 10 min, 30 min, 1 h, 2 h, 4 days, 7 days, and 10 days vs. incubator and ground controls), and pCO_2 /pO_2 (P = ns; 10 min, 30 min, 1 h, 2 h, 4 days, 7 days, and 10 days vs. incubator and ground controls) content of all EA.hy926 cell cultures.

Effect of Simulated Microgravity in Extracellular Matrix Proteins

After 10 days, static ground controls showed 10% collagen type I positive cells (Fig. 1A). After 10 days in simulated microgravity, we observed an eightfold increase in collagen type I (Fig. 1B,C; $P < 0.05$). In parallel, collagen type III was also increased significantly (threefold) in cultures grown under conditions of simulated microgravity as compared to static ground controls (Fig. 1D–F; $P < 0.05$). Using sirius red staining and polarization microscopy, we investigated endothelial cells growing in form of three-dimensional assemblies which secreted collagen type III and collagen type I (Fig. 1H–J). Ground control cells showed no color (Fig. 1G,I). According to Junqueira et al. [1978], the green

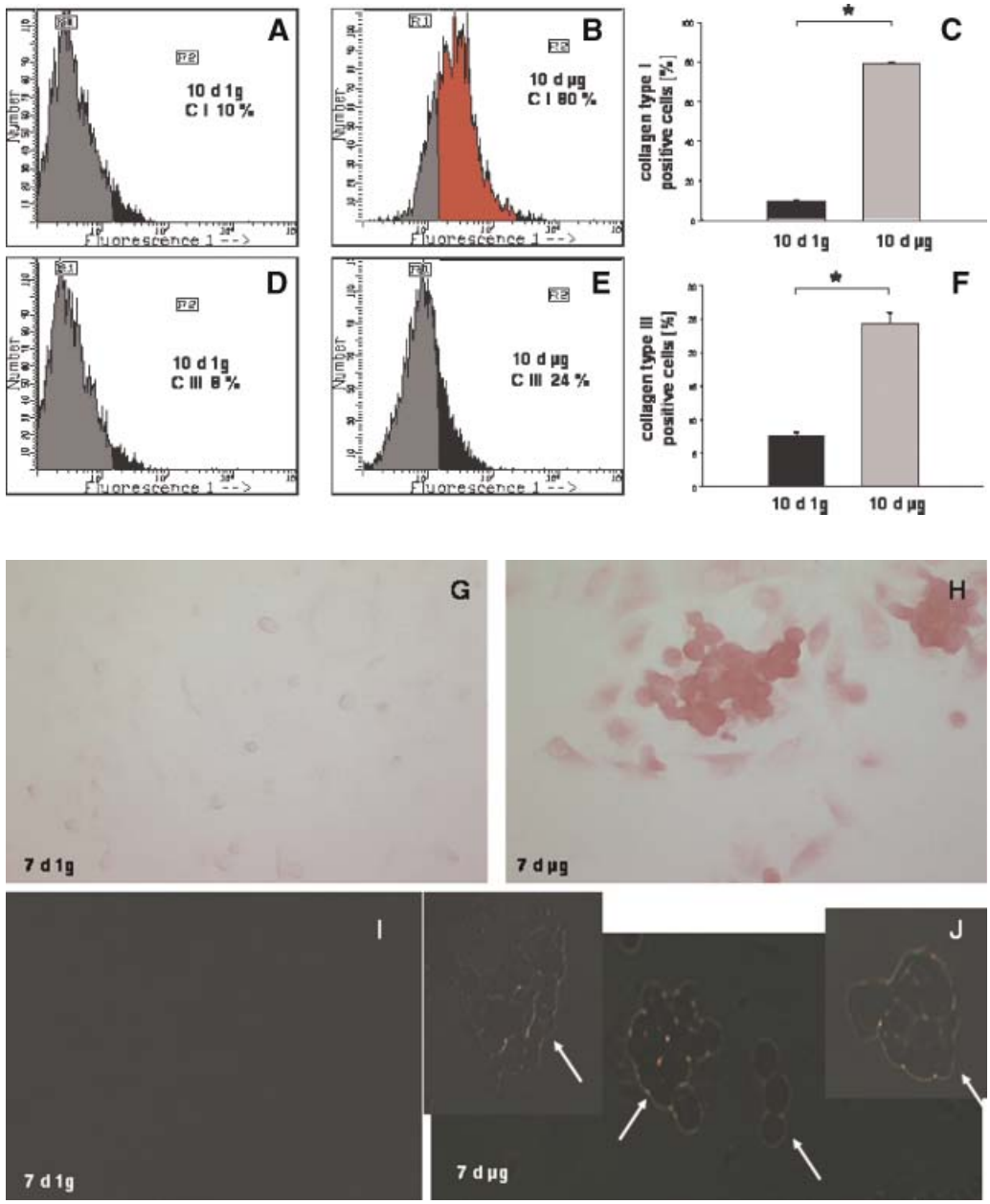

Fig. 1. Flow cytometric investigation of collagen type I (**A**–**C**) and collagen type III (**D**–**F**) revealed a clear increase of these ECM proteins in microgravity. Sirius red staining was negative in static 1*g* cells (**G**,**I**). EC cultured under microgravity for 7 days which were positive for sirius red (**H**). Polarization microscopy revealed red and yellow positive areas (collagen type I) as well as green color (collagen type III) in three-dimensional assemblies (**J**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

color is collagen type III and yellow, orange or red color collagen type I (Fig. 1J).

Time course of TGF-β_1 and osteopontin

We determined TGF-β_1 mRNA expression. The TGF-β_1 gene expression was significantly upregulated after 10 and 30 min up to 4 days as compared to static control cells. However, after 10 days of clinorotation TGF-β_1 gene expression was no longer upregulated in clinorotated cells (Fig. 2A).

Static ground control cell samples cultured for 10 days under 1*g* conditions exerted an elevated amount of osteopontin compared with

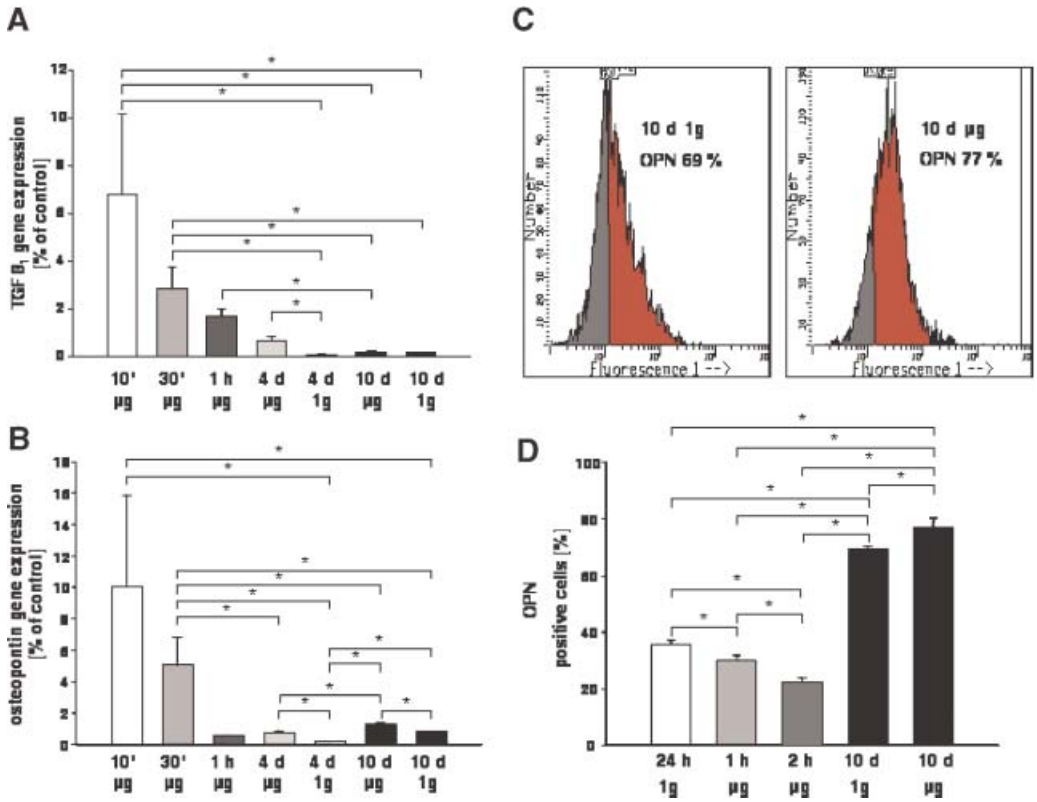

Fig. 2. TGF-β_1 gene expression (**A**) and osteopontin gene expression (**B**) were both early upregulated and declined with time. Osteopontin protein was significantly increased after a 10-day culture in microgravity compared to 1*g* 10 days-controls (**C**,**D**), 1*g* 24 h-controls as well as cell cultures grown for 1 h and 2 h under µg conditions (D). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

static ground control cultures which were incubated for 24 h as well as with cell cultures which were clinorotated for 1 and 2 h (Fig. 2D).

The percentage of osteopontin protein in 10-day-old 1*g* cultures was slightly but significantly lower than the amount of osteopontin-positive cells cultured under simulated microgravity as measured by flow cytometry (Fig. 2C,D; $P < 0.05$). At this time, the protein content is reflected in the respective mRNA content. There was a slight but significant upregulation after 4 and 10 days of culture under simulated microgravity compared to 1*g* controls (Fig. 2B). Interestingly, osteopontin gene expression was much more elevated after 10 and 30 min (Fig. 2B).

Apoptosis

Initiation of apoptosis has been repeatedly observed and proved when various types of cells have been exposed to microgravity conditions [Lewis et al., 1998; Grimm et al., 2002; Kossmehl et al., 2003; Maccarrone et al., 2003; Infanger et al., 2006].

We determined the time course of the gene expression of caspase-3, Fas and Fas-ligand. Fas ligand expression was at or below the detection limit with C_T-values above 40 in all samples. An accurate quantification was therefore impossible. The gene of caspase-3 was clearly upregulated after 10 min of clinorotation. After 4 and 10 days of clinorotation, caspase-3 gene expression was significantly elevated in cultures grown under conditions of simulated microgravity compared to 1*g* control cultures (Fig. 3A). In parallel, Fas gene expression was upregulated during the very early phase of exposure to simulated microgravity. It was still slightly but significantly increased after 4 days, while no significant difference could be detected after 10 days of culturing the cells under simulated microgravity conditions (Fig. 3B).

In ground controls, 8% of the cells contained caspase-3 protein after 24 h of cultivation. Fourteen percent of 10^6 1*g* control endothelial cells were positive for activated caspase-3 after

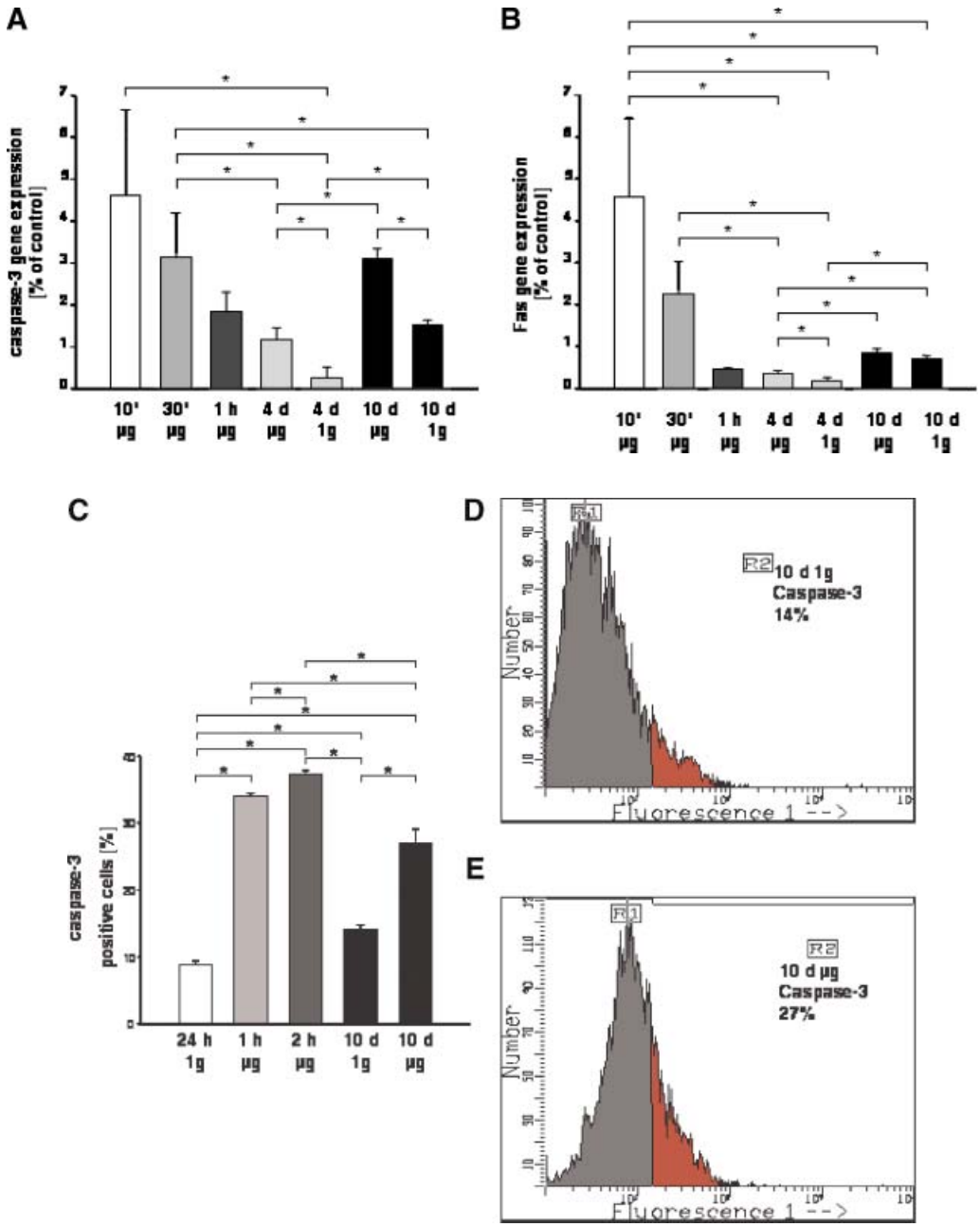

Fig. 3. Caspase-3 and Fas gene expression were both upregulated after 10 min of clinorotation (**A**,**B**). After 10 days, caspase-3 remained clearly upregulated in µg samples compared to 1*g* controls. Caspase-3 protein increased after 1 h and remained elevated up to the 10th day of microgravity (**C**–**E**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

10 days (Fig. 3C,D). Twenty-seven percent of endothelial cells cultured in simulated microgravity for 10 days were caspase-3-positive as measured by flow cytometry (Fig. 3E). Moreover, comparing endothelial cells from 10 days ground controls and the clinorotated specimens we found that the number of Bax protein-positive cells was increased under µg conditions by 6% (Fig. 4A–C; $P < 0.05$), and Bcl-2 proteins by 7% (Fig. 4D–F). In contrast, 4% fewer cells were Fas protein-positive after 10 days in simulated microgravity (Fig. 4G–I). After 1 and 2 h of culture under simulated microgravity, endothelial cells exerted a similar amount of Bax and Bcl-2 (Fig. 4C,F). In addition, Fas was slightly decreased after 2 h in simulated

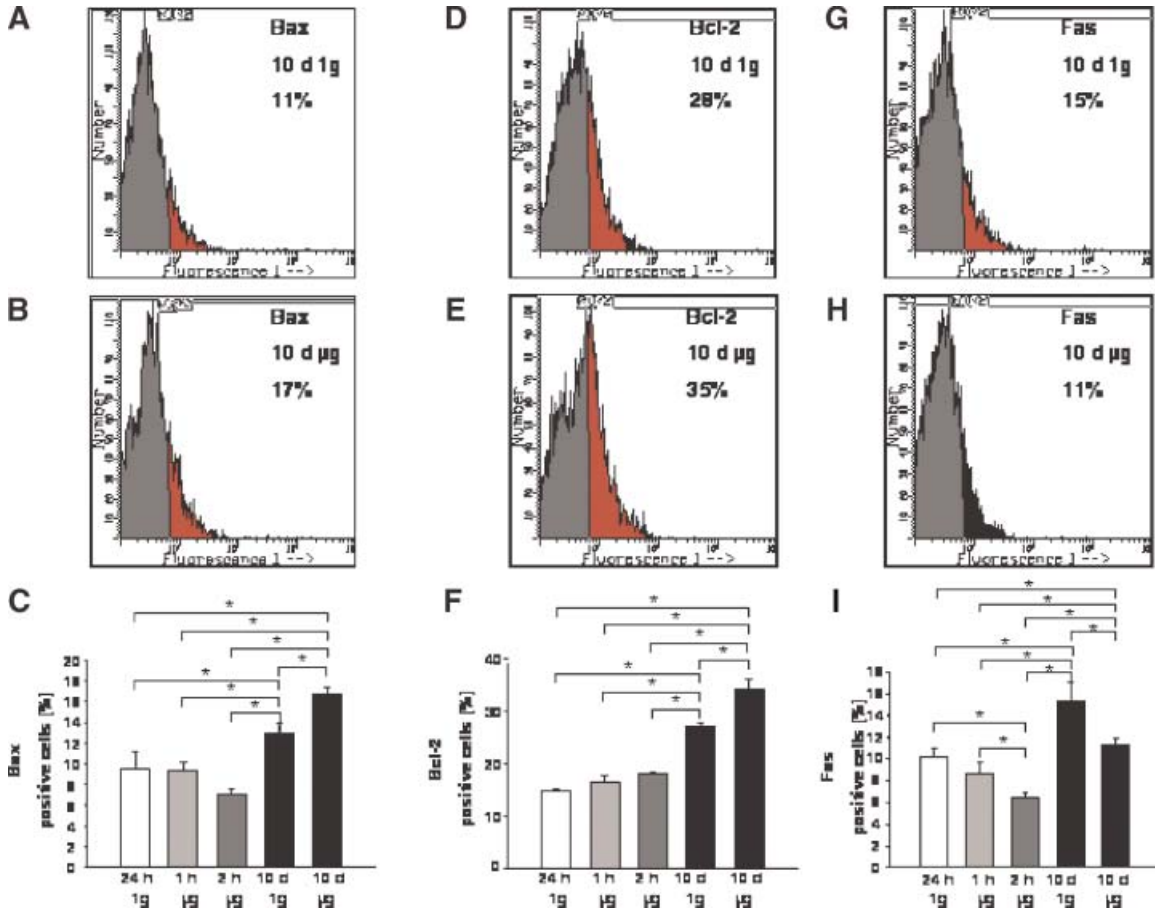

Fig. 4. Flow cytometric investigation of Bax (**A**–**C**), Bcl-2 (**D**–**F**), and Fas (**G**–**I**) measured after 1 and 2 h as well as 10 days of culture under microgravity and 1*g* conditions (24 h and 10 days). After 10 days, Bax and Bcl-2 protein were both increased under microgravity conditions whereas Fas protein was slightly decreased. After 1 and 2 h of µg, endothelial cells exerted a similar amount of Bax and Bcl-2 compared with static controls. Fas was slightly decreased after 2 h of clinorotation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

microgravity compared with corresponding static control cells. Clinorotation of endothelial cells for 10 days exerted a higher amount of Fas in contrast to short-term rotated cell cultures (Fig. 2I).

Changes of the Cytoskeleton

In addition to changes in gene expression and protein concentrations, we found that simulated microgravity also altered the structural arrangement of EA.hy926 F-actin filaments. After 30 min and 2 h of simulated microgravity, F-actin fibers had disappeared in endothelial cells (Fig. 5A,B). The normal structures of filaments clearly appeared to have changed in many but not all EA.hy926 cells after 4 days of exposure to simulated microgravity. Figure 5C clearly indicates an increased number of cells showing shortened and thickened filaments. After 10 days of cultivation, ground control cells showed thin elongated filaments, when F-actin was analyzed under a confocal scanning microscope (Figs. 5D and 6A). However, after 10 days under simulated microgravity, most cells showed perinuclear filament arrangements. All types of filaments studied had become radially arranged, greatly resembling the "stress fibers." The radial filaments extended to the cell membrane and were accumulated near the cellular membrane especially in endothelial cells growing in a three-dimensional assembly (Figs. 5E and 6B). In parallel, alpha- and beta-tubulin (microtubules) also showed long-lasting cytoskeletal alterations. Perinuclear accumulations of both tubulins were detectable (Fig. 6C,D).

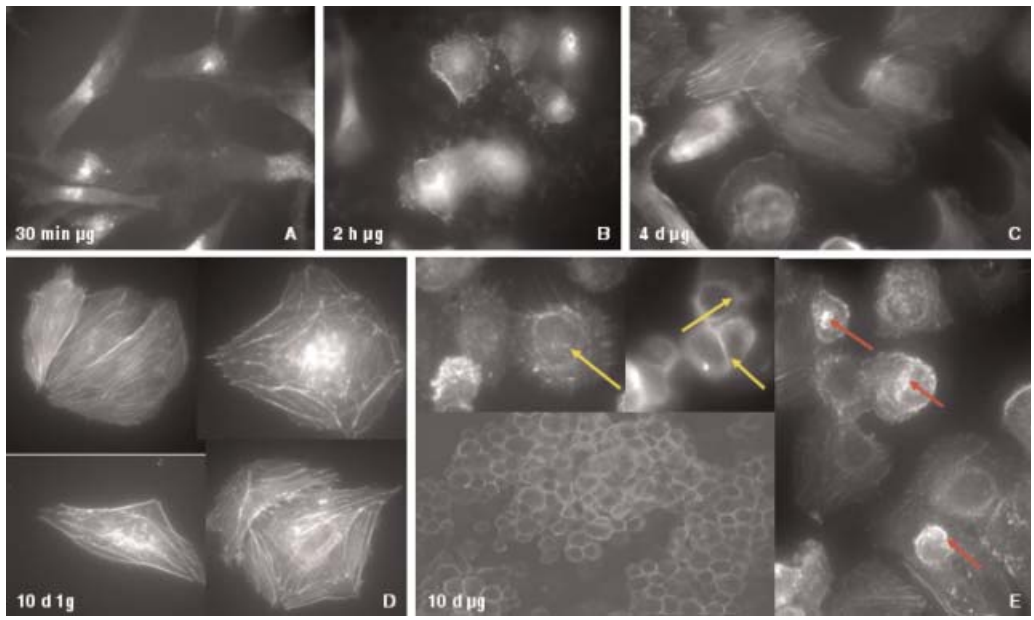

Fig. 5. F-actin staining of the cytoskeleton: After 30 min (**A**), 2 h (**B**) and 4 days (**C**), F-actin was decreased in microgravity. After 10 days of µg, F-actin accumulated at the cellular membrane (arrows) and perinuclear (**E**) compared to corresponding 1*g* control cells (**D**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

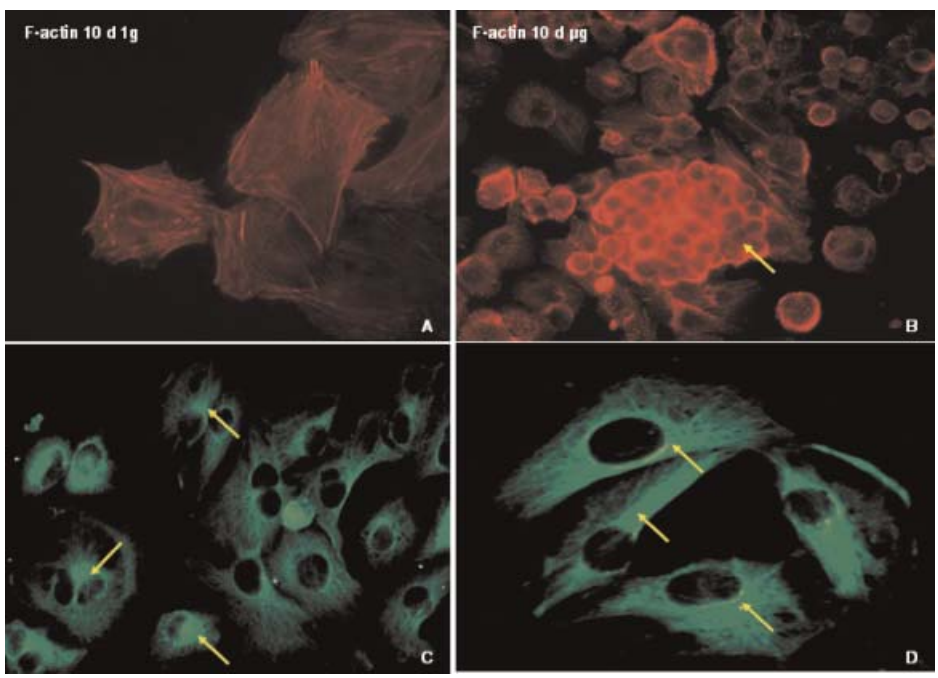

Fig. 6. F-actin staining: control endothelial cells at 1*g* (**A**) and 10 days clinorotated cells (**B**). F-actin accumulated at the cellular membrane (arrows) of multicellular spheroids (B). Alpha (**C**)- and beta (**D**)-tubulin immunofluorescences: In µg the microtubule filaments extended from poorly defined organizing centers and were coalesced, thicker, and shortened (arrows). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

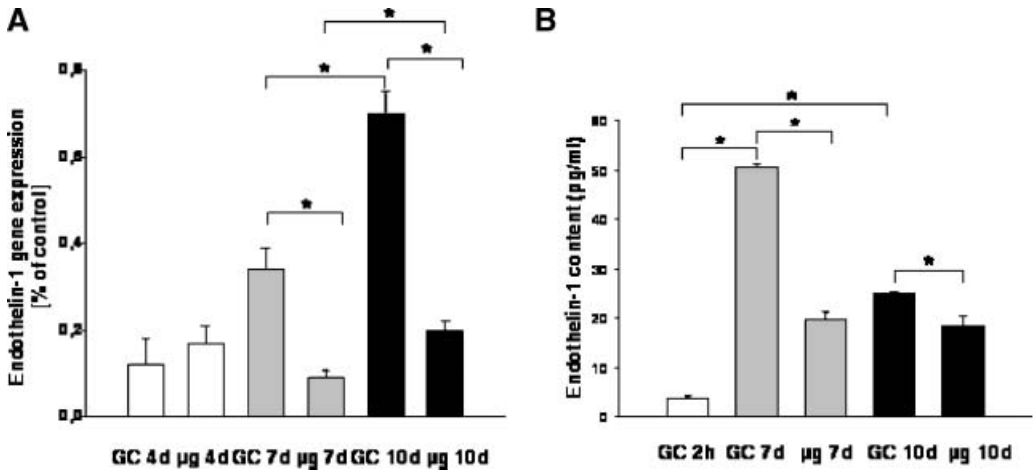

Fig. 7. **A**: Gene expression of endothelin-1 was significantly suppressed at days 7 and 10 in simulated microgravity condition. **B**: Endothelin-1 released in the supernatant after 2 h up to 10 days of culture under simulated µg conditions. Endothelin-1 release in the supernatant was significantly reduced in microgravity condition at days 7 and 10. $*P<0.05$.

Endothelin-1

ET-1 gene expression was significantly reduced in endothelial cells grown under simulated µg at days 7 and 10 (Fig. 7A). Comparing ground controls with clinorotated cultures, we could detect a significantly reduced release of ET-1 (Fig. 7B).

Cytokine Measurement by Multiplex Array Assay

In addition to cellular proteins, we also determined the quantities of soluble factors released into the culture supernatant during various periods of cell culture under both 1 and µg conditions. We analyzed numerous factors which might have been released into the supernatant by endothelial cells (see Materials and Methods). Of all these factors, brain-derived neurotrophic factor, IGF-1, Il-2, IL-3, Il-6, Il-8, MCP-1, MIP-1β, PAI-1, VEGF, TNF-alpha, TNF-R2, IFN-gamma, thrombopoietin, stem cell factor, tissue factor, von Willebrand Factor, TIMP-1, RANTES, and IgE were found to be released into the supernatant by EA.hy926 cells (Table II, Fig. 8). Comparing ground controls with clinorotated cultures, we could detect a significantly reduced release of brain-derived neurotrophic factor, tissue factor, and VEGF in simulated microgravity. A trend ($0.05 < P < 0.1$) to reduced secretion in simulated microgravity conditions was found for IGF-I and IL-10. No significant difference between ground controls and simulated microgravity conditions ($P > 0.1$) was measured for IFN-gamma, IgE, IL-2, IL-3, IL-8, MCP-1, MIP-1β, PAI-1, TIMP-1, RANTES, stem cell factor, TNF-alpha and TNF-R2, thrombopoietin, and von Willebrand factor. Most of the factors mentioned above, secreted from days 6 to 7 and from days 9 to 10, were enriched 3–7 times as compared to a 2 h short-term incubation supernatant (Table II, Fig. 8). But PAI-1 and VEGF were secreted at higher rates during the various periods of analysis (Table II, Fig. 8).

DISCUSSION

We decided to investigate human endothelial cells under conditions of simulated weightlessness using a three-dimensional RPM for a time period of 10 days. Amongst the observations demonstrated in this article, the most significant and novel finding is that we could demonstrate for the first time a very early upregulation of caspase-3, Fas, osteopontin, and TGF-β_1 gene expression in endothelial cells under conditions of simulated weightlessness. Moreover, we detected a continuing accumulation of extracellular matrix components and progressing structural rearrangements of cytoskeletal fibers throughout all the 10 days of clinorotation, while apoptosis did not exceed 30% when the cells were cultured in simulated microgravity for the same time period. Another result of our study warrants emphasis. It shows the first comprehensive

TABLE II. Soluble Factors Released by EA.hy926 cells

Factor	Detectable dose (ng/ml)	Day 0, 2 h GC (ng/ml)	Day 6–7, 24 h GC (ng/ml)	Day 6–7, 24 h μg (ng/ml)	Day 9–10, 24 h GC (ng/ml)	Day 9–10, 24 h μg (ng/ml)
IL-2	0.0012	n.d.	0.0020 ± 0.00015*	0.0015 ± 0.0007	0.00161 ± 0.0004	0.00133 ± 0.00005
IL-3	0.0350	0.021 ± 0.013	0.06 ± 0.01	0.047 ± 0.019	0.056 ± 0.018	0.033 ± 0.001
IL-6	0.0024	0.038 ± 0.0032	0.020 ± 0.0052	0.0169 ± 0.0087	0.0312 ± 0.0088	0.0376 ± 0.0005
IL-8	0.0007	0.101 ± 0.014	0.109 ± 0.023	0.0785 ± 0.0178	0.102 ± 0.011	0.118 ± 0.0064
von Willebrand factor	2	n.d.	3 ± 1	2 ± 1	11 ± 3	7 ± 1
RANTES	0.00024	0.0037 ± 0.00057	0.024 ± 0.007*	0.015 ± 0.006	0.022 ± 0.0054*	0.0212 ± 0.00014
IgE	2.8	20.2 ± 1.41	17.3 ± 3.11	20.9 ± 6.22	17.8 ± 2.90	14.95 ± 0.21
IFN-gamma	0.034	0.04 ± 0.007	0.098 ± 0.021	0.069 ± 0.029	0.084 ± 0.026	0.049 ± 0.005
Thrombopoietin	0.065	0.696 ± 0.122	1.17 ± 0.184	0.961 ± 0.31	1.001 ± 0.197	1.005 ± 0.395
Stem cell factor	0.011	0.00634 ± 0.003	0.0137 ± 0.0025	0.00119 ± 0.0039	0.0143 ± 0.0017	0.0107 ± 0.0014
Tissue factor	0.17	0.273 ± 0.054	0.395 ± 0.03*,[a]	0.029 ± 0.017	0.288 ± 0.142	0.273 ± 0.014

Values are given mean ± SD; GC, ground control; n.d., not detectable.
[a]GC 7 days versus μg 7 days; $P < 0.05$.
*$P < 0.05$ versus 2 h GC.

analysis of cytokines released into the supernatant from endothelial cells during a long-term period of simulated microgravity. The brain-derived neurotrophic factor, ET-1, tissue factor, and VEGF were secreted at a lower rate in simulated microgravity than in ground control cultures.

Long-term Effects of Simulated Microgravity on Gene Expression and Protein Content

It is well documented in the literature that the expression of genes may be enhanced within 10 min after a cell has received an adequate stimulatory signal which may be of chemical or mechanical nature [Ben Rejeb et al., 2004; Choi et al., 2004; Ortega et al., 2005; Varanasi and Datta, 2005]. Regarding the genes of those proteins we investigated the signal delivered to the cells by removing the gravity seems to induce such very fast upregulations of mRNA transcription. Like c-fos mRNA expression in mice after substance P administrations [Choi et al., 2004], the mRNA concentrations of the selected genes peaked at the 10th min and declined from the 10th to the 30th min. The maximum reached within the first 10 min is not exceeded up to the 10th day of incubation, although caspase-3 mRNA showed a second increase between the 4th day and the 10th day of cultivation. We conclude from our results that a major effect of simulated microgravity is due to a signal delivered to cells at the moment of transition from 1 to μg. However, a long-lasting additional influence of simulated microgravity seems to be present in addition as suggested by the caspase-3 gene expression pattern. Early transient and long-term effects of simulated microgravity were also observed when the alterations of cell-associated extracellular matrix components, apoptotic factors, and growth factors were investigated. It is known from the literature that an upregulation of mRNA within 10 min after receiving the activation signal may be followed by the production of the corresponding proteins after 24–72 h [Ortega et al., 2005; Varanasi and Datta, 2005].

Therefore, the enhancement of the various proteins within 4, 12 or 24 h which we observed on EA.hy926 cells in an earlier study could be related to a very early expression of the corresponding mRNA [Infanger et al., 2006]. However, enhanced amounts of activated caspase-3 found in clinorotated cells at day

10 as well as the strong accumulation of collagen type I and III after 10 days of clinorotation clearly indicate an additional long-term effect of simulated microgravity.

Changes of the Cytoskeleton

As observed by studies on other cells, endothelial cells also changed their structural arrangements of cytoskeletal filaments when exposed to simulated microgravity. The F-actin network transiently disappeared within a few early hours of clinorotation. Then, after 4 days, reorganization was visible in some but not all cells. After a 10-day growth period in simulated microgravity, we could detect a filament accumulation next to the cellular membrane as well as in the perinuclear region compared to static control cells. Disruption of cytoskeletal integrity has long been suggested as a gravity-sensing mechanism in single cells [Hoeger and Gruener, 1990; Hughes-Fulford, 2003]. Although the alteration of filament structure seems to be due to a direct effect of simulated microgravity on the proteins [Gruener et al., 1993], it cannot be ruled out that some factors that are overproduced during clinorotation may be involved [Pellegrino et al., 2004]. An intact cytoskeleton is necessary for signal transduction. Membrane cytoskeletal interactions are involved in transduction of second messengers by signal amplification [Carraway and Carraway, 1989]. Therefore, a relationship between cytoskeletal destruction and apoptosis may be postulated [Lewis et al., 1998]. Apoptosis is induced in many types of cells when they are grown under simulated microgravity [Lewis et al., 1998; Sakar et al., 2000; Maccarrone et al., 2003]. Similar changes of the

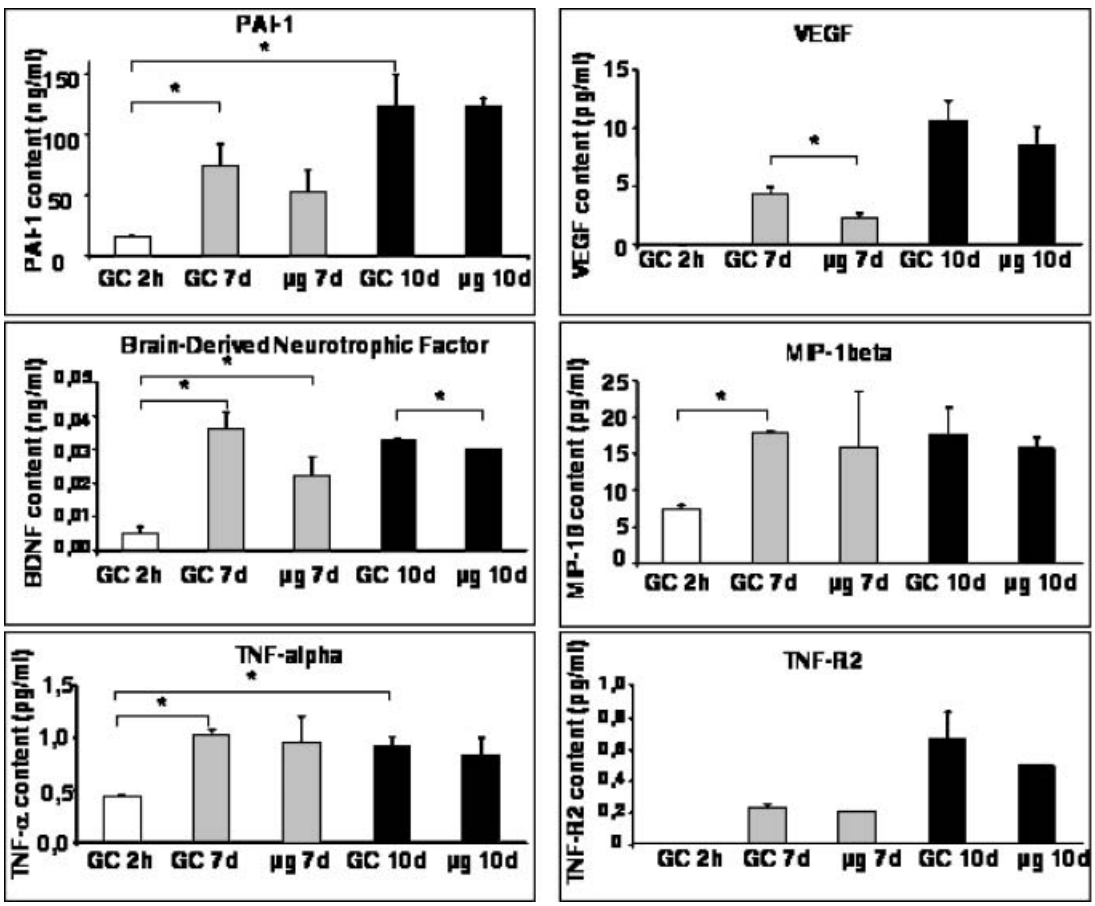

Fig. 8. Soluble factors released in the supernatant after 2 h up to day 10 of culture under µg conditions. PAI-1, VEGF, brain-derived neurotrophic factor, MIP-1beta, TNF-alpha, TNF-R2, MCP-1, IGF-I, and TIMP-1 were time-dependently increased in ground cultures compared to the 2 h supernatant. VEGF, brain-derived neurotrophic factor, and IGF-I release in the supernatant was significantly reduced in µg condition. $^*P < 0.05$.

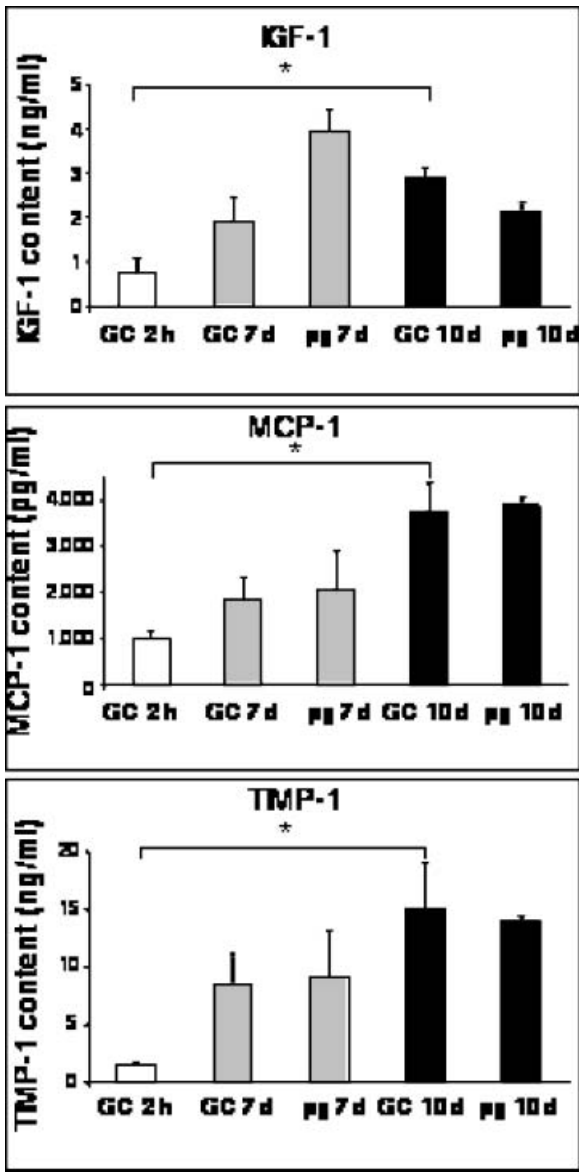

Fig. 8. (*Continued*)

cytoskeleton in different cell types were reported by others [Guignandon et al., 2001; Meloni et al., 2004; Meyers et al., 2005].

Factors Released into the Supernatant

Another important aspect of this study was the measurement of cytokines and soluble biological factors released into the supernatant. The factors which we detected corresponded very well with factors known to be produced by endothelial cells [Nakahashi et al., 2000; Shukaliak and Dorovini-Zis, 2000; Vischer et al., 2000; Volk et al., 2000; El Andaloussi et al., 2001; Jonsson and Palmblad, 2001; Wang et al., 2002; Yamaoka-Tojo et al., 2003; Devaraj et al., 2004; Felix et al., 2004; Pellegrino et al., 2004; Wondergem et al., 2004; Urbich et al., 2005; Mateo et al., 2006; Wang et al., 2006]. Matrix metalloproteinases MMP-3 and -9 were not released by endothelial cells under simulated microgravity and static 1*g* conditions. In contrast, TIMP-1 was secreted and significantly increased with time. Although no difference was detected between 0 and 1*g*, the high TIMP-1 concentration might explain the enormous increase in collagen production of endothelial cells in weightlessness. Cytokines such as IL-2, IL-3, IL-6, IL-8, TNF-alpha were secreted by EA.hy926 cells. Most of them remained unchanged in simulated microgravity. IL-2 and IL-3 were slightly decreased under clinorotation. VEGF secretion was reduced in simulated microgravity between the 6th and the 7th day, while after 10 days there was no significant difference in VEGF concentration between 1 and µg culture supernatants. Although animal space flight experiments may not be comparable to clinorotation experiments on cultured cells, it seems worth to mention that Felix et al. [2004] have already reported an altered cytokine expression in tissues of mice subjected to simulated microgravity. However, they detected no change for IL-3 and TNF-alpha serum levels. In their experiments, IFN-gamma and IL-2 were reduced in response to simulated microgravity and a variable response of TNF-alpha in different tissues indicated the tissue-specific effect of µg on the production of this cytokine. A very interesting finding of this study is the significant reduction in endothelin-1 in simulated microgravity. ET-1 is a vasoconstrictor secreted by endothelial cells. It is the natural counterpart of the vasodilator nitric oxide which has been shown to be increased under simulated microgravity [Cotrupi et al., 2005]. In addition, nitric oxide may inhibit the synthesis and hemodynamic effects of ET-1 [Cardillo et al., 2000]. ET-1 contributes to vascular tone and regulates cell proliferation through activation of ETA and ETB receptors [Marasciulo et al., 2006]. It is known that physical factors such as shear stress or stimuli including thrombin, epinephrine, Ang II, growth factors, cytokines, and free radicals enhance secretion of ET-1. Mediators such as nitric oxide, cyclic GMP, ANP and prostacyclin reduce the release of endogenous ET-1. Under normal conditions, the effects of the ET-1 are carefully regulated through

inhibition or stimulation of ET-1 release from endothelial cells [Marasciulo et al., 2006]. Imbalance in the production of vasodilator and vasoconstrictor agents may contribute to the onset of hemodynamic disorders [Marasciulo et al., 2006]. Since dysregulation of the endothelin system is important in the pathogenesis of several cardiovascular diseases [Vaneckova et al., 2005; Wainwright et al., 2005], the downregulation of ET-1 in microgravity may cause orthostatic intolerance and cardiovascular problems of astronauts.

Transient and Long-Term Effects of Simulated Microgravity

The results of this study indicate that a three-dimensional clinostat provides an important experimental system to culture endothelial cells grown as three-dimensional entities. We were able to demonstrate for the first time that the transition from gravity to simulated microgravity has a major effect on human endothelial cells. An important long-term effects may be on extracellular matrix proteins, the cytoskeleton network and microtubules. It may also be the cause of the sustained increase in the level of apoptotic cells. A variety of soluble factors, cytokines, and growth factors were observed to be released by cultured human endothelial cells. The majority of these cytokines was secreted at equal rates in simulated microgravity and 1*g* conditions, as determined by the immunological method applied, which does not necessarily exclude alterations of biological activities of the factors [Hymer et al., 1996]. However, ET-1 gene expression and release were clearly decreased under simulated weightlessness indicating a key role of this substance in endothelial dysfunction in space. The RPM has therefore again proved to be an important ground-based system for studying the effects of weightlessness on single cells on earth in preparation for future space missions to the *International Space Station*.

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