Yonsei Medical Journal
Vol. 42, No. 4, pp. 405 - 410, 2001

Original Article

Effect of Centrifugal Force on Cellular Activity of Osteoblastic MC3T3-E1 Cells *in vitro*

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The effects of centrifugal force on growth and differentiation of osteoblastic cells cultured in α-MEM containing 1% fetal bovine serum were investigated by assays of DNA synthesis, alkaline phosphatase activity and osteocalcin-production in osteoblastic MC3T3-E1 cells.

Centrifugation of the cells in low concentrations (1%) of fetal bovine serum caused a 1.9-fold increase of [^{3}H]thymidine incorporation on day 3 from the start of centrifugation, and gradually decreased with culture up to day 9. Alkaline phosphatase activity was not affected by centrifugal force until day 5, and increased rapidly after day 7. Stimulation of DNA synthesis by centrifugation was abolished in the presence of H-7, an inhibitor of protein kinase C. These results suggest that centrifugal force stimulates the proliferation of osteoblastic cells through an autocrine secretion of some diffusable growth-promoting activity. Additional centrifugation of the cells also slightly stimulated alkaline phosphatase activity, although this did not directly influence the cell's osteocalcin-production activity.

Key Words: Centrifugation, osteoblast, MC3T3-E1, alkaline phosphatase, DNA synthesis, fetal bovine serum

INTRODUCTION

Mechanical forces play an important role in the differentiation, growth, and remodeling of the skeleton. Prolonged space flight, bedrest, and immobilization all result in a negative calcium balance and an inhibition of bone formation.[1-4] On the other hand, mechanical stimulation of cultured bone cells leads to an increased intracellular cyclic AMP production and DNA synthesis.[5-7]

However, the cellular and molecular basis for these changes remains unclear. mRNA levels for type I collagen and osteopontin were up-regulated in chronic low frequency-strains of the OHS-4 osteosarcoma cell line[8] and β1-integrin mRNA was increased in human osteosarcoma TE-85 cells that were exposed to low frequency mechanical stimulation.[9] Mechanical loading of embryonic chick tibiotarsi that were maintained in an organ culture resulted in increased expression of type I collagen which was accompanied by an increase in alkaline phosphatase activity (ALP).[10]

In the lung, the fibroblast is a dynamic cell type. Fibroblasts, located in the interstitial space of the alveolar septal edges throughout the lung parenchyma and within the large vessels and airways, are high-collagen-producing cells. It has been hypothesized that pulmonary fibroblasts can sense changes in mechanical tension directly and they respond by altering the level of the gene expression of extracellular matrix (ECM). For example, the study by Berg et al.[11] indicates that a high lung inflation *in vivo* increases mRNA levels for ECM components and transforming growth factor-β1 in the outer parenchymal region of rabbit lungs. Furthermore, in an isolated perfused lung model, it has been demonstrated that increased airway pressures or lung overinflation results in increased mRNA levels of the ECM components α_1(I) and α_2(IV) procollagen and the laminin B chain.[12]

In this study, the effect of mechanical stress in osteoblasts was investigated. Understanding the mechanism behind changes in cellular activities in osteoblasts is important since this directly relates

Received June 8, 2001
Accepted July 18, 2001

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to the growth and healing of bones after fracture and may indicate the existence of a mechanical load sensing mechanism. In adult animals, bone formation and resorption are coupled. After immobilization or weightlessness, osteopenia occurs as a result of a relative decrease in bone formation and an increase in bone resorption. Both processes may actually be increased or decreased in absolute terms, but the net result is less bone formation than bone resorption. In the growing animal, bone formation exceeds bone resorption and may be less tightly coupled to it. In this case, immobilization or weightlessness results in bone loss which is caused primarily by the inhibition of bone formation. Therefore, activities of osteoblasts might be modulated by mechanical stresses. However, the mechanisms by which biomechanical forces change the cellular activities of osteoblasts remain to be elucidated.

In the present study, the effects of centrifugal force on proliferation and expression of the differentiated phenotype of clonal murine osteoblastic MC3T3-E1 cells were examined.

MATERIALS AND METHODS

Materials

Experimental materials were obtained as follows: Eagle's alpha minimum essential medium (α-MEM) was purchased from Sigma (St. Louis, MO, USA); fetal bovine serum (FBS) from Gibco (Grand Island, NY, USA); 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) from Sigma (St. Louis, MO, USA); and [^{3}H]thymidine from NEN Life Science Products (Boston, MA, USA).

Cell culture

MC3T3-E1 cells (RIKEN Cell Bank, Ibaraki, Japan) were inoculated in 96-well plates at a density of 2×10^4 cells/well and were maintained in α-MEM containing 10% FBS, antibiotic (100 U penicillin G/ml, 0.01 mg streptomycin/ml, 0.25 mg amphotericin B/ml in 0.85% saline), 20 mM L-glutamine, and 25 mM HEPES buffer at 37℃ in a humidified atmosphere (5% CO_2/95% air). The medium was changed every other day. Cultures were pre-incubated in α-MEM containing 1% FBS for 24 hrs.

Centrifugation

Centrifugal force was applied by a centrifuge (MF-550, Hanil, Inchun, Korea) that was equipped with a HM-2 micro titer rotor. The HM-2 rotor is a specially designed swing rotor for micro well plates. Application of centrifugal force was done by intermittent centrifugation (the cells were centrifuged for 10 min and returned for 7 hrs of culture, then the process was repeated). At the same time, control cultures were placed on the centrifuge apparatus, as a control for vibration effects.

[^{3}H]Thymidine incorporation

Cultures were applied with or without centrifugal forces (40 to 400g) and pulse-labeled with 10 μl of [^{3}H]thymidine (4×10^6 Bq/ml) for 4 hrs before the end of incubation.

Two groups of cultures were grown; one that was centrifuged at a centrifugal force of 40 to 400g and the other that wasn't. The tissue culture medium that they grew in was now considered to be conditioned and was transferred to a centrifuge tube and any precipitated debris was removed. Another group of cultures that were quiescent and confluent were prepared by pre-incubation in 0.2 ml of α-MEM containing 10% FBS for 24 hrs

Then the culture medium was replaced by 0.2 ml of α-MEM containing 50% conditioned medium and 1% FBS. All cultures were labeled with 10 μl [^{3}H]thymidine (4×10^6 Bq/ml) for 4 hrs. after addition of conditioned medium and grown for an additional 4 hrs. The cell layer was rinsed three times using an cold phosphate-buffered saline (PBS) and then homogenized with a electric blender (Biospec Products Inc., Switzerland) in 0.2 N NaOH/0.2% Triton X-100 at 4℃. The homogenate was treated with 10% trichloroacetic acid. The resultant precipitates were rinsed with ethanol/ethyl ether (3:1, v/v). The precipitates were solubilized in 0.2 N NaOH, and the radioactivity was measured by a liquid scintillation counter (LSC, 1450 MicroBeta TRILUX, Wallac, Truku, Finland).

Alkaline phosphatase activity

ALP activity was measured as described previously,[13] using p-nitrophenyl phosphate as the substrate. Briefly, cell layers were homogenized in 0.9% NaCl/0.2% Triton X-100. Aliquots were incubated with 0.5 M Tris-HCl (pH 9.0) containing 0.5 mM p-nitrophenyl phosphate and 1 mM $MgCl_2$ at 37℃ for 30 minutes. The absorbance of each well was measured at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The protein components of the treated cells were determined by the Lowrey method.[14] ALP specific activity was expressed as nmol p-nitrophenol (PNP) produced/min/mg protein.

Osteocalcin assay

Osteocalcin concentrations in conditioned medium samples were measured by radioimmunoassay using mouse osteocalcin reagents (Biomedical Technologies, Inc., MA, USA). The total DNA content of the confluent cells was determined by fluorometric assay as described.[15] In brief, the DNA concentration in 5% trichloroacetic acid-precipitated cell layers was determined against a calf thymus DNA standard using a fluorescence spectrometer (LS50B, Perkin-Elmer Ltd., Bucks, England) to measure the fluorescence emission at 500 nm during excitation at 408 nm. Osteocalcin production was expressed as nanograms of mouse osteocalcin in conditioned medium per microgram of cell DNA.

RESULTS AND DISCUSSION

The physiological significance of mechanical stress on bone metabolism has been shown by experiments *in vivo*.[16-19] However, it is difficult to evaluate a direct effect of mechanical force on bone metabolism because indirect effects exerted by other organs cannot be ruled out. Therefore, various kinds of mechanical forces have been applied to bone or cartilage cells in culture. These include a stretching force caused by a deformation of the culture dish,[8] the tensile force on collagen ribbons,[20] a hydrostatic compressive force,[21] and a centrifugal force.[22] Among these options, we chose to use the application of a centrifugal force on cultured cells which allows for a better control to be exercised. Fig. 1 shows the effect of centrifugation on DNA synthesis in MC3T3-E1 cells that were cultured using various serum concentrations. Cultures were pre-incubated in 0.2 ml of α-MEM containing 10% FBS for 24 hrs, and then the culture medium was replaced by α-MEM containing the stated concentration of FBS. Incorporation of [^{3}H]thymidine into DNA of the control cultures increased with the increase in serum concentrations (Data not shown). Centrifugation caused an increase in the incorporation of [^{3}H] thymidine into DNA compared with that of the controls that had only 1% FBS. However, this stimulatory effect could not be observed using 0 or 10% FBS. We compared the means ± SD of the two groups in three independent experiments.

The effect of centrifugal force on the expression of the differentiated phenotype of the cells was then examined. Cultures were pre-incubated for 24 hrs in 0.2 ml of α-MEM containing 10% FBS, and then the medium was replaced by 0.2 ml of α-MEM containing 0, 1, or 10% FBS. Centrifugation (40 to 400 g, 10 min) was performed three times daily immediately after replacement of the medium. Centrifugation increased the ALP activity compared with that of the controls that grew in 1% FBS (Fig. 1).

In the present study we found that the application of centrifugal force reproducibly enhanced DNA synthesis and ALP activity of MC3T3-E1 cells when the cells were stimulated by low concentrations (1%) of FBS. Since there was no significant increase in DNA synthesis and ALP activity of the cells in the absence of serum, the centrifugal force by itself did not trigger initiation of DNA synthesis and induction of ALP activity of quiescent MC3T3-E1 cells. When serum-growth factors that were sufficient for full stimulation of cellular growth were extracted from the culture medium containing 10% FBS, the application of centrifugal force resulted in no further increase of DNA synthesis and ALP-induction (Fig. 1). Thus, it is speculated that centrifugal force enhances DNA synthesis and ALP-induction of the serum-primed cells in a manner that compensates for insufficient serum availability.

The time course for DNA synthesis in centri-

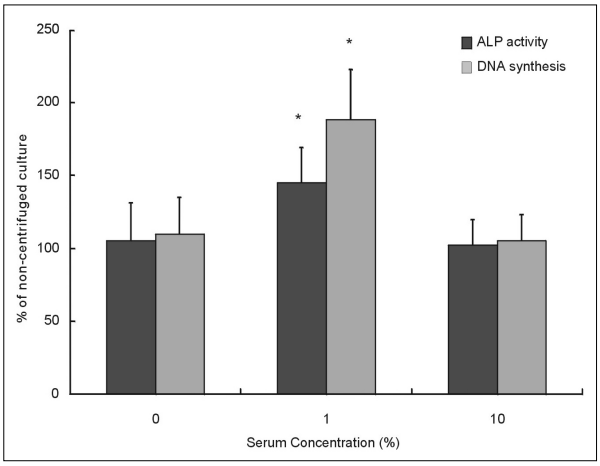

Fig. 1. Serum dependency of the effect of centrifugation on DNA synthesis and ALP activity. Confluent cultures were pre-incubated for 24 hrs in α-MEM containing 10% FBS, and then the culture medium was replaced by 0.2 ml of α-MEM containing the stated concentration of FBS. Values are mean ± SD for five wells. *p <0.005 vs. paired non-centrifuged culture.

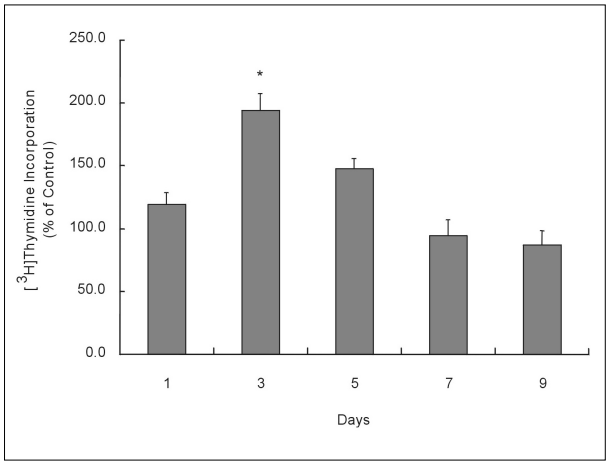

Fig. 2. Time course effect of centrifugation on DNA synthesis by cultured MC3T3-E1 cells. Confluent cultures were pre-incubated for 24 hrs in α-MEM containing 10% FBS, and then the culture medium was replaced by 0.2 ml of α-MEM with 1% FBS. Cultures were incubated with centrifugation at 400 g immediately after replacement of the medium. Values are the mean ± SD for five wells. *p <0.005 vs. paired non-centrifuged culture.

fugal force applied-MC3T3-E1 cells was examined. Cultures were pre-incubated in 0.2 ml of α-MEM containing 10% FBS for 24 hrs, and then the medium was replaced by 0.2 ml of α-MEM containing 1% FBS. Centrifugation (40 to 400 g, 10 min) was performed three times daily immediately after replacement of the medium. As summarized in Fig. 2, incorporation of [^{3}H]thymidine into DNA increased for 5 days (maximum at 3 days) and returned to control levels after 7 days of mechanical stimulation. There was only a 45% increase in [^{3}H]thymidine incorporation in mechanically stressed osteoblasts compared with control cells at 5 days, whereas there was a 1.9-fold increase at 3 days (Fig. 2). This stimulatory effect was increased by an increase in the centrifugal force applied (% of non-centrifuged culture at: 40 g, 111 ± 3; 100 g, 128 ± 11; 200 g, 164 ± 3; 400g, 194 ± 13; mean ± SD of three independent experiments). The early response to the mechanical load observed in the present study supports previous observations of an increased osteoblast proliferation that is seen both *in vivo* and *in vitro* following mechanical loading.[23,24]

As shown in Fig. 3, the stimulatory effect of centrifugal force on ALP activity reached a maximum at 100 g (ALP activity at: 40 g, 114.3 ± 8.3; 100 g, 125.5 ± 8.2; 200 g, 110 ± 10.8; 400 g, 92.5 ± 10.4; mean ± SD of three independent ex-

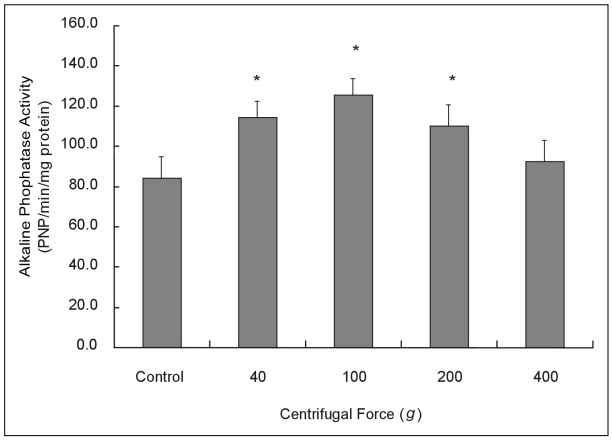

Fig. 3. The effect of centrifugal force on ALP activity of MC3T3-E1 cells. Confluent cultures were pre-incubated for 24 hrs in α-MEM containing 10% FBS, and then the culture medium was replaced by 0.2 ml of α-MEM with 1% FBS. Centrifugation (40 to 400 g, 10 min) was performed three times daily immediately after replacement of the medium. Values are mean ± SD for five wells. *p < 0.005 vs. non-centrifuged culture (control).

periments). In the time course effect of the centrifugal force experiment (centrifuged at 100 g in 1% FBS) on ALP activity, the centrifugation stimulated ALP activity by 1.5-fold greater than that of the control culture at 7 days (Fig. 4). Others have shown that dynamic cell stretching increased human osteoblast proliferation and carboxyter-

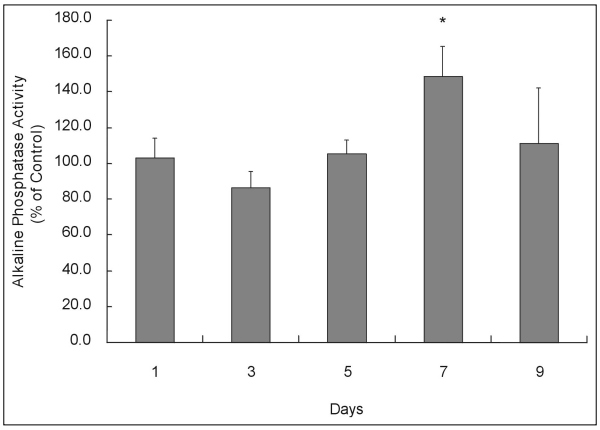

Fig. 4. The time course effect of centrifugation on ALP activity in cultured MC3T3-E1 cells. Confluent cultures were pre-incubated for 24 hrs in α-MEM containing 10% FBS, and then the culture medium was replaced by 0.2 ml of α-MEM with 1% FBS. Centrifugation (100 g, 10 min) was performed three times daily immediately after replacement of the medium. Values are mean ± SD for five wells. *p < 0.005 vs. paired non-centrifuged culture.

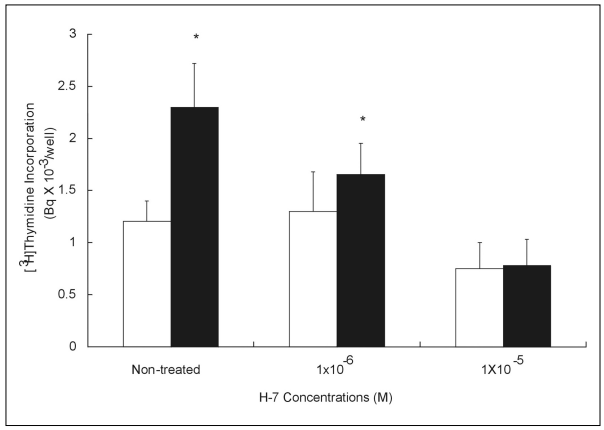

Fig. 5. The effect of H-7 on DNA synthesis of MC3T3-E1 cells. Confluent cultures were pre-incubated for 24 hrs in α-MEM containing 10% FBS, and then the culture medium was replaced by 0.2 ml of α-MEM containing the stated concentrations of H-7 in the presence of 1% FBS. Cultures were incubated with (■) or without (□) centrifugation at 400 g immediately after replacement of the medium. Values are mean ± SD for five wells. *p < 0.005 vs. paired non-centrifuged culture.

minal collagen type I propeptide synthesis but decreased osteocalcin synthesis and ALP activity.[25,26] However, our results demonstrated that mechanical stimulation increased DNA synthesis and enhanced ALP activity in the osteoblast cell lines. Nishioka et al.[27] reported that a cyclic stretching stimulated ALP actvity and collagenous protein synthesis. They claimed that these effects were regulated through prostagladin E2 production since this the stretch reduced the levels of prostaglandin E2 in the culture medium. When prostaglandin E2 was added exogenously, there was a significant decrease in ALP activity.

A further examination of osteocalcin production was studied in osteoblasts, which were subjected to an intermittent centrifugal force. Centrifugation for 1 to 9 days did not affect osteocalcin-production of the cells, regardless of the serum concentration (data not shown).

The stimulatory effect of centrifugation on DNA synthesis was inhibited by 10^{-6}-10^{-5} mol/L of H-7, which is a selective inhibitor of protein kinase (Fig. 5). These results suggested that possibility that activation of protein kinases mediated the effect of centrifugal force on DNA synthesis and a mechanical perturbation of the culture may be required at an early G1 phase in the cell-cycle progression of the cells, which exerts an active influence on an early growth-signal pathway.

Together these data suggest that our centrifuge system will be useful for the study of the effect of intermittent forces on osteoblast metabolism and growth. The present results also provide evidence that mechanical forces act directly on osteoblasts to modulate their proliferation and differentiation.

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