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Expression of neprilysin in periodontitis-affected gingival tissues

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ABSTRACT

Objective: Although the pathogeneses of Alzheimer's disease (AD) and periodontal diseases have overlapping features, including ageing and chronic inflammation, the association between AD and periodontitis remains unclear. To explore the pathogenesis of periodontitis, a comprehensive gene expression/transcriptome analysis in periodontitis-affected gingival tissues found that the AD pathway was significantly up-regulated in periodontitis-affected gingival tissues. AD-related genes, amyloid beta precursor protein (APP), interleukin-1 beta and compliment 1QA, were significantly elevated in periodontitis. In the present study, balance between mRNA expression of APP and a potent amyloid degradation enzyme, neprilysin (NEP), as well as protein localisation of APP and NEP were analysed. *Design:* Eighteen periodontitis-affected and 18 clinically healthy control gingival tissues were taken from patients with severe chronic periodontitis or undergoing tooth extraction. Total RNA was purified and used for quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR). The localisation of APP and NEP was analysed by immunohistochemistry (IHC).

Results: Both APP and NEP genes were up-regulated in periodontitis-affected gingival tissues. APP-expressing macrophages and NEP-expressing neutrophils and fibroblasts, reflecting inflammatory stages, were detected in inflamed gingival tissues by IHC.

Conclusion: The up-regulation of APP and NEP mRNA levels in periodontitis-affected gingival tissues compared with healthy controls was confirmed by qRT-PCR analyses. Since NEP is one of the primary enzymes that degrades amyloid beta, increased NEP mRNA levels in periodontitis may act as an inhibitor of amyloid beta accumulation in gingival tissues, balancing increased APP mRNA expression. However, NEP has several effects including degradation of vasoactive substances; therefore, further sresearch is needed.

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1. Introduction

Periodontal diseases such as periodontitis are caused by infection with periodontopathic bacteria. Repeated infection and the host immune response result in periodontal tissue destruction that is also associated with an increased risk of vascular diseases and mortality (Beck, Garcia, Heiss, Vokonas, & Offenbacher, 1996; DeStefano, Anda, Kahn, Williamson, & Russell, 1993; Grau et al., 2004; Wu et al., 2000). According to the World Health

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http://dx.doi.org/10.1016/j.archoralbio.2017.03.003 0003-9969/© 2017 Elsevier Ltd. All rights reserved. Organization, approximately 5–20% of older adults (aged \geq 65 years) suffer from severe forms of periodontal disease, which if untreated can result in tooth loss (Petersen, Bourgeois, Ogawa, Estupinan-Day, & Ndiaye, 2005). Patients with Alzheimer's disease (AD) and those with periodontal diseases often share common characteristics, including advanced age and chronic inflammation, though few studies have examined the relationship between oral health in early life and AD late in life (Gatz et al., 2006; Kamer et al., 2008; Kondo, Niino, & Shido, 1994; Stein, Desrosiers, Donegan, Yepes, & Kryscio, 2007). AD is a neurodegenerative disorder that causes degenerative change, including descent of cognitive function and memory impairment, and is the most common type of dementia. It was reported that the estimated prevalence of dementia was 5.2% and the number of people with dementia was 46.78 million in the world. The prevalence of AD was estimated



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around 60-70% of dementia, which was shown to increase with aging. Moreover, the population of dementia is expected to increase exponentially in future (Alzheimer's Disease International, 2015). AD is characterized by a variety of pathological features, such as extracellular senile plaques mainly composed of amyloid beta peptide $(A\beta)$, intracellular neurofibrillary tangles, synaptic loss and brain atrophy (Forman, Trojanowski, & Lee, 2004; Hardy & Selkoe, 2002; Selkoe, 2001). Inflammation is a crucial process in atherosclerosis and cardiovascular disease and believed to play a major role in both AD and periodontitis (Kamer et al., 2008; Rogers, 2008; Watts et al., 2008). Chronic inflammation, as measured by serum inflammatory markers including interleukin (IL)-1, IL-6, IL-10, tumour necrosis factor (TNF)- α , C-reactive protein, alpha 1antichymotrypsin, intracellular adhesion molecule-1 and vascular cell adhesion molecule-1, is associated with an increased risk of cognitive decline (Weaver et al., 2002; Yaffe et al., 2003) and dementia (Tan et al., 2007) as well as periodontitis (Nakajima & Yamazaki, 2009). The relationship between periodontitis and AD was suggested that periodontitis is a potential risk of reading AD. The pathogens and virulence factors of periodontitis and inflammatory mediators may cause persistent inflammation in the brain resulting neurological disorder (Gaur & Agnihotri, 2015; Watts et al., 2008). In animal experiment, tooth loss due to periodontitis cause mastication disorders and malnutrition, which accelerates brain memory impairment (Yamazaki, Wakabayashi, Kobayashi, & Suzuki, 2008). In contrast, the onset and progression of AD cause a decrease in manual dexterity and difficulty of oral self-plaque control leading to poor sanitation. AD patients also have a difficulty in consultation of dental clinic and reception of dental care. These are able to be contribution factors of the cause of periodontitis (Gaur & Agnihotri, 2015; Stein et al., 2007; Watts et al., 2008). Very recently, a systematic review with meta-analysis revealed that the presence of periodontal disease was epidemiologically associated with the presence of AD (Odds ratio: OR 1.69, 95% CI 1.21-2.35), when only severe form of periodontitis was evaluated, it was more significantly associated (OR 2.98, 95% CI 1.58–5.62) (Leira et al., 2017). Thus, periodontitis and AD could be related bidirectionally from the reasons above. However, whether periodontal disease is a factor preceding dementia is not clear. In addition, little biological evidence is available regarding the relationship between periodontitis and AD.

We previously reported differential gene expression profiles in periodontitis-affected gingival tissues compared with healthy gingival tissues using microarray analyses (Abe et al., 2011). Fifteen significantly increased pathways, including the AD pathway, and four significantly decreased pathways were found (Abe et al., 2011). Using microarray pathway frequency analyses and quantitative real-time reverse transcription polymerase chain reaction analysis (qRT-PCR), we found that components of the AD pathway were significantly elevated in inflamed human gingival tissues obtained from patients with generalized chronic periodontitis, including amyloid beta (A4) precursor protein (APP), a key gene in AD (Kubota et al., 2014).

A β accumulation, which is produced by amyloidogenic APP processing, is considered to be one of the principal causes of AD. An A β degradation enzyme, neprilysin (NEP), plays a key role in regulation of A β . NEP is an 85–110 kDa zinc-dependent membrane metalloprotease (also known as enkephalinase, neutral endopeptidase, common acute lymphoblastic leukaemia antigen and CD10) (Brown, Greaves, Lister, Rapson, & Papamichael, 1974; Letarte et al., 1988; Schwartz et al., 1980). NEP is reported to be widely expressed in several tissues and cells including human buccal mucosal epithelium, skin and lung fibroblasts and neutrophils in blood (Braun, Martin, Ledbetter, & Hansen, 1983; Connelly, Skidgel, Schulz, Johnson, & Erdös, 1985; Johnson, Ashton, Schulz, & Erdös, 1985; Kinoshita, Awano, Yoshida, Soh, & Ansai, 2013). We re-

analysed our previously reported microarray data (Abe et al., 2011) and found a tendency of increased NEP transcription levels in periodontitis-affected gingival tissues. To our knowledge, no quantitative analysis of NEP in periodontitis-affected gingival tissues has been reported. In addition, the cell type responsible for NEP expression in gingival tissues has not been determined.

The aim of the present study is to analyse the mRNA levels of APP and NEP using qRT-PCR and characterise the localisation of NEP protein in periodontitis-affected gingival tissues using immunohistochemistry (IHC). Simultaneous analysis of APP and NEP mRNA levels is valuable for consideration of their regional functions in gingival tissues.

2. Materials and methods

2.1. Participants

The study was approved by the regional ethics committee of the Faculty of Dentistry, Niigata University (27-R9-6-11), and all participants provided written informed consent prior to participating in the study. A total of 36 individuals were recruited from patients attending Niigata University Medical & Dental Hospital, Niigata, Japan. All participants were systemically healthy Japanese individuals, did not have diabetes, were not pregnant, were not current smokers and had taken no systemic antibiotics or antiinflammatory drugs within the previous 6 months. Women accounted for 41.7% of participants. Eighteen patients with generalised severe chronic periodontitis who had received conventional periodontal treatment more than 4–8 weeks before the study were selected (group P). Another 18 individuals who were clinically periodontally healthy and had no history of periodontal disease, impacted teeth or severe dental caries were also enrolled (group H) (Table 1). The classification of American Academy of Periodontology was used as the definition to classify periodontal patients in this study (Armitage, 1999).

2.2. Collection of gingival tissue samples

Gingival tissue sampling for qRT-PCR and IHC analyses was performed as previously described (Kubota, Nomura, Takahashi, & Hara, 1996; Kubota, Matsuki, Nomura, & Hara, 1997; Nakasone et al., 2009). A total of 18 periodontitis and 18 clinically healthy gingival tissue samples were harvested. Diseased sites showed bleeding on probing, a gingival index of ≥ 2 , a probing pocket depth

 Table 1

 Clinical characteristics of study participants.

	Group H (n = 18)	Group P (n = 18)
Gender (M:F)	11:7	10:8
Age (years)	$\textbf{27.5} \pm \textbf{4.3}$	$\textbf{62.9} \pm \textbf{11.0}$
GI	0.0 ± 0.0	2.0 ± 0.0
BOP	_	+
mean percentage of sites with BOP (%)	0.0	100.0
PPD mean \pm SD (mm) 5 mm (n/%) 6 mm (n/%) \geq 7 mm (n/%)	$\begin{array}{c} 2.2 \pm 0.8 \\ 0/0.0 \\ 0/0.0 \\ 0/0.0 \\ 0/0.0 \end{array}$	7.1 ± 2.2 2/11.1 8/44.4 8/44.4
CAL		
$mean \pm SD (mm)$	2.2 ± 0.8	7.4 ± 2.8
5 mm (n/%)	0/0.0	2/11.1
6 mm (n/%)	0/0.0	4/22.2
\geq 7 mm (n/%)	0/0.0	9/50.0

GI: gingival index, BOP: bleeding on probing, PPD: probing pocket depth, CAL: clinical attachment level, Values are presented as mean \pm standard deviation.

(PPD) of \geq 4 mm and clinical attachment level (CAL) of \geq 4 mm. Healthy sites had a PPD of \leq 3 mm with neither CAL nor gingival inflammation. The periodontitis-affected and healthy gingival (connective and epithelial) tissue samples were obtained during periodontal flap surgery and tooth extraction as previously described (Kubota et al., 2008). Immediately after surgery, half of the obtained samples were fixed for histological examination, while the other half were placed in 10 vol (10 ml reagent per 1 mg tissue) of RNAlater RNA Stabilisation Regent (Qiagen, Valencia, CA, USA) and stored at 4 °C for 24 h.

2.3. RNA extraction

The samples were thoroughly homogenised using a tissue homogeniser, and total RNA was isolated using 1 l of RNAiso[®] Plus (TaKaRa Bio Inc., Otsu, Japan). Two hundred millilitres of chloroform and 500 ml of isopropanol were added to the solution, and RNA was precipitated by centrifugation. The pellet was dissolved in 20 ml of RNase-free distilled water (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the concentration of the solutions was measured using a GENESYSTM 10S UV–vis spectro-photometer (Thermo Fisher Scientific Inc.).

2.4. cDNA synthesis

RNA was reverse transcribed into cDNA using Transcriptor Universal cDNA Master (Roche Diagnostics, Tokyo, Japan) and a Veriti[®] 60-well Thermal Cycler (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions and stored at -20 °C until use.

2.5. qRT-PCR

qRT-PCR analyses were carried out utilising EagleTaq Universal Master Mix (Roche Diagnostics) and a LightCycler[®] 96 System (Roche Diagnostics) as previously described (Kubota et al., 2008). The PCR primers for the target genes were as follows: APP (Hs01552283_ml) and NEP (Hs00153510_m1) (Applied Biosystems Inc., Foster, CA, USA). The qRT-PCR conditions were as follows: preincubation at 95 °C for 60 s, followed by 45 cycles of shuttle heating at 95 °C for 10 s and 60 °C for 30 s. All PCRs were run in duplicate. The quantity of each mRNA was normalised to that of glyceraldehyde 3phosphate dehydrogenase (GAPDH, Hs02786624_g1), which was amplified with specific endogenous control primers (Predeveloped TaqMan[®] Assay Reagent, Roche Diagnostics, Indianapolis, IN, USA)). The comparative threshold cycle (Ct: the number of PCR cycles necessary to obtain the threshold signal of fluorescence) method was used to quantify the amplified transcripts (Kubota et al., 2008). Sequence detection software (LightCycler[®] 96 version 1.1, Roche Diagnostics) was used to analyse the standards and carry out the quantification.

2.6. Histopathology

Immediately after the surgeries, half of the samples obtained from periodontitis patients were fixed, dehydrated, paraffin embedded and sliced at 5-mm thickness. The degree of inflammation was evaluated on haematoxylin and eosin (HE) stained sections prior to IHC examination as previously described (Tomita et al., 2013).

2.7. Antibodies

A rabbit polyclonal anti-APP antibody (clone RB-9023-P0) was obtained from Thermo Fisher Scientific (Runcorn, UK). A mouse monoclonal antibody against NEP (clone 56C6) was obtained from Leica Biosystems (Newcastle, UK). A mouse monoclonal antihuman CD68 antibody (clone PG-M1) and a mouse monoclonal anti-human neutrophil elastase antibody (clone NP57) were purchased from DakoCytomation (Glostrup, Denmark).

2.8. IHC

Paraffin sections were subjected to IHC analyses for APP. NEP. CD68 (to label macrophages) and neutrophil elastase (to label neutrophils) using the Envision+/HRP system (Dako). For APP and NEP staining, sections were not pretreated. For CD68 staining, sections were treated with 0.2% trypsin (type II, Sigma Chemical Co., St. Louis, MO, USA) in 10 mM Tris-HCl (pH 7.6) for 30 min at 37 °C. The sections were then rinsed in 0.01 M PBS containing 0.5% milk protein (Morinaga Milk Industry Co. Ltd., Tokyo, Japan) and 0.05% Triton X-100 (T-PBS) and subsequently treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activities. After rinsing in T-PBS, sections were incubated with 5% milk protein in T-PBS for 1 h at 37 °C in an incubator to block non-specific binding. The sections were then incubated overnight at 4°C in primary antibodies diluted 1:200 (anti-APP), 1:100 (anti-human neutrophil elastase) and 1:50 (anti-CD68) in T-PBS or undiluted (anti-NEP). After incubation, the sections were rinsed in T-PBS and incubated with polymer-immune complexes (EnVision+, peroxidase, rabbit/ mouse; Dako) for 1 h at room temperature. After rinsing with T-PBS, the sections were treated with 0.025% 3,3'-diaminobenzidine (Dohjindo Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.005% hydrogen peroxide. Finally, the



Fig. 1. Gene expression levels for amyloid beta precursor protein (APP) and neprilysin (NEP), which belong to the Alzheimer's disease pathway. Expression was standardised to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Statistical analysis was performed with the Mann-Whitney *U* test (p < 0.05). Gene expression in group P was significantly higher than that of group H for both APP and NEP.



Fig. 2. Histopathology of human gingival tissue. (a, b) Haematoxylin and eosin (H-E), 100×. The healthy gingival epithelium was flattened without rete ridges (a), while the periodontitis-affected gingival epithelium was thicker with longer rete ridges in the background of inflammatory cell infiltrates and submucosal fibrous tissue (b).

sections were counterstained with haematoxylin. For IHC control studies, the primary antibodies were replaced with pre-immune mouse IgG subclasses (Dako).

2.9. Statistical analysis

Diseased and control gingival tissues were compared using a two-sample paired *t*-test with a significance threshold P value of 0.05. Differences in the mRNA levels between healthy and periodontitis patients were checked with Shapiro-Wilks test and analysed with nonparametric Mann–Whitney *U* test. The null hypothesis was rejected when the two-tailed risk percentage was strictly below 5% (p < 0.05).

3. Results

3.1. APP and NEP mRNA expression in periodontitis-affected gingiva

To confirm our previous microarray results that indicated ADrelated genes were up-regulated with inflammation, APP and NEP mRNA levels were measured using qRT-PCR. APP and NEP mRNA expression levels in group P were significantly higher than that of group H (p = 0.0075 and 0.0003, respectively). Thus, APP and NEP were increased in periodontitis-affected gingival tissues compared with healthy gingival tissues (Fig. 1).

3.2. IHC

In healthy gingival tissues, neither infiltration of inflammatory cells nor epithelial proliferation was recognised (Fig. 2a). Conversely, periodontitis-affected gingival tissues showed infiltration of inflammatory cells, overgrowth of fibrous connective tissue and spindle-shaped cells and epithelial proliferation in the lamina propria (Fig. 2b). Healthy gingival tissues showed no histological findings suggestive of inflammation, while inflammatory cell infiltration was observed in periodontitis-affected gingival tissues, which confirm inflammatory status.

Most inflammatory cells infiltrated the lamina propria around the capillaries (Fig. 3a). APP localisation was observed in the cytoplasm of inflammatory cells (arrow) (Fig. 3b). Additionally, most APP-positive cells were simultaneously CD68-positive and therefore identified as macrophages (Fig. 3c).

Round cells with distinctly lobulated nuclei infiltrated the subepithelial stroma in periodontitis-affected gingiva (Fig. 4a). NEP and neutrophil elastase expression was observed in round cells infiltrating the stroma (arrows) (Fig. 4b and c). Additionally, spindle-shaped cells with elongated nuclei and fibrous tissue were observed (Fig. 5a). NEP expression mainly localised to the cytoplasm of spindle-shaped cells (arrows) (Fig. 5b). NEP-positive cells were identified as neutrophils and fibroblasts in the lamina propria.

4. Discussion

4.1. APP and NEP expression levels

The present study demonstrated that APP and NEP mRNA levels were significantly up-regulated in periodontitis-affected gingival tissues compared with healthy control tissues. This result revealed that the gene expressions of APP and NEP could be associated with periodontitis and increased by inflammation. The simultaneous up-regulation of APP and NEP genes could be explained by the fact



Fig. 3. Immunohistochemical profile for APP in periodontitis-affected gingival tissue. (a) H-E, (b) APP and (c) CD68, 400×. Inflammatory cells were histopathologically scattered in blood vessels in the lamina propria (a). APP was localised in the cytoplasm of most infiltrated inflammatory cells (b). APP (b) and CD68 (c) were similarly localised in most cells in the lamina propria. APP-positive cells in the lamina propria were identified as macrophages (arrows).



Fig. 4. NEP-expressing neutrophils are observed in periodontitis-affected gingival tissue. (a) H-E, (b) NEP and (c) neutrophil elastase, 400×. Inflammatory cells were histopathologically scattered in the lamina propria mucosae (a). NEP was localised in the cytoplasm of most infiltrated inflammatory cells (arrow) (b). NEP (b) and neutrophil elastase (c) were similarly localised in most cells in the lamina propria. NEP-positive cells were identified as neutrophils (arrows).



Fig. 5. NEP-expressing fibroblasts are observed in periodontitis-affected gingival tissue. (a) H-E and (b) NEP, 400×. Spindle-shaped cells were also histopathologically located in the stroma in the lamina propria. NEP was localized in the cytoplasm of most cells (a). NEP-positive cells in the lamina propria were identified as fibroblasts (arrows) (b).

that both genes are increased by regulation of APP intercellular domain, a metabolite of APP, in neurons and fibroblasts (Pardossi-Piquard et al., 2005; von Rotz et al., 2004).

Several studies reported that NEP levels in human brain physiologically decrease with age, irrespective of the presence of dementia (Hellström-Lindahl, Ravid, & Nordberg, 2008; Russo, Borghi, Markesbery, Tabaton, & Piccini, 2005). However, local expression of NEP was increased in periodontitis-affected gingival tissues in the present study. Given that NEP expression in gingival tissues should be down-regulated with age, similar to that in a healthy brain, inflammation in periodontitis might be more attributed to significant increases in APP and NEP gene transcription than ageing.

4.2. APP- and NEP-expressing cells

APP localisation was recognised in macrophages that infiltrated the lamina propria using IHC analysis, which corresponded to the results of our previous report (Kubota et al., 2014). Macrophages, which are immunocompetent cells, infiltrate tissues during the acute stage of inflammation and are involved in the production of matrix metalloproteinases such as collagenases (Kubota et al., 1996) and inflammatory cytokines such as IL-1 β (Matsuki, Yamamoto, & Hara, 1993) and modulate immune responses. Thus, the expression of APP could be up-regulated by inflammation and involved in the pathological condition of periodontitis.

NEP expression was detected in neutrophils and fibroblasts in the subepithelial area in periodontitis-affected gingival tissues, while NEP expression was also demonstrated in neutrophils isolated from peripheral blood (Braun et al., 1983; Connelly et al., 1985) and skin and lung fibroblasts (Johnson, Ashton et al., 1985; Xie et al., 2011). One of the functions of NEP is to degrade substance P (Skidgel, Engelbrecht, Johnson, & Erdös, 1984; Xie et al., 2011) and bradykinin (Erdös & Skidgel, 1989) and directly inactivate IL-1 β (Pierart, Najdovski, Appelboom, & Deschodt-Lanckman, 1988), which are molecules known to be crucial in inflammation, including periodontitis.

Substance P, a neuromessenger involved in the transmission of pain signals, influences the proliferation, activation and motility of keratinocytes and fibroblasts, which regulate the secretion of proinflammatory cytokines including IL-1 β from macrophages (Li et al., 2008). Given that NEP degrades and inactivates substance P and bradykinins (Matsas, Kenny, & Turner, 1984), NEP may be involved in the impairment of pain sensation in chronic periodontitis. Interestingly, IL-1 β is reported to induce exaggerated substance P production as well as NEP expression in fibroblasts (Xie et al., 2011). Since NEP degrades IL-1 β , a major cytokine in inflammation, up-regulated NEP expression in periodontitis-affected gingival tissues in acute to chronic stages of inflammation appears to contribute to the regulation of inflammation by controlling IL-1 β , substance P and bradykinins.

Recently, the up-regulation of NEP expression in buccal mucosal epithelium from periodontitis patients was reported (Kinoshita et al., 2013). This study is in agreement with our result that the severity of periodontal diseases may be associated with the expression of metalloendopeptidase genes including NEP. Therefore, NEP may act as a defence mediator in the inflammatory response in the oral mucosa as well as inflamed gingival tissues

during periodontitis. Hence, NEP expression in gingival tissue appears to be associated with the severity of periodontitis and may play a protective role.

4.3. The relationship between NEP and systemic status

The increased expression of NEP in periodontitis-affected gingival tissue may have an influence on systemic status including blood pressure. It was reported that continuous damage during adult respiratory distress syndrome (ARDS) leads to the leakage of NEP into the bloodstream (Johnson, Ashton et al., 1985). NEP was detected in alveolar epithelial cells and fibroblasts in the terminal airway and high levels of NEP were detected in the serum of patients with ARDS. The dislocation of NEP is believed to disrupt normal peptide metabolising functions and contribute to pathologic changes in the systemic circulation (Johnson, Coalson, Ashton, Larumbide, & Erdös, 1985). In the present study, NEP expression was found in fibroblasts in chronically inflamed periodontitis-affected gingival tissue. Because NEP can be readily disengaged from cell membranes and enter into circulation, excessive NEP concentrations in the bloodstream may cause a systemic influence on circulation including functional discordance of peptide metabolism and promotion of pathologic changes.

NEP also degrades several vasoactive peptides including natriuretic peptides (NPs) (Daniels & Maisel, 2007), which regulate blood pressure (Bavishi, Messerli, Kadosh, Ruilope, & Kario, 2015; Daniels and Maisel, 2007; McMurray et al., 2014; Solomon et al., 2012). Hence, NEP is an important enzyme in the control of blood pressure. Considering the up-regulated NEP expression in gingival tissues with inflammation, periodontitis could be pathologically influenced by systemic conditions including hypertension and cardiovascular disease (Beck and Offenbacher, 2005; Martin-Cabezas et al., 2016; Mattila et al., 1989). Similarly, NEP can degrade circulating Aβ. This finding may help to elucidate the association between periodontitis and systemic disorders including AD.

In summary, elevated NEP expression may act as a defence mediator for inflammatory reactions in gingival tissues, though NEP has numerous substrates and is involved in various complicated pathways. Further, increased NEP may move into the bloodstream and disrupt the normal metabolism of peptides, resulting in pathologic changes in systemic circulation. Therefore, further studies are needed to reveal the relationships between NEP in periodontitis-affected gingival tissues and systemic diseases. The epidemiological association between periodontitis and AD was previously reported (Ide et al., 2016; Stein et al., 2007), however, the pathological mechanism of these diseases is still unclear. A limitation of the study on the association of AD with periodontitis in humans may be ascribed to the difficulty in analysing human brain tissues from both AD and non-AD participants, which is now ethically impossible, however further comparative analysis of serum NEP levels of AD and periodontitis patients should be valuable. Nevertheless, the present study demonstrates a potential overlapping pathological mechanism of AD and periodontitis.

5. Conclusion

APP and NEP genes were simultaneously up-regulated in periodontitis-affected gingival tissues compared with healthy gingival tissues. APP expression was confirmed in macrophages in inflamed gingival tissues. Additionally, we revealed for the first time that NEP was expressed in periodontitis-affected gingival tissues, primarily in neutrophils and fibroblasts. Moreover, the finding that NEP expression was up-regulated with APP expression by inflammation suggested that NEP is involved in periodontitis pathogenesis and immune responses.

Competing interests

The authors report no conflicts of interest related to this study.

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Ethical approval

The protocol in this study approved by the Institutional Review Board of the Faculty of Dentistry, Niigata University.

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