**ORIGINAL ARTICLE**

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# A study on the regulatory effect of p-38 MAP kinase on nitric oxide and interleukin-6 in osteoblasts

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Tooth movement is the result of bone metabolism in the periodontium, where various cytokines take important roles. Interleukin-6(Il-6) and nitrous oxide (NO) were reported to be secreted from osteoblasts in the process of bone resorption. The mechanism of the process has not been clearly understood, but the activation of mitogen-activated protein kinase (MAPK) was known to be an important process in the release of the inflammatory cytokines in macrophages.

In this regard, to prove the role of MAPK in the release of IL-6 and NO in MC3T3E-1 osteoblasts, Northern blot analysis, Western blot analysis and immune complex kinase assay were used.

As a result, the treatment of MC3T3E-1 osteoblast cultures with combined interferon-$\gamma$ (IFN-$\gamma$), lipopolysaccharide (LPS) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) induces expressions of inducible nitric oxide synthase (iNOS) and IL-6, resulting in sustained releases of large amounts of NO and IL-6. However, IFN-$\gamma$, LPS, and TNF-$\alpha$ individually induce a non-detectable or small amount of NO and IL-6 in MC3T3E-1 osteoblasts. The role of MAPK activation in the early intracellular signal transduction involved in iNOS and IL-6 transcription in the combined agents-stimulated osteoblasts has been investigated. The p38 MAPK pathway is specifically involved in the combined agents-induced NO and IL-6 release, since NO and IL-6 release in the presence of a specific inhibitor of p38 MAPK, 4-(4-fluorophenyl)-2-(4-metylsulfinylphenyl)-5-(4-pyridyl)imidazole) (SB203580), were significantly diminished. In contrast, PD98059, a specific inhibitor of MEK1, had no effect on NO and IL-6 release. Northern blot analysis showed that the p38 MAPK pathway controlled the iNOS and IL-6 transcription level. These data suggest that p38 MAPK play an important role in the secretion of NO and IL-6 in LPS/IFN$\gamma$- or TNF-$\alpha$/IFN-$\gamma$-treated MC3T3E-1 osteoblasts.

**Key words :** Osteoblast , P-38 MAP kinase, Nitric oxide, Interleukin-6

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Bone metabolism is tightly regulated at the local level by networks of hormones, cytokines, and other factors. In pathological conditions of bone remodeling, including osteoporosis and periodontal diseases, inflammatory cytokines and local mediators are responsible for the enhancement of osteoclastic

resorption and inhibition of repair at the sites of bone resorption. It has been reported that pro-inflammatory agents, such as tumor necrosis factor-$\alpha$ *(TNF-$\alpha$)* and interferon-$\gamma$ (IFN-$\gamma$), function as modulators of bone remodeling *in vitro* and *in vivo*.[1,2] TNF-$\alpha$ is directly implicated in bone resorption by modulating the activities of osteoclasts and osteoblasts. These cytokines are also important for inducing cellular effector molecules, including oxygen free radicals and nitric oxide, which modulate the metabolism of bone tissue.

Nitric oxide (NO), a short-lived and highly reactive free radical, has been implicated in many biological functions in nervous, vascular, and immune systems.[3-5] NO-synthase (NOS) catalyses L-arginine to NO, which, when binding with guanylate cyclase, increases cGMP formation. Three distinct forms of NOS are identified: a neuronal form (nNOS) in the brain, an endothelial form (ecNOS) in vascular endothelium, and an inducible form (iNOS) in macrophages.[6-8] Both ecNOS and nNOS are constitutively expressed at low levels and modulate the entry of $Ca^{2+}$ into cells. However, iNOS is regulated by transcriptional activation of the gene. MacIntyre et al.[9] reported NO augmented osteoclast activity in a similar manner with calcium, which induced the retraction of cells by inhibiting their movement to a fresh site of bone resorption. However, NO has a preferential role as an inhibitor of osteoclast activity both *in vitro* and *in vivo*.[10,11] Primary osteoblast-like cells and osteosarcoma cell lines release NO upon the stimulation of inflammatory agents including IFN-$\gamma$, LPS and TNF-$\alpha$.[12-15] These data suggest that NO produced by osteoblasts may act on either osteoblasts in an autocrine manner, or on osteoclasts in bone remodeling.

Interleukin-6 (IL-6) is a multifunctional cytokine that regulates a variety of cellular functions.[16-18] In bone metabolism, IL-6 is recognized as an autocrine/paracrine factor, which induces osteoclast formation and stimulates the activity to reabsorb bone.[19] Bone resorptive agents, such as parathyroid hormone, tumor necrosis factor, and platelet-derived growth factor have been reported to stimulate IL-6 production and secretion in cultured osteoblasts.[20-22] Thus, accumulating evidence suggests that IL-6 secreted from osteoblasts acts as a downstream effect of diverse bone resorptive agents, resulting in the induction of bone resorption.

Bhat et al.[23] and Da Silva et al.[24] have recently shown that mitogen-activated protein kinase (MAPK) cascades are involved in cytokine and lipopolysaccharide (LPS)-mediated iNOS induction in astrocytes and microglial cells.

MAPK plays a role in mediating intracellular signal transduction and regulating cytokine production by mononuclear cells in response to a variety of extracellular stimuli. In response to appropriate stimuli, the MAPKs are activated by phosphorylation on both adjacent threonine and tyrosine residues that are separated by a single amino acid.[20,23] For extracellularly regulated kinases (ERK), the best studied of the MAPK families, this intervening amino acid is glutamate; for the p38 MAPK family, it is glycine. While ERK has been classically associated with growth- and differentiation-inducing signals, p38 MAPK is involved in inflammatory cytokines and environmental stress inducers.[25]

In this study, we would like to prove the expression of iNOS and IL-6 in MC3T3E-1 osteoblast treated with proinflammatory cytokines in relation to P38 MAPK activation.

## MATERIALS AND METHODS

### Materials

Murine interferon gamma and recombinant TNF-$\alpha$ were from R&D systems (Minneapolis, MN, USA). Anti-phospho-specific p38 MAPK ($Tyr^{182}$) antibody was purchased from New England Biolabs (Beverly, MA, USA). Anti-total p38 MAPK was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-iNOS and anti-IL-6 antibodies were obtained from Transduction Laboratories (Lexington, KY, USA). SB203580 was purchased from Calbiochem Corp. (San Diego, CA, USA). [$\gamma$-$^{32}$P] ATP was purchased from Du

Pont-NEN (Boston, MA, USA). Bovine serum (supplemented), fetal bovine serum (FBS), and α-modified Eagle medium (MEM) were from Gibco BRL (Grand Island, NY, USA). Six-well, 12-well, and 100-mm culture dishes were purchased from Corning-Costar (Cambridge, MA, USA).

### Cell culture

The MC3T3E-1 osteoblasts were maintained in α-MEM with 10% FBS, penicillin (100 U $ml^{-1}$), and streptomycin (100 g $ml^{-1}$). Cells were maintained at 37 ℃ in a humidified atmosphere of 95% air and 5% $CO_2$.

### Measurement of nitrite production

NO production was determined by measurement of nitrite in the medium as based on the Griess reaction[25]. An aliquot of the spent medium was mixed with an equal volume of a 1 : 1 mixture of 1% sulfanilamide in water and 0.1% N-1-naphthyl-ethylenediamine dihydrochloride in 5% phosphoric acid. The absorbance was then read at 570 nm. Sodium nitrite dissolved in the culture medium was used as the standard.

### Assay for IL-6

The cultured cells were pretreated with SB203580 for 1 h. The MC3T3E-1 osteoblasts were subsequently stimulated by TNF-α or LPS in the presence or absence of IFN-γ in 1 ml of α-MEM containing 10% FBS for the indicated periods. The conditioned medium was collected and IL-6 in the medium from MC3T3E-1 osteoblasts was measured by a mouse IL-6 ELISA kit, respectively.[26]

### Northern blot analysis of iNOS and IL-6 expression

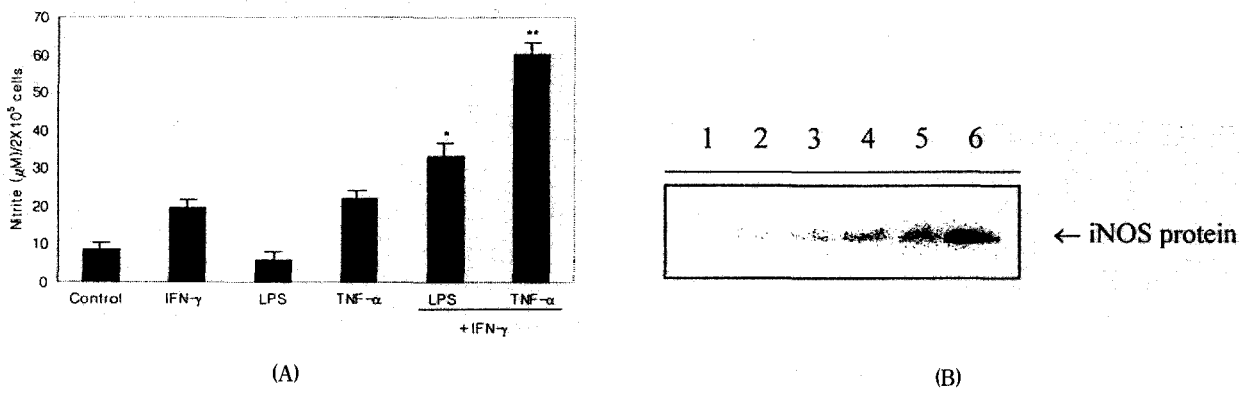
Total cellular RNA was prepared with a TRIzol reagent (Gibco BRL, Gaiterberg, MD, USA) according to the manufacturer's instructions. RNA (20 μg) was subjected to electrophoresis in 1% formaldehyde agarose gels, transferred to a nylon membrane by capillary blotting and fixed by UV irradiation. Hybridization was carried out 42℃ in 50 mM Tris-HCl, pH 7.4, 40% foramide, 4×SSC (15 mM sodium citrate, 150 mM NaCl). 10×Denhardt's solution, 0.1% $Na_4P_2O_7$, 1% sodium dodecyl sulfate (SDS) and 200 μg/ml herring sperm DNA. The blots were washed to a stringency of 2×SSC and 0.1% SDS at 42℃, and then exposed to an X-ray film at -70℃. A fragment of iNOS or IL-6 cDNA was labeled with α-$^{32}P$, using random primers (Boehringer, Mannheim, Germany), and was used as a hybridization probe.

### Western blot analysis

Western blot was performed for the analysis of p38 MAPK activation (using antibodies specific for the phosphorylated form of the kinase) and for the detection of iNOS and IL-6 protein. Protein samples (cell extracts, 50g) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% bovine serum albumin, 1% milk powder in 10 mM Tris-HCl containing 150 mM NaCl and 0.5% Tween-20 TBS-T for 1 h and incubated overnight with suitably diluted primary antibodies. Following extensive washing with TBS-T, the enhanced chemiluminescence method was used to detect the bands.

### *In vitro* immune complex kinase assay for p38

The phosphotransferase activity of p38 was measured by modification of the procedure previously described.[27] Briefly, MC3T3E-1 osteoblasts ($2\times10^6$ cells/each group) were treated with the indicated agents for various time periods. And then the cells were lysed in EB buffer (1% Triton X-100, 10 mM Tris, pH 7.6, 50 mM NaCl, 1 mg $ml^{-1}$ aprotinin, 5 mM EDTA, 50 mM NaF, 0.1 % 2-mercaptoethanol, and 100μM sodium orthovanadate). The cell lysates were subjected to centrifugation at




(A) (B)

**Fig. 1.** Cytokine stimulation of NO production and iNOS synthesis in MC3T3E-1 osteoblasts. MC3T3E-1 osteoblasts were treated with IFN-$\gamma$ (100 U ml$^{-1}$), LPS (1 $\mu$g ml$^{-1}$) and TNF-$\alpha$ (10 ng ml$^{-1}$) or in combinations : LPS+IFN-$\gamma$ or TNF-$\alpha$+IFN-$\gamma$ for 24 h. (A) The culture media were mixed with Griess reagent for the estimation of NO release (Materials and Methods). Data expressed the means±S.D. of four experiments. (B) The cell extracts were subjected to western blot analysis using antibodies specific iNOS. (1 : control, 2 : IFN-$\gamma$, 3 : LPS, 4 : TNF-$\alpha$, 5 : LPS+IFN-$\gamma$, 6 : TNF-$\alpha$+IFN-$\gamma$).
*, $P < 0.05$ vs. control; **, $P < 0.01$ vs. control.

12,000g at 4℃ for 30 min. The soluble fraction was collected and incubated with anti-p38 antibodies (Santa Cruz Inc. Santa Cruz, CA, USA). After incubation on ice for 3 h, 100μl of a 10 % solution of formalin-fixed *Staphylococcus aureus* (Calbiochem, La Jolla, CA) was added to anti-p38 immunoprecipitates and further incubated on ice for 1 h. The absorbed immune com-plex was washed twice with EB buffer and PAN buffer (10 mM PIPES buffer, pH 7.0, 1% aprotinin, 100 mM NaCl). The immune complex was mixed with 2 μg of GST-ATF21$_{-109}$ proteins as a substrate in 30 μl of the reaction buffer containing 2 μM cold ATP, 2 mM DTT, 20 mM $MgCl_2$, 2 μCi [$\gamma^{32}$-P] ATP, and 20 mM Tris-HCl, pH 7.5 at 30℃ for 20 min. The reaction was terminated by adding 15 μl of 3×SDS-PAGE sample buffer and boiled at 98℃ for 5 min. The proteins were separated on 12% SDS-PAGE and transferred onto a nitrocellulose membrane by the semi-dry electrotransfer system (Ellard Co, Seattle, WA, USA). The gel was dried under the vacuum. The phosphotransferase activity was visualized by autoradiography and quantified by PhosphoImager analyser (BAS, Fuji Co, Tokyo, Japan).

### Statistical treatment of data

The data were analyzed by Students' t-test and a $p < 0.05$ was considered to be significant. All data are presented as the mean±S.D. of triplicate independent cell preparations. Each experiment was repeated three times with similar results.

## RESULTS

### Production of NO and iNOS by MC3T3E-1 osteoblasts

We tested whether MC3T3E-1 osteoblasts produced nitric oxide (NO). MC3T3E-1 osteoblasts were stimulated with IFN-$\gamma$ (100 IU ml$^{-1}$), LPS (1 μg ml$^{-1}$), and TNF-$\alpha$ (10 ng ml$^{-1}$) for 24 h. After the stimula-tion of cells with inflammatory agents, NO released into culture medium was determined by using a colorimetric method. As shown in Fig. 1A, the individual agents, LPS had no apparent effects, IFN-$\gamma$ and TNF-$\alpha$ had only a slight stimulatory effect on the production of NO. LPS and TNF-$\alpha$, however, synergized with IFN-$\gamma$ to induce NO synthesis. TNF-$\alpha$, n particular, enhanced NO

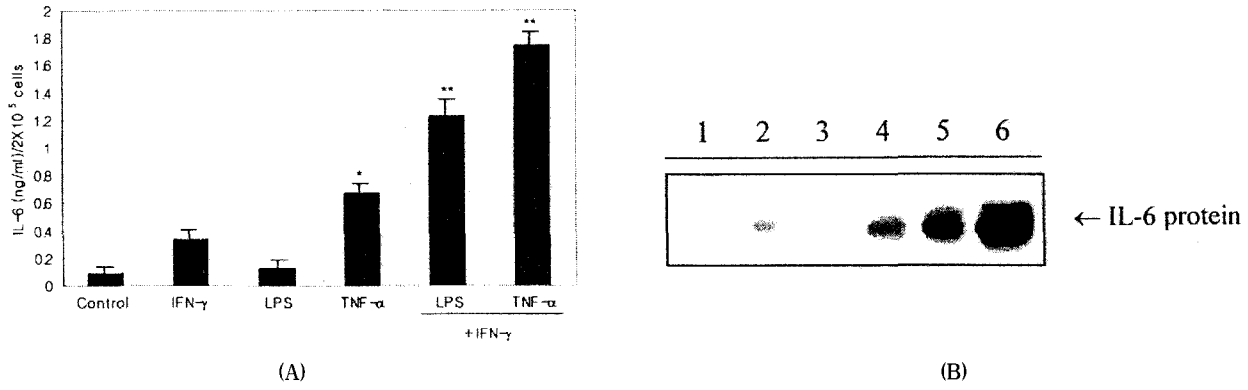



**Fig. 2.** Effects of agents on IL-6 secretion and IL-6 protein expression in MC3T3E-1 osteoblasts. MC3T3E-1 osteoblasts were treated with IFN- (100 U ml-1), LPS (1 μg ml$^{-1}$) and TNF-α (10 ng ml-1) alone or in combinations; LPS+IFN-γ or TNF-α+IFN-γ for 24 h and (A) the culture media were collected and IL-6 in the medium was measured by an IL-6 ELISA kit. Data showed the means±S.D. of four experiments. (B) The cell extracts were subjected to western blot analysis using antibodies specific for IL-6. (1: control, 2: IFN-γ, 3: LPS, 4: TNF-α, 5: LPS+IFN-γ, 6: TNF-α+IFN-γ).
*, P <0.05 vs. control ; **, P< 0.01 vs. control

synthesis by several fold. To determine the expression of iNOS that catalyzes the production of NO in inflammatory agents-treated cultures, we carried out western blot antibodies specific for iNOS. The data illustrated in Fig. 1B confirm the induction of iNOS synthesis in response to cytokine combination. The cultures treated with IFN-γ individually contained little detectable immunoreactive iNOS protein and those treated with TNF-α alone induced more detectable expressions of iNOS, relatively.

### IL-6 secretion and IL-6 protein in MC3T3E-1 osteoblasts

We also examined the effects of inflammatory cytokines on the production of IL-6 from MC3T3E-1 osteoblasts (Fig. 2A). Single treatment with LPS did not stimulate the MC3T3E-1 osteoblasts to produce IL-6. IFN-γ or TNF-α had a relatively stimulatory effect on the production of IL-6. However, in a similar manner to NO, MC3T3E-1 osteoblasts produced a significant amount of IL-6 secretion when stimulated with LPS or TNF-α in the presence of IFN-γ. To determine the expression of IL-6 protein, we performed western blot analysis using an antibody specific for IL-6. The data showed in Fig. 2B confirms the induction of IL-6 protein synthesis in response to the combined agents. The cultures treated with LPS individually did not contain detectable immuno-reactive IL-6 protein.

### Cytokine activation of p38 MAPK in MC3T3E-1 osteoblasts

Bhat et al.[23] suggested a key role of MAPK cascades, especially p38 MAPK, in the cytokine induction of iNOS in astrocytes and microglia. To test the activation of p38 MAPK in response to cytokine treatment, we studied the effect of LPS, TNF-α and IFN-γ alone or in combinations on the phosphorylation of p38 MAP kinase in MC3T3E-1 osteoblasts. Cell lysates were analyzed for p38 phosphorylation by immunoblot using an antibody that specifically recognizes that the phosphorylated form of the protein. As shown in Fig. 3B, C, LPS and TNF-α in the presence or absence of IFN-γ induced a transient increase in p38 MAPK phosphorylation that was visible as early as 30 and 60 min, respectively. The signals were significantly reduced after 120 min and were practically at background level after 240 min. The single

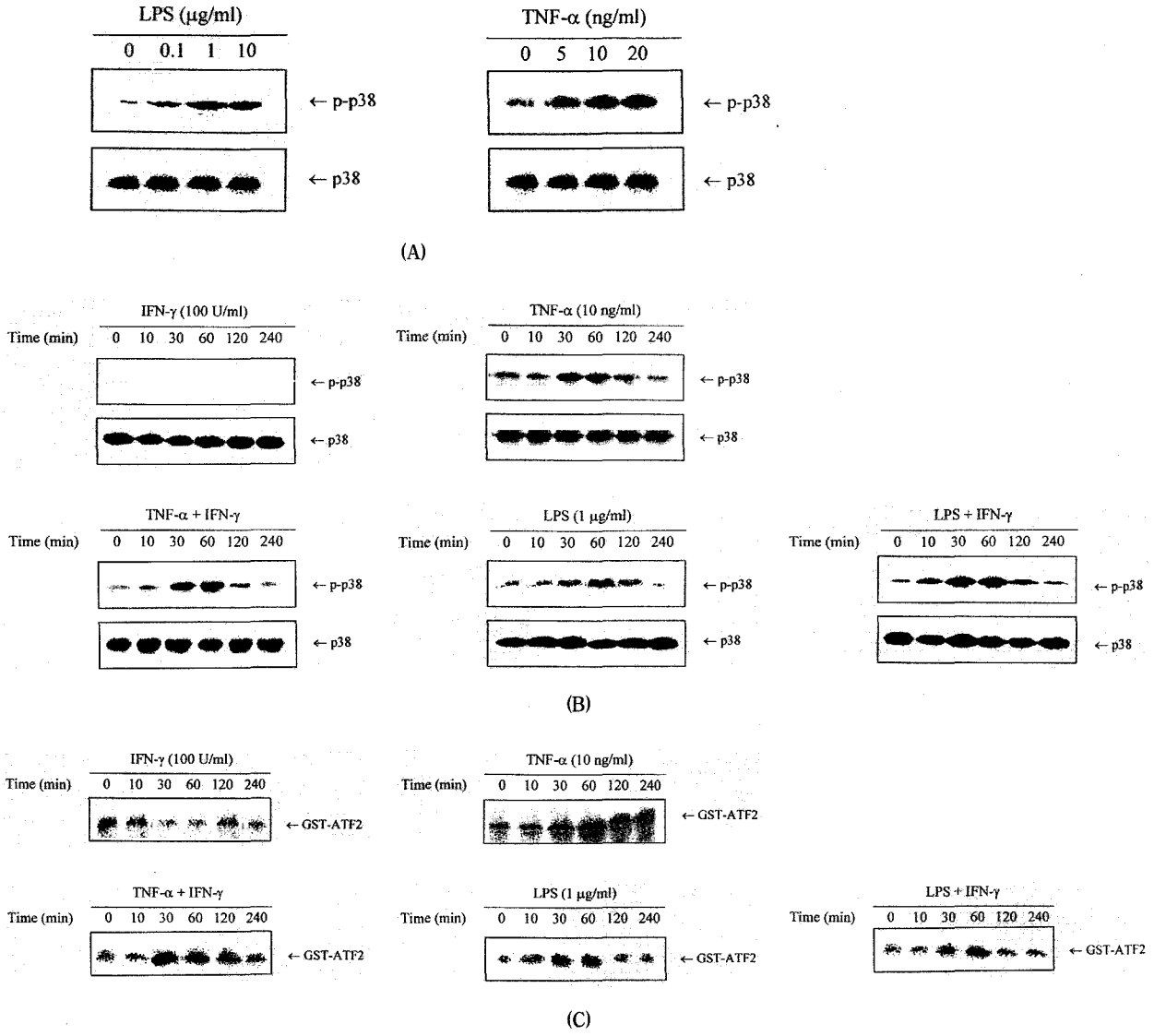
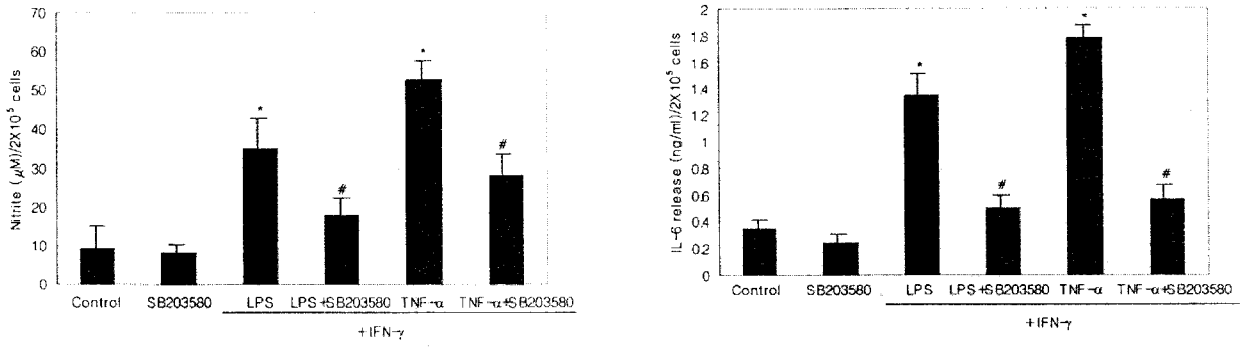



**Fig. 3.** Effect of agents on the activation of p38 MAPK in MC3T3E-1 osteoblasts. (A). MC3T3E-1 cells were exposed to LPS (0, 0,1, 1 or 10 $\mu g\ ml^{-1}$) or TNF-$\alpha$ (0, 5, 10 or 20 $ng\ ml^{-1}$) for 30 min. (B) The cells were treated with IFN-$\gamma$ (100 U ml-1), LPS (1 $\mu g\ ml^{-1}$) and TNF-$\alpha$ (10 $ng\ ml^{-1}$) alone or in combinations; LPS/IFN-$\gamma$ and TNF-$\alpha$/IFN-$\gamma$ for the indicated time intervals. The proteins were then subjected to immunoblot analysis, using antibodies specific for the active (phosphorylated) form of p38 MAPK. Parallel blots ran with anti-total p38 MAPK antibodies which served as controls. (C) Endogenous p38 MAP kinase activity was examined by immunocomplex assays. These are typical results from three independent experiments.

treatment of IFN-$\gamma$ had no effect on p38 MAPK phosphorylation.

To demonstrate that the various cytokines-mediated p38 phosphorylation is functional, the lysates were immunoprecipitated with p38 to activate ATF2, known as a substrate for p38 MAP kinase, being phosphorylated primarily on threonine residues 69 and 71, and this event increases its transactivating properties. ATF2 phosphorylation was then determined by p38 kinase assay. As shown in Fig. 3B, C, a significant increase in




(A) (B)

**Fig. 4.** SB203580, a specific inhibitor of p38 MAPK, inhibits the combined agents-induced NO and IL-6 production in MC3T3E-1 osteoblasts. MC3T3E-1 cultures were treated with LPS/IFN-$\gamma$, or TNF-$\alpha$/IFN-$\gamma$ in the presence or absence of the p38 MAPK inhibitor for 24 h. (A) Aliquots of the culture medium were analyzed for nitrite production. (B) The media were analyzed for IL-6 secretion. Data were means±S.D. of four experiments. *, P <0.05 vs. control ; #, P< 0.05 vs. without SB203580-treated.

ATF2 phosphorylation levels was noted at 30 min and peaked at 60 min of treatment, in parallel with p38 phosphorylation. However, a small stimulation in p38 kinase activity was then observed at 120 min of stimulation. 4-(4-fluorophenyl)-2-(4-metylsulfinyl-phenyl)-5-(4-pyridyl) imidazole (SB203580) (20 $\mu$M), the p38 MAPK specific inhibitor, reduced the stimulated p38 MAPK activity to the basal level (data not shown). These results indicate that the ATF2 phosphorylation and p38 phosphorylation were specific for p38 MAP kinase. The induction of iNOS and IL-6 expression in the MC3T3E-1 osteoblasts required combined LPS/IFN-$\gamma$, or TNF-$\alpha$/IFN-$\gamma$ stimulation (Fig. 1 and 2), yet LPS and TNF-$\alpha$ independently were able to strongly activate p38 MAPK. Furthermore, the treatment with combined agents did not significantly activate p38 MAPK in a quantitative or qualitative manner from single agent treatment.

### SB203580, a specific inhibitor of p38 MAPK, inhibits cytokine-induced NO and IL-6 production in MC3T3E-1 osteoblasts

SB203580 is a member of a new series of pyridinyl imidazole compounds that inhibit IL-1 and TNF-$\alpha$ production from LPS-stimulated human monocytes and the human monocyte cell line THP-1 28, 29). To clarify the role of p38 MAPK in cytokine-induced NO and IL-6 production, we then examined the effect of SB203580, a highly potent and specific inhibitor of p38 MAPK on the NO and IL-6 production induced by cytokines in MC3T3E-1 osteoblasts. SB203580 (20 $\mu$M) markedly inhibited cytokine-induced NO and IL-6 production (Fig. 4A and B).

### SB203580 inhibits cytokine-induced expression of iNOS and IL-6 protein in MC3T3E-1 osteoblasts

SB compounds were studied for their effect on iNOS and IL-6 protein in various cell lines activated with combined cytokines including TNF-$\alpha$/IFN-$\gamma$. The above results showed that the inclusion of SB203580 in the presence of LPS/IFN-$\gamma$ or TNF-$\alpha$/IFN-$\gamma$ drastically suppressed the induction of NO and IL-6 production. The block of NO and IL-6 synthesis correlated with a marked inhibition of the expression of iNOS and IL-6 protein as determined by western blot analysis (Fig. 5A and B).

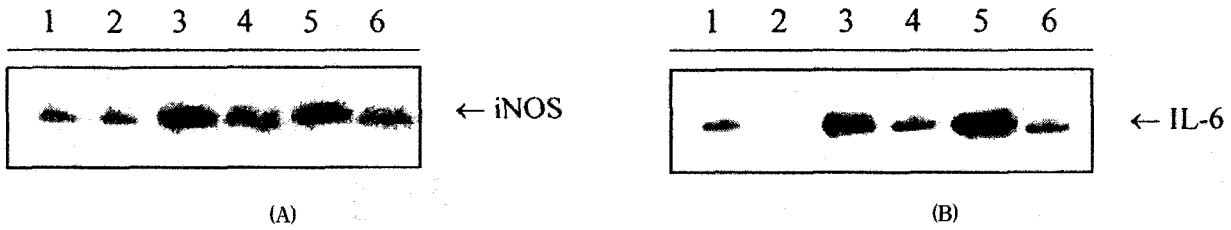



**Fig. 5.** Effect of SB203580 on combined agents-induced iNOS and IL-6 protein expression in MC3T3E-1 osteoblasts. (A) MC3T3E-1 osteoblasts were treated with LPS/IFN-$\gamma$, or TNF-$\alpha$/IFN-$\gamma$ in the presence or absence of the p38 MAPK inhibitor for 24 h. The cell extracts were then analyzed for iNOS protein expression. (B) Cell extracts from the cultures treated, as described above, were analyzed for IL-6 protein expression. (1 : control, 2 : SB203580, 3 : LPS+IFN-$\gamma$, 4 : LPS+IFN-$\gamma$+SB203580 5 : TNF-$\alpha$+IFN-$\gamma$, 6 : TNF-$\alpha$+IFN-$\gamma$+SB203580).

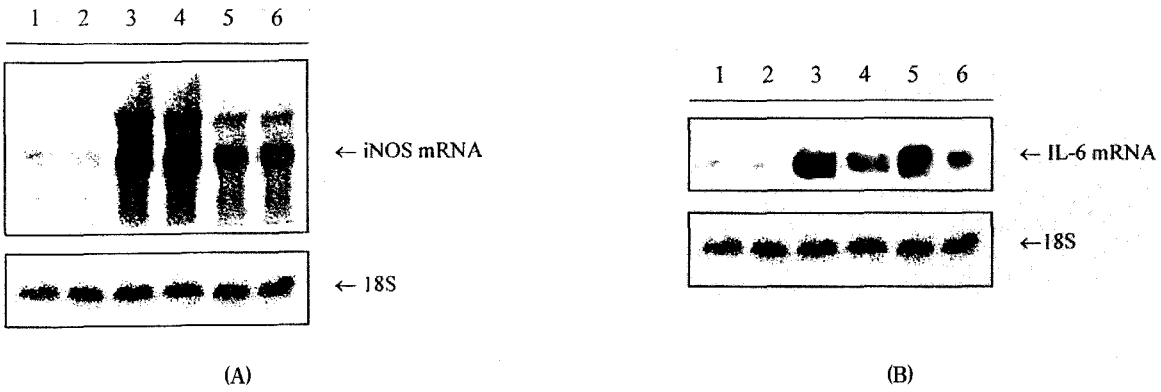



**Fig. 6.** Effect of SB203580 on the iNOS and IL-6 mRNA expression. MC3T3E-1 osteoblasts were stimulated with LPS/IFN-$\gamma$, or TNF-$\alpha$ /IFN-$\gamma$ in the presence or absence of the p38 MAPK inhibitor (20 $\mu$M). Total cellular RNA (20 $\mu$g) was then prepared, fractionated on a 1.2% (w/v) agarose gel, transferred onto Hybond N nylon membranes, and then hybridized to iNOS (A : 1 : control, 2 : SB203580, 3 : LPS+IFN-$\gamma$, 4 : TNF-$\alpha$+IFN-+, 5 : LPS+IFN-$\gamma$+SB203580, 6 : TNF-$\alpha$+IFN-$\gamma$+SB203580) and IL-6 cDNA probes (B : 1 : control, 2 : SB203580, 3 : LPS+IFN-0567, 4 : LPS+IFN-+SB203580 5 : TNF-$\alpha$+IFN-$\gamma$, 6 : TNF-$\alpha$+IFN-$\gamma$+SB203580).

### Effects of SB203580 on iNOS and IL-6 gene expression

In order to investigate whether the suppression of iNOS activity by SB203580 was due to reduced iNOS mRNA, a northern blot analysis for total mRNA samples extracted from MC3T3E-1 osteoblasts was carried out. As shown in Fig. 6A, SB203580 significantly reduced LPS/IFN-$\gamma$ or TNF-$\alpha$/IFN-$\gamma$-stimulated iNOS mRNA levels. Fig. 6B shows that significantly lower levels of IL-6 mRNA are expressed in the osteoblasts activated by LPS/IFN-$\gamma$, or TNF-$\alpha$/IFN-$\gamma$ in the presence of SB203580 than in its absence.

## DISCUSSION

Nitric oxide (NO) is a very small lipophilic molecule which rapidly diffuses and reaches the cytoplasmic compartments, and hence results in the activation of diverse biological functions. Interestingly, NO is a soluble gas in aqueous medium. Its biological functions are confined to adjacent cells since its ultrashort half-life limits the availability of newly synthesized NO to substances or cells nearby.[30] It has been reported that pro-inflammatory agents such as TNF-$\alpha$, IFN-$\gamma$ and LPS modulate the metabolism of bone remodeling in vitro and *in vivo*.[1,2] These agents also induce the production

of NO in various cell types of bone tissue. These reports suggest that it may be a modulator in these cytokines-induced processes of bone remodeling; especially it may function in the regulation of bone resorption.

Interleukin-6 (IL-6) is well known to be a pleiotropic cytokine that has important physiological effects such as promoting B-cell differentiation, T-cell activation, and inducing acute phase proteins.[17] As for bone metabolism, it has been reported that IL-6 stimulates bone resorption[21] and the induction of osteoclast formation.[31] Accumulating evidence indicates that IL-6 secreted from osteoblasts is an important downstream effector of bone resorptive agents.

In this study, we showed that MC3T3E-1 osteoblasts did not produce biologically significant amounts of NO and IL-6 upon single treatment with various agents including TNF-$\alpha$, IFN-$\gamma$, and LPS. However, the differently combined agents such as LPS/IFN-0567, or TNF-$\alpha$/IFN-$\gamma$ worked synergistically to generate NO and IL-6 in MC3T3E-1 osteoblasts.

Pyridinyl imidazoles have been shown to inhibit the p38 kinase-mediated synthesis of cytokines such as TNF-$\alpha$.[28,29] SB203580 as well as other members in this series of compounds have shown efficacy in several animal models of inflammation where cytokines play a definitive role.[32,33]

In addition, these compounds are active in the fetal rat long bone resorption assay, thus demonstrating a direct therapeutic effect on bone integrity.[34] SB203580 inhibits TNF-$\alpha$ synthesis,[35] and the target of this is known as CSBP/p38 kinase.[28] SB203580 is a highly selective and potent inhibitor of p38 MAP kinase.[29] One of the physiologic substrates of CSBP/p38 is MAPKAP kinase-2, which, in turn, phosphorylates hsp.[27]

The results presented in this study demonstrate the cytokine-mediated induction of NO and IL-6 in MC3T3E-1 osteoblasts and the key role of p38 MAPK signaling in this process. Specifically, the treatment of MC3T3E-1 osteoblasts with cytokine combinations, in particular, TNF-$\alpha$ plus IFN-$\gamma$, resulted in an induced production of NO and IL-6 and the expression of iNOS and IL-6 protein. SB203580, a specific inhibitor of p38 MAP kinase, inhibited the cytokine-induced production of NO and IL-6 and the expression of iNOS and IL-6 protein, thereby suggesting a role for this kinase cascade in cytokine-induced NO and IL-6 production. Inhibition of the regulation of NO and IL-6 synthesis in osteoblasts by SB203580 indicates that the p38 MAPK inhibitors may be useful for therapy of disease states in which NO or IL-6 has been shown to play a pro-inflammatory role. Additional evidence for the role of NO and IL-6 is provided by studies showing spontaneous production of these mediators from both rheumatoid arthritis and osteoarthritis patients, which may contribute to the pathology of these diseases.[36]

Our study also shows that LPS or TNF-$\alpha$ alone activates p38 MAPK, although the single treatment has no markedly regulatory effect on NO and IL-6 release (Fig. 3A). These results indicate that p38 MAPK plays a permissive role and that it acts in concert with other factors yet to be elucidated. In the case of IL-6 promoter, a variety of so-called responsive elements have been proposed.[37] Previous studies have shown a crucial role for NF-$\kappa$B as a transcriptional activator for IL-6 after pro-inflammatory cytokines-treatment.[38,39] Recently, it has been reported that the p38 MAP kinase inhibitor, SB203580, still inhibited the TNF-$\alpha$-induced expression of a reporter gene that is driven by a minimal promoter containing only two $\kappa$B-binding elements, suggesting that p38 MAP kinase is required for NF-$\kappa$B-mediated transcriptional activation.[40] The most abundant form of NF-$\kappa$B consists of p50 and p65 heterodimer is complexed to the inhibitory subunit, I$\kappa$B. Stimulation of cells with TNF-$\alpha$ or a number of other stress- or infection-dependent triggers leads to rapid phosphorylation of IB followed by proteolytic degradation. The released nucleophilic heterodimer then moves to the nucleus, where it binds to a specific DNA sequence and induces gene transcription. However, we could not observe any effect of SB203580 on IFN-$\gamma$, LPS or TNF-$\alpha$ alone treated or LPS/IFN-$\gamma$ or TNF$\alpha$/IFN-$\gamma$-induced DNA binding of NF-$\kappa$B, suggesting that the role of p38 MAP kinase in NF-$\kappa$B activation, if any, is at another level (data not shown). Recently, we

documented that these two TNF-$\alpha$-activated pathways-p38 MAPK and NF-$\kappa$B can be dissociated in other osteoblasts, MG-63 cells (not published). Therefore, it seems more likely that the p38 MAP kinase pathway regulates IL-6 transcription through the phosphorylation of another factor. Further studies on the interactions between NF-$\kappa$B co-activators and general transcription factors should elucidate the mechanism by which the p38 MAP kinase pathway affects the activity of NF-$\kappa$B-driven transcription.

Activation of the p38 MAP kinase pathway might be one of the earliest and possibly the central response after an insult causing stress to the body. Indeed, besides pro-inflammatory agents, heat shock, UV radiation and hyperosmolarity have been shown to activate the p38 MAP kinase pathway.[41,42] It is still unclear to what extent the signals going to the nucleus and responsible for gene activation are linked to cellular signals leading to cell death. This study shows that p38 MAPK inhibitors could be efficacious therapeutic agents by way of their ability to inhibit agents such as IFN-$\gamma$, LPS, and TNF-$\alpha$ and by inhibiting the downstream signal transduction pathways initiated by IFN-$\gamma$, LPS, and TNF-$\alpha$, including their ability to modulate iNOS and IL-6 protein expression. In this study, the p38 inhibitor inhibits the downstream signal transduction pathways initiated by IFN-$\gamma$, LPS, and TNF-$\alpha$, including their ability to modulate iNOS and IL-6 protein expression.

## CONCLUSIONS

Treatment of MC3T3E-1 osteoblast cultures with combined IFN-$\gamma$, LPS and TNF-$\alpha$ induces expressions of iNOS and IL-6, resulting in sustained releases of large amounts of nitric oxide and IL-6. However, IFN-$\gamma$, LPS, and TNF-$\alpha$ individually induced non-detectable or small amounts of NO and IL-6 in MC3T3E-1 osteoblasts. The role of MAPK activation in the early intracellular signal transduction involved in iNOS and IL-6 transcription in the combined agents-stimulated osteoblasts has been investigated. The p38 MAPK pathway was specifically involved in the combined agents-induced NO and IL-6 release, since NO and IL-6 release in the presence of a specific inhibitor of p38 MAPK, SB203580 was significantly diminished. Northern blot analysis showed that the p38 MAPK pathway controlled iNOS and IL-6 transcription levels. These data suggest that p38 MAPK plays an important role in the secretion of NO and IL-6 in LPS/IFN-$\gamma$ or TNF-$\alpha$/IFN-$\gamma$-treated MC3T3E-1 osteoblasts.

## REFERENCES


1. Gowen M, Wood DD, Ihrie EJ, McGuire MK, Russell RG. An interleukin 1 like factor stimulates bone resorption in vitro. Nature 1983 : 306 : 378-80.
2. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR. Stimulation of bone resorption and inhibition of bone formation in vitro by human tumor necrosis factors. Nature 1986 : 319 : 516-8.
3. Lowenstein CJ, Snyder SH. Nitric oxide, a novel biologic messenger. Cell 1992 : 70 : 705-7.
4. Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J 1992 : 6 : 3051-64.
5. Devlin AM, Brosnan MJ, Graham D, Morton JJ, McPhaden AR, McIntyre M, Hamilton CA, Reid JL, Dominiczak A. Vascular smooth muscle cell polyploidy and cardiomyocyte hypertrophy due to chronic NOS inhibition in vivo. Am J Physiol 1998 : 274 : H52-9.
6. Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 1991 : 351 : 714-8.
7. Lamas S, Michel T, Collins T, Brenner BM, Marsden PA. Effects of interferon-gamma on nitric oxide synthase activity and endothelin-1 production by vascular endothelial cells. J Clin Invest 1992 : 90 : 879-87.
8. Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan C. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 1992 : 256 : 225-8.
9. MacIntyre I, Zaidi M, Alam AS, Datta HK, Moonga BS, Lidbury PS, Hecker M, Vane JR. Osteoclastic inhibition : an action of nitric oxide not mediated by cyclic GMP. Proc Natl Acad Sci USA 1991 : 88 : 2936-40.
10. Kasten TP, Collin-Osdoby P, Patel N, Osdoby P, Krukowski M, Misko TP, Settle SL, Currie MG, Nickols GA. Potentiation of osteoclast bone-resorption activity by inhibition of nitric oxide synthase. Proc Natl Acad Sci USA 1994 : 91 : 3569-73.
11. Lwik CW, Nibbering PH, Van de Ruit M, Papapoulos SE. Inducible production of nitric oxide in osteoblast-like cells and in fetal mouse bone explants is associated with suppression of osteoclastic bone resorption. J Clin Invest 1994 : 93 : 1465-72.
12. Damoulis PD, Hauschka PV. Cytokines induce nitric oxide production in mouse osteoblasts. Biochem Biophys Res Commun

1994 : 201 : 924-31.
13. Damoulis PD, Hauschka PV. Nitric oxide acts in conjunction with proinflammatory cytokines to promote cell death in osteoblasts. J Bone Miner Res 1997 : 12 : 412-22.
14. Ralston SH, Grabowski PS. Mechanisms of cytokine induced bone resorption : role of nitric oxide, cyclic guanosine monophosphate, and prostaglandins. Bone 1996 : 19 : 29-33.
15. Chae HJ, Park RK, Chung HT. et. al. Nitric oxide is a regulator of bone remodelling. J Pharm Pharmacol 1997 : 49 : 897-902.
16. : 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.
17. Van Snick J. Interleukin-6 : an overview. Annu Rev Immunol 1990 : 8 : 253-78.
18. Hughes FJ, Howells GL. Interleukin-6 inhibits bone formation in vitro. Bone Miner 1993 : 21 : 21-8.
19. Roodman GD. Interleukin-6: an osteotropic factor? J Bone Miner Res 1992 : 7 : 475-8.
20. Feyen JH, Elford P, Di Padova FE, Trechsel U. Interleukin-6 is produced by bone and modulated by parathyroid hormone. J Bone Miner Res 1989 : 4 : 633-8.
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. Franchimont N, Canalis E. Platelet-derived growth factor stimulates the synthesis of interleukin-6 in cells of the osteoblast lineage. Endocrinology 1995 : 136 : 5469-75.
23. Bhat NR, Zhang P, Lee JC, Hogan EL. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-gene expression in endotoxin-stimulated primary glial cultures. J Neurosci 1998 : 18 : 1633-41.
24. Da Silva J, Pierrat B, Mary JL, Lesslauer W. Blockade of p38 mitogen-activated protein kinase pathway inhibits inducible nitric-oxide synthase expression in mouse astrocytes. J Biol Chem 1997 : 272 : 28373-80.
25. Stuehr DJ, Nathan CF. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med 1989 : 169 : 1543-55.
26. Kozawa O, Tokuda H, Matsuno H, Uematsu T. Involvement of p38 mitogen-activated protein kinase in basic fibroblast growth factor-induced interleukin-6 synthesis in osteoblasts. J Cell Biochem 1999 : 74 : 479-85.
27. MacKenna DA, Dolfi F, Vuori K, Ruoslahti E. Extracellular signal-regulated kinase and c-Jun NH2-terminal kinase activation by mechanical stretch is integrin-dependent and matrix-specific in rat cardiac fibroblasts. J Clin Invest 1998 : 101 : 301-10.
28. Lee JC, Laydon JT, McDonnell PC. et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994 : 372 : 739-46.
29. Cuenda A, Rouse J, Doza YN. et. al. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 1995 : 364 : 229-33.
30. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993 : 329 : 2002-12.
31. Chae HJ, Park RK, Kang JS. et. al. Effect of stem cell factor, interleukin-6, nitric oxide and transforming growth factor-beta on the osteoclast differentiation induced by 1,25-(OH)2D3 in primary murine bone marrow cultures. Pharmacol Toxicol 1998 : 82 : 223-9.
32. Griswold DE, Hillegass LM, Meunier PC, DiMartino MJ, Hanna N. Effect of inhibitors of eicosanoid metabolism in murine collagen-induced arthritis. Arthritis Rheum 1988 : 31 : 1406-12.
33. Badger AM, Bradbeer JN, Votta B. et. al. Pharmacological profile of SB203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. J Pharmacol Exp Ther 1996 : 279 : 1453-61.
34. Votta BJ, Bertolini DR. Cytokine suppressive anti-inflammatory compounds inhibit bone resorption in vitro. Bone 1994 : 15 : 533-8.
35. Young P, McDonnell P, Dunnington D. et. al. Pyridinyl imidazoles inhibit IL-1 and TNF production at the protein level. Agents Actions 1993 : C67-9.
36. McInnes IB, Leung BP, Field M. et. al. Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. J Exp Med 1996 : 184 : 1519-24.
37. Ray A, LaForge KS, Sehgal PB. On the mechanism for efficient repression of interleukin-6 promoter by glucocorticoids: enhancer, TaTa box, and RNA start site (Inr motif) occlusion. Mol Cell Biol 1990 : 10 : 5736-46.
38. Libermann TA, Baltimore D. Activation of interleukin-6 gene expression through the NF-B transcription factor. Mol Cell Biol 1990 : 10 : 2327-34.
39. Zhang Y, Lin JX, Vilcek J. Interleukin-6 induced by tumor necrosis factor and interleukine-1 in human fibroblasts involves activation of a nuclear factor binding to a B-like sequence. Mol Cell Biol 1990 : 10 : 3818-23.
40. Wim VB, Stephane P, Elke B, Karolien DBM, Lienhard, S, Walter F, Guy H. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-B p65 transactivation mediated by tumor necrosis factor. J Biol Chem 1998 : 273 : 3285-90.
41. Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 1994 : 265 : 808-11.
42. Rouse J, Cohen P, Trigon S. et. al. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphoryiation of the small heat shock proteins. Cell 1994 : 78 : 1027-37.

국문초록

# 조골세포에서 p-38 MAP kinase의 nitric oxide및 interleukin-6 생성조절에 관한 연구

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치아이동 시 발생하는 골흡수에서 이미 여러 cytokine의 중요성이 강조된 바 있으며 이 가운데 interleukin-6는 구강 및 연골조직 등에서 많은 연구의 초점이 되어 왔으나 확실한 기전은 아직까지 정확히 확립되어 있지 못하다. 골흡수 시 조골세포에서 유리되는 Interleukin-6 (IL-6)와 nitric oxide (NO) 등이 골흡수의 조절자로 최근 대두되고 있으며 Mitogen-activated protein kinase (MAPK)의 활성화로 인해 염증성 cytokine등이 유리될 수 있음이 최근 macrophage 등에서 증명된 바 있다. 그러므로 치아이동을 비롯한 구강 내 여러 염증의 조건에서 골흡수의 대표인자인 IL-6및 NO 유리가 MAPK등의 활성 등을 통해 조절될 수 있는 가능성을 시사하고 있다. 본 연구에서 조골세포 특징을 대부분 가지고 있는 조골세포주, MC3T3E1에서 p-38 MAP kinase을 매개로 NO와 IL-6가 유리됨을 확인하고자 하였다. 10% Fetal Bovine Serum이 첨가된 -MEM 배양액으로 배양한 조골세포주인 MC3T3E1 세포에 tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interferon-$\gamma$ (IFN-$\gamma$) 및 lipopolysacchalide(LPS) 등의 단독처리 시 NO와 IL-6의 증가는 확인되지 않았으나 TNF-$\alpha$/IFN-$\gamma$ 혹은 LPS/IFN-$\gamma$ 등의 처치시 NO와 IL-6의 유의한 증가를 보였으며, NO발현에 직접 관여하는 inducible nitric oxide synthase (iNOS)와 IL-6 단백질 및 mRNA의 발현을 관찰하였다. 또한 specific p-38 MAP kinase inhibitor인 SB203580의 NO와 IL-6의 생성 억제를 관찰하고 단백질과 mRNA 발현억제를 통해서도 확인함으로써 SB203580은 transcription 단계에서 NO와 IL-6의 생성을 조절하고 있음을 시사하여 주고 있다. TNF-$\alpha$/IFN-$\gamma$ 혹은 LPS/IFN-$\gamma$ 처치 시 p-38 MAP Kinase의 활성을 관찰하였으나 단독 처치 시 역시 p-38 MAP Kinase의 활성을 확인함으로써 NO와 IL-6생성기전에는 p-38 MAP Kinase이외에 다른 인자 역시 관여하고 있음을 보여주고 있다. 본 연구에서는 치아 등의 골조직의 구성 세포인 조골세포에서 NO와 IL-6유리를 확인하였으며, 또한 이들의 생성기전중의 하나로 p-38 MAP Kinase가 transcription 단계에서 관여하고 있음을 확인하였다.

**주요 단어 :** 조골세포, p-38 MAP Kinase, Nitric oxide, Interleukin-6