Journal of Cellular Biochemistry 104:1324–1341 (2008)

# Effects of Basic Fibroblast Growth Factor on Endothelial Cells Under Conditions of Simulated Microgravity

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**Abstract** Fibroblast growth factors interact with appropriate endothelial cell (EC) surface receptors and initiate intracellular signal cascades, which participate in modulating blood vessel growth. EC, upon exposure to basic fibroblast growth factors (bFGFs) undergo profound functional alterations, which depend on their actual sensitivity and involve gene expression and de novo protein synthesis. We investigated the effects of bFGF on signaling pathways of EA.hy926 cells in different environments. EC were cultured under normal gravity (1 g) and simulated microgravity (μg) using a three-dimensional (3D) clinostat. Microgravity induced early and late apoptosis, extracellular matrix proteins, endothelin-1 (ET-1) and TGF-$\beta_1$ expression. Microgravity reduced eNOS mRNA within 24 h. Moreover, a six- to eightfold higher amount of IL-6 and IL-8 was secreted within 24 h μg. In addition, microgravity induced a duplication of NF-kappaB p50, while p65 was quadrupled. At 1 g, bFGF application (4 h) reduced ET-1, TGF-$\beta_1$ and eNOS gene expression. After 24 h, bFGF enhanced fibronectin, VEGF, Flk-1, Flt-1, the release of IL-6, IL-8, and TGF-$\beta_1$. Furthermore, bFGF promoted apoptosis, reduced NFkB p50, but enhanced NFkB p65. After 4 h μg, bFGF decreased TGF-$\beta_1$, eNOS, and ET-1 gene expression. After 24 h μg, bFGF elevated fibronectin, Flk-1 and Flt-1 protein, and reduced IL-6 and IL-8 compared with vehicle treated μg cultures. In μg, bFGF enhanced NF-KappaB p50 by 50%, Bax by 25% and attenuated p65, activation of caspase-3 and annexin V-positive cells. bFGF differently changes intracellular signals in ECs depending whether it is applied under microgravity or normal gravity conditions. In microgravity, bFGF contributes to protect the EC from apoptosis. J. Cell. Biochem. 104: 1324–1341, 2008. © 2008 Wiley-Liss, Inc.

**Key words:** NFkappaB; basic fibroblast growth factor; cytokines; apoptosis

Abbreviations used: 1 g, normal gravity; μg, microgravity; bFGF, basic fibroblast growth factor; EC, endothelial cells; ECM, extracellular matrix; ET-1, endothelin-1; eNOS, endothelial nitric oxide synthase; Flk-1, fetal liver kinase 1; Flt-1, fms-like tyrosine kinase-1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; h, hour; IL-1β, interleukin-1 beta; IL-2, interleukin-2; IL-6, interleukin-6; IL-8, interleukin-8; Nf-kB, nuclear factor-kappaB; RPM, random positioning machine; RT, room temperature; SD, standard deviation; TNF-alpha, tumor necrosis factor-alpha; TGF-β1, transforming growth factor-beta1; VEGF, vascular endothelial growth factor; VEH, vehicle.

Grant sponsor: German Space Agency DLR; Grant number: 50WB0524; Grant sponsor: Programm zur Förderung von Forschung, Innovationen und Technologien (ProFIT); Grant number: 10135687; Grant sponsor: Bundesministerium für Bildung und Forschung Nationales Genomforschungsnetz; Grant numbers: KGCV1, 01GS0416.

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Received 24 September 2007; Accepted 27 December 2007
DOI 10.1002/jcb.21710

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Alterations of cellular signaling pathways due to for example aging, stress, or infections are frequent events. Recent studies have revealed that exposure of isolated cells to real or simulated microgravity conditions may change signal transduction pathways [Grimm et al., 2006]. The response of cells to changes in gravity varies among different cell types, especially when they are exposed to low gravity conditions [Hammond et al., 2000; Lewis et al., 2001; Hughes-Fulford et al., 2005; Grimm et al., 2006]. Two types of responses have been distinguished: A short-term response which occurs often during the transition from gravity to microgravity condition and a long-term response which can be observed even after 10 days of culturing the cells under microgravity condition [Infanger et al., 2006b, 2007b].

Endothelial cells (ECs) are highly sensitive to gravitational changes and show short-term as well as long-term responses [Sangha et al., 2001; Buravkova et al., 2005; Infanger et al., 2006b, 2007b]. The endothelium forms an interface between the blood and vessel wall in vivo. It plays a critical role in the blood flow dynamics, the regulation of coagulation, leukocyte adhesion, and vascular smooth muscle cell growth. Furthermore, it serves as a barrier for the transvascular diffusion process of liquids and solutes. The EC layers response to environmental changes due to its functional spectrum (endocrine, exocrine, cell adhesion, clotting, and transport functions) [Schiffrin, 1994; Aird, 2005]. Local alterations in blood pressure, oxygen tension, and blood flow lead to the secretion of particular substances or expression of selected receptor types.

Vascular endothelial growth factors (VEGFs) are the most important regulators of both physiological and pathological EC functions [Veikkola and Alitalo, 1999]. Vascular ECs are the prime targets of VEGFs, as they belong to the few cell types, which express the receptor tyrosine kinases VEGFR1 (also known as FLT-1) and VEGFR2 (KDR/FLK-1) [de Vries et al., 1992; Millauer et al., 1993]. Binding of VEGF to a VEGF-receptor may induce either proliferation, migration, or differentiation of ECs [Millauer et al., 1993] or release of enzymes and growth factors [Pepper et al., 1994]. Recently, we showed that VEGF counterbalances apoptosis and supports tube formation, if present in the supernatant of endothelial EA.hy926 exposed to simulated microgravity [Infanger et al., 2004, 2006a].

Further important regulators of EC functions are basic fibroblast growth factors (bFGFs). There are five bFGF isoforms described with different molecular masses each (18, 22, 23, 25, and 34 kDa) [Florkiewicz and Sommer, 1989]. Binding of bFGF to its receptor FGFR-1 exerts a mitogenic effect in ECs. However, in contrast to VEGF, this effect is non-specific because ectoderm- and mesoderm-derived cells are targeted too [Gospodarowicz et al., 1987; Fries and Maciag, 1995]. In addition, bFGF works synergistically with VEGF to induce angiogenesis [Asahara et al., 1995].

The principal aim of this study was to characterize the effects of bFGF on human ECs exposed to normal gravity or simulated microgravity. Applying molecular biological methods along with morphological techniques, we examined possible changes of selected genes, accumulation of extracellular matrix components, expression of various intracellular components such as VEGF, its receptors or TGF-$\beta_1$ as well as the elevation of apoptosis related factors and the release of soluble factors (cytokines) into the culture supernatant. The results revealed that bFGF may affect expression of some components of an EC equally under microgravity and gravity, others differently and some even in an opposite manner. Together, the cytokine's influence appears to be geared to protect the cells under conditions of simulated microgravity. A major significance of this study was the finding that bFGF exerted anti-apoptotic effects under conditions of simulated microgravity.

## MATERIALS AND METHODS

### Random Positioning Machine (RPM)

The RPM is a laboratory instrument to simulate microgravity conditions. Originally, the machine was developed by Hoson [Hoson et al., 1992] and manufactured by Dutch Space, Leiden, NL. Samples mounted on a platform randomly change the position in the 3D space on the machine controlled by dedicated software running on a personal computer. The movement of the experimental platform suspended in the center of two cardanic frames is realized by two independently running engines. These engines are controlled by feed-back signals from 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 $g = \omega^2 R/g0$ ($g0 = 9.81$ m/s$^2$), that provide guidelines for the design and lay-out of experiment packages and for the interpretation of the experimental results. The RPM was operated in a random walk (basic mode) with a rotation speed of 60°/s in a temperature controlled room ($37 \pm 1$°C).

### Cell Culture Procedure

The human endothelial EA.hy926 cells [Van Oost et al., 1986] were grown in DMEM (Invitrogen, Eggenstein, Germany) medium that was supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany), 100 units penicillin/ml, 100 μg streptomycin/ml, and 10 ng/ml bFGF (Chemicon, Hofheim, Germany) depending on the experimental design. 160 subconfluent monolayers ($10^6$ cells/flask) were cultured using cells of the same passage. Prior to each experiment culture flasks were randomized to the following study groups: (a) 80 static control cultures (cultures were kept in the same room as the RPM at 37°C) divided into one half bFGF test cultures ($n = 40$) and another half control cultures (vehicle $n = 40$; VEH, saline 0.9%) and (b) 80 samples for the RPM experiments (microgravity exposure) similarly subdivided into bFGF test cultures and control cultures. During each experiment samples were collected at 4 and 24 h, respectively. The samples of each group were investigated at the same time. The experiments were repeated four times at different days. In parallel, we analyzed 10 incubator control cultures. The cells grew in 25 cm$^2$ culture flasks (Sarstedt, Nümbrecht, Germany) filled with medium to the top by a syringe to avoid air bubbles. The filled culture flasks were fixed onto the clinostat. Many ECs grew in form of 3D structures, but also single cell suspensions and in greatest majority adherent cells were obtained. The latter ones were subjected to analysis. Three-dimensional aggregates were collected under microscopic control, seeded out in 8-well SuperCell chamber slides (BD Biosciences, Heidelberg, Germany) and stained by immunocytochemistry.

### Determination of Micromilieu

Small amounts of the EA.hy926 cell culture supernatants were immediately withdrawn for the measurement of pH, bicarbonate, sodium, potassium, $p0_2$ and $pC0_2$ content by a commercially available radiometer (EML 100, Radiometer Copenhagen, Denmark).

### Western Blot Analysis

SDS–PAGE, immunoblotting and densitometry were carried out on six replicates following routine protocols [Grimm et al., 2002; Kossmehl et al., 2003, 2005]. Antibodies against the following antigens were used for this study: Activated caspase-3, collagen type I, fibronectin, and laminin (dilution all 1:1,000; all Chemicon), ET-1 (1:1,000; Immunodiagnostics, Bensheim, Germany), VEGF, Flt-1, Flk-1, NFκb (1:1,000; Santa Cruz Biotechnology, Santa Cruz), β-actin (Sigma, Taufkirchen, Germany) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:4,000; ABR—Affinity BioReagents, Golden).

### 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., 2002; Infanger et al., 2007b]. The cells were incubated with unconjugated antibodies (Bax, Bcl-2, p53, caspase-3, and Fas, all Chemicon, and fibronectin, Sigma), 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., 2002; Infanger et al., 2007b]. Cells exerting fluorescence intensities above the upper limit of the negative control distribution were considered positive.

### Immunohistochemical Staining of Fibronectin and Haematoxylin–Eosin (HE) Staining

Immunocytochemistry was performed as recently described [Infanger et al., 2006a]. The monoclonal antibody was purchased from Sigma. HE staining was performed according to routine protocols.

### Annexin V-FITC/PI Assay

The Annexin V-FITC/PI assay was performed using the Annexin V-FITC apoptosis detection kit II (BD Pharmingen, San Diego, CA) according

to the instructions of the manufacturer. Annexin V-FITC is used to quantitatively determine the percentage of cells within a population that are actively undergoing programmed cell death. Briefly, $1 \times 10^6$ cells of 0 g samples were investigated as well as control cell cultures with and without bFGF ($n = 5$, each). The cells were labeled with Annexin V-FITC/PI. The Annexin V-FITC−/PI− population was regarded as normal healthy cells, while Annexin V-FITC+/PI− cells were taken as a measure of early apoptosis, Annexin V-FITC+/PI+ as necrosis. About $1 \times 10^4$ events were acquired for each experimental point via a Facscan flow cytometer (Becton Dickinson) equipped with an argon laser and analyzed with WinList software (Topsham, ME).

### Evaluation of Apoptosis: Acridine Orange/Ethidium Bromide

The monolayer cultures remaining under 1 g conditions at the bottom of the plastic culture flasks (BD Biosciences) during 24 h of clinorotation were examined by phase contrast microscopy. After culture, control and microgravity-exposed cells were stained with acridine orange/ethidium bromide as previously published [Zhou et al., 1997; Kossmehl et al., 2003].

### TUNEL Flow Cytometry

The TUNEL test was performed using the Apo-Direct™ Kit from Calbiochem (San Diego, CA), a two color TUNEL (Terminal deoxynucleotide transferase dUTPNick End Labeling) assay for labeling DNA breaks and total cellular DNA to detect apoptotic cells by flow cytometry. This kit utilizes terminal deoxynucleotidyl transferase (TdT) to catalyze incorporation of fluorescein-dUTP at the free 3′-hydroxyl ends of the fragmented DNA. The assay was performed according to the manufacturer's instructions.

### 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 $A_{260/280}$ ratio of >1.5. Quality control of the RNA by agarose gel electrophoresis showed no degradation. RNA concentrations were determined spectrophotometrically at 260 nm. Two micrograms total mRNA was added to 40 μl RT-mixture. One microliter of this dilution was used for each TaqMan reaction.

### Quantitative Real-Time PCR (TaqMan PCR)

We used the real-time quantitative PCR (RTq-PCR) to quantify expression levels of the genes of interest [Infanger et al., 2006a,b, 2007a,b]. Appropriate primers and fluorogenic probes were designed using 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 used 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).

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

| Gene | Primer name | Sequence |
|---|---|---|
| eNOS | eNOS-Tq-F | GCCAACGCCGTGAAGATCT |
| | eNOS-Tq-R | TCGGAGCCATACAGGATTGTC |
| | eNOS-Pr | CCTCGCTCATGGGCACGGTGAT |
| Endothelin-1 | EDN1-Tq-F | TGCCACCTGGACATCATTTG |
| | EDN1-Tq-R | CTCCAAGGCTCTCTTGGACCTA |
| | EDN1-Pr | ACACTCCCGAGCACGTTGTTCCG |
| TGF-beta1 | 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 and TAMRA at the 3′-end. If no probe is given, the assay is SYBR Green-based.

Relative quantification was performed 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 used 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 multiplex array assay analysis into a master microtiter plate. An aliquot of 50 μl for each sample was introduced into one of the capture microsphere multiplexes composed of IL-1β, IL-2, I-6, IL-8, TNF-α, TGF-$\beta_1$ and adiponectin bead coupled antibodies. These mixtures of sample and capture microspheres were thoroughly mixed and incubated at room temperature for 2 h. The microspheres were then washed and the volume reduced by vacuum filtration. A biotinylated reporter antibody was then added. After thorough mixing, they were incubated for an additional hour at room temperature in the dark. 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 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 Windows Excel. For each multiplex, standards were included on each microtiter plate in triplicates. Eight point standards were run in the first three columns of each plate. Each analytical condition was analyzed in four replicates. Unknown values for each of the analytes localized in a specific multiplex were determined by means of four and five parameter, weighted and non-weighted curve fitting algorithms included in the data analysis package. The least detectable dose for each cytokine was the following: IL-1 beta (0.033 ng/ml), IL-2 (12 pg/ml), IL-6 (2.4 pg/ml), IL-8 (0.7 pg/ml), TNF-alpha (4.4 pg/ml), adiponectin (10 pg/ml), and TGF-$\beta_1$ (5 pg/ml). For each cytokine, the lowest detectable dose was three 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 Gaussian distribution by Kolmogorov–Smirnov test and compared the cases by the use of independent—samples *t*-test or Mann–Whitney *U*-test (dependent on the results from the normality test). Differences were considered significant at the level of $P < 0.05$.

For the cytokine and growth factor measurements, comparisons between various groups were performed with the paired Student's *t*-test. A $P$ value $<0.05$ was considered statistically significant.

## RESULTS

During the first 24 h of clinorotation, the cell number of the subconfluent monolayers doubled and the monolayers became confluent. Although, proliferating cells detached from the culture dishes and formed 3D aggregates, the majority of the ECs remained adherent (Fig. 2G,H). In this time, neither clinorotation nor the presence of 10 ng/ml bFGF had an influence on the cellular micromilieu relative to pH ($P =$ ns; 4 and 24 h vs. 1 g controls), sodium and potassium ($P =$ ns; 4 and 24 h vs. 1 g controls), bicarbonate ($P =$ ns; 4 and 24 h vs. 1 g controls) and $pC0_2/p0_2$ ($P =$ ns; 4 and 24 h vs. 1 g controls).

### Effects of bFGF on Gene Expression of ET-1, eNOS and TGF-$\beta_1$

We quantitatively evaluated the mRNAs of the EC specific hormone ET-1, the enzyme endothelial nitric oxide synthase (eNOS) and TGF-$\beta_1$. After 4 and 24 h, the ET-1 mRNA quantity was increased in ECs cultured under simulated microgravity compared with controls. Application of bFGF for 4 h reduced the gene expression of ET-1 under μg and 1 g conditions (Fig. 1 A). After 24 h, when the monolayer had become confluent in static controls, the intracellular ET-1 mRNA concentration was reduced to 15% of the 4 h value. At

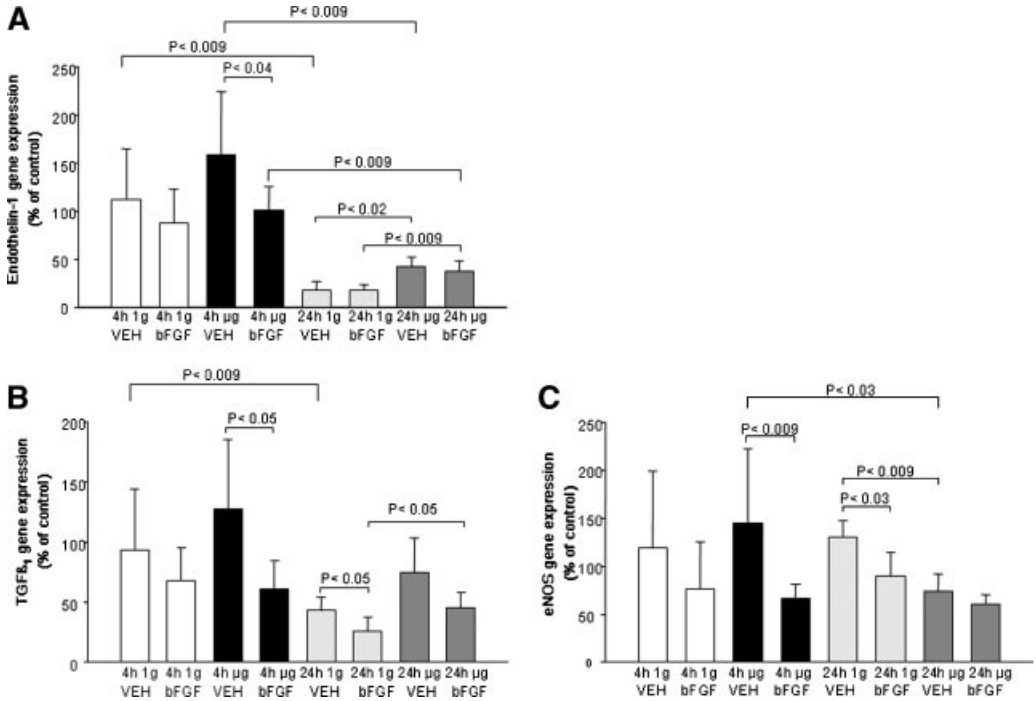


**Fig. 1.** Endothelin-1 gene expression (**A**), TGF-$\beta_1$ (**B**) and eNOS gene expression (**C**). All genes were detectable in static control cells. Microgravity early increased ET-1 as well as TGF-$\beta_1$ gene expression. bFGF reduced the expression of ET-1 after 4 h in 1 g and µg. TGF-$\beta_1$ gene expression was decreased by bFGF at both time points, irrespective of gravity. eNOS gene expression was decreased after 24 h under µg. bFGF reduced the amount of eNOS gene expression in 1 g and µg. Values are given as mean ± SD.

this time point, a significantly higher amount of ET-1 mRNA was found in clinorotated cells. However, after 24 h bFGF exerted no effect on ET-1 mRNA expression of ECs grown under conditions of both gravitational conditions (Fig. 1A).

In parallel, static control cells showed basic mRNA concentrations of TGF-$\beta_1$ when harvested 4 h after the start of the experiment. Clinorotated samples demonstrated a larger amount of TGF-$\beta_1$ mRNA. However, the bFGF treatment reduced the overall increase of TGF-$\beta_1$ mRNA expression in static and in clionorotated samples (Fig. 1B). One day after starting the experiment, the ECs contained about half the quantities of their 4 h TGF-$\beta_1$ mRNA under all experimental conditions, for example in static control and clinorotated cultures, irrespectively of bFGF treatment (Fig. 1B). bFGF application reduced the expression of TGF-$\beta_1$ in both µg and 1 g cultures after 4 and 24 h.

Concerning eNOS mRNA expression, the basic mRNA concentrations were equal after 4 and 24 h of incubation. However, clinorotation had slightly but not significantly upregulated the gene expression of eNOS during the early 4 h. Then after 24 h, the low gravity environment resulted in down-regulated levels which were comparable to those observed in static controls. In the presence of bFGF, the eNOS mRNA quantities were reduced, irrespectively of gravity (Fig. 1C).

### Effect of bFGF on Extracellular Matrix Proteins

Usually changes of protein concentrations follow the alterations of the respective mRNA concentrations with some delay. Therefore, cells were harvested after a 24 h exposure to simulated microgravity under either bFGF or VEH influence and compared with 1 g controls. Western blot analyses were performed in order to determine the quantities of selected extracellular matrix components. After 24 h, microgravity or bFGF application slightly increased ($P = \text{ns}$) the basic cellular amount of collagen type I to similar extents (Fig. 2A). The laminin and fibronectin production (Fig. 2B–D) was upregulated to a larger extent by microgravity

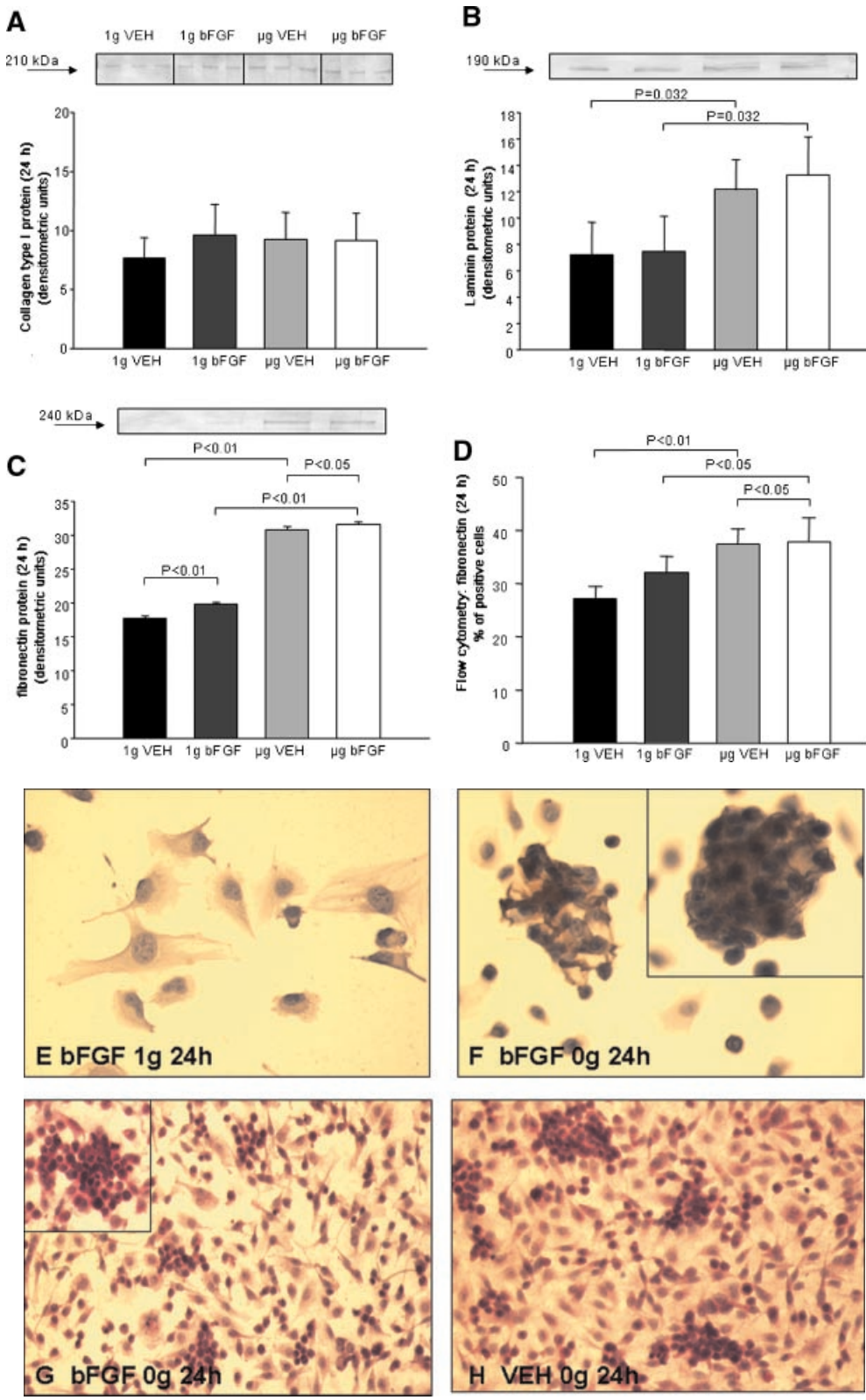


**Fig. 2.** Western blot analyses: **A**: Collagen type I was increased by bFGF alone, by microgravity and in combination of microgravity and bFGF. **B**: Laminin protein was significantly elevated by microgravity. **C**,**D**: Fibronectin was clearly enhanced by microgravity as measured by Western blotting and flow cytometry (D). bFGF exerted an effect in static controls and in µg samples. **E**: Fibronectin immunohistochemistry of bFGF treated EC at 1 g. Fibronectin-positive cellular cytoplasm was visible. **F**: Formation of multicellular aggregates was detectable within 24 h of clinorotation. These structures were fibronectin-positive. Magnification 200×. **G**: HE-staining of bFGF treated cells grown under microgravity. **H**: HE-staining of vehicle treated cells after 24 h simulated microgravity. Multicellular aggregates and adherent cells were shown. Magnification is 100× (insert 200). Values are given as mean ± SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

than by bFGF. Fibronectin was detected in multicellular aggregates obtained after 24 h of microgravity (Fig. 2F). Microgravity and bFGF in combination did exert a moderate synergistic effect on the cellular synthesis of fibronectin (Fig. 2).

### Effects of bFGF on Apoptosis Inducing Factors

The induction of apoptosis is a frequent early event when cells of various types are exposed to real and simulated microgravity. Flow cytometric analyses were applied in order to determine the percentage of cells expressing apoptosis related factors. After 24 h, 4% of the cells had expressed Bax in static controls and 8% in clinorotated cultures. bFGF had increased the percentage of Bax-positive cells by another 2% in ground controls and in clinorotated samples, respectively (Fig. 3A).

The amount of Bcl-2 protein increased in cultures grown under simulated microgravity. bFGF reduced the level of Bcl-2 in 1 g and μg samples (Fig. 3B).

Also Fas (9%)- and p53 (24%)-positive cells were detected in static control cultures, which had reached confluence within 24 h after the start of the experiment. The number of Fas- as well as of p53-positive cells was 1.5-fold higher in clinorotated samples after 24 h. bFGF application did not significantly change the respective percentage of Fas-positive cells in clinorotated samples (Fig. 3C). bFGF increased the amount of p53-positive cells cultured under μg (Fig. 3D).

Annexin V staining showed a decrease of viable adherent cells, as well as an increase in apoptotic and necrotic adherent EC, grown under μg. Application of bFGF significantly reduced the amount of apoptotic cells in μg (Fig. 4A). In parallel, an increase in viable cells was measured in the bFGF treated clinorotated cell population.

Using the TUNEL assay, the DNA fragment multimers of approximately 180–200 bp in length were fluorescently labeled and were quantitated by flow cytometry. Migrogravity as well as bFGF application to static control cells induced a slight increase in apoptotic cells. The addition of bFGF to clinostat-cultivated cells induced a decrease in TUNEL-positive cells indicating a cell-protective effect of bFGF against clinostat induced apoptosis (Fig. 4B).

Similarly, activated caspase-3 was detectable in static ground controls (Fig. 4C). Microgravity as well as bFGF application to static control cells induced augmentation of activated caspase-3 within 24 h. bFGF reduced the amount of caspase-3-protein in clinorotated cells (Fig. 4C,D).

Acridine-orange/ethidium bromide staining of EC control cells revealed that the 1 g control cells with and without bFGF treatment remained impermeable for the dyes (Fig. 4E,G). Clinorotated cells revealed nuclear condensation (Fig. 4F,H; phase II) and a few EC started to took up the dyes (phase III), indicating dead cells according to Zhou et al. [1997].

### Cellular Growth Factors and Hormones

ET-1 protein, VEGF, and VEGF-receptor proteins were measured after 24 h of clinorotation.

We detected a significant early enhancement of cellular ET-1 hormone (Fig. 5A), when we compared static controls with clinorotated cells, both harvested 1-day after the start of the experiment. The application of bFGF increased ET-1 protein expression in static controls, but exerted no further effect on elevated ET-1 in clinorotated cells (Fig. 5A).

In addition, bFGF clearly enhanced the amount of VEGF and of VEGF-receptors Flt-1 and Flk-1, when applied in static control cultures for 24 h (Fig. 5B–D). In the same way, microgravity induced VEGF and slightly Flk-1. On the RPM, however, bFGF did not affect VEGF production, but bFGF further increased Flk-1 and Flt-1 protein (Fig. 5C,D).

### Release of Soluble Factors in Culture Supernatants

Growth factors or interleukins released into the supernatant may affect cells by binding to their corresponding receptors which can be expressed on the surfaces of the releasing cells or neighboring cells in the intercellular medium. Therefore, 4 and 24 h after start of the experiment, we evaluated various soluble factors. IL-1β, TNF-α or adiponectin could not be detected in the medium surrounding the cells, neither after 4 h, nor after 24 h of culturing. But little amounts (between 0.05 and 0.1 pg/ml) of IL-2 were detectable (Fig. 6A). This interleukin was slightly enriched in the presence of bFGF, but did not respond to microgravity during the first 4 h of culturing. After 24 h, the IL-2 amount had slightly

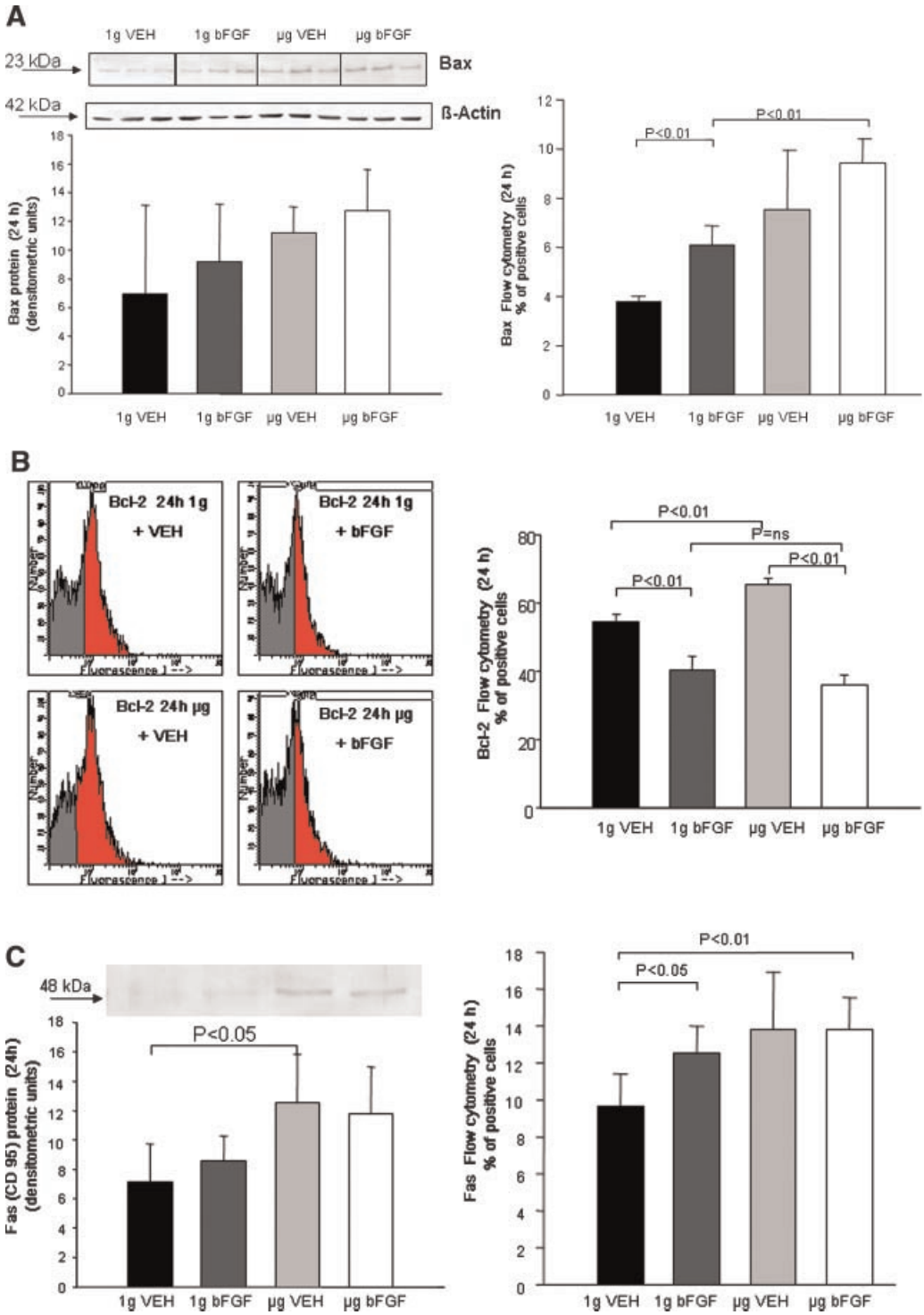


**Fig. 3.** Investigation of Bax (**A**), Bcl-2 (**B**), Fas (**C**), and p53 (**D**). These apoptotic factors were measured after 24 h under microgravity and 1 g conditions with and without bFGF treatment. After 24 h Bax, Bcl-2, Fas and p53 were increased in samples cultured under simulated microgravity. bFGF exerted synergistic effects on Bax, and p53. bFGF reduced Bcl-2 in µg cultures. Values are given as mean ± SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

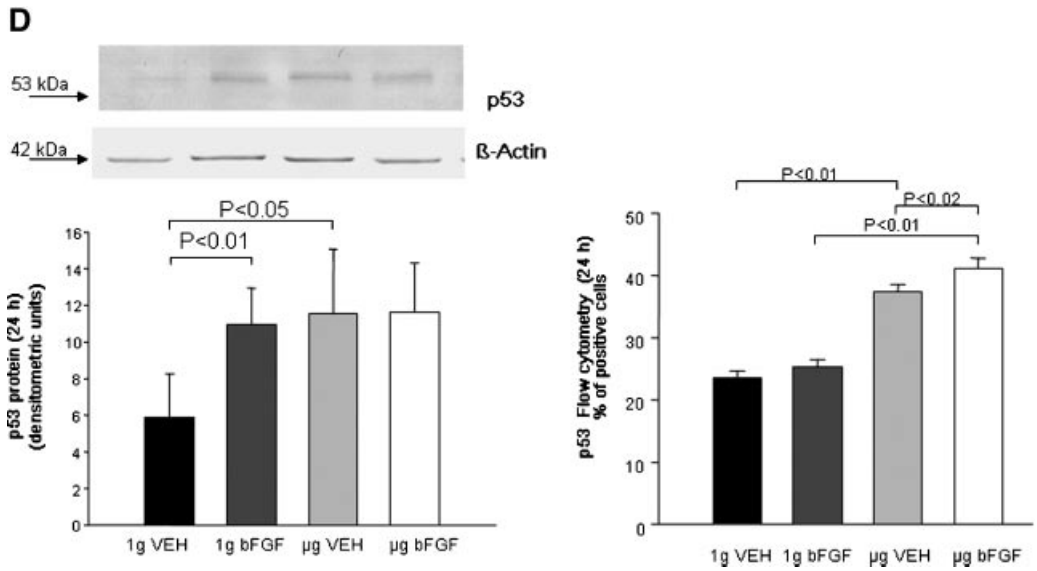


**Fig. 3.** (*Continued*)

increased in ground controls. The presence of bFGF in 1 g samples had attenuated IL-2 secretion at this time point (Fig. 6A). Microgravity significantly reduced the release of IL-2.

In contrast, we detected TGF-$\beta_1$ (4 pg/ml) in supernatants of static control and clinorotated cultures at 4 h after start of the experiments. In the presence of bFGF, this amount was doubled in control cultures, and enhanced by 50% in microgravity cultures. After 24 h, a promoting effect of bFGF could not be observed anymore, as 6 pg/ml were found in static control and 3 pg/ml in clinorotated culture supernatants, irrespectively whether or not bFGF was present in the cultures (Fig. 6B).

Similarly, after 4 h IL-6 (2 pg/ml) was detectable in bFGF-free culture supernatants. In static controls, the IL-6 concentration remained constant up to 1 day. In the presence of bFGF, the amount of IL-6 increased after 4 h and remained constant during the rest of the day, irrespectively whether the cells were cultured on ground or on the RPM. After 24 h of clinorotation in the absence of bFGF, however, the concentration of IL-6 was enhanced eightfold (Fig. 6C).

Investigating IL-8, we measured a concentration of 15 pg/ml, when the ECs had been cultured in the absence of bFGF for 4 h. In the same time, ground control cells had secreted 120 pg of the interleukin per ml supernatant, when bFGF was present. The bFGF dependent high concentration of IL-8 remained unchanged for the rest of the day, while the basic amount doubled. Under microgravity, the cells released a concentration of 30 pg/ml IL-8 and 75 pg/ml IL-8 in the presence of bFGF (4 h). After 24 h of clinorotation, an IL-8 concentration of 150 pg/ml was measured in the supernatants of bFGF-free cultures and 120 pg/ml when bFGF was present (Fig. 6D).

### NF-kappaB

Western blot analyses revealed that different concentrations of the two NF-kappaB subtypes p50 and p65 are detectable in cells cultured under different conditions for 24 h (Fig. 7). The basic amount of NF-kappaB p50 was reduced, when the ECs were cultured in the presence of bFGF under gravity for a day. However, after 24 h exposure to simulated microgravity, it was doubled and tripled when bFGF was present (Fig. 7A). Looking at the NF-kappaB subtype p65, we observed a different behavior. A 25% increase of basic concentration was achieved by adding bFGF to 1 g cultures for 24 h. Clinorotation alone caused a quadrupling of p65 concentrations during an equal time period. This was attenuated when bFGF was present in the cultures mounted on the working RPM (Fig. 7B).

## DISCUSSION

The results of our experiments clearly show that bFGF differently regulates several

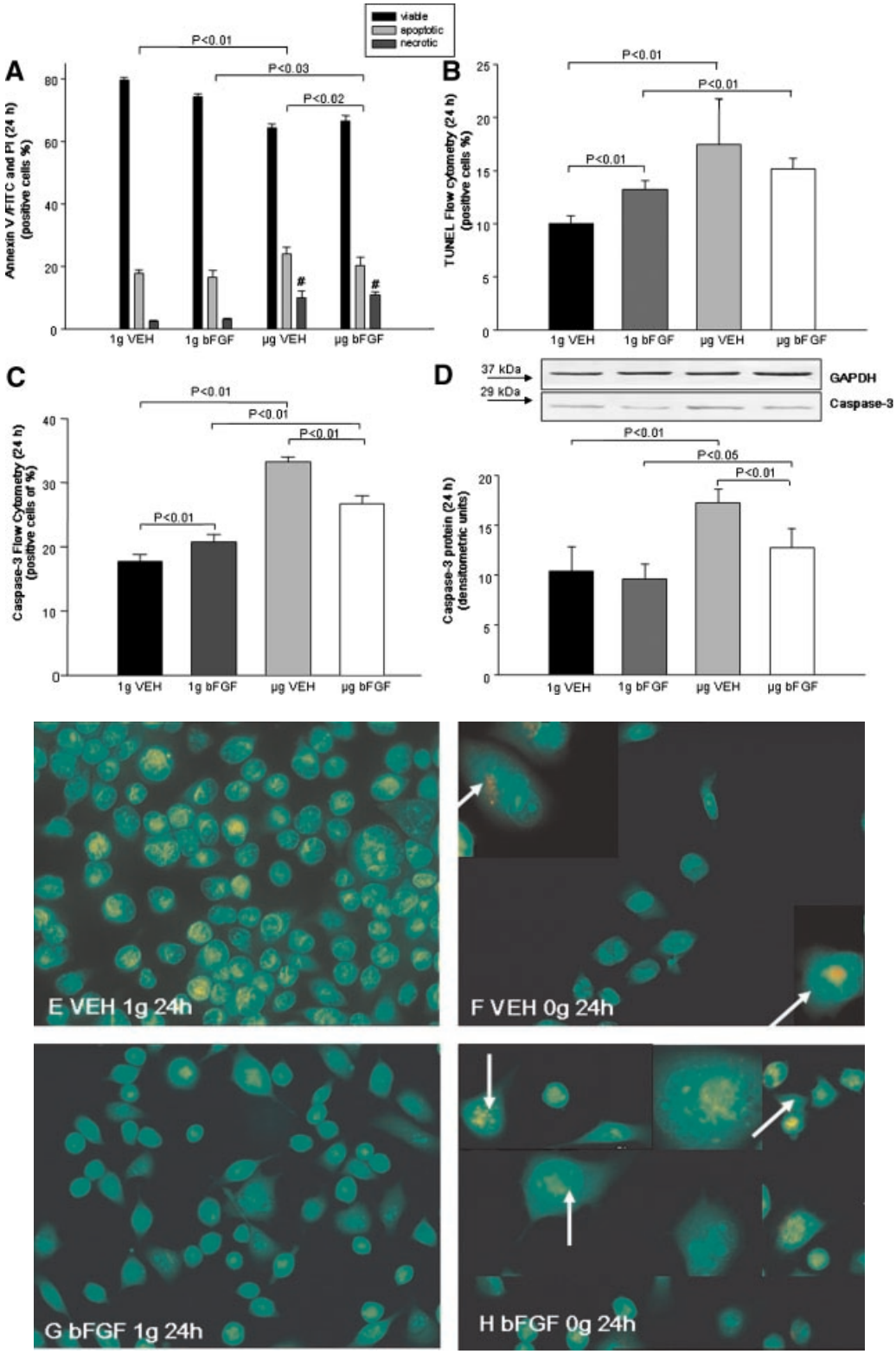


**Fig. 4.** **A**: Annexin V staining of adherent endothelial cells (ECs) revealed an increase of apoptotic cells by microgravity compared with 1 g controls. bFGF decreased the amount of early apoptosis. **B**: Comparison of the TUNEL assay of EA.hy926 cell line in different cultures: Microgravity increased TUNEL-positive ECs. bFGF reduced the amount of apoptotic cells. **C**,**D**: Western blot analysis and flow cytometry of activated caspase-3. Activated caspase-3 was elevated by microgravity, bFGF significantly the amount of caspase-3 compared with 1 g control cultures. Values are given as mean ± SD. **E**–**H**: Acridine orange staining of EC. Arrows indicate nuclear condensation (phase II) and formation of apoptotic bodies. Some cells took up the dyes (phase III), indicating dead cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

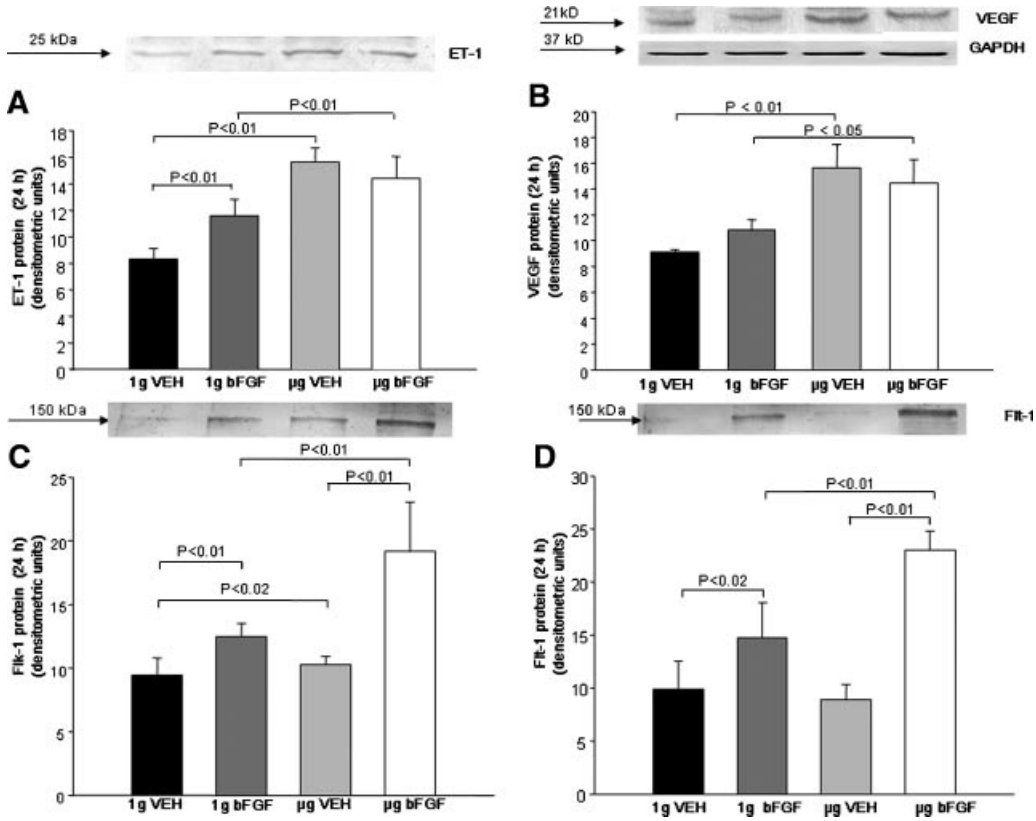


**Fig. 5.** **A**: ET-1 protein was detectable in static controls and further elevated by bFGF. ET-1 increased after 24 h of culture under microgravity. **B**: VEGF protein was sensitive to microgravity. bFGF elevated the level of VEGF protein in 1 g and µg conditions. Equal loading of proteins in each lane is indicated by GAPDH. **C**: Flk-1 protein was elevated by bFGF in 1 g and µg. **D**: Flt-1 was elevated by bFGF in cells cultured under 1 g and µg conditions. Western blots were analyzed by densitometry. Values are given as mean ± SD.

functions of EA.hy926 cells under gravity and simulated microgravity. We used the permanent EC line EA.hy926 and simulated microgravity using a RPM. Under these conditions EA.hy926 cells start tube formation like ECs in vivo [Infanger et al., 2006a]. The EA.hy926 cell line is a stable well-characterized EC line. The basic amount of extracellular matrix proteins, apoptotic factors, cytokines, cell adhesion molecules, cytoskeletal proteins, and many other factors was always comparable with previous data [Infanger et al., 2004, 2006a, 2007b]. In accordance to these results, we observed a microgravity-dependent regulation of ECM components. Microgravity promoted the production of fibronectin and laminin. However, only a moderate synergistic effect of bFGF and microgravity on fibronectin production could be observed. This suggests that bFGF has little influence on the ECM production under microgravity. bFGF is known to be produced by human ECs and is first bound to the extracellular matrix. Later, it is released into the surroundings to bind to its receptor [Flaumenhaft et al., 1989]. When released from the extracellular matrix of ECs, it may trigger the augmentation of ECM of various types of cells [Takayama et al., 2002]. No information could be found in the literature that bFGF triggers ECs in vivo to enhance ECM production. Only bovine corneal ECs have been described so far to be triggered by bFGF in vitro to produce ECM [Desgranges et al., 1992]. For testing bFGF actions, it appears that the µg situation, when ECs are forming 3D aggregates, may represent the in vivo situation better than monolayers under normal laboratory conditions.

According to other studies, we also observed an increase of apoptotic factors after the cells had been exposed to microgravity [Infanger et al., 2006a,b, 2007b]. At 1 g conditions, bFGF triggered an enhancement of production of Fas, p53, and Bax, too. In microgravity, however,

p53 and Bax production was further enhanced by bFGF. Annexin V, and caspase-3 were reduced, but independently of Fas which was not affected. Moreover, we detected a clear increase of apoptosis in clinorotated adherent cells as measured by TUNEL flow cytometry which was attenuated by bFGF. Here, we demonstrate for the first time an anti-apoptotic effect of bFGF on ECs in vitro under microgravity conditions. We could show a VEGF-like protective effect of bFGF. We detected that bFGF triggers VEGF, Flk-1 and Flt-1 production in static control cells. But, also microgravity challenges enhancement of VEGF even to a double extent, while it simultaneously promotes apoptosis. There is no further effect of microgravity and bFGF on VEGF but a clear one on Flt-1 and Flk-1. VEGF together with its receptors Flk-1 and flt-1 has been shown to stimulate proliferation, migration, and proteolytic activity of ECs. It also induces vascular permeability, vasodilatation, and angiogenesis [Ferrara and Davis-Smyth, 1997]. The expression of VEGF is controlled by differentiation, transformation, and oxygen supply. The effect of VEGF is regulated by two receptor tyrosine kinases VEGFR-1/Flt-1 (fms-like tyrosine kinase-1) and VEGFR-2/kinase insert domain-containing receptor (KDR), which are almost exclusively located on ECs [Infanger et al., 2007a,b]. The normal density of flt-1 and KDR is low in ECs of normal tissue. We recently demonstrated the cell-protective effect of externally applied VEGF to clinorotated ECs [Infanger et al., 2006a]. The up-regulation of the receptor tyrosine kinases plays an important role for the cell-protective effect of bFGF. Self-produced VEGF does not seem to

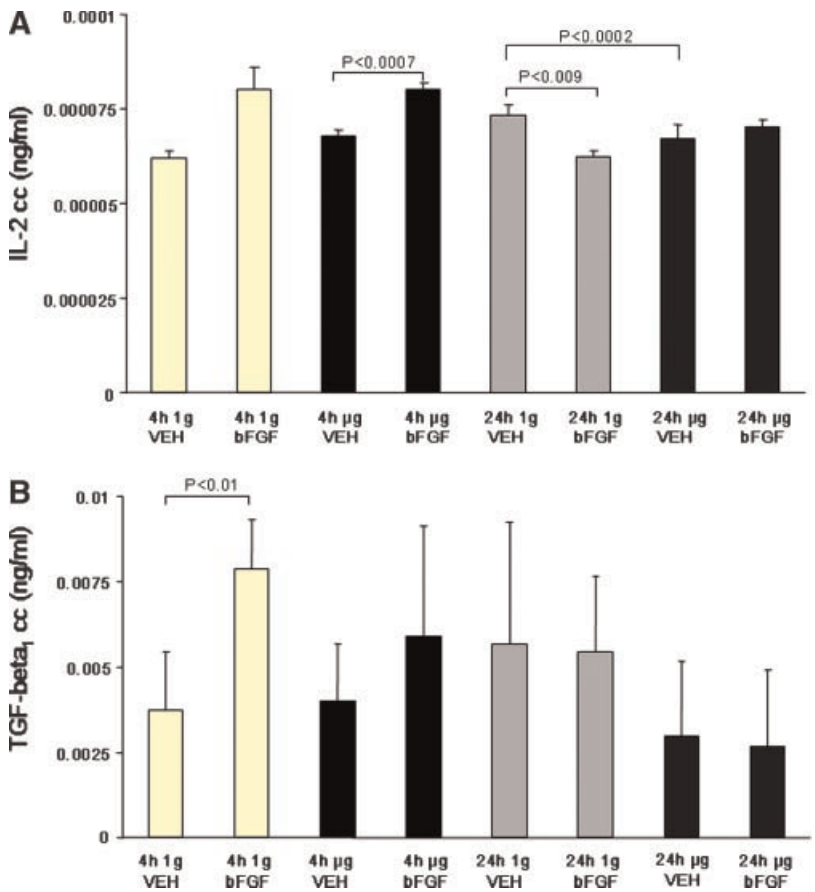


**Fig. 6.** Soluble factors released in the supernatant after 4 h up to 24 h of culture under µg conditions. **A**: Interleukin-2 release was increased by microgravity and bFGF application after 4 h of treatment. After 24 h, the IL-2 release was reduced by µg conditions. **B**: TGF-$\beta_1$ was clearly induced by bFGF in static controls after 4 h. There was no difference in all other conditions. **C**: IL-6 was elevated by bFGF in both 1 g and µg cultures after 4 h of treatment. After 24 h, bFGF significantly reduced the release of IL-6 under µg. **D**: IL-8 was clearly increased by bFGF after 4 h. After 24 h bFGF attenuated the release of IL-8 under µg conditions. Values are given as mean ± SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

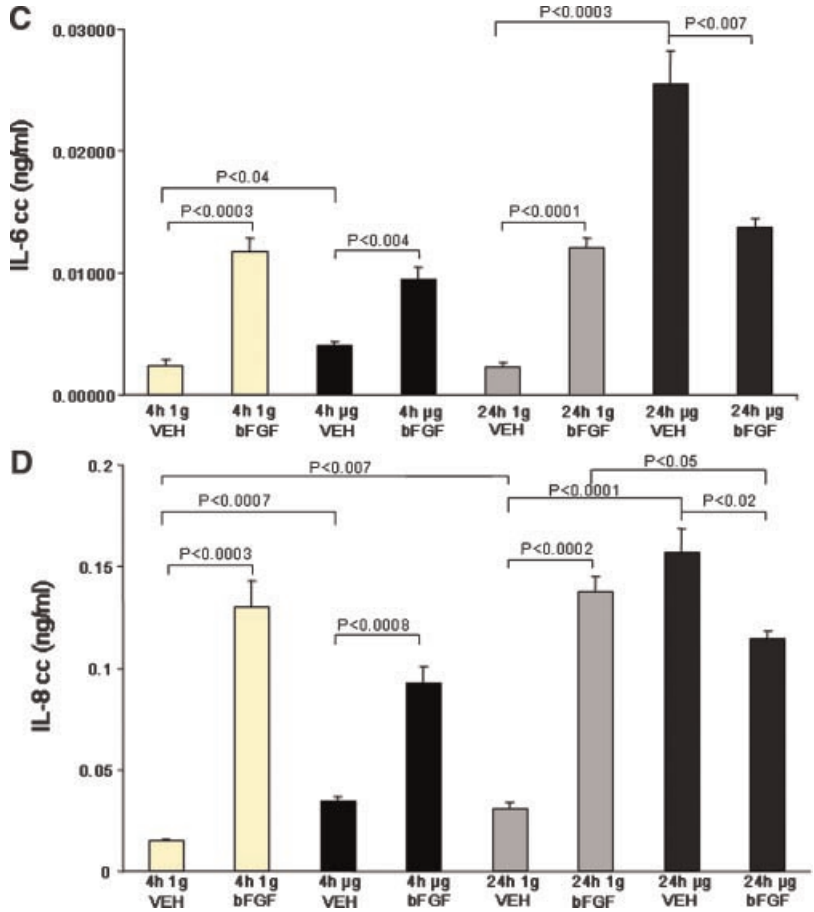


**Fig. 6.** (*Continued*)

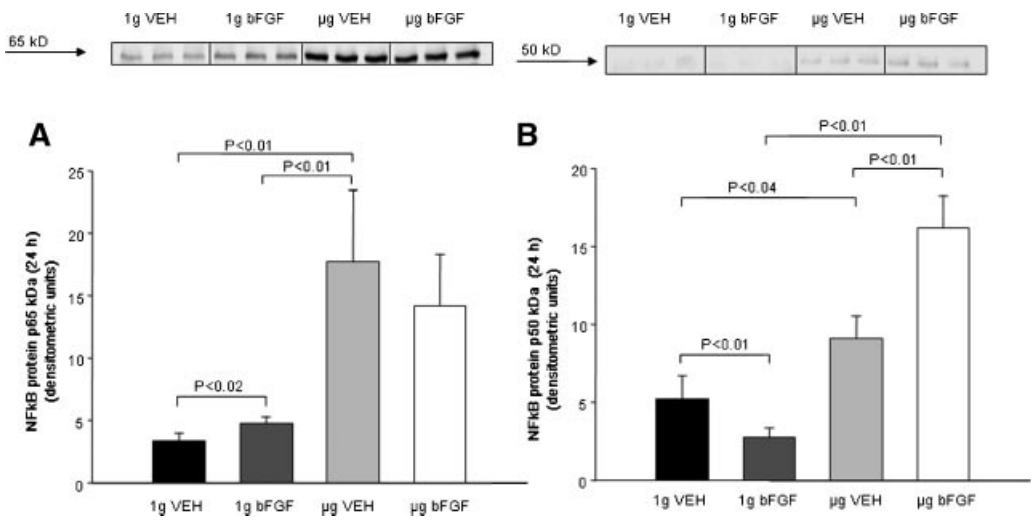


**Fig. 7.** Western blot analysis of NF-kB after 24 h of culture in 1 g and µg conditions: **A**: NF-kB p65 kDa was elevated by µg. bFGF increased NF-kB p65 in static controls and decreased it in µg cultures. **B**: NF-kB p50 kDa was increased by microgravity and further elevated by microgravity and bFGF treatment. Values are given as mean ± SD.

be sufficient to protect against apoptosis as externally added VEGF does [Infanger et al., 2006a].

The amount of ET-1 is known to be enhanced during the first 24 h of exposure to μg, but to be reduced below normal levels after 7 days under microgravity [Infanger et al., 2007b]. Here, we confirm the early increase of ET-1 mRNA and protein levels. bFGF induces ET-1 protein synthesis under 1 g. bFGF did not show any effect on ET-1. TGF-$\beta_1$ mRNA is upregulated under simulated microgravity, while eNOS mRNA is down-regulated. bFGF reduced the concentrations of both species of mRNA not only in normal gravity but also in microgravity. TGF-$\beta_1$ regulates the activation of eNOS via the cell surface receptor endoglin [Cheifetz et al., 1992; Gougos et al., 1992]. This receptor associates with eNOS and thereby upregulates its activity [Toporsian et al., 2005]. In our experiments, we observed that clinorotation causes opposite effects on the mRNAs of TGF-$\beta_1$ and eNOS. Thus, the eNOS mRNA does not seem to be affected by TGF-$\beta_1$ mRNA.

The secretion of IL-6 and Il-8 between the 4th and the 24th hour seems to be due to a specific activation, because other factors such as IL-1β, TNF-alpha, and adiponectin secretion are not enhanced. Furthermore, it took a day before microgravity induced secretion could be observed. This finding suggests that the release is a consequence of protein production. It is clearly different from the early bFGF-induced secretion activity, which could also be due to the fact that factors stored in the Weibel-Palade bodies are emptied under the influence of bFGF [van Mourik et al., 2002]. It is known that bFGF rapidly induced the release of glutamate from cultured neurons [Numakawa et al., 2002]. A direct effect of bFGF on the emptying activity of Weibel-Palade bodies has to our knowledge not yet been described in the literature.

A very interesting result was the differential regulation of two subtypes of Nuclear factor-κB (NF-κB). NF-κB transcription factors represent the terminal step in a signal transduction pathway leading from the cell surface to the nucleus. They comprise a family of structurally related eukaryotic transcription factors that promote the expression of more than 150 genes involved in a variety of cellular processes [Ghosh et al., 1998; Pahl, 1999]. Five members of the NF-κ≡ family, p50, p52, p65 (RelA), c-Rel, and RelB, form various homo- and heterodimers. Dimerization of various NF-κB subunits produces complexes with different DNA-binding specificities and transactivation potential [Li and Verma, 2002; Senftleben and Karin, 2002]. We observed that bFGF did virtually not change p50 and p65 concentrations under 1 g. In simulated microgravity, however, p65 is quadrupled in the absence of bFGF and p50 in the presence of bFGF. These results help to understand the Il-6 and Il-8 secretion activity. It is known that p65/p65 homodimers can activate the promoters of IL-8 and IL-6 [Miyazawa et al., 1998; Grassl et al., 2005]. As the relations of the concentrations of the various members may be assumed to influence dimerization, it seems reasonable that enhanced p65 may favor p65/p65 homodimer formation and may in this way enhance intracellular IL-6 and IL-8.

The idea appears reasonable, if one compares Figure 7A with Figure 2C,D (fibronectin) and Figure 7B with Figure 3A (Bax). Fibronectin expression is regulated by p65/p65 homodimers [Lee et al., 2002]. In our study, fibronectin is significantly enhanced by microgravity, while an additional bFGF treatment does not have any effect on the level of fibronectin. On the other hand, microgravity and bFGF acted synergistically on Bax content. Bax is known to be upregulated by p50/p50 [Grimm et al., 2005].

It is well known that high concentrations of proinflammatory cytokines (IL-6 and IL-8) induce EC apoptosis [Kofler et al., 2005]. We measured that bFGF elevated the release of IL-6 and IL-8 within 24 h. Microgravity elevated the amount of IL-6 and IL-8, but bFGF clearly reduced the release of IL-6 and IL-8 in the supernatant. This also may explain the cell-protective effect of bFGF under microgravity.

In summary, the results of this study indicate that the random positioning machine (3D clinostat) provides an important experimental system to culture adherent ECs under simulated microgravity as well as ECs grown as 3D aggregates (Fig. 2). We investigated the effects of bFGF under microgravity, which is simulated at minimal shear forces, as during the 1-min period of random rotation by the RPM shear forces are close to 0 for 43 s, 0.09–0.22 dyn/cm$^2$ for 13 s and 0.22–0.44 dyn/cm$^2$ for 4 s [Pardo et al., 2005]. Although it is known that shear stress can influence gene expression in ECs, the levels of forces required to affect expression

of factors such as VEGFR-2 or ET-1 are much higher [Sharefkin et al., 1991; Jin et al., 2003]. Even the most shear stress sensitive factor, the eNOS, is not significantly influenced in EAhy926 cells, when shear stress of 0.3 dyn/cm$^2$ is applied for 24 h [Ziegler et al., 1998]. So it appears justified to assume that the lack of gravity is the dominant factor, when the responsiveness to bFGF is changed, although some influence of the minimal shear forces cannot completely be ruled out.

Taken together, we were able to demonstrate that a transition from gravity to simulated microgravity modulates various effects of bFGF, in a similar way as drugs or gangliosides do [Meuillet et al., 1996; Izevbigie et al., 2000]. There are several cellular factors like Bax or eNOS which are either reduced or enhanced by bFGF, irrespectively whether the bFGF is added to cell cultures under 1 g or μg conditions (Fig. 8). There are other cellular factors such as collagen type I or Fas, which respond to bFGF under gravity but not under microgravity. A third group of components comprising important apoptotic factors such as caspase-3 or Annexin V-positive cells were enhanced by

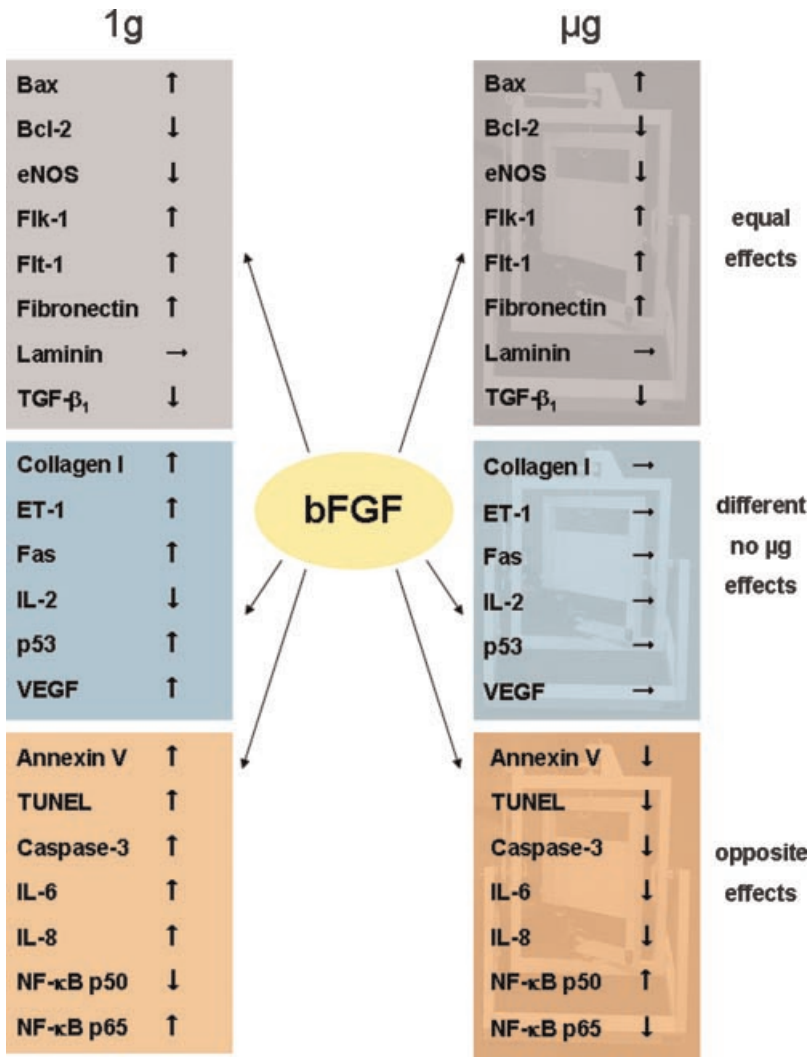


**Fig. 8.** Schematic diagram showing the summarized effects of bFGF under 1 g and simulated microgravity conditions on human ECs during a clinorotation experiment for 24 h. Under normal gravity conditions, bFGF induced a slight increase in apoptosis, which was the opposite in microgravity. In parallel, IL-6 and IL-8 decreased in microgravity, while Flt-1 and Flk-1 were further elevated, and TGF-$\beta_1$ mRNA more reduced by bFGF. These findings support the hypothesis that bFGF develops a cell-protective influence on ECs and acts first via the VEGF/VEGF receptor system, second via down-regulation of other pro-apoptotic growth factors such as caspase-3 and TGF-$\beta_1$ and third also by attenuation of the release of inflammatory cytokines. In addition, the Annexin V and TUNEL flow cytometry revealed a decrease in apoptosis in EC cultured under μg and the influence of bFGF. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

bFGF under 1 g, but significantly decreased under microgravity. This anti-apoptotic effect is a major finding of this study, which created the idea that abnormal physical conditions could influence cellular signaling like pharmacological medication.

In addition, these findings support the hypothesis that bFGF develops a cell-protective influence on ECs and acts via the VEGF/VEGF receptor system, via down-regulation of other pro-apoptotic growth factors such as TGF-$\beta_1$ and also by attenuation of the release of inflammatory cytokines.

## ACKNOWLEDGMENTS

Our research was supported by the German Space Agency DLR (grant 50WB0524), by the Programm zur Förderung von Forschung, Innovationen und Technologien—ProFIT (grant 10135687) and by a grant from the Bundesministerium für Bildung und Forschung (Nationales Genomforschungsnetz: KGCV1, 01GS0416). We thank the Space Biology group, ETH Zurich Switzerland, for providing the clinostat and lab facilities.

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