Methotrexate Suppresses Inflammatory Agonist Induced Interleukin 6 Synthesis in Osteoblasts

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ABSTRACT. ***Objective.*** Interleukin 6 (IL-6) is a pleiotropic cytokine that plays a crucial role in the pathogenesis of rheumatoid arthritis (RA). In bone metabolism, it is known that IL-6 is produced and secreted by osteoblasts, and that IL-6 induces osteoclast formation and stimulates bone resorption. Various bone inflammatory agonists such as tumor necrosis factor-α (TNF-α), IL-1α, prostaglandin D_2 (PGD_2), PGE_2, and $PGF_{2\alpha}$, which play important roles in the pathogenesis of RA, induce IL-6 synthesis in osteoblast-like MC3T3-E1 cells. Low dose methotrexate (MTX) is currently used for treatment of patients with RA. We investigated the effect of MTX on IL-6 synthesis induced by these agents in MC3T3-E1 cells.

Methods. Cultured cells were pretreated with various doses of MTX, and then stimulated by these inflammatory agonists. The IL-6 in the conditioned medium was measured by IL-6 enzyme immunoassay.

Results. MTX significantly suppressed IL-6 synthesis stimulated by these agonists in a dose-dependent manner, although MTX alone had no effect on the levels of IL-6. In addition, MTX significantly inhibited the enhancement by IL-17 of TNF-α-stimulated IL-6 synthesis. MTX reduced the levels of IL-6 induced by 12-*O*-tetradecanoylphorbol 13-acetate, a direct activator of protein kinase C (PKC), suggesting that MTX inhibits PKC signals for IL-6 synthesis.

Conclusion. MTX suppresses IL-6 synthesis stimulated by various inflammatory agonists in osteoblasts. (J Rheumatol 2005;32:787–95)

Key Indexing Terms:
METHOTREXATE INTERLEUKIN 6 OSTEOBLAST
RHEUMATOID ARTHRITIS INFLAMMATION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflamed synovial hyperplasia and cartilage destruction with excessive inflammatory cell infiltration[1]. In RA, various cytokines[2] or arachidonates[3] act as mediators and/or modulators of inflammation and joint destruction. Among them, tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1) are well recognized as inflammatory cytokines in the pathogenesis of RA[2]. Recent therapeutic interventions, including TNF-α antibodies and IL-1 receptor antagonists, strongly support the importance of these cytokines in RA[2]. It has been reported that IL-17, a T cell derived cytokine, contributes to the pathogenesis of RA, and shows additive or even synergistic effects with TNF-α and IL-1 in inducing joint pathology[4]. It is generally recognized that prostaglandins (PG) act as local modulators in osteoblasts and play a crucial role in the regulation of bone metabolism[5]. PG have been shown to be important mediators for inflammatory joint disorders such as RA[3,6].

Several disease modifying antirheumatic drugs are used for patients with RA[1]. Recently, methotrexate (MTX) has gained an important place among them for RA[7]. MTX, as a folate antagonist, was developed for the treatment of malignancies[8], and is useful for autoimmune inflammatory diseases such as RA in low dosage[9,10]. Antiinflammatory mechanisms for MTX have been reported mainly in the synovial cells and inflammatory cells: i.e., reduction of immunoglobulin[11], suppression of neutrophil chemotaxis[12], inhibition of IL-1 activity[13], increased adenosine release[14], inhibition of cyclooxygenase-2 (COX-2) activation[15], and suppression of lymphocyte proliferation[16]. Recently, it was reported that MTX inhibited IL-6 production by lipopolysaccharide (LPS) activated peripheral blood mononuclear cells (PBMC) obtained from patients with juvenile RA[17]. However, the mechanism underlying MTX induced suppression of IL-6 synthesis has not been precisely clarified; as well, the effect of MTX on osteoblasts around the diseased joint in RA remains to be elucidated.

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Bone metabolism is strictly regulated by osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption, respectively[5]. It is well known that receptor activator nuclear factor-κB ligand (RANKL) expressed on osteoblasts plays a pivotal role to transduce an essential differentiation signal to osteoclast lineage cells through binding to its receptor, RANK, expressed on the latter cells[18]. In addition, it has been reported that cytokines stimulating osteoclastogenesis, such as IL-1, IL-6, IL-11, IL-17, and TNF-α, increase the expression of RANKL with decrease of osteoprotegerin expression in osteoblast/stromal lineage cells[18].

IL-6 is a pleiotropic multifunctional cytokine that regulates diverse cell functions[19,20], and it has been reported that IL-6 stimulates bone resorption and induces osteoclast formation[21,22]. We have shown that TNF-α[23], IL-1[24], PGD_2[25], PGE_2[26], and $PGF_{2\alpha}$[27] stimulate IL-6 production and its secretion in cultured osteoblasts. Thus, accumulating evidence suggests that IL-6 secreted from osteoblasts plays an important role in bone resorption as a downstream effector of a variety of bone resorptive agents.

We investigated the effect of MTX on the synthesis of IL-6 induced by the various agonists such as TNF-α, IL-1α, PGD_2, PGE_2, and $PGF_{2\alpha}$ that have been shown to strongly affect the pathogenesis of RA in osteoblast-like MC3T3-E1 cells. We observed that, although MTX has little effect on IL-6 synthesis, it suppresses IL-6 synthesis induced by these agents.

MATERIALS AND METHODS

MTX was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). TNF-α and IL-1α were purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan). PGD_2, PGE_2, $PGF_{2\alpha}$, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse IL-6 and vascular endothelial growth factor (VEGF) enzyme immunoassay kits and IL-17 were obtained from R&D Systems Inc. (Minneapolis, MN, USA). Other materials and chemicals were obtained from commercial sources. PGD_2, PGE_2, and $PGF_{2\alpha}$ were dissolved in ethanol. TPA was dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of ethanol or DMSO was 0.1%, which did not affect the assay for IL-6 and VEGF.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria[28] were maintained as described[29]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells (5×10^4 cells) were seeded into 35 mm diameter dishes in 2 ml of α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 2 ml α-MEM containing 0.3% FCS. Cells were used for experiments after 48 h.

Primary culture mouse osteoblasts were prepared from neonatal balb/c mouse as described[30]. In brief, primary osteoblastic cells were prepared from calvariae of 2-day-old neonatal balb/c mice by digesting them with an enzyme solution containing 0.1% collagenase (Wako Pure Chemicals, Osaka, Japan) and 0.2% dispase (Godo Shusei, Tokyo, Japan). The isolated cells were pooled and seeded into 90 mm dishes in α-MEM containing 10% FCS at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells (5×10^4 cells) were seeded into 35 mm dishes in 2 ml α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 2 ml of α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

Assay for IL-6. The cultured cells were pretreated with various doses of MTX for 1 h, and then stimulated by the indicated doses of various agonists or vehicle in 1 ml α-MEM containing 0.3% FCS for the indicated periods. The conditioned medium was collected, and IL-6 in the medium was measured using an IL-6 enzyme immunoassay kit.

Assay for VEGF. Cultured cells were pretreated with various doses of MTX for 1 h, and then stimulated by the indicated doses of various agonists or vehicle in 1 ml α-MEM containing 0.3% FCS for the indicated periods. The conditioned medium was collected, and VEGF in the medium was measured by VEGF enzyme immunoassay kit.

Assay for cell viability. Cell viability was assessed as a function of NADH content using a TetraColor One [5 mM (2-(2-methoxy-4-nytrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt); 0.2 mM 1-methoxy-5-methylphenazinium methylsulfate; and 150 mM NaCl]-based assay according to the manufacturer's instructions (Seikagaku Inc., Nihonbashi, Tokyo, Japan)[31]. The cells (1.5×10^3 cells/well) were seeded into 96-well tissue culture plates in 100 µl of α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 100 µl of α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. Then some cells were pretreated with 1 µM of MTX for 1 h and then stimulated by 10 µM PGD_2 for 48 h; the other cells were not. After that, each well was washed once with α-MEM, and then 100 µl of α-MEM was added to each well without any supplement and incubated for 16 h. Finally, 10 µl of Tetracolor One solution was added to each well, and the cells were incubated for 1.5 h. A well for the negative control was prepared as described above without cells. The absorbance of each well was then determined at a wavelength of 450 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA expression. Cells were treated with 1 µM MTX for 1 h. Total RNA was isolated from the cells using Isogen (Nippon Gene, Tokyo, Japan), then a 1 µg sample was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Using 4% of the reverse-transcribed mix, cDNA fragments of test genes were amplified within the linear range by PCR using the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The specific primers were synthesized according to motifs: TTC ACA AGT CCG GAG AGG AG (IL-6, sense), TGG TCT TGG TCC TTA GCC AC (IL-6, antisense), TTC ATT GAC CTC AAC TAC ATG (GAPD), sense), and GTG GCA GTG ATG GCA TGG AC (GAPDH, antisense). PCR amplification of IL-6 cDNA (488 bp) for 33 cycles was 94°C denaturation (60 s), 55°C annealing (60 s), and 72°C extension (60 s). PCR amplification of GAPDH cDNA (443 bp) for 20 cycles was 94°C denaturation (60 s), 60°C annealing (60 s), and 72°C extension (60 s). Following these cycles of PCR amplifications, the amplified cDNA were further extended by additional incubation at 72°C for 10 min. Then equal amounts of each reaction were fractionated on 1% agarose gel in 1× TAE buffer, and the agarose gel was soaked in 1× TAE buffer containing ethidium bromide for 15 min with gentle agitation. The amplified cDNA fragments in the agarose gel were then visualized on an UV transilluminator and photographed.

Absorbance measurement. The absorbance of ELISA samples was measured at 450 nm with an EL340 Bio Kinetic Reader (Bio-Tek Instruments, Winooski, VT, USA).

Statistical analysis. Each experiment was repeated 3 times with similar results. The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs. $P < 0.05$ was considered significant. All data are presented as the mean ± SD of triplicate determinations.

RESULTS

Effect of MTX on TNF-α induced IL-6 synthesis in MC3T3-E1 cells. We previously reported that TNF-α significantly induced synthesis of IL-6 in a time-dependent manner up to

48 h[23]. We investigated the effect of MTX on TNF-α induced IL-6 synthesis. MTX (1 μM), which alone had little effect on IL-6 synthesis, significantly inhibited the TNF-α induced IL-6 synthesis in a time-dependent manner. The inhibitory effect of MTX reached a maximum at 48 h after stimulation (Figure 1A). The effect of MTX was dose-dependent in the range between 0.1 and 1.0 μM. The maximum effect of MTX on TNF-α induced IL-6 synthesis was observed at 1.0 μM, which caused about 30% decrease in the TNF-α effect (Figure 1B).

Effect of MTX on amplification by IL-17 of TNF-α induced IL-6 synthesis in MC3T3-E1 cells. It is well known that IL-17 is expressed in the synovium of patients with RA and contributes to the pathogenesis of arthritis, and expresses additive or even synergistic effects with TNF-α in inducing joint pathology[4]. We have reported that IL-17 markedly enhances the TNF-α induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells[32]. Here, we investigated the effect of MTX on the amplification by IL-17 of TNF-α induced IL-6 synthesis in these cells. MTX significantly suppressed the enhancement by IL-17 of TNF-α stimulated IL-6 synthesis in a dose-dependent manner in the range between 0.1 and 1.0 μM. The maximum effect of MTX was observed at 1.0 μM, which caused about 30% inhibition in the IL-17 enhanced TNF-α effect (Figure 2).

Effect of MTX on IL-1α induced IL-6 synthesis in MC3T3-E1 cells. We have reported that IL-1α induces IL-6 synthesis in a time-dependent manner up to 24 h in osteoblast-like MC3T3-E1 cells[24]. We examined the effect of MTX on the IL-1α induced IL-6 synthesis in osteoblasts. MTX (1 μM) decreased the IL-1α induced IL-6 synthesis in a time-dependent manner. The inhibiting effect by MTX reached a maximum at 24 h after stimulation (Figure 3A). The effect of MTX was dose-dependent in the range between 0.1 and 1.0 μM. The maximum effect of MTX was observed at 1.0 μM. MTX caused about 35% decrease in the IL-1α effect (Figure 3B).

Effect of MTX on PGD_2 induced IL-6 synthesis in MC3T3-E1 cells. We previously showed that PGD_2 stimulates IL-6 synthesis in MC3T3-E1 cells[25]. To clarify whether MTX affects the PGD_2 induced level of IL-6, we examined the effect of MTX on PGD_2 induced IL-6 synthesis. MTX (1.0 μM) significantly suppressed the PGD_2 stimulated IL-6 synthesis in a time-dependent manner. The inhibiting effect of MTX reached a maximum at 48 h after stimulation (data not shown). MTX significantly suppressed PGD_2 stimulated IL-6 synthesis in a dose-dependent manner, and the maximum effect of MTX was observed at 1.0 μM. MTX caused about 60% decrease in the PGD_2 effect (Figure 4A).

Effect of MTX on PGE_2 induced IL-6 synthesis in MC3T3-

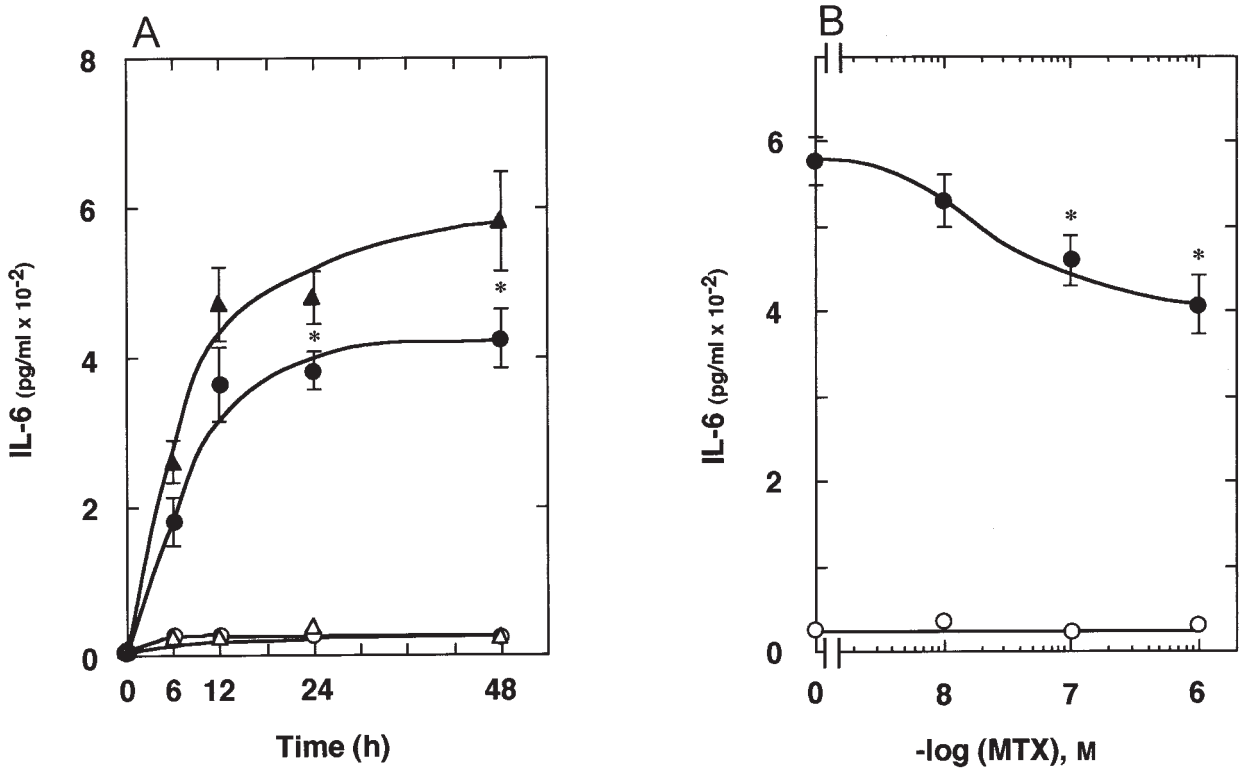

Figure 1. Effect of MTX on TNF-α induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with 1 μM MTX (●, ○) or vehicle (▲, △) for 1 h, and then stimulated with 10 ng/ml TNF-α (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs TNF-α alone. (B) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 ng/ml TNF-α (●) or vehicle (○) for 48 h. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs TNF-α without MTX.

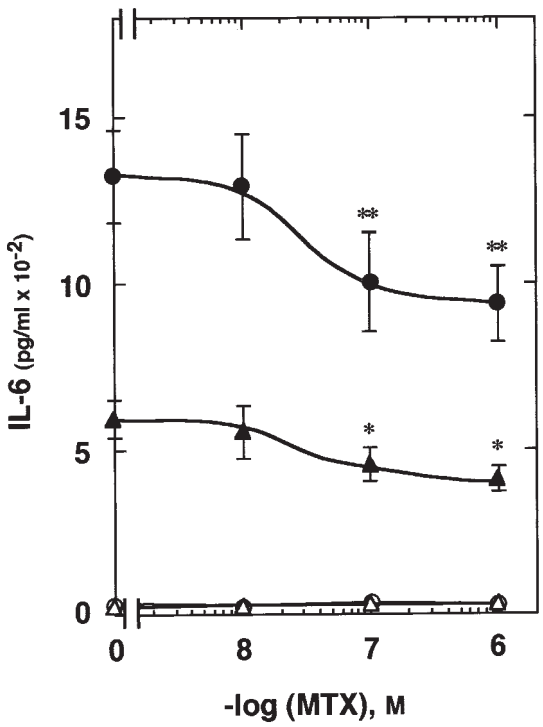

Figure 2. Effect of MTX on IL-17 amplification of TNF-α induced IL-6 synthesis in MC3T3-E1 cells. Cultured cells were pretreated with indicated doses of MTX for 1 h; cells were next treated with 10 ng/ml IL-17 (●, ○) or vehicle (▲, △) for 1 h, then stimulated with 10 ng/ml TNF-α (●, ▲) or vehicle (○, △) for 48 h. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *p < 0.05 vs TNF-α without IL-17 and MTX. **p < 0.05 vs TNF-α with IL-17 without MTX.

E1 cells. It is generally accepted that PGE_2 promotes inflammation and participates in destructive mechanisms in the rheumatoid joint[33]. In addition, it has been reported that PGE_2 and $PGF_{2\alpha}$ levels in the synovial fluid of patients with RA are significantly higher than the values obtained in patients with osteoarthritis (OA)[3]. As reported[26], PGE_2 stimulates IL-6 synthesis through both EP_1 receptor and EP_2 receptor in osteoblast-like MC3T3-E1 cells. We next examined the effect of MTX on the PGE_2 induced IL-6 synthesis in these cells. MTX (1.0 μM) inhibited PGE_2 stimulated IL-6 synthesis in a time-dependent manner. The inhibiting effect of MTX reached a maximum at 48 h after stimulation (data not shown). The effect of MTX was dose-dependent in the range between 0.1 and 1.0 μM. The maximum effect of MTX was observed at 1.0 μM and caused about 25% reduction in the PGE_2 effect (Figure 4B).

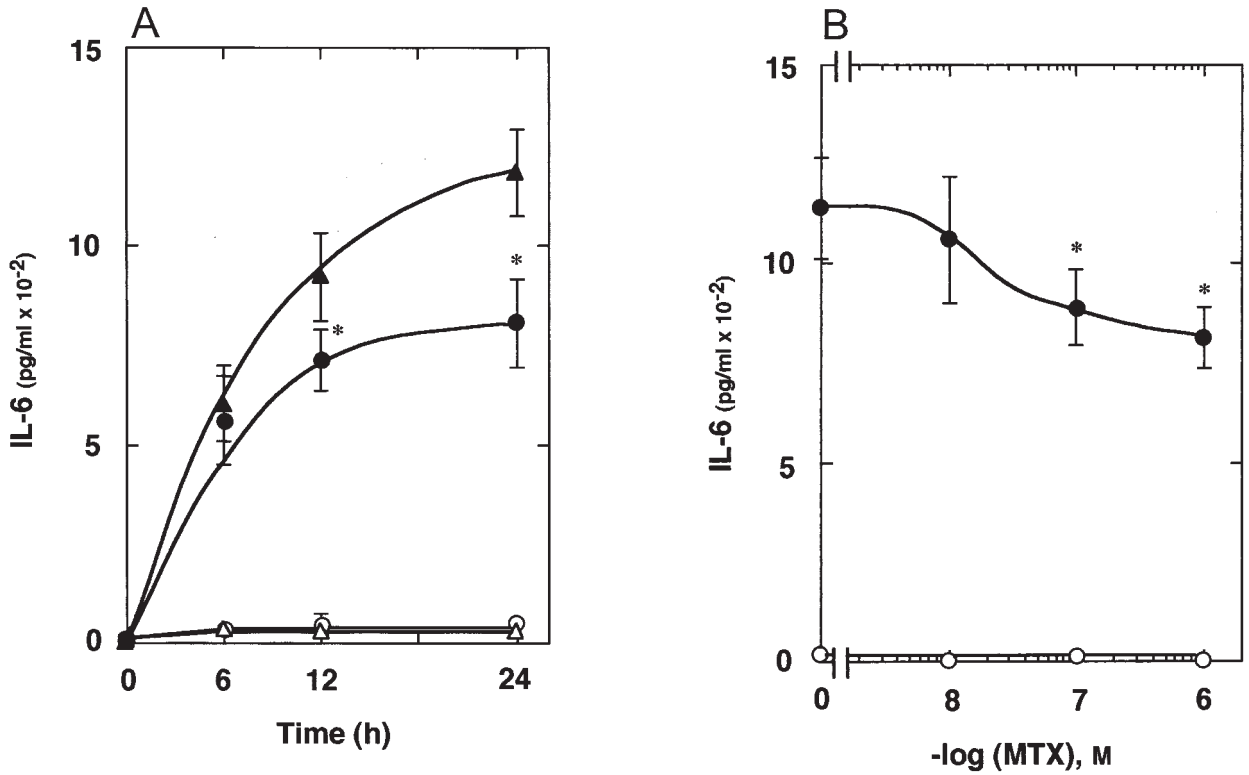

Figure 3. Effect of MTX on IL-1α induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with 1 μM MTX (●, ○) or vehicle (▲, △) for 1 h, and then stimulated with 30 ng/ml IL-1α (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *p < 0.05 vs IL-1α alone. (B) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 30 ng/ml IL-1α (●) or vehicle (○) for 24 h. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *p < 0.05 vs IL-1α without MTX.

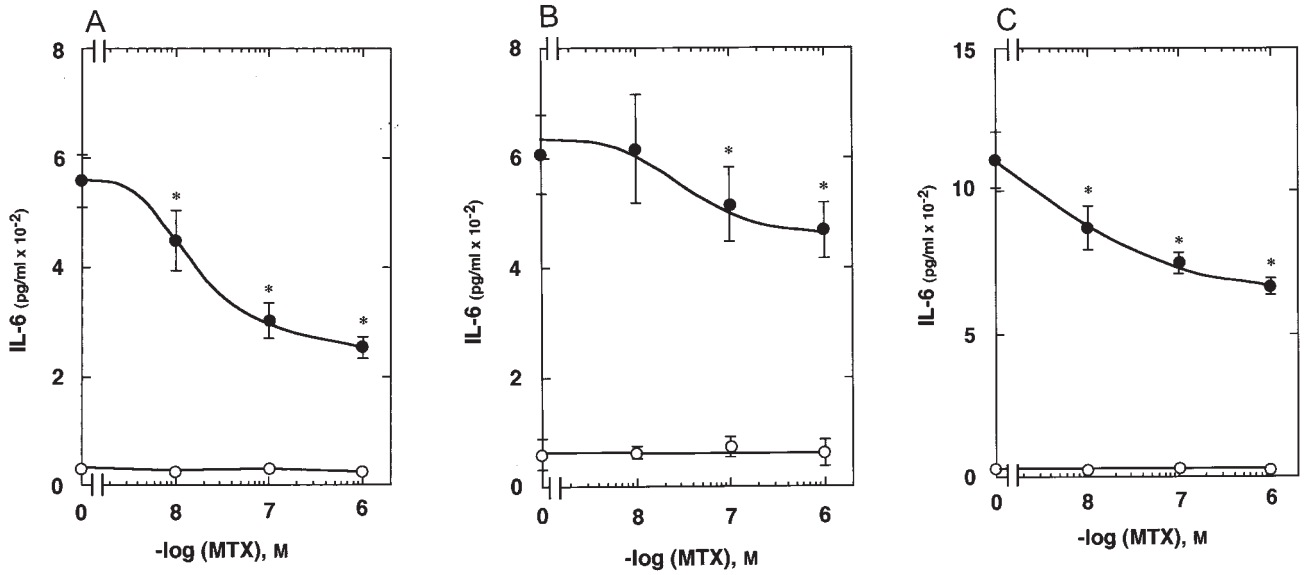

Figure 4. Effect of MTX on PGD_2, PGE_2, or $PGF_{2\alpha}$ induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 µM PGD_2 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs PGD_2 without MTX. (B) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 µM PGE_2 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs PGE_2 without MTX. (C) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 µM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs $PGF_{2\alpha}$ without MTX.

Effect of MTX on $PGF_{2\alpha}$ induced IL-6 synthesis in MC3T3-E1 cells. We have demonstrated[27,34] that $PGF_{2\alpha}$ induces IL-6 synthesis in MC3T3-E1 cells, and that protein kinase C (PKC) activation is involved in the mechanism. To clarify whether MTX affects the $PGF_{2\alpha}$ stimulated IL-6 synthesis, we examined the effect of MTX on $PGF_{2\alpha}$ induced IL-6 synthesis in MC3T3-E1 cells. MTX (1.0 µM) significantly suppressed the $PGF_{2\alpha}$ stimulated IL-6 synthesis in a time-dependent manner. The inhibiting effect of MTX reached a maximum at 48 h after stimulation (data not shown). MTX significantly suppressed $PGF_{2\alpha}$ stimulated IL-6 synthesis in a dose-dependent manner in the range between 0.01 and 1.0 µM. The maximum effect of MTX was observed at 1.0 µM. MTX caused about 40% decrease in the $PGF_{2\alpha}$ effect (Figure 4C).

Effect of MTX on TPA induced IL-6 synthesis in MC3T3-E1 cells. PKC is known to play a pivotal role in the regulation of various cellular functions[35]. We have demonstrated that activation of PKC directly activated by TPA, a PKC-activating phorbol ester[35], induces IL-6 synthesis in MC3T3-E1 cells[34]. We investigated the effect of MTX on IL-6 synthesis induced by TPA in these cells. MTX (1.0 µM) significantly suppressed TPA stimulated IL-6 synthesis in a time-dependent manner. The inhibiting effect of MTX reached a maximum at 48 h after stimulation (Figure 5A). The inhibitory effect of MTX was dose-dependent in the range between 0.1 and 1.0 µM. The maximum effect of MTX was observed at 1.0 µM, which caused about 30% decrease in the TPA effect (Figure 5B).

Effect of MTX on VEGF synthesis induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. It is recognized that angiogenesis is an important process in the development and perpetuation of RA[36]. VEGF is known as an essential mediator of angiogenesis[37]. It is reported that VEGF levels are markedly higher in the serum and synovial fluids of patients with RA than in patients with OA and healthy controls[38]. In osteoblasts, it has been reported that PGE_2 induces VEGF synthesis[39]. We have shown that $PGF_{2\alpha}$ induces VEGF synthesis[40]. We investigated the effect of MTX on VEGF synthesis induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. MTX alone had no effect on VEGF synthesis in these cells. Further, MTX did not affect the concentrations of VEGF induced by $PGF_{2\alpha}$ (Figure 6).

Effects of MTX on PGD_2, PGE_2, and $PGF_{2\alpha}$ induced IL-6 synthesis in primary osteoblastic cells. To clarify whether these effects of MTX are specific to MC3T3-E1 cells, we next investigated the effect of MTX on PGD_2, PGE_2, and $PGF_{2\alpha}$ induced IL-6 synthesis in primary mouse osteoblastic cells. MTX (0.1 or 1.0 µM) significantly suppressed IL-6 synthesis stimulated by these inflammatory agonists in a dose-dependent manner. The maximum effect of MTX was observed at 1.0 µM in each case (Figure 7).

Effect of PGD_2 with MTX treatment on viability of MC3T3-E1 cells. As shown in Figure 8, cell viability of PGD_2 with MTX treated cells was not lower than that of the untreated cells, and was instead significantly higher. Thus, it seems that PGD_2 with MTX treatment has no toxic effects on MC3T3-E1 cells.

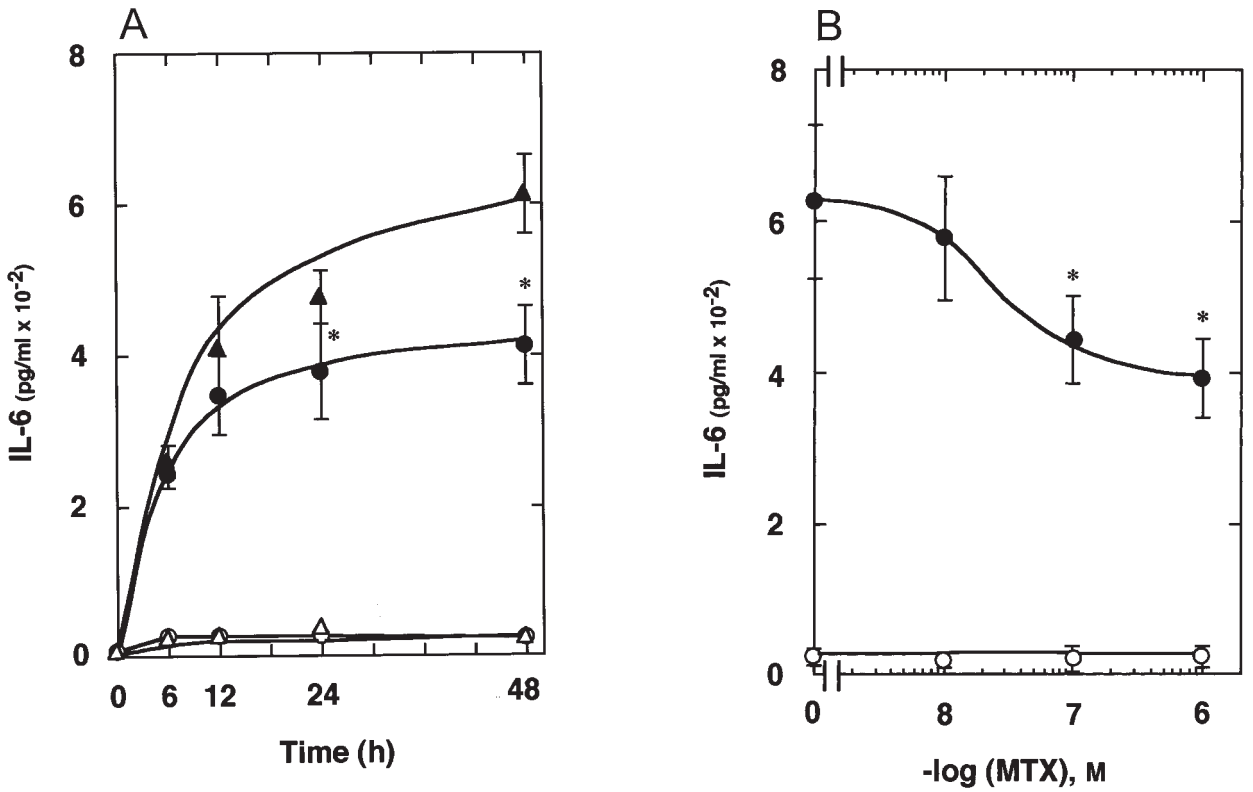

Figure 5. Effect of MTX on TPA induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with 1 μM MTX (●, ○) or vehicle (▲, △) for 1 h, then stimulated with 0.1 μM TPA (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *p < 0.05 vs TPA alone. (B) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 0.1 μM TPA (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *p < 0.05 vs TPA without MTX.

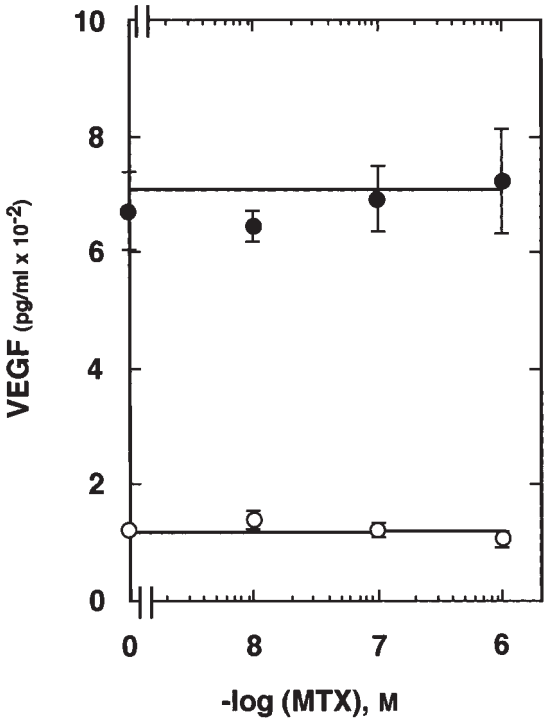

Figure 6. Effect of MTX on $PGF_{2\alpha}$ induced VEGF synthesis in MC3T3-E1 cells. Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations.

DISCUSSION

RA is one of the most common inflammatory diseases, and various inflammatory cytokines play crucial roles in its pathogenesis[2]. Among them, TNF-α and IL-1 are pivotal proinflammatory cytokines that have been shown to contribute to the clinical manifestations of RA[2,33,41]. TNF-α and IL-1 are reported to stimulate osteoblasts directly, and increase the expression of RANKL in these cells[18]. It is recognized that RANKL-RANK interaction is essential for osteoclast differentiation; TNF-α and IL-1 partially regulate osteoclastogenesis through osteoblast-osteoclast interaction[18,42]. RA is often complicated by generalized osteopenia due to increased bone resorption by osteoclasts[43]. It is known that TNF-α antibody[44] and IL-1 receptor antagonists[43] prevent bone loss caused by inflammatory arthritis, such as RA. PG are well known as important mediators of inflammation and joint destruction in RA[33]. COX-2

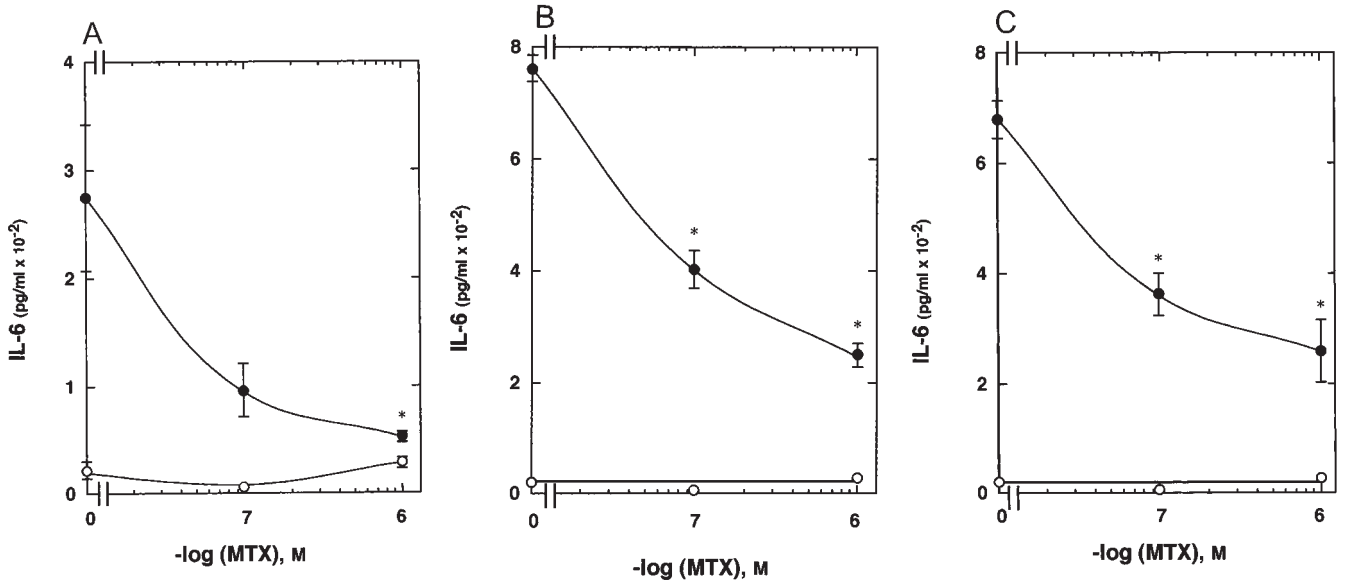

Figure 7. Effects of MTX on PGD_2, PGE_2, or $PGF_{2\alpha}$ induced IL-6 synthesis in primary osteoblastic cells. (A) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM PGD_2 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs PGD_2 without MTX. (B) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM PGE_2 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs PGE_2 without MTX. (C) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.05$ vs $PGF_{2\alpha}$ without MTX.

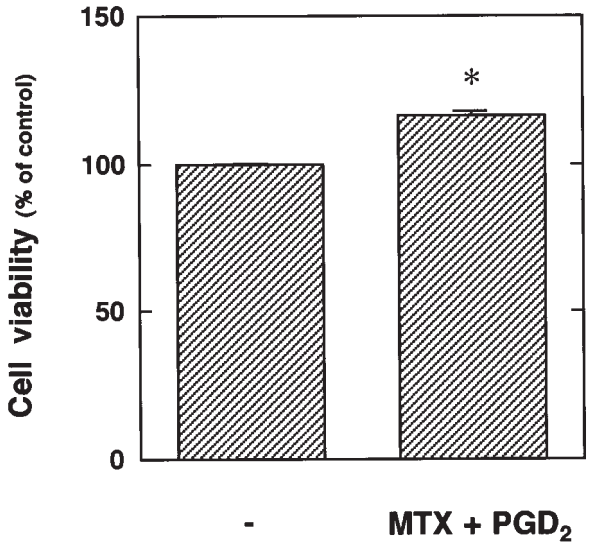

Figure 8. Effect of PGD_2 with MTX treatment on viability of MC3T3-E1 cells. Cells were treated first with MTX (1 μM) for 1 h and then with PGD_2 (10 μM) for 48 h (MTX + PGD_2, right bar), or not (left bar). After that, each culture was washed once with α-MEM, supplemented with 100 μl fresh α-MEM with no supplements, and then incubated 16 h. Finally, cell viability in each culture was assessed as a function of NADH content using TetraColor One solution. Absorbance of each well was determined at 450 nm. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with 2 different additional cell preparations. *$p < 0.01$ vs untreated cells.

inhibitor, one of the most common nonsteroidal antiinflammatory drugs routinely used in RA, blocks the conversion of arachidonic acid to PGH_2, subsequently converting to other PG, which results in preventing the inflammation of RA[45].

It is recognized that IL-6 stimulates bone resorption by inducing osteoclast formation, and IL-6 secreted from osteoblasts plays a key role in osteoclastogenesis[21,22]. In osteoblasts, we have shown that TNF-α[23], IL-1α[24], PGD_2[25], PGE_2[26], and $PGF_{2\alpha}$[27] stimulate IL-6 synthesis. In addition, we demonstrated that IL-17 enhanced TNF-α induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells[32].

It has recently been reported that MTX inhibits IL-6 production by LPS stimulated PBMC obtained from patients with juvenile RA[17]. However, the mechanism underlying MTX induced suppression of IL-6 synthesis is unknown. In addition, the effect of MTX on osteoblasts around the diseased joint in RA remains to be clarified. In this study, we showed that MTX significantly suppressed TNF-α stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells, although MTX by itself had little effect on IL-6 levels. It is likely that MTX significantly decreased IL-1α, PGD_2, PGE_2, or $PGF_{2\alpha}$ induced IL-6 synthesis in these cells. Moreover, MTX significantly suppressed the PG stimulated IL-6 synthesis in primary osteoblastic cells as observed in osteoblast-like MC3T3-E1 cells. Therefore, it is probable that this observation is common in osteoblastic lineage cells. These results strongly suggest that MTX suppresses inflammatory agonist induced IL-6 synthesis in osteoblasts.

We examined the level of IL-6 mRNA in MC3T3-E1 cells 1 h after addition of MTX alone using RT-PCR analysis. The level of IL-6 mRNA was not affected 1 h after addition of 1.0 μM MTX (data not shown), suggesting that MTX

might inhibit IL-6 accumulation at protein levels. Further investigations are required to examine specifically how MTX suppresses the IL-6 accumulation in osteoblast-like cells.

The suppressive effects of MTX on inflammatory agonist induced IL-6 synthesis at clinically relevant concentrations are not large (20%–30%). However, we have shown that MTX suppressed IL-6 synthesis stimulated by various bone inflammatory agonists, such as TNF-α, IL-1α, PGD_2, PGE_2, and $PGF_{2\alpha}$, that play important roles in the pathogenesis of RA in osteoblast-like MC3T3-E1 cells. In addition, MTX significantly inhibited the enhancement by IL-17 of TNF-α stimulated IL-6 synthesis. These results strongly suggest that MTX generally suppresses IL-6 synthesis stimulated by various inflammatory agonists in osteoblasts, although the effect is not large. Intriguingly, Nowak, *et al* recently reported that MTX in combination with prednisone decreases blood levels of IL-1ß and IL-6 and inhibits the intensity of free radical mediated processes in patients with RA[46]. Therefore, it is possible that prednisone may enhance the suppressive effect of MTX on IL-6 synthesis induced by various inflammatory agonists as described above in MC3T3-E1 cells. If so, such results justify the utility that the combination of MTX and prednisone decreases levels of IL-6 in patients with RA. We will next examine whether the combination of MTX and prednisone decreases IL-6 synthesis by the inflammatory agonists described above.

Previously, we showed that PGD_2 stimulates PKC activation through phosphoinositide hydrosis by phospholipase C (PLC) in osteoblast-like MC3T3-E1 cells[47]. Recently, we reported that PGD_2 stimulates HSP27 induction through p38 mitogen activated protein (MAP) kinase, p44/p42 MAP kinase, and stress activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), and that PKC acts at a point upstream from these MAP kinases[48,49]. Additionally, we have reported that MTX enhances PGD_2 stimulated heat shock protein 27 (HSP27) induction at a point downstream from MAP kinases in MC3T3-E1 cells[49]. MTX by itself had no effect on the formation of inositol phosphates, on activation of PKC, or on activation of the MAP kinases. Taking these results into account, it is most likely that MTX enhances the level of PGD_2 stimulated HSP27 at a point downstream from the 3 MAP kinases. On the other hand, we previously described that TNF-α and PGD_2 upregulate the level of IL-6 synthesis through activation of PKC[23,25], and that $PGF_{2\alpha}$ upregulates the level of IL-6 synthesis through p44/p42 MAPK[27] in MC3T3-E1 cells. In addition, MTX inhibited TPA induced IL-6 synthesis in osteoblasts. Thus, it is probable that MTX suppresses inflammatory agonist induced IL-6 synthesis at a point downstream from the MAP kinases in MC3T3-E1 cells. We also tried to examine how cycloheximide, an inhibitor of protein synthesis[50], affects the inhibitory activity of MTX on PGD_2 induced IL-6 accumulation. Pretreatment with 1.0 μM cycloheximide for 20 min completely suppressed PGD_2 induced IL-6 accumulation (data not shown). We were not able to evaluate how cycloheximide affects the inhibitory action of MTX on PGD_2 induced IL-6 accumulation. In addition, these results suggest that PGD_2 stimulates *de novo* synthesis of IL-6 protein in MC3T3-E1 cells.

MTX did not affect levels of VEGF induced by $PGF_{2\alpha}$, suggesting that MTX is unlikely to correlate with VEGF synthesis, and that the suppressive effect of MTX on IL-6 synthesis is not due to a cytotoxic effect of MTX on these cells, but to its specific inhibitory action on IL-6 synthesis in these cells.

In general, MTX is administered weekly in low doses (5–20 mg/week) to patients with RA[10]. It has been reported that weekly low dose pulse MTX therapy was performed routinely in patients with RA, resulting in serum concentrations of MTX up to 0.58 ± 0.2 μM[51]. Our results suggest that MTX, in the therapeutic doses for RA, suppresses IL-6 synthesis induced by all of these inflammatory agonists in osteoblast-like MC3T3-E1 cells. Taking these findings into account, it is probable that therapeutic dose MTX induced inhibition of IL-6 synthesis in osteoblasts takes part in the preventive effect of MTX on bone resorption by osteoclasts.

Our results suggest that low dose MTX therapy may prevent bone resorption by inhibiting IL-6 synthesis induced by various inflammatory agents (TNF-α, IL-1α, PGD_2, PGE_2, and $PGF_{2\alpha}$) in osteoblasts, resulting in suppression of osteoclast formation in the diseased joint in RA. These results raise the possibility that one of the therapeutic mechanisms of MTX for RA may inhibit osteopenia through the suppression of IL-6 synthesis induced by inflammatory agents.

REFERENCES

1. Lee DM, Weinblatt ME. Rheumatoid arthritis. Lancet 2001; 358:903-11.
2. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature 2003;423:356-61.
3. Egg D. Concentrations of prostaglandins D_2, E_2, $F_{2\alpha}$, 6-keto-$F_{1\alpha}$, and thromboxane B_2 in synovial fluid from patients with inflammatory joint disorders and osteoarthritis. Z Rheumatol 1984;43:89-96.
4. Lubberts E. The role of IL-17 and family members in the pathogenesis of arthritis. Curr Opin Invest Drugs 2003;4:572-7.
5. Nijweid PJ, Burger EH, Feyen JHM. Cells of bone: proliferation, differentiation and hormonal regulation. Physiol Rev 1986; 66:855-86.
6. Vergne P, Liagre B, Bertin P, et al. Methotrexate and cyclooxygenase metabolism in cultured human rheumatoid synoviocytes. J Rheumatol 1998;25:433-40.
7. Aletaha D, Smolen JS. The rheumatoid arthritis patient in the clinic: comparing more than 1,300 consecutive DMARD courses. Rheumatology Oxford 2002;41:1367-74.
8. Kaye SB. New antimetabolites in cancer chemotherapy and their clinical impact. Br J Cancer 1998;78 Suppl 3:1-7.
9. Williams HJ, Wilkens RF, Samuelson CO, et al. Comparison of low-dose oral pulse methotrexate and placebo in the treatment of rheumatoid arthritis: a controlled clinical trial. Arthritis Rheum 1985;28:721-30.
10. Bannwarth B, Pehourcq F, Schaeverbeke T, Dehais J. Clinical pharmacokinetics of low-dose pulse methotrexate in rheumatoid arthritis. Clin Pharmacokinet 1996;30:194-210.

11. Andersen PA, West SG, O'Dell JR, Via CS, Claypool RG, Kotzin BL. Weekly pulse methotrexate in rheumatoid arthritis. Ann Intern Med 1985;103:489-96.
12. Suarez CR, Pickett WC, Bell DH, McClintock DK, Oronsky AL, Kerwar SS. Effect of low dose methotrexate on neutrophil chemotaxis induced by leukotriene B_4 and complement C_{5a}. J Rheumatol 1987;14:9-11.
13. Segal R, Mozes E, Yaron M, Tartakovsky B. The effect of methotrexate on the production and activity of interleukin-1. Arthritis Rheum 1989;32:370-7.
14. Montesinos MC, Desai A, Delano D, et al. Adenosine A_{2A} or A_3 receptors are required for inhibition of inflammation by methotrexate and its analog MX-68. Arthritis Rheum 2003;48:240-7.
15. Mello SBV, Barros DM, Silva ASF, Laurindo IMM, Novaes GS. Methotrexate as a preferential cyclooxygenase 2 inhibitor in whole blood of patients with rheumatoid arthritis. Rheumatology Oxford 2000;39:533-6.
16. Hagel-Bradway S, Dziak R. Regulation of bone cell metabolism. J Oral Pathol Med 1989;18:344-51.
17. Aggarwal A, Misra R. Methotrexate inhibits interleukin-6 production in patients with juvenile rheumatoid arthritis. Rheumatol Int 2003;23:134-7.
18. Nakashima T, Kobayashi Y, Yamasaki S, et al. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappa B ligand: modulation of the expression by osteotropic factors and cytokines. Biochem Biophys Res Commun 2000;275:768-75.
19. Akira S, Hirano T, Taga T, Kishimoto T. Biology of multifunctional cytokines: IL-6 and related molecules (IL-1 and TNF). FASEB J 1990;4:2860-7.
20. van Snick JV. Interleukin-6: An overview. Ann Rev Immunol 1995;8:253-97.
21. Ishimi Y, Miyaura C, Jin CH, et al. IL-6 is produced by osteoblasts and induces bone resorption. J Immunol 1990;145:3297-303.
22. Roodman GD. Interleukin-6; an osteotropic factor? J Bone Miner Res 1992;7:475-8.
23. Kozawa O, Suzuki A, Kaida T, Tokuda H, Uematsu T. Tumor necrosis factor-α autoregulates interleukin-6 synthesis via activation of protein kinase C. J Biol Chem 1997;272:25099-104.
24. Miwa M, Kozawa O, Tokuda H, Uematsu T. Mitogen-activated protein (MAP) kinases are involved in interleukin-1 (IL-1)-induced IL-6 synthesis in osteoblasts: modulation not of p38 MAP kinase, but of p42/p44 MAP kinase by IL-1-activated protein kinase C. Endocrinology 1999;140:5120-5.
25. Tokuda H, Kozawa O, Harada A, Uematsu T. Prostaglandin D_2 induces interleukin-6 synthesis via Ca^{2+} mobilization in osteoblasts: regulation by protein kinase C. Prostaglandins Leukot Essent Fatty Acids 1999;61:189-94.
26. Kozawa O, Suzuki A, Tokuda H, Kaida T, Uematsu T. Interleukin-6 synthesis induced by prostaglandin E_2: cross-talk regulation by protein kinase C. Bone 1998;22:355-60.
27. Tokuda H, Kozawa O, Harada A, Uematsu T. p42/p44 mitogen-activated protein kinase activation is involved in prostaglandin $F_{2\alpha}$-induced interleukin-6 synthesis in osteoblasts. Cell Signal 1999;11:325-30.
28. Sudo H, Kodama AH, Amagi Y, Yamamoto S, Kasi S. In vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 1983;96:191-8.
29. Kozawa O, Tokuda H, Miwa M, Kotoyori J, Oiso Y. Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells. Exp Cell Res 1992;198:130-4.
30. Okahashi N, Nakamura I, Jimi E, Koide M, Suda T, Nishihara T. Specific inhibitors of vacuolar H(+)-ATPase trigger apoptotic cell death of osteoclasts. J Bone Miner Res 1997;12:1116-23.
31. Ishiyama M, Miyazono Y, Sakamoto K, Ohkura Y, Ueno K. A highly water-soluble disulfonated tetrazolium salt as a chromogenetic indicator for NADH as well as cell viability. Talanta 1997; 44:1299-305.
32. Tokuda H, Kanno Y, Ishisaki A, Takenaka M, Harada A, Kozawa O. Interleukin (IL)-17 enhances tumor necrosis factor-α-stimulated IL-6 synthesis via p38 mitogen-activated protein kinase in osteoblasts. J Cell Biochem 2004;91:1053-61.
33. Bingham CO III. The pathogenesis of rheumatoid arthritis: pivotal cytokines involved in bone degradation and inflammation. J Rheumatol 2002;30 Suppl 65:3-9.
34. Kozawa O, Suzuki A, Tokuda H, Uematsu T. Prostaglandin $F_{2\alpha}$ stimulates interleukin-6 synthesis via activation of PKC in osteoblast-like cells. Am J Physiol 1997;272:E208-11.
35. Carter CA. Protein kinase C as a drug target: implications for drug or diet prevention and treatment of cancer. Curr Drug Targets 2000;1:163-83.
36. Ewa MP. Angiogenesis in rheumatoid arthritis. Arthritis Res 2002;4:S81-S90.
37. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997;18:4-25.
38. Harada M, Mitsuyama K, Yoshida H, et al. Vascular endothelial growth factor in patients with rheumatoid arthritis. Scand J Rheumatol 1998;27:377-80.
39. Harada S, Nagy JA, Sullivan KA, et al. Induction of vascular endothelial growth factor expression by prostaglandin E_2 and E_1 in osteoblasts. J Clin Invest 1994;93:2490-6.
40. Tokuda H, Harada A, Hirade K, et al. Incadronate amplifies prostaglandin $F_{2\alpha}$-induced vascular endothelial growth factor synthesis in osteoblasts. Enhancement of MAPK activity. J Biol Chem 2003;278:18930-7.
41. Dayer JM. The pivotal role of interleukin-1 in the clinical manifestations of rheumatoid arthritis. Rheumatology Oxford 2003;42 Suppl 2:3-10.
42. Katagiri T, Takahashi N. Regulatory mechanisms of osteoblast and osteoclast differentiation. Oral Dis 2002;8:147-59.
43. Hirayama T, Danks L, Sabokbar A, Athanasou NA. Osteoclast formation and activity in the pathogenesis of osteoporosis in rheumatoid arthritis. Rheumatology Oxford 2002;41:1232-9.
44. Demis E, Roux C, Breban M, Dougados M. Infliximab in spondylarthropathy — influence on bone density. Clin Exp Rheumatol 2002;20:S185-6.
45. Smith WL, Garavito RM, De Witt DL. Prostaglandin endoperoxide synthase (cyclooxygenase)-1 and -2. J Biol Chem 1996; 271:33157-60.
46. Nowak D, Lewandowicz J, Dbkowska B, Marczak J. Combination of methotrexate and prednisone decreases circulating concentrations of interleukin 1 beta and interleukin 6 in patients with rheumatoid arthritis. Poor correlation of cytokine suppression with clinical improvement. Int J Immunopathol Pharmacol 1999;12:13-21.
47. Tsushita K, Kozawa O, Tokuda H, Oiso Y, Saito H. Proliferative effect of PGD_2 on osteoblast-like cells; independent activation of pertussis toxin-sensitive GTP-binding protein from PGE_2 or $PGF_{2\alpha}$. Prostaglandins Leukot Essent Fatty Acids 1992;45:267-74.
48. Kozawa O, Otsuka T, Hatakeyama D, et al. Mechanism of prostaglandin D_2-stimulated heat shock protein 27 induction in osteoblasts. Cell Signal 2001;13:535-41.
49. Yoshida M, Niwa M, Ishisaki A, et al. Methotrexate enhances prostaglandin D_2-stimulated heat shock protein 27 induction in osteoblasts. Prostaglandins Leukot Essent Fatty Acids 2004; 71:351-62.
50. Obrig TG, Culp WJ, McKeehan WL, Hardesty B. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J Biol Chem 1971;246:174-81.
51. Kremer JM, Galivan J, Streckfuss A, Kamen B. Methotrexate metabolism analysis in blood and liver of rheumatoid arthritis patients. Arthritis Rheum 1986;29:832-5.