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# Periodontal disease and gene-expression levels of metalloendopeptidases in human buccal mucosal epithelium

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*Background and Objective:* Endopeptidases, such as neutral endopeptidase (NEP), endothelin-converting enzyme-1 (ECE-1) and a disintegrin and metalloprotease 17 (ADAM17), are believed to have various important roles in oral mucosal and epidermal tissue for the regulation of defensive biological responses in the oral cavity, and their expression and activity are influenced by various factors, including oral diseases. However, knowledge concerning these endopeptidases in the oral cavity has been minimal until now. This study focused on three metalloendopeptidases – NEP, ECE-1 and ADAM17 – in the oral buccal mucosal epithelium of patients with periodontal diseases and investigated the relationship between their gene-expression levels and periodontal disease.

*Material and Methods:* The levels of expression of *NEP*, *ECE-1* and *ADAM17* mRNAs in tissue samples collected from the oral buccal mucosal epithelium of 61 patients were investigated by relative quantification using real-time RT-PCR analysis. information on oral and systemic health was obtained from the clinical record of each patient.

*Results:* Among the three groups, classified based on the diagnosis of periodontal diseases (healthy/gingivitis, early periodontitis and moderate/advanced periodontitis), the relative expression level of *NEP* mRNA was significantly increased in the early periodontitis group and in the moderate/advanced periodontitis group compared with that in the healthy/gingivitis group. Moreover, the relative expression levels of *ECE1* and *ADAM17* mRNAs were significantly increased in the moderate/advanced periodontitis group compared with those in the healthy/gingivitis group. The correlation coefficients between the mean relative expression levels of *NEP* and *ECE1* mRNAs, *NEP* and *ADAM17* mRNAs, and *ECE1* and *ADAM17* mRNAs were $r = 0.758$, $r = 0.707$ and $r = 0.934$, respectively ($p < 0.001$). Furthermore, among the oral-related factors, there was a significant correlation between the number of sites with probing pocket depths of more than 4 mm and of more than 6 mm and the relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs. In stepwise logistic regression models, high relative expression levels of *ECE1* and *ADAM17* mRNAs were significantly associated with moderate/advanced periodontitis.

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*Conclusion:* The present study suggests that the severity of periodontal disease may be associated with the expression of metalloendopeptidase genes, including *NEP*, *ECE1* and *ADAM17*, in the buccal mucosal epithelium.

Zinc-dependent metalloendopeptidases, such as neutral endopeptidase (NEP; also known as EC 3.4.24.11, neprilysin or CD10), endothelin-converting enzyme (ECE) and proteins of a disintegrin and metalloprotease (ADAM) family, play vital roles in cellular processes, from fertilization through development and death. Many of the physiological roles of these enzymes have yet to be identified. They may provide potential therapeutic targets in the treatment of human diseases (1,2).

NEP is a cell-surface aminopeptidase that is present on the epithelial cells of various tissues such as the lung, kidney, intestine, prostate, endometrium and placenta, and plays an important role in the maintenance of homeostasis in normal tissues (1,3). In oral tissue, the expression of NEP is enhanced in oral squamous cell carcinoma, and it was suggested that NEP might be an indicator of a poor prognosis in oral tumor development (4).

ECE is a type II integral membrane-bound peptidase with considerable sequence similarity to NEP, and is the rate-limiting enzyme in the production of mature endothelin-1 (ET-1) (5). ECE has three identified isoforms: ECE-1; ECE-2; and ECE-3 (6,7). ECE-1 is present in a variety of tissues that produce ET-1, which is implicated in the pathogenesis of many diseases, such as hypertension, pulmonary hypertension, acute renal failure, chronic heart failure, gastric mucosal inflammation, rhinitis and cancer (8–13). Recently, we reported that the expression of ECE-1 was increased, along with ET-1, in oral squamous cell carcinoma cells compared with normal human epidermal keratinocytes (14).

The ADAMs are a family of multidomain proteins involved in both proteolysis and cell adhesion. Although they are primarily located on the cell membrane, soluble forms of some ADAMs have been described (15,16). Recently, specific ADAMs have been implicated in a number of diseases, including rheumatoid arthritis, Alzheimer's disease, atherosclerosis, asthma and cancer (16,17). ADAM17, also known as tumor necrosis factor-α (TNF-α)-converting enzyme, is the most extensively investigated ADAM protease and is known to release soluble TNF-α from its membrane precursor, pro-TNF-α, thus permitting paracrine TNF-α signaling (18). In the oral cavity, the level of ADAM17 was shown to be elevated in the gingival crevicular fluid of patients with chronic and aggressive periodontitis (19). Furthermore, a recent study demonstrated that treatment of oral fibroblasts with ET-1 activates ADAM17-mediated release of epidermal growth factor receptor (EGFR) ligands, triggering EGFR signaling and increased motility in neighboring head and neck cancer cells (20). Thus, there is a suggestion that ADAM17, along with NEP and ECE-1, is associated with the progression of oral diseases, such as periodontal diseases and oral cancer.

The oral cavity is constantly exposed to a variety of biological, chemical and mechanical insults. The oral mucosa is a critical protective interface between the external and internal environments and must serve as a barrier to a myriad of microbial species (21,22). Moreover, oral mucosal epithelial cells (i.e. oral keratinocytes) actively participate in immune responses and inflammation by secreting a variety of cytokines, chemokines, growth factors and neuropeptides in response to biological, chemical and mechanical stimulation in the oral cavity (23,24). Keratinocytes are thought to participate in the regulation of the immune response through the enzymatic action of various metalloendopeptidases, including NEP, ECE and ADAMs, which cleave bioactive peptides such as chemokines, cytokines and neuropeptides, thus affecting their activity and specificity (25–28). In oral keratinocytes, these metalloendopeptidases may also regulate biological activities throughout the progression of oral diseases. Knowledge concerning NEP, ECE and ADAMs in the oral cavity has been minimal until now. Therefore, we were interested in the presence and roles of NEP, ECE and ADAMs in the oral cavity.

This study focused on three metalloendopeptidases – NEP, ECE-1 and ADAM17 – in the oral buccal mucosal epithelium of patients with periodontal diseases. We hypothesized that NEP, ECE-1 and ADAM17 have various important roles in oral mucosal and epidermal tissue (specifically in the regulation of defensive biological responses in the oral cavity) and that their expression and activity are influenced by periodontal diseases. Therefore, this study aimed to investigate whether their gene-expression levels in human buccal mucosal epithelium were related to periodontal diseases.

## Material and methods

### Study population

Human oral buccal mucosal tissue samples were obtained from 61 patients (27 male patients and 34 female patients) without periodontitis and with generalized chronic periodontitis, randomly selected from among patients who visited the Hospital of Kyushu Dental College for dental treatment and check-ups. The mean age ± standard deviation of the patients was 60.3 ± 18.5 years (male patients: 65.6 ± 11.3 years; female patients: 56.0 ± 21.9 years) (Table 1). This study was conducted with the approval of the Kyushu Dental College Research Ethics Committee.

Informed written consent was obtained from all patients.

### Clinical examination

A dentist performed periodontal examinations at six sites per tooth. All examinations were carried out by the same dentist, who measured the clinical periodontal probing depth and bleeding on probing (BOP), related to the gingival inflammation, and counted the sites the number of sites in which a periodontal pocket (probing depths $\geq$ 4 mm and $\geq$ 6 mm) and BOP was present. All diagnoses of periodontal diseases based on the periodontal examinations were confirmed by the presence of horizontal or vertical bone loss in radiographic findings.

The subjects were classified into three groups [healthy/gingivitis group ($n$ = 8), early periodontitis group ($n$ = 28) and moderate/advanced periodontitis group ($n$= 25)] based on differences in periodontal diseases, diagnosed in accordance with the radiographic findings of the alveolar bone in the American Dental Association classification (29). The clinical periodontal parameters used for the classification of the three groups were as follows: the healthy/gingivitis group included the subjects with no radiographic evidence of bone loss; the early periodontitis group included the subjects with horizontal bone loss in which the alveolar bone level was 3–4 mm from the cemento–enamel junction area; and the moderate/advanced periodontal group included the subjects with horizontal or vertical bone loss in which the alveolar bone level was 4 mm or more from the cemento–enamel junction area.

*Table 1.* Profile of the subjects in the different classes based on the diagnosis of periodontal diseases

| Parameters | All ($n$ = 61) | Healthy/ gingivitis ($n$ = 8) | Early periodontitis ($n$ = 28) | Moderate/ advanced periodontitis ($n$ = 25) |
|---|---|---|---|---|
| Age (years)* | 60.3 (18.5) | 21.9 (3.6) | 66.0 (10.5) | 66.0 (12.8) |
| Female (%)** | 34 (55.7) | 8 (100) | 13 (46.4) | 13 (52.0) |
| Lifestyle habits | | | | |
| Smoking | | | | |
| Number of smokers | 8 (13.1) | 1 (12.5) | 4 (14.3) | 3 (12.0) |
| Number of presmokers | 15 (24.6) | 0 (0.0) | 10 (35.7) | 5 (20.0) |
| Number of nonsmokers | 38 (62.3) | 7 (87.5) | 14 (50.0) | 17 (68.0) |
| Number of alcohol consumers | 31 (50.8) | 5 (62.5) | 11 (39.3) | 15 (60.0) |
| Systemic diseases | | | | |
| Hypertension (+) | 18 (29.5) | 0 (0.0) | 8 (28.6) | 10 (40.0) |
| Diabetes (+) | 4 (6.6) | 0 (0.0) | 3 (10.7) | 1 (4.0) |
| Cerebrovascular diseases (+) | 4 (6.6) | 0 (0.0) | 1 (3.6) | 3 (12.0) |
| Heart diseases (+) | 7 (11.5) | 0 (0.0) | 6 (21.4) | 1 (4.0) |
| Liver diseases (+) | 3 (4.9) | 0 (0.0) | 2 (7.1) | 1 (4.0) |
| Cancer (+) | 9 (14.8) | 0 (0.0) | 3 (10.7) | 6 (24.0) |
| Oral cancer (+) | 3 (4.9) | 0 (0.0) | 0 (0.0) | 3 (12.0) |

Values are given as mean (standard deviation) or number (%).
Cancer (+): number of subjects with a medical history of cancer. Cerebrovascular diseases (+): number of subjects with a medical history of cerebrovascular diseases. Diabetes (+): number of subjects undergoing treatment for diabetes. Heart diseases (+): number of subjects with a medical history of heart diseases. Hypertension (+): number of subjects undergoing treatment for hypertension. Liver diseases (+): number of subjects with a medical history of liver diseases.
*$p < 0.05$, analysis of variance with the Scheffé test (healthy/gingivitis group vs. early periodontitis group and moderate/advanced periodontitis group).
**$p < 0.05$, chi-square test.

### Buccal epithelial cell swabs

Tissue samples were collected from the oral buccal mucosal epithelium using a cotton swab. The oral buccal mucosal epithelium on the left and on the right was rubbed 30 times with a cotton swab, and the tissue obtained was placed in phosphate-buffered saline.

Total RNA from the tissue samples was isolated using an RNeasy mini kit (QIAGEN, Tokyo, Japan), and complementary DNA was synthesized using the SUPERSCRIPT™ first-strand synthesis system (Invitrogen, Tokyo, Japan) for RT-PCR, according to the manufacturer's instructions. The integrity of the total RNA was verified by performing RT-PCR for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene.

### Real-time quantitative RT-PCR

The levels of expression of *NEP*, *ECE1* and *ADAM 17* mRNAs were quantified using a Roche Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany). Each reverse transcriptase reaction was amplified in a 20-μL PCR mixture using the LightCycler® FastStart DNA Master SYBR Green Master Mix (Roche Molecular Biochemicals). The following primers, designed from each known sequence, were used: NEP, 5′-ATG ACA TTG GCC CAG ATC C-3′ (sense) and 5′-CAC CAG CTC CTA AAC TAA CG-3′ (antisense); ECE-1, 5′-CTG AGA CAG GAG GCA GC-3′ (sense) and 5′-CTG TTG GAG TTC TTG GAA TC-3′ (antisense); ADAM17, 5′-CAG CAC AGC TGC CAA GTC ATT-3′

(sense) and 5′-CCA GCA TCT GCT AAG TCA CTT CC-3′ (antisense); and GAPDH, 5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′ (sense) and 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′ (antisense). Expression of mRNA was quantified using the second derivative maximum method, which determines the crossing points of individual samples using an algorithm that identifies the first turning point of the fluorescence curve. The expression level of each metalloendopeptidase mRNA was calculated relative to the expression of *GAPDH* mRNA using the $^{\Delta\Delta}C_t$ (cycle number at threshold) method, normalizing the $C_t$ values of the mRNA of each metalloendopeptidase to the $C_t$ values of *GAPDH* mRNA relative to a control sample. The values are shown in arbitrary units. Amplification of specific transcripts was confirmed by melting-curve profiles.

### Statistical analyses

Variables between the three periodontal groups (healthy/gingivitis group, early periodontitis group and moderate/advanced periodontitis group) were compared using an analysis of variance with a Scheffé test or using a Kruskal–Wallis test followed by a Scheffé test. The quantitative variables were converted to categorical variables, as follows: (i) levels of expression of *NEP*, *ECE1* and *ADAM17* mRNAs [NEP classes, ECE-1 classes and ADAM17 classes (two classes each): low levels (0–50 percentile) and high levels (51–100 percentile); (ii) number of teeth [teeth classes (three classes): 1–9 teeth; 10–19 teeth; and $\geq$ 20 teeth]; (iii) number of sites with a probing depth of more than 4 mm (PD4) [PD4 classes (three classes): no PD4 sites; 1–9 PD4 sites; and $\geq$ 10 PD4 sites]; (iv) number of sites with a probing depth of more than 6 mm (PD6) [PD6 classes (two classes): no PD6 sites; and $\geq$ 1 PD6 sites]; (v) number of sites with BOP [BOP classes (three classes): no BOP; 1–9 BOP sites; and $\geq$ 10 BOP sites. Additionally, according to a history of smoking habits, the subjects were classified into three groups: subjects who were current smokers (smokers); subjects who were not current smokers, but were former smokers (presmokers); and subjects who had never been smokers (nonsmokers). The distributions between the three periodontal classes and the categorized variables and between the NEP, ECE-1 and ADAM17 classes and the PD4 and PD6 classes were compared using a chi-square test.

The systemic diseases and the three periodontal classes were coded for Spearman's correlation or stepwise logistic regression analyses, as follows: (i) hypertension, 0 (subjects not undergoing treatment for hypertension) and 1 (subjects undergoing treatment for hypertension); (ii) diabetes mellitus (DM), 0 (subjects not undergoing treatment for DM) and 1 (subjects undergoing treatment for DM); (iii) cerebrovascular diseases (CVD), 0 (subjects without a medical history of CVD) and 1 (subjects with a medical history of CVD); (iv) heart diseases, 0 (subjects without a medical history of heart diseases) and 1 (subjects with a medical history of heart diseases); (v) liver diseases, 0 (subjects without a medical history of liver diseases) and 1 (subjects with a medical history of liver diseases); (vi) cancer, 0 (subjects without a medical history of cancer) and 1 (subjects with a medical history of cancer); (vii) oral cancer, 0 (subjects without a medical history of oral cancer) and 1 (subjects with a medical history of oral cancer); and (viii) periodontal classes, 0 (healthy/gingivitis group), 1 (early periodontitis group) and 2 (moderate/advanced periodontitis group). The correlations between age, and oral- and systemic-related parameters and the relative levels of expression of *NEP*, *ECE1* and *ADAM17* mRNAs were analyzed using Spearman's correlation analyses. Independent variables related to high relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs were verified by stepwise logistic-regression models using the related parameters considered, based on the results of the Spearman's correlation analyses. Statistical analyses were performed using IBM SPSS statistics software (IBM Japan, Tokyo, Japan).

## Results

### Profile of the subjects

The mean age $\pm$ standard deviation of subjects in the healthy/gingivitis group (21.9 $\pm$ 3.6 years) was significantly lower than that of subjects in the early periodontitis and moderate/advanced periodontitis groups (66.0 $\pm$ 10.5 years and 66.0 $\pm$ 12.8 years, respectively) ($p < 0.05$, analysis of variance with Scheffé test), and all subjects in the healthy/gingivitis group were female. Regarding systemic diseases, 18 (29.5%) and four (6.6%) subjects were undergoing treatment for hypertension and DM, respectively, and four (6.6%), seven (11.5%) and three (4.9%) had a medical history of CVD, heart diseases and liver diseases, respectively. Nine (14.8%) subjects had a medical history of cancer, and three subjects had a medical history of oral cancer (4.9%). Although the subjects in the healthy/gingivitis group had no systemic diseases, there were no statistically significant differences in the distribution of each systemic disease between the three groups. There were no significant differences in the proportions of smokers and alcohol consumers between the three groups (Table 1).

The mean numbers $\pm$ standard deviation of sites with PD4, PD6 and BOP (13.2 $\pm$ 13.3, 3.6 $\pm$ 5.0 and 18.7 $\pm$ 17.9, respectively) in the moderate/advanced periodontitis group were significantly higher compared with those in the healthy/gingivitis and early periodontitis groups (PD4: 0.1 $\pm$ 0.2 and 1.7 $\pm$ 1.7; PD6: 0.0 $\pm$ 0.0 and 0.0 $\pm$ 0.0; and BOP: 3.9 $\pm$ 6.4 and 9.7 $\pm$ 9.1, respectively). There was no significant difference in the numbers of teeth among the three groups (Table 2).

### Comparison of the relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs between the three classes based on the diagnosis of periodontal diseases

The mean $\pm$ standard deviation relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs were 67.0 $\pm$

213.6, 3.0 ± 7.3 and 0.4 ± 0.9, respectively, and dispersion of their values varied widely. The correlation coefficients between the mean ± standard deviation relative expression levels of *NEP* and *ECE1* mRNAs, *NEP* and *ADAM17* mRNAs, and *ECE1* and *ADAM17* mRNAs were $r = 0.758$, $r = 0.707$ and $r = 0.934$, respectively (all coefficients, $p < 0.001$, Spearman's correlation analysis). The bar charts of the relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs in the three periodontal disease groups are shown in Fig. 1. The relative expression level of *NEP* mRNA was significantly enhanced in the early periodontitis group and in the moderate/advanced periodontitis group compared with the healthy/gingivitis group. The relative expression levels of *ECE1* and *ADAM17* mRNAs were significantly enhanced in the moderate/advanced periodontitis group compared with the healthy/gingivitis group.

### Correlations between parameters related to periodontal disease and the relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs

Periodontal classes were significantly correlated with the relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs ($r = 0.503$, $p < 0.001$; $r = 0.563$, $p < 0.001$; and $r = 0.546$, $p < 0.001$, respectively). Additionally, there were significant correlations between the number of PD4 sites and the relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs ($r = 0.391$, $p < 0.05$; $r = 0.473$, $p < 0.001$; and $r = 0.479$, $p < 0.001$, respectively) and between the number of PD6 sites and the relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs ($r = 0.269$, $p < 0.05$; $r = 0.386$, $p < 0.01$; and $r = 0.387$, $p < 0.01$, respectively, Spearman's correlation analysis). There was no significant correlation between the number of BOP sites and the relative mRNA expression levels of the three metalloendopeptidases (Table 3). Although the correlations between other parameters and the relative mRNA expression levels of the three metalloendopeptidases are not shown, age was significantly correlated with the relative expression levels of *ECE1* and *ADAM17* mRNAs ($r = 0.374$, $p < 0.01$ and $r = 0.403$, $p < 0.01$, respectively, Spearman's correlation analysis), and a medical history of CVD was significantly correlated with the expression level of *ADAM17* mRNA ($r = 0.256$, $p < 0.05$).

*Table 2.* Oral characteristics of the subjects in the different classes based on the diagnosis of periodontal diseases

| Parameters | All ($n = 61$) | Healthy/ gingivitis ($n = 8$) | Early periodontitis ($n = 28$) | Moderate/advanced periodontitis ($n = 25$) |
|---|---|---|---|---|
| Number of teeth | 24.5 (2.9) | 27.3 (1.5) | 23.4 (5.9) | 25.0 (4.1) |
| Subjects with 1–9 teeth | 1 (1.6) | 0 (0.0) | 1 (3.6) | 0 (0.0) |
| Subjects with 10–19 teeth | 7 (11.5) | 0 (0.0) | 5 (17.9) | 2 (8.0) |
| Subjects with ≥20 teeth | 53 (86.9) | 8 (100) | 22 (78.5) | 23 (82.0) |
| Number of PD4 sites*, ** | 8.3 (12.4) | 0.1 (0.3) | 2.5 (2.5) | 17.5 (15.2) |
| Subjects with no PD4 sites | 15 (24.6) | 7 (87.5) | 7 (25.0) | 1 (4.0) |
| Subjects with 1–9 PD4 sites | 30 (49.2) | 1 (12.5) | 21 (75.0) | 8 (32.0) |
| Subjects with ≥ 10 PD4 sites | 16 (26.2) | 0 (0.0) | 0 (0.0) | 16 (64.0) |
| Number of PD6 sites*,*** | 1.5 (3.5) | 0 (0.0) | 0 (0.0) | 3.6 (5.0) |
| Subjects with no PD6 sites | 44 (72.1) | 8 (100.0) | 28 (100.0) | 8 (32.0) |
| Subjects with ≥ 1 PD6 site | 17 (27.9) | 0 (0.0) | 0 (0.0) | 17 (68.0) |
| Number of BOP sites*, ** | 18.3 (18.3) | 6.3 (10.0) | 14.4 (13.3) | 26.6 (21.8) |
| Subjects with no BOP | 7 (11.5) | 3 (37.5) | 3 (10.7) | 1 (4.0) |
| Subjects with 1–9 BOP sites | 16 (26.2) | 3 (37.5) | 10 (35.7) | 3 (12.0) |
| Subjects with ≥ 10 BOP sites | 38 (62.3) | 2 (25.0) | 3 (12.0) | 21 (84.0) |

Values are given as mean (standard deviation) or number (%).
BOP, bleeding on probing; PD4, probing depth of more than 4 mm; PD6, probing depth of more than 6 mm.
*$p < 0.05$, analysis of variance with the Scheffé test (healthy/gingivitis group and early periodontitis group vs. moderate/advanced periodontitis group).
**$p < 0.05$, chi-square test.
***$p < 0.001$, chi-square test.

### Comparison between the three classes based on the diagnosis of periodontal diseases and endopeptidase classes, based on the relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs

The proportion of subjects in the healthy/gingivitis group increased more in the classes with low levels of expression of *NEP*, *ECE1* and *ADAM17* mRNAs than in the classes with high levels of expression [*NEP* (low and high levels): 26.7% and 0.0%; *ECE1* classes: 22.6% and 3.3%; and *ADAM17*: 23.3% and 3.2%]. The proportion of subjects in the moderate/advanced periodontitis group increased more in the high-level classes than in the low-level classes [*NEP* (low and high levels): 30.0% and 51.6%; *ECE1* classes: 16.1% and 66.7%; and *ADAM17* classes: 16.7% and 64.5%]. There were significant differences in the proportion of subjects

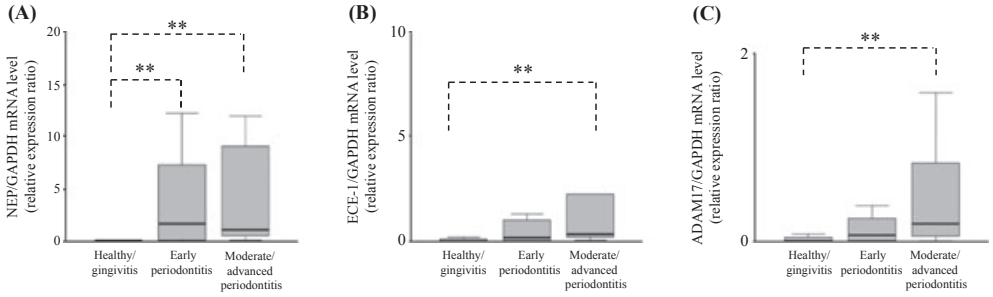


*Fig. 1*. Comparison between the classes based on the diagnosis of periodontal diseases and the relative mRNA expression levels of neutral endopeptidase (*NEP*), endothelin-converting enzyme-1 (*ECE1*) and a disintegrin and metalloprotease 17 (*ADAM17*). (A) *NEP*, (B) *ECE1*, (C) *ADAM17*. Healthy/gingivitis: eight subjects with normal periodontal health or gingivitis. Early periodontitis: 28 subjects with early periodontitis. Moderate/advanced periodontitis: 25 subjects with moderate periodontitis or advanced periodontitis. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. $^{**}p < 0.01$ (Kruskal–Wallis test followed by the Scheffé test).

*Table 3*. Correlations between parameters related to periodontal disease and the relative expression levels of neutral endopeptidase (*NEP*), endothelin-converting enzyme-1 (*ECE1*) and a disintegrin and metalloprotease 17 (*ADAM17*) mRNAs

| Parameters | *NEP* | *ECE1* | *ADAM17* |
|---|---|---|---|
| Periodontal classes | 0.503*** | 0.563*** | 0.546*** |
| Number of PD4 sites | 0.391** | 0.473*** | 0.479*** |
| Number of PD6 sites | 0.269* | 0.386** | 0.387** |
| Number of BOP sites | 0.180 | 0.238 | 0.143 |

Periodontal classes: subjects were coded as 0 (healthy/gingivitis group), 1 (early periodontitis group) and 2 (moderate/advanced periodontitis group).
BOP, bleeding on probing; PD4, probing depth of more than 4 mm; PD6, probing depth of more than 6 mm.
$^{*}p < 0.05$, Spearman's correlation analysis.
$^{**}p < 0.01$, Spearman's correlation analysis.
$^{***}p < 0.001$, Spearman's correlation analysis.

between the three classes and the low-level and high-level *NEP*, *ECE1* and *ADAM17* classes ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively, chi-square test) (Table 4).

### Relationship between periodontal diseases and high relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs in stepwise logistic regression models

Periodontal classes, numbers of PD4 and PD6 sites and age were used as independent variables in the stepwise logistic regression models for ECE-1, as determined by Spearman's correlation analysis. CVD was added as an independent variable in the logistic regression model for ADAM17, together with periodontal classes, numbers of PD4 and PD6 sites and age (Table 5). Finally, moderate/advanced periodontitis was significantly associated with high relative expression levels of *ECE1* and *ADAM17* mRNAs [odds ratio = 28.0 (95% confidence interval: 2.8–282.3) and odds ratio = 28.0 (95% confidence interval: 2.8–282.3), respectively]. Furthermore, no significant independent variables were determined in the logistic regression model for high relative expression levels of *NEP* mRNA.

## Discussion

The present study is the first to show a relationship between periodontal disease and the gene-expression levels of *NEP*, *ECE1* and *ADAM17* in the oral buccal mucosal epithelium. It showed that the exacerbation of periodontal health status correlated with increases in the expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs in the oral buccal mucosal epithelium. The oral fluids of patients with chronic periodontitis, including saliva and gingival crevicular fluid, contain specific locally and systemically derived mediators of periodontal disease, including microbial, host-response and bone-resorption-specific markers (30). The cells that comprise the innate immune response are primarily phagocytes, including neutrophils and macrophages, and the cells that line the epithelial mucosa. Although it was originally thought that the epithelium provided defenses primarily as a barrier against microbial invasion, it has been more recently recognized that these cells play an important active role in the recognition of microbes, eliciting a defensive response (31). Therefore, the changes in the mRNA expression of the three metalloendopeptidases in this study may reflect the responses of cells in the oral mucosal epithelium to the inflammatory mediators induced in the oral fluids by the progression of periodontitis, although this was not demonstrated in the present study.

In this study, all subjects with PD6 at one site or more and those with PD4 at 10 sites or more were included in the moderate/advanced periodontitis group. Moreover, increases in the numbers of PD4 and PD6 sites along with progress of periodontal diseases correlated with the increases in the levels of *NEP*, *ECE1* and *ADAM17* mRNAs in the oral buccal mucosal epithelium. However, an increase in the number of BOP sites, an indicator of locally inflamed periodontal sites, did not correlate with increases in the expression of mRNA for the three me-

*Table 4*. Comparison between the three classes based on the diagnosis of periodontal diseases and the endopeptidase classes, based on the relative mRNA expression levels of neutral endopeptidase (*NEP*), endothelin-converting enzyme-1 (*ECE1*) and a disintegrin and metalloprotease 17 (*ADAM17*) mRNAs

| | Endopeptidase classes | | | | | |
|---|---|---|---|---|---|---|
| | *NEP** | | *ECE1*** | | *ADAM17*** | |
| Parameters | Low levels | High levels | Low levels | High levels | Low levels | High levels |
| Healthy/gingivitis ($n$ = 8) | 8 (26.7) | 0 (0.0) | 7 (22.6) | 1 (3.3) | 7 (23.3) | 1 (3.2) |
| Early periodontitis ($n$ = 28) | 13 (43.3) | 15 (48.4) | 19 (61.3) | 9 (30.0) | 18 (60.0) | 10 (32.3) |
| Moderate/advanced Periodontitis ($n$ = 25) | 9 (30.0) | 16 (51.6) | 5 (16.1) | 20 (66.7) | 5 (16.7) | 20 (64.5) |

Values are given as $n$ (%).
Endopeptidase classes (high and low levels): subjects were categorized into two classes at the 50th percentile of the relative mRNA expression level of each endopeptidase. %: percentage of all subjects in each endopeptidase class.
*$p < 0.01$, chi-square test.
**$p < 0.001$, chi-square test.

*Table 5*. Relationship between periodontal diseases and high relative expression levels of endothelin-converting enzyme-1 (*ECE1*) and a disintegrin and metalloprotease 17 (*ADAM17*) mRNAs in stepwise logistic regression models

| Dependent variables | Independent variables | Odds ratio | 95% confidence interval | $p$-value |
|---|---|---|---|---|
| High levels of *ECE1* | Early periodontitis (vs. healthy/gingivitis) | 3.3 | 0.4–31.1 | 0.295 |
| | Moderate/advanced periodontitis (vs. healthy/gingivitis) | 28.0 | 2.8–282.3 | 0.005 |
| High levels of *ADAM17* | Early periodontitis (vs. healthy/gingivitis) | 3.9 | 0.4–36.2 | 0.234 |
| | Moderate/advanced periodontitis (vs. healthy/gingivitis) | 28.0 | 2.8–282.3 | 0.005 |

Classes of endopeptidase expression (high and low levels): subjects were categorized into two classes at the 50th percentile of the relative mRNA expression level of each endopeptidase. Age and periodontal classes, determined by Spearman's correlation analysis, were used as independent variables in the logistic regression models for ECE-1, and cardiovascular disease (CVD) was added as an independent variable in the logistic regression model for ADAM17, together with age and periodontal classes.

talloendopeptidases, although the development of periodontitis seemed to be linked to an increase in locally inflamed periodontal sites. This may be because some of the subjects with extensive gingival inflammation were included in the healthy/gingivitis and early periodontitis groups as well as in the moderate/advanced periodontitis group.

The present study used exfoliated epithelial cells obtained using a cotton swab from the oral buccal mucosal epidermis because the buccal mucosa is an easily accessed tissue and has been used successfully to obtain gene samples and because this study investigated comprehensive influences on oral keratinocytes, including differences in lifestyle and systemic diseases in addition to periodontal diseases. Although we recognize that the oral sample should be of periodontal tissue rather than of buccal mucosal epithelium for the investigation of sites localized with periodontal disease, it was more reasonable in this study to use oral buccal epithelium rather than periodontal tissue, which may be more strongly linked to local periodontal health. Thus, this study suggests that the levels of mRNA for the three metalloendopeptidases in oral buccal epithelium may reflect differences in comprehensive periodontal health seen in various local periodontal pathological states.

NEP is reported to inactivate a variety of peptides, such as enkephalins, substance P, bradykinin and interleukin-1beta (32). NEP is present on epidermal keratinocytes of inflamed skin, and it was suggested that NEP may interfere with the inflammatory response by degrading the proinflammatory neuropeptide substance-P (33). Therefore, in the oral cavity, NEP is thought to regulate proinflammatory signals on the keratinocytes of the oral mucosal epithelium, in the same manner as it regulates those of skin cells. In this study, the trend toward an increase in the expression of *NEP* mRNA in patients with periodontitis may reflect an enhanced regulation of proinflammatory signals for the cellular response in oral fluids during periodontal inflammation. Furthermore, because there is no significant relationship between the levels of expression of *NEP* mRNA and periodontal parameters in the logistic regression model in this study, the expression of NEP in the oral mucosal epithelium may not be directly related to the development of periodontitis.

A recent study established the presence of ECE-1 in the oral mucosa and demonstrated that an increase in ECE-1 expression and in ET-1 generation correlates with the onset of oral mucosal ulceration (34,35). Furthermore, we previously reported that ET-1, the biologically active form derived from

inactive big-ET-1 through the catalytic activity of ECE-1, was strongly expressed in gingival epithelial cells and endothelial cells of inflamed gingival tissue (36). In another of our recent studies, we showed that ET-1 was involved in the regulation of interleukin-1β expression in gingival tissues and it was suggested that ET-1 signaling to the cells might be a therapeutic target for treating the interleukin-1β-dependent inflammatory response (37). Although the relationship between ECE-1 and periodontal diseases has not been previously demonstrated, the action of ECE-1 is thought to be associated with the inflammatory process in inflamed periodontal tissue, as the expression levels of ECE-1 are correlated to those of ET-1. Thus, our findings that the expression of *ECE1* mRNA in the oral buccal mucosal epithelium increases in patients with moderate or advanced periodontitis may indicate that the increase in the expression of *ECE1* mRNA is influenced by changes in the oral environment that are linked to the progression of periodontal disease.

ADAM17 is ubiquitously expressed and cleaves membrane proteins, such as EGFR ligands, L-selectin and TNF-α, from the surface, thus regulating responses to tissue injury and inflammation. A recent study identified a critical role of the ADAM17–EGFR signaling axis in maintaining the homeostasis of the postnatal epidermal barrier and suggested that this pathway could represent a good target for the treatment of epidermal barrier defects (28). Until now, there has been no report on the ADAM17 levels in the oral mucosal epithelium, whilst recent studies reported that an increased level of TNF-α-converting enzyme (ADAM17) in the gingival crevicular fluid of patients with periodontitis may be associated with alveolar bone loss and worsening of the outcome of periodontitis (19,38). TNF-α-converting enzyme (ADAM17) can promote the expression and release of ligands and receptors that are strongly associated with inflammation, including TNF-α and interleukin-1 receptor II (19). Therefore, the increase in the expression of *ADAM17* mRNA in the oral buccal mucosal epithelium of patients with moderate or advanced periodontitis, as found in the present study, may be related to the role of the ADAM17–EGFR signaling axis in maintaining the homeostasis of the oral mucosal epidermal barrier and may additionally be influenced by the increase in inflammatory mediators, such as ECE-1, in oral fluids, which arises from the deterioration caused by periodontal diseases.

In this study, the mean age of subjects in the healthy/gingivitis group was younger than in the early and moderate/advanced periodontitis groups, and all of the subjects in the healthy/gingivitis group were women. Therefore, the comparison between the relative expression levels of *NEP*, *ECE1* and *ADAM17* mRNAs among the healthy/gingivitis, early and moderate/advanced periodontitis groups needs to consider the differences in the mean age and gender between the groups. The present study investigated the relationship between the levels of *NEP*, *ECE1* and *ADAM17* mRNAs expressed in the oral buccal mucosal epithelium and differences in the age, gender, lifestyle (such as cigarette smoking and alcohol drinking) and systemic health of the subjects, including a full medical history, in addition to the oral-related parameters. Consequently, no obvious relationship was found between the levels of mRNAs for these metalloendopeptidases and differences in age, gender and lifestyle, including cigarette smoking and systemic health, in this study, although the levels of expression of *NEP*, *ECE1* and *ADAM17* mRNAs tended to correlate with an increase in age.

We recognize the limitations of this study in demonstrating the roles of NEP, ECE-1 and ADAM17 in the oral cavity, as this study did not confirm the protein expression levels and the enzyme activities of NEP, ECE-1 and ADAM17 in the oral buccal mucosal epithelium. Moreover, the subjects in this study constituted too small a population size to verify the relationship between systemic diseases and expression of metalloendopeptidase genes, and the collected exfoliated epithelial cell samples might not reflect the exact state of the cells *in vivo* because the biological signals in these cells might change compared with those in live epithelial cells of the oral mucosal epithelium. However, we consider that the present study succeeds in showing novel results to elucidate the defensive responses of cells in the oral mucosal epithelium and the roles of metalloendopeptidases such as NEP, ECE-1 and ADAM17 in the oral cavity. Moreover, the present study suggests that the expression of *NEP*, *ECE1* and *ADAM17* mRNAs may be linked because there was a very high positive correlation between the levels of expression of mRNAs of the three metalloendopeptidases. In particular, the actions of ECE-1 and ADAM17 may be a target to regulate the pathological inflammatory processes caused by periodontal diseases, as cytokines, such as ET-1 and TNF-α, which are produced by these enzymes, act as important modulators of inflammation (37,39). Additionally, the gene-expression levels of *NEP*, *ECE1* and *ADAM17* in the buccal mucosal epithelium may be used as a screening marker for patients with periodontitis and to evaluate the prognosis for periodontitis, as there are clear differences in the gene-expression levels of the three metalloendopeptidases between patients with a healthy periodontal status or gingivitis and patients with moderate or advanced periodontitis.

In conclusion, the present study suggests that the severity of periodontal disease may be associated with the expression of metalloendopeptidase genes, including *NEP*, *ECE1* and *ADAM17*, in the buccal mucosal epithelium, and that NEP, ECE-1 and ADAM17 may act together as defensive mediators in the inflammatory response in the oral mucosa.

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## References


1. Turner AJ, Brown CD, Carson JA, Barnes K. The neprilysin family in health and disease. *Adv Exp Med Biol* 2000;**477**:229–240.
2. Turner AJ. Exploring the structure and function of zinc metallopeptidases: old enzymes and new discoveries. *Biochem Soc Trans* 2003;**31**:723–727.
3. Erdos EG, Skidgel RA. Human neutral endopeptidase 24.11 (NEP, enkephalinase); function, distribution and release. *Adv Exp Med Biol* 1988;**240**:13–21.
4. Piattelli A, Fioroni M, Iezzi G *et al*. CD10 expression in stromal cells of oral cavity squamous cell carcinoma: a clinic and pathologic correlation. *Oral Dis* 2006;**12**:301–304.
5. Yanagisawa M, Kurihara H, Kimura S *et al*. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;**332**:411–415.
6. Emoto N, Yanagisawa M. Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. *J Biol Chem* 1995;**270**:15262–15268.
7. Hasegawa H, Hiki K, Sawamura T *et al*. Purification of a novel endothelin-converting enzyme specific for big endothelin-3. *FEBS Lett* 1998;**428**:304–308.
8. Douglas SA. Clinical development of endothelin receptor antagonists. *Trends Pharmacol Sci* 1997;**18**:408–412.
9. Haynes WG, Webb DJ. Endothelin as a regulator of cardiovascular function in health and disease. *J Hypertens* 1998;**16**:1081–1098.
10. Slomiany BL, Piotrowski J, Slomiany A. Up-regulation of endothelin-converting enzyme-1 in gastric mucosal inflammatory responses to Helicobacter pylori lipopolysaccharide. *Biochem Biophys Res Commun* 2000;**267**:801–805.
11. Mullol J, Picado C. Endothelin in nasal mucosa: role in nasal function and inflammation. *Clin Exp Allergy* 2000;**30**:172–177.
12. Bagnato A, Rosano L. The endothelin axis in cancer. *Int J Biochem Cell Biol* 2008;**40**:1443–1451.
13. Zhang JS, Tan YR, Xiang Y, Luo ZQ, Qin XQ. Regulatory peptides modulate adhesion of polymorphonuclear leukocytes to bronchial epithelial cells through regulation of interleukins, ICAM-1 and NF-kappaB/IkappaB. *Acta Biochim Biophys Sin (Shanghai)* 2006;**38**:119–128.
14. Awano S, Dawson LA, Hunter AR, Turner AJ, Usmani BA. Endothelin system in oral squamous carcinoma cells: specific siRNA targeting of ECE-1 blocks cell proliferation. *Int J Cancer* 2006;**118**:1645–1652.
15. Blobel CP. ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* 2005;**6**:32–43.
16. Edwards DR, Handsley MM, Pennington CJ. The ADAM metalloproteinases. *Mol Aspects Med* 2008;**29**:258–289.
17. O'Shea C, McKie N, Buggy Y *et al*. Expression of ADAM-9 mRNA and protein in human breast cancer. *Int J Cancer* 2003;**105**:754–761.
18. Black RA. Tumor necrosis factor-alpha converting enzyme. *Int J Biochem Cell Biol* 2002;**34**:1–5.
19. Bostanci N, Emingil G, Afacan B *et al*. Tumor necrosis factor-alpha-converting enzyme (TACE) levels in periodontal diseases. *J Dent Res* 2008;**87**:273–277.
20. Hinsley EE, Hunt S, Hunter KD, Whawell SA, Lambert DW. Endothelin-1 stimulates motility of head and neck squamous carcinoma cells by promoting stromal-epithelial interactions. *Int J Cancer* 2012;**130**:40–47.
21. Sahasrabudhe KS, Kimball JR, Morton TH, Weinberg A, Dale BA. Expression of the antimicrobial peptide, human beta-defensin 1, in duct cells of minor salivary glands and detection in saliva. *J Dent Res* 2000;**79**:1669–1674.
22. Dale BA, Fredericks LP. Antimicrobial peptides in the oral environment: expression and function in health and disease. *Curr Issues Mol Biol* 2005;**7**:119–133.
23. Stadnyk AW. Cytokine production by epithelial cells. *FASEB J* 1994;**8**: 1041–1047.
24. Hedges SR, Agace WW, Svanborg C. Epithelial cytokine responses and mucosal cytokine networks. *Trends Microbiol* 1995;**3**:266–270.
25. Lendeckel U, Kahne T, Riemann D, Neubert K, Arndt M, Reinhold D. Review: the role of membrane peptidases in immune functions. *Adv Exp Med Biol* 2000;**477**:1–24.
26. Pernet I, Mayoux C, Trompezinski S, Schmitt D, Viac J. Modulation of endothelin-1 in normal human keratinocytes by UVA1/B radiations, prostaglandin E2 and peptidase inhibitors. *Exp Dermatol* 2000;**9**:401–406.
27. Steinhoff M, Brzoska T, Luger TA. Keratinocytes in epidermal immune responses. *Curr Opin Allergy Clin Immunol* 2001;**1**:469–476.
28. Franzke CW, Cobzaru C, Triantafyllopoulou A *et al*. Epidermal ADAM17 maintains the skin barrier by regulating EGFR ligand-dependent terminal keratinocyte differentiation. *J Exp Med* 2012;**209**:1105–1119.
29. American Dental Association. *Risk Management Series: Diagnosing and Managing the Periodontal Patient*. Chicago: American Dental Association, 1986: 12–16.
30. Kinney JS, Ramseier CA, Giannobile WV. Oral fluid-based biomarkers of alveolar bone loss in periodontitis. *Ann N Y Acad Sci* 2007;**1098**:230–251.
31. Diamond G, Beckloff N, Ryan LK. Host defense peptides in the oral cavity and the lung: similarities and differences. *J Dent Res* 2008;**87**:915–927.
32. Erdos EG, Skidgel RA. Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. *FASEB J* 1989;**3**:145–151.
33. Xie L, Takahara M, Nakahara T *et al*. CD10-bearing fibroblasts may inhibit skin inflammation by down-modulating substance P. *Arch Dermatol Res* 2011;**303**:49–55.
34. Slomiany BL, Piotrowski J, Slomiany A. Suppression of endothelin-converting enzyme-1 during buccal mucosal ulcer healing: effect of chronic alcohol ingestion. *Biochem Biophys Res Commun* 2000;**271**:318–322.
35. Slomiany BL, Slomiany A. Nonsteroidal anti-inflammatory drugs impair oral mucosal repair by eliciting disturbances in endothelin-converting enzyme-1 and constitutive nitric oxide synthase. *J Physiol Pharmacol* 2001;**52**:81–92.
36. Yamamoto E, Awano S, Koseki T, Ansai T, Takehara T. Expression of endothelin-1 in gingival epithelial cells. *J Periodontal Res* 2003;**38**:417–421.
37. Rikimaru T, Awano S, Mineoka T, Yoshida A, Ansai T, Takehara T. Relationship between endothelin-1 and interleukin-1beta in inflamed periodontal tissues. *Biomed Res* 2009;**30**:349–355.
38. Lee JH, Choi YJ, Heo SH, Lee JM, Cho JY. Tumor necrosis factor-alpha converting enzyme (TACE) increases RANKL expression in osteoblasts and serves as a potential biomarker of periodontitis. *BMB Rep* 2011;**44**:473–477.
39. Garlet GP. Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. *J Dent Res* 2010;**89**:1349–1363.