Methotrexate (MTX) Inhibits Osteoblastic Differentiation *in Vitro:* Possible Mechanism of MTX Osteopathy

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ABSTRACT . ***Objective***. To clarify the mechanism of impaired bone formation during low dose methotrexate (MTX) therapy.

Methods. The *in vitro* effects of MTX on the function and differentiation of osteoblastic cells were investigated using (1) a mouse osteogenic cell line (MC3T3-E1) with the capacity to differentiate into osteoblastic or osteocytes, (2) a human osteoblastic osteosarcoma cell line (SaOS-2) with a mature osteoblastic phenotype, and (3) mouse bone marrow stromal cells containing osteoblast precursors. Osteoblast function was assessed by measuring the cellular activity of alkaline phosphatase (ALP) and the mineralization capacity of cultures.

Results. MTX suppressed ALP activity dose-dependently in growing MC3T3-E1 cells, but proliferation of these cells was only inhibited by a high concentration of MTX. In contrast, inhibition of ALP activity in MC3T3-E1 cells of mature osteoblastic phenotype was only observed with 10^{-8} M and 10^{-7} M MTX, and proliferation was not influenced. ALP activity and the proliferation of SaOS-2 cells were not inhibited by MTX, even when growing cells were treated. However, both ALP activity and formation of calcified nodules in bone marrow stromal cell cultures were significantly suppressed by MTX at concentrations between 10^{-10} and 10^{-7} M.

Conclusion. These results suggest that MTX suppresses bone formation by inhibiting the differentiation of early osteoblastic cells. (J Rheumatol 2001;28:251–6)

Key Indexing Terms:
METHOTREXATE OSTEOPATHY OSTEOBLAST DIFFERENTIATION

Methotrexate (MTX) is a folate antagonist that has been widely used in the treatment of rheumatoid arthritis (RA). MTX osteopathy, which is characterized by bone pain, osteoporosis, and fractures, is well recognized to occur in association with high dose therapy for the treatment of childhood malignancy[1-3]. However, Preston has also reported 2 cases of osteopathy, including fractures, in postmenopausal women taking low dose MTX for rheumatic disease[4]. In addition, a recent study showed that prolonged low dose MTX therapy can cause osteopenia in animals by increasing bone resorption and decreasing bone formation[5–8]. Although several clinical studies have shown that low dose MTX therapy does not induce generalized bone loss[9-11], there have been increasing reports of osteopathy associated with low dose MTX[12,13]. In addition, bone biopsy specimens taken from patients with MTX osteopathy have revealed decreased bone formation[4].

To clarify the mechanism underlying impaired bone formation during low dose MTX therapy, we investigated the *in vitro* effects of MTX on the function and differentiation of osteoblastic cell lines and bone marrow osteoblast precursors.

MATERIALS AND METHODS

Methotrexate. Purified MTX was a generous gift from Laderle Japan (Wyeth Laderle Japan, Tokyo, Japan), and was dissolved in phosphate buffered saline (pH 7.4) before use. MTX was added to cell cultures at concentrations between 10^{-10} and 10^{-7} M because a previous study indicated that a concentration of 0.6 µM was similar to the serum levels attained in patients following standard treatment for RA[5,14].

Osteoblastic cells. MC3T3-E1 mouse osteoblastic cells. The mouse osteoblastic clone MC3T3-E1 is an osteogenic cell line with the capacity to differentiate into osteoblasts or osteocytes *in vitro*[15]. MC3T3-E1 cells were generously provided by Dr. H. Kodama (Ohu University, Koriyama, Japan) and were seeded into 96 well culture plates at a density of 2×10^3/well in 200 µl of α-minimal essential medium (αMEM) supplemented with 5% fetal bovine serum (FBS), a 1% penicillin-streptomycin mixture, 50 µg/ml ascorbic acid, and 10 mM ß-glycerophosphate. Culture was done in a 5% CO_2 atmosphere at 37°C and the medium was changed every 3 days. To investigate the effect of MTX on the cells at different stages of differentiation, it was added to cultures during the growth phase or after confluence (5 days and 8 days after seeding, respectively).

SaOS-2 human osteoblastic osteosarcoma cells. SaOS-2 is a clonal human osteosarcoma cell line with a mature osteoblastic phenotype that has a high

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alkaline phosphatase (ALP) activity and shows a high level of collagen synthesis[16]. The cells were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan) and were cultured in McCoy's medium supplemented with 5% FBS and antibiotics. Cells were cultured for 5 days until a semiconfluence and then were cultured for 2 more days in the presence of MTX.

Bone marrow stromal cells. Bone marrow stromal cells were isolated from 6-week-old female Lewis rats (Nippon SLC Co., Kanagawa, Japan), as described[17,18]. Briefly, the bilateral femora were resected and the bone marrow was flushed out into a 15 ml conical culture tube with 1 ml of αMEM using an 18 G needle. The harvested marrow was filtered through a stainless steel mesh (50 µm pore size) and then was centrifuged at 1000 rpm for 5 min.

The cell pellet was resuspended in fresh αMEM and the number of viable cells was counted in a hemocytometer using the trypan-blue dye exclusion technique. Then the cells were seeded in a 25 cm^2 culture flask at a density of 1×10^6/ml and were cultured in αMEM supplemented with 5% FBS, antibiotics, 50 µg/ml ascorbic acid, and 10 mM ß-glycerophosphate. Adherent cells were harvested before confluence and were used as marrow stromal cells[19].

Cellular proliferation. Cellular proliferation was assessed by the MTT [3-(4,5-dimethyl thial-2-yl)-2,5-diphenyl-tetrazolium bromide] assay (Sigma Chemical Co., St. Louis, MO, USA). At the end of culture, 10 µl of MTT solution was added to each well and the cells were incubated for 2 h at 37°C. After aspiration of the supernatant, solvent (0.04 N-HCl and 90% isopropyl alcohol) was added to dissolve the crystals and the absorbance was measured at 595 nm on a microplate reader (Benchmark microplate reader, Bio-rad Laboratories, Japan).

Markers of osteoblastic function. ALP activity. ALP activity was assessed by a calorimetric assay using *p*-nitrophenyl phosphate as the substrate. Briefly, the cells were incubated with 0.1 M aminoethanol solution (pH 10.5) containing 150 mM NaCl, 2 mM $MgCl_2$, and 2.5 mg/ml of *p*-nitrophenyl phosphate for 1 h at 37°C. Production of *p*-nitrophenol was quantified by measuring the absorbance at 405 nm using a microplate reader.

Formation of calcified nodules. Bone marrow stromal cells were plated into a 48 well culture plate at a density of 4×10^3 cells/well and were grown in αMEM supplemented with 10% FBS, 10^{-6} M dexamethasone, 10 mM ß-glycerophosphate, and 50 µg/ml ascorbic acid. After overnight culture, the cells were cultured for a further 2 weeks in the presence or absence of MTX. Then the cells were fixed in 10% formaldehyde and stained with alizarin red to detect calcified tissue, after which the red-stained calcified nodules were counted under a stereomicroscope.

Statistical analysis. Differences from control values were determined by one-way analysis of variance and subsequent Fisher's least significant difference multiple-comparison test. The probability value of $p < 0.05$ was taken to indicate statistical significance.

RESULTS

Effect of MTX on MC3T3-E1 cells. Since MC3T3-E1 cells differentiate into mature osteoblasts during culture, ALP activity was not detected for 3 days after seeding, but increased rapidly thereafter. When MTX was added to cultures at 5 days after seeding (when cells were in the logarithmic growth phase), concentration dependent suppression of ALP activity was observed and significant suppression was obtained at a concentration as low as 10^{-10} M (Figure 1A). In contrast, suppression of ALP activity was only observed at higher MTX concentrations (10^{-8} and 10^{-7} M) when the cells were cultured for 8 days (near confluence) before exposure to the drug (Figure 1B).

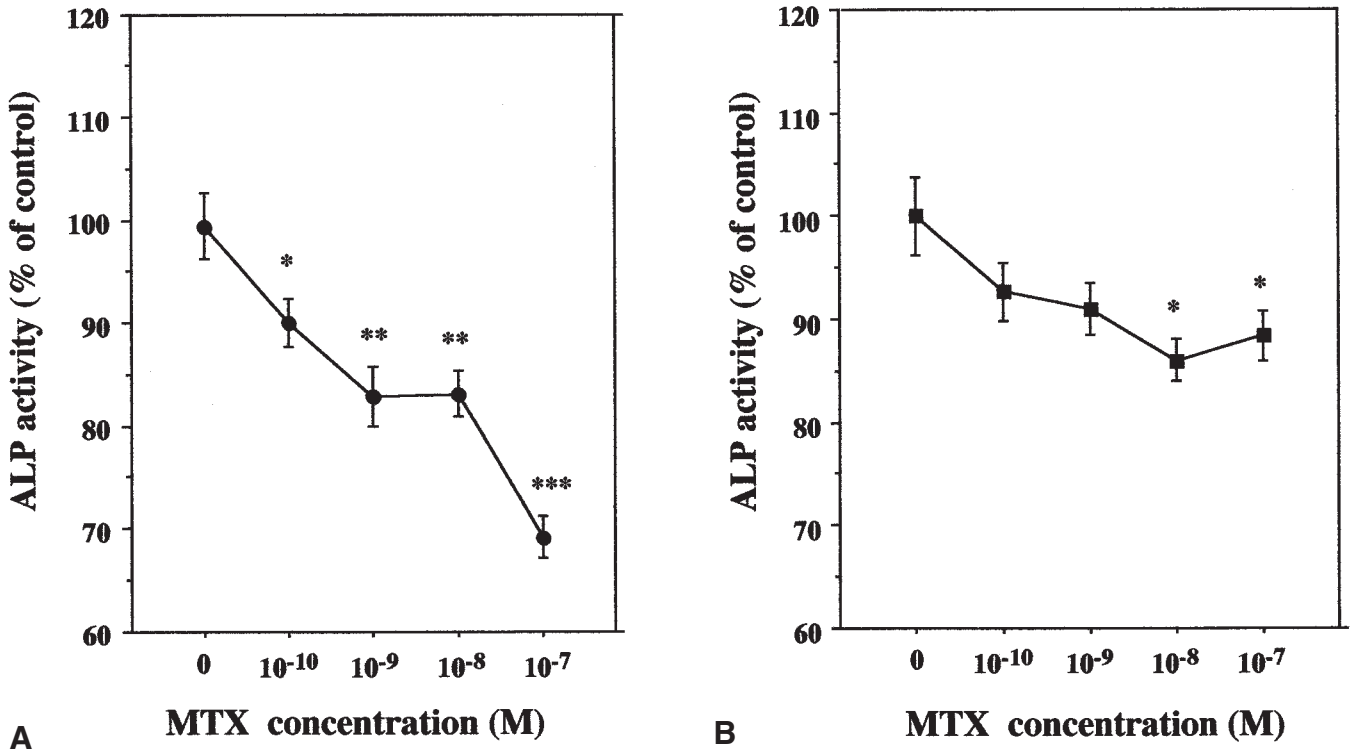

Figure 1. Effect of methotrexate (MTX) on alkaline phosphatase (ALP) activity in mouse osteoblastic MC3T3-E1 cells at different stages of differentiation. Concentration dependent suppression of ALP activity was observed when MTX was added to the cells in the logarithmic growth phase (A). Suppression of ALP activity in cells near confluence was only observed at higher MTX concentrations (10^{-8} and 10^{-7} M) (B). Data are expressed as the mean ± SEM; *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ vs control.

Proliferation of growing MC3T3-E1 cells was also inhibited by MTX, but only at the highest concentration of 10^{-7} M (Figure 2A). MTX did not inhibit the proliferation of MC3T3-E1 cells in the confluent state even at a high concentration (Figure 2B).

Effect of MTX on SaOS-2 cells. SaOS-2 cells are human osteosarcoma cells with an osteoblastic phenotype, so ALP activity could be detected on the day after seeding. MTX concentrations ranging from 10^{-10} to 10^{-7} M had no inhibitory effect on either the proliferation or ALP activity of these cells (Figure 3A, B).

Effect of MTX on ALP activity and mineralization in marrow stromal cell cultures. Using marrow stromal cells, the effect of MTX on 2 specific markers of osteoblasts was investigated. ALP activity was used as a marker of early osteoblast differentiation, and mineralizing capacity was used as a marker of late differentiation[15]. When marrow stromal cells were cultured in the presence of ß-glycerophosphate, ascorbic acid, and dexamethasone, most of the cells showed ALP activity. MTX significantly inhibited ALP activity at concentrations ranging from 10^{-10} to 10^{-7} M (Figure 4A). When formation of calcified nodules was observed in 14 day cultures of marrow stromal cells under the same conditions (Figure 5), the number of calcified nodules was significantly decreased in a dose dependent fashion by the addition of MTX (Figures 4B and 5).

DISCUSSION

Low dose MTX is considered to be a useful treatment for RA, because it rapidly suppresses joint inflammation, pain, and stiffness and is able to significantly retard bone destruction[20,21]. Generalized osteoporosis is well documented to occur in RA. Regarding the mechanism of this osteoporosis, 3 major factors have been proposed: (1) systemic rheumatoid inflammation, (2) immobility, and (3) drugs such as corticosteroids[21-28]. Since rapid suppression of rheumatoid inflammation by MTX allows an increase of physical activity, this drug might potentially improve osteoporosis associated with RA. However, a deleterious effect of MTX on bone has been reported in animal models and in patients with RA. Sally, *et al* first reported 2 cases of MTX osteopathy with fractures in patients with rheumatic disease receiving longterm treatment with low dose MTX[4]. There has been an increasing number of reports about osteopathy during low dose MTX therapy[12,13]. Although glucocorticoids have a predilection for cancellous bone, MTX induced bone loss and fractures occurred predominantly in cortical bone[13]. Histological examination of patients with rheumatic disease has shown that MTX osteopathy features decreased bone formation characterized by a decreased osteoblast surface and a decreased mineral apposition rate. In addition, May, *et al* reported that low dose MTX causes osteopenia in normal and ovariectomized animals by decreasing bone formation and increasing bone resorption[5]. However, the

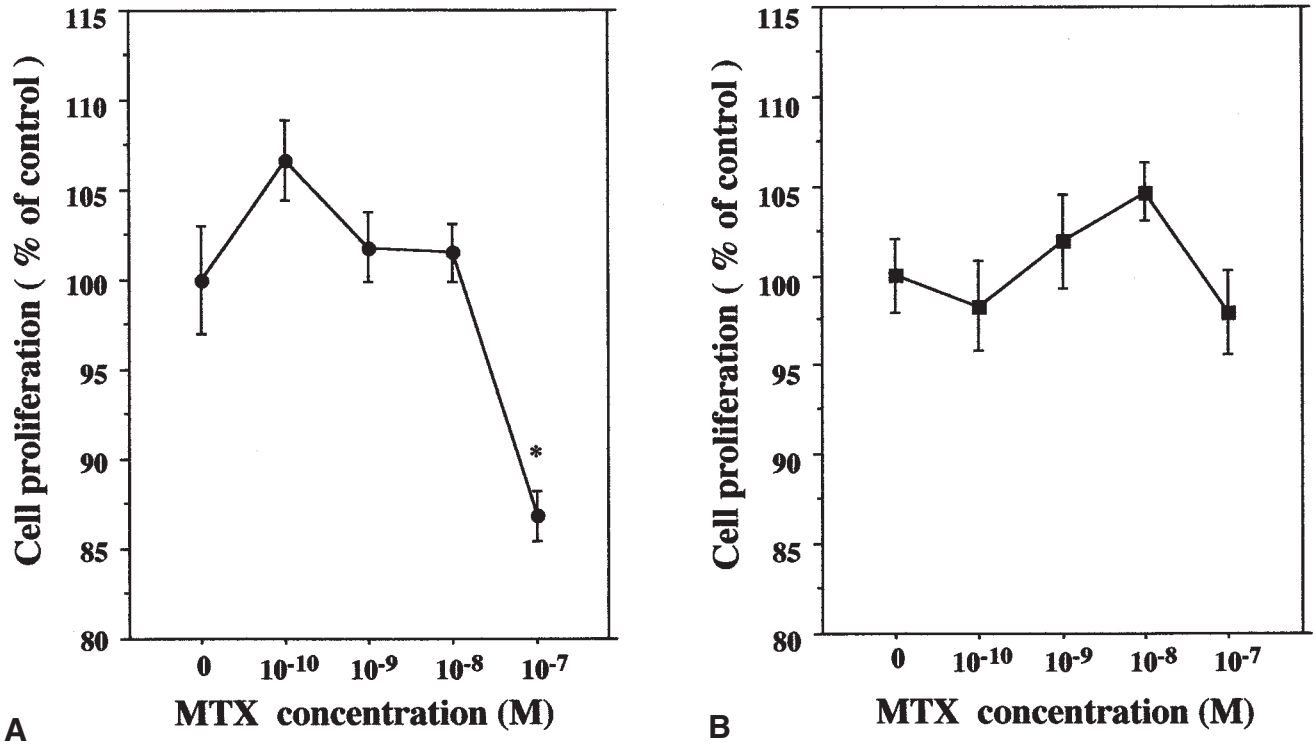

Figure 2. Effect of methotrexate (MTX) on the proliferation of mouse osteoblastic MC3T3-E1 cells at different stages of differentiation. MTX did not inhibit proliferation of MC3T3-E1 cells, except when cells in the growth phase were exposed to the highest concentration of MTX. Data are expressed as the mean ± SEM; *p < 0.0001 vs control.

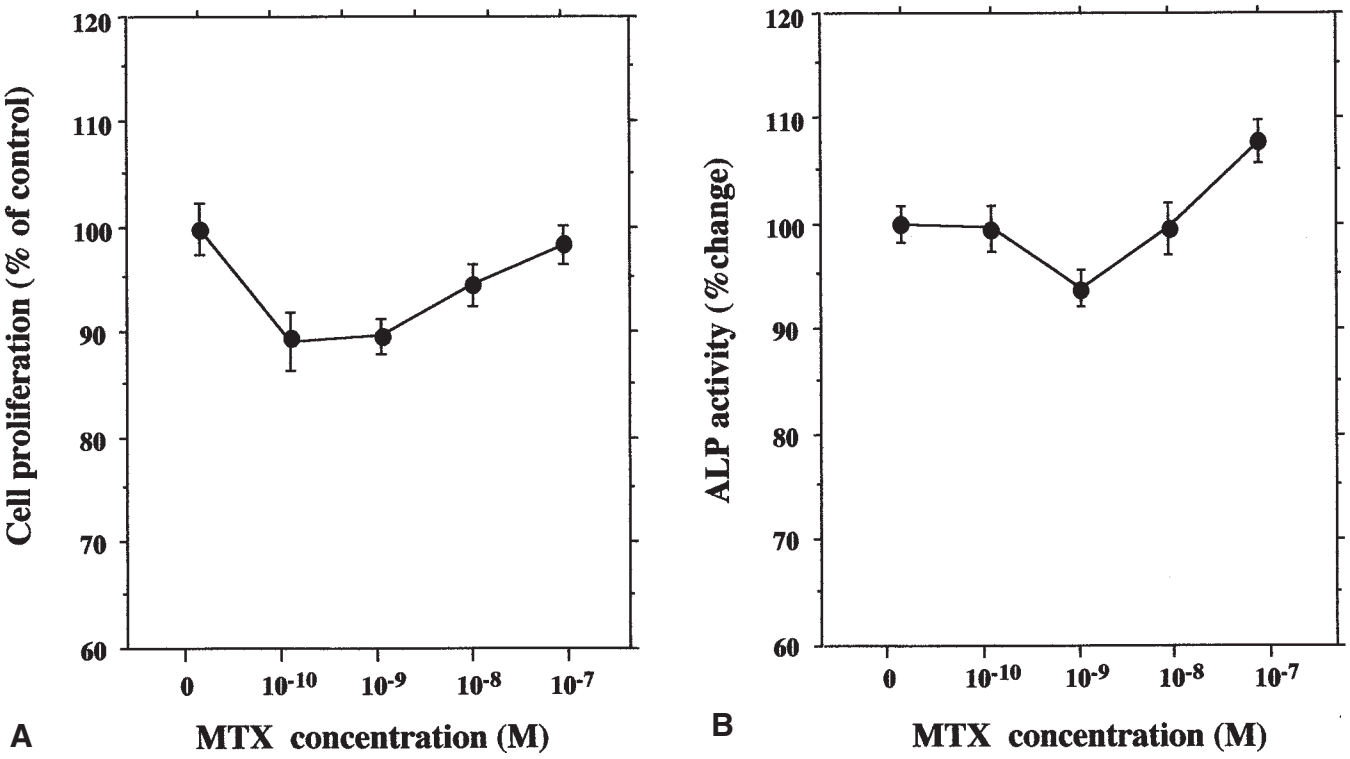

Figure 3. Effect of methotrexate (MTX) on the proliferation (A) and alkaline phosphatase (ALP) activity (B) of SaOS-2 human osteoblastic osteosarcoma cells. No suppression of ALP activity or cell proliferation was observed. Data are expressed as mean ± SEM.

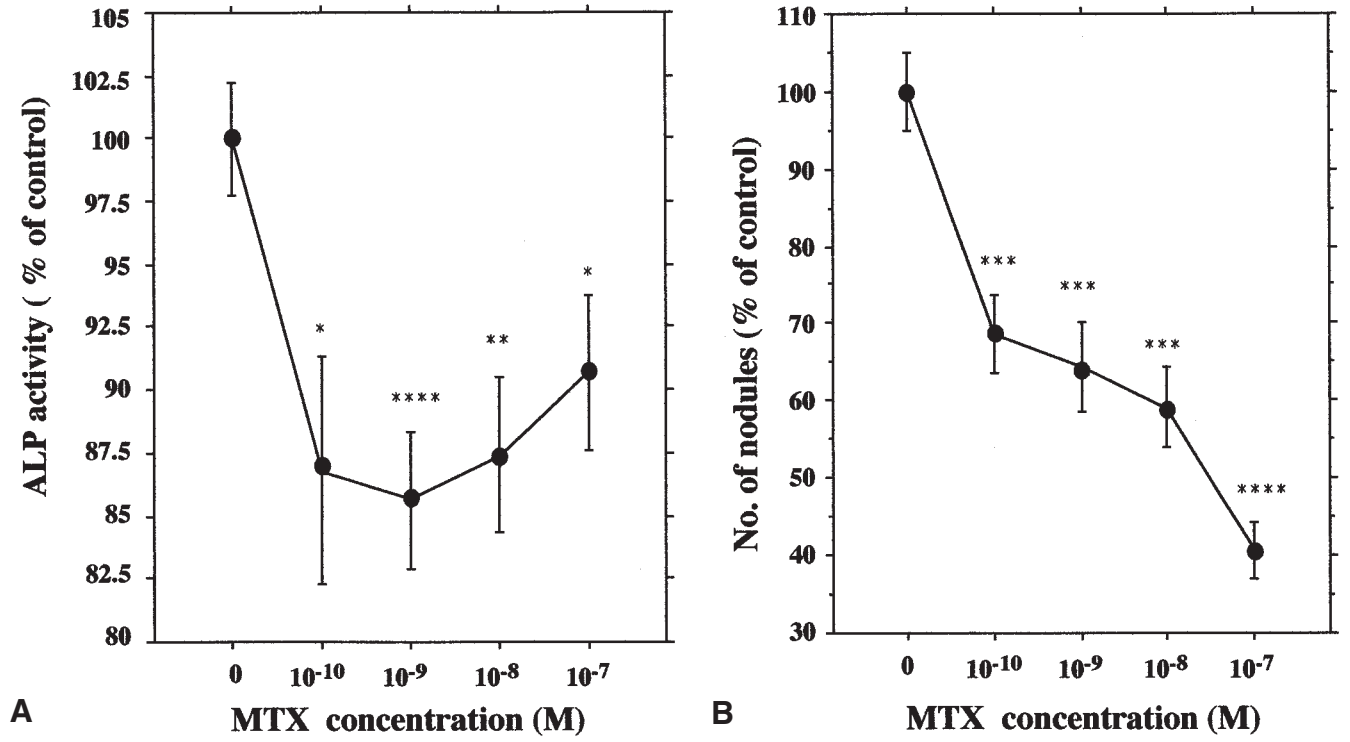

Figure 4. Effect of methotrexate (MTX) on alkaline phosphatase (ALP) activity (A) and formation of calcified nodules (B) in bone marrow stromal cell cultures. MTX inhibited both ALP activity and mineralization in a concentration dependent fashion. Data are mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs control.

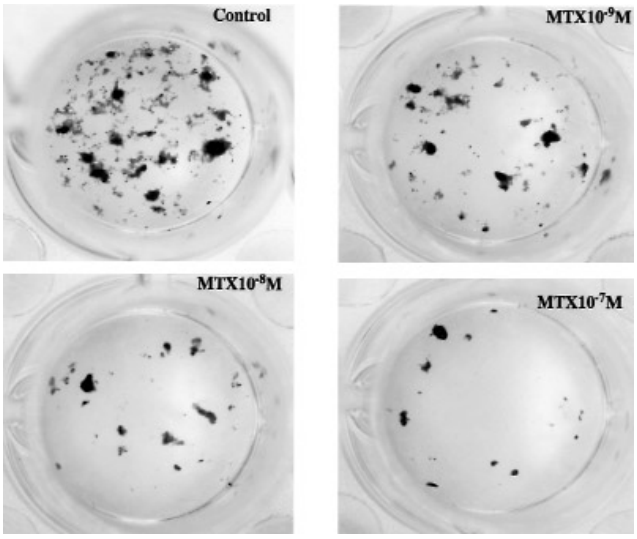

Figure 5. Calcified nodules formed by cultured rat bone marrow cells in the presence or absence of methotrexate (MTX). MTX caused a concentration dependent decrease of nodule formation.

precise mechanism by which MTX decreases bone formation has been unclear.

Our study observed an inhibitory effect of MTX on the differentiation of MC3T3-E1 cells and marrow osteoblast precursor cells. MC3T3-E1 cells have the capacity to differentiate into osteoblasts or osteocytes[29]. ALP activity was undetectable in these cells for the initial 2 days after seeding, but increased gradually as the culture period became longer. By about 8 days after seeding, most of the cells expressed ALP activity. In this study, MTX inhibited ALP activity when it was added to cultures on the 5th day after seeding, but the inhibitory effect on 8-day cells was very weak. Similarly, no suppression of ALP in mature osteoblastic SaOS-2 cells was observed. In addition, MTX strongly inhibited ALP activity and the formation of calcified nodules in cultures of marrow stromal cells. These results suggest that MTX affects early osteoblastic cells that do not express ALP and inhibits their differentiation into osteoblasts, but has little influence on either the function or proliferation of late-stage osteoblasts.

The effects of MTX on osteoblastic cells have been reported previously by May, *et al*[5] and Preston, *et al*[7]. Osteoblasts show phenotypic differences according to the stage of maturation[30]. May, *et al* used mouse osteoblastic cells that expressed ALP and osteocalcin and had the ability to calcify matrix, suggesting these cells were in the final stage of differentiation. On the other hand, Preston, *et al* used clonal rat osteosarcoma (UMR-106) cells that expressed ALP, but not osteocalcin, suggesting an earlier stage of differentiation[6]. MTX did not inhibit ALP activity in either study. These results are consistent with our findings that MTX has little effect on the ALP-expressed osteoblastic cells. However, we cannot exclude the possibility that MTX also suppresses mature osteoblasts, since its inhibition of matrix mineralization by MTX was observed by May, *et al* using terminally differentiated osteoblasts[6].

Recently, the transcription factor Cbfa1 has been reported to be an important regulator of osteoblastic differentiation[29]. Inhibition of Cbfa1 is thought to be one of the mechanisms of glucocorticoid induced osteoporosis[31]. Therefore, study of the effect of MTX on Cbfa1 expression by osteoprogenitor cells may be helpful for understanding the molecular mechanism of MTX osteopathy.

Since MTX rapidly suppresses rheumatoid synovitis and decreases the production of proinflammatory cytokines, thus improving physical activity, this drug may also exhibit a beneficial effect on the bones in patients with active RA. We previously reported that short term MTX had a favorable effect on abnormal bone metabolism and osteopenia in rats with adjuvant induced arthritis. Some recent studies have also shown that there is no deleterious effect of MTX on axial bone mass[10,32,33]. However, patients usually continue to take disease modifying antirheumatic drugs, including MTX, even after achieving remission of RA. Although there are limitations to generalizing from the results of our *in vitro* study to the clinical situation, longterm MTX therapy might suppress osteoblastic differentiation followed by a decrease in bone formation and therefore increase the risk of osteopathy in such patients. Accordingly, a longitudinal study is necessary to elucidate the longterm effects of MTX on the density of both axial and limb bones.

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