Expression and effect of tumor necrosis factor-α in human dental follicle cells in vitro

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Purpose: To investigate (1) the effect of interleukin-1α (IL-1α) on tumor necrosis factor-α (TNF-α) expression, (2) the induced effect of TNF-α on the expression of monocyte chemoattractant protein-1 (MCP-1), and (3) the corresponding signal transduction pathway in the human dental follicle cells (HDFCs).
Materials and Methods: Primary HDFCs were cultured. The fifth passaged HDFCs were incubated with IL-1α. Then examined the expression of TNF-α by reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assays (ELISA). The expression of MCP-1 was examined after HDFCs incubatied with TNF-α by ELISA and RT-PCR. Furthermore, the induction of MCP-1 mRNA expression by TNF-α was inhibited by SB203580, SP600125, and PD98059.
Results: The results of RT-PCR and ELISA revealed that IL-1α increases the TNF-α gene expression and secretion significantly at concentration of 25-100 ng/mL ($p<0.05$). Furthermore, TNF-α enhances MCP-1 gene expression and secretion at concentration of 10-100 ng/mL ($p<0.05$) and the action is inhibited by SP600125, the special inhibitor of c-Jun N-terminal kinase (JNK).
Conclusion: IL-1α induces expression of TNF-α by HDFCs. One of the actions of TNF-α in tooth eruption may be that it enhances MCP-1 gene expression and secretion and these events require JNK activity, which recruits monocytes into the dental follicle, where these cells are fused to form osteoclasts to resorb alveolar bone for the formation of an eruption pathway. **(Int Chin J Dent 2007; 7: 35-42.)**
Key Words: dental follicle, interleukin-1α, monocyte chemoattractant protein-1, tumor necrosis factor-α.

Introduction

A critical cellular event of tooth eruption is the influx of mononuclear cells into the dental follicle, the loose ectomesenchyme derived connective tissue surrounding the enamel organ and the dental papilla of the developing tooth germ prior to eruption. The mononuclear cells recruited into dental follicle are fused to form osteoclasts needed for bone resorption.[1] Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that often is overexpressed in a number of disease states such as sepsis syndrome, rheumatoid arthritis, inflammatory bowel disease,[2] and periodontitis.[3] TNF-α induces osteoclast differentiation and bone resorption by stimulating osteoclast precursors. It gains importance as a local regulator of bone cell function for good reason. TNF-α treatment of cultured bone explants or cell cultures of mineralizing osteoblasts caused increases calcium release and suppression of matrix protein production, suggesting stimulation of bone resorption and inhibition of formation.[4-6] In addition, increased production of TNF-α is demonstrated as part of the overall cytokine stimulus, and blockade of TNF-α action is shown to alleviate bone loss.[7-8] It was reported that TNF-α is maximally expressed in dental follicle of postnatal day 9 rats regulated by interleukin-1α (IL-1α) and TNF-α stimulates the expression of potential tooth eruption genes monocyte chemoattractant protein-1 (MCP-1) and vascular endothelial growth factor (VEGF). There is a second minor burst of osteoclasts seen on the alveolar bone surrounding the first molar and we have proposed that the maximum expression of TNF-α at day 9 may be involved to up-regulate the second round of osteoclastogenesis.[9]

Recent studies have shown no single mechanism is responsible for TNF-α action on bone cell; activation of a complex signal pathway drives gene transcription in a direction that produces a skeletal catabolic state. Kumar

et al. demonstrated that in osteoblasts significant activation of p38 mitogen-activated protein kinase (MAPK) was observed following treatment with TNF-α while inhibitors of p38 MAPK have been shown to reduce TNF stimulation of osteoclastogenesis.[10] Using a human peripheral blood mononuclear cells (PBMC) culture model, HB Kwak et al. reported that TNF-α regulates osteoclast differentiation through $p21^{WAF1/Cip1}$ expression and further shows that these events require c-Jun N-terminal kinase (JNK) activity.[11] However, even the regulation of TNF-α has been studied in different cell types, no studies to date have addressed intracellular signaling and molecular regulation mechanisms on human dental follicle cells (HDFCs).

In this study, we investigated whether TNF-α is normally expressed in the dental follicle cells, and the levels of the effect of TNF-α itself on the expression of genes in HDFCs that may relate to tooth eruption. In addition, signal transduction pathways that TNF-α regulates in HDFCs were also investigated using specific inhibitors.

Materials and Methods

Culture of HDFCs

HDFCs were established and cultured as previously described.[12] Prior to commencement, the study protocol was approved by Ethics Committee of the Fourth Military Medical University. Normal adolescents impacted third molars were surgically removed and collected which needed for orthodontics treatment after informed consent. The age of the patients varied between 11 and 15 years. Attached dental follicles were separated from the mineralized tooth. The surfaces of the follicle tissues were cleaned and minced by using a sterilized scalpel. Also dental papilla tissue was discarded. Tissues were digested in a solution of 0.1 U/mL collagenase type I and 1 U/mL dispase (Roche, Mannheim, Germany) for 1 hour at 37˚C. Minced and digested tissues of dental follicle explants were seeded into 60 mm plates with 25 mL Dulbecco's modified Eagle medium (DMEM, HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) and 1 mmol/L sodium pyruvate, in an incubator at 37˚C in an atmosphere of 5% CO_2. After single cells had been attached to the plastic surface, non-adherent cells were removed by change of medium. Plastic adherent cells were continuously propagated until confluence. HDFCs of passage 5 were grown in 250 mL T-flasks until confluence. Prior to treatments, cells were serum-deprived DMEM containing 0.2% fatty-acid-free and growth-factor-depleted bovine serum albumin (BSA) for 12 hours.

Incubation of HDFCs with IL-1α

The cells were incubated in serum-free minimal essential medium containing recombinant human IL-1α (PeproTech Rock Hill, NJ, USA) for the designated concentration-dependent studies. The cells were incubated with IL-1α 0, 5, 10, 25, 50, and 100 ng/mL[9] for 6 hours. After the treatments, the cells were collected for RNA isolation and cell culture supernates were collected for TNF-α level measured by enzyme-linked immunosorbent assays (ELISA). Each experiment was repeated at least three times. Controls were incubated in serum-free DMEM containing BSA only.

Isolation of total RNA

Total cellular RNA was isolated from each culture using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA), according to protocols provided by the manufacturer. To remove contaminating genomic DNA, the RNA samples were treated with RNase-free DNase I (Qiagen) at 37°C for 30 minutes.

Reverse transcription polymerase chain reaction

First, 2 μg of total RNA was used as a template to synthesize first-strand complementary DNA (cDNA) with

oligo (dT) primer and reverse transcriptase, using the Omniscript RT Kit (Qiagen). Then 1 μL of the cDNA mixture was subjected to polymerase chain reaction (PCR) amplification using specific primers. The specific primers for the genes of TNF-α, MCP-1 and β-actin were designed using Primer Premier 5 (Premier Biosoft Ltd., Palo Alto, CA, USA). Primers were synthesized by BioAsia Corp. (Shanghai, China). The primer sequences used in this study are shown in Table 1.

Table 1. Primer sequences used in semi-quantitative RT-PCR.

	Forward	Reverse
TNF-α (product 491 bp)	5′-GAAATGGAGGCAATAGGTT-3′	5′-AGCCGTGGGTCAGTATGT-3′
MCP-1 (product 308 bp)	5′-CTTCTGTGCCTGCTGCTC-3′	5′-AGATTCTTGGGTTGTGGA-3′
bactin (product 326 bp)	5′-CCGGATCCATCTCTTGCTCGAAGTCCA-3′	5′-AGAGGCATACAGGGACAACACA-3′

A total of 40 cycles of PCR was performed by mixing 3 μL cDNA with dNTP, primers, PCR buffer, and Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA) to 25 μL in each reaction with the amplification protocol of 94˚C for 30 s (denatureation), 58.5˚C for 2 s (annealing), and 74˚C for 30 s (extention). The amplification products were electrophoresed on 2% agarose gels (Amresco, Solon, OH, USA) and visualized by ethidium bromide staining. The relative intensities of the gel bands were measured using NIH Image software, and the results were normalized to the mRNA level of actin, a housekeeping enzyme. We performed these experiments using samples from at least three different cell preparations, and quantification of mRNA was confirmed using the same cell sample at least in triplicate.

ELISA for the quantitative determination of TNF-α/MCP-1 concentration

In order to quantify the concentration of TNF-α secreted after incubating the dental follicle cells with TNF-α and that of MCP-1 secreted after incubating the dental follicle cells with TNF-α, ELISA kits (R&D Systems, Minneapolis, MN, USA) were employed using a monoclonal antibody for human TNF-α and MCP-1 that had been precoated onto micro-plates. Measurements were performed in triplicate on samples diluted 1:10 according to the manufacturer's instructions. In brief, 100 μL assay buffer, 50 μL sample, and 50 μL detection antibody were added to each well. After incubation for 16 hours at 4°C, the wells were washed with wash buffer, then 200 μL streptavidin-horseradish peroxidase conjugate was added to each well followed by incubation for 60 minutes at room temperature. After further washing, substrate was added to all wells and incubated for 20 minutes, followed by stop solution. The absorption was determined with a micro-plate reader model 550 (Bio-Rad, Hercules, CA, USA) at 450 nm against 570 nm as a reference. The intensity of the blue color measured was in proportion to the amount of human TNF-α and MCP-1 bound. All experiments were repeated three times.

Inhibitors

Signal transduction pathways that mediated cellular responses to TNF-α were investigated using specific inhibitors: SB203580 (25 μmol/L) to inhibit p38MARK, SP600125 (15 μmol/L) to inhibit JNK, and PD98059 (50 μmol/L) for specific inhibition of a extracellular signal-regulated kinase (ERK). All inhibitors were obtained from Sigma (St. Louis, MO, USA) and the concentrations used were the effective doses previously reported.[13] After the HDFCs had been pre-incubated in the presence of each inhibitor for 30 minutes to permit these compounds to penetrate the cells and block their respective pathways, 10 ng/mL TNF-α was applied in culture

for 6 hours. Total RNA was extracted from the cells, and the expression levels of MCP-1 mRNA were determined by RT-PCR.

Statistical analysis

Mean values with standard deviations (SD) were calculated. Some data were subjected to multiple measurement analysis of variances (ANOVA), and Student's t test was used to analyze differences between the cultures tested. A p-value of less than 0.05 was accepted as significant.

Results

Effect of IL-1α on expression of TNF-α mRNA and protein in HDFCs IL-1α

We first determined the TNF-α mRNA espression in HDFCs in response to IL-1α Cells were treated with IL-1α, the cell were harvested, and the level of expression mRNA was determined by RT-PCR. The expression of TNF-α mRNA was increased significantly by IL-1α with concentration of 25, 50, and 100 ng/mL in comparison with the control (IL-1α, 0 ng/mL) cultures (Fig. 1A). When the level of TNF-α protein in the induced cultures was analyzed by ELISA, the increase of TNF-α protein (Fig. 1B) correlated with the accumulation in TNF-α mRNA expression.

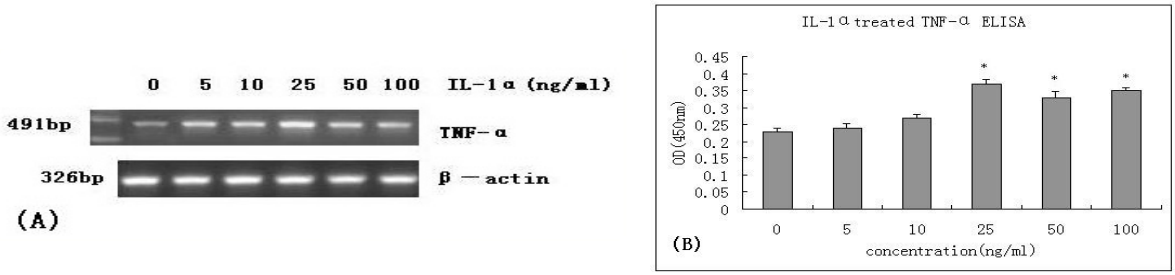

Fig. 1. Effect of IL-1α on expression of TNF-α mRNA and protein in HDFCs.
In a concentration-dependent manner, cells were stimulated for 6 hours in the IL-1α concentration indicated (A and B). Cells were collected for examination of TNF-α mRNA by RT-PCR and supernatants were analyzed for TNF-α concentration by ELISA. Mean values with SDs of three independent experiments are presented. *Statistically significant difference is shown above compared to control group (IL-1α, 0 ng/mL), p<0.05.

Effect of TNF-α on expression of MCP-1 mRNA and protein in HDFCs

Fig. 2A shows how different concentration of TNF-α affect the MCP-1 mRNA expression level in HDFCs. TNF-α concentration at 10, 25, 50, or 100 ng/mL caused a significant increase in MCP-1 mRNA expression compared to that in control (0 ng/mL) cultures (Fig. 2A). These results were confirmed by ELISA for MCP-1 protein in the conditioned medium from these cells (Fig. 2B).

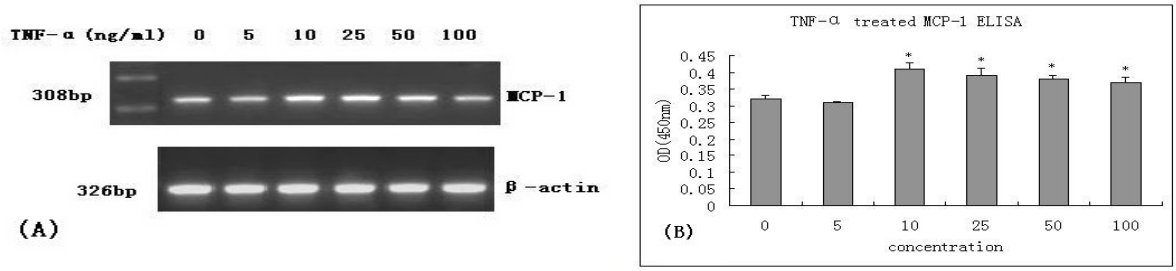

Fig. 2. Effect of TNF-α induced expression of MCP-1 protein (B), mRNA (A) in HDFCs.
The cells were stimulated for 6 hours in the TNF-α concentration indicated (A and B). Total RNAs were extracted, and the levels of mRNA were analyzed by RT-PCR (A). MCP-1 protein levels in the supernatants were measured by ELISA (B). Mean values with SDs of three independent experiments are presented. *Statistically significant difference is shown above compared to control group (TNF-α, 0 ng/mL), p<0.05.

Effects of inhibitors on induction of MCP-1 mRNA expression by TNF-α in HDFCs

The mechanisms of the translating signal induced by TNF-α which activates increased MCP-1 gene expression are as yet unidentified. To investigate whether the up-regulation of MCP-1 mRNA by TNF-α was dependent on de novo protein synthesis, we treated the cells with aforementioned special inhibitor in Materials and Methods. The effect was inhibited by SP600125 but not by SB203580, or PD98059, suggesting that it depended on JNK pathway (Fig. 3).

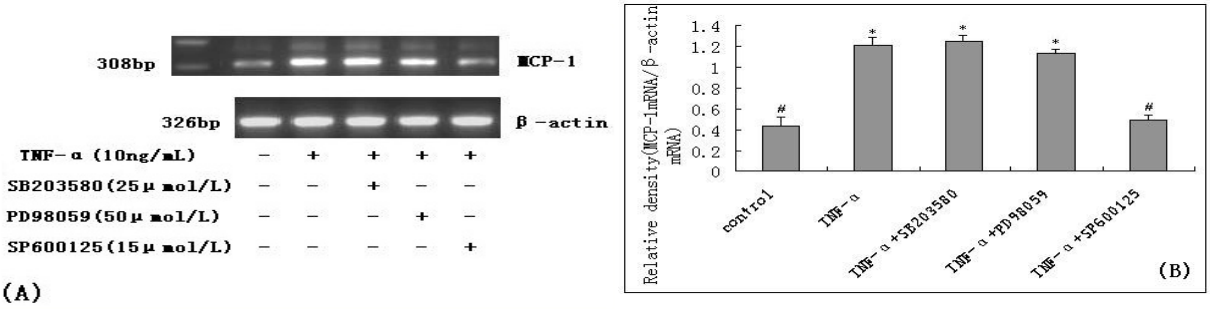

Fig. 3. Effects of inhibitors on induction of MCP-1 mRNA expression in TNF-α stimulated HDFCs.
The cells were pretreated with SB203580 (25 μmol/L), SP600125 (15 μmol/L), and PD98059 (50 μmol/L) 30 minutes, post-treated with or without TNF-α (10 ng/mL) 6 hours. Total RNAs were extracted from the cells, and expression levels of MCP-1 mRNA were determined by RT-PCR as described in Materials and Methods. (A) Agarose gel electrophoresis of the products of PCR using specific primers for MCP-1 or β-actin. (B) Relative density of the MCP-1 mRNA to β-actin mRNA. Mean values with SDs of three independent experiments are presented. *Statistically significant difference is shown above compared to control group (TNF-α, 0 ng/mL), $p<0.05$. #Statistically significant difference is shown above compared to TNF-α (10 ng/mL) group.

Discussion

Tooth eruption is a localized event and a cascade of molecular signals generated in the dental follicle and stellate reticulum appears to initiate its onset. Consequently, mononuclear cells are recruited into the follicle and, in turn, fuse to become osteoclasts needed to resorb the alveolar bone to form an eruption pathway. IL-1α is present early postnatally in the stellate reticulum of the first mandibular molars.[14] In tooth eruption, IL-1α may play an important role that injection of IL-α accelerated eruption by two days in rats.[15] In knockout mice lacking a functional type I receptor for interleukin-1α (IL-1R) tooth eruption is delayed for two days in molars and one day in incisors.[16] Recent researches show that IL-1α enhances CSF-1,[17,18] MCP-1,[19] and NF-κB[20] expression which are responsible for tooth eruption. In addition, Kawashima et al. reported that IL-1α plays a crucial part in periapical bone resorption and high correlation with TNF-α, which seems to relate to their co-stimulatory effects.[21]

TNF-α is one of well recognized members of the TNF family that play an important physiological and/or pathological role in bone metabolism. The predominant action of TNF-α in osteoclast formation and function is likely mediated by directly targeting osteoclast precursors and mature osteoclasts, i.e., the binding of TNF-α to its receptors on osteoclast precursors/osteoclasts initiates various signaling cascades shown in, leading to the modulation of osteoclast formation, function and survival.[22,23] TNF-α is expressed slightly in the dental follicle of the first mandibular molar of the rat beginning at day 3 postnatally, but maximal expression is seen at day 9, a time that correlates with a slight burst of osteoclast formation seen at day 10 postnatally.[9] In our study, HDFCs in vitro does not express constitutively the TNF-α but does express it after incubation with IL-1α. There is a maximal expression of TNF-α at 25 ng/mL IL-1α. The expression of TNF-α decreased at 50-100 ng/mL IL-1α.

But statistically significant difference is shown above compared to control group (IL-1α, 0 ng/mL). The result is similar to that of Wise.[9] Although the chronology of the localization of the potential eruption molecules are not clear during human tooth eruption, we speculate that IL-1α in the stellate reticulum enhances the expression of TNF-α in the dental follicle which participates in the formation of osteoclasts. Thus paracrine signalling may occur, whereby molecules in the stellate reticulum affect the cells in the adjacent dental follicle (or vice versa) to initiate eruption.

MCP-1 is a member of the chemokine family of cytokines. MCP-1, a single polypeptide chain (148 aa) with a signal peptide domain (23 aa residues), is present and is secreted as mature MCP-1 (125 aa) in several cells such as osteoblasts,[24] mesangial cells,[25] endothelial cells and fibroblasts,[26] alveolar macrophages,[27] and gingival tissues.[28] The fact that fibroblasts express the MCP-1 gene is particularly interesting because the dental follicle is primarily fibroblastic in nature. The principal function of MCP-1 is thought to be the stimulation of monocyte recruitment. Que et al. reported that in vivo MCP-1 is expressed to recruit the mononuclear cells to the dental follicle to initiate eruption, with maximal gene expression at day 3 in the rat.[29] However, comparing MCP-$1^{-/-}$ mice with wild-type controls during tooth eruption Graves et al demonstrated that at early time points, monocyte recruitment occurs by MCP-1 independent mechanisms while at a later time point, MCP-1 may play a contributory role in the recruitment of monocytic cells.[30] Many of the eruption molecules, such as IL-1α, TGF-β_1,[31] parathyroid hormone-related protein (PTHrP),[32] TNF-α,[9] in turn, have been shown to enhance the gene expression of MCP-1. In our experiment there is a maximal increase in expression of MCP-1 at 10 ng/mL concentration of TNF-α, followed by a progressive decrease at higher concentration. This study shows that TNF-α can enhance the expression of the MCP-1 gene to bring about the cellular events of eruption -recruitment of monocytes into the dental follicle, their fusion to form osteoclasts and bone resorption by these osteoclasts to form an eruption pathway.

A major mechanism through which signals are transmitted from environmental stimuli to the nucleus involves activation of kinase related to the mitogen-activated protein (MAP) kinase superfamily. To date, at least three subgroups of MAP kinase family members have been identified to be involved in a wide range of cellular responses to extracellular signals. They are extracellular signal-regulated kinases (ERKs), 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. Our studies are based on the observation that TNF-α causes p38, JNK, and ERK activation. In our study, inhibition of MCP-1 mRNA expression by TNF-α is abrogated by pretreatment with SP600125 (Fig. 3). This experiment indicates that the JNK-MAPK pathway is involved in MCP-1 release. Westra et al.[33] reported that TNF-α is the major inducer of endothelial cell activation and is the stimulator of inflammatory signal transduction pathway involving p38 MAPK but the p38 MAPK inhibitor significant reduces mRNA expression and protein production of IL-6, IL-8 and MCP-1, which shows that TNF-α induces MCP-1 by p38 MAPK signaling pathways in endothelial cells. However, Hanazawa et al.[34] reported that the biological responses of MCP-1 synthesis in osteoblasts MC3T3-El cells induced by TNF-α are regulated via *c-fos* pathway. We obtain the same results as that Chen et al.[35] reported that both the p42/44 MAPK and the JNK pathways coordinately modulate TNF-α induced CCL2/MCP-1 expression in rat vascular smooth muscle cells (VSMCs). However, in other rat cell types or in human cell systems, p38 MAPK was reported to mediate the induction of MCP-1 elicited by TNF-α.[36-38] Thus, the MAPK signals mediating TNF-α dependent MCP-1 expression may

vary by cell types and species.

We finally conclude that IL-1α induces an increase of TNF-α synthesis and mRNA expression in cultured HDFCs. TNF-α enhances MCP-1 synthesis and mRNA expression. Furthermore, we have evidenced that SP600125, the special inhibitor of JNK, suppresses the TNF-α induced MCP-1 mRNA expression. These findings may be important for understanding the effect of TNF-α in tooth eruption.

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