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Inhibitory effect of simulated microgravity on differentiating preosteoblasts

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Abstract

The bone loss induced by microgravity is partly due to the decrease of mature osteoblasts. In the present study, we employed the random positioning machine (RPM) to simulate microgravity and investigated the acute effects of simulated microgravity on the differentiation of 2T3 preosteoblasts. Following 7 days' culture under normal (1 *g*) condition, cells were exposed to simulated microgravity for 24 h. The results showed that 24 h treatment of simulated microgravity significantly decreased alkaline phosphatase (ALP) activity without changing the cell morphology. In addition, the mRNA expressions of osteogenic genes, including runt-related gene 2 (Runx2), osterix, osteocalcin (OC), type I collagen (Col I) and bone morphogenetic protein (BMP), were dramatically downregulated. Moreover, western blot analysis of total extracellular signal-regulated kinase (Erk) and phosphorylated Erk (p-Erk) indicated that p-Erk level, which represents the Erk activation status, was increased. Taken together, our results suggested that acute exposure to simulated microgravity inhibited osteoblast differentiation through modulating the expression of osteogenic genes and the Erk activity. These findings provide new insight for bone loss due to microgravity and unloading.

Keywords: Bone loss; Simulated microgravity; Random positioning machine; Osteoblast differentiation

1. Introduction

As vertebrate animals evolving and living in 1 *g* gravity environment, the formation and function of bone are affected by 1 *g* gravity. Manned spaceflights have indicated the profound effects of gravity on bone (Vernikos, 1996; Vernikos and Schneider, 2010). Previous spaceflight experiments demonstrated that absence of 1 *g* gravity resulted in both reduction of bone mass and induction of bone loss (Bloomfield, 2010; Caillot-Augusseau et al., 1998, 2000; Collet et al., 1997; Lang et al., 2004; Vico et al., 2000). Microgravity condition in space may increase fracture risk for astronauts after returning to Earth (Keyak et al., 2009). Based on previous studies, it has been suggested that the inhibition of osteoblast differentiation and bone formation is responsible for this effect (Carmeliet et al., 2001; Morey and Baylink, 1978; Oganov, 2004; Turner et al., 1995; Zerath et al., 2000).

Many cytokines, transcription factors and signaling pathways have been implicated in regulation of osteoblast differentiation (Ge et al., 2007; Kong et al., 2010; Nakashima and de Crombrugghe, 2003; Yamaguchi et al., 2000). Alkaline phosphatase (ALP), osteocalcin (OC) and type I collagen (Col I) are important markers for osteoblast maturation and indicators for osteoblast differentiation. Runt-related gene 2 (runx2)/Core-binding factor 1 (Cbfa1), functioning to regulate osteocalcin expression (Ducy et al., 1997), and osterix, downstream of runx2 (Komori et al., 1997; Nakashima et al., 2002), are essential transcription factors for osteoblast differentiation and bone formation.

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Recent studies also show that MAPK signaling pathway is involved in activation of runx2 and plays an important role in regulating osteoblast differentiation and in mechanotransduction (Matsushita et al., 2009; Papachristou et al., 2009; Xiao et al., 2000). If these factors and pathways are affected, the process of osteoblast differentiation will be abnormal and may result in bone loss. Space experiments indicated that microgravity induced bone loss was partly due to the inhibition of osteoblast differentiation (Carmeliet et al., 1997; Garetto et al., 1990; Vico et al., 2000).

Due to the limited opportunity and expensive cost for carrying out space flight experiments, ground-based simulation methods, including parabolic flight, clinostat, rotary wall vessel (RWV), random positioning machine (RPM) and large gradient high magnetic field (LGHMF), were developed to study the mechanism related to the biological effects of microgravity (Di et al., 2011; Hammond and Hammond, 2001; Qian et al., 2009; van Loon, 2007). Consistent with the results of spaceflight experiments, ground-based simulated microgravity showed inhibitory effect on osteoblast differentiation. Long-term treatment of simulated microgravity using RPM inhibited ALP activity and downregulated osteogenic genes such as runx2, osteomodulin and parathyroid hormone receptor 1 (Pardo et al., 2005). Using RWV, 24 h exposure to simulated microgravity has been shown to inhibit markers of osteoblast differentiation and suppress runx2 expression and AP-1 transactivation (Ontiveros and McCabe, 2003). Although these findings indicated that simulated microgravity has inhibitory effect on osteoblast differentiation, the cellular and molecular mechanism has not been fully understood.

Based on the above findings, we speculated that runx2 is a key regulator for osteoblast differentiation under simulated microgravity and extracellular signal-regulated kinase (Erk) pathway may be involved in this process. The present study was conducted to determine the acute effects of simulated microgravity, by using RPM, on the differentiation of differentiating 2T3 preosteoblasts.

2. Materials and methods

2.1. Chemicals and antibodies

Alpha-Minimum Essential Medium (α-MEM) was purchased from Invitrogen (USA). Fetal bovine serum (FBS) was obtained from HyClone (China). Ascorbic acid, β–glycerophosphate (β-GP), p-nitrophenol solution and Alkaline Phosphatase Yellow (pNPP) Liquid Substrate system for ELISA were purchased from Sigma–Aldrich (USA). BCIP/NBT Alkaline phosphatase Color Development Kit was obtained from Beyotime (China). Trizol reagent was purchased from Invitrogen (USA). Anti-Erk antibody and anti-phospho-Erk antibody were obtained from Cell Signaling Technology (USA). Anti-GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) antibody was purchased from Merck (Germany).

2.2. Cell culture

Murine 2T3 preosteoblast cells were kindly provided to us by Dr. Jean X. Jiang, University of Texas Health Science Center. The cells were cultured in α-MEM medium containing 10% FBS at 37 °C, 5% CO_2 incubator. For differentiation assay, cells were allowed to grow to confluence, and the medium was changed to osteogenic medium (α-MEM containing 10% FBS and supplemented with 100 μg/ml ascorbic acid and 5 mM β-GP), which was changed every 2–3 days.

2.3. Simulated microgravity using random positioning machine

A desktop random positioning machine (RPM) purchased from the Center for Space Science and Applied Research of Chinese Academy of Sciences was used to simulate microgravity, as described previously (Luo et al., 2011; Xiang et al., 2010). Briefly, the RPM contains inner and outer frames that can rotate independently in random directions controlled by computer system (van Loon, 2007). Cell culture vessel was fixed on the inner frame and the RPM was placed inside a 37 °C incubator.

For simulated microgravity studies, 2T3 cells were seeded on glass slides at a density of 2×10^4 cells/cm^2 and cultured with α-MEM medium containing 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. When cells grew to confluence, the medium was changed to osteogenic medium and cells were cultured for 7 days. Then, cells were placed into cell culture flasks which were fulfilled with osteogenic medium without bubbles and fixed into the culture vessel of RPM. The RPM were rotated randomly at range of 0–8 rpm to simulate microgravity (van Loon, 2007) for 24 h. Cells of control group were cultured in the same 37 °C incubator without rotating.

2.4. Cell morphology observation

Cell morphology was observed using Olympus inverted phase contrast microscope (Japan) and images were taken by a camera attached to the microscope.

2.5. Alkaline phosphatase staining

ALP of 2T3 cells was stained with BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime) according to the manufacturer's instruction. Briefly, cells were fixed in 10% formalin for 15 min, followed by washing with phosphate buffered saline (PBS, pH7.4) for three times, then were stained using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) solution. The staining reaction was stopped by washing with distilled water and the cells were photographed by the Nikon digital camera attached to the 80i microscope (Japan).