The Impact of Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor on Cardiac Fibroblasts Grown under Altered Gravity Conditions

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Key Words

Cardiac fibroblasts • Extracellular matrix • Apoptosis • Growth factors • Microgravity • Random positioning machine

Abstract

Background: Myocardium is very sensitive to gravitational changes. During a spaceflight cardiovascular atrophy paired with rhythm problems and orthostatic intolerance can occur. The aim of this study was to investigate the impact of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) on cardiac fibroblasts (CF) grown under altered gravity conditions. **Methods:** We examined the influence of exposure to a Random Positioning Machine (RPM) on CF, derived from porcine hearts. We focused on growth, extracellular matrix protein (ECMP) synthesis and apoptosis. **Results:** When cultured on a RPM, CF began to form 3D spheroids within 24h, irrespective of growth factor treatment. Exposure to RPM induced an increased synthesis of ECMP and also resulted in elevated apoptosis in adherent CF as measured by terminal deoxynucleotidyl transferase-mediated dUTP digoxigenin

nick end labeling (TUNEL) analysis, 4',6-diamidino-2-phenylindole (DAPI) staining, and caspase-3 detection. bFGF and VEGF significantly decreased the amount of ECMP (collagen type I, III, chondroitin sulfate) in 1g and RPM cultures, and also significantly reduced the amount of apoptotic CF as well as caspase-3. **Conclusions:** Altered gravity conditions on a RPM induced 3D growth, elevated ECMP synthesis and apoptosis in cardiac fibroblasts. Growth factor treatment attenuated programmed cell death and ECMP secretion.

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Introduction

A long-term spaceflight can cause a variety of health problems in humans and mammals [1-3]. Astronauts and cosmonauts suffer from bone [4] and muscle loss [5], nephrolithiasis [3] and cardiovascular problems [6]. Sensory input changes, cardiovascular atrophy paired with rhythm problems as well as orthostatic intolerance

can occur [1]. Therefore, it is of great interest to know how cardiac cells do respond to altered gravity conditions and which signal molecules are involved in possible changes.

Cardiac fibroblasts (CF) are the most abundant cell type in the heart and play a major role in synthesizing extracellular matrix proteins (ECMP). Cellular behavior and the function of CF are crucial determinants affecting structural changes within the myocardium. Focusing on cellular and molecular mechanisms that mediate cardiac tissue remodeling is important in light of the fact that the abnormal accumulation of ECMP in the interstitium (fibrosis) has been associated with the development of extensive left ventricular (LV) hypertrophy in the myocardium. These structural alterations, the so-called pathological remodeling process, can induce LV dysfunction and heart failure [7, 8].

Usually, ECMP form an organized network that surrounds and interconnects cellular structures [9]. It includes interstitial collagens, proteoglycans, glycoproteins, cytokines, growth factors and proteases [10, 11]. The density of the extracellular matrix is an important factor that is intimately tied to cardiac function [12, 13]. Collagen expression and accumulation are increased in a number of cardiovascular diseases [7, 14-17]. The density of the ECM affects compliance and the movement of cells and fluids within the ECM [9].

Growth factors influence different kinds of cells. Vascular endothelial growth factor (VEGF) mostly affects endothelial cells and is a potent mediator of vascular regulation in angiogenesis and vascular permeability [18-21]. Moreover, VEGF also influences a number of other cell types (e.g. stimulation monocyte/macrophage migration, neurons, cancer cells, kidney epithelial cells). We recently demonstrated that the VEGF/VEGF receptor and IGF-1-IGF-1R systems are activated by ischemia/reperfusion (I/R) [22]. There are several therapeutic strategies using VEGF to treat myocardial infarction and reduce infarct size and increase LV function [23-25]. Therefore, we investigated the effects of this growth factor on CF *in vitro* to analyze its effects under different gravitational conditions. VEGF had a cell-protective effect on endothelial cells cultured under conditions of simulated microgravity and inhibited apoptosis in adherent endothelial cells [26].

The basic fibroblast growth factor (bFGF) belongs to the fibroblast growth factor family. This family is involved in wound healing, angiogenesis and embryonic development. It is already known that bFGF has a direct protective effect against ischemia/reperfusion injury [27].

Other research groups have demonstrated positive effects of bFGF after myocardial infarction in animal models [28-30], however little is known about the effects of bFGF on CF. bFGF reduced apoptosis induced by simulated microgravity in endothelial cells [31].

The so-called 'Random Positioning Machine' is a well established method to culture cells in altered gravity conditions [32-34]. A principal aim of this study is to answer the question how these conditions influence CF. Exposure to a RPM alters gene signaling [35], ECM production and induces cytoskeletal alterations [36] in different cell types [37]. Before doing an experiment in real microgravity, which is expensive and time consuming, one should do extensive ground based studies by altering the influence of gravity. Thus, we decided to use the RPM for simulating weightlessness on Earth. In order to prove the functionality of CF in this experimental setup, we investigated growth, the process of apoptosis and the synthesis of ECMP after 24 hours of culturing. In addition, we focused on the impact of growth factor treatment (VEGF, bFGF) on CF grown under altered gravity conditions.

Materials and Methods

Random Positioning Machine

The influence of gravity was altered by using a desktop RPM manufactured by Dutch Space, an EADS Astrium company (Leiden, The Netherlands). The RPM was developed by Hosen and investigated in detail by van Loon [38, 39]. Under the control of an associated software, the RPM enables a random 3D position change of the biological specimen. The hardware dimensions are 30 x 30 x 30 cm³. As indicated by the manufacturer, two operational modes are available: real time generation of a random profile with user defined control parameters or execution of a previously calculated random path using a constant rotation rate (ω). For the last mode, which was used here, changes in 'random walk direction' can be limited in order to suppress spurious (random) accelerations. Rotation rate ω and geometrical distance from the center of rotation (R) yield 'g-contours', through $g = \omega^2 R / g_0$ ($g_0 = 9.81 \text{ m/s}^2$), that provide guidelines for the design and lay-out of experiment packages and for the interpretation of the experimental results.

On the RPM, the samples were mounted close to the center of the platform. The RPM was placed in an incubator under standard cell culture conditions with 37°C and 5% CO₂. To start a RPM experiment, the culture flasks containing subconfluent CF monolayers were completely filled with medium, taking care to avoid air bubbles. This feeding was done just before start of the experiment. Rotation is 60°/min. Air exchange is assured through a waterproofed but air permeable membrane in the cap of the T25 cm² culture flasks

(Sarstedt, Nümbrecht, Germany). Investigation-time on the RPM was 24 hours. Fixation of the cells was performed directly after stopping the RPM. The method was published recently [26, 31, 32].

Cell isolation and culture procedure

Left ventricular myocardium from adult female German Landrace pigs (body weight range of 60-80 kg) was prepared and mechanically dissociated by ripping up the tissue with needles and canulating the resulting very small shreds until single cells could be filtered through gauze. Cells were treated with 2 ml of 0.1% collagenase type IV (Roche, Mannheim, Germany) for 30 min at 37°C in 5% CO₂ to obtain single cell suspensions. After incubation, the enzymatic action was stopped by adding RPMI 1640 medium containing 10% fetal calf serum (FCS; Invitrogen, Eggenstein, Germany). Cells were spun down (Greiner, Frickenhausen, Germany) for 10 min at 2000 rpm. The supernatant was discarded and the pellet was resuspended in RPMI 1640 medium supplemented with 10% FCS, 100 µM sodium pyruvate and 2 µM glutamine (Sigma, Deisenhofen, Germany), 100 IU/ml penicillin and 100 µg/ml streptomycin (Biochrom, Berlin, Germany), plated in culture flasks and incubated under standard cell culture conditions. The CF of passages 1 were seeded out in BD Falcon™ culture slides (BD Heidelberg, Germany) and characterized.

For the stimulation tests 10⁶ CF of passages 3-6 were seeded out in T25 cm² cell culture flasks from Sarstedt. After reaching subconfluence, the culture medium was removed and the cell culture flasks were randomized to the different groups: 1g vehicle, 1g VEGF, 1g bFGF, RPM vehicle, RPM VEGF and RPM bFGF (n=6 cultures, each group). The flasks were completely filled with the corresponding stimulation medium just before start of the experiment (time of the experiment: 24h). The following growth factors were used: VEGF-165 (10 ng/mL; Chemicon, Hofheim, Germany), bFGF (10 ng/mL; Chemicon, Hofheim, Germany).

Identification of cells and immunohistochemical analysis

Morphologic characteristic features of the CF *in vitro* were examined by phase contrast light microscopy (Olympus, Hamburg, Germany). The nature of these cells was determined by immunofluorescence staining with anti-vimentin, anti-desmin, anti-fibroblast surface protein marker and anti-factor VIII antibodies (Sigma, Deisenhofen, Germany) for the detection of fibroblasts, muscle cells and endothelial cells, respectively [40-42]. Subsequently, the monolayers were washed twice in phosphate-buffered saline (PBS), fixed with methanol and ethanol (1:2, room temperature, 30 min) or acetone (-20°C, 15 min). Then they were incubated for 24 h with antibodies at optimal concentrations. They were then rinsed twice in PBS and incubated for 24 h with an anti-mouse/rabbit FITC-conjugated immunoglobulin antibody (DAKO, Hamburg, Germany). After washing in PBS, the cells were incubated with Propidium iodide (Molecular Probes, Eugene, OR, USA) for 1 h at 37 °C. Then, they were rinsed again in PBS, mounted with Vectarshield immunofluorescence mounting medium (Vector, Burlingame, CA, USA) covered with glass slides and sealed with nail polish.

Viability staining

10⁶ CF were seeded out in a special flask on slides (NUNC, Thermo Scientific, Roskilde Denmark). After 24 h, the flasks were completely filled without air bubbles (n=6 each group) and randomized for RPM exposure or 1g condition (RPM experiment and 1g: 24h). After the experiment, they were examined by phase-contrast microscopy. After culture, control and microgravity-exposed cells remained unfixed, were washed with PBS and were stained with acridine orange/ethidium bromide at room temperature (Invitrogen/ Molecular Probes, Darmstadt, Germany) according to the method of Zhou et al. [43]. Immediately, after 30 seconds, the stained slides were investigated by fluorescence microscopy.

Cells for 4',6-diamidino-2-phenylindole (DAPI) staining (n=6 each group) were fixed with 3.7 % formaldehyde (room temperature, 10 min) and incubated in 1 µg/mL DAPI in PBS for 15 min (Invitrogen/Molecular Probes, Darmstadt, Germany). Stained nuclei were viewed with a fluorescence microscope.

Flow cytometric analysis of cellular antigens

In each test, monoclonal antibodies were added to 10⁵ single cells that had been prepared and fixed in ethanol (70%) as previously described [44]. The cells were incubated with unconjugated antibodies (chondroitin sulfate, fibronectin, collagen type I (all Sigma), collagen type III (Monosan), caspase-3 (Santa Cruz), osteopontin (MPIIB101, University of Iowa, Hybridoma Bank), and laminin (Millipore, Billerica, MA, USA) for 60 min at room temperature in darkness and washed three times with Phosphate Buffered Saline (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 FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany) equipped with an argon laser. Cells exerting fluorescence intensities above the upper limit of the negative control distribution were considered positive.

Evaluation of apoptosis by TUNEL flow cytometry

To evaluate the rate of apoptosis terminal deoxynucleotidyltransferase-dUTP nick end labeling (TUNEL) test was performed by flow cytometry using the MEBSTAIN Apoptosis Kit Direct (Medical and Biological Laboratories, Immunosource, Halle-Zoersel, Belgium). TUNEL staining is based on the specific labeling of terminal deoxynucleotidyltransferase (TdT) to 3'-OH ends of fragmented DNA generated by apoptosis. Adherent CF cultures (each group: n=6; each culture contains 5 x 10⁶ cells) were washed twice with calcium/magnesium-free PBS, trypsinized with 0.05% Trypsin-EDTA (Invitrogen) for 3-5 min, then the reaction was stopped with calcium/magnesium containing PBS, the cells were spun down with a centrifuge (1000 rpm, 10 min, 4°C) and finally according to the manufacturer fixed with 4% paraformaldehyde at 4 °C for 10 min and then stored in 80% ethanol at 4°C overnight. Then cells were washed twice with PBS containing 0.2% Bovine Serum Albumin (BSA). For permeabilization cells were incubated for 30 min at -20 °C with 70% ethanol and were washed twice as described before. Terminal deoxynucleotidyl transferase (TdT) reaction reagent was added to the cell pellet and incubated for 1 h at 37°C.

Fig. 1. Phase contrast microscopy, acridine orange/ethidium bromide or DAPI stained cardiac fibroblasts cultured under 1g conditions, comparison of growth factor effects: A, B, E, F, I, J: Phase contrast microscopy of isolated CF grown under conditions of 1g. No morphological differences were found between the different groups. C, G, K: Acridine orange/ethidium bromide staining revealed viable healthy cells (green fluorescence) and no signs of necrosis (red fluorescence). Both, bFGF (G) or VEGF (K) showed no difference compared with vehicle treatment. D, H, L: A few apoptotic cells were visible (yellow arrows) in cultures grown under 1g conditions. A reduced amount of apoptotic cells was found in CF treated with bFGF (H) or VEGF (L). Magnification x200.

Fig. 2. Phase contrast microscopy, acridine orange/ethidium bromide or DAPI stained cardiac fibroblasts cultured on a RPM, comparison of growth factor effects: A, B, E, F, I, J: Phase contrast microscopy of isolated CF. After 24h of culturing on the RPM three-dimensional aggregates were detectable (red arrows). CF grew in form of 3D spheroids, irrespective of growth factor treatment (bFGF E, F; VEGF I, J). C, G, K: Acridine orange/ethidium bromide staining revealed no necrotic cells in all three groups. All fibroblasts exerted a green fluorescence. D, H, L: DAPI staining showed that culture in microgravity clearly elevated the amount of CF with chromatin condensation (yellow arrows). Magnification x200.

Using PBS containing BSA the cells were washed twice. After resuspension in 700 μ l of the buffer (BD FACSTFlow™ Sheath Fluid, BD, Heidelberg, Germany) cells were analyzed by flow cytometry according to the routine protocol as previously described [31]. For each sample 10.000 cells were recorded. All experiments were conducted in triplicates.

Negative controls (cells incubated in the absence of TdT) and positive controls (cells treated with DNase I (100 μ g/ml) for 1 h at 37°C) were used for voltage and gain adjustments and assignment of histogram regions.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). All data are expressed as means

$\pm$ standard deviation (SD). Statistical analysis was performed using one-way ANOVA with post-hoc Bonferroni corrections. Differences were considered significant at the level of $p < 0.05$.

Results

Characterization of cardiac fibroblasts (CF) cultured under 1g and altered gravity conditions and the effects of bFGF and VEGF on cell growth

Characterized cell cultures were positive for vimentin (100%), a member of the intermediate

filament family of proteins that is especially found in connective tissue. Vimentin is the most important fibroblast marker. In addition, the fibroblast surface protein is also a reliable marker for fibroblast cell cultures. The CF were anti-fibroblast surface protein-positive (100%). All CF were negative for desmin, a marker for skeletal muscle tissue, smooth muscle tissue, and cardiac muscle tissue. To exclude a contamination with endothelial cells, we used Factor VIII related antigen, which was negative in all cultures.

To characterize the morphology, the cells were investigated by phase contrast light microscopy of subconfluent cultures. They exerted the morphological features characteristic of fibroblastic cells, including an irregularly branched cytoplasm and a large ovoid nucleus (Fig. 1A, B). In the initial phases of cell culturing, the cells were spindle-shaped and after reaching confluence they developed a stellar shape.

There was no difference in the growth behavior of the cell groups under 1g (Fig. 1A, B, E, F, I, J). Growth factor treated and untreated cells remained healthy and showed no necrosis (Fig. 1C, G, K) as detectable by acridine orange/ethidium bromide staining (green fluorescence). DAPI staining revealed chromatin condensation in some CF. Apoptosis was reduced in cells treated with VEGF and bFGF in comparison to the untreated group (Fig. 1D, H, L).

When cultured on a RPM, CF grew in form of multicellular spheroids (MCS) and adherently. This effect was independent of growth factor treatment (Fig. 2A, B, E, F, I, J). All three groups were negative for necrosis, as shown by acridine orange/ethidium bromide staining (Fig. 2C, G, K). Chromatin condensation, however, was increased in all three groups cultured under altered gravity conditions compared to similarly treated 1g-cells (Fig. 2D, H, L).

Increase of apoptosis under exposure to a RPM and effects of growth factors

Caspase-3, a signaling marker of apoptosis, is usually expressed in low amounts in healthy cells. Under 1g, about 21 % of CF were found to be expressing caspase-3, and both bFGF as well as VEGF reduced these rates (bFGF -62,3%; VEGF -85,7%). Culturing cells on a RPM clearly induced the amount of caspase-3-positive CF, while growth factor treatment suppressed or even reversed this effect (Fig. 3A). VEGF in particular showed anti-apoptotic properties.

Similar data were obtained by TUNEL flow cytometry which revealed an increase in apoptosis of

Fig. 3. Analysis of growth factor effects on apoptosis in CF cultured under 1g and altered gravitational conditions. Caspase-3 flow cytometry (A) revealed a significant increase in caspase-3 positive cells in CF cultured under altered gravity conditions. bFGF and VEGF significantly attenuated the rate of apoptotic cells. TUNEL flow cytometry (B) revealed a similar amount of apoptotic cells at 1g. Apoptosis was increased in cells cultured on the RPM, but the absolute difference between the groups was moderate. bFGF and VEGF reduced the percentage of apoptotic cells in CF exposed to the RPM. Values are given as mean $\pm$ SD. * $P < 0.05$ vs. μ g vehicle (veh); # $P < 0.05$ vs. 1g veh; + $P < 0.05$ vs. 1g bFGF, § $P < 0.05$ vs. 1g VEGF.

adherent CF cultured on a RPM. bFGF significantly reduced the amount of apoptotic cells under altered gravity conditions and under 1g. Moreover, VEGF decreased the number of apoptotic cells in samples grown on a RPM to a lower level (13%) compared with those cultured under 1g (18%) (Fig. 3B).

Fig. 4. Collagen type I (A) and collagen type III (B) flow cytometry revealed a clear increase in these collagen subtypes when CF are cultured on a RPM. Both growth factors reduced these structural ECMP. Values are given as mean $\pm$ SD. * $P < 0.05$ vs. μ g vehicle (veh); # $P < 0.05$ vs. 1g veh; + $P < 0.05$ vs. 1g bFGF, § $P < 0.05$ vs. 1g VEGF.

Altered synthesis of extracellular matrix proteins

Collagen type I: bFGF and VEGF both reduced the number of collagen-type I-positive CF at 1g. Exposure to a RPM elevated the amount of collagen type I-positive cells, whereas growth factor treatment only slightly elevated the percentage of collagen type I-positive cells compared with corresponding 1g controls. Both, bFGF and VEGF reduced the amount of collagen type I-positive cells (Fig. 4A).

Fig. 5. The cell adhesion molecules osteopontin (A) and laminin (B) were both elevated in CF cultured on a RPM. bFGF and VEGF slightly reduced the amount of these cell adhesion molecules of CF cultured on the RPM. Values are given as mean $\pm$ SD. * $P < 0.05$ vs. μ g vehicle (veh); # $P < 0.05$ vs. 1g veh; + $P < 0.05$ vs. 1g bFGF, § $P < 0.05$ vs. 1g VEGF.

Collagen type III

More than 30% of CF expressed collagen type III. Growth factor treatment significantly reduced the number of collagen III expressing cells, which was also the case under altered gravity conditions. These conditions alone slightly increased the amount of collagen type III-positive cells (Fig. 4B).

Fig. 6. Fibronectin (A) was clearly induced by VEGF under 1g conditions, whereas bFGF reduced it. Under conditions of altered gravity, both growth factors bFGF and VEGF decreased the amount of fibronectin. The proteoglycan chondroitin sulfate (B) was induced by exposure to a RPM and reduced by both growth factors. Values are given as mean $\pm$ SD. * $P < 0.05$ vs. μ g vehicle (veh); # $P < 0.05$ vs. 1g veh; + $P < 0.05$ vs. 1g bFGF, § $P < 0.05$ vs. 1g VEGF.

Osteopontin

Using flow cytometry, we measured a percentage of 90% osteopontin-positive CF. bFGF application reduced the amount of this cell adhesion molecule in fibroblasts under 1g conditions, whereas VEGF had no effect at 1g (Fig. 5A). In RPM-cultured samples, osteopontin was significantly elevated, whereas both growth factors significantly reduced the number

of osteopontin-positive cells (bFGF: -7.6%; VEGF: -4.8%).

Laminin

Laminin was significantly reduced by VEGF in CF cultured under normal gravity conditions. RPM exposure increased the amount of laminin-positive cells. Both growth factors reduced the percentage of laminin-positive CF in altered gravity conditions (Fig. 5B).

Fibronectin

In parallel, we also observed 80% fibronectin-positive CF. This amount was further elevated by VEGF. In contrast, bFGF slightly reduced the amount of fibronectin-positivity at 1g. Culture on a RPM resulted in an increase in fibronectin which was slightly decreased by both growth factors (Fig. 6A).

Chondroitin sulfate

Chondroitin sulfate was highly expressed in CF isolated from the left ventricle. bFGF and VEGF reduced the amount of this proteoglycan under both gravity conditions (Fig. 6B). Culture on a RPM only moderately elevated the synthesis of chondroitin sulfate of fibroblasts.

Discussion

Myocardium is well-regulated in response to changes in loading conditions. An increase in cardiac muscle mass is the basic adaptive response to increased volume or pressure loading, which can be seen as specific molecular changes in different proteins. The cardiac fibroblast's reaction to chronic pressure-overload, hypoxia or other diseases of the heart is the increased production of extracellular matrix proteins [7, 8, 13-17, 22, 40-42]. In the absence of a manifest cardiovascular disease, one model of chronic circulatory unloading in humans is sustained exposure to microgravity, either directly (i.e., spaceflight) or as simulated by bed rest. Spaceflight results in decreased relative (to the supine position) volume loading of the heart and may leads to cardiac atrophy [1, 2, 4, 6].

Cardiac fibroblasts situated in the cardiac interstitium produce extracellular matrix proteins. This cell type is very sensitive to external changes such as hypoxia induced by ischemia/reperfusion. In addition, it was shown that fibroblasts also react on gravitational

changes [45]. Therefore, we decided to investigate isolated cardiac fibroblasts on a Desktop RPM to clarify the impact of altered gravity conditions on growth, apoptosis and on the synthesis behaviour of extracellular matrix proteins.

The Desktop RPM is a small size 'Random Positioning Machine' that can be used as 'microgravity simulator' in the bioscience laboratory by randomly rotating the accommodated experiment package around the Earth's gravity vector. Other artificial microgravity models are the 2D or 3D clinostat as well as the Rotating Wall Vessel which give us the possibility to simulate microgravity on Earth for short- and long-term studies to prepare a space project and to increase our knowledge in Life Sciences under microgravity conditions. In addition, these devices can be used for tissue engineering of intima constructs and of cartilage [26, 31, 32].

In this study, we demonstrate that CF derived from normal porcine hearts revealed a significant increase in the synthesis of collagen I and III, laminin, fibronectin, osteopontin, and chondroitin sulfate when cultured on a RPM. Compared with previous results [41], we detected that the increased amount of collagen after culturing the cells up to passage 6 remained stable. This finding indicates that CF play a crucial role in regulating ECM remodeling. There is an extensive interaction between CF and cardiomyocytes allowing both cell types to influence their morphology and function. It is known that CF are responsible for the synthesis and accumulation of ECM components in the cardiac interstitium [46] and that the amount of ECM deposited *in vivo* usually changes dramatically post-MI. Until now, the mechanisms inducing the ECM production of CF *in vitro* are unknown. Bradykinin significantly reduced the production of fibronectin and collagen types I and III in normal adult rat CF *in vitro* [47]. Non-selective beta-receptor blockade with carvedilol and interferon-gamma also attenuated the synthesis of ECMP in normal rat CF and fibroblasts deriving from hypertrophic myocardium [40].

This is the first study demonstrating that CF when exposed to a RPM increased their ECM synthesis. The cells try to reorganize themselves in a new environment. They start growing in form of three-dimensional spheroids, irrespective of growth factor treatment. Little is known about fibroblasts cultured under microgravity and expression of extracellular matrix proteins. Basile et al. [48] observed an increase in fibronectin production in human fetal fibroblasts

cultured under hypergravity conditions, whereas collagen type I was only slightly elevated. This increase was not significant compared with control samples. Loesberg et al. investigated rat dermal fibroblasts with combined simulated microgravity (RPM) and microgrooved surface topography. They detected a decrease in fibronectin gene expression under microgravity [49]. Ingram et al. [50] used the high aspect ratio vessel bioreactor (HARV) developed by NASA and examined normal prostate fibroblasts (NPF 209) for periods of a few days to approximately a month. Collagen IV was expressed by fibroblast cell lines cultured either in monolayer or in the bioreactor. By the fourth day in culture, normal prostate fibroblasts produced compact spheroids approximately 0.5 mm in diameter that stained intensely throughout for collagen IV. These fibroblast spheroids were used for coculture experiments with epithelial carcinoma cells. Another study examined collagen synthesis under microgravity, normal gravity, and hypergravity conditions by incubating human fibroblast cultures with [3H]-proline for 4, 7, 10, and 20 h during the Spacelab D2-mission in 1993. Quantitative analysis revealed an increase of collagen synthesis under microgravity conditions, being up to 143% higher than in 1 g controls. In contrast, hypergravity samples showed a decrease in collagen synthesis with increasing g, being at the 13% level at 10 g [51].

The influence of the investigated growth factors was different. Although, bFGF seems to down-regulate most of the ECMP with the exception of laminin which remained unaltered in cardiac fibroblasts under normal gravity conditions. In the 3D system, bFGF and VEGF receptors might be differently accessible under 1g and on the RPM. This is a subject of future studies on this experimental system. VEGF sometimes had the opposite effect, and the production of fibronectin and osteopontin was even enhanced under 1g. VEGF reduced the amount of collagen subtypes under 1g. RPM-culture significantly induced the amount of extracellular matrix proteins. VEGF application to cells grown under these conditions reduced this increase in ECMP.

Padua et al. showed that bFGF might have a direct protective effect against I/R injury on adult cardiomyocytes [27]. But the question how bFGF interacts with the interstitial cells remains. One reason might be that bFGF induces the steady-state level of collagenase mRNA up to threefold [52]. When collagenase activity is upregulated then the collagen protein must be found in lower amounts. Further

investigations need to be performed to investigate MMPs and TIMPs as well as the influence of bFGF and VEGF on their action.

In our investigation, we detected a strong downregulation of collagen type III by both growth factors at 1g and on a RPM. Other authors reported about the influence of VEGF on collagen as a therapeutic treatment. Zhang et al. [53] discussed the difficulty of applying VEGF without implicating side effects. These authors produced a collagen-targeting VEGF that induced an improved cardiac function in rats with experimental acute myocardial infarction [53]. Furthermore, a decay of the VEGF concentration during the experiment has to be considered so that an initial effect may be different from a long-term effect [26, 54].

A very interesting result of this study was the finding that cardiac fibroblasts start growing as 3D aggregates (multicellular spheroids) after 24 hours. 3D growth of normal cells such as endothelial cells, chondrocytes and others as well as tumor cells *in vitro* is an established cell culture model with a variety of advantages in many research applications. 3D cultures of tumor cells are often referred to as multicellular spheroids; they resemble small microtumors *in vivo* rather than cells grown in 2D monolayers [55]. The model of multicellular spheroids has been developed to represent an experimental model being intermediate in its complexity between monolayer cultures and normal tissue or tumors growing *in vivo*. 3D cell culture systems better reflect the *in vivo* behavior of most cell types. The 3D intima constructs of EAhy926 endothelial cells or the cartilage tissue-engineered after several days of culture on the RPM were a good example [32, 56]. These aggregates deliver new information about the expression of genes and proteins, signal transduction and metabolism of human cells growing *in vitro* three-dimensionally. They can be used for pharmacological studies and may help to reduce animal studies.

Normally, 1g control cells grew in a 2D pattern. Under altered gravity conditions, cells keep floating without stirring so that initial cell-cell interactions required for spheroid formation will be induced by forces exclusively due to biochemical components actually expressed on surfaces of cells, whereas gravity-related push or shear events will not influence spheroid formation. Ingram et al. had demonstrated 3D growth in breast, bladder and prostate cancer cells, glioma cells but also in fibroblasts [50]. They used a HARV (high aspect ratio vessel) NASA rotary cell culture system for testing

the growth behavior of various human tumor cell lines in simulated microgravity [50].

TUNEL flow cytometry revealed a slight increase in apoptosis in cells cultured on a RPM. 17% of the CF were apoptotic under 1g conditions which is comparable to the basal apoptosis rate of chondrocytes [32] and endothelial cells [56]. A moderate increase in programmed cell death after RPM exposure was also detected in other cell types grown under similar conditions such as endothelial cells [26], thyroid cancer cells [55] or lymphocytes [57]. Both growth factors showed a cell-protective effect under altered gravity conditions which was also detected in endothelial cells [31]. The effects of VEGF were different in RPM cultures compared with 1g cultures. A reason for this finding may be the 3D growth behavior of CF after RPM exposure. In future studies we will focus on flk1 and flt1 receptor regulation of 3D spheroids.

In parallel, we detected an increase in caspase-3 which was attenuated by both growth factors under altered gravity conditions. VEGF nearly suppressed the amount of caspase-3 in RPM exposed samples. The reason for this cell-protective effect on CF remains unclear. Acridine orange/ethidium bromide staining revealed no signs of necrosis (red stained cells) in 1g cultures and RPM exposed samples, but 4', 6-diamidino-2-phenylindole (DAPI) staining showed nuclei with chromatin condensation indicating a significant increase in programmed cell death under RPM-culture. The amount of nuclei with chromatin condensation was reduced by both growth factors.

Taken together the results of this study, we investigated cardiac fibroblasts for the first time under altered gravity conditions. Cardiac fibroblasts adapted early to this changed environment and started to form 3D spheroids. A moderate increase in programmed cell death was detectable in these cells and was blunted by growth factor application. All fibroblasts showed an increased positivity for extracellular matrix proteins. Both growth factors reduced the amount of extracellular matrix proteins under exposure to a RPM indicating their influence in extracellular matrix remodeling under different gravity conditions. The development of cardiac atrophy or cardiac function and morphology should be investigated in future spaceflight *in vivo* experiments on mice. These animals can live in a special mouse habitat for 14 days or longer during an orbital flight in an unmanned recoverable capsule of the Foton type, a microgravity platform for physics and life science studies, so that after these long-term flights their

hearts can deliver us more information about cardiac function in space.

Abbreviations

β (Beta); bFGF (Basic fibroblast growth factor); BSA (Bovine serum albumin); CF (Cardiac fibroblasts); 2D (Two-dimensional); 3D (Three-dimensional); DAPI (4',6-Diamidino-2-phenylindole); ECMP (Extracellular matrix proteins); ESA (European Space Agency); FCS (Fetal calf serum); FITC (Fluorescein isothiocyanate); Fig (Figure); h (hour); IgG (Immunoglobulin G); I/R (Ischemia/reperfusion); μg (Microgravity); mL (Milliliter). PBS (Phosphate buffered

saline); RPM (Random Positioning Machine); s (Second); s-μg (Simulated microgravity); TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP digoxigenin nick end labeling); VEGF (Vascular endothelial growth factor); VEH (Vehicle).

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References

- White RJ, Averner M: Humans in space. *Nature* 2001;22;409:1115-1118.
- Convertino VA: Status of cardiovascular issues related to space flight: Implications for future research directions. *Respir Physiol Neurobiol* 2009;169:34-37.
- Pietrzyk RA, Jones JA, Sams CF, Whitson PA: Renal stone formation among astronauts. *Aviat Space Environ Med* 2007;78:9-13.
- McCarthy ID: Fluid shifts due to microgravity and their effects on bone: a review of current knowledge. *Ann Biomed Eng* 2005;33:95-103.
- Sekiguchi C: Issues of health care under weightlessness. *Acta Physiol Scand Suppl* 1994;616:89-97.
- Rossum AC, Ziegler MG, Meck JV: Effect of spaceflight on cardiovascular responses to upright posture in a 77-year-old astronaut. *Am J Cardiol* 2001;88:1335-1337.
- Weber KT, Brilla CG: Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation* 1991;83:1849-1865.
- Weber KT, Brilla CG, Janicki JS: Myocardial fibrosis: functional significance and regulatory factors. *Cardiovasc Res* 1993;27:341-348.
- Baudino TA, Carver W, Giles W, Borg TK: Cardiac fibroblasts: friend or foe? *Am J Physiol Heart Circ Physiol* 2006;291:1015-1026.
- Corda S, Samuel JL, Rappaport L: Extracellular matrix and growth factors during heart growth. *Heart Fail Rev* 2000;5:119-130.
- Holmes JW, Borg TK, Covell JW: Structure and mechanics of healing myocardial infarcts. *Annu Rev Biomed Eng* 2005;7:223-253.
- Lindsey ML, Mann DL, Entmann ML, Spinale FG: Extracellular matrix remodeling following myocardial injury. *Ann Med* 2003;35:316-326.
- Weber KT, Jalil JE, Janicki JS, Pick R: Myocardial collagen remodeling in pressure overload hypertrophy. A case for interstitial heart disease. *Am J Hypertens* 1989;2:931-940.
- Chapman D, Weber KT, Eghbali M: Regulation of fibrillar collagen types I and III and basement membrane type IV collagen gene expression in pressure overloaded rat myocardium. *Circ Res* 1990;67:787-794.
- Rothermund L, Kreutz R, Kossmehl P, Fredersdorf S, Shakibaei M, Schulze-Tanzil G, Paul M, Grimm D: Early onset of chondroitin sulfate and osteopontin expression in Angiotensin II-dependent left ventricular hypertrophy. *Am J Hypertens* 2002;15:644-652.
- Riecke K, Grimm D, Shakibaei M, Kossmehl P, Schulze-Tanzil G, Paul M, Stahlmann R: Low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin increase transforming growth factor β and cause myocardial fibrosis in marmosets (*Callithrix jacchus*). *Arch Toxicol* 2002;76:360-366.
- Grimm D, Cameron D, Griesse DP, Riegger GA, Kromer EP: Differential effects of growth hormone on cardiomyocyte and extracellular matrix protein remodeling following experimental myocardial infarction. *Cardiovasc Res* 1998;40:297-306.
- Bates DO, Harper SJ: Regulation of vascular permeability by vascular endothelial growth factors. *Vasc Pharmacol* 2003;39:225-237.
- Ferrara N: VEGF: An update on biological and therapeutic aspects. *Curr Opin Biotechnol* 2000;11:617-624.
- Ferrara N, Gerber HP, LeCouter J: The biology of VEGF and its receptors. *Nat Med* 2003;9:669-976.
- Ferrara N: Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 2001;280:1358-1366.

- 22 Infanger M, Faramarzi S, Grosse J, Kurth E, Ulbrich C, Bauer J, Wehland M, Kreutz R, Kossmehl P, Paul M, Grimm D: Expression of Vascular endothelial growth factor and receptor tyrosine kinases in cardiac ischemia-reperfusion injury. *Cardiovasc Pathol* 2007;16:291-299.
- 23 Reilly JP, Grise MA, Fortuin FD, Vale PR, Schaer GL, Lopez J, VAN Camp JR, Henry T, Richenbacher WE, Losordo DW, Schatz RA, Isner JM: Long-term (2-year) clinical events following transthoracic intramyocardial gene transfer of VEGF-2 in no-option patients. *J Interv Cardiol* 2005;18:27-31.
- 24 Losordo DW, Vale PR, Hendel RC, Milliken CE, Fortuin FD, Cummings N, Schatz RA, Asahara T, Isner JM, Kuntz RE: Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. *Circulation* 2002;105:2012-2018.
- 25 Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, Giordano FJ, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson CM, Bajamonde A, Rundle AC, Fine J, McCluskey ER: VIVA Investigators: The VIVA trial: Vascular endothelial growth factor in ischemia for vascular angiogenesis. *Circulation* 2003;107:1359-1365.
- 26 Infanger M, Kossmehl P, Shakibaei M, Baatout S, Witzing A, Grosse J, Bauer J, Cogoli A, Faramarzi S, Derradji H, Neefs M, Paul M, Grimm D: Induction of three-dimensional assembly and increase in apoptosis of human endothelial cells by simulated microgravity: impact of vascular endothelial growth factor. *Apoptosis* 2006;11:749-764.
- 27 Padua RR, Sethi R, Dhalla NS, Kardami E: Basic fibroblast growth factor is cardioprotective in ischemia-reperfusion injury. *Mol Cell Biochem* 1995;143:129-135.
- 28 Yanagisawa-Miwa A, Uchida Y, Nakamura F, Tomaru T, Kido H, Kamudo T, Sugimoto T, Kaji-Utsuyama M, Kurashima C: Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. *Science* 1992;257:1401-1403.
- 29 Battler A, Scheinowitz M, Bor A, Hasdai D, Vered Z, Di Segni E, Nass D, Engelberg S, Eldar M, Savion N: Intra-coronary injection of basic fibroblast growth factor enhances angiogenesis in infarcted swine myocardium. *J Am Coll Cardiol* 1993;22:2001-2006.
- 30 Uchida Y, Yanagisawa-Miwa A, Nakamura F, Yamada K, Tomaru T, Kimura K, Morita T: Angiogenic therapy of acute myocardial infarction by intrapericardial injection of basic fibroblast growth factor and heparin sulphate: An experimental study. *Am Heart J* 1995;130:1182-1188.
- 31 Ulbrich C, Westphal K, Baatout S, Wehland M, Bauer J, Flick B, Infanger M, Kreutz R, Vadrucchi S, Egli M, Cogoli A, Derradji H, Pietsch J, Paul M, Grimm D: Effects of basic fibroblast growth factor on endothelial cells under conditions of simulated microgravity. *J Cell Biochem* 2008;104:1324-1341.
- 32 Ulbrich C, Westphal K, Pietsch J, Winkler HD, Leder A, Bauer J, Kossmehl P, Grosse J, Schoenberger J, Infanger M, Egli M, Grimm D: Characterization of human chondrocytes exposed to simulated microgravity. *Cell Physiol Biochem* 2010;25:551-560.
- 33 Versari S, Villa A, Helder MN, Doulabi BZ, van Loon J, Bradamante S: Effects of gravity on proliferation and differentiation of adipose tissue-derived stem cells. *J Gravit Physiol* 2007;14:127-128.
- 34 Meloni MA, Galleri G, Pippia P, Cogoli-Greuter M: Cytoskeleton changes and impaired motility of monocytes at modelled low gravity. *Protoplasma* 2006;229:243-249.
- 35 Ward NE, Pellis NR, Risin SA, Risin D: Gene expression alterations in activated human T-cells induced by modeled microgravity. *J Cell Biochem* 2006;99:1187-1202.
- 36 Infanger M, Kossmehl P, Shakibaei M, Schulze-Tanzil G, Cogoli A, Faramarzi S, Bauer J, Curcio F, Paul M, Grimm D: Longterm conditions of mimicked weightlessness influences the cytoskeleton in thyroid cells. *J Gravit Physiol* 2004;11:169-172.
- 37 Boonstra J: Growth factor-induced signal transduction in adherent mammalian cells is sensitive to gravity. *FASEB J* 1999;13 Suppl:S35-42.
- 38 Hoson T, Kamisaka S., Masuda Y, Yamashita M: Changes in plant growth processes under microgravity conditions simulated by a three-dimensional clinostat. *Bot Mag* 1992;105:53-70.
- 39 van Loon, J.J.W.A: Some history and use of the random positioning machine, RPM, in gravity relayed research. *Adv Space Res* 2007;39:1161-1165.
- 40 Grimm D, Huber M, Jabusch HC, Shakibaei M, Fredersdorf S, Paul M, Riegger GA, Kromer EP: Extracellular matrix proteins in cardiac fibroblasts derived from rat hearts with chronic pressure-overload: effects of beta-receptor blockade. *J Mol Cell Cardiol* 2001;33:487-501.
- 41 Kossmehl P, Schönberger J, Shakibaei M, Faramarzi S, Kurth E, Habighorst B, von Bauer R, Wehland M, Kreutz R, Infanger M, Schulze-Tanzil G, Paul M, Grimm D: Increase of fibronectin and osteopontin in porcine hearts following ischemia and reperfusion. *J Mol Med* 2005;83:626-637.
- 42 Eghbali M, Czaja MJ, Zeydel M, Weiner FR, Zern MA, Seifter S, Blumenfeld OO: Collagen chain mRNAs in isolated heart cells from young and adult rats. *J Mol Cell Cardiol* 1988;20:267-276.
- 43 Zhou P, Qian L, Kozopas KM, Craig RW: Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. *Blood* 1997;89:630-643.
- 44 Infanger M, Ulbrich C, Baatout S, Wehland M, Kreutz R, Bauer J, Grosse J, Vadrucchi S, Cogoli A, Derradji H, Neefs M, Küster S, Spain S, Paul M, Grimm D: Modeled gravitational unloading induced downregulation of endothelin-1 in human endothelial cells. *J Cell Biochem* 2007;101:1439-1455.
- 45 Loesberg WA, Walboomers XF, van Loon JJ, Jansen JA: Simulated microgravity activates MAPK pathways in fibroblasts cultured on microgrooved surface topography. *Cell Motil Cytoskeleton* 2008;65:116-129.
- 46 Neuss M, Regitz-Zagrosek V, Hildebrandt A, Fleck E: Isolation and characterization of human cardiac fibroblasts from explanted adult hearts. *Cell Tissue Res* 1996;286:145-153.
- 47 Kim NN, Villegas S, Summerour SR, Villarreal FJ: Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide. *J Mol Cell Cardiol* 1999;31:457-466.
- 48 Basile V, Romano G, Fusi F, Monici M: Comparison between the effects of hypergravity and photomechanical stress on cells producing ECM. *Microgravity Sci Technol* 2009;21:151-157.
- 49 Loesberg WA, Walboomers XF, Bronkhorst EM, van Loon JJ, Jansen JA: The effect of combined simulated microgravity and microgrooved surface topography on fibroblasts. *Cell Motil Cytoskeleton* 2007;64:174-185.
- 50 Ingram M, Techy GB, Saroufeem R, Yazan O, Narayan KS, Goodwin TJ, Spaulding GF: Three-dimensional growth patterns of various human tumor cell lines in simulated microgravity of a NASA bioreactor. *In Vitro Cell Dev Biol Anim* 1997;33:459-466.
- 51 Seitzer U, Bodo M, Müller PK, Açil Y, Bätge B: Microgravity and hypergravity effects on collagen biosynthesis of human dermal fibroblasts. *Cell Tissue Res* 1995;282:513-517.

- 52 Chua CC, Chua BHL, Zhao ZY, Krebs C, Diglio C, Perrin E: Effect of growth factors on collagen metabolism in cultured human heart fibroblasts. *Connect Tissue Res* 1991;26:271-281.
- 53 Zhang J, Ding L, Zhao Y, Sun W, Chen B, Lin H, Wang X, Zhang L, Xu B, Dai J: Collagen-targeting vascular endothelial growth factor improves cardiac performance after myocardial infarction. *Circulation* 2009;119:1776-1784.
- 54 Infanger M, Schmidt O, Kossmehl P, Grad S, Ertel W, Grimm D: Vascular endothelial growth factor serum level is strongly enhanced after burn injury and correlated with local and general tissue edema. *Burns* 2004;30:305-311.
- 55 Grimm D, Bauer J, Kossmehl P, Shakibaei M, Schönberger J, Pickenhahn H, Schulze-Tanzil G, Vetter R, Eilles C, Paul M, Cogoli A: Simulated microgravity alters differentiation and increases apoptosis in human follicular thyroid carcinoma cells. *FASEB J* 2002;16:604-606.
- 56 Grimm D, Bauer J, Ulbrich C, Westphal K, Wehland M, Infanger M, Aleshcheva G, Pietsch J, Ghardi M, Beck M, El-Saghire H, de Saint-Georges L, Baatout S: Different responsiveness of endothelial cells to vascular endothelial growth factor and basic fibroblast growth factor added to culture media under gravity and simulated microgravity. *Tissue Eng Part A* 2010;16:1559-1573.
- 57 Maccarrone M, Battista N, Meloni M, Bari M, Galleri G, Pippia P, Cogoli A, Finazzi-Agrò A: Creating conditions similar to those that occur during exposure of cells to microgravity induces apoptosis in human lymphocytes by 5-lipoxygenase-mediated mitochondrial uncoupling and cytochrome c release. *J Leukoc Biol* 2003;73:472-481.