

Tumor necrosis factor- α converting enzyme (TACE) increases RANKL expression in osteoblasts and serves as a potential biomarker of periodontitis

Ji-Hyun Lee¹, Young-Jin Choi¹, Sun-Hee Heo¹, Jae-Mok Lee² & Je-Yoel Cho^{1,*}

Departments of ¹Biochemistry, ²Periodontology, School of Dentistry, Kyungpook National University, Daegu 700-422, Korea

Periodontitis is a very prevalent disease. Therefore, biomarkers for the early and standard diagnoses of periodontitis are urgently needed. TACE is a membrane-bound metalloprotease. Although a recent study suggested that TACE levels in GCF are elevated during periodontal disease, the levels of TACE in GCF at different stages of chronic periodontitis have not been determined. Here, we analyzed the protein levels of TACE in GCF from periodontal disease subjects and confirmed that the protein levels of TACE were higher in the moderate periodontitis groups. TACE is known to be a NF- κ B ligand that stimulates RANKL secretion in osteoblasts. To understand the effects of TACE on RANKL and OPG in osteoblasts, we treated MG63 cells with TACE. We observed an increase in RANKL protein expression but a decrease in OPG protein expression. Our data suggest that TACE can induce RANKL expression and promote osteoclastogenesis, thus worsening the outcome of periodontitis. [BMB reports 2011; 44(7): 473-477]

INTRODUCTION

Periodontitis is one of the most common inflammatory diseases and is characterized by the presence of (mainly gram-negative) anaerobes, accumulation of immune cells, formation of periodontal pockets, and loss of tooth attachment (1, 2). Recent studies have investigated the roles of cytokines and secreted proteinases in periodontitis (3).

Tumor necrosis factor alpha (TNF- α), a cytokine involved in the inflammatory response, is synthesized as a 26 kDa membrane-anchored protein, from which an active soluble 17 kDa extracellular domain is proteolytically released. This proteolytic release event is catalyzed by TNF- α converting enzyme (TACE), a membrane-bound metalloproteinase (4). TACE has

been implicated in the pathogenesis of periodontal destruction and is also known to be a stimulator of bone resorption. A recent study also suggested that TACE may be of relevance to periodontitis. TACE can promote the expression and release of ligands and receptors that are strongly associated with inflammation, including TNF- α and interleukin-1 receptor II (5).

Receptor activator of NF- κ B ligand (RANKL) is a 317 amino acid peptide produced by osteoblastic lineage cells and activated T-cells. RANKL exists in two forms: one presents as a cell surface protein with a molecular weight of 35.5 kDa while the other presents as a soluble form with a molecular weight of 27.2 kDa. The osteoclastogenic action of RANKL can be blocked by the soluble decoy receptor osteoprotegerin (OPG) (6). Together, these two molecules play key roles in bone resorption (7). The role of RANKL and OPG expression in the regulation of bone destruction has also been demonstrated in periodontitis (8-10). RANKL can be also cleaved and released from the cell surface by TACE (7, 11, 12). However, the relationship between TACE and periodontal disease has not been studied enough to make definitive conclusions.

In the present study, we investigated whether or not TACE can serve as a biomarker in different stages of periodontitis and determined the effect of TACE on RANKL and OPG expression in osteoblastic cells.

RESULTS

TACE protein levels in gingival tissues and GCF from periodontitis patients

TACE protein levels were first assessed in the gingival tissues of periodontitis patients by Western blot analysis. Elevated levels of TACE were detected in the gingival tissues of patients with gingivitis, moderate periodontitis, and severe periodontitis compared to that of the healthy group (Fig. 1A). Relative protein levels were measured by densitometry analysis of the TACE bands using the Scion image analysis program. The levels of TACE in the gingival tissues of the healthy control, gingivitis, moderate periodontitis, and severe periodontitis groups were 0.27 ± 0.18 , 0.47 ± 0.35 , 0.71 ± 0.42 , and 0.61 ± 0.41 , respectively (Fig. 1B). ANOVA analysis revealed that TACE expression was significantly higher in the moderate

*Corresponding author. Tel: 82-53-420-4997; Fax: 82-53-421-1417; E-mail: jeycho@knu.ac.kr
DOI 10.5483/BMBRep.2011.44.7.473

Received 25 April 2011, Accepted 24 May 2011

Keywords: GCF, OPG, Periodontal disease, RANKL, TACE

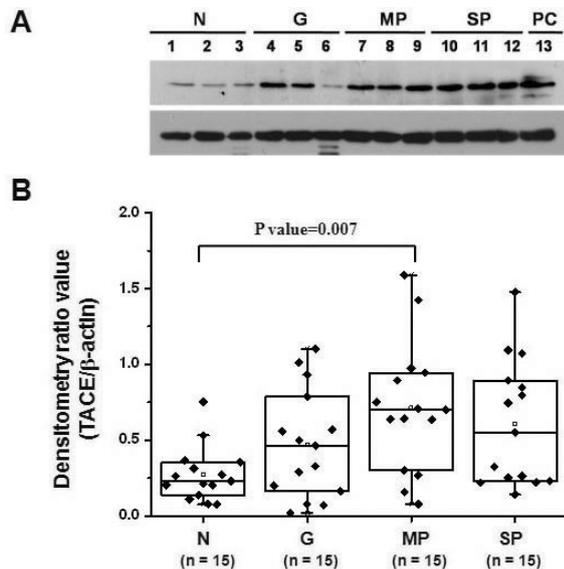


Fig. 1. Validation of tumor necrosis factor- α converting enzyme (TACE) in gingival tissues. (A) TACE protein expression in gingival tissues from periodontitis patients as assessed by Western blotting with anti-TACE antibody. Equivalent amounts of protein (20 μ g) were loaded into each lane. N, Normal control; G, Gingivitis; MP, Moderate periodontitis; SP, Severe periodontitis; PC, positive control. (B) Densitometry analysis of corresponding bands was carried out using the Scion image program. The boxes represent the upper 75% and lower 25% quartiles of the measurements with respect to the median value (middle line). Small open rectangles indicate the mean values for each of the groups. TACE expression was significantly different between the moderate periodontitis group and the healthy group.

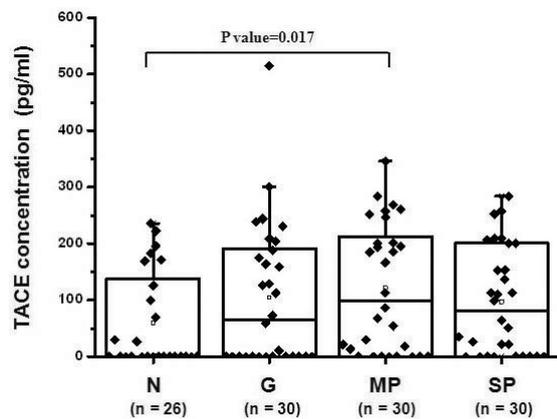


Fig. 2. TACE protein levels in gingival crevicular fluid (GCF). The concentrations of TACE in the GCF from 26 healthy controls, 30 patients with gingivitis, 30 patients with moderate periodontitis, and 30 patients with severe periodontitis were measured by ELISA. The individual values represent the total TACE concentration (pg/ml) in the GCF from each subject. The TACE protein concentration was higher in patients with moderate periodontitis compared to the healthy controls.

periodontitis group compared to the healthy group (P value = 0.07).

To measure TACE protein expression in GCF, a sensitive, quantitative ELISA was used. TACE expression was detected in 11 out of 26 healthy samples, and the average concentration was 58.8 ± 84.2 pg/ μ l. However, TACE were detected in 17 out of 30 gingivitis patients, 22 out of 30 moderate periodontitis patients, and 21 out of 30 in severe periodontitis patients. The average concentrations of TACE in GCF were 100.3 ± 130.2 pg/ μ l in the gingivitis group, 109.3 ± 116.0 pg/ μ l in the moderate periodontitis group, and 88.4 ± 96.0 pg/ μ l in the severe periodontitis group. ANOVA analysis showed that the levels of TACE in GCF slightly increased in the moderate periodontitis group compared to the healthy group (P value = 0.017). There was no significant difference between the gingivitis or severe periodontitis group and the healthy group (Fig. 2).

Expression of RANKL and OPG by TACE in MG63 cells

Since TACE levels were elevated in the GCF from periodontitis patients, we investigated whether or not TACE has any effect on regulation of the osteoclastic differentiation factors RANKL and OPG in osteoblasts. Human osteoblastic MG63 cells were treated with recombinant TACE, and the expression levels of

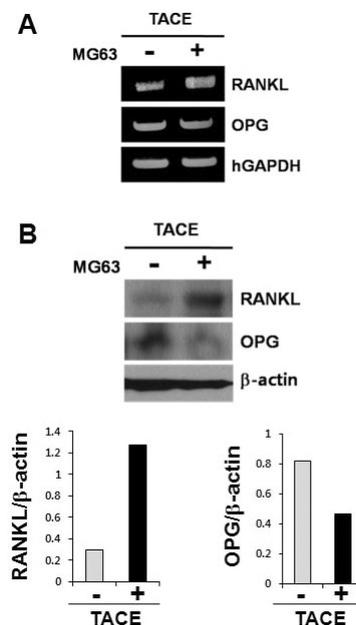


Fig. 3. Effect of TACE on RANKL and OPG expression in MG63 cells. MG63 cells were treated with 100 ng/ml of TACE for 36 h, and total lysates were prepared. (A) RANKL and OPG mRNA levels were assessed by RT-PCR. (B) RANKL and OPG protein levels were determined by Western blotting (top). RANKL and OPG protein expression levels were quantified by densitometry and normalized to β -actin expression by Scion image analysis (bottom).

RANKL and OPG were examined. The mRNA expression of RANKL slightly increased while the level of OPG slightly decreased in response to TACE treatment for 36 h (Fig. 3A). However, the protein level of RANKL was significantly elevated while the OPG protein level was significantly decreased in MG63 cells treated with TACE for 36 h (Fig. 3B).

DISCUSSION

In this study, we observed an increase in TACE protein levels in moderate periodontitis groups compared with healthy groups. Recent studies have shown that the total amount of TACE in GCF is elevated in patients with chronic and aggressive periodontitis compared with healthy controls (5). However, no report has yet examined the levels of TACE in both the gingival tissues and GCF at different stages of periodontitis. This is also the first study that has investigated the levels of TACE protein in gingival tissues from patients with gingivitis and different stages of periodontitis. TACE levels were elevated in the periodontitis groups compared with the healthy groups (Fig. 1). Densitometry analysis of TACE Western blots showed a significant increase in TACE expression in the moderate periodontitis groups (Fig. 1B).

TACE expression was also increased in GCF from patients with moderate periodontitis compared with the healthy groups (Fig. 2). Although the gingivitis and severe periodontitis groups showed slightly increased levels of TACE, only the moderate periodontitis group showed a statistically significant increase. We hypothesize that the level of TACE during moderate periodontitis becomes statistically high enough to be detected due to increased inflammation. However, later on during severe periodontitis, when bleeding is apparent in the GCF, anti-inflammatory signals appear and reduce the presence of inflammatory molecules such as TACE. This could explain why the level of TACE was slightly lower in the severe periodontitis group. Besides, we also observed that other biomarkers such as azurocidin were also lower in the severe periodontitis group compared to the moderate periodontitis group (manuscript in revision).

TACE is a membrane-bound metalloproteinase that can cleave RANKL into its product, soluble RANKL, which is a key factor for osteoclastogenic activity and bone resorption (11). Recent studies have shown that *P. gingivalis* stimulates the mRNA expression and production of TACE by host cells (10). Previously, stimulation of PDL cells with a high concentration of purified *P. gingivalis* LPS led to an increase in RANKL expression, though lower concentrations had no effect (7). Here, we demonstrated that exposure of MG63 cells to TACE resulted in increased RANKL protein expression, although mRNA expression only slightly changed (Fig. 3). The fact that TACE had a more dramatic effect on the protein expression of RANKL and OPG than on mRNA expression indicates that TACE might participate in posttranscriptional regulation of RANKL and OPG (e.g., down-regulation of RANKL-targeted

micro-RNA and/or RANKL protein stabilization by down-regulation of RANKL-targeted E3 ligases). Further investigation is needed to evaluate these possibilities. It is possible that recombinant TACE could stimulate protein production of RANKL but not that of OPG by increasing cytokine activation. However, the cytokines involved in this process are yet to be identified, and elucidation of the mechanism requires further study. IL-1 β and TNF- α are known to activate NF- κ B in MG63 cells, resulting in induction of target genes (13). A report that exposure of osteoblast-like cells to IL-1 β and TNF- α results in increased IL-6R production mediated by TACE activation (14) also provides evidence that TACE might reversibly activate these cytokines to increase RANKL protein expression.

In conclusion, our studies demonstrated that TACE treatment leads to increased RANKL expression in osteoblast cells. It was also found that the use of drugs that suppress TACE could be a new therapeutic strategy for inhibiting bone destruction in patients with periodontitis.

MATERIALS AND METHODS

Materials

Recombinant human TACE and human Adam17/TACE ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, USA). TRIZOL Reagent and DMEM medium were purchased from Invitrogen (Grand Island, NY). Sequencing grade modified trypsin and complete protease inhibitor cocktail tablets were purchased from Roche (Mannheim, Germany). TACE, RANKL, anti-goat IgG, and OPG antibodies were obtained from Santa Cruz Biotechnology (California, USA). β -actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The 5% premixed stacking gel solutions, 12 and 15% premixed running gel solutions, and RIPA lysis buffer were purchased from Elpis-Biotech (Daejeon, Korea).

Cell culture

MG63 human osteosarcoma cells were grown in DMEM supplemented with 10% dialyzed fetal calf serum and 100 units/ml of penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. For treatment with TACE recombinant protein, confluent MG63 cells were washed twice with serum-free medium and treated with TACE at a concentration of 100 ng/ml.

Gingival tissues biopsies

Gingival tissues were obtained from periodontal biopsies taken from 45 periodontitis patients and 15 healthy control subjects (Table 1). Samples of diseased gingival tissue were obtained from periodontitis tissues resected during periodontal surgery. Healthy gingival tissues were obtained during the third molar extraction. All of the procedures were approved by the IRB (#

Table 1. Clinicopathological characteristics of periodontitis patients included in the study

Gingival tissue	N	G	MP	SP
Gender F : M	9 : 6	9 : 6	11 : 4	7 : 8
Age	41.6 ± 14.8	49.2 ± 13.4	48.3 ± 7.7	47.4 ± 8.2

GCF	N	G	MP	SP
Gender F : M	15 : 11	14 : 16	15 : 15	11 : 19
Age	31.2 ± 11.1	44.0 ± 11.4	50.9 ± 8.6	52.9 ± 9.0

KNU 74005-830) of Kyungpook National University, and consent forms were obtained from each donor. Base incisions were made 1-2 mm subgingivally; therefore, the specimens consisted of the gingival margin, sulcular epithelium, and gingival connective tissue. Soft tissue biopsy specimens were washed extensively with PBS. The tissues were stored at -70°C prior to analysis.

GCF collection

GCF samples were obtained from 116 adult periodontitis patients (Table 1). For the healthy group, GCF samples were collected from the sulcus of multi-rooted teeth from four posterior areas with a pocket depth (PD) of up to 3 mm without bleeding upon probing. For the gingivitis group, GCF samples were collected from teeth with a 3 mm PD. For the moderate periodontitis group, GCF samples were collected from teeth with a 4-6 mm PD with bleeding upon probing. For the severe periodontitis group, GCF samples were collected from teeth with a $6 \geq$ mm PD with bleeding on probing. The selected sites were cleared of supragingival plaques, isolated with cotton rolls, and dried with a gentle stream of air to prevent saliva contamination. Samples were lyophilized and re-eluted in 20 μl of saline, followed by storage at -70°C prior to analysis.

Western blot analysis

Western blot analysis was performed as previously reported (15). Briefly, the blots were incubated overnight at 4°C with goat polyclonal anti-TACE (1 : 750), mouse monoclonal anti-RANKL (1 : 500), rabbit polyclonal anti-OPG (1 : 500), and mouse monoclonal anti- β -actin (1 : 2,000) antibodies. After washing in TBS containing 0.05% Tween 20, blots were incubated for 1 h at room temperature with anti-rabbit, anti-goat, or anti-mouse IgG horseradish peroxidase-linked secondary antibody (1 : 1,000). Protein bands were visualized by ECL detection.

Enzyme-linked immunosorbant assay (ELISA)

GCF samples were analyzed for TACE using a commercially available ELISA kit (R&D systems, Minneapolis, USA). This assay comprised a sandwich ELISA and was performed according to the manufacturer's instructions using human recombi-

nant standards. Results are reported as the total amount of TACE per GCF sample and are expressed as pg/ml. Whenever TACE levels are compared among patient groups, mean whole mouth TACE values are used.

Determination of mRNA levels

RT-PCR analysis was performed as previously reported (15). Total cellular RNA was extracted from cells using TRIZOL Reagent, and RNA concentrations were measured by spectrophotometry. To determine the mRNA expression of RANKL and OPG, RT-PCR was performed using the primers as previously reported (16).

Statistical analysis

All data are presented as means \pm standard deviations (SD). Densitometry and ELISA results of the groups were analyzed for statistical significance by one-way ANOVAs of variance with Tukey's test. The analyses were performed using Origin-Pro 8. Probability (P value) of less than 0.05 was considered to be significant.

Acknowledgements

This work was supported by the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (Grant No. A090610), and by the National Research Foundation of Korea (NRF) grant funded by the Korean Government (MEST) (Grants No. 2010-0029494).

REFERENCES

1. Listgarten, M. A. (1986) Pathogenesis of periodontitis. *J. Clin. Periodontol.* **13**, 418-430.
2. Mezyk-Kopec, R., Bzowska, M., Potempa, J., Bzowska, M., Jura, N., Sroka, A., Black, R. A. and Bereta, J. (2005) Inactivation of membrane tumor necrosis factor alpha by gingipains from *Porphyromonas gingivalis*. *Infect. Immun.* **73**, 1506-1514.
3. Sorsa, T., Suomalainen, K. and Uitto, V. J. (1990) The role of gingival crevicular fluid and salivary interstitial collagenases in human periodontal diseases. *Arch. Oral Biol.* **35** (Suppl), S193-196.
4. Primakoff, P. and Myles, D. G. (2000) The ADAM gene family: surface proteins with adhesion and protease activity. *Trends Genet.* **16**, 83-87.
5. Bostanci, N., Emingil, G., Afacan, B., Han, B., Ilgenli, T., Atilla, G., Hughes, F. J. and Belibasakis, G. N. (2008) Tumor necrosis factor-alpha-converting enzyme (TACE) levels in periodontal diseases. *J. Dent. Res.* **87**, 273-277.
6. Schoppet, M., Preissner, K. T. and Hofbauer, L. C. (2002) RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. *Arterioscler. Thromb. Vasc. Biol.* **22**, 549-553.
7. Krajewski, A. C., Biessei, J., Kunze, M., Maersch, S., Perabo, L. and Noack, M. J. (2009) Influence of lipopolysaccharide and interleukin-6 on RANKL and OPG expression and release in human periodontal ligament

- cells. *Apmis*. **117**, 746-754.
8. Bostanci, N., Ilgenli, T., Emingil, G., Afacan, B., Han, B., Toz, H., Atilla, G., Hughes, F. J. and Belibasakis, G. N. (2007) Gingival crevicular fluid levels of RANKL and OPG in periodontal diseases: implications of their relative ratio. *J. Clin. Periodontol.* **34**, 370-376.
 9. Belibasakis, G. N., Meier, A., Guggenheim, B. and Bostanci, N. (2011) Oral biofilm challenge regulates the RANKL-OPG system in periodontal ligament and dental pulp cells. *Microb. Pathog.* **50**, 6-11.
 10. Bostanci, N., Reddi, D., Rangarajan, M., Curtis, M. A. and Belibasakis, G. N. (2009) Porphyromonas gingivalis stimulates TACE production by T cells. *J. Oral Microbiol.* **24**, 146-151.
 11. Lum, L., Wong, B. R., Josien, R., Becherer, J. D., Erdjument-Bromage, H., Schlondorff, J., Tempst, P., Choi, Y. and Blobel, C. P. (1999) Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. *J. Biol. Chem.* **274**, 13613-13618.
 12. Pan, B., Farrugia, A. N., To, L. B., Findlay, D. M., Green, J., Lynch, K. and Zannettino, A. C. (2004) The nitrogen-containing bisphosphonate, zoledronic acid, influences RANKL expression in human osteoblast-like cells by activating TNF-alpha converting enzyme (TACE). *J. Bone Miner. Res.* **19**, 147-154.
 13. Brechter, A. B., Persson, E., Lundgren, I. and Lerner, U. H. (2008) Kinin B1 and B2 receptor expression in osteoblasts and fibroblasts is enhanced by interleukin-1 and tumor necrosis factor-alpha. Effects dependent on activation of NF-kappaB and MAP kinases. *Bone* **43**, 72-83.
 14. Franchimont, N., Lambert, C., Huynen, P., Ribbens, C., Relic, B., Chariot, A., Bours, V., Piette, J., Merville, M. P. and Malaise, M. (2005) Interleukin-6 receptor shedding is enhanced by interleukin-1beta and tumor necrosis factor alpha and is partially mediated by tumor necrosis factor alpha-converting enzyme in osteoblast-like cells. *Arthritis Rheum.* **52**, 84-93.
 15. Lee, J. H., Kim, B. G., Ahn, J. M., Park, H. J., Park, S. K., Yoo, J. S., Yates, J. R., 3rd and Cho, J. Y. (2010) Role of PI3K on the regulation of BMP2-induced beta-Catenin activation in human bone marrow stem cells. *Bone* **46**, 1522-1532.
 16. Lau, Y. S., Adamopoulos, I. E., Sabokbar, A., Giele, H., Gibbons, C. L. and Athanasou, N. A. (2007) Cellular and humoral mechanisms of osteoclast formation in Ewing's sarcoma. *Br. J. Cancer* **96**, 1716-1722.