J Periodont Res 2008; 43: 379–385

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.01033.x

Significance of thrombomodulin release from gingival epithelial cells in periodontitis patients

T. Matsuyama[1], M. Tokuda[2], Y. Izumi[1]

Departments of [1]Periodontology and [2]Restorative dentistry and Endodontology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

Matsuyama T, Tokuda M, Izumi Y. Significance of thrombomodulin release from gingival epithelial cells in periodontitis patients. J Periodont Res 2008; 43: 379–385.

Background and Objective: Thrombomodulin, a cell transmembrane glycoprotein, binds to thrombin and converts it from a procoagulant protease to an anticoagulant enzyme that activates protein C. Thrombomodulin is very important in regulating the function of thrombin. Elevated soluble thrombomodulin is present in the gingival crevicular fluid of subjects with periodontitis. The objective of the present study was to investigate the mechanisms about the elevated soluble thrombomodulin in gingival crevicular fluid.

Material and methods: Gingival sections from six patients with chronic periodontitis and from three periodontally healthy subjects were immunostained for thrombomodulin detection. Thrombomodulin levels were investigated in the gingival crevicular fluid of 11 subjects with chronic periodontitis. The effects of neutrophil enzymes on thrombomodulin release and on thrombomodulin in the gingival crevicular fluid were examined by an enzyme-linked immunosorbent assay or by Western blotting.

Results: The expression of gingival epithelial thrombomodulin was lost or decrease near infiltrating neutrophils. Thrombomodulin was rapidly released from gingival epithelial cells by neutrophil enzymes, and gingival crevicular fluid with periodontitis included the proteolytic cleavage thrombomodulin using immunoblotting analysis. The thrombomodulin release was not caused by rapid cell damage, on lactate dehydrogenase assay. There were significant differences in thrombomodulin content between gingival crevicular fluid samples from healthy and diseased sites, regardless of the degree of probing depth.

Conclusion: Neutrophil enzymes induced rapid thrombomodulin release from the membrane surface of gingival epithelial cells. This might explain the thrombomodulin increase in gingival crevicular fluid with local diseased gingiva. Elevation of thrombomodulin in gingival crevicular fluid may be a potential marker of epithelial cell membrane injury.

Takashi Matsuyama, Department of Periodontology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima, 890-8544, Japan
Tel: +81 99 2756202
Fax: +81 99 2756208
e-mail: takashi@dentb.hal.kagoshima-u.ac.jp

Key words: gingival epithelial cells; neutrophil enzyme; periodontitis; thrombomodulin

Accepted for publication July 6, 2007

The gingival and periodontal bleeding and coagulation characteristics of periodontal disease indicate that thrombin is likely to be generated via the coagulation cascade in periodontal tissues (1). Besides its central role in homeostasis, thrombin has been shown to have important pro-inflammatory cellular effects relevant to the pathogenesis of periodontal disease (2,3).

Thrombomodulin was first identified as an endothelial cell surface transmembrane glycoprotein. It forms a high-affinity complex with thrombin and converts it from a procoagulant

protease to an anticoagulant enzyme that activates protein C (4). Thrombomodulin is also known to be expressed by suprabasal keratinocytes within the human epidermis (5,6). In addition to its anticoagulant role, thrombomodulin on keratinocytes has been shown to regulate thrombin at epidermal sites of cutaneous injury and is believed to play a role in epidermal differentiation (7–9). Furthermore, the N-terminal lectin-like domain of thrombomodulin has recently been demonstrated to have anti-inflammatory properties (10,11). Thrombomodulin is also a cofactor for thrombin-mediated activation of the thrombin-activation fibrinolysis inhibitor (12).

The soluble form of thrombomodulin is found in serum, plasma and urine, and it is the most released following endothelial cell damage. Therefore, soluble serum thrombomodulin is an accepted established marker of disease-affected patients with multisystemic autoimmune vasculitis including those with systemic lupus erythematosus, Wegner's granulomatosis and other systemic vasculitides (13–15). Recently, plasma-soluble thrombomodulin is potential marker for predicting pancreatic necrosis within the first 48 h (16). Factors that may contribute to endothelial damage are hypoxia or microcirculatory abnormalities, release of proteinase from activated granulocytes, activation of mediator systems and activation of platelets (17).

We previously showed that gingival epithelial cells expressed thrombomodulin, and gingival crevicular fluid at bleeding sites in periodontitis patients without systemic disease contained high levels of soluble thrombomodulin (18), suggesting that the thrombomodulin content in gingival crevicular fluid was associated with local inflammation. However, the source of the soluble form of thrombomodulin in gingival crevicular fluid remains unknown in periodontitis. Therefore, the present study aimed to investigate the possible mechanisms about the elevated soluble thrombomodulin in gingival crevicular fluid.

Material and methods

Tissue samples

Healthy ($n = 3$, probing depth < 3 mm, no bleeding on probing and no bone loss) or inflamed ($n = 6$, probing depth 3–6 mm, bleeding on probing and bone loss) gingival tissues were obtained from nine patients who had no history or current signs of systemic disease and had received no medication within the past 6 mo (six women and three men; mean age, 46.8 ± 7 years) when their teeth were extracted for therapeutic reasons. They were taken from the marginal gingiva near the extracted socket after obtaining the patient's informed consent, according to guidelines approved by the Ethical Committee at Kagoshima University Graduate School of Medical and Dental Sciences.

Gingival crevicular fluid sampling

In 11 chronic periodontitis subjects (six women and five men; mean age, 47.3 ± 7 years), gingival crevicular fluid samples were collected from periodontal pockets with 1–10 mm probing depth after obtaining the patient's informed consent, according to guidelines approved by the Ethical Committee at Kagoshima University Graduate School of Medical and Dental Sciences. In healthy sites ($n = 10$), gingival crevicular fluid sampling was collected from periodontal pockets with < 3 mm probing depth, no bleeding on probing and no bone loss. In diseased sites ($n = 19$), gingival crevicular fluid samples were collected from periodontal pockets with 3–5 mm probing depth ($n = 10$) or ≥ 6 mm probing depth ($n = 9$) with bleeding on probing and bone loss. All study subjects were in good general health and had not received periodontal treatment or medication during the previous 6 mo (Table 1). Gingival crevicular fluid was sampled using a filter paper after removing all supragingival plaque and keeping the area dry with cotton wool. Paper strips were carefully inserted into the crevice up to 1 mm depth and left there for 30 s to avoid mechanical injury, and strips contaminated with blood were discarded. The gingival crevicular fluid volume from each strip was determined by electronic impedance (Periotoron 8000; IDE Interstate, Amityville, NY, USA), placed into a sterile polypropylene tube and kept for 1 mo at −80°C until further analysis.

Measurement of thrombomodulin in gingival crevicular fluid

Thrombomodulin in gingival crevicular fluid was measured using a two-site enzyme-linked immunosorbent assay (ELISA) (Prototype thrombomodulin ELISA kit; Diagnostica Stago, Asnieres, France) according to the manufacturer's protocol. Gingival

Table 1. Total amounts of thrombomodulin in gingival crevicular fluid samples and measures of periodontal status at the healthy sites (PD < 3 mm) and diseased sites (PD ≥ 3 mm) in periodontitis subjects

	Healthy sites ($n = 10$)	Diseased sites ($n = 19$)	
	< 3 mm PD	3–5 mm PD	> 6 mm PD
Probing depth (PD) (mm)	1.80 ± 0.42	4.86 ± 0.38	7.17 ± 1.19
Gingival index	0	1.00 ± 0.58	1.42 ± 0.67
Plaque index	0.4 ± 0.52	0.33 ± 0.50	1.42 ± 1.00
Gingival crevicular fluid volume (μL)	0.07 ± 0.01	0.65 ± 0.18	0.86 ± 0.28
Thrombomodulin (ng)	3.91 ± 2.21	156.75 ± 24.01	201.24 ± 69.83
Concentration of thrombomodulin in gingival crevicular fluid (ng/μL)	59.36 ± 32.02	255.95 ± 81.38[a]	248.39 ± 103.48[a]

Data are expressed as mean ± standard deviation.
[a]Statistically significantly higher than healthy sites ($p < 0.01$).

crevicular fluid samples were eluted in 100 μL of phosphate-buffered saline including 2% bovine serum albumin and protease inhibitors (×100; Sigma, St Louis, MO, USA) and then incubated for 60 min at 4°C. The ELISA plate was precoated with the $F(ab')_2$ fragment of human thrombomodulin. Serial dilutions of human thrombomodulin were used as reference standards, and 200-μL aliquots of the standard or the test sample (1 : 3) were tested in each well. The concentration of thrombomodulin in gingival crevicular fluid was calculated according to a standard curve constructed with data for the reference standards. The results of thrombomodulin were expressed as nanograms for total amounts and as ng/mL for concentrations when adjusted for gingival crevicular fluid volume (Table 1). Experiments were performed in duplicate, and mean values were obtained.

Immunohistochemistry

Cryostat sections (4 μm) of gingival tissues were fixed in 10% formaldehyde at 4°C for 10 min. They were washed in phosphate-buffered saline and incubated with mouse anti-human thrombomodulin IgG (5 μg/mL; Fuji Chemical Corp., Toyama, Japan). After washing in phosphate-buffered saline, sections were further stained immunohistochemically using the LSAB kit (Dako, Kyoto, Japan) according to the manufacturer's protocols. Color development was performed with the diaminobenzidine substrate kit (Dako). Counterstaining with 10% Harris hematoxylin was performed before coverslipping. Negative control slides were prepared by substitution with the isotype-matched control as primary antibody.

Proteinases of neutrophils

Commercially available human neutrophil elastase (Elastin Products Company, Inc., Owensville, MO, USA) and cathepsin G (Athens Research & Technology, Athens, GA, USA) were used. The following final concentrations were used according to previous data (data not shown): 10 μg/mL elastase; 10 μg/mL cathepsin G. As a protease inhibitor, α1-anti-trypsin (Calbiochem, Cambridge, MA, USA; 10 μg/mL) was used.

Isolation and culture of human gingival epithelial cells, and detection of thrombomodulin in culture supernatants

Gingival epithelial cells were obtained from healthy gingival tissues using an explant culture method (18). Cells were maintained in a serum-free keratinocyte medium (Gibco, Grand Island, NY, USA) supplemented with epidermal growth factor (5 ng/mL) and bovine pituitary extracts (35–50 μg/mL) containing 0.09 mM Ca^{2+}. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. The third or fourth passage of gingival epithelial cells was used for the respective experiments. Cells were subcultured on 96-well plates under optimal culture conditions, up to near-confluent cell density. After treatment with neutrophil elastase or cathepsin G for 0, 0.5, 1, 2, 4, 8 or 24 h, culture supernatants were collected and stored at −20°C. In addition, supernatants for treatment with elastase or cathepsin G alone, and those for the use of each neutrophil enzyme and α1-anti-trypsin for 6 h were also collected and stored. Thrombomodulin in supernatant was measured using the prototype thrombomodulin ELISA kit.

Western blotting

Cells (10^6 cells/mL) were treated with cell lysate buffer (1% Triton X-100, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 40 mM HEPES pH 7.4) at 4°C for 30 min or with elastase (10 μg/mL) for 6 h. After centrifugation, each sample was diluted 1 : 3 or 1 : 1 in sample buffer (10% sodium dodecyl sulfate, 100 mmol β-mercapthoethanol, 80 mmol 10% glycerol, Tris/HCl pH 6.8). Furthermore, a gingival crevicular fluid sample with 6 mm probing depth from periodontitis patient was also prepared for western blot analysis, and recombinant human thrombomodulin (5 μg/mL; American Diagnostica Inc., Greenwich, CT, USA) was used as a positive control. Samples were prepared by boiling in sample buffer for 5 min. The enhanced chemiluminescence protein molecular weight marker (Amersham Pharmacia Biotech, Bucks., UK), or each sample, was loaded onto a sodium dodecyl sulfate polyacrylamide gel (4–20%) and transferred to a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech). Following membrane transfer, the membrane was blocked and incubated with anti-thrombomodulin (Fuji Chemical Corp.). Blots were visualized using the ECL-plus western blotting detection reagents (Amersham Biosciences).

Lactate dehydrogenase assay

Lactate dehydrogenase activity is used as a marker of cellular injury and was determined in the collected supernatants and cellular extracts. Cells were subcultured on 96-well plates under optimal culture conditions, up to near-confluent cell density. They were treated with elastase or cathepsin G from human neutrophils for 0, 0.5, 1, 2, 4, 6, or 24 h. At the end of these incubation times, the supernatants were collected and stored at −20°C. Cells in wells were simultaneously lysed by adding Triton X-100 (Sigma) at a final concentration of 2% in the medium while being treated with the proteases. Lactate dehydrogenase activities in both samples were determined using a commercial kit (Roche, Mannheim, Germany) that detects lactate dehydrogenase activity according to the disappearance of NADH in the presence of pyruvate and lactate dehydrogenase. From the absorbance at 490 nm, lactate dehydrogenase activity was expressed as the percentage of enzyme activity in the culture medium relative to the total activity in the samples of lysed cells.

Statistical analysis

A Student's *t*-test for unpaired samples was used with a two-tailed test. Unless stated otherwise, a p-value of < 0.05 was considered significant.

Results

Loss of epithelial thrombomodulin in inflamed gingival tissue, and presence of thrombomodulin in gingival crevicular fluid

The expression of epithelial thrombomodulin was examined immunohistochemically using healthy or diseased gingival tissues. Thrombomodulin presented in the epithelium and endothelium of healthy gingival tissue. In particular, thrombomodulin was located in the cell–cell space of the suprabasal layer in the gingival epithelium (Fig. 1B). In diseased tissue, inflammatory cells infiltrated into the epithelium and thrombomodulin expression was lost or decreased (Fig. 1C). However, the endothelium in the diseased tissue clearly expressed thrombomodulin (Fig. 1E). Neutrophils within the epithelium appeared to affect the expression of thrombomodulin (Fig. 1D). The negative control with nonimmune IgG did not show non specific staining (Fig. 1A).

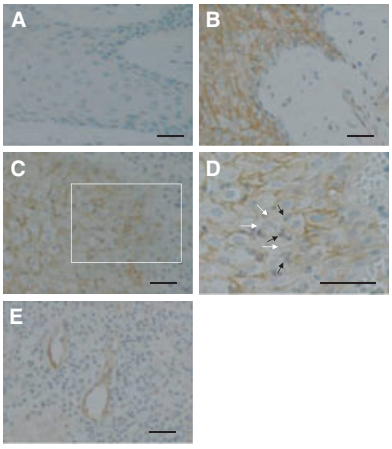

Fig. 1. Immunohistochemical staining for thrombomodulin in healthy (A,B) or inflamed (C,D,E) gingiva from periodontitis patients. (A) The negative control. No positive staining was observed with the isotype-control IgG. (B) Positive staining in the cellular membrane of suprabasal and basal layers and vessels were uniformly seen in healthy gingival tissue. (C) The immunoreactivity for thrombomodulin in inflamed gingiva was lost or decreased in the boxed area. (D) The boxed area in panel C was magnified. Neutrophils (black arrows) were detected at the intercellular space, which lost or decreased thrombomodulin (white arrows). (E) Blood vessels in inflamed gingiva showed clear immunoreactivity for thrombomodulin. Bars: 50 μm.

In addition, gingival crevicular fluid from diseased sites (Gingival index > 1) contained significantly higher amounts of the soluble form of thrombomodulin than gingival crevicular fluid from healthy sites (Gingival index = 0) (Table 1). There were no differences in thrombomodulin content from gingival crevicular fluid between the 3–5 mm groups and ≥6 mm groups in probing depth.

Release of epithelial thrombomodulin

Thrombomodulin was very rapidly detected in the supernatants of gingival epithelial cell cultures under serum free-culture conditions by the Thrombomodulin-specific ELISA, as soon as 30 min after the start of incubation with elastase. Thrombomodulin was also released after 4 h of incubation with cathepsin G (Fig. 2A). The release of thrombomodulin was blocked by adding α1-anti-trypsin complex, indicating that neutrophil proteases caused thrombomodulin release (Fig. 2B).

Western blot analysis

Thrombomodulin in supernatants of cultured cells treated with neutrophil elastase was analyzed using western blotting. Thrombomodulin degradation products were present in cultures treated with neutrophil elastase. The major molecular-weight form of thrombomodulin was 56,000 Da, using anti-thrombomodulin for detection, compared with the intact form of thrombomodulin (105,000 Da) (Fig. 3A). A gingival crevicular fluid sample from a ≥6 mm probing depth pocket of a periodontitis patient was also analyzed. There were more thrombomodulin-degradation products in the gingival crevicular fluid sample compared with that *in vitro* (Fig. 3B).

Cytotoxicity

Finally, we investigated the occurrence of cell injury after the incubation of oral epithelial cells with neutrophil proteases. Incubation with elastase or cathepsin G seemed not to lead to rapid cell damage (Fig. 3C). Cell injury was less than 10% up to 1 h after incubation with proteases. The kinetics of thrombomodulin release was not necessarily related to increasing cell injury by the effects of proteases, indicating that neutrophil proteases appeared to cleave thrombomodulin on the cell membrane and to preserve viability.

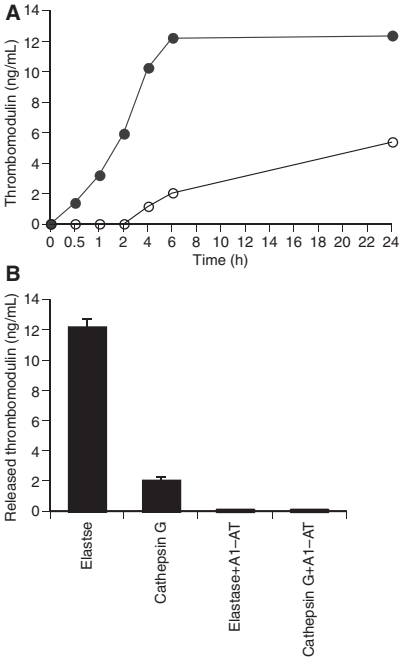

Fig. 2. (A) Increase in surface thrombomodulin in gingival epithelial cell cultures after 0.5, 1, 2, 4, 8 or 24 h of incubation with 10 μg/mL of elastase (•) and 10 μg/mL of cathepsin G (○) in serum-free medium. The amount of thrombomodulin in cultured supernatants was determined by enzyme-linked immunosorbent assay. The mean of three experiments is shown. (B) Inhibition, by α1-anti-trypsin treatment (10 μg/mL), of thrombomodulin release after elastase (10 μg/mL) or cathepsin G (10 μg/mL) treatment for 6 h. The concentration of thrombomodulin amounts in the culture supernatants were determined by enzyme-linked immunosorbent assay. Each value represents the mean ± standard deviation of three independent determinations. A1-AT, α1-anti-trypsin.

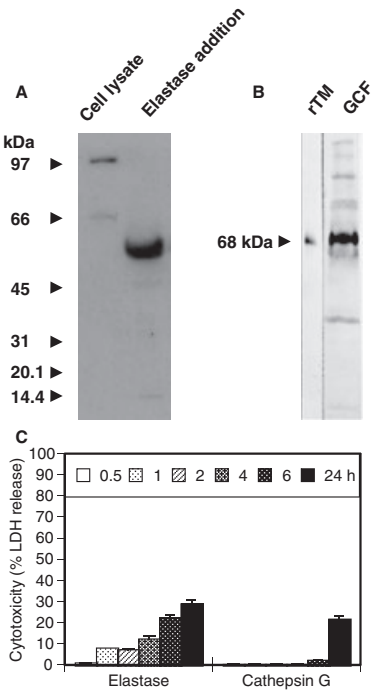

Fig. 3. (A) Supernatants of gingival epithelial cells treated with elastase for 6 h were used for western blot analysis. Western blot analysis of appropriate culture supernatants confirmed proteolytic thrombomodulin cleavage by elastase treatment. The cell lysate without any enzymatic treatment was blotted for comparison with the supernatant that was treated with elastase. (B) A gingival crevicular fluid sample from a 6-mm probing depth pocket of a periodontitis patient was immunoblotted for thrombomodulin. Recombinant thrombomodulin was used as a control. The gingival crevicular fluid sample included the thrombomodulin degradation products. (C) Cytotoxicity of neutrophil elastase (10 μg/mL) or cathepsin G (10 μg/mL) on gingival epithelial cells after incubation for the indicated times. Gingival epithelial cell injury was quantified by the release of the cytosolic enzyme lactate dehydrogenase. Each value represents the mean ± standard deviation of three independent determinations. GCF, gingival crevicular fluid; LDH, lactate dehydrogenase; rTM, recombinant thrombomodulin.

Discussion

We examined the release of thrombomodulin related to thrombin regulation from the gingival epithelium *in vitro and in vivo*. In the present study, we showed that thrombomodulin in periodontitis-diseased gingival epithelium was lost or decreased, although thrombomodulin in the endothelium was expressed. Thrombomodulin release from gingival epithelial cells was caused by neutrophil enzymes, mainly elastase *in vitro* and supernatants from cultured cells or gingival crevicular fluid with periodontitis included proteolytic thrombomodulin fragments. However, the rapid thrombomodulin release *in vitro* was not caused by cell damage. Moreover, the thrombomodulin content in gingival crevicular fluid did not correlate with the degree of probing depth, showed the highest level at diseased site with a probing depth from 3 mm. These results suggest that the epithelial cell membrane injury caused by neutrophil enzymes may result in thrombomodulin increase in the gingival crevicular fluid from patients with periodontitis.

Thrombin has been proposed to enhance granulation tissue formation by stimulating mitogenic responses in fibroblasts, macrophages and endothelial cells (19). Thrombin has also been reported to stimulate phosphoinositide hydrolysis in keratinocytes (20). Thrombomodulin by differentiating keratinocytes can regulate these cellular effects of thrombin by directly competing for thrombin binding with signal-transducing thrombin receptors and by promoting protein C-mediated inhibition of thrombin production (21). Thus, thrombomodulin regulates thrombin at sites of coagulation and inflammation. Accordingly, the decrease in thrombomodulin amount on the cell surface, in some cases may cause pro-inflammatory effects by thrombin that contribute to the pathogenesis of periodontal diseases (3).

Immunohistochemical observation of thrombomodulin in periodontitis patients showed that thrombomodulin was lost or decreased within the gingival epithelium, and an increasing number of neutrophils were observed at the periphery of this area (Fig. 1). The inflamed site is characterized by an infiltration of neutrophils. Infiltration of neutrophils into gingival tissues is an early event in gingival inflammation (22). Neutrophils play a destructive role in periodontal tissue breakdown because of high levels of lysosomal enzymes, generation of superoxides and the presence of reactive oxygen derivatives (23). Previous studies clearly show that human leukocyte elastase and cathepsin G are found in gingival tissues and that the concentration of these proteinases increases in inflamed gingival tissues during periodontal diseases (24–27). The elastase level in periodontitis sites (1.24 ± 0.13 Absorbance sites, probing depth = 5.7 ± 0.2 mm) from periodontitis was significantly higher than at healthy sites (0.18 ± 0.06 Absorbance sites, probing depth = 2.4 ± 0.1 mm) and gingivitis sites (0.42 ± 0.90 Absorbance sites, probing depth = 2.8 ± 0.2 mm) (26). The mean concentration of elastase in gingival crevicular fluid from patients with periodontitis (probing depth = 5.67 mm) was reported to be 1430 nM (27) and cathepsin G activity in gingival crevicular fluid was measured between adults with periodontitis and periodontally healthy controls (24). Significantly increased cathepsin G activities were found in seven of nine gingival crevicular fluid samples from adult patients with periodontitis. In gingival crevicular fluid samples from periodontally healthy controls, cathepsin G activity was found in only two of five samples and the activities was relatively low. A cathepsin G concentration of 5–25 ng/mL in gingival crevicular fluid samples from adults with periodontitis has been detected using dot immunoblot analysis. Accordingly, high levels of elastase and cathepsin G in gingival crevicular fluid with periodontitis is thought to affect thrombomodulin release into the gingival crevicular fluid.

Next, we examined the influence of neutrophil elastase or cathepsin G on thrombomodulin release using cultured gingival epithelial cells. We showed that neutrophil elastase induced the release of thrombomodulin from gingival epithelial cells after only 30 min of incubation, and that thrombomodulin release peaked after 6 h (Fig. 2A). The effect of elastase on thrombomodulin release was stronger than that of cathepsin G (Fig. 2B). Elastase is

very specific, preferentially cleaving target proteins on the C-terminal side of small aliphatic amino acids (28). Cathepsin G has a different specificity; it preferentially cleaves after phenylalanine residues (29). The differences in thrombomodulin release, as shown in Fig. 2, may be caused by the different target points of cleavage for the two enzymes. The effect of elastase on thrombomodulin release was similar to its effect on endothelial cells (30,31). These results indicated that neutrophil activation in periodontitis lesions correlated with gingival epithelial thrombomodulin release into gingival crevicular fluid.

In addition, we also examined the extent of epithelial cell injury by neutrophil enzymes *in vitro*. Immunoblotting analysis of thrombomodulin release into culture supernatants or gingival crevicular fluid showed the presence of proteolytic cleavage, indicating that thrombomodulin was affected by enzymatic action (Fig. 3A, B). Using a cell cytotoxicity assay, lactate dehydrogenase release into cultured supernatants was found to be $< 10\%$ in the initial 1 h, and was $22.379 \pm 1.21\%$ after 6 h of elastase treatment, or $2.187 \pm 0.02\%$ after 6 h of treatment with cathepsin G (Fig. 3C). It is well known that lactate dehydrogenase is a stable cytoplasmic enzyme (32). It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. Therefore, we showed that the increase in soluble thrombomodulin was not only an early event, but was also independent of the late death or destruction of epithelial cells.

Molecules in the gingival crevicular fluid might be useful predictors of periodontal disease activity (33,34). The monitoring of microbial infection and the analysis of the host response using gingival crevicular fluid have been suggested as means of identifying individuals at risk for future periodontal breakdown (26,35,36). There were no difference in thrombomodulin content from gingival crevicular fluid between the 3–5 mm groups and ≥ 6 mm in probing depth at the diseased sites, however, the groups at diseased sites showed clearly significantly higher levels of thrombomodulin compared with the healthy group, indicating that the thrombomodulin increase in gingival crevicular fluid may not be related to the degree of probing depth.

In conclusion, the present study provides further evidence on the release of thrombomodulin from injured gingival epithelial cells by neutrophil enzymes in periodontitis. This might explain the increase of thrombomodulin in gingival crevicular fluid from local diseased gingiva with no systemic disease. Elevation of thrombomodulin in gingival crevicular fluid may be a potential marker of epithelial cell membrane injury. Further research is required to elucidate the significance of soluble thrombomodulin in gingival crevicular fluid on periodontal disease.

Acknowledgements

This work was supported by Grants in Aid for Scientific Research (#13672193 and #15592193) from the Ministry of Education, Science, Sports, and Culture, Japan.

References

1. Tatakis DN. Blood coagulation factors in periodontal pathophysiology: a review with emphasis on the role of thrombin. *Semin Thromb Hemost* 1992;**18:**28–33.
2. Lerner UH, Sahlberg K, Ljunggren O. Thrombin and bradykinin enhance prostaglandin production in human peripheral blood monocytes. *J Oral Pathol Med* 1989;**18:**246–250.
3. Hou L, Ravenall S, Macey MG *et al.* Protease-activated receptors and their role in IL-6 and NF-IL-6 expression in human gingival fibroblasts. *J Periodont Res* 1998; **33:**205–211.
4. Esmon CT. Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. *Faseb J* 1995;**9:**946–955.
5. Jackson DE, Mitchell CA, Bird P, Salem HH, Hayman JA. Immunohistochemical localization of thrombomodulin in normal human skin and skin tumours. *J Pathol* 1995;**175:**421–432.
6. Lager DJ, Callaghan EJ, Worth SF, Raife TJ, Lentz SR. Cellular localization of thrombomodulin in human epithelium and squamous malignancies. *Am J Pathol* 1995;**146:**933–943.
7. Raife TJ, Lager DJ, Madison KC *et al.* Thrombomodulin expression by human keratinocytes. Induction of cofactor activity during epidermal differentiation. *J Clin Invest* 1994;**93:**1846–1851.
8. Raife TJ, Demetroulis EM, Lentz SR. Regulation of thrombomodulin expression by all-trans retinoic acid and tumor necrosis factor-alpha. differential responses in keratinocytes and endothelial cells. *Blood* 1996;**88:**2043–2049.
9. Peterson JJ, Rayburn HB, Lager DJ *et al.* Expression of thrombomodulin and consequences of thrombomodulin deficiency during healing of cutaneous wounds. *Am J Pathol* 1999;**155:**1569–1575.
10. Conway EM, Van de Wouwer M, Pollefeyt S *et al.* The lectin-like domain of thrombomodulin confers protection from neutrophil-mediated tissue damage by suppressing adhesion molecule expression via nuclear factor kappaB and mitogen-activated protein kinase pathways. *J Exp Med* 2002;**196:**565–577.
11. Abeyama K, Stern DM, Ito Y *et al.* The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel antiinflammatory mechanism. *J Clin Invest* 2005;**115:**1267–1274.
12. Nesheim M. Thrombin and fibrinolysis. *Chest* 2003;**124:**33S–39S.
13. Boehme MW, Raeth U, Galle PR, Stremmel W, Scherbaum WA. Serum thrombomodulin – a reliable marker of disease activity in systemic lupus erythematosus (SLE): advantage over established serological parameters to indicate disease activity. *Clin Exp Immunol* 2000;**119:**189–195.
14. Boehme MW, Schmitt WH, Youinou P, Stremmel WR, Gross WL. Clinical relevance of elevated serum thrombomodulin and soluble E-selectin in patients with Wegener's granulomatosis and other systemic vasculitides. *Am J Med* 1996; **101:** 387–394.
15. Blann A, Seigneur M. Soluble markers of endothelial cell function. *Clin Hemorheol Microcirc* 1997;**17:**3–11.
16. Lu XL, Cai JT, Lu XG, Si JM, Qian KD. Plasma level of thrombomodulin is an early indication of pancreatic necrosis in patients with acute pancreatitis. *Intern Med* 2007;**46:**441–445.
17. Boldt J, Wollbruck T, Sonneborn S, Welters A, Hempelmann G. Thrombomodulin in intensive care patients. *Intensive Care Med* 1995;**21:**645–650.
18. Matsuyama T, Izumi Y, Shibatate K *et al.* Expression and activity of thrombomodulin in human gingival epithelium: in vivo and in vitro studies. *J Periodont Res* 2000;**35:**146–157.
19. Stiernberg J, Redin WR, Warner WS, Carney DH. The role of thrombin and thrombin receptor activating peptide

(TRAP-508) in initiation of tissue repair. *Thromb Haemost* 1993;**70:**158–162.
20. Talwar HS, Fisher GJ, Harris VA, Voorhees JJ. Agonist-induced hydrolysis of phosphoinositides and formation of 1,2-diacylglycerol in adult human keratinocytes. *J Invest Dermatol* 1989;**93:**241–245.
21. Esmon NL, Carroll RC, Esmon CT. Thrombomodulin blocks the ability of thrombin to activate platelets. *J Biol Chem* 1983;**258:**12238–12242.
22. Suchett-Kaye G, Morrier JJ, Barsotti O. Interactions between non-immune host cells and the immune system during periodontal disease: role of the gingival keratinocyte. *Crit Rev Oral Biol Med* 1998;**9:**292–305.
23. Battino M, Bullon P, Wilson M, Newman H. Oxidative injury and inflammatory periodontal diseases. the challenge of antioxidants to free radicals and reactive oxygen species. *Crit Rev Oral Biol Med* 1999;**10:**458–476.
24. Tervahartiala T, Konttinen YT, INgmane T, Häyrinen-Immonen R, Ding Y, Sorsa T. Cathepsin G in gingival tissue and crevicular fluid in adult periodontitis. *J Clin Periodontol* 1996;**23:**68–75.
25. Wolff LF, Koller NJ, Smith QT, Mathur A, Aeppli D. Subgingival temperature: relation to gingival crevicular fluid enzymes, cytokines, and subgingival plaque micro-organisms. *J Clin Periodontol* 1997;**24:**900–906.
26. Jin L, Yu C, Corbet EF. Granulocyte elastase activity in static and flow gingival crevicular fluid. *J Periodont Res* 2003; **38:**303–310.
27. Cox SW, Rodriguez-Gonzalez EM, Booth V, Eley BM. Secretory leukocyte protease inhibitor and its potential interactions with elastase and cathepsin B in gingival crevicular fluid and saliva from patients with chronic periodontitis. *J Periodont Res* 2006;**41:**477–485.
28. Rao NV, Wehner NG, Marshall BC *et al*. Characterization of proteinase-3 (PR-3), a neutrophil serine proteinase. Structural and functional properties. *J Biol Chem* 1991;**266:**9540–9548.
29. Nakajima K, Powers JC, Ashe BM, Zimmerman M. Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. Studies with peptide substrates related to the alpha 1-protease inhibitor reactive site. *J Biol Chem* 1979;**254:**4027–4032.
30. Boehme MW, Deng Y, Raeth U *et al*. Release of thrombomodulin from endothelial cells by concerted action of TNF-alpha and neutrophils: in vivo and in vitro studies. *Immunology* 1996;**87:**134–140.
31. Boehme MW, Galle P, Stremmel W. Kinetics of thrombomodulin release and endothelial cell injury by neutrophil-derived proteases and oxygen radicals. *Immunology* 2002;**107:**340–349.
32. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* 2003;**24:**1121–1131.
33. Fine DH, Mandel ID. Indicators of periodontal disease activity: an evaluation. *J Clin Periodontol* 1986;**13:**533–546.
34. Figueredo CM, Gustafsson A. Increased amounts of laminin in GCF from untreated patients with periodontitis. *J Clin Periodontol* 2000;**27:**313–318.
35. Jin LJ, Soder PO, Leung WK *et al*. Granulocyte elastase activity and PGE2 levels in gingival crevicular fluid in relation to the presence of subgingival periodontopathogens in subjects with untreated adult periodontitis. *J Clin Periodontol* 1999;**26:**531–540.
36. Jin L, Soder B, Corbet EF. Interleukin-8 and granulocyte elastase in gingival crevicular fluid in relation to periodontopathogens in untreated adult periodontitis. *J Periodontol* 2000;**71:**929–939.