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Abstract

Physiological strain plays an important role in maintaining the normal function and metabolism of bone cells. It is well known that the mineral content of astronauts' bones decreases during spaceflight. Thus, gravity is one of the important factors in the muscloskeletal system. The vector-free horizontal clinostat has been used to simulate conditions of microgravity for examining such effects on cells in culture. We analyzed the effects of simulated microgravity using a horizontal clinostat on cultured osteoblast-like cells (HuO9 cell line). Total cellular protein, which was measured as an indication of cell proliferation, was not significantly inhibited under simulated microgravity conditions. No morphological changes were detected under microgravity conditions by phase-contrast microscopy. However, the alkaline phosphatase (ALP) activity and osteocalcin production of the HuO9 cells decreased significantly under microgravity conditions. Our data indicate that simulated microgravity directly inhibits some differentiation phenotypes and some functions of osteoblasts. On the other hand, the addition of 1,25-dihydroxy-vitamin D_3 (1,25-$(OH)_2$-D_3) increased ALP activity under simulated microgravity conditions, although the total activity of ALP in the cells treated with 1,25-$(OH)_2$-D_3 was still lower under simulated microgravity conditions than that in the control cells. However, the cells under simulated microgravity conditions showed a greater enhancement of ALP activity by treatment with 1,25-$(OH)_2$-D_3.

KEYWORDS: microgravity, osteoblast, alkaline phosphatase, osteocalcin

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Effects of Simulated Microgravity on Human Osteoblast-Like Cells in Culture

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Physiological strain plays an important role in maintaining the normal function and metabolism of bone cells. It is well known that the mineral content of astronauts' bones decreases during spaceflight. Thus, gravity is one of the important factors in the muscloskeletal system. The vector-free horizontal clinostat has been used to simulate conditions of microgravity for examining such effects on cells in culture. We analyzed the effects of simulated microgravity using a horizontal clinostat on cultured osteoblast-like cells (Hu09 cell line). Total cellular protein, which was measured as an indication of cell proliferation, was not significantly inhibited under simulated microgravity conditions. No morphological changes were detected under microgravity conditions by phase-contrast microscopy. However, the alkaline phosphatase (ALP) activity and osteocalcin production of the Hu09 cells decreased significantly under microgravity conditions. Our data indicate that simulated microgravity directly inhibits some differentiation phenotypes and some functions of osteoblasts. On the other hand, the addition of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3) increased ALP activity under simulated microgravity conditions, although the total activity of ALP in the cells treated with 1,25-$(OH)_2$-D_3 was still lower under simulated microgravity conditions than that in the control cells. However, the cells under simulated microgravity conditions showed a greater enhancement of ALP activity by treatment with 1,25-$(OH)_2$-D_3.

Key words: microgravity, osteoblast, alkaline phosphatase, osteocalcin

Physiological strain plays an important role in the growth and maintenance of skeletal systems. In some recent studies, bed rest, immobilization and hindlimb suspension resulted in a negative calcium balance and an inhibition of bone formation (1, 2). In another study, centrifugal force activated alkaline phosphatase (ALP) activity and osteocalcin (bone γ-carboxyglutamic acid-containing protein, BGP) synthesis of human osteoblast-like cells *in vitro* (3). Other investigators have reported that cyclic and/or continuous stretching stress affects the proliferation and metabolism of cultured human osteoblasts (4).

Gravity affects all living things and exerts an important physiological stress on the muscloskeletal system. It is well known that the bone mineral content of astronauts decreases during spaceflight (5). Previous studies have shown a decrease in bone formation and mineralization (6-9), and an increase in bone resorption under microgravity conditions (10). However, there have been few studies on the effects of microgravity on bone cell functions in culture.

Because of the difficulty of performing experiments in space, simulated experiments of microgravity on earth have been carried out to study the effects of reduced gravity on cells and animals. The clinostat has been used to produce a vector-free gravitational environment for over 100 years (11). Previous investigators have demonstrated that microgravity simulated by clinostat inhibits cell proliferation (12), disrupts synapse formation (11), and causes morphological changes in myocytes and neurons (13). Furthermore, it has been shown that the proliferation of mouse osteoblastic cells decreases under clinostat-simulated microgravity (14).

Since ALP and BGP are known to be associated with

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bone formation, they are good phenotype markers for the functions of osteoblastic cells in culture. Using clinostat simulation, we report here the effects of microgravity on cell proliferation, ALP activity and BGP production of cultured human osteoblast-like cells.

Materials and Methods

Cell culture. The human osteosarcoma cell line HuO9 (15), which retains a high ALP activity and secretes BGP into culture medium, was grown in RPMI-1640 medium (Nissui, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Intergen, New York, USA) and 100 μg/ml kanamycin in a 5% CO_2 incubator. After 6×10^4 cells were cultured on a 12×32 mm cover glass (Matsunami Glass Ind., Ltd., Osaka, Japan) for 24 h, the cover glass was transferred into a cuvette (No. 67.749, Sarstedt, Germany) filled with RPMI-1640 containing 10% FBS, 15 mM Hepes and 100 μg/ml kanamycin. In order to simulate microgravity, the cuvette was set on a horizontal plane in the clinostat (Kohzuseiki Co., Ltd., Tokyo, Japan), where the axis of rotation was perpendicular to the vector of gravity. As a control, another cuvette was set on a control plane, where the axis of rotation was parallel to the vector of gravity. These two axes were rotated by the same motor. The clinostat system rotated 10–30 times per min at 37°C.

In order to examine the effect of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3), the medium was replaced at the start of the clinostat experiments with the same type of medium containing 10^{-7} M 1,25-$(OH)_2$-D_3 (Calcitriol, Wako Pure Chemical Ind., Osaka, Japan) in 0.1% ethanol, differing only in the concentration of FBS, which was reduced to 1%.

Alkaline phosphatase activity. ALP activity was determined by modifying the method of Lowry *et al.* (16), using p-nitrophenylphosphate as a substrate. Cell extracts were prepared as follows: The cells were washed three times with phosphate-buffered saline solution, transferred into 0.1% Triton X-100 (Millipore Co., Bedford, MA, USA) with a cell scraper, disrupted with a sonicator, and centrifuged for 5 min at $180 \times g$ to separate the supernatant for enzyme assay. The specific ALP activity was expressed in units (micromoles per hour) per milligram of protein.

Secretion of BGP. To determine the secretion of BGP, the concentration of FBS in the culture medium was reduced to 1%. The medium from the clinostat rotation culture was collected from the cuvette, and the BGP concentration was measured by two-site immunoradiometric assay (IRMA) using a BGP-IRMA kit (Mitsubishi Chemical Co., Tokyo, Japan).

Protein concentration. The protein concentration of the cell extracts was estimated using Bradford's method (17).

Statistical evaluation. The data were analyzed using the Student's t-test. The level of significance was $P < 0.05$.

Results

The protein concentration on days 4 and 7 under microgravity conditions tended to decrease compared with those of the rotational controls, but there was no statistically significant difference between the two cultures (Fig. 1) indicating no measurable inhibition of cell proliferation under microgravity conditions. By phase contrast microscopy, the HuO9 cells showed polygonal morphology under microgravity conditions, but no evident morphological changes in the cells were observed between the simulated microgravity and control cultures (Fig. 2).

The ALP activity of the HuO9 cells increased time-dependently. The ALP activity of the rotational controls (20 rotations per min, rpm) was 16.18 unit/mg protein on day 4 and 31.27 unit/mg protein on day 7. Under

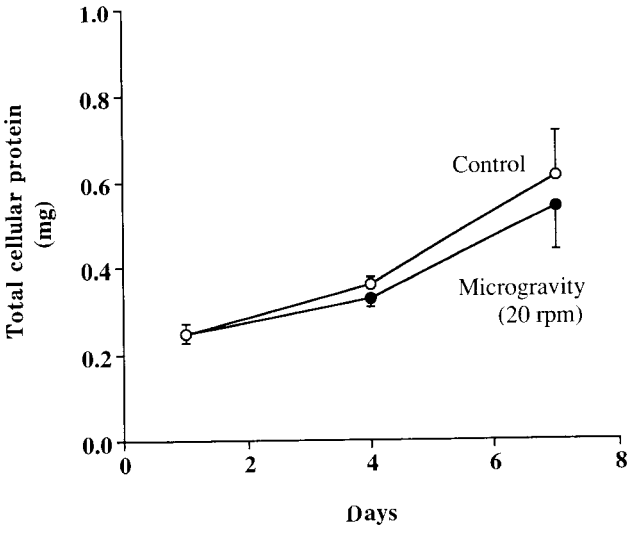

Fig. 1 Time-course increase in total cellular protein under control and simulated microgravity conditions. Each point is expressed as the mean ± SD from triplicate determinations.

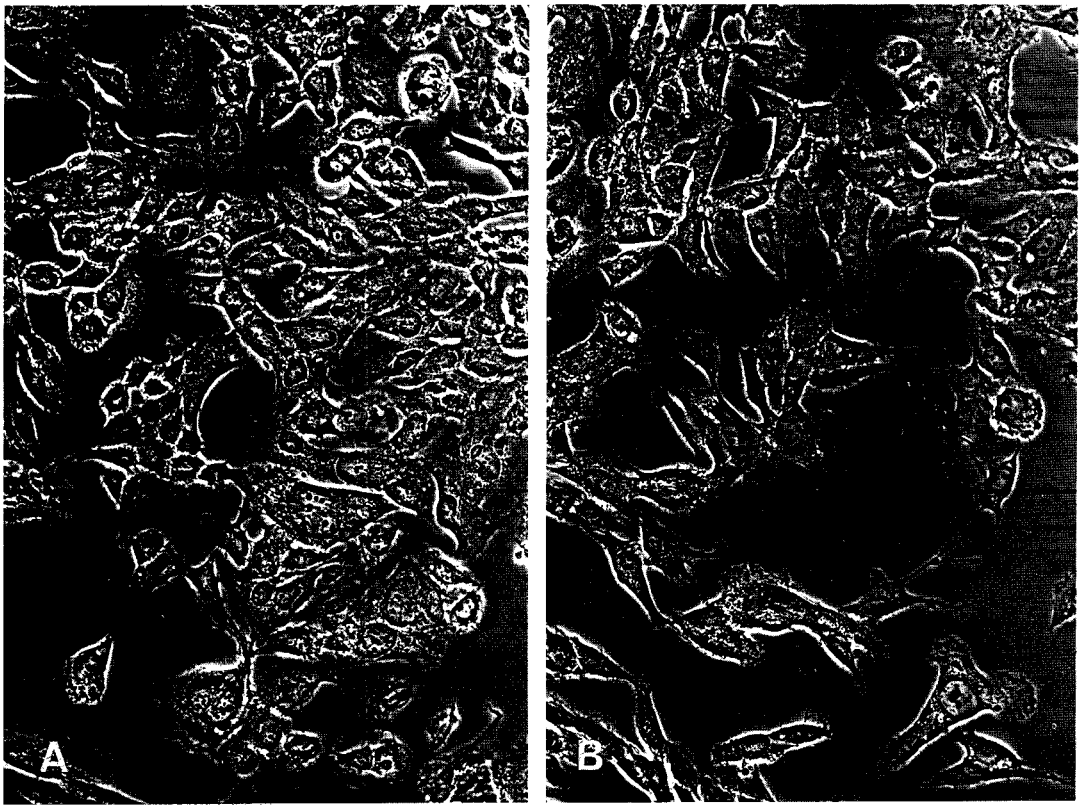

Fig. 2 Phase-contrast appearance of HuO9 cells on day 7. **A**: control, **B**: simulated microgravity culture. Magnification: × 200.

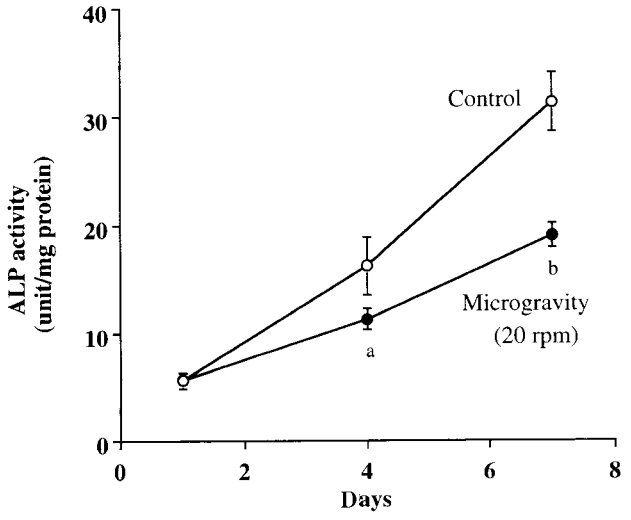

Fig. 3 Time-course changes in alkaline phosphatase (ALP) activity under control and simulated microgravity conditions. Each point is expressed as the mean ± SD from triplicate determinations. a: $P < 0.05$, b: $P < 0.005$.

conditions of microgravity, however, the ALP activity of the cells was 11.21 unit/mg protein on day 4 and 18.92 unit/mg protein on day 7, showing a statistically significant reduction compared with those of the controls (Fig. 3). The difference between the microgravity and the control cultures was more magnified on day 7 than on day 4. The addition of 10^{-7}M 1,25-dihydroxyvitamin D_3 (1, 25-$(OH)_2$-D_3) to the cultures increased the ALP activity of both populations of HuO9 cells, although the total activity of ALP in the cells treated with 1,25-$(OH)_2$-D_3 was still lower under simulated microgravity conditions than that in the control cells (Fig. 4). However, the cells under simulated microgravity conditions showed a greater enhancement of ALP activity by the treatment with 1, 25-$(OH)_2$-D_3 than the control cells. Figure 5 shows the relationship between the rate of clinostat rotation and the decrease in the ALP activity of the HuO9 cells under simulated microgravity. The decrease in ALP activity under these conditions was independent of the clinostat rotation rate (10 to 30 rpm).

No BGP was detected by IRMA in the culture

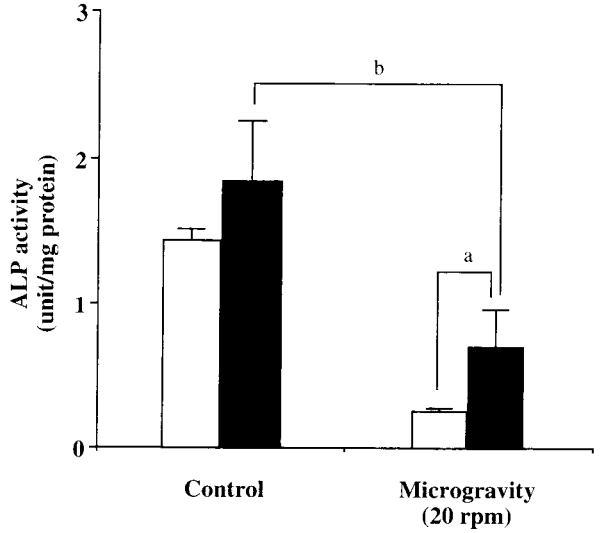

Fig. 4 Effects of 10^{-7}M 1,25-$(OH)_2$-D_3 on control and simulated microgravity cultures. ALP activity was measured on day 4. Open columns show cultures without 10^{-7}M 1,25-$(OH)_2$-D_3, and closed columns with 10^{-7}M 1,25-$(OH)_2$-D_3. The ALP activity is expressed as the mean ± SD from triplicate determinations. a: $P < 0.001$, b: $P < 0.05$. ALP: See legend to Fig. 3.

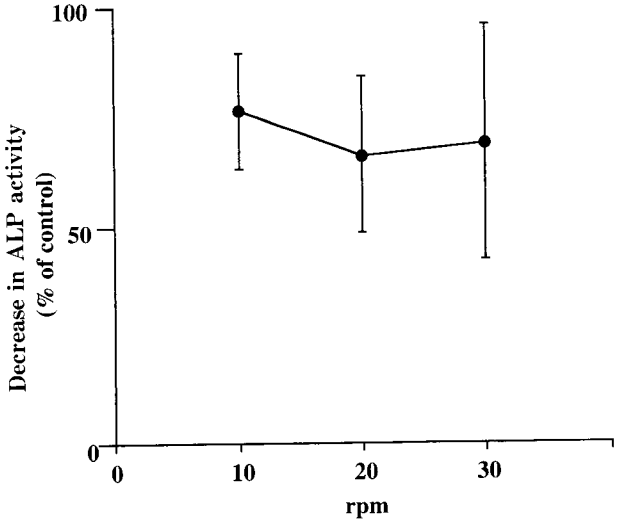

Fig. 5 Relationship between the rate of clinostat rotation and the decrease in ALP activity under simulated microgravity conditions. The data (mean ± SD) are expressed as a percentage of the control cultures. ALP: See legend to Fig. 3.

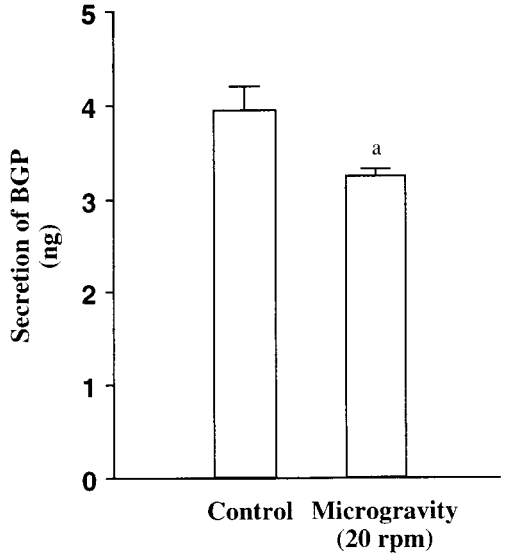

Fig. 6 Effects of simulated microgravity on the secretion of bone γ-carboxyglutamic acid-containing protein (BGP) into the culture medium in the presence of 10^{-7}M 1,25-$(OH)_2$-D_3. The BGP in the medium collected on day 4 was measured by IRMA. The data are expressed as the mean ± SD from triplicate determinations. a: $P < 0.01$.

medium in which the HuO9 cells had been cultured for 4 days, but when 10^{-7}M 1,25-$(OH)_2$-D_3 was added to the culture, the BGP secreted by HuO9 cells became detectable by IRMA. However, even in the presence of 10^{-7}M 1,25-$(OH)_2$-D_3, the secretion of BGP significantly decreased under microgravity conditions compared with that of the control culture (Fig. 6).

Discussion

Gravity is a universal strain on all organs. Physiological stress changes various characteristics of osteoblastic cells in culture. The physiological force of stretching increases the number of cultured bone cells synthesizing DNA (18). Compressive pressure decreases ALP activity and the mineralization of MC3T3-E1 cells (19). Hypergravity induced by centrifugal force activates ALP activity and the BGP synthesis of human osteoblast-like cells (3). However, the precise effects of gravity on bone metabolism are still unclear. Bed rest (1) and hind limb suspension (2), which induce simulated microgravity, result in a negative calcium balance and decreased bone formation. It is apparent that the skeletal system requires various strains to maintain its normal metabolic activities. We analyzed the effects of simulated microgravity on human osteoblast-like cells in culture using a horizontal clinostat system.

It is well known that ALP activity reflects bone formation and/or mineralization (20). Thus, ALP activity is considered to be a good marker of osteoblastic cell function in culture. In the present study, simulated microgravity decreased the ALP activity of HuO9 cells. This indicates that microgravity substantially affects one of the differentiated characteristics of osteoblastic cells.

Like ALP, BGP is related to bone formation and/or mineralization and is an osteoblast marker in culture. In this study, the secretion of BGP into the medium also decreased under simulated microgravity in the presence of 1,25-$(OH)_2$-D_3. During spaceflight, BGP content was found to be reduced in the humerus and vertebrae of rats (21). Other investigators found that a 10-day spaceflight decreased the mRNA levels of BGP in the long bone and calvarial periosteum of rats (22). The findings that BGP production of osteoblasts decreases under simulated microgravity suggest that microgravity inhibits some differentiated phenotypes and the functioning of osteoblasts.

The addition of 1,25-$(OH)_2$-D_3 increased ALP activity under microgravity and the cells under simulated microgravity conditions showed a greater enhancement of ALP activity by the treatment with 1,25-$(OH)_2$-D_3. In senile osteoporosis, 1,25-$(OH)_2$-D_3 is often useful and effective. Our results indicate that 1,25-$(OH)_2$-D_3 may be useful for preventing osteoporosis induced under microgravity.

Spaceflight affects the skeletal system morphologically, physiologically and biochemically. A statistically significant loss of bone mineral was found in Skylab astronauts (23). A decreased mass of mineralized tissue and an increased fat content of the bone marrow in the tibia and humerus of rats were detected after an 18.5-day spaceflight (24). These results may be due to mechanical unloading, but endocrine and other systemic factors cannot be ruled out. Spaceflight also results in a decreased secretion of biologically active growth hormone (25) and testosterone (26), which are known to affect bone and muscle metabolisms. In addition, changes in blood flow and/or fluid shifts may influence the musculoskeletal system. However, our results showing that simulated microgravity decreased the ALP activity and the secretion of BGP of osteoblast-like cells demonstrate that microgravity directly affects osteoblasts themselves and decreases some osteoblast functions.

Although many experiments have been performed in space to study the effects of microgravity on bone function, the precise metabolic behavior of bone cells during spaceflight has not yet been determined. Our experiments show that microgravity directly affects osteoblastic functions, resulting in decreased ALP activity and BGP secretion of osteoblasts. Further experiments using osteoblasts with more differentiated characteristics are necessary to learn how gravity influences osteoblast activity.

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