Possible Involvement of Palmitate in Pathogenesis of Periodontitis

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Type 2 diabetes (T2D) is characterized by decreased insulin sensitivity and higher concentrations of free fatty acids (FFAs) in plasma. Among FFAs, saturated fatty acids (SFAs), such as palmitate, have been suggested to promote inflammatory responses. Although many epidemiological studies have shown a link between periodontitis and T2D, little is known about the clinical significance of SFAs in periodontitis. In this study, we showed that gingival fibroblasts have cell-surface expression of CD36, which is also known as FAT/fatty acid translocase. Moreover, CD36 expression was increased in gingival fibroblasts of high-fat diet-induced T2D model mice, compared with gingival fibroblasts of mice fed a normal diet. DNA microarray analysis revealed that palmitate increased mRNA expression of pro-inflammatory cytokines and chemokines in human gingival fibroblasts (HGF). Consistent with these results, we confirmed that palmitate-induced interleukin (IL)-6, IL-8, and CXCL1 secretion in HGF, using a cytokine array and ELISA. SFAs, but not an unsaturated fatty acid, olate, induced IL-8 production. Docosahexaenoic acid (DHA), which is one of the omega-3 polyunsaturated fatty acids, significantly suppressed palmitate-induced IL-6 and IL-8 production. Treatment of HGF with a CD36 inhibitor also inhibited palmitate-induced pro-inflammatory responses. Finally, we demonstrated that Porphyromonas gingivalis (P.g.) lipopolysaccharide and heat-killed P.g. augmented palmitate-induced chemokine secretion in HGF. These results suggest a potential link between SFAs in plasma and the pathogenesis of periodontitis.

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Materials and Methods
Reagents
Reagents were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. Sulfosuccimidyl oleate sodium (SSO), which specifically binds to cell surface CD36 (Campbell et al., 2004), was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). SSO was dissolved in dimethyl sulfoxide (DMSO). TRITON X-100 was purchased from MP Biomedicals (Aurora, OH). P.g. LPS (tlrl-pglps), HKPG (tlrl-hkpg), and LPS-RS Ultrapure (tlrl-prslps) were purchased from InvivoGen (San Diego, CA). For cytotoxicity analysis, we used an “In Vitro Toxicology Assay Kit, Lactic Dehydrogenase (LDH) based” (TOX7). A stock solution of FFA was prepared, conjugated with FFA-free bovine serum albumin (BSA), and checked for LPS contamination as described previously with modification (Cousin et al., 2001; Shikama et al., 2013; Shikama et al., 2015). Briefly, a 100 mM FFA stock solution of palmitic acid, stearic acid, and oleic acid was prepared by dissolving them in 0.1 M NaOH. A 100 mM FFA stock solution of docosahexaenoic acid (DHA) was prepared by dissolving it in ethanol. The 100 mM FFA solution was mixed with 10% BSA solution to obtain a 5 mM FFA working solution. The 100 mM FFA solutions were prepared just before examinations, and the working solution was sterile filtered through a 0.2 μm pore size membrane filter and used immediately. Cells were treated with FFAs and/or bacterial components with 1% BSA and without fetal bovine serum (FBS). For control incubation, 1% BSA was used.

Cells and cell culture
THP-1 human acute monocytic leukemia cells (TIB-202) were purchased from American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium supplemented with 10% FBS. A Simian virus-40 (SV40) antigen immortalized gingival epithelial cell line (OBA-9), which was generated by Prof. Shinya Murakami (Kusumoto et al., 2004), was maintained in Defined Keratinocyte-SFM (Gibco, Carlsbad, CA). Normal human lung fibroblasts (HFL-III) were provided by the Japanese Collection of Research Bioresources Cell Bank. HGF was obtained from a healthy subject using standard explant techniques (Schor et al., 1985). Gingival tissues were obtained from patients undergoing routine dental surgery in the Department of Oral Surgery (Hiroshima University Hospital), after obtaining approval from the Ethical Committee of Hiroshima University Hospital. Informed consent was obtained from the subjects. A population doubling level of HGF of less than 25 was used in this study. HGF and HFL-III were maintained in DMEM supplemented with 10% FBS.

Mice
Twelve-week-old male C57BL/6j mice and diet-induced obesity (DIO) mice which had been fed a commercial high-fat diet from 4 weeks of age were obtained from Charles River Japan (Kanagawa, Japan). DIO mice were used as a mouse model of T2D (Collins et al., 2004; Petro et al., 2004). Mice were maintained in...
accordance with the principles and guidelines established by the University of Tokushima.

RNA isolation
Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Total RNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, NC), and cDNA was synthesized with a first-strand cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN) from 1 mg of total RNA for RT-PCR. For DNA microarray, RNA quality was determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

RT-PCR
Primers were as follows: (CD36) forward, 5'-GAGAAGTTATGGGCTAT-3', and reverse, 5'-TCCAAGGAGGCAAAAGG-3'; (TLR4) forward, 5'-AGGATCTGGAGGCT-3', and reverse, 5'-GAAATGGAGGCACCCCTT-3'; (GAPDH) forward, 5'-GCCACATCGCTCAGACAC-3', and reverse, 5'-GAGAAGTTATGGGCTAT-3'. PCR products were analyzed by agarose gel electrophoresis, and bands were visualized using a LAS-3000 UV Lumino-image analyzer (Fujifilm, Tokyo, Japan).

Gene expression profiles
Gene expression profiles were analyzed by Hokkaido System Science (Hokkaido, Japan) using a SurePrint G3 Human GE Microarray kit 8 × 60K (Agilent Technologies). Statistical analysis after data acquisition and normalization of expression data was performed using GeneSpring GX 12.1 (Agilent Technologies), and per-chip normalization to the 75th percentile was performed. After this normalization, extremely low intensity probes were excluded, and differentially expressed genes were identified by applying a cut-off of ≥1.5-fold. The microarray data will be available from the Gene Expression Omnibus database under accession number GSE62761 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62761).

Cell viability assay
One day before the experiment, HGF were seeded in a 96-well flat-bottomed plate (OrangeScientific, Braine-l’Alleud, Belgium) at 2 × 10^4 cells/well. After treatment of HGF with 25 μM SSO in DMEM with 1% BSA for 1 h, cell viability was evaluated by the mitochondrial conversion of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) to a formazan using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).
Japan) according to the manufacturer’s instructions. Formazan was quantified using a microplate reader at absorbance 450 nm (BioRad, Hercules, CA).

Cytokine array
The profile of 36 cytokines secreted by Pal-stimulated HGF was analyzed using a human cytokine array kit (ARY005; R&D Systems, Minneapolis, MN). HGF (3 × 10^5 cells/ml/well in 6-well plate) were stimulated with 1% BSA or 100 μM Pal for 36 h, and cell culture supernatants were collected and analyzed according to the manufacturer’s instructions. Chemiluminescent membrane images were captured with a LAS-3000 UV Lumino-image analyzer (Fujiﬁlm). Densitometric analysis was performed with ImageJ software.

Detection of CD36 and GAPDH expression by Western blot
After treatment, cells were washed twice with ice-cold PBS, and RIPA buffer (Thermo Scientiﬁc) with 1% protease inhibitor cocktail was added. The lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated with antibodies against CD36 (#14347) or GAPDH (#2118) (Cell Signaling Technology, Danvers, MA). Immunoblotting was performed using an ECL Plus system (GE Healthcare, Piscataway, NJ) according to the manufacturer’s instructions. Blots were visualized using an LAS-3000 UV Lumino-image analyzer (Fujiﬁlm).

Measurement of IL-6, IL-8, and CXCL1 in culture supernatants by ELISA
Cells were seeded into 96-well plates and incubated overnight. Details are given in the ﬁgure legends. The amounts of IL-6, IL-8, and CXCL1 in the culture supernatant was analyzed using an 1% TRITON X-100-treated samples as positive controls. LDH release is shown as a percentage of that of triton X-100-treated samples. The results are expressed as mean ± SD from triplicate cultures. (D) Effects of DHA on Pal-induced IL-6 and IL-8 secretion. HGF were treated with 100 μM Pal in combination with DHA at the indicated concentrations for 48 h. The concentrations of IL-6 and IL-8 in the medium were determined by ELISA. The results are expressed as mean ± SD of triplicate cultures. "P < 0.01 versus 100 μM Pal group (Dunnett’s multiple-comparison test). Experiments were performed three times to conﬁrm the reproducibility of the results.

Detection of cell-surface CD36 expression by ﬂow cytometry
After treatment, cells were detached with Accutase, incubated for 10 min with TruStain FcX (BioLegend, San Diego, CA), and then stained with PE mouse IgG2a (400211; BioLegend) or PE anti-human CD36 (BD Biosciences). Membranes were incubated with antibodies against CD36 (#14347) or GAPDH (#2118) (Cell Signaling Technology, Danvers, MA). Immunoblotting was performed using an ECL Plus system (GE Healthcare, Piscataway, NJ) according to the manufacturer’s instructions. Blots were visualized using an LAS-3000 UV Lumino-image analyzer (Fujiﬁlm).
CD36 antibody (336205; BioLegend). Expression was detected using a FACSVerse and analyzed with FACSuite (BD Biosciences).

Sample preparation for immunohistochemical (IHC) staining

Archived paraffin-embedded tissue specimens from patients with inflammatory gingival polyps used for IHC analysis were obtained from the Division of Pathology, Tokushima University Hospital. We also used mouse tissues obtained from 3 C57BL/6J mice and 3 DIO mice for IHC analysis. After mice were anesthetized and killed, tissues were fixed in 10% formalin, followed by decalcification with KCX. Fixed tissues were embedded in paraffin and cut into 4.5-μm-thick sections. For IHC staining, tissue sections were deparaffinized in xylene, and rehydrated in descending grades of ethanol. Endogenous peroxidase activity was blocked with methanol containing 0.3% H2O2 for 30 min. Microwave pretreatment in citrate buffer was performed for 10 min, three times, to retrieve the antigenicity. Then the sections were treated with a monoclonal anti-CD36 antibody (ab17044; Abcam, Cambridge, MA) for human gingival tissue or a polyclonal anti-CD36 antibody (ab78054; Abcam) for murine periodontal tissue at 4°C overnight. After incubation with secondary antibody, the reaction was detected by diaminobenzidine (Dako, Tokyo, Japan) staining. Then the sections were counterstained with hematoxylin, dehydrated in ascending grades of ethanol, and finally mounted on slides. CD36 staining was evaluated as the percentage of stained gingival fibroblasts within areas of gingival connective tissue. The percentage of CD36-positive gingival fibroblasts was determined by counting the cells in three randomly selected fields in each mouse under a microscope at ×100 magnification.
Statistical analysis
The statistical significance of differences was evaluated by Student’s unpaired t test or Dunnett’s multiple-comparison test after analysis of variance (ANOVA) using IBM SPSS Statistic software 19.0 (IBM). Values of $P < 0.05$ were considered significant.

Results
Histological analysis of CD36 expression in human gingival tissue and murine periodontal tissue
First, using IHC staining, we examined whether gingival and periodontal tissues express the CD36 molecule. In human gingival tissues, CD36 expression was observed in gingival epithelial cells, gingival fibroblasts, and vascular endothelial cells (Fig. 1A). Next, to clarify the involvement of T2D in CD36 expression of cells composing the periodontium, periodontal tissues of control mice and DIO mice (Collins et al., 2004; Petro et al., 2004) were also stained with CD36 antibody. CD36 was expressed not only in gingival epithelial cells and fibroblasts, but also in periodontal ligament fibroblasts. Interestingly, the percentage of CD36-positive gingival fibroblasts in DIO mice was significantly higher than that in control mice (Fig. 1B and C). These data suggest that Pal may induce pro-inflammatory responses in periodontal tissues, and that periodontal tissues of patients with obesity-induced insulin resistance may be sensitive to Pal-induced pro-inflammatory responses.

IL-6 and IL-8 secretion were induced by Pal treatment in HGF, but not in gingival epithelial cells
We next examined whether HGF and gingival epithelial cells in culture express CD36 mRNA and cell-surface CD36 by RT-PCR and flow cytometry, respectively. It was found that HGF expressed CD36 mRNA at a similar level to that observed in THP-1 cells, whereas it was weakly expressed, if at all, in OBA-9 cells (Fig. 2A). In flow cytometric analysis, cell-surface expression of CD36 was detected in HGF, but not in OBA-9 cells (Fig. 2B). CD36 is a transmembrane glycoprotein consisting of 472 amino acids with a predicted molecular weight of approximately 54 kDa (Van Nieuwenhoven et al., 1998; Hoosdally et al., 2009). However, the molecular weight of cell-surface and functional CD36 is more than 77 kDa, depending on the cell type and on posttranslational glycosylation (Greenwalt et al., 1990; Tang et al., 1994). Western blotting analysis revealed that the glycosylated CD36 protein was detected in HGF, but not in OBA-9 cells (Fig. 2C). Together with the results shown in Figure 1, these results suggest that CD36 is functionally expressed on the cell surface in HGF, but is only expressed intracellularly in gingival epithelial cells. Consistent with these
results, although Pal treatment at levels as high as 500 μM failed to induce IL-6 and IL-8 secretion in OBA-9 cells (Fig. 2D), it dose-dependently induced both IL-6 and IL-8 secretion in HGF (Fig. 2E). To examine whether cell-surface expression of CD36 and Pal-induced IL-6 and IL-8 secretion are general characteristics of fibroblasts, we re-examined these findings using another human fibroblasts (HFL-III). Although HFL-III also expressed CD36 mRNA, they did not show cell-surface expression of CD36 (Appendix Fig. 1A and B). Although cell-surface expression of TLR4, which is known to be involved in Pal-induced pro-inflammatory responses (Maloney et al., 2009; Eguchi et al., 2012), has been reported in HGF (Hatakeyama et al., 2003; Kiji et al., 2007), its mRNA was hardly expressed in HFL-III compared with that in HGF (Appendix Fig. 1C). Moreover, Pal stimulation did not induce IL-6 or IL-8 secretion in HFL-III (Appendix Fig. 1D). These results suggest that HGF are susceptible to the Pal concentration in plasma, which would lead to pro-inflammatory cytokine secretion.

SFA-induced IL-8 secretion by HGF and its inhibition by DHA

We next investigated whether the ability to induce IL-8 secretion by HGF would depend on the types of FFAs. Treatment with either Pal or Ste, but not with oleate (Ole), which is an unsaturated fatty acid, significantly induced IL-8 secretion. Moreover, the amount of Pal-induced IL-8 secretion was about 3.5-fold greater than that with Ste stimulation (Fig. 3A). Pharmacological inhibition of p38 mitogen-activated protein kinase (MAPK) or nuclear factor-κB (NF-κB) significantly suppressed Pal-induced IL-6 and IL-8 secretion (Fig. 3B). Recently, it was reported that supplementation of DHA, which is an omega-3 polyunsaturated fatty acid, improved periodontal outcomes in people with periodontitis (Naqui et al., 2014), and resolvin D1, which is a lipid mediator derived from DHA, reduced P. g-induced chemokine secretion in HGF (Khaleed et al., 2013). Thus, we examined the effects of DHA on Pal-induced IL-6 and IL-8 secretion. We first confirmed that 10 μM DHA treatment had no cytotoxic effect (Fig. 3C), and then showed that DHA dose-dependently inhibited Pal-induced IL-6 and IL-8 secretion in HGF (Fig. 3D). These data suggest that SFAs in plasma may increase IL-6 and IL-8 production through activation of p38 MAPK and NF-κB in periodontal tissues, and DHA supplementation may improve periodontitis and gingival inflammation in patients with T2D.

Pal-induced gene expression and involvement of CD36 molecule in Pal-induced IL-6, IL-8, and CXCL1 production in HGF

As Pal induces various cytokines and chemokines in vivo and in vitro (Haavsen et al., 2009; Eguchi et al., 2012), we next used DNA microarray analysis to investigate the increased expression of genes by Pal stimulation in HGF. Cytokine (IL5, IL6, IL12,4), chemokine (Cxcl1, Cxcl2, Cxcl26), and cytokine receptor (Ii17r, Il12r) genes were up-regulated compared with controls (Fig. 4A). Moreover, protein arrays revealed that secretion of not only IL-6 and IL-8, but also CXCL1 was increased by Pal stimulation in HGF (Fig. 4B). To evaluate their secretion more quantitatively, we performed ELISA analysis. Pal stimulation time-dependently and significantly induced IL-6, IL-8, and CXCL1 secretion compared with the control group (Fig. 4C).

As CD36 has established roles in the endocytic uptake of endogenous ligands such as FFAs (Campbell et al., 2004), oxidized phospholipids, and amyloid proteins (Moore and Freeman, 2006), and CD36-mediated signaling has also been implicated in the pro-inflammatory effects of these ligands (Stewart et al., 2010), we next investigated whether CD36 is involved in the Pal-induced pro-inflammatory response in HGF. A CD36-specific inhibitor, SSO, significantly inhibited Pal-induced IL-6, IL-8, and CXCL1 secretion (Fig. 4D), though SSO at this concentration had no effect on viability of HGF (Fig. 4E). These data suggest that Pal in plasma may induce cytokines and chemokines associated with the CD36 molecule in gingival fibroblasts, resulting in exacerbation of inflammation in periodontitis.

Augmentation of Pal-induced chemokines by P.g. LPS and HKPG

Finally, to elucidate synergistic effects of P.g. on Pal-induced chemokine secretion, we examined IL-8 and CXCL1 production in HGF stimulated with Pal in combination with P.g. LPS or HKPG. In this experiment, P.g. LPS alone did not induce IL-8 secretion in HGF due to lack of FBS (Appendix Fig. 2). P.g. LPS and HKPG augmented Pal-induced IL-8 secretion (Fig. 5A), and P.g. LPS augmented Pal-induced CXCL1 secretion (Fig. 5B), in HGF. These data indicate that Pal in plasma may potentially exacerbate the pathogenesis of periodontitis.

Discussion

The present study demonstrated that Pal is an endogenous ligand that induces pro-inflammatory responses in HGF. Since fibroblasts are the major constituent of gingival connective tissue and are capable of producing various pro-inflammatory cytokines, it has been speculated that gingival fibroblasts actively participate in the inflammatory processes associated with periodontal diseases (Takada et al., 1991; Kusumoto et al., 2004). However, at present there is no evidence on whether Pal level in plasma is correlated with the pathogenesis of periodontitis. Through further studies, we would like to obtain evidence that a high plasma level of Pal exacerbates the pathogenesis of periodontitis.

Although epidemiological studies have demonstrated an association between obesity-related metabolic disorders and periodontitis (Nelson et al., 1990; Saito et al., 1998), the molecular mechanisms are poorly understood. Several in vitro studies showed that a high-glucose condition induces pro-inflammatory cytokines in HGF (Jiang et al., 2012), and high-glucose treatment increases the invasive ability of P.g. in HGF (Chang et al., 2013). Moreover, in vivo analysis recently revealed that a high-fat diet exacerbates alveolar bone loss induced by Aggregatibacter actinomycetemcomitans (another major periodontopathogen) infection (Chen et al., 2014). Given these reports and our findings in this study, we speculate that not only a high glucose concentration but also an abnormal lipid profile in plasma may exacerbate the pathogenesis of periodontitis. Pal concentration in plasma of patients with T2D was significantly higher than that of control subjects, with mean nocturnal values of 127 ± 13 and 80 ± 10 μM, respectively (Isley et al., 2006). In this study, we mainly used 100 μM Pal, which is a reasonable concentration to reproduce the pathophysiological concentration in the plasma of T2D patients, to induce IL-6, IL-8, and CXCL1 production in HGF.

As described in the Introduction, several papers have shown that SFAs induce inflammatory responses via TLR-dependent (Maloney et al., 2009; Snodgrass et al., 2013) and -independent (Anderson et al., 2012) signaling. In this study, a TLR4 inhibitor dose-dependently inhibited Pal-induced IL-6 and IL-8 secretion (Appendix Fig. 3), suggesting involvement of TLR4 in Pal-induced pro-inflammatory responses in HGF. Previous studies have shown anti-inflammatory effects of DHA (Oh et al., 2010;
Boden G. 1997. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Phipps et al., 1997. It is also reported that subsets on the basis of morphological features, size, and molecule expression. This study, we could not distinguish HGF by their expression in HGF (Appendix Fig. 4). This result implies suppression of CD36 expression (Pietsch et al., 1995; Jiang SY, Wei CC, Deng JY. 2012). High glucose induces inflammatory cytokine and chemokine expression, via TLR4 and T2D model mice. In HGF, but not in human gingival epithelial cells. Palmitate induces pro-inflammatory cytokine and chemokine expression, via TLR4 and CD36-mediated pathway. (iv) Co-stimulation with P.g.-infected gingival fibroblasts. This work was supported by a research sponsorship from Tokushima Prefecture, Japan, the Knowledge Cluster from the Ministry of Education, Science, and Culture of Japan (M.F.), and JSPS KAKENHI Grant Number 26462880 (Y.S.).

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Fibroblasts derived from either gingival tissue (HGF) or lung (HFL-III) were distinct in their expression of CD36 and TLR4, and in Pa-induced cytokine production (Fig. 2 and Appendix Fig. 1). It has been shown that fibroblasts from various tissues, such as lung, skin, and periodontium, are heterogeneous and that these cells can be separated into subsets on the basis of morphological features, size, and function (Phipps et al., 1997). It is also reported that these cells can be separated into subsets on the basis of morphological features, size, and function (Phipps et al., 1997). It is also reported that pertussis toxin and β2-adrenergic receptor agonists inhibit the expression of CD36 in human fibroblasts (Riar JF, Ceballos GP, Bonen A. 2004). A novel function for fatty acid translocase (FAT/CD36) in inflammatory response to periodontal pathogen challenge: Implications in acute and chronic infections. Arch Oral Biol 59:1075–1084.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web-site.