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Impact of simulated microgravity on human bone stem cells: New hints for space medicine

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ABSTRACT

Bone loss is a well known early event in astronauts and represents one of the major obstacle to space exploration. While an imbalance between osteoblast and osteoclast activity has been described, less is known about the behavior of bone mesenchymal stem cells in microgravity.

We simulated microgravity using the Random Positioning Machine and found that mesenchymal stem cells respond to gravitational unloading by upregulating HSP60, HSP70, cyclooxygenase 2 and superoxyde dismutase 2. Such an adaptive response might be involved in inducing the overexpression of some osteogenic transcripts, even though the threshold to induce the formation of bone crystal is not achieved. Indeed, only the addition of an osteogenic cocktail activates the full differentiation process both in simulated microgravity and under static 1G-conditions.

We conclude that simulated microgravity alone reprograms bone mesenchymal stem cells towards an osteogenic phenotype which results in complete differentiation only after exposure to a specific stimulus.

1. Introduction

Human bone mesenchymal stem cells (bMSC) possess capacity for self-renewal and multilineage differentiation potential into osteoblasts, chondrocytes and adipocytes [1]. Because of these properties, bMSC are crucial for tissue homeostasis and for repair after injury, and are emerging as attractive tools for tissue engineering and regenerative medicine.

Upon specific stimuli, bMSC are induced to differentiate into osteoblasts, which deposit new bone matrix. Dysfunction of bMSC in terms of self-renewal and differentiation potential has been described in senile and post-menopausal osteoporosis. bMSC derived from postmenopausal osteoporotic patients tend to differentiate in adipocytes rather than osteogenic cells [2]. In aging, alterations of the cytoskeleton and increased oxidative stress seem to be implicated in reducing bMSC response to signals from the microenvironment [3].

Bone loss (1–2% a month) is also well documented in astronauts [4] and it represents a key concern and a limiting factor for space exploration. Space-associated osteopenia is a very early event which affects especially weight-bearing bones and requires a very long time for recovery after return to earth [5]. It is linked to the impaired activity of osteoblasts and the increased function of osteoclasts as the result of gravitational unloading due to microgravity [6]. Since space flights are infrequent and expensive, several devices have been invented to simulate microgravity on earth, even though they mimic only some aspects of real microgravity. Thanks to these bioreactors, significant alterations in the behavior of osteoclasts and osteoblasts have been described [7]. Contrasting results are reported about bMSC exposed to simulated microgravity. Indeed, some authors report the inhibition of bMSC differentiation [8,9], while others demonstrate their increased differentiation [10,11] to the point of suggesting the use of bioreactors simulating microgravity for tissue engineering [12]. These discrepancies might be ascribed to the use of different microgravity bioreactors, protocols to culture the cells and induce their differentiation, and analytical procedures.

Recently, we have contributed to an experiment onboard the International Space Station (ISS) to test the behavior of cultured bMSC in space. While waiting for the cells to return to earth, we investigated the effects of simulated microgravity generated by the Random Positioning Machine (RPM) on the same bMSC that were space-flown. RPM is a 3D clinostat widely used before and after space flight experiments [13], which has yielded challenging insights into the behavior of cells and simple organisms. In particular,

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RPM proved to be a valuable tool for simulating microgravity in adherent cells [14].

We examined the expression of some genes involved in osteogenic differentiation in bMSC cultured in the RPM or in static 1G-conditions, with and without the addition of an osteogenic medium. In particular, we focused our studies on i) *RUNX2*, which is known to be the master switch of osteogenesis, since complete absence of ossification was observed in *RUNX2* knockout mice [15]; ii) Osterix (*OSX*), which is fundamental in promoting the early stages of osteogenesis but it is not sufficient to achieve a full differentiation into osteoblast; iii) collagen 1A1 (*COL1A1*), essential for progression of differentiation at early stages [15]; iv) osteocalcin (*OSC*) and osteopontin (*OSP*), the most abundant non collagenous components of bone extracellular matrix, both crucial for the osteogenic phenotype [15].

We show that culture of bMSC in the RPM i) activates the stress response and ii) accelerates the expression of several osteogenic genes, apart from *COL1A1*. Only the addition of an osteogenic cocktail containing vitamin D allows the differentiation of bMSC with deposition of bone crystals.

2. Materials and methods

2.1. Culture of bMSC

bMSC were isolated from adult human bone marrow withdrawn from bilateral punctures of the posterior iliac crests of a normal volunteer [16] and tested for purity by flow cytometry at the Policlinico in Milan according to institutional guidelines approved by the IRCCS Policlinico (donor 1). These cells were cultured at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium with 1000 mg/L glucose and containing 10% fetal bovine serum and 2 mM glutamine (culture medium, CM). All the reagents for cell culture were from Sigma–Aldrich, St. Louis, Missouri, USA. The behavior of these primary cells was compared with that of bMSC purchased from Lonza, Basel, Switzerland (donor 2) which were cultured according to manufacturer's instructions. The cells were used between passage 2 and 5.

2.2. Culture in the RPM

Microgravity conditions were simulated using the RPM (Dutch Space, Leiden, Netherlands) [13]. The RPM provides continuous random change in orientation relative to the gravity vector of an accommodated experiment. Culture flasks containing confluent monolayers 72 h after seeding were completely filled with medium supplemented with 12.5 mM HEPES (Sigma–Aldrich) devoid of air bubbles and fixed in the RPM, as close as possible to the centre of the platform, which was then rotated using the real random mode (random speed and random direction) of the machine. The RPM operated at 37 °C. 1G ground control cultures, treated in parallel in identical equipment, were placed on the basis of the RPM.

2.3. In vitro osteogenic differentiation of bMSC

The cells were seeded in T25 flasks. Once the cells were confluent, an osteogenic induction cocktail was added to the medium (osteogenic medium, OM). The osteogenic cocktail contains 2×10^{-8} M 1α,25-Dihydroxyvitamin D_3, 10 mM β-glycerolphosphate and 0.05 mM ascorbic acid (Sigma–Aldrich).

To analyze calcium (Ca) deposition by bMSC, the cells were rinsed with PBS, fixed (70% ethanol, 1 h) and stained for 10 min with 2% Alizarin Red S (pH 4.2, Sigma–Aldrich) [17]. Alizarin Red S staining was released from the cell matrix by incubation in 10% cetylpyridinium chloride (Sigma–Aldrich) in 10 mM sodium phosphate (pH 7.0), for 15 min and the absorbance measured at 562 nm.

2.4. Real-Time PCR

Total RNA was extracted by the PureLink RNA Mini kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). Single-stranded cDNA was synthesized from 0.2 μg RNA in a 40 μl final volume using High Capacity cDNA Reverse Transcription Kit, with RNase inhibitor (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time PCR was performed three times in triplicate on the 7500 FAST Real Time PCR System instrument using TaqMan Gene Expression Assays (Life Technologies, Monza, Italy): Hs00231692_m1 (*RUNX2*), Hs01866874_s1 (*OSX*), Hs00164004_m1 (*COL1A1*), Hs01587814_g1 (*OSC*) and Hs00959010_m1 (*OSP*). The housekeeping gene *GAPDH* (Hs99999905_m1) was used as an internal reference gene. Relative changes in gene expression were analyzed by the $2^{-\Delta\Delta Ct}$ method [18].

2.5. Protein array

After 24 h of culture in the RPM or in static 1G-conditions, bMSC were lysed in lysis buffer (50 mM TrisHCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40). Protein concentrations were determined using the Bradford protein assay (Sigma–Aldrich). Cell extracts (80 μg) were utilized to incubate the membranes on which 26 antibodies against human cell stress-related proteins were spotted in duplicate (R&D systems, Space Import Export, Milan, Italy). The array was performed according to the manufacturer's instructions. Densitometry was performed by the ImageJ software. Two separate experiments were performed and data are expressed as % of the variation in the signal intensity of RPM *vs* static 1G-conditions.

2.6. Statistical analysis

Statistical significance was determined using Student's t test and set as following: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Culture in the RPM induces the expression of RUNX2 and OSX in bMSC from two donors

Because of individual biologic variability, initially we investigated the effects of simulated microgravity on the expression of osteogenic markers using bMSC from two healthy male donors. The cells were cultured in CM for 4 days in the RPM or in static 1G-conditions as a control. By RT-PCR a significant increase of the transcripts for *RUNX2* and *OSX* was detected in bMSC from both donors when cultured in the RPM (Fig. 1A). We found no modulation of *PPARγ*, a master regulator of adipogenesis (Fig. 1A) [19].

To understand whether the overexpression of *RUNX2* and *OSX* suffices osteogenesis, we cultured bMSC in the RPM for 10 days, which is the time usually necessary to detect calcium deposition. By Alizarin Red S staining, which reveals the formation of calcium nodules, we did not observe any deposition of calcium in the matrix of bMSC from the two donors (Fig. 1B).

Since similar results were obtained from the two donors, we continued our studies on bMSC of donor 1, which were used for experiments onboard the ISS.

3.2. Culture in the RPM activates stress response

After 24 h of culture in the RPM, no major modifications of cell