Porphyromonas gingivalis Induces Apoptosis and Autophagy via ER Stress in Human Umbilical Vein Endothelial Cells

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It has been reported that periodontitis is associated with an increased risk of atherosclerosis. Accumulating evidence suggests that endothelial dysfunction is an early marker for atherosclerosis. To determine how periodontal infections contribute to endothelial dysfunction, we examined the effect of Porphyromonas gingivalis on human umbilical vein endothelial cells (HUVEC). P. gingivalis significantly suppressed the viability of HUVEC, induced DNA fragmentation and annexin V staining, and increased caspase-3, caspase-8, and caspase-9 activities. P. gingivalis also increased the expression of GADD153 and GRP78 and caspase-12 activity. Further, P. gingivalis induced autophagy, as evidenced by increased LC3-II and Beclin-1 levels. The suppression of P. gingivalis-induced autophagy by silencing of LC3 with siRNA significantly increased P. gingivalis-induced apoptosis. ER stress inhibitor, salubrinal, suppressed apoptosis and autophagy by inhibiting P. gingivalis-induced DNA fragmentation and LC3-II expression. These data suggest that P. gingivalis infection induces ER stress-mediated apoptosis followed by autophagic response that protects HUVEC from P. gingivalis-mediated apoptosis, potentially amplifying proatherogenic mechanisms in the perturbed vasculature.

1. Introduction

Periodontal disease is highly prevalent, affecting up to 90% of the global population [1]. Porphyromonas gingivalis, a major periodontal pathogen, was recently implicated in the pathogenesis of atherosclerosis [2]. P. gingivalis can directly access the systemic circulation and the endothelium in patients with periodontitis, as transient bacteremias are common [3]. Indeed, P. gingivalis has been detected in human atherosclerotic plaques [4, 5], and it can both invade endothelial cells (EC) and persist therein [6]. Further, P. gingivalis elicits a proatherogenic response in EC in the form of increased leukocyte adhesion with concomitant upregulation of adhesion molecules and proinflammatory cytokines and chemokines [7, 8]. Interestingly, these effects require the invasion of EC by viable bacteria [8].

EC are key cellular components of blood vessels that function as a selectively permeable barrier between blood and tissue. It is believed that atherogenic risk factors induce apoptosis in EC, leading to the denudation or dysfunction of the intact endothelial monolayer, with subsequent atherosclerotic lesion formation as a result of lipid accumulation, monocyte adhesion, and inflammatory reactions [9, 10]. Endothelial cell apoptosis has several potential deleterious effects, including plaque erosion and thrombosis [11]. Recent studies have demonstrated increased endoplasmic reticulum (ER) stress protein expression in the vascular cells of atherosclerotic lesions and regulation of the protein in the endothelium by several atherosclerotic stressors [12].

Autophagy is a cellular defense mechanism involving degradation and recycling of cytoplasmic components. Autophagy can protect cells from apoptosis; thus, it is said to sit at the crossroad between cell death and survival. However, excessive autophagy can destroy essential cellular components and cause cell death [13]. Accumulating evidence has also suggested that ER stress is linked to autophagy [14]. In the present study, we examined the capacity of whole, viable P. gingivalis to induce cell death via apoptosis, ER stress, and autophagy in human umbilical vein endothelial cells (HUVEC).
2. Materials and Methods

2.1. Bacterial Strains and Culture Methods. P. gingivalis strain 381 and KDP136 (gingipain-null mutant) were cultured on anaerobic blood agar plates (Becton Dickinson Co., Sunnyvale, CA) in a model 1024 anaerobic system (Forma Scientific, Marietta, OH) under 10% H2, 80% N2, and 10% CO2 for 3–5 days. The cells were then inoculated into brain heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 5 μg/ml hemin and 0.4 μg/ml menadione and cultured to an OD600 of 0.8 (109 cfu/ml). The cells were then centrifuged at 8000 × g for 20 min at 4°C and diluted in phosphate-buffered saline (PBS).

2.2. Cell Line and Reagents. HUVEC (provided by Lonza-Takara, Tokyo, Japan) were cultured at 37°C in a humidified atmosphere with 5% CO2 in endothelial cell culture medium (EGM-2 BulletKit, Lonza-Takara). All experiments were performed on cells at passages 4–8 at approximately 80% confluence.

2.3. Cell Viability Assay and Cytotoxicity Assay. Cell viability was determined using a Cell Counting Kit-8 (CCK-8; Wako, Osaka, Japan). Briefly, cells (1.0 × 105/well) were cultured in 100 μl of endothelial cell culture medium in a 96-well plate and stimulated with P. gingivalis at the indicated multiplicity of infection (MOI). After 19 h, 10 μl of CCK-8 solution was added, and the cells were incubated at 37°C for 2 h, followed by measurement of A450 using a spectrophotometer.

2.4. Measurement of Cell Death. Cellular apoptosis was quantified by DNA fragmentation using the Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Mannheim, Germany). Briefly, HUVEC (5 × 105/dish) were cultured with P. gingivalis 381 and KDP136 strains at the indicated MOI. After 7, 16, and 24 h, the cells were lysed in 200 μl of lysis buffer, and 20 μl of the supernatant was reacted with 80 μl of anti-DNA immunocomplex conjugated with peroxidase, which interacts with streptavidin-coated wells, in a microtiter plate for 2 h. At the end of the incubation, 100 μl of substrate was added, and color development was quantified as a wavelength of 405 nm. The results were calculated as the ratio of the absorbance of the P. gingivalis-treated cells to the absorbance of the nontreated control cells. For the apoptosis inhibition assays, cells were preincubated for 1 h with caspase-12 inhibitor Z-VAD-FMK (MBL, Nagoya, Japan) or ER stress inhibitor, salbrinal (50 μM) (Sigma-Aldrich, St. Louis, MO, USA), before stimulation with the bacteria. Apoptosis of the P. gingivalis-treated HUVEC was assessed by annexin V-EnzoGold and 7-amino-actinomycin D (7-AAD) staining (Enzo Life Sciences Inc., Farmingdale, NY) and flow cytometric analysis using a FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.5. Caspase Assay. After incubation (5 × 105 cells/dish) for 16 h with P. gingivalis 381, the cells were harvested, and the caspase-1, caspase-3, caspase-8, caspase-9, and caspase-12 activities were measured using a caspase fluorometric protease assay kit (MBL). The amount of 7-amino-4-trifluoromethylcoumarine (AFC) released was measured using an ARVO multilabel/luminescence counter with excitation and emission at 400 and 505 nm, respectively. 2.6. Real-Time Quantitative RT-PCR. Quantitative RT-PCR was performed using primers specific for C/EBP homologous protein (CHOP)/growth arrest and DNA damage 153 (GADD153) (GGCAGCTGAGTCATTGCC and GCAGATTCCACCATCGGTCA), glucose-regulated protein-78 (GRP78) (CTCTACGTGTCAAGATCTCCATCC and GTTTCAAATGTCACCATATCAGTCC), Beclin-1 (CCAGATGGTTATGCCCAGAC and CATTCCATCCACGGGAAACAC), microtubule-associated protein 1 light chain 3B (MAP1LC3B) (ACCGATTTGCACATACGTTG and GGACCCTTCAGCTTACATCGTAC), and β-actin (CATCCGTAAAGACCTCATTGCAAC and ATGGAGCCACCGATCCACA). The thermal cycling profile was as follows: 95°C for 10 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, with a final dissociation at 95°C for 5 s, 60°C for 30 s, and 95°C for 15 s. The starting amount of RNA was quantified using a standard curve; fold changes in the expression of CHOP/GADD153, GRP78, Beclin-1, and LC3B relative to β-actin were determined in triplicate.

2.7. Antibodies and Western Blot Analysis. Rabbit antibodies against GADD153, GRP78, Beclin-1, and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), while those against LC-3 were purchased from MBL. Secondary horseradish peroxidase- (HRP-) conjugated goat anti-rabbit and goat anti-mouse antibodies were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Cells were lysed in a buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin, and 2 μg/ml leupeptin (pH 7.4). For immunoblotting, proteins resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), which were then exposed to primary and then secondary antibodies. Chemiluminescence detection was performed with an ECL™ Western Blotting Detection Kit (Amer sham). The signal intensities of the corresponding bands were measured by a Light Capture equipped with a computer software (ATTO, Osaka, Japan).

2.8. siRNA Knockdown. Knockdown of endogenous LC3 with siRNA was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Briefly, HUVEC grown to 50–80% confluence in a 6-well plate were transfected with either LC3B siRNA (sc-43391, Santa Cruz Biotechnology) or control siRNA (sc-37007, Santa Cruz Biotechnology). The final concentration of respective siRNA was 50 nM for each transfection, and the experiments were carried out 24 h after transfection.

2.9. Acridine Orange Staining. Cells were treated with P. gingivalis 381 at an MOI 1:105 for 8 h and stained with 1 ng/ml acridine orange at room temperature for 20 min. Then, cells were washed with PBS and visualized by fluorescence microscopy.
2.10. Statistical Analysis. All data are presented as means ± SEM. Multiple-group comparisons were made by one-way analysis of variance, followed by post hoc intergroup comparison by the Bonferroni-Dunn test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. P. gingivalis 381 Inhibits Cell Proliferation and Induces Cell Death through Apoptosis. P. gingivalis 381 significantly diminished the proliferation of HUVEC at higher MOI (Figure 1(a)). Next, we investigated whether the suppression of cell proliferation observed in HUVEC treated with P. gingivalis 381 was dependent on apoptosis. Incubation with P. gingivalis 381 strongly induced apoptosis in the HUVEC in a dose- and time-dependent manner (Figure 1(b)). Higher apoptosis rates were observed at MOI of 1 : 5 × 10^2 after 16 h and 1 : 10^2 and 1 : 5 × 10^2 after 24 h compared to the non-treated control group. In contrast, KDP 136 strain did not cause DNA fragmentation in any MOI and 24 h culture. In addition, we confirmed the possible apoptotic effect of P. gingivalis 381 by annexin V and 7-AAD staining and flow cytometric analysis (Figure 1(c)). The early apoptotic cells represented by the lower right quadrant (7-AAD negative and annexin V positive) and the nonviable necrotic and late-state apoptotic cells represented by the upper right quadrant (positive for annexin V binding and 7-AAD uptake). After 21 h of treatment with P. gingivalis 381 at an MOI of 1 : 10^2, the number of early-stage apoptotic cells was significantly increased by up to 47.8 ± 2.0% (p < 0.01). These results indicated that the decrease in viable cells induced by P. gingivalis 381 treatment was secondary to apoptosis. Apoptotic cell death typically occurs via the stimulation of caspase activity [15]. Caspase-8 and caspase-9 appear to be activated in the death receptor- and mitochondrial-dependent apoptotic pathways, respectively, and caspase-3, which is common to both pathways, induces DNA fragmentation. Pyroptosis, which is uniquely dependent on caspase-1, has been described in monocytes, macrophages, and dendritic cells infected with a range of microbial pathogens such as Salmonella, Francisella, and Legionella [16]. Therefore, we assessed the activities of caspase-1, caspase-3, caspase-8, and caspase-9. P. gingivalis 381 enhanced caspase-3, caspase-8, and caspase-9 activities, suggesting involvement of the mitochondria-mediated intrinsic pathway and death receptor-induced extrinsic pathway (Figure 1(d)). On the other hand, caspase-1 activity was not enhanced by P. gingivalis 381 within the range of the MOI investigated (data not shown). Therefore, cell death induced by P. gingivalis 381 was unrelated to pyroptosis.

3.2. ER Stress Is Involved in P. gingivalis 381-Induced Apoptosis. To determine whether ER-mediated events contribute to P. gingivalis 381-induced apoptosis, we examined the levels of several substances reported to participate in ER-induced apoptosis and the unfolded protein response (UPR). Real-time PCR and Western blot analysis demonstrated that the ER stress-induced proteins or genes GADD153 and GRP78 were upregulated by treatment with P. gingivalis 381 (Figures 2(a) and 2(b)), although there was not so drastic but still significant difference in GRP78 mRNA level. A significant increase in caspase-12 activity at 21 h after the addition of P. gingivalis 381 was also observed at MOI of 1 : 10 and 1 : 10^2 (Figure 2(c)). Furthermore, DNA fragmentation induced by P. gingivalis 381 infection was significantly suppressed by pretreatment with salubrinal and a caspase-12 inhibitor (Figure 2(d)). P. gingivalis 381-induced caspase-3 and caspase-12 activities were also completely abrogated by pretreatment with salubrinal (Figure 2(e)). These data suggest that the ER stress response functions prior to P. gingivalis 381-mediated apoptosis.

3.3. P. gingivalis 381 Contributes to ER Stress-Induced Autophagy. Autophagy is essential for the removal of damaged organelles and long-lived cytosolic macromolecules to maintain energy homeostasis, and hence cell survival, under starvation conditions. When excessive, however, autophagy results in autophagic cell death. To examine the connection between P. gingivalis 381 infection and the stimulation of autophagy, we examined the expression of Atg6 (Becn1-1) and the colocalization of LC3-II (both are autophagosome markers). Real-time PCR and Western blot analysis demonstrated that LC3-II and Beclin-1 were upregulated by treatment with P. gingivalis 381 (Figures 3(a) and 3(b)). In order to examine if autophagic signaling contributes to the protection against cell death, autophagy (a ratio of LC3-II/LC3-I) was suppressed by siRNA specific for LC3 (Figure 3(c)). P. gingivalis 381 significantly increased apoptosis in autophagy-deficient HUVEC. These results suggest that autophagy induced by P. gingivalis 381 protects HUVEC from apoptosis caused by the same bacteria.

Autophagy is also characterized by acidic vesicular organelle (AVO) formation, which can be assayed by acridine orange staining [17]. AVOs accumulated in the cytoplasm of HUVEC exposed to P. gingivalis 381 (Figure 3(d)); however, this was inhibited by the addition of salubrinal (Figure 3(d)). Furthermore, salubrinal suppressed P. gingivalis 381-induced LC3-II expression (Figure 3(e)). These data suggest that the ER stress response functions prior to autophagy induced by P. gingivalis 381 infection.

4. Discussion

In this study, we found that HUVEC challenge with high doses of P. gingivalis 381 for 21 h significantly exhibited suppression of viability. And it was found that this decrease in viability is cell death caused by endothelial apoptosis as evidenced by DNA fragmentation, annexin V staining, and caspase activity (Figures 1(a) and 1(d)). Furthermore, since the challenge with KDP136 did not induce DNA fragmentation, it was suggested that the apoptosis-inducing factor in P. gingivalis 381 may be caused by gingipain (Figure 1(b)). Caspase-3, caspase-8, and caspase-9 were significantly activated by intermediate high doses of P. gingivalis 381 infection, suggesting that multiple signaling pathways were involved. Therefore, activation of these caspases may
contribute to endothelial dysfunction. Pyroptosis is a more recently recognized form of regulated cell death with morphological and biochemical properties distinct from necrosis and apoptosis [16]. However, the death of the HUVEC in this study was unrelated to pyroptosis since caspase-1 activity was not changed at any P. gingivalis dose examined (data not shown).

In this study, P. gingivalis 381 induced the expression of a number of ER stress markers, including GADD153, GRP78, and caspase-12, indicating that P. gingivalis 381 induces ER stress. The mRNA and protein levels of GADD153 and GRP78 were increased in MOI concentration dependently except for the mRNA level of GADD153, but the caspase-12 activity was maximized at MOI (10) and decreased at MOI (10^2). Because gingipain of P. gingivalis can cleave caspase-3 [18], cleavage of caspase-12 may be occurring at high concentration of MOI. Furthermore, since addition of either an ER stress inhibitor (salburinal) or an anti-caspase-12 reagent significantly decreased P. gingivalis 381-induced apoptosis in HUVEC, ER stress may occur upstream rather than downstream of apoptosis in cells exposed to high doses of P. gingivalis 381. Therefore, the activation of ER stress markers such as GADD153, GRP78, and caspase-12 will likely to happen at an early stage compared with the factors related to apoptosis. Induction of the mammalian UPR involves, in part, enhanced transcription of genes encoding ER chaperones, including BiP/GRP78, which serves to correct protein misfolding. The UPR, which serves to restore cellular homeostasis, induces the transcription of genes encoding antiapoptotic and proapoptotic proteins. Thus, severe or prolonged ER stress may induce apoptosis. Again, our data suggest that P. gingivalis 381 induces apoptosis after

![Figure 1](image_url)
ER stress. ER stress has been demonstrated at all stages of atherosclerotic lesion development in ApoE knockout mice, and it is evident in human atherosclerotic lesions [12, 19, 20]. However, the relationship between \textit{P. gingivalis} 381 infection and ER stress is yet to be determined. Our preliminary data indicated that the infection with a higher \textit{P. gingivalis} 381 MOI induced reactive oxygen species (ROS) in HUVEC (data not shown). It is known that ROS induces the ER stress [21, 22]. Further, it was also found that lipopolysaccharide (LPS) could activate the ER stress [23]. Therefore, ROS and LPS release in HUVEC by infection with \textit{P. gingivalis} might induce the ER stress to HUVEC. Indeed, the expression levels of UPR-related genes were significantly higher in periodontitis compared with gingivitis lesions [24].

Accumulating evidence suggests that the ER plays an essential role not only in apoptosis but also in the regulation of autophagy [14, 25]. However, whether ER stress-mediated autophagy contributes to cell survival or cell death remains unclear. Here, we found that \textit{P. gingivalis} 381-induced ER stress enhanced autophagy. The process of autophagosome formation depends on several autophagy proteins [26]. By translational modification of LC3, LC3-II (16 kDa) localizes exclusively to autophagosomal membranes and has been used as an autophagy marker [27]. We demonstrated that \textit{P. gingivalis} 381 induced the expression of autophagy markers (e.g., Beclin-1, LC3-II, and AVOs), and that LC3-II and AVO expressions were inhibited by salubrinal. In addition, since specific suppression of LC3 by siRNA effectively downregulated LC3-II/LC3-I ratio and significantly increased the apoptosis in HUVEC upon stimulation with \textit{P. gingivalis} 381, the autophagy may protect \textit{P. gingivalis} 381-induced apoptosis. Most likely, autophagy under basal conditions plays an important role in cellular housekeeping, whereas induced autophagy may function as a death pathway, as suggested by our results.
In summary, our data highlight the important relationships of ER stress and autophagy with apoptosis in HUVEC during P. gingivalis 381 exposure. Our data also provide putative mechanisms for P. gingivalis 381-induced endothelial dysfunction, as well as suggesting potential strategies for the prevention of P. gingivalis 381-induced endothelial impairment.

5. Conclusions

The experiments revealed that P. gingivalis 381 infection induces ER stress-mediated apoptosis followed by autophagic response that protects HUVEC from P. gingivalis 381-mediated apoptosis, potentially amplifying proatherogenic mechanisms in the perturbed vasculature.
Data Availability
The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure
This work was presented at the following link: https://www.researchgate.net/publication/266772287_Porphyromonas_gingivalis_induces_ER_stress-induced_apoptosis_and_autophagy_in_HUVEC.

Conflicts of Interest
The authors report no conflicts of interest.

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