ハビリは，ごく限られたものが対象で，大多数の高齢者では身体的および精神的な機能の回復を最大限に図り，可能な限り独立して生活し得る能力を取り戻すことが目標となる。このために高齢者リハビリの流れから，第1に寝たきりや要介護状態を予防する予防的リハビリ，第2に疾病の治療とともに早期に開始される急性期リハビリ，第3に急性期から機能回復を目指した回復期リハビリへのスムースな移行，第4に地域との連携が重要で，維持期リハビリが必要となる。以下に高齢者リハビリの理念について概説する。

1．高齢者の残存機能を引き出し，自立した生活を目標とする

従来の医療は，臓器疾患の治療を第一義的に考えた延命のための医療が中心で，急性期疾患の治療や救命に多大な貢献を果たしてきたが，高齢者医療ではこの先進的臓器別診療では高齢患者のQOLを損なう場合すらある。例えば，高齢者医療では複数の疾患を有していたり，慢性疾患を合併していた場合，臓器別の診療により単一の疾患の治療に専念するばかりに，関節拘縮，筋力低下などの廃用症候群を引き起こし，かえって生活機能低下をもたらし，疾患は治ったが自宅での自立した生活ができなくなるという高齢者にとって不幸な事態を生じることがある。そのためには高齢者のQOLの向上を目指し，自立した生活を送ることに着目する医療の必要性が問われるようになってきた。高齢者医療では，単なる臓器の疾患の診断と治療ではなく全人的な包括医療が必須であり，患者の日常生活動作，精神機能，および社会的状況の三つを総合的に評価して効果的な医療を行うことが必要である。従来よりリハビリは，包括医療を実践してきた診療科であり，医療の中に日常生活動作の観点を取り入れてきた学問である。そのためリハビリでは，高齢者の残存機能を引き出し，生活の自立機能の障害予防を最終目的とした医療は得意とする領域であり，高齢者医療の中で自立機能障害の予防を果たすのにリハビリの位置づけは非常に重いといえる。

2．予防医学としてのリハビリテーションを重視する

加齢とともに，さまざまな疾患や障害を有するようになるが，高齢者が障害に悩まされることなく，老後に活力のある生活を送れるようにするためには，高齢期，特に75歳以上の後期高齢者に発症する疾患や障害の発症をいかに予防していくかが特に重要な課題となる。成人期からの不適切な生活習慣が主要な発症原因であるといわれている脳血管障害，虚血性心疾患，閉塞性動脈硬化症などの動脈硬化性血管障害や骨粗鬆症などは，不適切な生活習慣を適正化することにより，これらの疾患の多くを予防あるいは発症を遅らせることができる[2]。また現在，介護を必要とするようになる原因としては，脳血管疾患，高齢による衰弱，転倒・骨折，認知症が頻度の高いものであり，これらを予防することが，要介護状態を予防することになる。そのために，高齢による衰弱を除いた脳血管疾患，転倒による骨折，認知症などの予防対策が必要である。要介護状態および寝たきりを予防するためにも，リハビリの果たす役割は重要である。しかし，リハビリの手法も従来の方法では対応困難な疾患もみられる。例えば，認知症のリハビリはまだ十分に確立しているとは言い難い。

3．高齢者の特性を踏まえたリハビリテーションが必要性である[9]

リハビリを行っていく場合，高齢者の特性を理解したうえで実施していかなくてはいけない。高齢者に多発する疾患の多くは老化を基盤にしており，いくつかの特徴を兼ね備えている。高齢者では多臓器にわたる疾患が認められ，症状が非定型的である。そして慢性化しやすいし，機能障害につながりやすく，合併症を併発しやすい。さらに社会的要因や環境により症状が変動しやすい。例えば，高齢者が入院により夜間せん妄を引き起こすことがある。リハビリを実施するうえで注意を払う必要があることは，まず第1に疾病の治療とともに身体活動性を保つことを優先することであ

る。高齢者ではいったん疾病に罹患すると若年者に比べ重症化，長期化しやすく，廃用症候群に陥りやすい。そのために，疾病の治療だけが優先されるのではなく，日常生活動作能力を維持し，改善しながらQOLを高めることが求められる。高齢者医療では病気は治ったが入院前の生活に戻れないようではいけないのである。また高齢者には，一度に複数の慢性疾患を抱えている可能性がある。医療技術の進歩により，数々の病気を早期に発見，治療できるようになったが，すべての疾患を完全治癒にもっていくことが困難な場合が多い。疾病を根治するのではなく，慢性疾患をもちながら，自立した生活を維持することも大切である。高齢者リハビリでは，複数存在する慢性疾患の増悪や合併症の発症に注意を払いながらリハビリを実施していく必要がある。

4．高齢者医療では，より一層のチーム医療が必要とされている

高齢者医療は，包括的医療といわれている。高齢者はいったん疾病に罹患すると，廃用症候群に陥りやすく，疾病の治療ばかりに目を向けていると，すぐに日常生活動作能力が低下し，入院も長期化する可能性が高い。そのため，高齢者リハビリでは，より一層早期からのリハビリを開始することが必要で，生活機能を維持するために，医師のみならず，看護師，理学療法士，作業療法士，言語聴覚士，薬剤師，ケースワーカーと協力しあい，情報交換を密に行いながら，疾病の治療とともに日常生活動作能力を保持しなければならない。チーム医療を進める方法として，さまざまな専門家やコメディカルが一同に会して合同カンファレンスを開くのが一つの方法である。いくら熟練した医師でも，すべての専門性を網羅することは不可能であり，時間と能力には限りがあるので，周囲に専門職が複数いて，互いのコミュニケーションをよくすることで，よりよい医療が患者に提供できる。各科医師とコメディカルが情報を共有し，適切に育成された人的資源を有効に活用する。

表1．老年症候群

老年症候群
意識障害
認知症，せん妄
不眠，うつ状態
めまい
言語障害，聴覚障害，視力障害
骨関節変形，骨粗鬆症，骨折
尿失禁，夜間頻尿
誤嚥，便秘，下痢
脱水，発熱
低体温
浮腫，肥満，るいそう，低栄養
褥瘡
喘鳴，喀痰咳嗽，呼吸困難
手足のしびれ，間欠性跛行
動脈硬化，不整脈
痛み
出血傾向，吐血，下血
ADL低下

5．高齢者リハビリテーションでは，特有の疾患を対象とする[10)11)]

老化により，骨関節・筋などの運動器，呼吸・循環器系，神経系，精神機能，代謝機能など多くの臓器に形態的変化と機能的変化をもたらす。これらのことが，高齢者に特有の疾病を発生させることになる。具体的には，高齢者では脳血管障害，認知症，パーキンソン症候群を中心とした中枢神経障害，転倒による大腿骨頸部骨折，脊椎圧迫骨折，変形性関節症，変形性脊椎症，骨粗鬆症などの整形外科疾患，肺炎や慢性呼吸不全などの呼吸器疾患，心筋梗塞や胸部手術後，血管手術，悪性腫瘍などの腹部手術後，糖尿病による神経障害もリハビリ対象疾患として多くみられる。また高齢者に多くみられ，原因はさまざまであるが治療と同時に介護およびケアを必要とする一連の症状や所見を老年症候群と呼称する。具体的な老年症候を表1に示す[12)]。これらの老年症候を多くもつことはリハビリの阻害因子を多く抱えていることになり，リハビリを施行するうえで注意を払わなければならないものである。その中でも嚥下障害，骨折などは，リハビリ対象疾患の大きな部分を占

めている。

Ⅲ．高齢者リハビリテーションの課題

1．予防的リハビリテーションへの取り組み

寝たきりまたは要介護状態の予防に対しても，積極的に対応していかなければならない。例えば，認知症の進行を予防する有効なリハビリプログラムを確立することも急務である。認知症高齢者に対する非薬物的療法としては，具体的には，回想法，リアリティオリエンテーション，行動療法，sensory stimulation，音楽療法，理学療法（筋力強化，バランス訓練，関節可動域訓練），作業療法（家事・家庭内役割作業，手工芸・工作），レクリエーション，園芸療法，演芸療法，社会心理療法，散歩，ラジオ体操，リズム体操，民謡体操，ストレッチ体操，肩こり体操，ダンスなどがあり，環境の整備，介護者への教育・指導なども含めると多岐にわたる。しかしながら，まだ有効性が確立した認知症に対するリハビリプログラムはみられない[13]。また，大腿骨頸部骨折の90%は転倒に起因するともいわれるが，高齢者の転倒には筋力の低下，視力の低下，認知症，生活様式など多くの要因が関与している。大腿骨頸部骨折患者の生命予後はよいが，約半数は歩行能力が低下し，約20%は寝たきり状態に陥るといわれている。転倒予防が骨折予防につながると考えられ，この目的で転倒予防および骨粗鬆症に対してもリハビリは対応していかなければいけない[14][15]。高齢者リハビリでは，より一層の予防目的のリハビリが重視されることになる。

2．高齢者の特性を踏まえたリハビリテーションプログラムの確立[16]

高齢者は，体力低下を伴っていることが多く，耐久性も低下しているために，長時間でかつ複雑な訓練内容は導入できないことがあり，単純で慣れた動作を中心とした訓練計画を立てる必要がある。例えば高齢者では，加齢とともに減少する筋肉量や平衡感覚機能は容易に回復しない。また，筋力増強の重要性は認識されていても，高齢者が無理なく行える筋力増強の方法はまだ確立されていない。ただ高齢者では，運動量の多い激しい筋力トレーニングが必要なわけではない。各自が自分に合った運動をして，身体活動性を維持すればよい。高齢者には高齢者に適したリハビリプログラムが必要である。また高齢者では，他の年齢層に比べライフスタイルの違い，住む環境状況の違い，過去に経験してきたことの違いにより個人差が非常に大きいことと，家族制度など社会構造の変化がきて，家族構成上核家族化が進み，単独世帯，夫婦のみの世帯，夫婦ともに65歳以上の世帯などが増加していて，家庭での介護能力が減少している現状がある。それゆえに高齢者のリハビリを実施するうえで，社会的背景が個人で大きく異なるため部分的で一方的な見方では偏りがあり，広い視野に立って，リハビリプログラムの立案と最終的な目標を個別に設定する必要がある。

3．地域連携システムの確立

高齢者医療では，病院と地域の連携が密接である必要がある。このためには，医療と看護，介護の垣根を越えた連携が必要である。日本ではリハビリも多様化しており，医療保険下で行われる入院および通院でのリハビリおよび訪問リハビリと訪問看護があり，介護保険の導入後，特に訪問リハビリ，通所リハビリなど，医療で行われるリハビリとの境界が不明瞭となっている。高齢者の介護状態の軽減や在宅での自立した日常生活を重視するためには，高齢者が長年住み慣れた地域において満足できるリハビリが受けられる体制をつくる必要があり，医療保険と介護保険でのリハビリの役割分担を明確にして，スムースな移行ができるように体制を整える必要がある[17]。

4．証拠に基づいたリハビリテーション

医学界では，証拠に基づいた医療が求められており，リハビリ医学でも例外でなく，適切な無駄のない医療は，客観的な証拠があって初めて可能となる。客観的な証拠とは，リハビリとしてどの種類の運動を，どの頻度で，どのくらいの期間行

うと最良の効果が得られるかを科学的に明らかにすることである。例えば，高齢者での効果的な筋力強化のプログラムや，認知症に対する効果的なプログラムに対する証拠はまだ不十分であり，今後の報告が待たれるところである。

5．最新の医療に対応したリハビリテーションの必要性

現代医療は着実に進歩しており，高齢者にもその適応範囲は徐々に広がっていくことが予想される。先端医療としては，皮膚，筋肉，骨，軟骨などの移植医療，ES 細胞や組織幹細胞を用いた再生医療，遺伝子治療など急速な医療の進歩がみられていく中で，それに応じたリハビリプログラムなどの開発が必要と考えられる。

おわりに

高齢化社会を迎えて，老化によりさまざまな疾患や障害を有する可能性が高くなり，疾患を治療するためにリハビリを含めた医療的ニーズはさらに高まることが推測できる。その一方で，医療費の高騰という社会・経済的な問題が起きているのも事実であり，医療費を抑えるためにも医療供給側は，より的確な治療を選択する必要があると同時に，疾患の治療だけでなく生活自立支援，健康増進や予防医学の側面にも積極的に介入する必要がある。リハビリの立場としては，これら生活自立支援，健康増進や予防医学にも対応していかなければならないために，求められる役割は非常に重要である。

文献

1) 折茂　肇：21世紀の老人医療のあり方とその将来展望．Geriatric Medicine 37(3)：333－337, 1999.
2) 折茂　肇：序論．老年医療の歩みと展望，日本老年医学会編，メジカルビュー社，2003，pp 2－5.
3) 黒川幸雄，大西秀明：高齢社会と理学療法の展望．高齢者の理学療法，森本　榮，黒川幸雄編，三輪書店，2002，pp 2－6.
4) 井口昭久：老年医学とは何か．日老医誌 40：439－444，2003.
5) 内閣府（編）：平成15年版高齢社会白書．2004, p13.
6) 厚生労働省（監修）：厚生労働白書．平成15年版，活力ある高齢者像と世代間の新たな関係の構築，2004，p366.
7) 生活情報センター（編）：介護サービス統計資料年報2003，文栄社，2003，p45.
8) 柳澤信夫：高齢化社会における寝たきり予防の重要性．寝たきりの予防と治療，財団法人長寿科学振興財団編，保健同人社，2001，pp 9－16.
9) 下方浩史：高齢者の疫学．これからの老年学，井口昭久編，名古屋大学出版会，2000，pp41－56.
10) 鳥羽研二：高齢者の特徴と診療上の留意事項．老年医学，萩原俊男編，朝倉書店，2003，pp24－34.
11) 村上恵一：身体疾患のリハビリテーション．老年者のリハビリテーション，平井俊策，江藤文夫編，ワールドプランニング，1997，pp 9－15.
12) 鳥羽研二：老年症候群．老年医学テキスト，日本老年医学会編，メジカルビュー社，2002, pp98－103.
13) 長屋政博：痴呆疾患における理学療法の意義．老年期痴呆の克服をめざして，財団法人長寿科学振興財団編，保健同人社，2002，pp251－257.
14) 長屋政博，原田　敦，奥泉宏康：高齢者の骨折と予防のための運動療法．現代医学 45：329－333，1997.
15) 長屋政博，荒川幸子：転倒予防教室の効果．愛知県理学療法士会誌 15（1）：1－8，2003.
16) 長屋政博：リハビリテーション．Geriatric Medicine 39（11）：1757－1764，2001.
17) 石川　誠：リハビリテーション．寝たきりの予防と治療，財団法人長寿科学振興財団編，保健同人社，2001，pp117－132.

Methotrexate Suppresses Inflammatory Agonist Induced Interleukin 6 Synthesis in Osteoblasts

MINORU YOSHIDA, YOSUKE KANNO, AKIRA ISHISAKI, HARUHIKO TOKUDA, KOUSEKI HIRADE, KEIICHI NAKAJIMA, YOSHIHIRO KATAGIRI, KATSUJI SHIMIZU, and OSAMU KOZAWA

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.

From the Departments of Pharmacology and Orthopaedic Surgery, Gifu University Graduate School of Medicine; and Department of Pharmacy, Gifu University Hospital, Gifu, Japan.

M. Yoshida, MD, Fellow, Departments of Pharmacology and Orthopaedic Surgery; Y. Kanno, PhD Student, Department of Pharmacology; A. Ishisaki, PhD, Assistant Professor, Department of Pharmacology; H. Tokuda, MD, PhD, Department of Clinical Laboratory, National Center for Geriatrics and Gerontology; K. Hirade, PhD, Fellow, Department of Pharmacology; Y. Katagiri, PhD, Professor, Department of Pharmacy; K. Nakajima, PhD, Assistant Professor, Department of Pharmacology; K. Shimizu, MD, PhD, Professor, Department of Orthopaedic Surgery; O. Kozawa, MD, PhD, Professor, Department of Pharmacology.

Address reprint requests to Dr. O. Kozawa, Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan. E-mail: okozawa@cc.gifu-u.ac.jp

Accepted for publication December 13, 2004.

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-

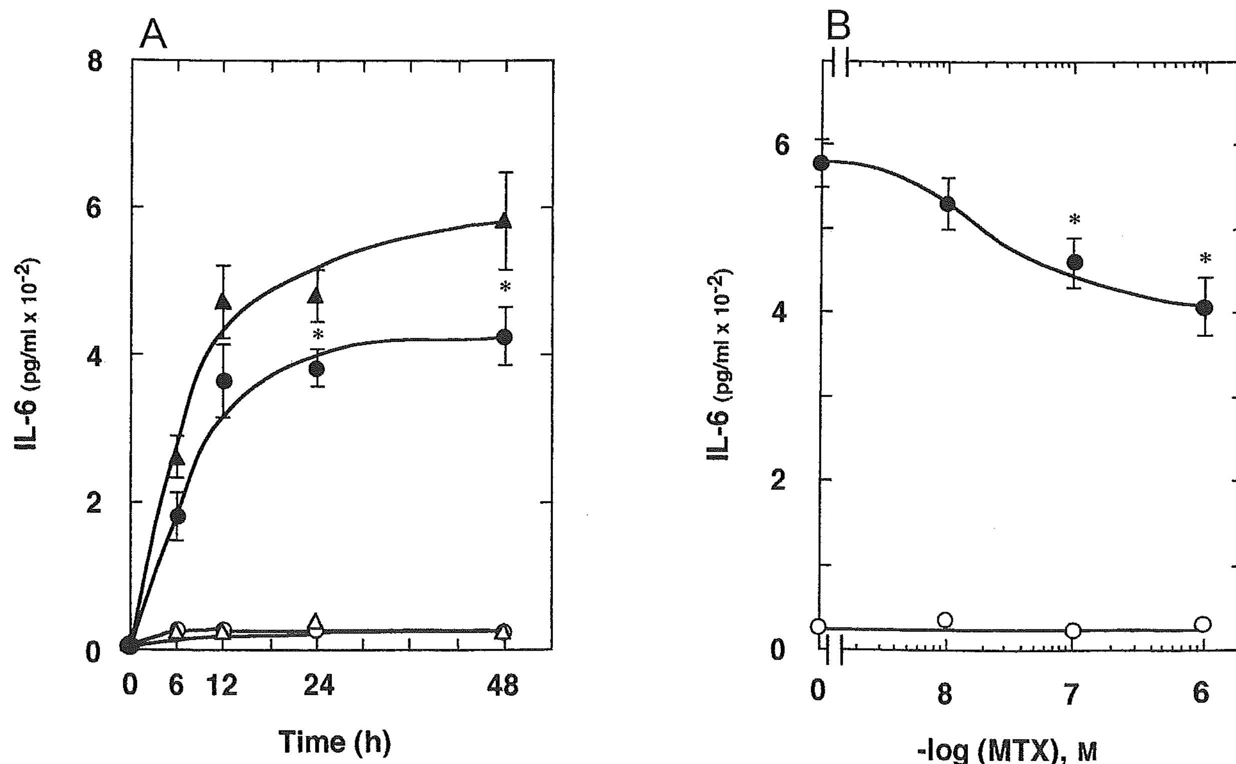


Figure 1. Effect of MTX on TNF-α induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with 1 μM MTX (●, ○) or vehicle (▲, △) for 1 h, and then stimulated with 10 ng/ml TNF-α (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents the mean ± SD of triplicate determinations. 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.

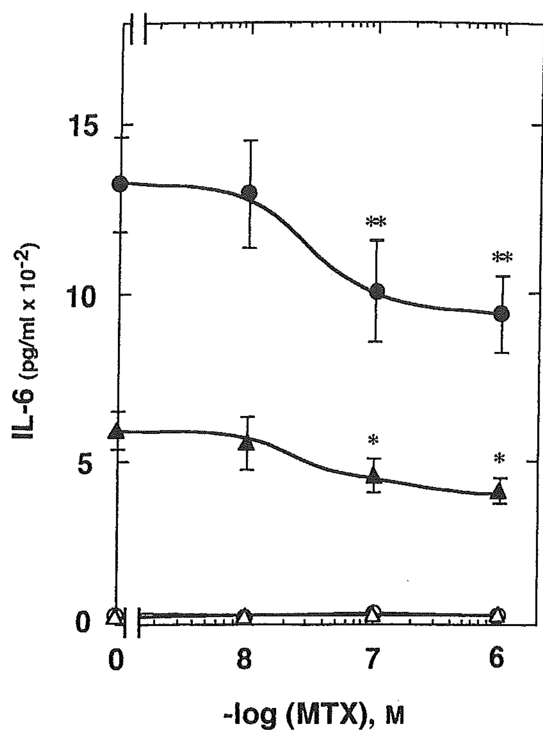
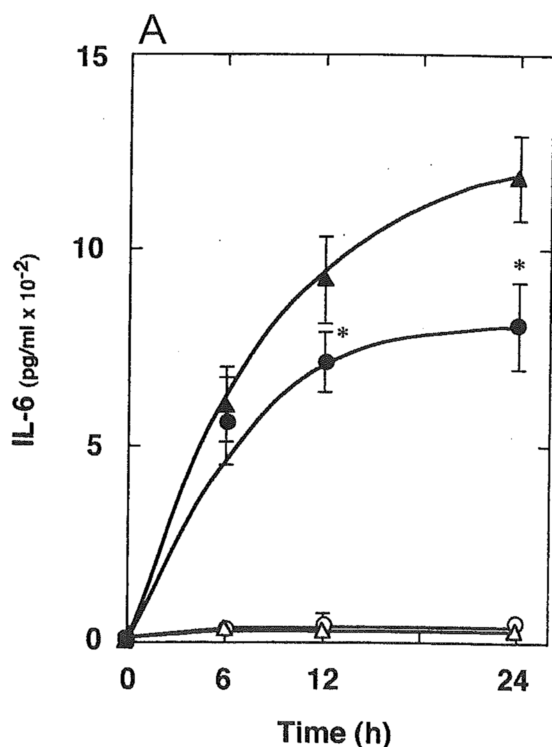


Figure 2. Effect of MTX on IL-17 amplification of TNF-α induced IL-6 synthesis in MC3T3-E1 cells. Cultured cells were pretreated with indicated doses of MTX for 1 h; cells were next treated with 10 ng/ml IL-17 (●, ○) or vehicle (▲, △) for 1 h, then stimulated with 10 ng/ml TNF-α (●, ▲) or vehicle (○, △) 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).



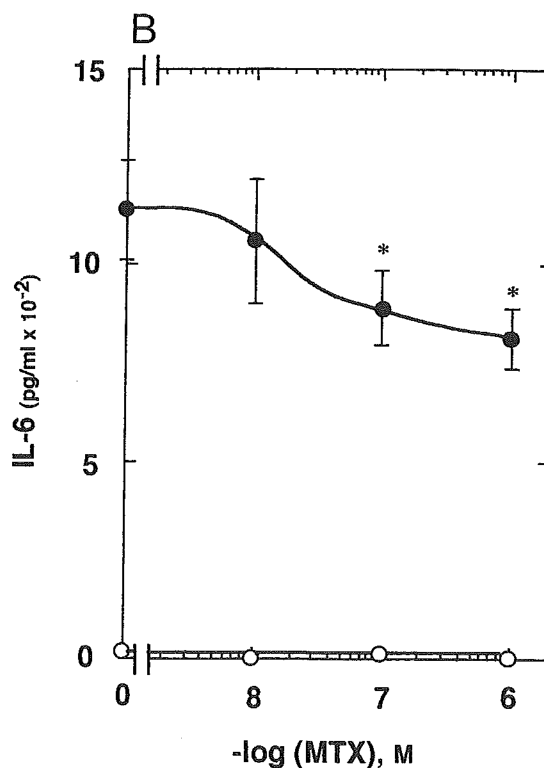


Figure 3. Effect of MTX on IL-1α induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with 1 μM MTX (●, ○) or vehicle (▲, △) for 1 h, and then stimulated with 30 ng/ml IL-1α (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents mean ± SD of triplicate determinations. 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.

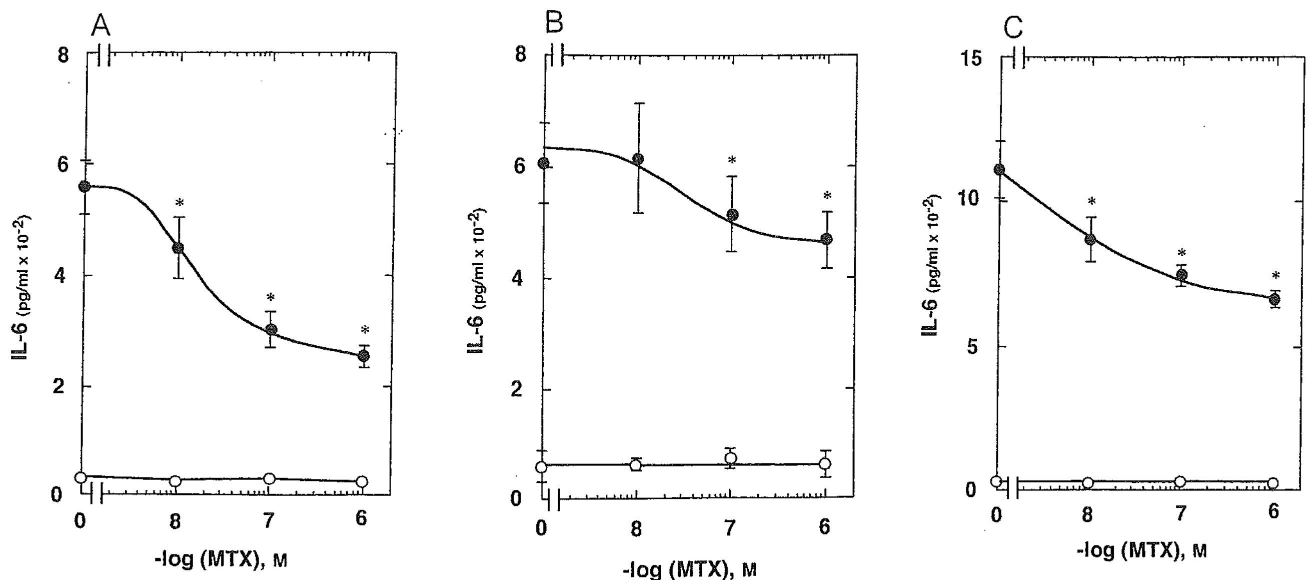


Figure 4. Effect of MTX on PGD_2, PGE_2, or $PGF_{2\alpha}$ induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM PGD_2 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. 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.

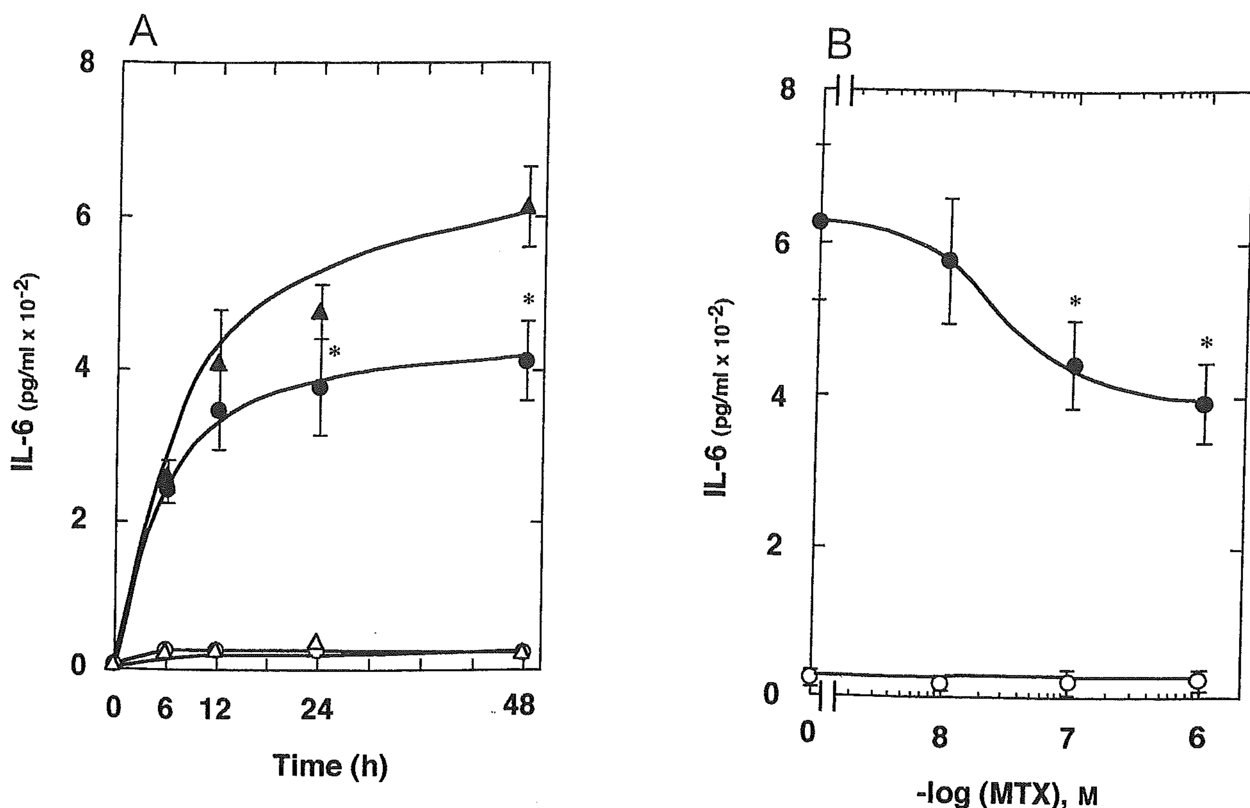


Figure 5. Effect of MTX on TPA induced IL-6 synthesis in MC3T3-E1 cells. (A) Cultured cells were pretreated with 1 µM MTX (●, ○) or vehicle (▲, △) for 1 h, then stimulated with 0.1 µM TPA (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents the mean ± SD of triplicate determinations. 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.

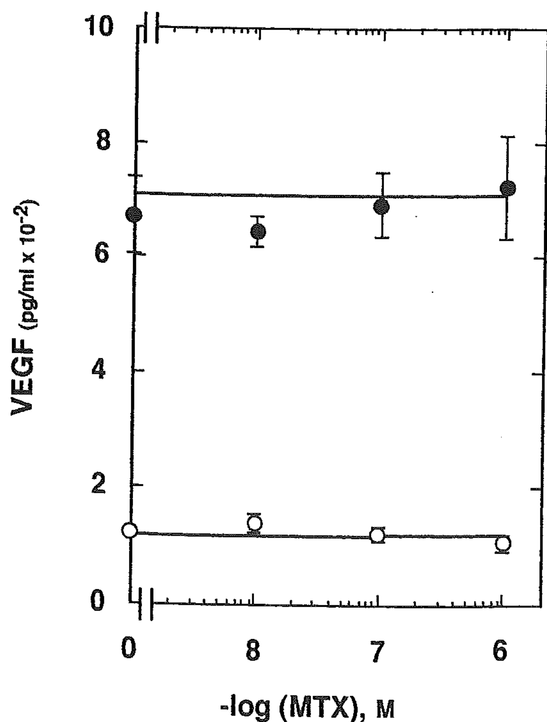


Figure 6. Effect of MTX on $PGF_{2\alpha}$ induced VEGF synthesis in MC3T3-E1 cells. Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 µM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results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

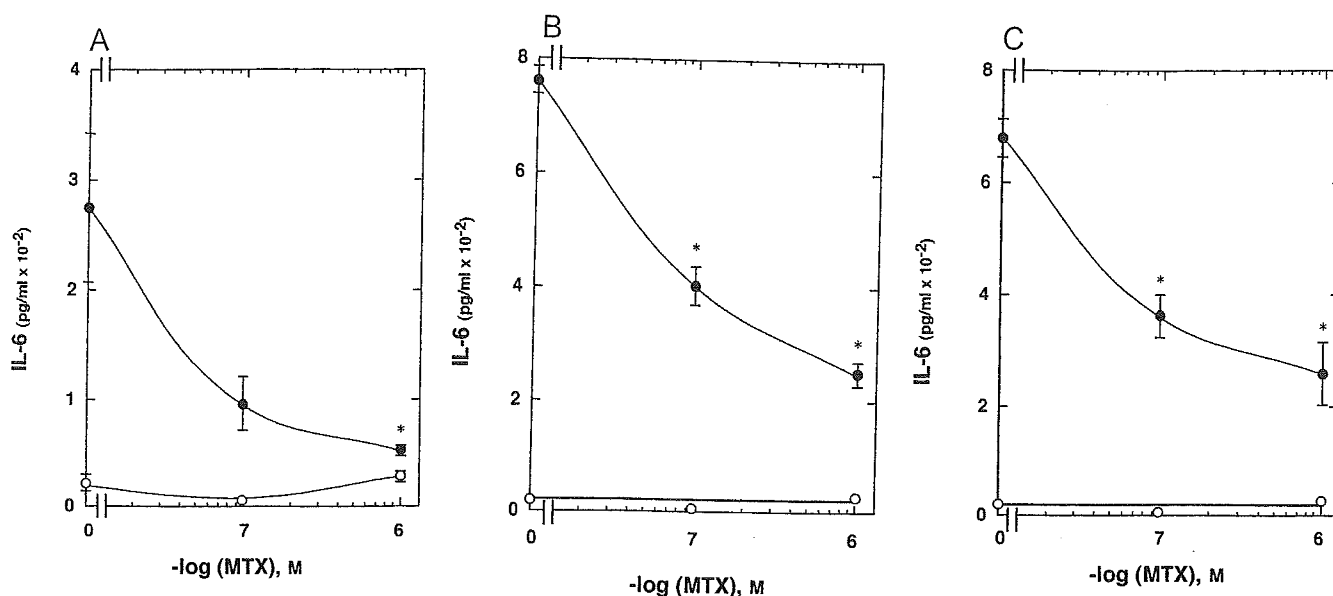


Figure 7. Effects of MTX on PGD_2, PGE_2, or $PGF_{2\alpha}$ induced IL-6 synthesis in primary osteoblastic cells. (A) Cultured cells were pretreated with indicated doses of MTX for 1 h, then stimulated with 10 μM PGD_2 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. 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.

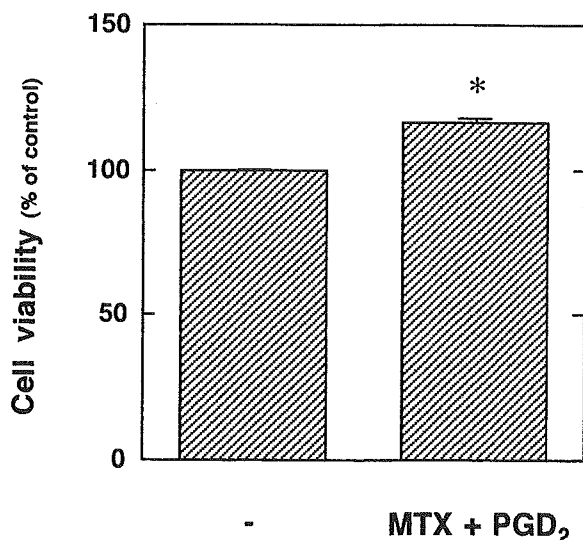


Figure 8. Effect of PGD_2 with MTX treatment on viability of MC3T3-E1 cells. Cells were treated first with MTX (1 μM) for 1 h and then with PGD_2 (10 μM) for 48 h (MTX + PGD_2, right bar), or not (left bar). After that, each culture was washed once with α-MEM, supplemented with 100 μl fresh α-MEM with 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.

SAPK/JNK Plays a Role in Transforming Growth Factor-β-induced VEGF Synthesis in Osteoblasts

Y. Kanno[1,2]
A. Ishisaki[1]
M. Yoshida[1]
H. Tokuda[1,3]
O. Numata[2]
O. Kozawa[1]

Abstract

We previously reported that transforming growth factor-β (TGF-β) activates p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase, resulting in the stimulation of vascular endothelial growth factor (VEGF) synthesis in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the involvement of stress-activated protein kinase/c-*Jun N*-terminal kinase (SAPK/JNK), another member of the MAP kinase superfamily, in TGF-β-induced VEGF synthesis in these cells. TGF-β markedly induced SAPK/JNK phosphorylation. SP600125, a specific inhibitor of SAPK/JNK, markedly reduced TGF-β-induced VEGF synthesis. SP600125 suppressed TGF-β-induced SAPK/JNK phosphorylation. PD98059, an inhibitor of upstream kinase of p44/p42 MAP kinase and SB203580, an inhibitor of p38 MAP kinase, each failed to reduce TGF-β-induced SAPK/JNK phosphorylation. A combination of SP600125 and PD98059 or SP600125 and SB203580 suppressed TGF-β-stimulated VEGF synthesis in an additive manner. These results strongly suggest that TGF-β activates SAPK/JNK in osteoblasts, and that SAPK/JNK plays a role in addition to p42/p44 MAP kinase and p38 MAP kinase in TGF-β-induced VEGF synthesis.

Key words
TGF-β · VEGF · SAPK/JNK · Osteoblast

Introduction

Vascular endothelial growth factor (VEGF) has been characterized as a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells [1]. VEGF, which is produced and secreted from a variety of cell types, is known to increase capillary permeability and stimulate proliferation of endothelial cells [1]. As for bone metabolism, inactivation of VEGF has been shown to cause complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate [2]. Accumulating evidence indicates that osteoblasts among bone cells produce and secrete VEGF in response to various humoral factors such as insulin-like growth factor I and vitamin D_3 [1,3–5]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts; the former is responsible for bone formation and the latter for bone resorption [6]. During bone remodeling, capillary endothelial cells provide the microvasculature as well as osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, and migrate into the resorption lacuna. Osteoblast, osteoclast and capillary endothelial cell activity is recognized to be closely coordinated via humoral factors and direct cell-to-cell contact, and these cells cooperatively regulate bone metabolism [7]. Thus, there is no doubt that VEGF secreted from osteoblasts plays an important role in bone metabolism regulation. It has recently

Affiliation
[1] Department of Pharmacology, Gifu University School of Medicine, Japan
[2] Institute of Biological Science, University of Tsukuba, Ibaraki, Japan
[3] Department of Internal Medicine, Chubu National Hospital, National Institute for Longevity Sciences, Obu, Aichi, Japan

Correspondence
Dr. Osamu Kozawa · Department of Pharmacology · Gifu University Graduate School of Medicine · Gifu 501-1194 · Japan · Phone: +81 (58) 230-6214 · Fax: +81 (58) 230-6218 · E-Mail: okozawa@cc.gifu-u.ac.jp

Received 25 May 2004 · **Accepted after revision** 20 September 2004

Bibliography
Horm Metab Res 2005; 37: 140–145 © Georg Thieme Verlag KG Stuttgart · New York ·
DOI 10.1055/s-2005-861291 · ISSN 0018-5043

been reported that transforming growth factor-β (TGF-β) induces the expression of VEGF in osteoblast-like MC3T3-E1 cells [8,9]. However, the mechanism behind VEGF synthesis in osteoblasts has not yet been precisely clarified.

TGF-β is a multifunctional cytokine that regulates cell growth, differentiation and extracellular matrix production [10]. TGF-β, which is abundantly stored in bone matrix tissue, stimulates the recruitment and proliferation of osteoblasts [11]. As for intracellular signaling, TGF-β binds to the TGF-β type II receptor, which activates TGF-β type I receptor [12,13]. Activated type I receptor is known to phosphorylate Smad 2 and Smad 3, resulting in their translocation into the nucleus, where they can bind to DNA in the promoters of TGF-β target genes [14]. In addition to the Smad signaling pathway, other signaling pathways such as the mitogen-activated protein (MAP) kinase superfamily have recently been shown to mediate TGF-β signaling [14]. Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and c-*Jun* *N*-terminal kinase, are known to be central elements used by mammalian cells to transduce the diverse messages [15]. TGF-β-activated kinase (TAK1), a member of the MAP kinase kinase kinase family, has been identified as an upstream kinase for MAP kinase [16]. TAK1 kinase activity is stimulated by TGF-β in osteoblast-like MC3T3-E1 cells [16]. The p44/p42 MAP kinase pathway has been reported to participate in the upregulation of β1(I) collagen gene expression by TGF-β in ROS17/2.8 osteosarcoma cells [17]. We previously reported that TGF-β activates p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells resulting in VEGF synthesis [18,19]. However, the involvement of SAPK/JNK in TGF-β signaling in osteoblasts has not yet been elucidated.

In the present study, we investigated the involvement of SAPK/JNK in TGF-β-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. Here, we demonstrate that TGF-β activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in these cells, and that SAPK/JNK plays a role as a positive regulator in TGF-β-induced VEGF synthesis.

Materials and Methods

Materials

TGF-β and mouse VEGF enzyme immunoassay kit were purchased from R&D Systems, Inc. (Minneapolis, MN). SP600125, SB203580 and PD98059 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phosphospecific SAPK/JNK antibodies and SAPK/JNK antibodies were purchased from New England BioLabs, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. SP600125, SB203580 or PD98059 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the VEGF assay or the analysis of SAPK/JNK.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [20] were maintained as previously described [21]. 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 were seeded into 35 mm diameter dishes or 90 mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

VEGF assay

The cultured cells were stimulated by TGF-β in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with SP600125, PD98059 or SB203580 for 60 min. The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF enzyme immunoassay kit.

Analysis of SAPK/JNK

The cultured cells were stimulated by TGF-β in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 × g for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli [22] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [23] by using phosphospecific SAPK/JNK antibodies or SAPK/JNK antibodies with peroxidase-labeled antibodies raised in goat anti-rabbit IgG used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film using the ECL Western blotting detection system. When indicated, the cells were pretreated with SP600125, PD98059 or SB203580 for 60 min.

Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT). Densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

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 ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Effect of TGF-β on SAPK/JNK phosphorylation in MC3T3-E1 cells

To clarify whether TGF-β activates SAPK/JNK in osteoblast-like MC3T3-E1 cells, we examined the effect of TGF-β on SAPK/JNK phosphorylation. TGF-β markedly induced SAPK/JNK phosphorylation in a time-dependent manner (Fig. **1**). The maximum effect of TGF-β on phosphorylation was observed at 60 min after stimulation.

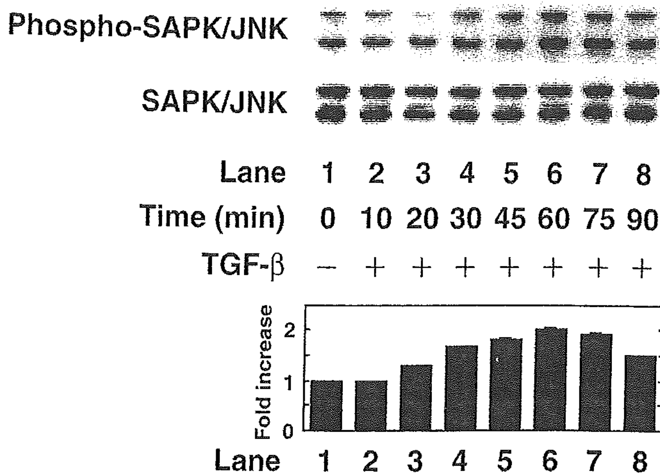


Fig. 1 Effect of TGF-β on SAPK/JNK phosphorylation in MC3T3-E1 cells. The cultured cells were stimulated by 5 ng/ml of TGF-β for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific SAPK/JNK or SAPK/JNK. The upper bands and the lower ones indicated p54 and p46 SAPK/JNK, respectively. Lane 1, control cells.

Effect of SP600125 on TGF-β-induced VEGF synthesis in MC3T3-E1 cells

We have recently shown that TGF-β stimulates VEGF synthesis by activating p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells [19]. To investigate whether SAPK/JNK is involved in TGF-β-induced VEGF synthesis in MC3T3-E1 cells, we examined the effect of SP600125, a highly specific inhibitor of JNK [24], on VEGF synthesis. SP600125, which alone hardly affected basal VEGF levels, significantly reduced TGF-β-stimulated VEGF synthesis (Fig. **2A**). The inhibitory effect of SP600125 on TGF-β-induced VEGF synthesis was dose-dependent in the range between 1 and 30 µM (Fig. **2B**). The maximum inhibitory effect of SP600125 on synthesis was observed at 30 µM, which caused a 44% reduction in the effect of TGF-β.

Effect of SP600125 on SAPK/JNK phosphorylation induced by TGF-β in MC3T3-E1 cells

We found that SP600125 truly inhibited SAPK/JNK phosphorylation induced by TGF-β (Fig. **3**). According to the densitometric analysis, SP600125 caused a reduction of around 50% in the effect of TGF-β on SAPK/JNK phosphorylation.

Effect of PD98059 or SB203580 on TGF-β-induced SAPK/JNK phosphorylation in MC3T3-E1 cells

PD98059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase [25], failed to affect TGF-β-induced SAPK/JNK phosphorylation (Fig. **4a**). Additionally, SB203580, a specific inhibitor of p38 MAP kinase [26], did not reduce TGF-β-induced SAPK/JNK phosphorylation (Fig. **4b**).

Combined effect of SP600125 and PD98059 or that of SP600125 and SB203580 on TGF-β-stimulated VEGF synthesis in MC3T3-E1 cells

We further examined the effect of a combination of SP600125 and PD98059 on TGF-β-stimulated VEGF synthesis in MC3T3-E1 cells. Combining SP600125 and PD98059 significantly reduced TGF-β-stimulated VEGF synthesis in an additive manner (Table **1**). Moreover, a combination of SP600125 and SB203580 also suppressed VEGF synthesis induced by TGF-β in an additive manner (Table **2**).

Discussion

The MAP kinase superfamily is currently known to mediate intracellular signaling of extracellular agonists and play a crucial role in cellular function including proliferation, differentiation, and cell death in a variety of cells [15]. Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK function as central elements used by mammalian cells to transduce a variety of messages from extracellular agonists to nucleus [15]. In the present study, we demonstrated that TGF-β induces SAPK/JNK phosphorylation in osteoblast-like MC3T3-E1 cells. We have previously shown that TGF-β activates both p44/p42 MAP kinase

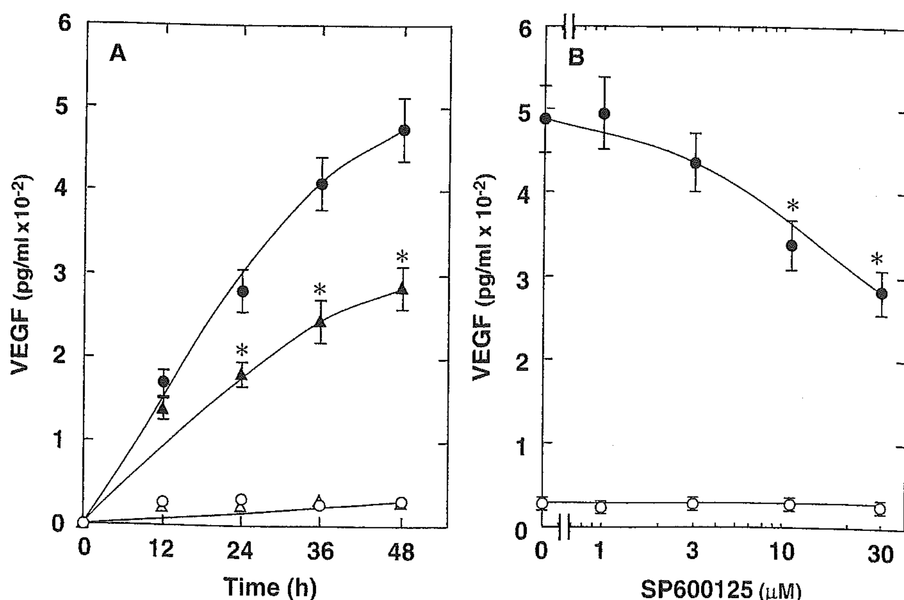


Fig. 2 Effect of SP600125 on TGF-β-induced VEGF synthesis in MC3T3-E1 cells. **a** The cultured cells were pretreated with 30 µM SP600125 (triangles) or vehicle (circles) for 60 min, and then stimulated by 5 ng/ml TGF-β (solid symbols) or vehicle (open symbols) for the indicated periods. **b** The cultured cells were pretreated with various doses of SP600125 for 60 min, and then stimulated by 5 ng/ml TGF-β (solid circles) or vehicle (open circles) for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared with the value of TGF-β alone.

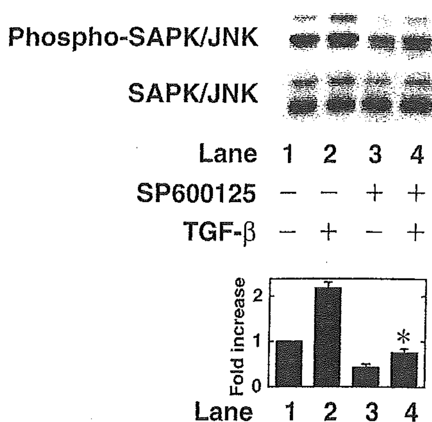
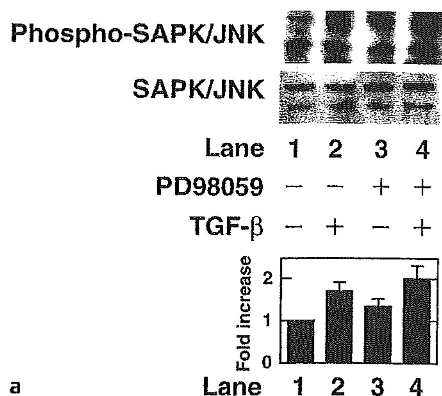


Fig. 3 Effect of SP600125 on TGF-β-induced SAPK/JNK phosphorylation in MC3T3-E1 cells.

The cultured cells were pretreated with 30 μM SP600125 or vehicle for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of TGF-β-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared with the value of TGF-β alone.



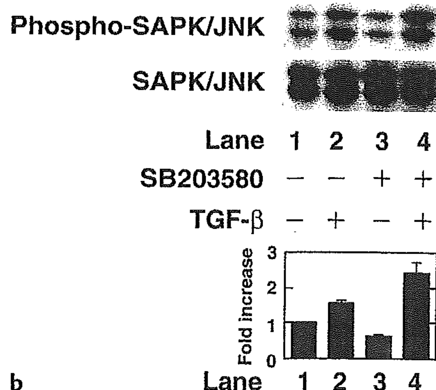


Fig. 4 Effect of PD98059 or SB203580 on TGF-β-induced SAPK/JNK phosphorylation in MC3T3-E1 cells.

a The cultured cells were pretreated with 50 μM PD98059 or vehicle for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 20 min. **b** Cultured cells were pretreated with 30 μM SB203580 or vehicle for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phosphospecific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of TGF-β-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

Table 1 Effect of a combination of SP600125 and PD98059 on TGF-β-stimulated VEGF synthesis in MC3T3-E1 cells

SP600125	*PD98059*	*TGF-β*	*VEGF (pg/ml)*
−	−	−	28 ± 3
−	−	+	487 ± 40
−	+	−	24 ± 3
−	+	+	320 ± 35*
+	−	−	21 ± 2
+	−	+	281 ± 29*
+	+	−	28 ± 5
+	+	+	136 ± 18**

The cultured cells were pretreated with 30 μM SP600125, 10 μM PD98059 or vehicle for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared with the value of TGF-β alone. $^{**}p < 0.05$, compared with the value of SP600125 and TGF-β.

Table 2 Effect of a combination of SP600125 and SB203580 on the TGF-β-stimulated VEGF release in MC3T3-E1 cells

SP600125	*SB203580*	*TGF-β*	*VEGF (pg/ml)*
−	−	−	25 ± 3
−	−	+	505 ± 49
−	+	−	28 ± 3
−	+	+	316 ± 30*
+	−	−	21 ± 2
+	−	+	323 ± 29*
+	+	−	24 ± 5
+	+	+	115 ± 18**

The cultured cells were pretreated with 30 μM SP600125, 10 μM SB203580 or vehicle for 60 min, and then stimulated by 5 ng/ml TGF-β or vehicle for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared with the value of TGF-β alone. $^{**}p < 0.05$, compared with the value of SP600125 and TGF-β.

and p38 MAP kinase in MC3T3-E1 cells [18]. MAP kinases are known to be activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase [15,27]. Therefore, these results strongly suggest that TGF-β independently activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells.

TGF-β reportedly induces the expression of VEGF in osteoblast-like MC3T3-E1 cells [8,9]. Here, we have shown that SP600125, a specific inhibitor of SAPK/JNK [24], significantly suppresses TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. These results strongly suggest that the activation of SAPK/JNK is involved in TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. In addition, we found that SP600125 truly attenuated SAPK/JNK phosphorylation induced by TGF-β in these cells. Thus, it is probable that suppression of TGF-β-stimulated VEGF synthesis by SP600125 is due to the inhibition of SAPK/JNK activation. We have recently reported that activating both p44/p42 MAP kinase and p38 MAP kinase play important

roles as positive regulators in TGF-β-induced VEGF synthesis in MC3T3-E1 cells [19]. In addition, we have demonstrated that PD98059, a specific inhibitor of MEK [25] or SB203580, a specific inhibitor of p38 MAP kinase [26], did not reduce TGF-β-induced SAPK/JNK phosphorylation. Therefore, SAPK/JNK would seem to participate at least partly in TGF-β-stimulated VEGF synthesis, independently of p44/p42 MAP kinase or p38 MAP kinase. Moreover, TGF-β-stimulated VEGF synthesis was suppressed additively by a combination of SP600125 and PD98059 or SP600125 and SB203580. Based on these findings, it is most likely that VEGF synthesis stimulated by TGF-β is mediated through p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK activation in osteoblast-like MC3T3-E1 cells. We have recently reported that SAPK/JNK is involved in prostaglandin E1-induced VEGF synthesis in MC3T3-E1 cells [28]. Interestingly, not p44/p42 MAPK but p38 MAPK is involved in PGE1-induced VEGF synthesis in these cells [29]. In addition, activating p44/p42 MAPK and JNK but not p38 MAPK by TGF-β reportedly negatively regulates Smad3-induced alkaline phosphatase activity and mineralization in MC3T3-E1 cells [30]. Thus, it is likely that each of these MAP kinases plays a separate role and finely regulates the cellular function of osteoblasts. Further investigations on other signaling systems of TGF-β such as Smad 2 and Smad 3 are required to clarify the detail regulatory mechanism of VEGF synthesis induced by TGF-β in osteoblasts. Regarding the involvement of MAP kinases in VEGF synthesis stimulated by TGF-β, we previously reported that p44/p42 MAP kinase and p38 MAP kinase, but not SAPK/JNK, participate in TGF-β-induced VEGF synthesis in aortic smooth muscle A10 cells [31]. The involvement of SAPK/JNK in TGF-β-induced VEGF synthesis may not be a general phenomenon, but specific to osteoblasts.

Capillary network-providing microvasculature expansion is an essential process in bone remodeling [7]. Since VEGF is a specific mitogen of vascular endothelial cells [1], it is probable that VEGF synthesized by osteoblasts acts as an important intercellular mediator between osteoblasts and vascular endothelial cells. It has been reported that VEGF is involved in trabecular bone formation and expansion of the hypertrophic chondrocyte zone in epiphyseal growth plate of mouse [2], supporting the importance of VEGF in bone metabolism. Flt-1, known as VEGF receptor 1, has been shown as a novel cell surface marker for the lineage of monocyte-macrophages in humans, which was found to differentiate efficiently into multinuclear osteoclasts [32]. Moreover, VEGF has recently been reported to induce osteoclast chemotaxis [33]. These evidences strongly suggest that VEGF secreted from osteoblasts also plays a role in the regulation of osteoclastic bone resorption. On the other hand, TGF-β is know to be produced by osteoblasts, stored abundantly in bone matrix in the latent form, and activated in the bone microenviroment [11]. During bone resorption, there is speculation that TGF-β in extracellular bone matrix is released, affecting osteoblasts and osteoprogenitor cells existing in the neighborhood. In addition, a recent study has reported that TGF-β expression is regulated by IGF-I receptor signaling in Ewing's osteosarcoma cells, suggesting the involvement of tumor angiogenesis [34]. Taking these reports into account, it seems that TGF-β-induced VEGF synthesis by osteoblasts plays a pivotal role in the process of bone remodeling through regulating the capillary endothelial cells proliferation and osteoclastic bone resorption. Further investigations would be necessary to clarify the exact role of VEGF in bone metabolism.

In conclusion, our present results strongly suggest that TGF-β activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in osteoblasts, and that SAPK/JNK plays at least a contributory role as a positive regulator in TGF-β-induced VEGF synthesis.

Acknowledgement

This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and the Research Grants for Longevity Sciences (15A-1 and 15C-2) from the Ministry of Health, Labor and Welfare of Japan.

References

1 Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997; 18: 4–25
2 Gerber H-P, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 1999; 5: 623–628
3 Goad DL, Rubin J, Wang H, Tashijian AH Jr, Patterson C. Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinology 1996; 137: 2262–2268
4 Wang DS, Yamazaki K, Nohtomi K, Shizume K, Ohsumi K, Shibuya M, Demura H, Sato K. Increase of vascular endothelial growth factor mRNA expression by 1,25-dihydroxyvitamin D_3 in human osteoblast-like cells. J Bone Miner Res 1996; 11: 472–479
5 Schalaeppi JM, Gutzwiller S, Finlenzeller G, Fournier B. 1,25-dihydroxyvitamin D_3 induces the expression of vascular endothelial growth factor in osteoblastic cells. Endocr Res 1997; 23: 213–229
6 Nijweide PJ, Burger EH, Feyen JHM. Cells of bone: proliferation, differentiation, and humoral regulation. Physiol Rev 1986; 66: 855–886
7 Erlebacher A, Filvaroff EH, Girelman SE, Derynck R. Toward a molecular understanding of skeletal development. Cell 1995; 80: 371–378
8 Saadeh PB, Mehrara BJ, Steinbrech DS, Dudziak ME, Greenwald JA, Luchs JS, Spector JA, Ueno H, Gittes GK, Longaker MT. Transforming growth factor-β1 modulates the expression of vascular endothelial growth factor by osteoblasts. American J Physiol 1999; 277: C628–C637
9 Chua CC, Hamdy RC, Chua BH. Mechanism of transforming growth factor-β-induced expression of vascular endothelial growth factor in murine osteoblastic MC3T3-E1 cells. Biochim Biophys Acta 2000; 1497: 69–76
10 Massague J, Blain SW, Lo RS. TGF-β signaling in growth control, cancer, and heritable disorders. Cell 2000; 103: 295–309
11 Bonewald LF. Transforming growth factor-beta. In: Bilezikian JP, Raisz LG, Rodan GA (eds). Principles of Bone Biology, 2nd ed. San Diego: Academic Press, 2002: 903–918
12 Heldin CH, Miyazono K, ten Dijke P. TGF-β signalling from cell membrane to nucleus through SMAD proteins. Nature 1997; 390: 465–471
13 Massague J. TGF-β signal transduction. Ann Rev Biochem 1998; 67: 753–791
14 Miyazono K, Kusanagi K, Inoue H. Divergence and convergence of TGF-β/BMP signaling. J Cell Physiol 2001; 187: 265–276
15 Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 1999; 79: 143–180
16 Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K. Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. Science 1995; 270: 2008–2011
17 Palcy S, Goltzman D. Protein kinase signalling pathways involved in the up-regulation of the rat alpha1(I) collagen gene by transforming

growth factor β1 and bone morphogenetic protein 2 in osteoblastic cells. Biochem J 1999; 343: 21–27

[18] Hatakeyama D, Kozawa O, Niwa M, Matsuno H, Ito H, Kato K, Tatematsu N, Shibata T, Uematsu T. Upregulation by retinoic acid of transforming growth factor-β-stimulated heat shock protein 27 induction in osteoblasts: involvement of mitogen-activated protein kinases. Biochim Biophys Acta 2002; 1589: 15–30

[19] Tokuda H, Hatakeyama D, Akamatsu S, Tanabe K, Yoshida M, Shibata T, Kozawa O. Involvement of MAP kinases in TGF-β-stimulated vascular endothelial growth factor synthesis in osteoblasts. Arch Biochem Biophys 2003; 415: 117–125

[20] Sudo H, Kodama H, Amagai Y, Yamamoto S, Kasai S. In vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 1983; 96: 191–198

[21] 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: 130–134

[22] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680–685

[23] Kato K, Ito H, Hasegawa K, Inaguma Y, Kozawa O, Asano T. Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 glioma cells. J Neurochem 1996; 66: 946–950

[24] Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal Kinase. Proc Natl Acad Sci USA 2001; 98: 13681–13686

[25] Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. J Biol Chem 1995; 270: 27489–27494

[26] Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 1995; 364: 229–233

[27] Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, David RJ. Pro-inflammatory cytokines and enviromental stress cause p38 MAP kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 1995; 270: 7420–7426

[28] Kanno Y, Tokuda H, Nakajima K, Ishisaki A, Shibata T, Numata O, Kozawa O. Involvement of SAPK/JNK in prostaglandin E(1)-induced VEGF synthesis in osteoblast-like cells. Mol Cell Endocrinol. 2004; 220: 89–95

[29] Tokuda H, Kozawa O, Miwa M, Uematsu T. p38 mitogen-activated protein (MAP) kinase but not p44/p42 MAP kinase is involved in prostaglandin E1-induced vascular endothelial growth factor synthesis in osteoblasts. J Endocrinol. 2001; 170: 629–638

[30] Sowa H, Kaji H, Yamaguchi T, Sugimoto T, Chihara K. Activations of ERK1/2 and JNK by transforming growth factor beta negatively regulate Smad3-induced alkaline phosphatase activity and mineralization in mouse osteoblastic cells. J Biol Chem. 2002; 277: 36024–36031

[31] Yamamoto T, Kozawa O, Tanabe K, Akamatsu S, Matsuno H, Dohi S, Uematsu T. Involvement of p38 MAP kinase in TGF-beta-stimulated VEGF synthesis in aortic smooth muscle cells. J Cell Biochem. 2001; 82: 591–598

[32] Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, Shibuya M. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. Blood 2001; 97: 785–791

[33] Henriksen K, Karsdal MA, Delaissé JM, Engsig MT. RANKL and VEGF induce osteoclast chemotaxis through an ERK1/2 dependent mechanism. J Biol Chem 2003; 278: 48745–48753

[34] Strammiello R, Benini S, Manara MC, Perdichizzi S, Serra M, Spisni E, Picci P, Scotlandi K. Impact of IGF-I/IGF-IR circuit on the angiogenetic properties of Ewing's sarcoma cells. Horm Metab Res. 2003; 35: 675–684

Possible Involvement of Phosphatidylinositol 3-Kinase/ Akt Pathway in Insulin-like Growth Factor-I-induced Alkaline Phosphatase Activity in Osteoblasts

T. Noda[1]
H. Tokuda[1,2]
M. Yoshida[1]
E. Yasuda[1]
Y. Hanai[2]
S. Takai[1]
O. Kozawa[1]

Abstract

In the present study, we investigated whether Akt is involved in insulin-like growth factor-I (IGF-I)-stimulated activity of alkaline phosphatase, a marker of mature osteoblast phenotype, in osteoblast-like MC3T3-E1 cells. IGF-I induced the phosphorylation of Akt in these cells. Akt inhibitor significantly suppressed the IGF-I-stimulated alkaline phosphatase activity. The phosphorylation of Akt induced by IGF-I was reduced by the Akt inhibitor. LY294002 and wortmannin, inhibitors of phosphatidylinositol 3-kinase, significantly suppressed the IGF-I-induced alkaline phosphatase activity. The phosphorylation of Akt induced by IGF-I was markedly reduced by LY294002 and wortmannin. These results strongly suggest that phosphatidylinositol 3-kinase/Akt plays a role in the IGF-I-stimulated alkaline phosphatase activity in osteoblasts.

Key words
IGF-I · Alkaline phosphatase · Akt · Osteoblast

Introduction

Insulin-like growth factor-I (IGF-I) is generally known to play an important role in bone metabolism [1,2]. Mainly synthesized and secreted from liver, IGF-I mediates a variety of the actions of growth hormone secreted from pituitary gland under the control of the hypothalamus. Evidence is accumulating that IGF-I is necessary for bone-fracture healing [3]. Bone metabolism is recognized to be mainly regulated by two functional cells, osteoblasts and osteoclasts, with the former responsible for bone formation and the latter for bone resorption [4]. As for osteoblasts, IGF-I has been reported to stimulate the proliferation of these cells and synthesize bone matrix proteins [1]. We have previously demonstrated that IGF-I induces DNA synthesis synergistically with protein kinase C activation in osteoblast-like MC3T3-E1 cells [5]. In addition, IGF-I reportedly stimulates alkaline phosphatase activity, a marker of mature osteoblast phenotype [6], in osteoblasts [7]. IGF-I is also produced by osteoblasts [2]. In a previous study [8], we have shown that osteoblast-like MC3T3-E1 cells secrete IGF-I resulting in stimulation of mineralization, and protein kinase C activation suppresses the secretion of IGF-I. These findings gave rise to our speculation that IGF-I secreted from osteoblasts may play a crucial role in the regulation of bone metabolism.

Akt, also known as protein kinase B, is a serine/threonine protein kinase that plays a crucial role in mediating intracellular signaling of variety of agonists including IGF-I, platelet derived growth factor (PDGF), and cytokines [9]. Akt has been shown to regulate biological functions such as gene expression, survival and oncogenesis [9]. Akt has been identified as a downstream target of phosphatidylinositol 3-kinase [10,11]. Akt containing a pleckstrin homology domain is recruited to the plasma membrane by the lipid product of phosphatidylinositol 3-kinase, and activated. As for osteoblasts, IGF-I and PDGF have been reported to induce translocation of Akt to the nucleus [12]. In addition, cyclic stretch or androgen was recently reported to activate Akt [13,14]. How-

Affiliation
[1] Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu, Japan
[2] Department of Clinical Laboratory, National Hospital for Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan

Correspondence
Dr. Osamu Kozawa · Department of Pharmacology, Gifu University Graduate School of Medicine · Gifu 501-1194 · Japan · Phone: +81 (58) 230 62 14 · Fax: +81 (58) 230 62 15 · E-Mail: okozawa@cc.gifu-u.ac.jp

Received 3 November 2004 · **Accepted after revision** 10 March 2005

Bibliography
Horm Metab Res 2005; 37: 270–274 © Georg Thieme Verlag KG Stuttgart · New York ·
DOI 10.1055/s-861468 · ISSN 0018-5043