Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2010;25:551-560

Accepted: December 23, 2009

Characterization of Human Chondrocytes Exposed to Simulated Microgravity

Claudia Ulbrich[1], Kriss Westphal[1,4], Jessica Pietsch[1], Henrik D.F. Winkler[1], Annekatrin Leder[1], Johann Bauer[3], Peter Kossmehl[1], Jirka Grosse[5], Johann Schoenberger[5], Manfred Infanger[6], Marcel Egli[4] and Daniela Grimm[1,2]

[1]Institute of Clinical Pharmacology and Toxicology, Charité-Universitätsmedizin Berlin, Berlin, Germany, [2]Department of Pharmacology, University of Aarhus, Århus, Denmark, [3]Max-Planck Institute of Biochemistry, Martinsried, Germany, [4]Space Biology Group, ETH, Zurich, Switzerland, [5]Department of Nuclear Medicine, University of Regensburg, Regensburg, Germany, [6]Department of Plastic Surgery, Charité-Universitätsmedizin Berlin, Berlin, Germany

Key Words

Chondrocytes • Apoptosis • Cytoskeleton • Extracellular matrix • Growth factors • Microgravity

Abstract

Background: Tissue engineering is a strategy of cartilage regeneration, but scaffolds, required for 3D growth of chondrocytes, are still a problem. Methods: Searching for possibilities to improve scaffold-free engineering of cartilage, we characterized human chondrocytes incubated on a random positioning machine (RPM) to simulate microgravity (µg). Results: When cultured in simulated µg, human chondrocytes start forming 3D cell assemblies within 5 days. After 24h, we could not detect caspase-3, Fas, p53 or Bcl-2 proteins in these cells, Annexin V flow cytometry, however, revealed 18% of apoptotic chondrocytes in 1g cultures but only 10% on the RPM. Both rates of apoptosis were not changed, when vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) was added. 24 h, simulated microgravity also had significantly decreased collagen type I and X, but did not change collagen type IV and laminin, while collagen type II, chondroitin sulfate and aggrecan were elevated as compared with 1g controls. The production of collagen type II/X, chondroitin sulfate and aggrecan was modified, when external bFGF or VEGF had been applied. Conclusion: Chondrocytes exposed to simulated µg seem to change their extracellular matrix production behavior, while they rearrange their cytoskeletal proteins prior to forming 3D aggregates.

Introduction

In human organisms, chondrocytes are the only cell type that is found in cartilage. They produce and maintain the cartilaginous matrix, which consists mainly of different types of collagens and proteoglycans [1]. Loss of cartilage frequently occurs, especially in elderly people. It leads to diseases such as osteoarthritis, which are a serious problem in modern medicine [2]. Therefore, culturing of chondrocytes with the aim to engineer cartilage tissue either *in vivo* at the site of damage [3, 4], or *in vitro* for subsequent implantation [5] is an up to date field to explore [6, 7]. Most investigators use scaffolds to support three-dimensional growth of chondrocytes [8]. But scaffolds have the disadvantage that they cannot be separated from

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/10/0255-0551$26.00/0

Accessible online at:
www.karger.com/cpb

Associate Professor Daniela Grimm, MD
Department of Pharmacology, Aarhus University
Wilhelm Meyers Allé 4, DK-8000 Århus C (Denmark)
Tel. +45-89-421720, Fax: +45-86128804
E-Mail daniela.grimm@farm.au.dk

the cells anymore before the substitute cartilage should be functioning. Therefore, investigation of scaffold-free methods of culturing chondrocytes in a three-dimensional manner appears to be worthwhile.

Incubation of cells under real or simulated microgravity has great impact on their growth and physiology. Amongst many other effects, microgravity triggers many cells to grow within three-dimensional aggregates. Spheroids were formed, when follicular thyroid cancer ML-1 cells were cultured on a Random Positioning Machine (RPM) [9]. In contrast, adherent human endothelial EA.hy926 cells, which grow in commercially available culture flasks as monolayers, formed elongated or tube-like structures, when cultured on a RPM [10]. Furthermore, bladder cells assembled to organoids that displayed characteristics of *in vivo* tissue-specific differentiation and Sertoli cells together with neuron precursor cells formed tissue constructs showing intra-aggregate cellular organization with distinct intercellular junction-like densities between adjacent cells [11, 12]. Also aggregates resembling cartilage were formed, when chondrocytes were exposed to microgravity [13, 14].

Several studies on chondrocytes cultured either in space or on a machine simulating microgravity [15, 16] revealed that these cells are very resistant to adverse effects and stress induced by microgravity [13, 15, 17]. As exposure of cells to real weightlessness is time-consuming and expensive, we decided to apply the RPM for simulating microgravity. We used human hip joint chondrocytes to study and improve their three-dimensional cell aggregation *in vitro* and were especially interested in early cellular events which may initiate the formation of cartilage. To prove the functionality of chondrocytes in simulated microgravity first, we examined the rate and process of apoptosis changes, and the accumulation of extracellular matrix components after 24 hours of culturing. Then the cell-cell aggregation behavior and the rearrangement of cytoskeletal proteins were investigated in order to see the microgravity dependent causes of the three-dimensional growth of chondrocytes.

Materials and Methods

Random positioning machine (RPM)

Microgravity conditions were simulated by a Desktop RPM manufactured by Dutch Space, an EADS Astrium company, Leiden, NL [18, 19]. The RPM is a laboratory instrument enabling the position of an accommodated (biological) experiment in three-dimensional space to be randomly changed under the control of dedicated software running on a personal computer. To start a RPM experiment, the culture flasks containing subconfluent chondrocyte monolayers were completely filled with medium, taking care to avoid air bubbles. The filled culture flasks were fixed onto the RPM as close as possible to the center of the platform, which was rotated at a speed of 60°/s. The RPM was located in a commercially available incubator providing 37°C and 5% CO_2. 50% of the culture supernatant was replaced by fresh medium every day. Air exchange is assured because of a waterproofed but air permeable membrane in the cap of the flasks.

Cell culture procedure

The human chondrocytes derived from hip joint cartilage were purchased from Provitro (Berlin, Germany). They were grown in complete chondrocyte growth medium supplemented with 10% FCS (Provitro, Berlin, Germany), 100 IU penicillin/mL and 100 µg streptomycin/mL (Biochrom, Berlin, Germany). Subconfluent monolayers (10^6 cells/flask) were randomized to the following study groups: 60 static control cultures (1g ground controls are cultures kept in the same incubator as the RPM at 37°C; vehicle-treated (VEH), bFGF, VEGF; n = 20 each group), and 60 samples for the simulated microgravity experiments (VEH, bFGF, VEGF; n = 20 each group). The cells grew in 25 cm^2 culture flasks (Sarstedt, Nümbrecht, Germany). When indicated, we added 10 ng/mL of vascular endothelial growth factor (VEGF) and 10 ng/mL of basic fibroblast growth factor (bFGF) to the medium (both Chemicon, Hofheim, Germany). Ten additional cell culture flasks were used for an experiment duration time up to 18 days, whereas five flasks were cultured under 1g conditions and the other five under µg conditions using the RPM. For investigating changes in apoptosis and ECM we used the adherent cells.

Histochemistry

Chondrocytes were stained with hematoxylin and eosin for histological analysis as described by Grimm et al. [20]. Samples were analyzed with the help of a microscope (Zeiss, Germany) and the image analyses program Scion Image (Version 1.63 Mac Os, Scion Corporation, USA).

Immunofluorescence

For immunofluorescence staining [9, 21], cells were seeded out into one of a 4-chamber supercell chamber slide (BD, Heidelberg, Germany) until they attached to the slides (4h). Subsequently, the chondrocytes were washed twice in PBS and fixed with 4% paraformaldehyde (4°C). Then cells were washed twice in PBS again and incubated with the first antibody for 24h at room temperature (RT). The morphology of the microtubule cytoskeleton (alpha-tubulin; 1:100; Sigma, Taufkirchen, Germany) and intermediate filaments (vimentin, 1:50, Sigma, Taufkirchen, Germany) were evaluated by indirect immunofluorescence and analyzed with a Zeiss 510 META inverted confocal laser scanning microscope equipped with a Plan-Apochromat 63×1.4 objective. Excitation and emission wave lengths were: λ_{exc} = 488 nm and λ_{em} = ≥505 nm for FITC.

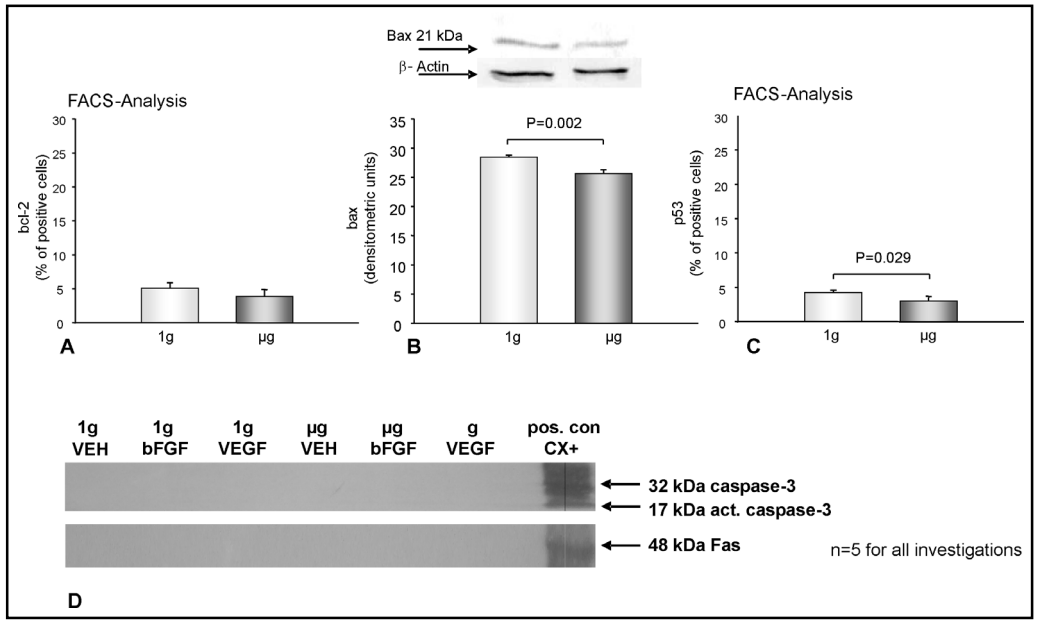


Fig. 1. FACS-analyses of Bcl-2 and p53 (A, C) and Western Blot analyses of bax (B) as well as of activated caspase-3 and Fas, which could not be detected in chondrocytes exposed to 1g or µg conditions but in colon carcinoma CX+ cells (D).

Western blot analysis

SDS-PAGE and immunoblotting were carried out following routine protocols [10, 22, 23]. Six replicates have been performed. Antibodies against the following antigens were used for this study: Fas, Bax, activated caspase-3, collagen type I, collagen type II, and laminin (dilution all 1:1000; all Chemicon, Hofheim, Germany), aggrecan (1:1000; Santa Cruz Biotechnology, Santa Cruz, USA), collagen type IV (DAKO, Hamburg, Germany), chondroitin sulfate, β-Actin (Sigma-Aldrich, Taufkirchen, Germany), collagen type X (Abcam, Cambridge, UK) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:4.000; ABR-Affinity BioReagents, Golden). We used protein from the colon carcinoma cell subline CX+ with high HSP70 as positive control for Fas and caspase-3 [24].

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 [25, 26]. The cells were incubated with unconjugated antibodies (Bcl-2 and p53; all Chemicon, Hofheim, Germany; collagen type I; Sigma-Aldrich, Taufkirchen, Germany), for 60 min at RT in the 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 [9]. Cells exerting fluorescence intensities above the upper limit of the negative control distribution were considered positive.

Annexin V-FITC/PI assay

The Annexin V-FITC/PI assay [27] was performed using the Annexin V-FITC apoptosis detection kit II (BD Pharmingen, San Diego, CA, USA) 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, 1×10^6 cells of a sample were investigated. 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 1×10^4 events were acquired for each experimental point via a Facscan flow cytometer (Becton Dickinson, Heidelberg, Germany) equipped with an argon laser and analyzed with WinList software (Topsham, ME, USA).

Statistics

Statistical analysis was performed using SPSS 12.0 (SPSS, Inc., Chicago, IL, USA). All data are expressed as means±standard deviation. We tested all parameters for deviations from the Gaussian distribution using the Kolmogorov-Smirnov test and compared the cases with the controls with the independent-sample *t* test or the Mann-Whitney test (depending on the results of the normality test). Differences were considered significant at the level of $p<0.05$.

Results

Apoptosis

In order to estimate the vitality of the cells of interest, we tried to compare the rate of apoptosis within the various cell cultures after incubation of 24 hours either under 1g control or µg conditions. To our surprise, we could neither detect caspase-3 nor Fas in any type of the human hip joint chondrocytes used. The failure to see caspase-3 or Fas was due to the absence of these proteins, as the proteins were clearly seen, when the colon carcinoma CX+ cells were analyzed (Fig. 1D). Also Bcl-2 and p53 were expressed at very low levels, as we found by flow cytometry that less than 5% of the chondrocytes of a

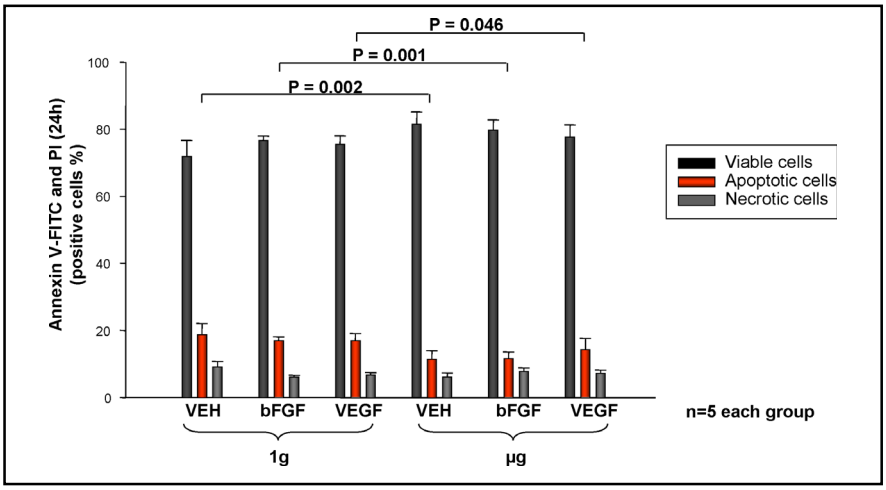


Fig. 2. Annexin V-FITC flow cytometry of µg cultures indicated viable (black bars), apoptotic (red bars) and necrotic cells (grey bars). Growth factor treatment exerted no effect at 1g, but VEGF increased Annexin V-positive cells (red bar) under simulated µg. VEH=vehicle.

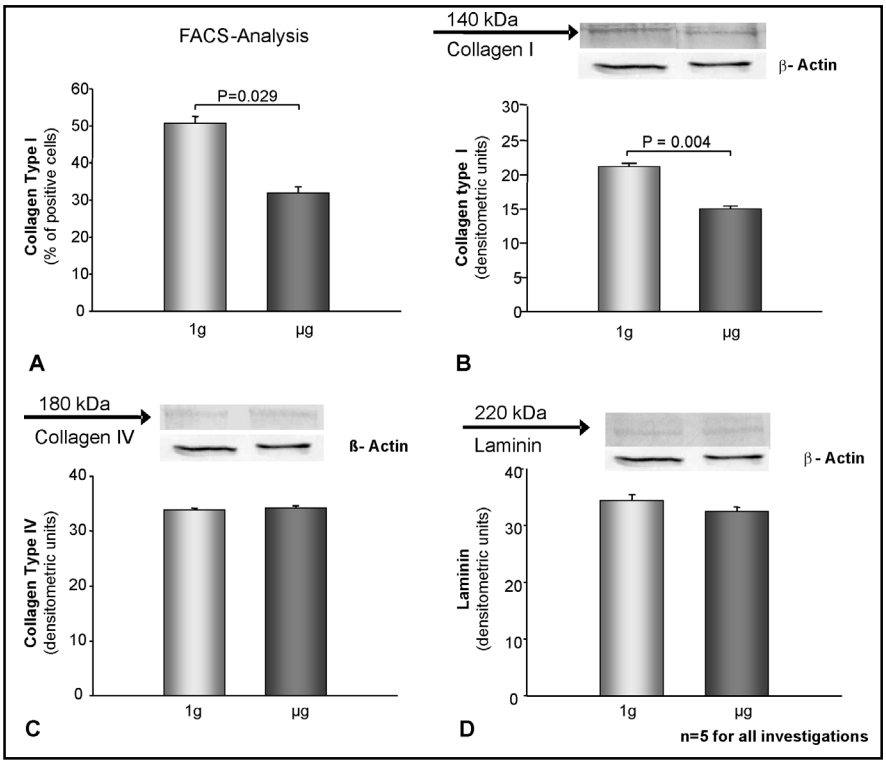


Fig. 3. Flow cytometric analyses revealed a significant decrease in the number of collagen type I positive cells in the simulated µg group (A). A reduced amount of collagen type I was also observed by Western blot analyses (B). Additional Western blots showed no difference in collagen type IV (C) and laminin (D) protein content in both groups of chondrocytes. As loading control β-actin was used.

population were stained positive for Bcl-2 or p53, irrespectively whether the cells had been incubated on the RPM or at 1g (Fig. 1A,C). Only bax was expressed in significant quantities, while slightly less bax was found, when the chondrocytes had been cultured on the RPM (Fig. 1B).

In order to see caspases/Fas independent apoptosis, we applied the Annexin V method recently developed in our laboratory. By flow cytometry, we found that 18% of the 1g control chondrocytes but only 10% of the µg exposed cells showed signs of apoptosis after 24 h of culturing (Fig. 2, red bars). The results were similar; when bFGF or VEGF had been added to the culture medium at the start of the experiment. The percentage of necrotic cells was below 10% in all samples (Fig. 2, grey bars). So we could calculate that 80% of the chondrocytes, remained viable during growth under simulated microgravity, while only about 75% did so under 1g conditions (Fig. 2, black bars).

Effects of microgravity on the production of extracellular matrix proteins

The chondrocytes cultured for 24h under conditions of simulated µg showed a reduced collagen type I expression. By flow cytometry we found only 30% of the cells being stained positive after exposure to s-µg, while 50% of the 1g control cells were positive (Fig. 3A). The decreased number of collagen type I positive cells in

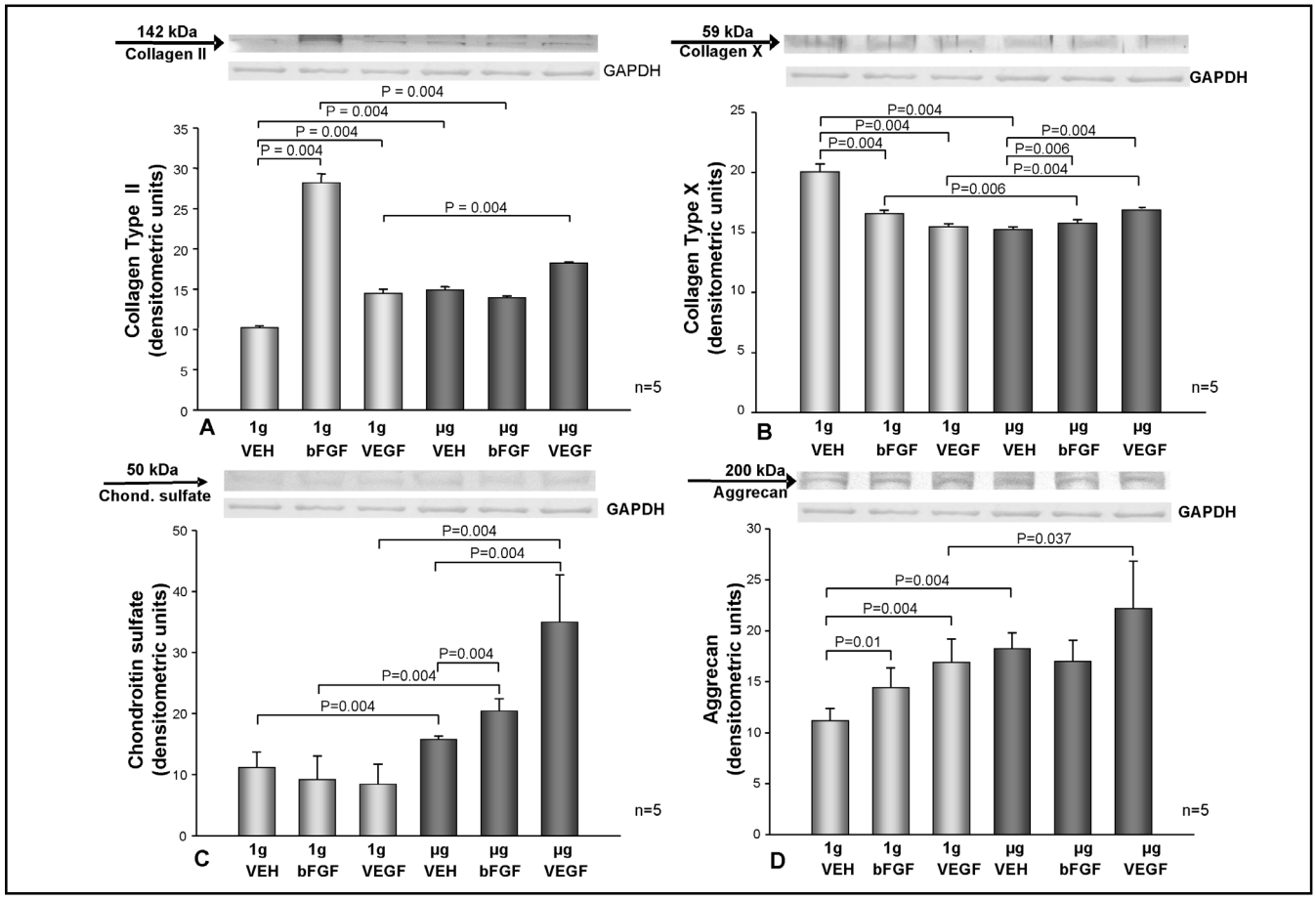


Fig. 4. Western blot analyses of collagen types II (A) and X (B) and of chondroitin sulfate (C) and aggrecan (D). The chondrocytes had been incubated under 1g and s-µg conditions. When indicated, 10 ng/ml of VEGF or bFGF had been added to the medium prior to the experiments. As loading control GAPDH was used.

µg were reflected by reduced total amount of collagen type I found analyzing the cell populations by the Western blot method (Fig. 3B). In contrast, microgravity did not influence collagen type IV and laminin expression, as the amount of these proteins were equal in all the analyzed cell populations (Fig. 3C, 3D).

The contents of collagen type II, chondroitin sulfate and aggrecan were higher in chondrocytes exposed to µg (Fig. 4A, 4C, 4D), while collagen type X was lower (Fig. 4B) as compared with static 1g controls. This basic ECM production was affected by addition of external bFGF or VEGF. bFGF and VEGF significantly elevated collagen type II production of human chondrocytes grown at 1g conditions. VEGF, but not bFGF also increased collagen type II in µg cultures. External bFGF as well as VEGF down-regulated collagen type X production in the 1g control cells but reduced the decrease of collagen type X concentration, when the chondrocytes were cultured on the RPM (Fig. 4B). But neither bFGF nor VEGF had influence on chondroitin sulfate production in static control cultures, although they elevated the accumulation of this ECM component significantly, when added to cultures exposed to simulated µg. Aggrecan was enhanced by bFGF and VEGF in 1g control cells, but only VEGF further increased aggrecan, when chondrocytes were cultured on the RPM.

Morphological changes

In order to investigate how morphology and growth behavior of the human chondrocytes is changing under s-µg, we performed a long-term experiment. After 5 days of incubation, the chondrocytes formed monolayers under 1g conditions (Fig. 5A). However, when equal samples of chondrocytes were cultured in parallel on a RPM for 5 days, some of the cells started to form three-dimensional structures (Fig. 5B). At this time, the three-dimensional aggregates may reach a length of 100 µm and a thickness of about four cellular sheets stacked up (Fig. 5B). During the next 2 days, the three-dimensional cell clusters elongated in the cultures exposed to simulated

Fig. 5. Phase contrast microscopy of human chondrocytes after 5d (A, B), 7d (C, D) and 10d (E, F). Comparing 1g controls (A, C, E) with simulated µg samples (B, D, F), we detected the growth of three-dimensional aggregates (B, D, F).

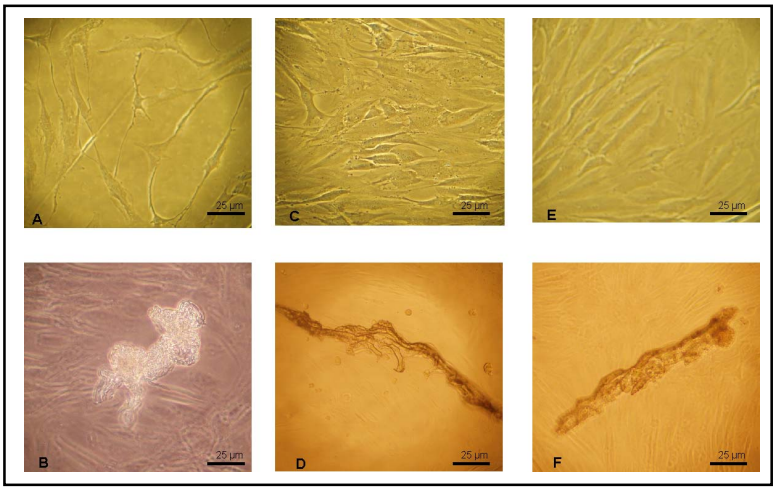


Fig. 6. Hematoxylin-eosin staining of chondrocytes, which had grown within a monolayer under static 1g conditions (A) or had formed aggregates under simulated µg conditions (B-D).

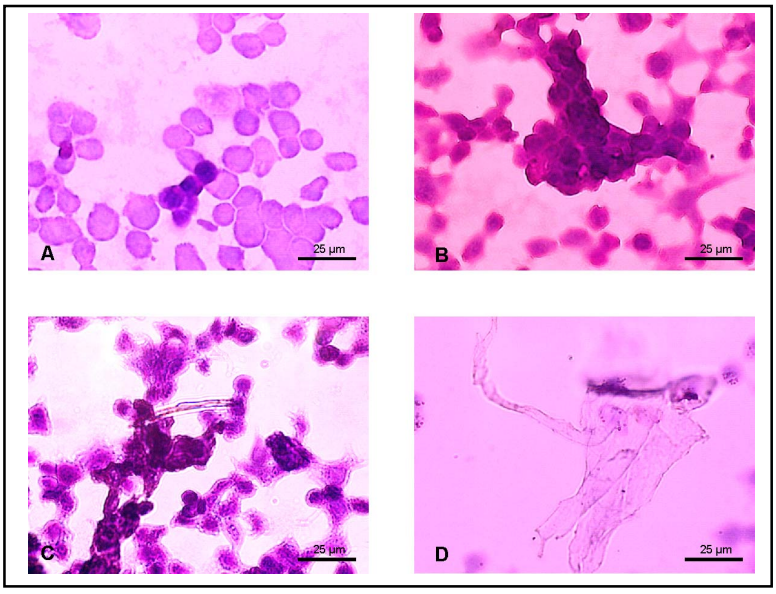
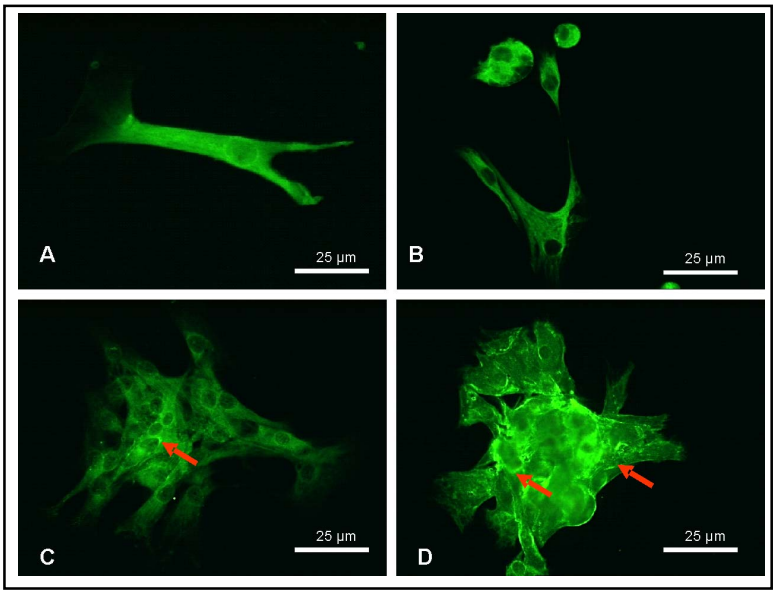


Fig. 7. Cultured at 1g for 18 d, control chondrocytes showed even distribution of α-tubulin (A), while exposed to simulated µg, they exhibited a perinuclear accumulation of α-tubulin (B, red arrow). When chondrocytes had been exposed to simulated µg for 18 d, vimentin staining exerted an accumulation around the nucleus and in the outer cellular membrane (D, red arrows), while a rather even distribution of vimentin can be seen in chondrocytes cultured at 1g control (C).



microgravity (Fig. 5D), while a confluent monolayer remained visible in the 1g ground control cells (Fig. 5C). The three-dimensional cell aggregates thickened, when exposed to simulated microgravity for another

three days (Fig. 5F), while the 1g ground control chondrocytes remained within monolayers (Fig. 5E).

The aggregates contained cells, as proved, when human chondrocytes were stained by the HE method after 18 days. The cells which had been incubated on ground under 1g conditions remained single cells (Fig. 6). But those chondrocytes which had been exposed to simulated μg, had formed three-dimensional aggregates (Fig. 6B-D). At this time the cells still contain normal nuclei indicating their non-apoptotic status.

As earlier studies have shown that a change in growth behavior is often accompanied by alterations of the cytoskeleton, we performed immunofluorescence staining. After 18d of culturing under simulated microgravity and static 1g conditions, the cells were treated with antibodies against alpha-tubulin or against vimentin and the bound antibodies were visualized according to the technique described above. In this way, we detected that alpha-tubulin was distributed over the total body of control chondrocytes as long as the cell grew under normal 1g conditions (Fig. 7A), but was accumulated around the nuclei in cells exposed to simulated μg (Fig. 7B). In contrast, vimentin was also distributed over the whole control chondrocytes with little accumulation around the nucleus (Fig. 7C). However, this cytoskeletal protein had re-arranged near the outer cellular membrane, when the chondrocytes had assembled to three dimensional clusters under μg conditions (Fig. 7D).

Discussion

This study proves the earlier observation [13-17, 28] that chondrocytes of different species like other human cells such as thyroid cancer cells and endothelial cells form 3D cell aggregates when being cultured on a random positioning machine. Already during the first day of incubation on the RPM, the human chondrocytes seem to switch to a path, which guides them to start three-dimensional cell-cell aggregation and rearrangement of the cytoskeletal proteins alpha-tubulin and vimentin. This conclusion appears reasonable, because after the first day of culturing i) apoptosis in cell populations incubated on a RPM was lower than in 1 g control cultures, while the chondrocytes did not express caspase-3 and Fas under any condition; and ii) chondrocytes produced extracellular matrix components under simulated μg in a similar way as chondrocytes do, which can be found in the resting or proliferative zone of a growth plate [29].

Apoptosis

Surprising results were obtained when we investigated the vitality of the cells. In contrast to other studies [30, 31], neither caspase-3 nor Fas were detectable in the type of the human chondrocytes investigated. Still apoptosis was observed at a rate of 18% in monolayer cultures and 10% in microgravity cultures. This means that apoptosis observed in our study is proceeding via a pathway, which is independent of caspases and Fas like it was described by Whiteman et al. [32], who investigated peroxynitrite initiated apoptosis of chondrocytes. The alternative apoptotic pathway observed here may include bax, which is present at a reasonable concentration, while p53 and Bcl-2 may not play a role either, because these proteins are present in only 5% of the cells.

The finding that the rate of apoptosis was slightly higher in 1g control cultures than in microgravity cultures contrasts earlier investigations on adherent endothelial cells [10, 25], thyroid cancer cells [9], or cultured glial cells [33]. These cells expressed caspase-3 and Fas and their tendency to enter apoptosis was higher under microgravity than under gravity. So it appears that chondrocytes, which do not switch on a caspase-3/Fas dependent apoptotic pathway, have a high capability to withstand the stress of transition from gravity to microgravity.

Changes of the extracellular matrix

After 24h of culturing, laminin and collagen type IV, both surrounding chondrocytes of the cartilage extracellularly *in vivo* were expressed in μg cells like in 1g control cells [34, 35]. At the same time, however, the quantity of collagen type II was enhanced and that of collagen type I diminished. Collagen type I is produced by those chondrocytes, which have already changed to "fibroblastoid like" cells [36]. *In vivo*, such a change does not occur in growth plate cartilage [37]. Only *in vitro*, when chondrocytes grow as monolayers, they strongly upregulate collagen type I and simultaneously down-regulate collagen type II [38]. Therefore, the lower quantity of collagen type I suggests that chondrocytes do not differentiate to "fibroblastoid like" cells under simulated μg as they usually do *in vitro* growing as a monolayer under 1g conditions.

The observed changes in collagen production point to the possibility that chondrocytes in microgravity behave like chondrocytes in growth-plates, where they produce high amounts of collagen II and low amounts of collagen type I (Fig. 3, 4). However, our data also show that

collagen type X was down-regulated, when the chondrocytes had been exposed to simulated microgravity. Collagen type X is usually presented by prehypertrophic and hypertrophic chondrocytes [39], which can be found in the hypertrophic region of the growth plate. But, it is absent on chondrocytes found in the resting and proliferative zones of the growth plate [40]. Therefore, one might conclude that the chondrocytes incubated on the RPM resemble the chondrocytes of the resting or proliferative zone of a cartilage growth plate producing high amounts of collagen type II and low amounts of collagen type I and type X. The conclusion is supported by a significant increase of chondroitin sulfate and aggrecan in the µg group compared with the static 1g group. Chondroitin sulfate provides the source molecules for proper synthesis of aggrecan [41], which is produced by chondrocytes of the resting and proliferative zone of the growth plate [40] and plays a key role as a chondro-protective and anti-inflammatory factor that inhibits apoptosis [42].

Growth factors

A difference between chondrocytes incubated on the RPM and those cultured in 1g was also seen investigating the effects of VEGF and bFGF. VEGF is an essential coordinator of chondrocyte death, chondroclast function, extracellular matrix remodeling in different cell types, angiogenesis, bone formation in the growth plate and also plays a key role in burn injury [10, 43-45]. VEGF is usually detectable in hypertrophic chondrocytes that undergo apoptosis [46]. Furthermore, VEGF receptor 2 was colocalized with VEGF both in hypertrophic cartilage *in vivo* and hypertrophic cartilage engineered *in vitro* [47]. As in our study VEGF had virtually no effect on the number of apoptotic human chondrocytes, one might also suggest that chondrocytes with hypertrophic features were not present in one of the two different kinds of cultures.

But VEGF further enhanced the effect of microgravity on collagen type II, chondroitin sulfate and aggrecan production, while it increased only collagen type II and aggrecan production in control cultures (Fig. 4). In contrast, bFGF supported the microgravity effect on chondroitin sulfate only, while it strongly enhanced type II collagen and slightly aggrecan production in 1g control cultures. bFGF and VEGF reduced collagen type X production in ground controls but retarded the collagen type X decrease triggered by microgravity. These findings suggest that chondrocytes incubated under 1g may change the collagen type II and type X as well as aggrecan production under the influence of VEGF and bFGF [48] like chondrocytes exposed to microgravity do. But they never form three-dimensional cell aggregates. Therefore, a chondrocyte differentiation state characterized by enhanced expression of collagen type II and aggrecan and suppression of collagen type X does not seem to be the only cause of induction of three-dimensional cell growth of chondrocytes under conditions of simulated microgravity. It remains to be of interest which role chondroitin sulfate plays in cell aggregation, of which the expression is affected by simulated microgravity but not by bFGF and VEGF under normal gravity. This molecule is negatively charged and may change the whole surface potential of a cell [49].

Morphology

Three-dimensional cell aggregation became detectable after 5 days of exposure of cells to simulated microgravity, but never in 1g control cells. The early 3D aggregates first elongate (Fig. 5B, 5D) and then thicken (Fig. 5F). After 18 days of culturing chondrocytes on the RPM, we investigated the cytoskeleton of the cells and found a rearrangement of alpha-tubulin and vimentin. The cytoskeletal proteins, which are distributed over the whole cell in monolayer chondrocytes, aggregated either around the nucleus or in peripheral near membrane zones. Similar observations have been made before in different cell types such as endothelial cells [10, 50], glial cells [51] and monocytes [52]. This rearrangement seems to be caused by microgravity. A possible link between rearrangement of cytoskeletal components and three-dimensional cell aggregation remains to be determined.

In summary, three-dimensional cell aggregation of chondrocytes begins to develop during the first day of exposure to simulated microgravity. On the RPM the cells start to produce similar patterns of ECM components, which distinct chondrocytes of growth plate's secret *in vivo*. Therefore, the RPM may become a tool, which is useful to trigger chondrocytes to growth in a three-dimensional manner so that cartilage may be formed *in vitro* without a scaffold.

Abbreviations

β (beta); Bcl-2 (B-cell lymphoma-2); bFGF (basic fibroblast growth factor); 3D (three-dimensional); ECM (extracellular matrix); FCS (fetal calf serum); FITC (Fluorescein isothiocyanate); GAPDH (Glycerinaldehyd-3-phosphat-Dehydrogenase); h (hour); IgG (immu-

noglobulin G); kD (kilodalton); µg (microgravity); mL (millilitre); PBS (phosphate buffered saline); PI (propidium iodide); RPM (Random Positioning Machine); s (second); s-µg (simulated microgravity); VEH (vehicle); VEGF (vascular endothelial growth factor).

Acknowledgement

We like to thank Norbert Hinz for his excellent technical assistance.

References

1 Muir H: The chondrocyte, architecture of cartilage – Biomechanics, structure, function and molecular-biology of cartilage matrix macromolecules. Bioessays 1995;17:1039-1048.

2 Verzijl N, Bank RA, TeKoppele JM, DeGroot J: AGEing and osteoarthritis: a different perspective. Curr Opinion Rheumatol 2003;15:616-622.

3 Suh JKF, Matthew HWT: Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials 2000;21:2589-2598.

4 Marijnissen WJCM, van Osch GJVM, Aigner J, Verwoerd-Verhoef HL, Verhaar JAN: Tissue-engineered cartilage using serially passaged articular chondrocytes. Chondrocytes in alginate, combined in vivo with a synthetic (E210) or biologic biodegradable carrier (DBM). Biomaterials 2000;21:571-580.

5 Brun P, Abatangelo G, Radice M, Zacchi V, Guidolin D, Gordini DD, Cortivo R: Chondrocyte aggregation and reorganization into three-dimensional scaffolds. J Biomed Mat Res 1999;46:337-346.

6 Schulz RM, Bader A: Cartilage tissue engineering and bioreactor systems for the cultivation and stimulation of chondrocytes. Eur Biophys J 2007;36:539-568.

7 Pound JC, Green DW, Roach HI, Mann S, Oreffo RO: An ex vivo model for chondrogenesis and osteogenesis. Biomaterials 2007;28:2839-2849.

8 Hutmacher DW: Scaffolds in tissue engineering bone and cartilage. Biomaterials 2000;21:2529-2543.

9 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 ad increases apoptosis in human follicular thyroid carcinoma cells. FASEB J 2002;16:604-614.

10 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.

11 Smith YC, Grande KK, Rasmussen SB, O'Brien AD: Novel three-dimensional organoid model for evaluation of the interaction of uropathogenic Escherichia coli with terminally differentiated human urothelial cells Infect Immun 2006;74:750-757.

12 Cameron DF, Hushen JJ, Colina L, Mallery J, Willing A, Sanberg PR, Saporta S: Formation and structure of transplantable tissue constructs generated in simulated microgravity from sertoli cells and neuron precursors Cell Transplant 2004;13:755-763.

13 Freed LE, Langer R, Martin I, Pellis NR, Vunjak-Novakovic G: Tissue engineering of cartilage in space. Proc Natl Acad Sci U S A 1997;94:13885-13890.

14 Hsu SH, Kuo CC, Yen HJ, Whu SW, Tsai CL: The effect of two different bioreactors on the neocartilage formation in type II collagen modified polyester scaffolds seeded with chondrocytes. Artifical Organs 2005:29:467-474.

15 Klement BJ, Spooner DS: Mineralization and growth of cultured embryonic skeletal tissue in microgravity. Bone 1999;24:349-359.

16 Duke PJ, Daane EL, Montufar-Solis D: Studies of chondrogenesis in rotating systems. J Cell Biochem 1993;51:274-282.

17 Montufar-Solis D, Duke PJ: Gravitational changes affect tibial growth plates according to Hert's curve. Aviation Space Environ Med 1999;70:245-249.

18 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.

19 van Loon JJWA: Some history and use of the random positioning machine, RPM, in gravity relayed research. Adv Space Res 2007;39:1161-1165.

20 Kossmehl P, Kurth E, Faramarzi S, Habighorst B, Shakibaei M, Wehland M, Kreutz R, Infanger M, J Danser AH, Grosse J, Paul M, Grimm D: Mechanisms of apoptosis after ischemia and reperfusion: role of the renin-angiotensin system. Apoptosis 2006;11:347-358.

21 Grimm D, Jabusch HC, Kossmehl P, Huber M, Fredersdorf S, Griese DP, Krämer BK, Kromer EP: Experimental diabetes and left ventricular hypertrophy: effects of beta-receptor blockade. Cardiovasc Pathol 2002;11:229-237.

22 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 beta and cause myocardial fibrosis in marmosets (Callithrix jacchus). Arch Toxicol 2002;76:360-366.

23 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.

24 Gehrmann M, Marienhagen J, Eichholtz-Wirth H, Fritz E, Ellwart J, Jäättelä M, Zilch T, Multhoff G: Dual function of membrane-bound heat shock protein 70 (Hsp70), Bag-4, and Hsp40: protection against radiation-induced effects and target structure for natural killer cells. Cell Death Differ 2005;12:38-51.

25 Infanger M, Ulbrich C, Baatout S, Wehland M, Kreutz R, Bauer J, Grosse J, Vadrucci S, Cogoli A, Derradji H, Neefs M, Küsters S, Spain M, Paul M, Grimm D: Modeled gravitational unloading induced downregulation of endothelin-1 in human endothelial cells. J Cell Biochem 2007;101:1439-1455.
26 Ulbrich C, Westphal K, Baatout S, Wehland M, Bauer J, Flick B, Infanger M, Kreutz R, Vadrucci S, Egli M, Cogoli A, Derradji H, Pietsch J, Paul M, Grimm D: Effects of basic Fibroblast Growth Factor on endothelial cells under conditions of simulated microgravity. J Cell Biochem 2008;104:1324-1341.
27 Grosse J, Grimm D, Westphal K, Ulbrich C, Moosbauer J, Pohl F, Koelbl O, Infanger M, Eilles C, Schoenberger J: Radiolabeled annexin V for imaging apoptosis in radiated human follicular thyroid carcinomas - is an individualized protocol necessary? Nucl Med Biol 2009;36:89-98.
28 Conza N, Mainil-Varlet P, Rieser F, Kraemer J, Bittmann P, Huijser R, van den Bergh L, Cogoli A: Tissue engineering in space. J Gravit Physiol 2001; 8: P17-20.
29 Poole AR, Pidoux I, Rosenberg L: Role of Proteoglycans in Endochondral Ossification: Immunofluorescent Localization of Link Protein and Proteoglycan Monomer in Bovine Fetal Epiphyseal Growth Plate J Cell Biol 1982;92:249-260.
30 Lo MY, Kim HAT: Chondrocyte apoptosis induced by hydrogen peroxide requires caspase activation but not mitochondrial pore transition. J. Orthopaedic Res. 2004;22:1120-1125.
31 Chen JH, Cao JL, Chu YL, Wang ZL, Yang ZT, Wang HL: T-2 toxin-induced apoptosis involving Fas, p53, Bcl-xL, Bcl-2, Bax and caspase-3 signaling pathways in human chondrocytes. J Zhejiang Univ-Sci B 2008;9:455-463.
32 Whiteman M, Armstrong JS, Cheung NS, Siau JL, Rose P, Schantz JT, Jones DP, Halliwell B: Peroxynitrite mediates calcium-dependent mitochondrial dysfunction and cell death via activation of calpains. FASEB J 2004;18:1395-1397.
33 Uva BM, Masini MA, Sturla M, Buzzone F, Giuliani M, Tagliaferro G, Strollo F: Microgravity-induced apoptosis in cultured glial cells. Eur J Histochem 2002;46:209-214.
34 Dürr J, Lammi P, Goodman SL, Aigner T, von der Mark K: Identification and immunolocalization of laminin in cartilage. Exp Cell Res 1996;222:225-333.
35 Kvist AJ, Nystrom A, Hultenby K, Sasaki T, Talts JF, Aspberg A: The major basement membrane components localize to the chondrocyte pericellular matrix - A cartilage basement membrane equivalent? Matrix Biol 2008;27:22-33.
36 Frondoza C, Sohrabi A, Hungerford D: Human chondrocytes proliferate and produce matrix components in microcarrier suspension culture. Biomaterials 1996;17:879-188.
37 Mundlos S, Zabel B: Developmental expression of human cartilage matrix proteins. Dev Dyn 1994;199:241-152.
38 Schnabel M, Marlovits S, Eckhoff G, Fichtel L, Gotzen L, Vécsei V, Schlegel J: Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis Cartilage 2002;10:62-70.
39 Valle PL, Iwamoto M, Fanning P, Pacifici M, Olson BR: Multiple negative elements in a gene that codes for an extracellular-matrix protein, collagen x, restrict expression to hypertrophic chondrocytes. J Cell Biol 1993;121:1173-1179.
40 Vornehm SI, Dudhia J, VonderMark K, Aigner T: Expression of collagen types IX and XI and other major cartilage matrix components by human fetal chondrocytes in vivo. Matrix Biol 1996;15:91-98.
41 Lemons ML, Sandy JD, Anderson DK, Howland DR: Intact aggrecan and chondroitin sulfate-depleted aggrecan core glycoprotein inhibit axon growth in the adult rat spinal cord. Exp Neurol 2003;184:981-990.
42 Fioravanti A, Collodel G: In vitro effects of chondroitin sulfate. Adv Pharmacol 2006;53:449-465.
43 Gerber HP, Vu T, Ryan A, Kowalski J, Werb Z, Ferrara N: VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during enchondral bone formation. Nature Med 1999;5:623-638.
44 Horner A, Bishop NJ, Bord S, Beeton C, Kelsall AW, Coleman N, Compston JE: Immunolocalisation of vascular endothelial growth factor (VEGF) in human neonatal growth plate cartilage. J Anat 1999;194:519-524.
45 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.
46 Harper J, Klagsbrun M: Cartilage to bone - angiogenesis leads the way. Nature Med 1999;5: 623-628.
47 Carlevaro MF, Cermelli S, Cancedda R, Cancedda FD: Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci 2000;113:59-69.
48 Schmal H, Zwingmann J, Fehrenbacher M, Finkenzeller G, Stark GB, Südkamp NP, Hartl D, Mehlhorn AT: bFGF influences human articular chondrocyte differentiation. Cytotherapy 2007;9:184-193.
49 Mehrishi JN, Bauer J: Electrophoresis of cells and the biological relevance of surface charge. Electrophoresis 2002;23:1984-1994.
50 Grimm D, Infanger M, Ulbrich C, Pietsch J, Kossmehl P, Vadrucci S, Baatout S, Flick B, Paul M, Bauer J: A delayed type of three-dimensional growth of human endothelial cells under simulated weightlessness. Tissue Eng Part A 2009;15: 2267-2275.
51 Uva BM, Masini MA, Sturla M, Prato P, Passalacqua M, Giuliani M, Tagliafierro G, Strollo F: Clinorotation-induced weightlessness influences the cytoskeleton of glial cells in culture. Brain Res 2002;934:132-139.
52 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.