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Roles of p38 and ERK MAP kinases in IL-8 expression in TNF-α- and dexamethasone-stimulated human periodontal ligament cells

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Abstract

Orthodontic tooth movement is recognized as a pro-inflammatory stressor of human periodontal ligament (hPDL) cells. However, the cell-signaling pathways linking interleukin-8 (IL-8), intercellular adhesion molecule-1 (ICAM-1), pro-inflammatory cytokines, and dexamethasone in hPDL cells have not been well elucidated. In this study, we investigated the role of mitogen-activated protein (MAP) kinases in dexamethasone- and TNF-α-induced IL-8 and ICAM-1 expression in hPDL cells. IL-8 production was measured by enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. MAP kinase activation and IκB degradation were determined by Western blot analysis, and ICAM-1 expression was determined by RT-PCR and FACS analysis. TNF-α increased IL-8 mRNA expression and protein secretion in a dose- and time-dependent manner. Dexamethasone suppressed TNF-α-induced IL-8 production in a dose-dependent manner. In addition, dexamethasone inhibited TNF-α-induced phosphorylation of p38 MAP kinase and extracellular-regulated kinases (ERKs), IκB degradation, and NF-κB activation. Selective inhibitors for ERKs and p38 attenuated TNF-α-induced IL-8 and ICAM-1 expression in the presence and absence of dexamethasone, indicating that MAP kinases play a role in the response of hDPL cells to TNF-α. Furthermore, these results suggest that inflammatory cytokine- and dexamethasone-induced IL-8 and ICAM-1, produced via a MAP kinase pathway, may serve as an important mediator of PDL immunoregulation involved in bone remodeling during orthodontic tooth movement.

Keywords: IL-8; ICAM-1; TNF-α; Dexamethasone; Human periodontal ligament cells; MAP kinase; NF-κB

1. Introduction

During orthodontic tooth movement, applied mechanical loading induces inflammation in the periodontal ligament (PDL). The cells of the PDL respond to these mechanical signals by synthesizing cytokines and growth factors, both of which regulate alveolar bone resorption and formation [1,2]. Osteoclast precursors derived from bone marrow migrate through the blood to the periodontal space, where they differentiate into mature osteoclasts. The osteoclasts then resorb alveolar bone upon stimulation by PDL-derived cytokines [3]. The mechanisms by which inflammatory cytokines mediate osteoclast formation and bone resorption are not completely understood, however, particularly in human PDL (hPDL) cells.

The chemotactic cytokine known as interleukin 8 (IL-8) and the cell-surface adhesion molecule known as intercellular adhesion molecule-1 (ICAM-1) are major mediators of inflammation that contribute to sequestration of neutrophils at sites of tissue injury [4]. IL-8 is a member of the CXC chemokine family of cytokines that were originally identified as monocyte-derived factors capable of attracting

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and activating neutrophils [5,6]. IL-8 is produced by a variety of cell types, such as macrophages, neutrophils, and endothelial cells, in response to inflammation and injury [7]. IL-8 is increasingly being recognized as a multifunctional cytokine with other activities, in addition to its activity as a chemokine.

In bone, IL-8 is synthesized by osteoblasts and osteoclasts [8]. Bendre et al. [9] demonstrated that the actions of IL-8 related to bone resorption are both indirect and direct. IL-8 indirectly stimulates expression of osteoblast receptor activator of NF-κB ligand (RANKL), and it directly affects the osteoclastogenic response of human osteoclast precursors. Recently, Tuncer et al. [10] observed that orthodontic forces lead to an increase in IL-8 and subsequent accumulation of neutrophils, suggesting that IL-8 triggers bone remodeling. Thus, IL-8 may be important in regulation of bone formation and resorption during orthodontic tooth movement.

Tumor necrosis factor (TNF)-α is an inflammatory cytokine that appears to be an important mediator of bone destruction in inflammatory disease [11]. TNF-α is primarily produced by activated monocytes, macrophages, and osteoblasts, and it stimulates osteoclast differentiation and osteoclastic bone resorption *in vitro* and *in vivo* [12]. Various studies have shown that prostaglandin E (PGE), IL-1β, and TNF-α are increased in human gingival crevicular fluid during orthodontic tooth movement [13,14]. Furthermore, TNF-α has recently been reported to enhance expression of IL-8 mRNA and secretion of IL-8 protein in various cell types [8,15,16]. Therefore, TNF-α is believed to play a key role in the biological processes involved in orthodontic tooth movement.

The roles of glucocorticoids in osteoclast formation and activation have been extensively examined *in vivo* and *in vitro*, with conflicting results. These osteotropic factors are thus assumed to play different roles in different microenvironments and cell types [17,18]. In a study by Pharoah and Heersche [19], dexamethasone, a synthetic glucocorticoid analog, inhibited the formation of osteoclast-like cells in bone marrow cultures. However, in other studies, glucocorticoids increased osteoclast formation and activity through both direct and indirect mechanisms [17,18,20]. Hirayama et al. [21] reported that dexamethasone directly affected osteoclast formation and activity, stimulating the proliferation and differentiation of human osteoclast precursors, and inhibiting the bone resorption activity of mature osteoclasts. In the present study, we investigated the effects of dexamethasone in the presence of various osteotropic factors to gain additional insight regarding its role in hDPL cells.

The mitogen-activated protein (MAP) kinases (MAPKs) comprise well-conserved signaling pathways and include extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK. These activated kinases initiate a cascade of protein phosphorylation that activates nuclear transcription factors, such as NF-κB, and AP-1. These transcription factors subsequently promote expression of inflammatory cytokines, chemokines, and ICAMs [22,23].

We hypothesized that orthodontic tooth movement causes inflammation and release of IL-8- and ICAM-1-inducing cytokines by activating MAPK signaling pathways in hPDL cells. In the present study, we focused on IL-8 secretion and ICAM-1 expression because these molecules are known to play important roles in the recruitment of circulating inflammatory cells. We investigated the effects of TNF-α and dexamethasone on the production of chemokines and ICAM-1 via activation of MAPK signaling pathways in primary cultured hPDL cells.

2. Materials and methods

2.1. Reagents

TNF-α and polyclonal goat anti-human IL-8 antibodies (Abs) were obtained from R&D Systems (Minneapolis, MN, USA). SB203580 and PD98059 were purchased from Calbiochem (La Jolla, CA, USA). Polyclonal rabbit anti-human IL-8 Abs was purchased from Endogen (Woburn, MA, USA). Abs against p38 kinase, ERK1/2, and the Abs specific to the phosphorylated forms of these proteins were purchased from Cell Signaling Technology (Beverly, MA, USA). Horse radish peroxidase (HRP)-conjugated anti-rabbit IgG was from Amersham Biosciences (Little Chalfont, UK). Anti-human I-κBα or antiphospho-I-κBα were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium (DMEM), KGM medium (Clonetics), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA). Dexamethasone and all other chemicals were bought from Sigma Chemical Co. (St. Louis, MO, USA) unless indicated otherwise.

2.2. Primary culture of hPDL cells

hPDL cells were isolated with an explant culture technique from patients, with an age under 25 years undergoing orthodontic treatment by following described methods [24]. Informed written consent from donors were obtained for use of the tissues. Patients signed the corresponding informed consent approved by the Institutional Review Board at Wonkwang University for used of the tissues. Briefly, these tissues were cut into 1 mm^2 explants and placed on a 100 mm culture dishes (Nunc, Naperville, IL, USA) containing 10,000 U/ml of penicillin G sodium, 10,000 μg/ml of streptomycin sulphate, 25 μg/ml of amphotericin B, and 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL, Grand island, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After 2 or 3 days, cells started to outgrow from the explants. When the primary cell culture reached confluence, cells were detached with 0.025% trypsin and 0.05% EDTA, diluted with culture medium, and then subcultured in a ratio of 1:4. Cell cultures between the 5th and 7th

passage were used in this study. Alkaline phosphatase activity for 3 days was checked before the experiments.

2.3. ELISA for IL-8 protein measurement

hPDL cells were seeded at 2×10^5 into 24-well plates (Nalge Nunc International, Rochester, NY, USA) and grown until formation of confluent monolayers, reaching a final density of 5×10^5 cells/well. Monolayers were then incubated for 4–48 h in a fresh medium containing stimuli as indicated. The supernatants were collected, cleared by centrifugation, and kept at −20 °C until evaluation by ELISA. For measurement of IL-8 concentrations in cell culture supernatants, 96-well microtiter plates (MaxiSorp; Nunc) were coated with 0.2 μg/well goat anti-human IL-8 Abs (R&D Systems) in 50 μl of PBS at 4 °C overnight. All further steps were conducted at room temperature. After washing three times with PBS, nonspecific binding sites were blocked by incubation with 150 μl PBS + 1% BSA/0.05% Tween 20/well for 2 h. After three washes with PBS, 50 μl of samples or IL-8 standards were added and incubated for 2 h. As a second Ab, 0.05 μg/well polyclonal rabbit anti-human IL-8 (Endogen) was added and incubated for 2 h. As a third Ab, alkaline phosphatase-labeled monoclonal mouse anti-rabbit IgG (Sigma–Aldrich) was diluted in 50 μl of PBS + 0.1% BSA/0.05% Tween 20 to 1:50,000 and incubated for 2 h. Finally, alkaline phosphatase substrate *p*-nitrophenyl phosphate (Sigma–Aldrich) was added at a concentration of 1 mg/ml in 0.1 M glycine buffer (pH 10.4) containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. After overnight incubation, plates were read at 405 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The detection limit of the ELISA was 30 pg/ml.

2.4. Cell viability assay

Viable cells were detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, which forms blue formazan crystals that are reduced by mitochondrial dehydrogenase present in living cells. Briefly, 2×10^4 cells were seeded in a 96-well plate and cultured overnight for cell attachment. After cell treatment, 50 μl of MTT solution (0.5 mg/ml in PBS) were added to each well and incubated for 4 h. To each well, 50 μl of DMSO were added. The plates were then shaken until the crystals had dissolved. Reduced MTT was then measured spectrophotometrically in a dual beam microtiter plate reader at 570 nm.

2.5. RNA isolation and RT-PCR

Cells were grown in 60-mm culture dishes and incubated for 4–24 h in a fresh medium containing stimuli as indicated. After discarding growth medium, total RNA was isolated from cells using easy-Blue (iNtRON Biotechnology, Daejon, Korea), following the manufacturer's instructions. Reverse transcription of the RNA was performed using AccuPower RT PreMix (Bioneer, Daejon, Korea). One microgram of RNA and 20 pmol/μl primers were preincubated at 70 °C for 5 min and transferred to a mixture tube. The reaction volume was 20 μl. cDNA synthesis was performed at 42 °C for 60 min, followed by RT inactivation at 94 °C for 5 min. Thereafter, the RT-generated DNA (2–5 μl) was amplified using AccuPower PCR PreMix (Bioneer). The primers used for cDNA amplification and PCR conditions were as follows:

IL-8, 289 bp	(F) 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′
	(R) 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′
ICAM-1, 238 bp	(F) 5′-TATGGCAACGACTCCTTCT-3′
	(R) 5′-CATTCAGCGTCACCTTGG-3′
GAPDH, 306 bp	(F) 5′-CGGAGTCAACGGATTTGGTCGTAT-3′
	(R) 5′-AGCCTTCTCCA TGGTGGTGAAGAC-3′

IL-8; an initial denaturation at 94 °C for 5 min; 25 cycles at 94 °C for 30 s, at 62 °C for 30 s, and at 72 °C for 30 s, and ICAM-1; 30 cycles at 94 °C for 45 s, at 58 °C for 1 min, and at 72 °C for 1 min, GAPDH (glyceraldehyde-3-phosphate dehydrogenase); 25 cycles at 94 °C for 30 s, at 62 °C for 30 s, at 72 °C for 30 s. PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

2.6. Phosphorylation of p38 and ERK MAP kinase

Cells were stimulated according to designated experimental protocols. Proteins were extracted with an extraction buffer (50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 1% nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM 4-nitrophenyl phosphate, 10 mg/ml leupeptin, 10 mg/ml pepstatin A, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride). Activation of MAP kinase was examined by determining its phosphorylation state using the antibodies specific to the phosphorylated forms of p38 MAP kinase and ERK-1 and ERK-2.

2.7. Western blot analysis

Protein samples (50 μg) were mixed with an equal volume of 2× SDS sample buffer, boiled for 5 min, and then separated through 8–15% SDS–PAGE gels. After electrophoresis, proteins were transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% dry milk (1 h), rinsed, and incubated with primary antibodies (diluted at 1:500 or 1:1000) in TBS overnight at 4 °C. Primary antibody was then removed by washing

the membranes four times in TBS, and labeled by incubating with 0.1 mg/ml peroxidase-labeled secondary antibodies (against mouse and rabbit) for 1 h. Following three washes in TBS, bands were visualized by ECL and exposed to X-ray film.

2.8. Surface ICAM-1 expression by flow cytometry

Monolayers of PDL cells were detached by incubation with trypsin 0.25% and EDTA 0.25% in calcium- and magnesium-free PBS (pH 7.2). For single-label flow cytometric analysis of ICAM-1 expression, 2×10^5 cells/ml were incubated for 30 min on ice with 2.5 μl of anti-human ICAM-1 MAb in a total volume of 500 μl of PBS containing 0.05 mM EDTA, BSA 0.1% and FBS 0.05%. After washing twice, cells were incubated for 30 min on ice with 500 μl of a dilution (1 in 100) of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG1 (DAKO). Cells were washed twice with PBS then the immunofluorescence of the FITC single label was measured with a flow cytometer (Beckton–Dickinson, CS, USA).

2.9. Statistical analysis

The results were expressed as means ± SD of three independent experiments. Statistical significances were compared between each treated group and control by the Student's *t*-test. Each experiment was repeated at least three times and yielded comparable results. Values with $p < 0.05$ were considered significant.

3. Results

3.1. Effect of TNF-α on expression of IL-8 mRNA and protein in hPDL cells

We first determined the time course for IL-8 secretion in hPDL cells in response to TNF-α, as well as the optimal TNF-α concentration for this response. Cells were treated with TNF-α, the cell culture media were harvested, and the level of secreted IL-8 protein was determined by ELISA. TNF-α-induced IL-8 secretion was found to steadily increase in a time- and dose-dependent manner (Fig. 1A and C). Maximal induction was seen after 48 h of exposure to 20 ng/ml TNF-α. When the level of IL-8 mRNA in the induced cultures was analyzed by RT-PCR, the increase in IL-8 protein secretion correlated with the accumulation of IL-8 mRNA (Fig. 1B and D).

3.2. Effects of dexamethasone on TNF-α-induced expression of IL-8 mRNA, IL-8 protein and cell viability in hPDL cells

We next investigated the effect of dexamethasone on TNF-α-induced production of IL-8 and cell viability in hPDL cells. Treatment of cell cultures with dexamethasone (10^{-9}, 10^{-8}, and 10^{-7} M) significantly inhibited the induction of IL-8 mRNA expression, protein secretion and cell viability by TNF-α (10 ng/ml), in a concentration-dependent manner (Fig. 2A–C). In particular, treatment with 10^{-7} M dexamethasone dramatically decreased levels of IL-8 protein, mRNA and cell viability. These results show

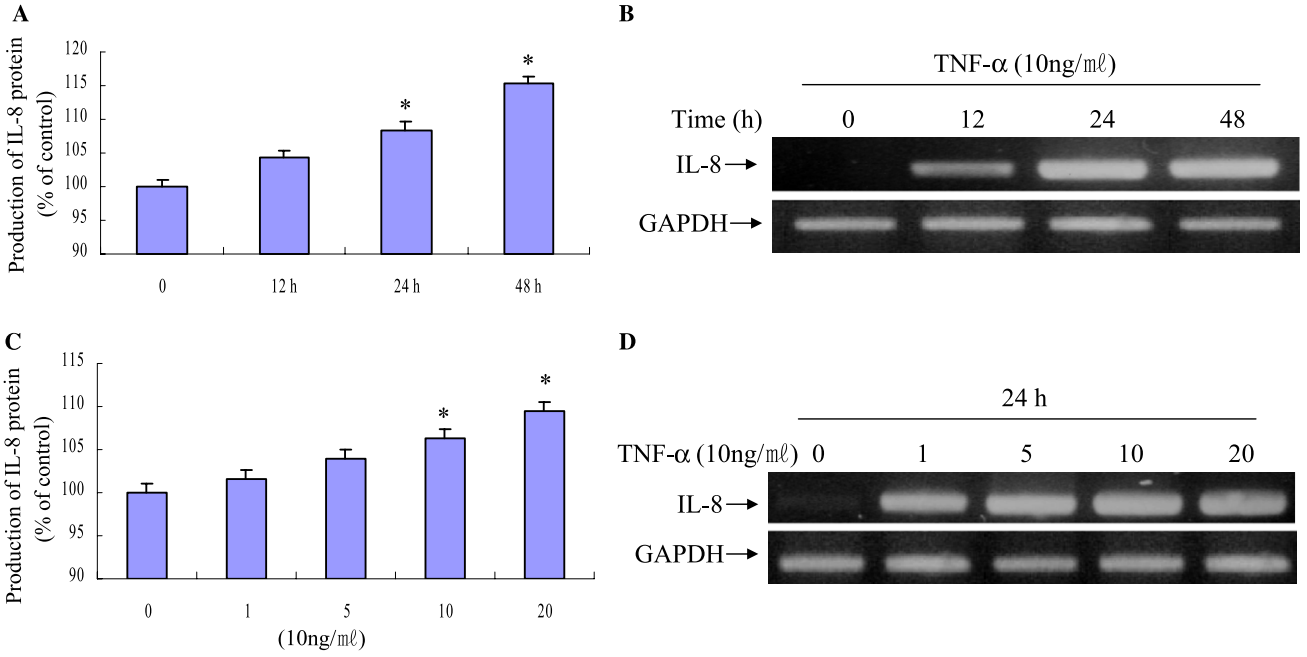

Fig. 1. Effect of TNF-α on expression of IL-8 mRNA and protein in hPDL cells. In a time-dependent manner were stimulated with 10 ng/ml of TNF-α for the time indicated (A and B). In a concentration-dependent manner, cells were stimulated for 24 h in the TNF-α concentration indicated (C and D). Supernatants were analyzed for IL-8 concentration by ELISA. Values are means ± SD of three independent experiments. *Statistically significant difference compared to control group, $p < 0.05$.

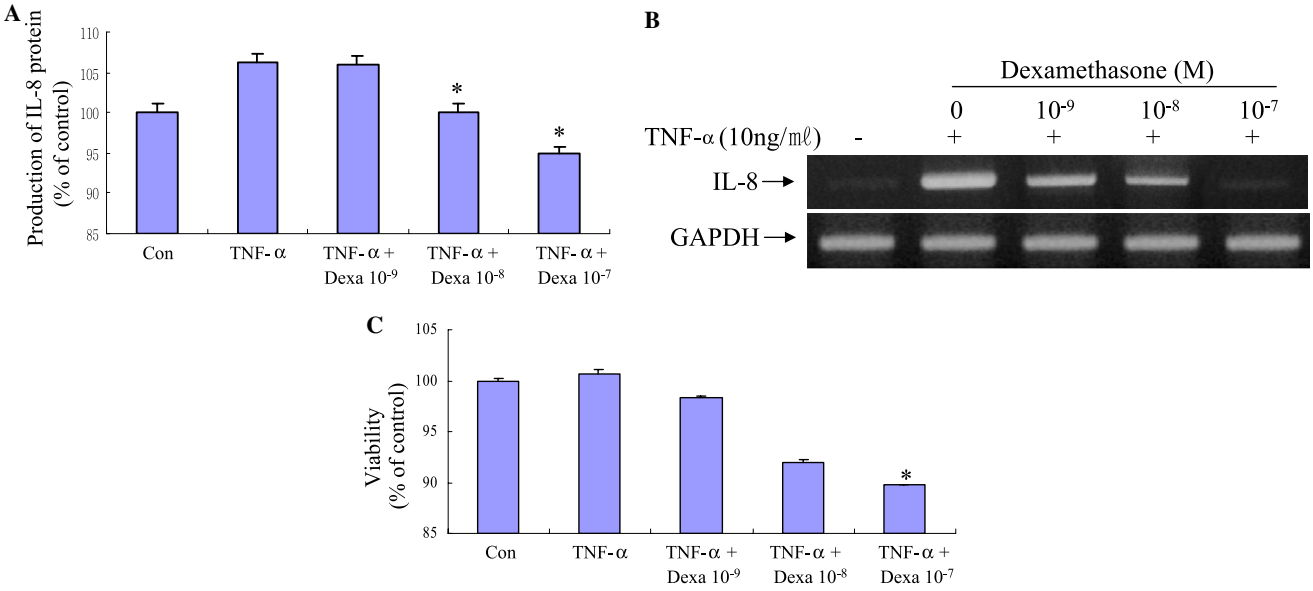

Fig. 2. Effect of dexamethasone on TNF-α-induced expression of IL-8 protein (A), mRNA (B) and cell viability (C) in hPDL cells. The cells were treated with TNF-α (10 ng/ml) and dexamethasone (10^{-9}, 10^{-8}, 10^{-7} M) for 24 h. IL-8 protein levels in the supernatants were measured by ELISA (A). Total RNAs were extracted, and the level of mRNA were analyzed by RT-PCR (B). Cell viability on PDL cells as measured by MTT assay (C). Values are means ± SD of three independent experiments. *Statistically significant difference compared to TNF-α group, $p < 0.05$.

that dexamethasone blocks TNF-α-induced IL-8 production and cell growth in hPDL cells.

3.3. Effects of dexamethasone on phosphorylation of p38 and ERK1/2 MAPKs in TNF-α-stimulated hPDL cells

Recent studies have demonstrated that inhibition of the ERK/p38 pathway in HT-29, HeLa, human airway epithelial, and mast cells attenuates IL-8 secretion [25–27]. In the present study, we examined the effects of dexamethasone on TNF-α-induced MAPK phosphorylation by incubating hPDL cells with TNF-α (10 ng/ml) and dexamethasone (10^{-9}, 10^{-8}, and 10^{-7} M) for 30 or 60 min. Phosphorylation of p38 and ERK1/2 MAPKs was then assessed by Western blot analysis. Dexamethasone treatment was found to significantly inhibit TNF-α-induced phosphorylation of p38 MAPK and ERK1/2 in a dose-dependent manner, without affecting the total levels of these kinases (Fig. 3). These results indicate that the inhibitory effect of dexamethasone on TNF-α-induced MAPK phosphorylation may suppress IL-8 production in hPDL cells.

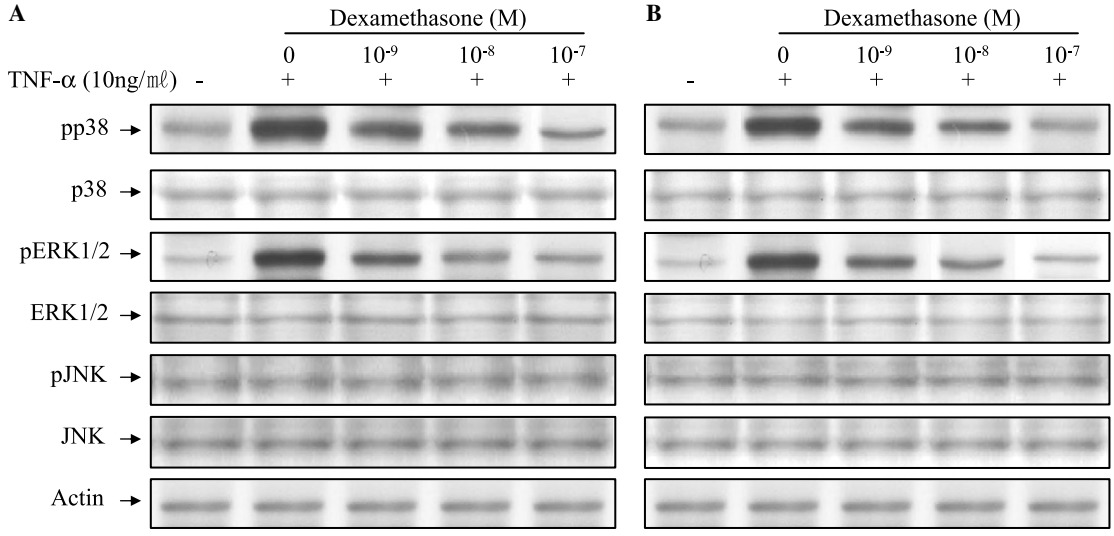

Fig. 3. Effect of dexamethasone on phosphorylation of p38, ERK and JNK MAPKs in TNF-α-stimulated hPDL cells. The cells were treated with TNF-α (10 ng/ml) and dexamethasone (10^{-9}, 10^{-8}, 10^{-7} M) for 30 min (A) and 60 min (B). Cell lysates were analyzed by Western blot. This similar data were obtained in three independent experiments.

3.4. Effects of dexamethasone on phosphorylation and degradation of inhibitor-κB (IκB) in TNF-α-stimulated hPDL cells

Stimulation of cells with inducers such as TNF-α leads to rapid phosphorylation, ubiquitination, and degradation of IκB. Degradation of IκB allows NF-κB to translocate to the nucleus, bind to its specific promoter elements, and activate gene transcription. Since NF-κB activation is important for the regulation of IL-8 [28], we examined the effect of dexamethasone on TNF-α-induced NF-κB activation. Cells were exposed to TNF-α for 15 min, cell lysates were prepared, and IκB degradation and phosphorylation were analyzed by Western blotting with anti-IκBα and anti-phospho-IκBα antibodies, respectively. IκBα became almost undetectable 15 min after TNF-α treatment, and it returned to the original level 1 h after TNF-α treatment (Fig. 4A).

We next examined whether dexamethasone had any effect on TNF-α-induced phosphorylation of IκBα protein, inasmuch as IκBα phosphorylation is reported to be important for IκB degradation [28]. Phosphorylation of IκB protein after TNF-α treatment was dramatically inhibited by dexamethasone, in a concentration-dependent manner (Fig. 4B). These results indicate that dexamethasone may suppress TNF-α-induced IL-8 gene expression in PDL cells by inhibiting degradation and phosphorylation of IκB.

3.5. Effects of p38 and ERK inhibitor on IL-8 expression in dexamethasone- and TNF-α-stimulated hPDL cells

Since our results indicated that p38 and ERK are inhibited by TNF-α exposure, we next examined whether these MAPKs are required for induction of pro-inflammatory gene expression by TNF-α. Cells were pre-treated with 20 μM SB203580 (a specific inhibitor of p38 MAPK) or 20 μM PD98059 (a specific inhibitor of ERK kinase) for 1 h. They were then treated with 10 ng/ml TNF-α and 10^{-7} M dexamethasone for 24 h, supernatant fractions were collected, and the amount of IL-8 protein secreted was determined by ELISA (Fig. 5A).

Pre-treatment of hPDL cells with either of the selective inhibitors for p38 (SB203580) or ERK kinase (PD98059) blocked TNF-α-induced IL-8 production (Fig. 5A). As seen in Fig. 5B, the level of secreted IL-8 protein correlated with the level of accumulated IL-8 mRNA. With dexamethasone (10^{-7} M) treatment, the inhibitory effect of pre-treatment on IL-8 mRNA and protein expression was also noted (Fig. 5A and B). These results implicate the tyrosine p38 and ERK1/2 pathways in the mechanism by which dexamethasone and TNF-α induce IL-8 expression.

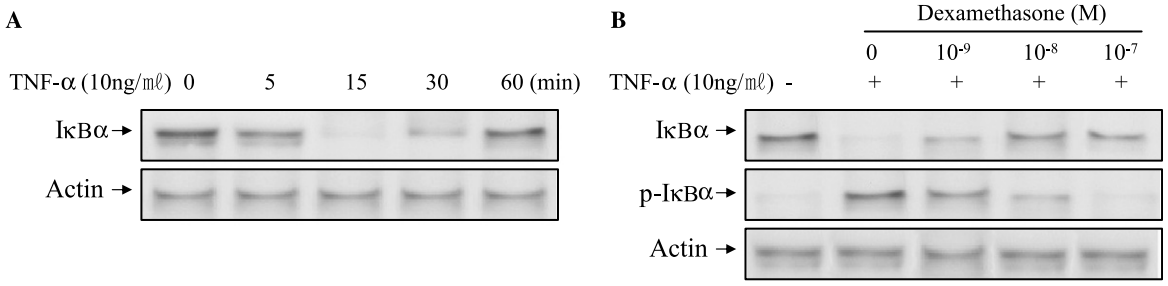

Fig. 4. Effect of dexamethasone on phosphorylation and degradation of IκB in TNF-α stimulated hPDL cells. Cells were stimulated with TNF-α (10 ng/ml) for time indicated (A). Cells were treated with three different concentration of dexamethasone (10^{-9}, 10^{-8}, 10^{-7} M) for 30 min (B). To analyze the level of IκB and phospho-IκB, cells were stimulated with TNF-α for 15 min, respectively. Cell lysates were analyzed by Western blot using anti-IκBα (37 kD) and antiphospho-*IκBα* antibodies. This similar data were obtained in three independent experiments.

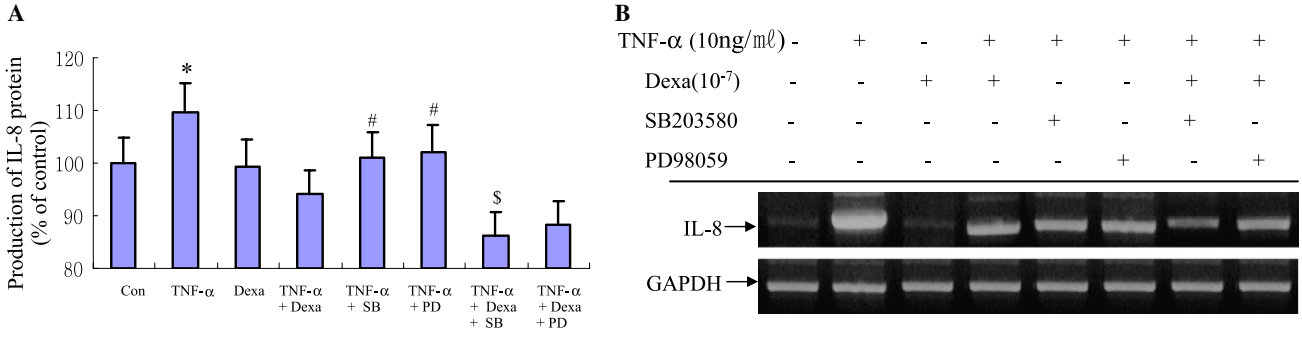

Fig. 5. Effect of p38 and ERK inhibitor on IL-8 protein (A) and mRNA (B) expression in dexamethasone and TNF-α stimulated PDL cells. The cells were pretreated with SB203580 (20 μM) and PD98059 (20 μM) for 1 h, post-treated with TNF-α (10 ng/ml) and dexamethasone (10^{-7} M) for 24 h. IL-8 protein levels in the supernatants were measured by ELISA (A). Total RNAs were extracted, and the level of mRNA were analyzed by RT-PCR (B). Values are means ± SD of three independent experiments. *Statistically significant difference compared to control group, $p < 0.05$. #Statistically significant difference compared to TNF-α group, $Statistically significant difference compared to TNF-α plus dexamethasone group, $p < 0.05$.

3.6. Effects of p38 and ERK inhibitor on ICAM-1 expression in dexamethasone- and TNF-α-stimulated hPDL cells

Because of similarities in the kinetics of induction of IL-8 and ICAM-1 mRNA, and in the structures of the IL-8 and ICAM-1 gene promoters, we hypothesized that MAPKs may also regulate ICAM-1 expression. Both IL-8 and ICAM-1 mRNA were rapidly up-regulated in hPDL following stimulation with TNF-α (Fig. 6A), but simultaneous treatment with dexamethasone suppressed the induction of ICAM-1 by TNF-α (Fig. 6A–C).

RT-PCR analysis indicated that pre-treatment of hPDL cells with SB203580 or PD98059 inhibited induction of ICAM-1 mRNA expression by TNF-α, with or without dexamethasone (Fig. 6A). The effects of MAPK inhibitors on the surface expression of ICAM-1 were also examined by FACS analysis. The results of the FACS analysis, which are shown in a conventional flow cytometric histogram on a log scale in Fig. 6B and C, are consistent with the RT-PCR analysis. SB203580 inhibited TNF-α-induced surface expression of ICAM-1 in the presence and absence of dexamethasone (Fig. 6B). PD98059 also blocked TNF-α-induced surface expression of ICAM-1 in the absence of dexamethasone, but not in its presence (Fig. 6C). These results suggest that the mechanisms by which dexamethasone and TNF-α induce ICAM-1 expression do not involve the tyrosine p38/ERK1/2 pathway in the same way.

4. Discussion

The PDL is a highly vascularized and cellularized connective tissue that attaches tooth root to the surrounding alveolar bone. The fibroblastic PDL cells in this tissue present an osteoblast-like phenotype that includes alkaline phosphatase activity and expression of osteocalcin. However, in the presence of inflammatory cytokines, such as IL-1β, PDL cells undergo transient phenotypic changes. They lose their osteoblast-like characteristics [29] and express genes that are associated with catabolic processes, including bone resorption, as evidenced by studies on the mechanism of orthodontic tooth movement [29,30].

Orthodontic tooth movement activates the secretion of osteolytic cytokines, such as IL-1, IL-6, and TNF-α that are produced by macrophages, fibroblasts, and osteoblasts [13,14]. IL-8, which was originally identified as a monocyte-derived factor capable of attracting and activating neutrophils, is increasingly being recognized as multifunctional. It appears to be important in the regulation of bone resorption, and its diverse functions have been investigated in a variety of cell types [5,6]. However, the effects of pro-inflammatory cytokines and glucocorticoids on IL-8 production in hPDL cells have not previously been studied in detail.

In the present study, we investigated the production and secretion of IL-8 from hPDL cells that were treated with the pro-inflammatory cytokine TNF-α in the presence and absence of dexamethasone. These treatments were chosen to mimic the inflammatory responses of bone resorption during orthodontic tooth movement. IL-8 secretion was significantly enhanced by treatment with TNF-α, in a dose- and time-dependent manner (Fig. 1A and B), and expression of IL-8 mRNA was also enhanced, in a time-dependent manner (Fig. 1C and D). These results are in accordance with those of previous studies using various human cell types, including osteoclasts [8], endothelial cells

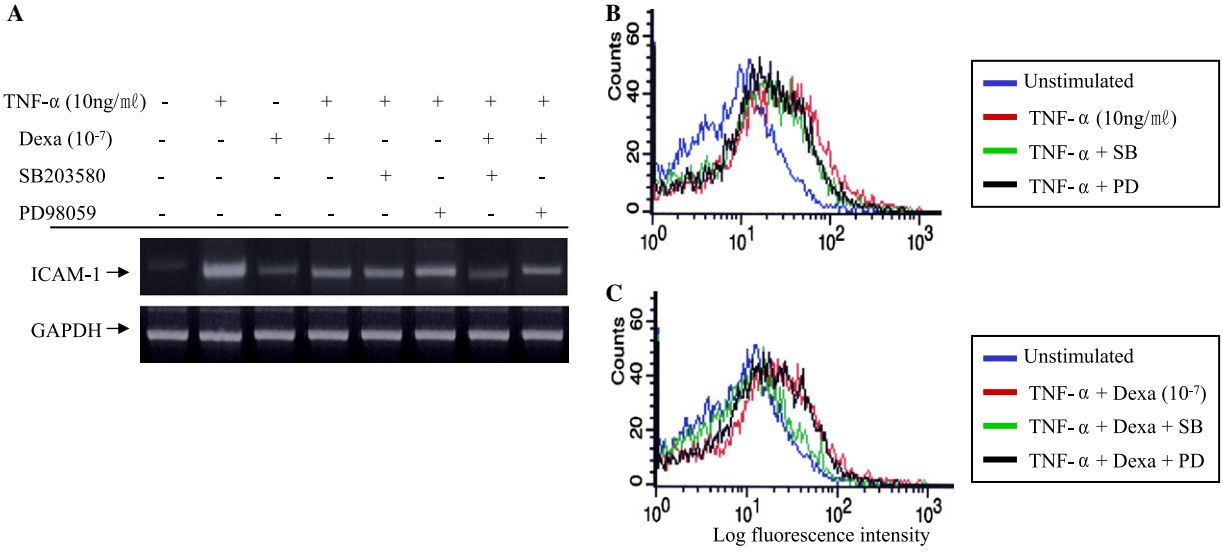

Fig. 6. Effect of p38 and ERK inhibitor on ICAM-1 expression in dexamethasone and TNF-α stimulated PDL cells by RT-PCR (A), and FACS-analysis (B). The cells were pretreated with SB203580 (20 μM) and PD98059 (20 μM) for 1 h, post-treated with TNF-α (10 ng/ml) and dexamethasone (10^{-7} M) for 24 h. Total RNAs were extracted, and the level of mRNA were analyzed by RT-PCR. Surface ICAM-1 expression by flow cytometry was analyzed by fluorescence-activated cell sorting by using anti-human ICAM-1 and FITC-labeled IgG. Nontreated cells were used as a control. The experiments were performed three times, and representative data are shown.

[15], and airway smooth-muscle cells [16], and they indicate that TNF-α-dependent up-regulation of IL-8 is due to an increase in transcription.

One characteristic feature of many cytokines is that their actions involve interactive networks or cascades that are often stimulatory. However, our present data demonstrate that dexamethasone inhibits TNF-α-induced secretion of IL-8 (Fig. 2A and B), providing an example, therefore, of an equally interesting inhibitory interaction. In a previous study by Fujisawa et al. [31], dexamethasone was found to have no effect on TNF-α-induced IL-8 production, but this lack of effect may be related to the low concentration of dexamethasone used. Additionally, we did not observe a significant effect for dexamethasone at the concentration (10^{-9} M) used in their study; however, at higher concentrations (10^{-8} and 10^{-7} M), it significantly inhibited TNF-α-induced IL-8 production in our study, and in those of Rothe et al. [8], Nyhlen et al. [15], and Pang et al. [16]. These results suggest that dexamethasone plays a important role in the regulation of bone remodeling and that this effect is due to inhibition of TNF-α-induced IL-8 secretion during orthodontic tooth movement.

IL-8 production in response to pro-inflammatory stimuli is dependent on MAPK- and NF-κB-mediated pathways [32]. NF-κB comprises specific, heterodimeric complexes that are inactive in the cytoplasm of resting cells, where each NF-κB complex is bound to one of the IκB proteins. Stimulus-induced activation of the NF-κB-inducing kinase causes it to phosphorylate the IκB kinase complexes [33]. Ubiquitination and proteasome-mediated degradation of IκB follows, freeing NF-κB to translocate to the nucleus, where it interacts with its target DNA motifs and regulates the secretion of various chemokines, including IL-8 [33,34].

IκB kinases are phosphorylated by NF-κB-inducing kinases, which activate various MAPKs, including the stress-activated protein kinase JNK [35]. Recently, the IL-6 and IL-8 genes have been identified as new targets regulated by JNK [36]. Other studies have shown that (i) ERK1/2 and JNK play an important role in streptococcal protein I/II-mediated synthesis of IL-6 and IL-8 in fibroblast-like synoviocytes [37]; (ii) p38 MAPK plays a crucial role in IL-6 and IL-8 production by TNF-α- or IL-1β-stimulated rheumatoid synovial fibroblasts [38]; and (iii) p38 and ERK MAPKs contribute to TNF-α-stimulated IL-8 secretion by intestinal epithelial cells [39].

To investigate the connection between the activation of p38 and ERK and induction of IL-8, we evaluated the effects of JNK and ERK pathway inhibitors (SB203580 and PD98059, respectively) and an anti-inflammatory agent (dexamethasone) on NF-κB, MAPK activation, and IL-8 production in hPDL cells exposed to TNF-α. Dexamethasone inhibited TNF-α-mediated phosphorylation of p38 and ERK1/2 (Fig. 3), indicating a possible specific interaction with the p38/ERK1/2 signal pathway. Moreover, dexamethasone inhibited TNF-α-mediated IκB degradation (Fig. 4) and NF-κB binding to DNA, providing evidence that dexamethasone regulates expression of the pro-inflammatory target gene, IL-8, by suppressing the NF-κB-activating signal pathway in hPDL cells.

Although the importance of ERK1/2 in IL-8 expression has been described in previous studies [40,41], the role of p38 MAPK in IL-8 expression is unclear. In a study by Ridley et al. [42], SB203580 had no effect on IL-8 expression in IL-1-stimulated human fibroblast or umbilical vein endothelial cells. On the other hand, in a study by Zu et al. [43], activation of p38 MAPK was required for stimulation of IL-8 production by granulocyte-macrophage colony-stimulating factor, lipopolysaccharide, or TNF-α in neutrophils. In the present study, both the p38 inhibitor and the ERK inhibitor efficiently blocked IL-8 protein production in response to TNF-α/dexamethasone, as shown by ELISA (Fig. 5A) and semi-quantitative RT-PCR (Fig. 5B). Thus, the effect of stimulating agents on IL-8 regulation appears to vary with cell type and the particular agent used.

ICAM-1, a cell surface adhesion molecule, is involved in many biological responses, including leukocyte adhesion to endothelial cells. It is expressed on several types of cells, including endothelial cells, epithelial cells, and leukocytes, and it is up-regulated at sites of inflammation by pro-inflammatory cytokines [44–46]. Evidence for ICAM-1 expression in the PDL was reported by Toms et al. [47], who used immunohistochemical analysis to examine the effect of endotoxin exposure on rat PDL blood vessels. Furthermore, Joe et al. [48] demonstrated that IL-1β causes up-regulation of ICAM-1 in PDL cells. However, whether ICAM-1 functions as a signaling molecule to transmit biochemical signals in hPDL cells remains unknown. In the present study using hDPL cells, we found that TNF-α induces ICAM-1 expression, that dexamethasone significantly inhibits this response, and that this response is consistent with the effect of TNF-α on IL-8 (Fig. 6). Hence, this report constitutes the first description of a signaling role for ICAM-1 in hPDL cells.

Transformed intestinal epithelial cells have been shown to express IL-8 and ICAM-1 both constitutively and upon treatment with TNF-α [44,45], and p38 MAPK plays a role in the regulation of pro-inflammatory mediators, such as IL-8, and adhesion molecules, such as ICAM-1 [23,46]. Thus, we examined whether MAPKs regulate ICAM-1 expression in dexamethasone- and TNF-α-stimulated hPDL cells at the level of translation or transcription. Interestingly, we found that SB203580 dramatically suppresses ICAM-1 expression by hPDL cells exposed to TNF-α with or without dexamethasone, as shown by semi-quantitative RT-PCR (Fig. 6A) and FACS analysis. PD98059 pretreatment blocked TNF-α-induced ICAM-1 expression, whereas did not affect ICAM-1 expression in cells simultaneously treated with TNF-α and dexamethasone. According to Jijon et. al. [26], neither PD98059 nor SB203580 reduced the number of ICAM-1 molecule following TNF-α treatment on the intestinal epithelium. The reason for this difference may reflect compensatory effect of dexamethasone for TNF-α, and may activate different target MAP kinase in different cell types.

The present study demonstrates that hPDL cells secrete the potent pro-inflammatory cytokine IL-8, and that TNF-α exposure stimulates production of IL-8 by these cells. Furthermore, dexamethasone inhibits the effect of TNF-α on IL-8 expression in these cells by blocking MAPKs and the IκB/NF-κB pathway. The up- or down-regulation of both ICAM-1 and IL-8 in response to TNF-α or dexamethasone appears to be mediated by p38 and ERK MAPKs. Thus, these factors may play an important role in the immunoregulation of bone remodeling during orthodontic tooth movement. Further studies elucidating the interactions among other osteotropic factors in hDPL cells should lead to techniques that will facilitate effective and rapid orthodontic tooth movement.

References

[1] Davidovitch Z. Tooth movement. Crit Rev Oral Biol Med 1991;2:411–50.

[2] Lekic P, McCulloch CA. Periodontal ligament cell population : the central role of fibroblasts in creating a unique tissue. Anat Rec 1996;245:327–41.

[3] Roberts WE, Goodwin Jr WC, Heiner SR. Cellular response to orthodontic force. Dent Clin North Am 1981;25:3–17.

[4] Wagner JG, Roth RA. Neutrohil migration mechanisms, with an emphasis on the pulmonary vascualture. Pharmacol Rev 2000;52:349–74.

[5] Yoshimura T, Matsushima K, Tanaka S, Robinson E, Appella E, Oppenheim J, et al. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. Proc Natl Acad Sci USA 1987;84:9233–7.

[6] Sozzani S, Locati M, Allavena P, Van Damme J, Mantovani A. Chemokines: a superfamily of chemotactic cytokines. Int J Clin Lab Res 1996;26:69–82.

[7] Zwahlen R, Walz A, Rot A. In vitro and in vivo activity and pathophysiology of human interleukin-8 and related peptides. Int Rev Exp Pathol 1993;34B:27–41.

[8] Rothe L, Collin-Osdoby P, Chen Y, Sunyer T, Chaudhary L, Tsay A, et al. Human osteoclasts and osteoclast like cells synthesize and release high basal and inflammatory stimulated levels of the potent chemokine interleukin-8. Endocrinology 1998;139:4353–63.

[9] Bendre MS, Montague DC, Peery T, Akel NS, Gaddy D, Suva LJ. IL-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease. Bone 2003;33:28–37.

[10] Tuncer BB, Ozmeric N, Tuncer C, Teoman I, Cakilci B, Yucel A, et al. Level of interleukin-8 during tooth movement. Angle Orthod 2005;75:631–6.

[11] Roodman GD. Advances in bone biology: the osteoclast. Endocr Rev 1996;17:308–32.

[12] Azuma Y, Kaji K, Katigi R, Takeshita S, Kudo A. Tumor necrosis factor-alpha induces differentiation of and bone resorption by osteoclasts. J Biol Chem 2000;275:4858–64.

[13] Lowney JJ, Norton LA, Shafer DM, Rossomando EF. Orthodontic force increases tumor necrosis factor-α in the human gingival sulcus. Am J Orthod Dentofacial Orthop 1995;108:519–24.

[14] Uematsu S, Mogi M, Deguchi T. Interleukin (IL)-1 beta, IL-6, tumor necrosis factor-alpha, epidermal growth factor, and beta 2-microglobulin levels are elevated in gingival crevicular fluid during human orthodontic tooth movement. J Dent Res 1996;75:562–7.

[15] Nyhlen K, Linden M, Andersson R, Uppugunduri S. Corticosteriod and interferon inhibit cytokine-induced production of IL-8 by human endothelial cells. Cytokine 2000;12:355–60.

[16] Pang L, Knox AJ. Synergic inhibition by beta(2)-agonist and corticosteroids on tumor necrosis factor-alpha-induced interleukin-8 release from cultured human airway smooth-muscle cells. Am J Respir Cell Mol Biol 2000;23:79–85.

[17] Hofbauer LC, Gori F, Riggs BL, Lacey DL, Dunstan CR, Spelsberg TC, et al. Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. Endocrinology 1999;140:4382–9.

[18] Richards CD, Langdon C, Deschamps P, Pennica D, Shaughnessy SG. Stimulation of osteoclast differentiation in vitro by mouse oncostatin M, leukaemia inhibitory factor, cardiotrophin-1 and interleukin 6: synergy with dexamethasone. Cytokine 2000;12:613–21.

[19] Pharoah M, Heersche J. Dexamethasone inhibits formation of osteoclast-like cells in bone-marrow cultures. J Dent Res 1986;65:1006–9.

[20] Kaji H, Sugimoto T, Kanatani M, Nishiyama K, Chihara K. Dexamethasone stimulates osteoclast-like cell formation by directly acting on hemopoietic blast cells and enhances osteoclast-like cell formation stimulated by parathyroid hormone and prostaglandin E2. J Bone Miner Res 1997;12:734–41.

[21] Hirayama T, Sabokbar A, Athanasou NA. Effect of corticosteroids on human osteoclast formation and activity. J Endocrinol 2002;175:155–63.

[22] Gupta T, Barrett AJ, Whitmarsh J, Cavanagh HK, Sluss B, Davis RJ. Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J 1996;15:2760–70.

[23] Roebuck KA, Finnegan A. Regualtion of intercellular adhesion molecule-1 (CD54) gene expression. J Leuko Biol 1999;66:876–88.

[24] Somerman MJ, Archer SY, Imm JR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. J Dent Res 1988;67:66–70.

[25] Imasato A, Desbois-Mouthon C, Han J, Kai H, Cato AC, Akira S, et al. Inhibition of p38 MAPK by glucocorticoids via Induction of MAPK phosphatase-1 enhances nontypeable *Haemophilus influenzae*-induced expression of Toll-like receptor. J Biol Chem 2002;277:47444–50.

[26] Jijon HB, Panenka WJ, Mansen K, Parson HG. MAP kinases contributes IL-8 secretion by intestinal epithelial cells via a posttranscriptional mechanism. Am J Physiol Cell Physiol 2002;283:C31–41.

[27] Hiragun T, Peng Z, Beaven MA. Dexamethasone up-regulates the inhibitory adaptor protein Dok-1 and suppresses downstream activation of the mitogen-activated protein kinase pathway in antigen-stimulated RBL-2H3 mast cells. Mol Pharmacol 2005;67:598–603.

[28] Baldwin Jr AS. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649–83.

[29] Agarwal S, Chandra CS, Piesco NP, Langkamp HH, Bowen L, Baran C. Regulation of periodontal ligament cell functions by interleukin-1beta. Infect Immun 1998;66:932–7.

[30] Kanzaki H, Chiba M, Shimizu Y, Mitani H. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. J Bone Miner Res 2002;17:210–20.

[31] Fujisawa K, Nasu K, Arima K, Sugano T, Narahara H, Miyakawa I. Production of interleukin (IL)-6 and IL-8 by a choriocarcinoma cell line, BeWo. Placenta 2000;21:354–60.

[32] Holtmann H, Winzen R, Holland P, Eickemeier S, Hoffmann E, et al. Induction of interleukin-8 synthesis integrates effects of transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. Mol Cell Biol 1999;19:6742–53.

[33] Karin M, Ben-Nariah Y. Phosphorylation meets ubiquitination: the control of NF-kappa-B activity. Annu Rev Immunol 2000;18:621–63.

[34] Thomas LH, Friedland JS, Sharland M, Becker S. Respiratory syncytial virus-induced RANTES production from human bronchial epithelial cells is dependent on NF-κB nuclear binding and inhibited

by adenovirus-mediated expression of IB. J Immunol 1998;161:1007–16.
[35] Lin A, Minden A, Martinetto H, Claret FX, Lange-Carter C, Mercurio F, et al. Identification of a dual specificity kinase that activates the jun kinases and p38-mpk2. Science 1995;268:286–90.
[36] Krause A, Holtmann H, Eickemeier S, Winzen R, Szamel M, Resch K, et al. Stress-activated protein kinase/Jun-N-terminal kinase is required for interleukin-1-induced IL-6 and IL-8 gene expression in the human epidermal carcinoma cell line KB. J Biol Chem 1998;273:23681–9.
[37] Neff L, Zeisel M, Sibilia J, Scholler-Guinard M, Klein JP, Wachsmann D. NF-κB and the MAP kinases/AP-1 pathways are both involved in interleukin-6 and interleukin-8 expression in fibroblast-like synoviocytes stimulated by protein I/II, a modulin from oral streptococci. Cell Microbiol 2001;3:703–12.
[38] Suzuki M, Tetsuka T, Yoshida S, Watanabe N, Kobayashi M, Matsui N, Okamoto T. The role of p38 mitogen-activated protein kinase in IL-6 and IL-8 production from the TNF-α or IL-1 stimulated rheumatoid synovial fibroblast. FEBS Lett 2000;465:23–7.
[39] Jijon HB, Panenka WJ, Madsen KL, Parsons HG. MAP kinases contribute to IL-8 secretion by intestinal epithelial cells via a posttranscriptional mechanism. Am J Physio Cell Physiol. 2002;283:C31–41.
[40] Liu R, O'Connell M, Johnson K, Pritzker K, Mackman N, Terkeltaub R. Extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 mitogen-activated protein kinase signaling and activation of activator protein 1 and nuclear factor κB transcription factors play central roles in interleukin-8 expression stimulated by monosodium urate monohydrate and calcium pyrophosphate crystals in monocytic cells. Arthritis Rheum 2000;43:1145–55.
[41] Bruder JT, Kovesdi I. Adenovirus infection stimulates the Raf/MAPK signaling pathway and induces interleukin-8 expression. J Virol 1997;71:398–404.
[42] Ridley SH, Sarfield SJ, Lee JC, Bigg HF, Cawston TE, Taylor DJ, et al. Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase: regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. J Immunol 1997;158:3165–73.
[43] Zu YL, Qi J, Gilchrist A, Fernandez GA, Vazquez-Abad D, Kreutzer DL, et al. p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-α or FMLP stimulation. J Immunol 1998;160:1982–9.
[44] Dippold W, Wittig B, Schwable W, Mayet W, Meyer zum Buschenfekde KH. Expression of intracellular adhesion molecule 1 (ICAM-1 CD54) in colonic epithelial cells. Gut 1993;34:1593–7.
[45] Eckmann L, Jung HC, Schirer-Maly C, Panja A, Morzycka-Wroblewska E, Kangnoff MF. Differential cytokine expression by human intestinal epithelial cell lines : regulated expression of interleukin-8. Gastroenterology 1993;105:1689–97.
[46] Saccani S, Pantano S, Natoli G. p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. Nat Immunol 2002;3:69–75.
[47] Toms A, Gannon B, Carati C. The immunohistochemical response of the rat periodontal ligamanet endothelium to an inflammatory stimulus. Aust Orthod 2000;16:61–8.
[48] Joe BH, Borke L, Keskintepe M, Hanes PJ, Mailhot JM, Singh BB. Interleukin-1 beta regulation of adhesion molecules on human gingival and periodontal ligament fibroblasts. J Periodontol 2001;72:865–70.