*J Oral Tissue Engin 2023;**21**(2): 59-65*

ORIGINAL ARTICLE

Effects of Tumor Necrosis Factor-α on Interleukin-8 Production in Human Gingival Epithelial Cells

Feng ZHENG, Hiroshi INOUE, and Seiji GODA

Department of Physiology, Osaka Dental University, Osaka, Japan

SYNOPSIS

Tumor necrosis factor (TNF)-α is an inflammatory mediator that is released during the early stages of the inflammatory response and plays an important role in the development of periodontitis. Interleukin-8 (IL-8) is associated with the attraction and activation of neutrophils into the area of inflammation, which is considered an important factor in the regulation of inflammatory responses. In this study, we investigated the effect of TNF-α stimulation on IL-8 production in Ca9-22 cells, a human gingival epithelial cell line. Stimulation with TNF-α enhanced IL-8 production and ERK1/2 phosphorylation. U0126, a potent and selective MEK1/2 inhibitor, inhibited phosphorylation of ERK1/2 but not p38. These findings suggest that the enhancement of IL-8 production by TNF-α-stimulated Ca9-22 may lead to the induction and activation of neutrophils into inflamed tissues and worsen periodontitis.

***Key words**: TNF-α, IL-8, human gingival epithelial cells*

INTRODUCTION

Periodontal disease progresses as a result of interactions among reactive oxygen species, growth factors, cytokines, and pro-inflammatory mediators such as matrix metalloproteinases (MMPs), and in severe cases it causes tooth loss[1,2]. The gingival epithelial barrier is exposed to numerous microbes and its state is based on a homeostatic balance between host resistance and colonization by the microbiota. Therefore, it is important to examine the response of inflammatory stimuli to gingival epithelial cells.

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that plays a major role in tissue destruction[3]. TNF-α is produced by epithelial cells, macrophages, and fibroblasts, and is associated with the promotion of bone resorption, increased vascular permeability, and activation of leukocytes[3,4]. TNF-α acts on osteoblasts and periodontal ligament cells, which constitute the periodontal tissue, and promotes the expression of RANKL, resulting in the differentiation and formation of osteoclasts[5]. Osteoclasts are further activated by IL-1β and TNF-α and absorb alveolar bone, resulting in periodontal disease progression[5].

Patients with gingivitis or periodontitis have been reported to produce higher levels of inflammatory mediators, such as TNF-α and IL-6, than healthy individuals[6]. IL-8 is a potent inflammatory mediator present in periodontal disease tissues, leading to angiogenesis, tissue remodeling, and neutrophil and lymphocyte recruitment[7,8]. It has been reported that IL-8 levels in inflamed gingival tissue are higher than in normal tissue and that crevicular fluid is correlated with disease severity[9,10]. IL-8 is associated with chronic leukocyte recruitment, leading to periodontal tissue destruction. Therefore, regulating IL-8 production may have anti-inflammatory effects on gingival epithelial cells attacked by periodontal pathogens.

In this study, we used Ca9-22 cells, a human gingival epithelial cell line, to examine IL-8 production induced by TNF-α stimulation and its activation signal transduction pathway.

MATERIALS AND METHODS

1. Cell culture

Human gingival epithelial cell line, Ca9-22 cells, were used as representative gingival epithelial cell[11], purchased from the JCRB Cell Bank (ID: JCRB0625; Osaka, Japan).

Cells were cultured in Dulbecco's modified Eagle's Medium (D-MEM; FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal calf serum (Biowest, Nuaillé, France), 2 mM L-glutamine (FUJIFILM Wako Pure Chemical), 1% penicillin, and 1% streptomycin (FUJIFILM Wako Pure Chemical). Ca9-22 cells were maintained at 37°C containing 5% CO_2 and 95% air humidity.

2. Reagents, antibodies, and inhibitors

We purchased Human TNF-α from Miltenyi Biotec (Bergisch Gladbach, Germany); U0126 from LC Laboratories (Woburn, MA, USA); anti-phospho ERK1/2, ERK1/2, and anti-phosphop38 from Cell Signaling Technology (Beverly, MA, USA); and anti-p38 from Santa Cruz Biotechnology (Dallas, TX, USA). HRP-labeled anti-mouse IgG and anti-rabbit IgG were used as secondary antibodies (Jackson ImmunoResearch, West Grove, PA).

3. Enzyme-linked immunosorbent assay

Ca9-22 was seeded on a 96-well plate at a density of 1×10^5 cells/well, and cultured for 24 hours in D-MEM containing 10% FBS, 2 mM L-glutamine, 1% penicillin, and 1% streptomycin. Then, Ca9-22 was incubated in serum-free D-MEM containing 2 mM L-glutamine, 1% penicillin, and 1% streptomycin for 24 hours. After incubation, Ca9-22 was stimulated with or without 2 ng/mL TNF-α at for 24 hours. The supernatants were collected and centrifuged to remove debris. IL-8 production in the culture supernatant was quantified using enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol. ELISA MAX Deluxe Set Human IL-8 was purchased from BioLegend (San Diego, CA, USA). Assays were performed in triplicate in three independent experiments, and the means ± standard deviations were calculated.

4. Western blotting

Ca9-22 was seeded on a 12-well plate at a density of 1.0×10^5 cells/well and cultured for 4 days to 90% confluency in D-MEM containing 10% FBS. Ca9-22 was then incubated in serum-free D-MEM for 1 hour. After incubation, Ca9-22 was stimulated with 2 ng/mL of TNF-α, and time-dependent changes in the phosphorylation of ERK1/2 for up to 30 minutes after the stimulation were examined. After the stimulation, total protein was isolated in a buffer sample supplemented with Halt protease and

phosphatase inhibitor cocktail (Thermo Fisher Scientific). Equal amounts of each sample were then separated by 10% SDS/PAGE, transferred to PVDF membranes, and blocked with 20% Blocking One in 50 mM Tris–HCl at pH 7.5 and 150 mM NaCl overnight. Membranes were incubated with 1:1,000-diluted primary antibodies against anti-phospho ERK1/2 antibody at RT for 1 hour. Membranes were then incubated with the matching secondary antibody (HRP-labeled anti-rabbit IgG; 1:2,000) at RT for 1 hour. The same membrane was stripped and reprobed with an anti-ERK1/2 antibody at 1:1,000. Blot images were acquired using ChemiDoc MP.

To confirm whether TNF-α stimulation is involved in phosphorylation of ERK1/2, we treated Ca9-22 with U0126, a potent and selective MEK1/2 inhibitor. Ca9-22 was cultured and treated without serum, as described in previous experiments. Ca9-22 was pretreated with U0126 (1, 5, 10 μM) for 1 hour and then stimulated by with 2 ng/mL of TNF-α for 10 minutes. After the stimulation, ERK 1/2 phosphorylation and p38 phosphorylation were detected by Western blotting.

5. Statistical analysis

Student's *t*-test was used to determine the statistical significance of the differences between two groups. All analyses were performed using GraphPad software (San Diego, CA, USA). Values are expressed as mean ± standard deviation, and all experiments were performed at least thrice. *P* values < 0.01 were considered statistically significant.

RESULTS

1. TNF-α increased IL-8 production in Ca9-22.

We initially examined the effect of TNF-α on IL-8 production in Ca9-22 using ELISA. There was no production of IL-8 in the unstimulated control group; however, 2 ng/mL TNF-α stimulation markedly enhanced the production of IL-8 at the protein level in Ca9-22 (Fig. 1).

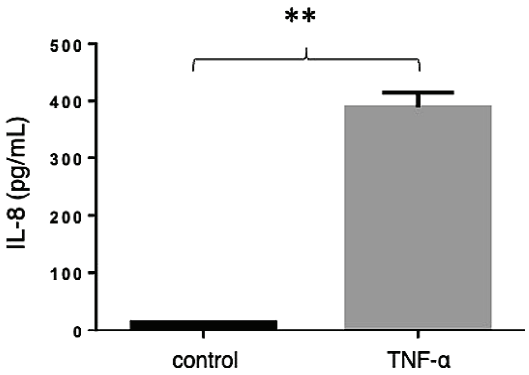

Figure 1 TNF-α increases IL-8 production in Ca9-22

Ca9-22 (1×10^4 cells/well) are seeded on a 96-well plate and incubated with or without 2 ng/mL TNF-α at 37 ℃ for 24 hours. The production of IL-8 is examined using ELISA MAX Deluxe Set Human IL-8. Each sample is tested in triplicate. All values are expressed as means ± standard deviations (**P < 0.01). Experiments are performed independently three times. IL-8, interleukin-8, ELISA, enzyme- linked immunosorbent assay

2. TNF-α stimulation enhanced the phosphorylation levels of ERK1/2 in Ca9-22 cells.

The role of ERK1/2 in enhancing the secretion of IL-8 by TNF-α stimulation was examined using Western blotting. Time-dependent changes in ERK1/2 phosphorylation up to 30 minutes after stimulation were then examined. ERK1/2 was constitutively phosphorylated even without stimulation; however, there was less ERK1/2 phosphorylation without stimulation. The phosphorylation of ERK1/2 was slightly enhanced after 1

minute, and markedly increased from 10 to 30 minutes (Fig. 2, upper panel). We observed a peak in ERK1/2 phosphorylation after 15 min. To ensure that equal amounts of ERK1/2 were obtained from the lysates, membranes were stripped and reprobed with anti-ERK1/2 antibody. We found that equal amounts of ERK1/2 were produced by lysates obtained from each sample (Fig. 2, lower panel).

3. U1206 inhibited TNF-α-induced phosphorylation of ERK1/2 but not p38 in Ca9-22 cells

To confirm whether TNF-α stimulation is involved in phosphorylation of ERK1/2, we treated Ca9-22 with U0126 and performed Western blotting. Ca9-22 receiving U0126 treatment was preincubated for 1 hour before TNF-α stimulation. TNF-α stimulation enhanced the phosphorylation of ERK1/2. TNF-α-stimulated enhancement of ERK1/2 phosphorylation was suppressed by treatment with U0126. TNF-α-stimulated ERK1/2 phosphorylation was completely inhibited even at a concentration of 1μM U0126. However, U0126 treatment had no effect on the enhancement of p38 phosphorylation induced by TNF-α stimulation. To ensure that equal amounts of ERK1/2 and p38 were obtained from the lysates, membranes were stripped and reprobed with anti-ERK1/2 or anti-p38 antibody. We found that equal amounts of ERK1/2 and p38 were produced by lysates obtained from each sample (Fig.3 middle upper panel, lower panel).

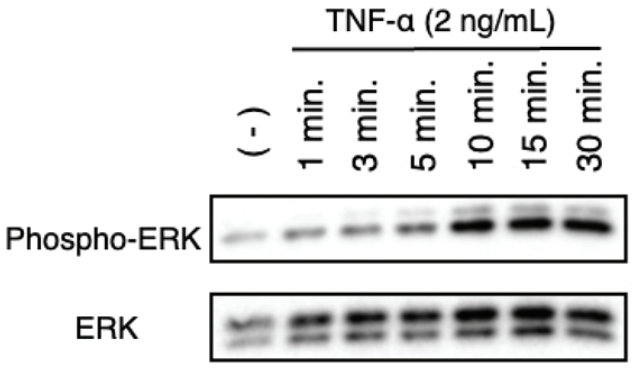

Figure 2 TNF-α stimulation enhances the phosphorylation levels of ERK1/2 in Ca9-22 cells.
Ca9-22 are stimulated by TNF-α (2 ng/mL) for 0 to 30 minutes. Cell lysate samples are prepared. The phosphorylation level of ERK1/2 is examined by Western blotting (upper). To ensure that equal amounts of ERK1/2 are obtained from lysates, the membranes are stripped and reprobed with anti-ERK1/2 antibodies (lower). Data presented are representative of four independent experiments. TNF-α, tumor necrosis factor-α

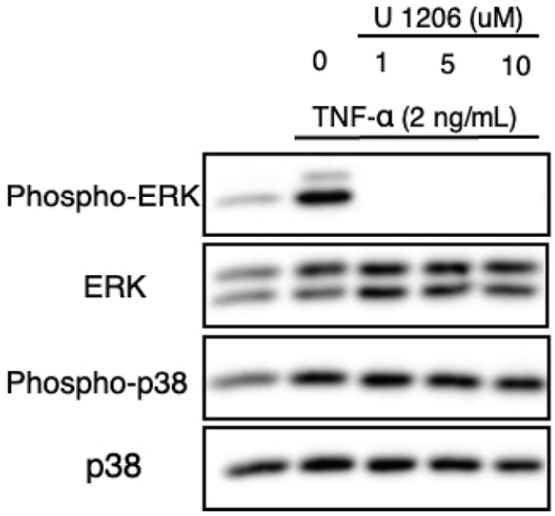

Figure 3 U1206 inhibits TNF-α-induced phosphorylation of ERK1/2 but not p38 in Ca9-22 cells.
Ca9-22 are pretreated with various concentrations of U0126 for 1 hour at 37℃ before incubation with 2 ng/mL of TNF-α for 10 min. Cell lysate samples are prepared. The phosphorylation level of ERK1/2 or p38 are examined by Western blotting. To ensure that equal amounts of ERK1/2 or p38 are obtained from lysates, membranes are stripped and reprobed with anti-ERK1/2 or p38 antibodies. Data presented are representative of four independent experiments. TNF-α, tumor necrosis factor-α

DISCUSSION

In the present study, we reported that the stimulation of TNF-α in the human gingival epithelial cell line Ca9-22 induced production of IL-8 via ERK1/2 phosphorylation of MAP kinase signaling. Human gingival epithelial fibroblasts release various inflammatory factors, such as IL-1β and IL-6, and chemokines, such as IL-8, in response to stimulation from periodontopathogenic bacteria[12]. The oral mucosa of the gingiva creates a protective barrier between the host and its environment. As the oral mucosa is constantly exposed to numerous microorganisms, and the thick, multi-layered, and keratinized gingiva epithelium is one of its most important defense mechanisms The host response and immune reaction to infectious organisms play an important role in the development of periodontitis[13]. Similar to other inflammatory diseases, proinflammatory cytokines, such as TNF-α, IL-6, and IL-8, are significant mediators in periodontitis[14,15].

TNF-α is an important inflammatory mediator and is released during the early stages of the inflammatory response[3]. TNF-α is an inflammatory cytokine produced by macrophages, epithelial cells, and fibroblasts and is involved in the progression of periodontal disease[3,4]. In this study, we investigated the response of Ca9-22 to inflammation by stimulating it with TNF-α. We found that Ca9-22 increased the production of IL-8 upon TNF-α stimulation. IL-8 has been detected in the periodontal tissue of patients with periodontal disease, and it has been reported that there is a correlation between disease severity and IL-8 levels in the gingival tissue[16,17]. IL-8 promotes migration, proliferation, and decreased gap junctional intercellular communication in human gingival epithelial cells[18]. These findings suggest that IL-8 is involved in the initiation and progression of periodontal disease in the gingival epithelium. Therefore, our finding that TNF-α-stimulated Ca9-22 enhanced IL-8 suggests that gingival epithelial cells attacked by periodontal pathogens and inflammatory cytokines may progress inflammation. Accordingly, we hypothesized that regulating TNF-α-stimulated IL-8 production has an anti-inflammatory effect on periodontal disease. To clarify the mechanism of IL-8 production induced by TNF-α stimulation, we investigated the intracellular signaling pathway of TNF-α-stimulated Ca9-22 using Western blotting. A previous study reported that TNF-α induces increased gingival epithelial permeability via p38 and ERK phosphorylation in human gingival epithelial cells[19].

MAPKs constitute a group of serine/threonine protein kinases that are subdivided into three subfamilies: p38, JNK, and ERK1/2. MAPKs are activated by various extracellular stimuli and induce the phosphorylation of important signaling molecules associated with cell proliferation, inflammation, and apoptosis[20]. In this study, we found that TNF-α stimulation increased ERK1/2 phosphorylation levels in Ca9-22. To confirm that ERK1/2 is phosphorylated by TNF-α stimulation, we inhibited the cascade using U0126. U0126 is a chemically synthesized organic compound that inhibits activation of ERK1/2 by inhibiting the kinase activity of MEK1/2. Crosstalk occurs between different MAPKs[21]; hence, we examined the possibility that crosstalk occurs between ERK1/2 and p38 and that the two protein kinases influence each other. We found that inhibition of ERK1/2 activation using the MEK inhibitor U0126 did not affect p38 phosphorylation; thus, this suggests that crosstalk between ERK1/2 and p38 is not involved in the enhancement of IL-8 production by TNF-α-stimulated Ca9-22. The intracel-

lular signaling mechanisms that cause crosstalk between the ERK1/2 and p38 pathways remain to be elucidated.

The basic mechanism of IL-8 production induced by TNF-α stimulation in Ca9-22 has not yet been characterized. The present results suggest that the MAPK pathway (particularly ER1/2) may play an important role in TNF-α-stimulated Ca9-22-induced IL-8 production. However, the mechanism of increased phosphorylation levels of MAP kinase remains to be elucidated. In addition, further studies are required to investigate the influence of MAPKs other than ERK1/2, such as p38 and JNK, and signaling pathways downstream of MAPKs such as c-Fos and c-Jun. Our findings suggest that TNF-α-stimulated IL-8 production is important in the regulation of the development of inflammation in the gingival epithelium.

CONCLUSION

We found that TNF-α induced IL-8 production in Ca9-22 via the ERK1/2-activated signaling pathway. Our findings suggest that the inflammatory response of gingival epithelial cells is activated by TNF-α stimulation to produce IL-8, which develops inflammation. Therefore, regulating IL-8 production may have anti-inflammatory effects on gingival epithelial cells. As intracellular signaling mechanisms form a complex network, further studies are required on the signal control mechanisms for TNF-α-stimulated Ca9-22 to produce IL-8.

ACKNOWLEDGMENTS

We are grateful to Dr. Mao from the Graduate School of Dentistry, Department of Physiology, Osaka Dental University.

This work was supported by the Japan Society for the Promotion of Science KAKENHI Grant Number JP21K10269 (Grant-in-Aid for Scientific Research (C)).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

1) Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. Lancet 2005; 366: 1809-1820.
2) Sapna G, Gokul S, Bagri-Manjrekar K. Matrix metalloproteinases and periodontal diseases. Oral Dis 2014; 20: 538-550.
3) Parikh AA, Moon MR, Kane CD, Salzman AL, Fischer JE, Hasselgren PO. Interleukin-6 production in human intestinal epithelial cells increases in association with the heat shock response. J Surg Res 1998; 77: 40-44.
4) Assuma R, Oates T, Cochran D, Amar S, Graves DT. IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. J Immunol 1998; 160: 403-409.
5) Belibasakis GN, Bostanci N. The RANKL-OPG system in clinical periodontology. J Clin Periodontol. 2012; 39: 239-248.
6) Zhu J, Guo B, Fu M, Guo W, Yuan Y, Yuan H, Zhang S, Yu, H. Interleukin-6-174G/C polymorphism contributes to periodontitis susceptibility: an updated meta-analysis of 21 case-control studies. Dis Markers 2016; 2016: 9612421. doi: 10.1155/2016/9612421.
7) Darveau RP. Periodontitis: A polymicrobial disruption of host homeostasis. Nat Rev Microbiol 2010; 8: 481-490.
8) Fujita T, Yoshimoto T, Kajiya M, Ouhara K, Matsuda S, Takemura T, Akutagawa K, Takeda K, Mizuno N, Kurihara H. Regulation of defensive function on gingival epithelial cells can prevent periodontal disease. Jpn Dent Sci Rev 2018; 54: 66-75.
9) Finoti LS, Nepomuceno R, Pigossi SC, Corbi SC, Secolin R, Scarel-Caminaga RM. Association between interleukin-8 levels and chronic periodontal disease. Medicine (Baltimore) 2017; 96: e6932. doi: 10.1097/MD.0000000000006932.
10) Imai H, Fujita T, Kajiya M, Ouhara K, Miyagawa T, Matsuda S, Shiba H, Kurihara H. Amphotericin B down-regulates Aggregatibacter actinomycetemcomitans- induced production of IL-8 and IL-6 in human gingival epithelial cells. Cell Immunol 2014; 290: 201-208.

11) Kurosawa Y, Yamaguchi H, Uemichi K, Shinozuka K, Kirihara Y, Tsuda H. Butyrate-treatment induces gingival epithelial cell death in a three-dimensional gingival-connective tissue hybrid co-culture system. J Dent Sci. 2023; 18: 893–897.
12) Liu J, Wang Y, Ouyang X. Beyond toll-like receptors: Porphyromonas gingivalis induces IL-6, IL-8, and VCAM-1 expression through NOD-mediated NF-κB and ERK signaling pathways in periodontal fibroblasts. Inflammation 2014; 37: 522-533.
13) Van Dyke TE, Serhan CN. Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. J Dent Res 2003; 82: 82-90.
14) Noh MK, Jung M, Kim SH, Lee SR, Park KH, Kim DH, Kim HH, Park YG. Assessment of IL-6, IL-8 and TNF-α levels in the gingival tissue of patients with periodontitis. Exp Ther Med 2013; 6: 847-851.
15) Bastos MF, Lima JA, Vieira PM, Mestnik MJ, Faveri M, Duarte PM. TNF-alpha and IL-4 levels in generalized aggressive periodontitis subjects. Oral Dis 2009; 15: 82-87.
16) Garlet GP, Martins W Jr, Ferreira BR, Milanezi CM, Silva JS. Patterns of chemokines and chemokine receptors expression in different forms of human periodontal disease. J Periodontal Res 2003; 38: 210-217.
17) Tsai CC, Ho YP, Chen CC. Levels of interleukin-1 beta and interleukin-8 in gingival crevicular fluids in adult periodontitis. J Periodontol 1995; 66 :852-859.
18) Fujita T, Ashikaga A, Shiba H, Uchida Y, Hirono C, Iwata T, Takeda K, Kishimoto A, Hirata R, Kawaguchi H, Shiba Y, Kurihara H. Regulation of IL-8 by Irsogladine maleate is involved in abolishment of Actinobacillus actinomycetemcomitans-induced reduction of gap-junctional intercellular communication. Cytokine. 2006; 34: 271-277.
19) Miyagawa T, Fujita T, Yumoto H, Yoshimoto T, Kajiya M, Ouhara K, Matsuda S, Shiba H, Matsuo T, Kurihara H. Azithromycin recovers reductions in barrier function in human gingival epithelial cells stimulated with tumor necrosis factor-α. Arch Oral Biol 2016; 62: 64-69.
20) Kim EK, Choi EJ: Pathological roles of MAPK signaling pathways in human diseases. Biochem Biophys Acta 2010; 1802: 396-405.
21) Digicaylioglu M, Lipton SA. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature 2001; 412: 641-647.

(Received, September 22, 2023/
Accepted, November 24, 2023)

Corresponding author:
Hiroshi INOUE, D.D.S., Ph.D.
Department of Physiology,
School of Dentistry, Osaka Dental University
Osaka Japan
8-1, Kuzuhahanazono-cho, Hirakata,
Osaka 573-1121, Japan
Tel: +81-72-864-3054
Fax: +81-72-864-3154
E-mail : inoue-hi@cc.osaka-dent.ac.jp