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PRELIMINARY REPORT

Effects of Vitamin B_{12} on Cell Proliferation and Cellular Alkaline Phosphatase Activity in Human Bone Marrow Stromal Osteoprogenitor Cells and UMR106 Osteoblastic Cells

G.S. Kim, C.-H. Kim, J.Y. Park, K.-U. Lee, and C.S. Park

Pernicious anemia has recently been recognized as one of the risk factors for osteoporosis and bone fractures, but the underlying pathophysiologic mechanism is still unknown. To determine whether vitamin B_{12} has any direct effect on osteoblasts, we studied the effects of vitamin B_{12} on the proliferation and alkaline phosphatase activity in human bone marrow stromal osteoprogenitor cells (hBMSC) and UMR106 osteoblastic cells. Vitamin B_{12} at concentrations as low as 10^{-12} mol/L significantly stimulated [^{3}H]-thymidine incorporation in both types of cells, but concentrations higher than 10^{-12} mol/L did not produce a greater effect. Vitamin B_{12} in the concentration range from 10^{-12} to 10^{-8} mol/L concentration-dependently increased alkaline phosphatase activity in both hBMSC and UMR106 cells. Based on these results, we suggest that a suppressed activity of osteoblasts may contribute to osteoporosis and fractures in patients with vitamin B_{12} deficiency.

PERNICIOUS ANEMIA has recently been recognized as a risk factor for osteoporosis and bone fractures,[1,2] but the mechanism of bone loss has not been established. Some investigators[3,4] have attributed it to long-standing calcium deficiency, since achlorhydria has been thought to impair the absorption of dietary calcium. But Eastell et al[1] showed that patients with achlorhydria had a normal fractional absorption of calcium. Melton and Kochman[5] recently reported a case of pernicious anemia with severe osteoporosis. The patient exhibited a dramatic response in bone density to treatment with vitamin B_{12} and cyclic etidronate therapy. Such dramatic increases in bone mineral density have not been reported in patients given etidronate therapy alone,[6] suggesting the possibility that vitamin B_{12} might have a direct effect on bone. Carmel et al[7] reported that serum levels of skeletal alkaline phosphatase and osteocalcin were low in vitamin B_{12}–deficient patients, and that these values were normalized after vitamin B_{12} replacement. In addition, they showed that alkaline phosphatase content in calvarial osteoblastic cells from chicken embryos is vitamin B_{12}–dependent, thus suggesting that vitamin B_{12} plays an important role in functional maturation of osteoblasts. However, there have been few, if any, studies on the effects of vitamin B_{12} on human osteoblastic cells. Furthermore, the effect of vitamin B_{12} on osteoblast proliferation is not yet established. Therefore, we studied the effects of vitamin B_{12} on the proliferation and alkaline phosphatase activity in human bone marrow stromal osteoprogenitor cells (hBMSC) and UMR106 rat osteoblastic osteosarcoma cells.

MATERIALS AND METHODS

hBMSC Culture

hBMSC were isolated from ribs obtained at the time of open thoracotomy. The ribs were excised aseptically, cleaned of soft tissues, and opened longitudinally. The exposed bone marrow was flushed several times using a culture medium. The medium with flushed bone marrow was centrifuged at 1,400 rpm for 10 minutes. Cell pellets were resuspended in the culture medium, and enriched bone marrow stromal cells were obtained by Ficoll-Hypaque (NYCOMED, Oslo, Norway) gradient centrifugation. The cells were seeded in a 75-cm^2 plastic culture flask at a density of 4×10^5 cells/cm^2 and cultured in alpha-modified minimum essential me-

From the Division of Endocrinology, Asan Medical Center, Seoul; and the Department of Physiology, University of Ulsan College of Medicine, Seoul, Korea.

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Address reprint requests to G.S. Kim, MD, Division of Endocrinology, Department of Medicine, Asan Medical Center, Song-Pa PO Box 145, Seoul 138-600, Korea.

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dium ([α-MEM] Sigma, St Louis, MO) containing 10% fetal bovine serum ([FBS] GIBCO, Grand Island, NY), and penicillin and streptomycin (100 U/mL and 100 μg/mL, respectively). The medium was changed twice per week from the second week on. When the bone marrow stromal cells were grown to approximately 80% confluency, they were subcultured using conventional techniques with 0.01% trypsin and 0.05% EDTA. All assays were performed after the second passage of the cells.

UMR106 Osteoblastic Cell Culture

UMR106 osteoblastic cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and penicillin-streptomycin until confluency. Cells were then subcultured using the same technique as described for hBMSC. Experiments were performed after four to five subcultures.

Proliferation Assay

Cell proliferation was assessed by measurement of [^{3}H]-thymidine (New England Nuclear, Boston, MA) incorporation. hBMSC and UMR106 osteoblastic cells were seeded in 24-well plates at a density of 2×10^4 cells/well. After 48 hours' culture of hBMSC in α-MEM supplemented with 10% FBS, the media were changed to vitamin B_{12}–free DMEM containing 2% FBS. Since [^{3}H]-thymidine incorporation of hBMSC was low in the absence of serum, the experiments were performed in the presence of 2% FBS in the medium. Vitamin B_{12} was added to the media to produce a final concentration ranging from 10^{-14} to 10^{-8} mol/L, and the cells were cultured for an additional 24 hours. [^{3}H]-thymidine was added to the media at 1.0 μCi/mL for the final 4 hours, and incorporation of [^{3}H]-thymidine into trichloroacetic acid–precipitable material was measured by standard methods.[8]

UMR106 osteoblastic cells in DMEM were cultured until 20% to 30% confluency. They were then washed with phosphate-buffered saline and cultured in serum-free, vitamin B_{12}–free DMEM containing 0.1% BSA with or without vitamin B_{12} for 24 hours. [^{3}H]-thymidine was added to the media at 0.5 μCi/mL for the final 90 minutes, and incorporation of [^{3}H]-thymidine was measured.

Cellular Alkaline Phosphatase Activity

UMR106 cells and hBMSC were seeded into 12-well plates at a density of 4×10^4 cells/well and grown to 30% confluency. The media were then changed with fresh serum-free, vitamin B_{12}–free DMEM containing 0.1% BSA with or without vitamin B_{12}. After 72 hours of culture, the medium was removed, and the alkaline phosphatase activity of the cell layer was measured by the *p*-nitrophenyl phosphate hydrolysis method.[9] The data were normalized to represent alkaline phosphatase activity as nanomoles per milligram protein per minute.

Statistical Analysis

Data were analyzed by ANOVA, and post hoc analysis by Duncan's multiple-range test was used to test for differences between means.

RESULTS AND DISCUSSION

Vitamin B_{12} deficiency has well-known hematologic and neurologic effects, but its effects on bone metabolism are less well documented. The present study shows that vitamin B_{12} has a direct stimulating effect on osteoblastic cells from both rats and humans. It should be pointed out that UMR106 cells are a rat osteosarcoma cell line that may respond to stimuli that normal osteoblasts may or may not respond to. In addition, the hBMSC are not mature osteoblasts. However, previous studies have shown that when cultured to confluence in the presence of serum, these cells possess many of the phenotypic characteristics of differentiated osteoblasts, including deposition of mineralized matrix.[10,11] They produce type I procollagen and osteocalcin in response to 1,25-dihydroxyvitamin D_3[10] and also increase cyclic adenosine monophosphate in response to parathyroid hormone.[11] With these limitations in mind, vitamin B_{12} at 10^{-14} mol/L did not have any significant effect, but did significantly stimulate both the proliferation and functional maturation as assessed by [^{3}H]-thymidine incorporation (Fig 1) and alkaline phosphatase activity (Fig 2), respectively, at 10^{-12} mol/L in both types of cells.

In agreement with a previous study by Carmel et al,[7]

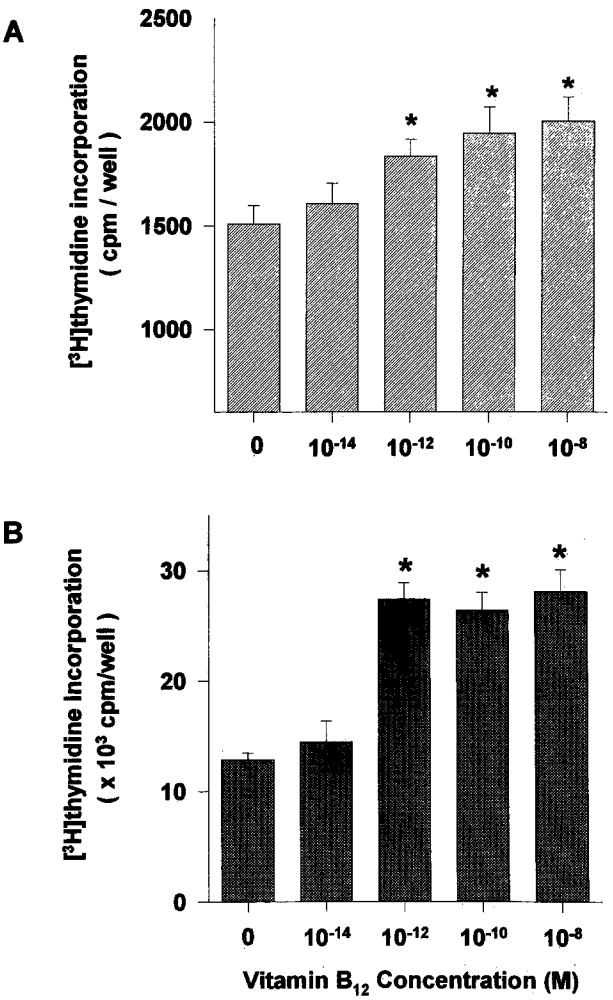

Fig 1. Effect of vitamin B_{12} on [^{3}H]-thymidine incorporation in hBMSC (A) and UMR106 osteoblastic cells (B). Values are the mean ± SE of 8 determinations (*$P < .001$ *v* control).

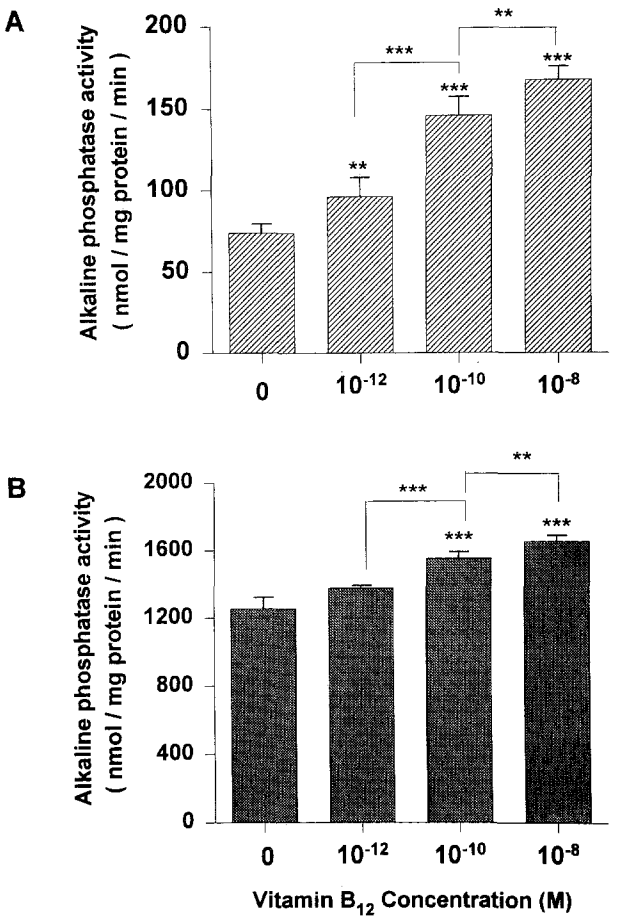

Fig 2. Effect of vitamin B_{12} on alkaline phosphatase activity in hBMSC (A) and UMR106 osteoblastic cells (B). Alkaline phosphatase activity is expressed as nmol/mg protein/min. Values are the mean ± SE of 6 determinations (P < .01, ***P < .001: v control).**

alkaline phosphatase activity was increased by vitamin B_{12} in a concentration-dependent manner (Fig 2). Since the normal serum concentration of vitamin B_{12} in humans is in the range of 1.5 to 6.6 × 10^{-10} mol/L, it can be speculated that serum vitamin B_{12} concentrations found in clinical vitamin B_{12} deficiency (< 1.0 × 10^{-10} mol/L) might be associated with defective functional maturation of osteoblasts.

The effect of vitamin B_{12} on osteoblast proliferation is still controversial. Carmel et al[7] reported that the total number of cells was not affected by vitamin B_{12}. In our study, we found a significant stimulating effect of vitamin B_{12} on osteoblast proliferation as measured by [^{3}H]-thymidine uptake (Fig 1). This discrepancy in results could be related to the differences in measurement methods, dosage of vitamin B_{12}, stage of cell differentiation, or cell source.

Nevertheless, our data show that osteoblast proliferation was stimulated at a low concentration of vitamin B_{12} (10^{-12} mol/L), but concentrations above this level did not further increase proliferation. Although the clinical meaning of this finding is not yet clear, it can be speculated that vitamin B_{12} at a minimum concentration is required to exert a permissive effect on osteoblast proliferation.

It has long been known that osteoporosis frequently occurs in patients following gastrectomy or gastric bypass procedures.[12-14] The pathogenesis of osteoporosis in these conditions is not clear although some investigators[3,4] have attributed it to long-standing calcium deficiency. Vitamin B_{12} deficiency is found in 10% to 80% of patients after gastrectomy.[12,15,16] Taken together with our in vitro findings, it is suggested that vitamin B_{12} deficiency may contribute to osteoporosis and bone fractures in conditions such as pernicious anemia, gastrectomy, or gastric bypass. Since vitamin B_{12} deficiency is also a common occurrence in the elderly population, with a prevalence up to 10%,[17] it is conceivable that such a deficiency may also contribute to the development of senile osteoporosis, at least in part.

In summary, our results have shown that vitamin B_{12} has significant and direct stimulating effects on the proliferation and alkaline phosphatase activity of both hBMSC and UMR106 cells. These results support the possibility that suppressed activity of osteoblasts under vitamin B_{12} deficiency may contribute to osteoporosis and bone fractures in patients with vitamin B_{12} deficiency.

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