Secreted gingipains from *Porphyromonas gingivalis* increase permeability in human cerebral microvascular endothelial cells through intracellular degradation of tight junction proteins

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Despite a clear correlation between the infiltration of periodontal pathogens in the brain and cognitive decline in Alzheimer’s disease (AD), the precise mechanism underlying bacteria crossing the blood-brain barrier (BBB) remains unclear. The periodontal pathogen *Porphyromonas gingivalis* produces a unique class of cysteine proteases termed gingipains. Gingipains appear to be key virulence factors that exacerbate sporadic AD. We herein report that gingipains are involved in increasing permeability of hCMEC/D3 cell monolayer, human cerebral microvascular endothelial cell lines, through degradation of tight junction proteins including Zonula occludens-1 (ZO-1) and occludin. There was a significant decrease in the mean protein levels of ZO-1 and occludin after infection of hCMEC/D3 cells with wild-type (WT) *P. gingivalis*. However, infection of these cells with a gingipain-deficient *P. gingivalis* strain showed significantly lower reduction of the mean protein levels of either ZO-1 and occludin, compared to the WT strain. Similar results were obtained after treatment with culture supernatant from WT and gingipain-deficient *P. gingivalis* strains. *In vitro* digestion of human recombinant ZO-1 and occludin by WT *P. gingivalis* culture supernatant in the absence or presence of gingipain inhibitors indicated that gingipains directly degraded these tight junction proteins. A close immunohistochemical examination using anti-gingipain antibody further revealed that gingipains localized in the cytosol and nuclei of hCMEC/D3 cells after infection with WT *P. gingivalis* and treatment with its culture supernatant. Furthermore, intracellular localization of outer membrane vesicles (OMVs) bound gingipains from WT *P. gingivalis* and OMV-induced degradation of ZO-1 and occludin were also observed in hCMEC/D3 cells. Thus, the delivery of gingipains into the cerebral microvascular endothelial cells, probably through OMV, may be responsible for the BBB damage through intracellular degradation of ZO-1 and occludin.

**1. Introduction**

Many efforts have been made to develop therapeutic agents for Alzheimer’s disease (AD) based on the amyloid cascade hypothesis, but there is no effective therapeutic agent available at present. Now much attention has been paid to infiltrated pathogens of periodontitis in the brain as a trigger of AD (Kamer et al., 2008a, 2008b; Singhrao et al., 2014; Wu and Nakanishi, 2014; Nakanishi et al., 2020). Lipopolysaccharide (LPS) and gingipains of *Porphyromonas gingivalis*, two major pathogens of chronic periodontitis, have been detected in AD brain specimens (Poole et al., 2013; Dominy et al., 2019). Furthermore, there is growing clinical evidence of a correlation between periodontitis and cognitive decline in AD patients (Ide et al., 2016; Holmer et al., 2018). Recent studies have further revealed that gingipains play critical roles in neuroinflammation and cognitive decline in mice (Dominy et al., 2019). Therefore, *P. gingivalis* itself and/or its virulence factors, *Z. gingivalis*, *P. gingivalis*, and *Z. gingivalis*, are unique cysteine proteases that are key virulence factors in periodontal disease and AD.

**Abbreviations:** AD, Alzheimer’s disease; ANOVA, analysis of variance; BBB, blood-brain barrier; DIC, differential interference contrast; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; HA, hemagglutinin; Kgp, lysine-specific gingipain; MMP, matrix metalloprotease; MOI, multiplicity of infection; NaF, fluorescein sodium; OD, optical density; OMVs, outer membrane vesicles; PAR2, protease-activated protease 2; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; F. gingivalis, *Porphyromonas gingivalis*; Rgp, arginine-specific gingipain; SDS, sodium dodecyl sulfate; ZO-1, zonula occludens-1.

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including LPS and gingipains, may access the AD brain through the blood–brain barrier (BBB), leading to the induction of neurological damage via their neurotoxic effects and/or induction of neuro-inflammation. However, little is known concerning the precise mechanism underlying infiltration of P. gingivalis itself and/or its virulence factors in the brain parenchyma.

Gingipains can be divided into two categories: arginine-specific gingipain (Rgp) and lysine-specific gingipain (Kgp). Rgp has been further subdivided into RgpA and RgpB based on the structure. Gingipains are primarily composed of a signal peptide, an N-terminal domain, a catalytic domain, an immunoglobulin superfamily-like domain, a hemagglutinin (HA)/adhesion domain and a C-terminal domain (Kadowaki et al., 2000). RgpB is devoid of the HA domain, while RgpA contains four HA domains. Gingipains are produced as a secreted or membrane-associated form on the cell surface. Both Rgp and Kgp activate the extracellular signal-regulated kinase 1/2 (ERK1/2) through proteolytic processing of PAR2 in microglia to promote migration (Tsu et al., 2017; Nonaka and Nakanishi, 2020). PAR2 is also localized in cerebral microvascular endothelial cells, the major components of BBB, and ERK1/2 plays divergent roles in microvascular endothelial cell permeability and cytokine production (Zhou et al., 2018). In contrast, Kgp increases the vascular permeability in skin, probably through the degradation of extracellular proteins, including type I collagen and fibronectin (Kadowaki et al., 2004). More recently, it was shown that infection of P. gingivalis disrupted the barrier functions of intestinal epithelial cells (Tsuchino et al., 2021) and oral epithelial cells (Ben Lagha et al., 2021) through the degradation of tight junction proteins. Furthermore, P. gingivalis invades and survives in cultured neurons and produces intraneuronal gingipains that are proteolytically active (Haditsch et al., 2020). Moreover, the increased expression of interleukin-6 in gingival tissues in a ligature-induced periodontitis model in mice caused neuroinflammation-mediated BBB disruption (Furutama et al., 2020), and brain microvascular endothelial cells after P. gingivalis LPS treatment induced neuroinflammation (Sato et al., 2021). However, whether or not gingipains disrupt the BBB function remains unclear.

We therefore examined the effects of gingipains on two key regulatory factors of the brain microvascular endothelial cell barrier: PAR2 and tight junction proteins.

2. Materials and methods

2.1. Cell culture

A human cerebral endothelial cell line (hCMEC/D3) was obtained from Merck (Darmstadt, Germany) and maintained on 50 μg/mL Rat tail collagen I (Thermo Fisher Scientific Inc., Waltham, MA, USA)-coated dishes at 37 °C in EBM-2 endothelial cell growth basal medium-2 (Lonza, Basel, Switzerland) supplemented with an EGM-2 MV microvascular endothelial singlekots kit (Lonza).

2.2. Bacteria culture and preparation of culture supernatant

P. gingivalis ATCC33277 (WT), a Kgp-deficient mutant strain (KDP129), and a Kgp- and Rgp-deficient mutant strain (KDP136) were maintained on blood agar plates containing 40 mg/mL trypto-soya agar (Nissui Pharmaceutical, Tokyo, Japan), 5 mg/mL brain heart infusion (BHI) medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 1 mg/mL cysteine (Wako Pure Chemical Industries, Osaka, Japan), 5 μg/mL hemin (Sigma-Aldrich, St. Louis, MO, USA), 0.5 μg/mL menadione (Sigma-Aldrich), and 5% defibrinated sheep blood (Nippon Bio-Test Laboratories, Tokyo, Japan) in a Bactron anaerobic chamber (Shel Lab, Cornelius, OR, USA) at 5% CO2, 5% H2, and 90% N2. For experiments, these bacteria were grown in enriched BHI broth containing 37 mg/mL BHI medium, 2.5 mg/mL yeast extract, 1 mg/mL cysteine, 5 μg/mL hemin, and 0.5 μg/mL menadione.

After growth, the optical density (OD) measurement was used to estimate the number of cultured bacteria. After determining the number of bacteria by adjusting the OD value, the bacterial suspension was centrifuged at 6000×g for 10 min at 4 °C. The supernatant was discarded, and the bacteria pellets were suspended in EBM-2 endothelial cell growth basal medium-2 without fetal bovine serum (FBS) and gentamycin and then used for infection experiments. To prepare the culture supernatant, WT P. gingivalis, KDP129, or KDP136 was grown in enriched BHI medium. Culture supernatants were collected after centrifugation (6000-10000×g for 10–20 min at 4 °C). To prepare a 10-fold solution, the collected culture supernatant was filtrated with an ultrafiltration column, 10 K (Apro Science, Naruto, Japan). Uncondensed (Fig. 1B, Fig. 2B and C) or condensed P. gingivalis culture supernatant (Fig. 5) was added to EBM-2 medium without FBS at a ratio of 1:3 and used for each experiment.

2.3. Measurement of the permeability of the hCMEC/D3 monolayer

For the fluorescein sodium (Na–F) permeability assay, hCMEC/D3 (3.3×104 cells in 250 μL) were cultured on the upper side of a collagen I-coated transwell membrane (pore size 0.4 μm, for 24 well plate; Corning Incorporated, Corning, NY, USA) in EBM-2 medium at 37 °C until becoming confluent and then incubated with FBS-free EBM-2 medium containing WT P. gingivalis culture supernatant or BHI medium (Control medium) in the absence or presence of 1 μM KYT1 (a Rgp inhibitor; Peptide Institute Inc., Osaka, Japan) + 1 μM KYT36 (a Kgp inhibitor; Peptide Institute Inc.) for 6 h. After replacing the medium in the upper side with assay buffer (10 mM Hepes, pH 7.4, 136 mM NaCl, 0.9 mM CaCl2, 0.5 mM MgCl2, 2.7 mM KCl, 1.5 mM KH2PO4, 25 mM glucose) containing 10 μg/mL Na–F, transwells were placed in 500 μL of assay buffer in a 24-well plate for 5 min. Fluorescence (excitation: 485 nm, emission: 535 nm) in the bottom compartment was measured with a plate reader to determine the amount of Na–F that had passed through the hCMEC/D3 monolayer.

2.4. Immunoblotting analyses

To detect phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) and total ERK1/2, semi-confluent hCMEC/D3 cells were incubated with FBS-free EBM-2 medium containing WT P. gingivalis culture supernatant or BHI medium (Control medium) in either the absence or presence of 10–50 μM KYT1 + 10–50 μM KYT36 or 50 μg/mL PAR2 antagonist peptides (FSLLRY-NH2, Sigma-Aldrich, Tokyo, Japan) for 15 min. The cells were then lysed with 10 mM Tris (hydroxymethyl)aminomethane, 0.15 M NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) NP-40, protease inhibitor (Nacalai Tesque, Kyoto, Japan), and phosphate inhibitor (Nacalai Tesque) or RIPA buffer (10 mM Tris-HCl, pH 7.5, 1% [v/v] NP-40, 0.1% [w/v] sodium deoxycholate, 0.1% [w/v] SDS, 150 mM NaCl, 1 mM EDTA, protease inhibitor), and cell lysates were electrophoresed using 10–12% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gels. To detect PAR2, semi-confluent hCMEC/D3 cells were lysed with 10 mM Tris (hydroxymethyl)aminomethane, 0.15 M NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) NP-40, protease inhibitor, and cell lysates were subjected to electrophoresis using 10–12% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE).

To examine the effect of PAR2 tethered ligands, semi-confluent hCMEC/D3 cells were incubated with 600 μM synthetic peptides, whose sequences correspond to candidates of tethered ligands of PAR2—SLJGKV (residues 37–42; possible tethered ligand exposed by Rgp; R&D Systems, Minneapolis, MN, USA) and GVTVE (residues 52–56; possible tethered ligand exposed by Kgp; Biologica Co., Nagoya, Japan)—for 3 days. For infection experiments, semi-confluent hCMEC/D3 cells that had been incubated with WT P. gingivalis, KDP129, or KDP136 in the absence or presence of 50 μg/mL PAR2 antagonist, 10–100 nM metalloprotease (MMP) inhibitor I (Merck), and 100 μM CL82198 (Santa Cruz, Biotecnology, Inc., Dallas, TX, USA) for 4 h.
(multiplicity of infection (MOI): 1000) were lysed with RIPA buffer. In some experiments, semi-confluent hCMEC/D3 cells were incubated with FBS-free EBM-2 medium containing culture supernatant from WT *P. gingivalis*, KDP129, or KDP136 (2 × 10⁹ CFU of each) or BHI medium (Control medium), or WT *P. gingivalis* outer membrane vesicles (OMVs) dissolved in BHI medium, or OMV-free WT *P. gingivalis* culture supernatant for 4 h. Lysates were electrophoresed using 10% (w/v) SDS–polyacrylamide gels. The proteins on the SDS–polyacrylamide gels were then transferred to nitrocellulose membranes. After blocking with Blocking one (Nacalai Tesque), the membranes were incubated with rabbit anti-ERK1/2 antibodies (1:1000; Cell Signaling Technology), rabbit anti-PAR2 antibodies (1:300, Biopass, Boston, MA, USA), rabbit anti-Zonula occludens-1 (ZO-1) antibodies (1:500; Thermo Fisher Scientific Inc.), mouse anti-occludin antibodies (1:1000; Thermo Fisher Scientific Inc.), rabbit anti-VE-cadherin antibodies (1:1000, Cell signaling Technology), rabbit anti-GAPDH antibodies (1:1000; Proteintech, Tokyo, Japan), or rabbit anti-β-actin antibodies (1:500; Genetex, Los Angeles, CA, USA) at 4 °C overnight.

| Control | *Pg* culture supernatant |
|---------|--------------------------|
| none    | WT                       |
|         | KDP129                   |
|         | KDP136                   |
| ZO-1    | n.s.                     |
|         | p=0.0021                 |
| Occludin| n.s.                     |
|         | p=0.0015                 |
| VE-cadherin | n.s.                  |
|         | p=0.0011                 |

**Fig. 1.** Gingipain-dependent increases in the permeability of hCMEC/D3 monolayers accompanied by a reduction in tight junction protein levels. (A) The immunoblots show ZO-1, occludin, and VE-cadherin in hCMEC/D3 cells after incubation with WT and gingipain-mutant *P. gingivalis* strains for 4 h (MOI: 1000). GAPDH was the loading control. (B) The immunoblots show ZO-1 and occludin in hCMEC/D3 cells after incubation with culture supernatant from WT and gingipain-mutant *P. gingivalis* strains (2 × 10⁹ CFU of each) for 4 h. GAPDH was the loading control. (C–D) The mean intensities of ZO-1, occludin, and VE-cadherin, which were detected by the immunoblots shown in A (C) or B (D), were measured and normalized against those of GAPDH signals. They are shown here relative to the values in uninfected cells (C) or cells after incubation with Control medium (D). (E) An *in vitro* permeability assay of Na–F in hCMEC/D3 monolayers after incubation with WT *P. gingivalis* culture supernatant in the absence or presence of KYT1 + KYT36 (1 μM/each) for 6 h. The data are presented as the mean ± SE of three independent experiments, and p values were calculated using a one-way ANOVA with a post-hoc Tukey’s test. A value of p < 0.05 was considered to indicate statistical significance.

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2.5. Degradation of human recombinant ZO-1 and occludin by the bacterial culture supernatant

To prepare the protein extract from WT P. gingivalis culture supernatant, overnight culture of WT P. gingivalis in enriched BHI medium was harvested by centrifugation at 10,000 × g for 20 min at 4 °C. Ammonium sulfate was added to the supernatant to give 75% saturation. The precipitated proteins were collected by centrifugation at 10,000 × g for 20 min at 4 °C and suspended in 10 mM sodium phosphate buffer pH 7.0. After dialysis against the same buffer, the insoluble materials were removed by centrifugation at 26,700 × g for 30 min at 4 °C. The resulting supernatant was used for experiments.

Human recombinant ZO-1-Myc/DDK (400 ng: ORIGENE, Rockville, MD, USA) and GST-occludin (400 ng: abcam, Cambridge, UK) were incubated with protein extract from WT P. gingivalis culture supernatant in 20 mM sodium phosphate buffer, containing 1 mM DTT, pH 7.5, at 25 °C (ZO-1) or 37 °C (occludin) for 30 min in the absence or presence of 10^{-4} M KYT1 and/or KYT36. Recombinant ZO-1-Myc/DDK and GST-occludin was incubated with 170 and 340 ng of protein from WT P. gingivalis culture supernatant, respectively. The reactions were terminated by adding protease inhibitor cocktail (Nacalai Tesque), and total ERK1/2 in hCMEC/D3 cells after incubation with BHI medium (Control medium) or KYT1 and/or KYT36 was collected as pellet (MOI: 1000) or incubated with FBS-free EBM-2 medium containing 1:100 diluted (w/v) BSA for blocking and then incubated with mouse monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) overnight. Subsequently, they were incubated with anti-mouse IgG-Alexa-488 (1:200; Thermo Fisher Scientific Inc.) for 45 min to detect gingipain signals. Incubation with anti-p-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA) was carried out at room temperature, and PBS was used for all washing steps after incubation with antibodies. Nuclei were detected by incubation with Hoechst 33342 (1:10,000, Thermo Fisher Scientific Inc.) for 15 min. Fluorescence and differential interference contrast (DIC) images were taken using a confocal microscope (FW1000; Olympus, Tokyo, Japan).

2.6. Isolation of OMVs

OMVs were isolated as described in (Seyama et al., 2020). Briefly, WT P. gingivalis culture medium was centrifuged at 2800 × g for 15 min at 4 °C to remove bacterial cells. The supernatant was filtered with a Millipore filter (pore size, 0.22 μm). The supernatant (8 ml) was concentrated to 1 ml by using an ultrafiltration column, 10 K (Apro Science). The concentrate was mixed with Total Exosome Isolation Reagent (Thermo Fisher Scientific Inc.) and incubated overnight at 4 °C. Afterwards, the samples were centrifuged at 10,000 × g for 60 min at 4 °C. The OMV fraction was dissolved in 100 μL PBS. In some experiments, WT P. gingivalis OMV and OMV-free WT P. gingivalis supernatant were prepared as described in (Furuta et al., 2009). Briefly, WT P. gingivalis were removed from the culture by centrifugation at 10,000 × g for 30 min at 4 °C. The supernatant was filtered with Millipore filter (pore size, 0.22 μm). WT P. gingivalis OMVs were collected as pellet by ultracentrifugation of 0.5 ml of P. gingivalis culture supernatant at 100,000 × g for 3 h at 4 °C and dissolved in 0.5 ml of BHI medium. The supernatant was also collected as OMV-free WT P. gingivalis culture supernatant, and both were used for experiments (Fig. 6 B and C). After preparation, we confirmed that supernatant after ultracentrifugation didn’t contain OMVs by observing under the microscope. There were not Cy5-labeled OMVs in them.

2.7. Immunofluorescent staining

hCMEC/D3 cells (9.5 × 10^4 cells/well) were cultured on collagen-coated cover glass in 24-well plates at 37 °C for 3 days and then infected (MOI: 1000) or incubated with FBS-free EBM-2 medium containing culture supernatant from WT P. gingivalis for 4 h. Cells were fixed with 4% (w/v) paraformaldehyde for 15 min and then permeabilized with phosphate-buffered saline (PBS) containing 0.2% (v/v) Triton X-100. After washing with PBS, cells were incubated with PBS containing 3% (w/v) BSA for blocking and then incubated with mouse monoclonal antibodies that recognize hemagglutinating proteases of WT P. gingivalis, including RgpA and Kgp (1:100; 61BG1.3, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) overnight. Subsequently, they were incubated with anti-mouse IgG-Alexa-488 (1:200; Thermo Fisher Scientific Inc.) for 45 min to detect gingipain signals. Incubation with antibodies was carried out at room temperature, and PBS was used for all washing steps after incubation with antibodies. Nuclei were detected by incubation with Hoechst 33342 (1:10,000; Thermo Fisher Scientific Inc.) for 15 min. Fluorescence and differential interference contrast (DIC) images were taken using a confocal microscope (FV1000; Olympus, Tokyo, Japan).

To visualize WT P. gingivalis OMVs, OMVs (75 μg of total protein) prepared with Total Exosome Isolation Reagent were incubated with 5 μM Cy5 Mono NHS Ester (Lumiprobe Corporation, Cockeyville, MD,
USA) for 90 min at 37 °C. The labeled OMVs were collected by centrifugation (10,000 × g for 30 min at 4 °C), and dissolved in PBS, and then centrifugated at 10,000 × g for 30 min at 4 °C to remove unincorporated dye. Precipitated labeled OMV were dissolved in 0.5 mL of FBS-free EBM2 medium. To observe the internalization of Cy5 labeled WT P. gingivalis OMV, hCMEC/D3 cells (9.5 × 10⁴ cells/well) were cultured on collagen-coated cover glass in 24-well plates at 37 °C for 3 days and then 200 μL of Cy5-labeled WT P. gingivalis OMVs (150 μg of total protein/mL) were incubated at 37 °C for 4 h or 6 h. Cells were fixed with paraformaldehyde for 15 min and then stained with mouse monoclonal antibodies that recognize hemagglutinating proteases of WT P. gingivalis, including RgpA and Kgp as described above. Cellular localization of Cy5-labeled OMVs and gingipains in hCMEC/D3 cells was analyzed using a confocal microscope (FV1000).

2.8. Data processing and statistical analyses

The data are represented as the mean ± standard error. The statistical analyses, which included a two-tailed unpaired Student’s t-test (for comparison of two groups) and a one-way analysis of variance (ANOVA) (for comparison of more than three groups), were conducted with the Graph Pad Prism 8 software package (Graph Pad Software Inc., San Diego, CA, USA). A value of *p* < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Effects of gingipains on tight junction proteins in hCMEC/D3 cells

Infection of hCMEC/D3 cells with WT P. gingivalis significantly reduced the mean protein levels of both ZO-1 and occludin as compared to the none-treated control (Fig. 1A, C). On the other hand, the mean protein level of VE-cadherin was not significantly changed after infection with WT. We also found that infection of these cells with either Kgp-deficient strain (KDP129) or Kgp and Rgp-deficient strain (KDP136) showed a significant reduction of the mean protein levels of both ZO-1 and occludin as compared to the none-treated control. The mean ZO-1 level in cells infected with KDP129 was not significantly different from that infected with WT, whereas the mean ZO-1 level in cells infected with KDP136 was significantly higher than that infected with WT. On the other hand, the mean protein levels of occludin in cells infected with either KDP129 or KDP136 were significantly higher than that infected with WT and didn’t have significant difference between these two mutant strains.

We next examined the effects of culture supernatants from WT and gingipain-deficient P. gingivalis strains on the tight junction protein levels of hCMEC/D3 cells, because gingipains can exist in membrane-bound and soluble forms (Guo et al., 2010). Treatment with culture supernatant from WT P. gingivalis significantly reduced the mean protein levels of both ZO-1 and occludin as compared to the none-treated control (Fig. 1B, D). In contrast, treatment with culture supernatant from either KDP129 or KDP136 exerted no significant effect on their mean protein levels (Fig. 1B, D), and mean ZO-1 and occludin level didn’t have significant difference between two mutant strains.

These results suggest that Rgp and Kgp are involved in the WT P. gingivalis infection–induced reduction of ZO-1 and occludin in hCMEC/D3 cells, respectively. On the other hands, Kgp is mainly responsible for the culture supernatant-induced reduction of both tight junction proteins.

3.2. Increase in the permeability of hCMEC/D3 cell monolayer by WT P. gingivalis culture supernatant

We next examined the effects of WT P. gingivalis culture supernatant on the permeability of the hCMEC/D3 cell monolayer. The permeability of hCMEC/D3 cells cultured in collagen-coated transwells was quantified using Na-F. The Na-F permeability was significantly increased in hCMEC/D3 cell monolayer after treatment with WT P. gingivalis culture supernatant. This effect was completely inhibited by combined treatment with the Rgp inhibitor KYT1 and Kgp inhibitor KYT36 (Fig. 1E), suggesting that Rgp and Kgp are responsible for increased permeability of the hCMEC/D3 cell monolayer after treatment with WT P. gingivalis culture supernatant.

3.3. Possible involvement of PAR2 activation by gingipains in the decrease in ZO-1 and occludin of hCMEC/D3 cells after treatment with WT P. gingivalis culture supernatant

To address the mechanism underlying P. gingivalis induced decrease in ZO-1 and occludin, we first focused on the PAR2-mediated signaling, as the activation of PAR2 plays divergent roles in the brain microvascular endothelial cell permeability (Zhou et al., 2018). A major band corresponding to PAR2 was detected in the soluble fraction of hCMEC/D3 cells (Fig. 2A). Treatment with WT P. gingivalis culture supernatant significantly increased the mean phosphorylation level of ERK1/2, which is a downstream molecule of PAR2, in soluble fractions of hCMEC/D3 cells (Fig. 2B). WT P. gingivalis culture supernatant-induced ERK1/2 phosphorylation was significantly suppressed by combined treatment with the gingipain inhibitors KYT1 and KYT36 or PAR2 antagonist (Fig. 2C). These observations indicate that secreted gingipains induced phosphorylation of ERK1/2 through the activation of PAR2. Therefore, it may be concluded that gingipains induce a decrease in ZO-1 and occludin through PAR2, which subsequently activates the ERK1/2 pathway.

We therefore next examined the effects of inhibitor of PAR2 on P. gingivalis infection and culture supernatant-induced decreases in ZO-1 and occludin of hCMEC/D3 cells. We also examined the effects of inhibitors of MMP9 and MMP13, as PAR2 induces the secretion of MMP9 through the induction of the expression of proMMP9 in an oral squamous cell carcinoma cell line (Inaba et al., 2014). Furthermore, MMP13 was also expressed in a human bone chondrosarcoma cell line through PAR2-ERK2 signaling (Falconer et al., 2019), and MMPs are involved in the degradation of ECM molecules and surface proteins related in cell-to-cell junctions (Li et al., 2018; Lu et al., 2009). A PAR2 peptide antagonist (FSLLRY-NH2, 50 μg/ml), an MMP9 inhibitor (MMP9 inhibitor I, 10 and 100 nM), and an MMP13 inhibitor (CL82198, 100 μM) had no significant effect on the P. gingivalis infection and culture supernatant-induced decrease in ZO-1 and occludin of hCMEC3 cells (Fig. 3A-D). We further examined the effects of two synthetic peptides whose sequences correspond to candidates of tethered ligands of PAR2 after cleavage of the N-terminus of PAR2 by Rgp and Kgp: SLIGKV (residues 37–42), which corresponds to the possible tethered ligand exposed by Rgp, and GVTVE (residues 52–56), which corresponds to the possible tethered ligand exposed by Kgp (Nonaka and Nakanishi, 2020). Neither SLIGKV nor GVTVE had any significant effect on the mean protein level of ZO-1, occludin, or VE-cadherin (Supplementary Fig. 1).

These observations suggest that activation of PAR2 by gingipains are probably not involved in WT P. gingivalis infection and culture supernatant-induced decrease in ZO-1 and occludin of hCMEC3 cells. Therefore, we next focused on the direct degradation of ZO-1 and occludin by gingipains.

3.4. Possible involvement of direct proteolytic degradation by gingipains in P. gingivalis induced decrease in ZO-1 and occludin

We then examined whether or not secreted gingipains could degrade ZO-1 and occludin using WT P. gingivalis culture supernatant. Both human recombinant ZO-1-Myc/DDK and GST-occludin were extensively degraded by protein extracts from P. gingivalis culture supernatant (Fig. 4). The degradation of ZO-1 was not significantly inhibited by either an Rgp inhibitor (KYT1) or a Kgp inhibitor (KYT36) alone, but their combined treatment completely inhibited the degradation of ZO-1.
In contrast, the degradation of occludin was not inhibited by KYT1 but was markedly inhibited by KYT36 alone and the combined treatment of KYT1 and KYT36 (Fig. 4B). Therefore, it is considered that Kgp and Rgp collaboratively induce direct degradation of ZO-1. Furthermore, Kgp is mainly responsible for the degradation of occludin. Unfortunately, however, ZO-1 is an intracellular protein, whereas occludin is a type II transmembrane protein, although there are no lysine residues, which are potential cleavage sites of Kgp, in the extracellular domain of occludin (Supplementary Fig. 2). Given the above, Kgp and Rgp seem able to degrade ZO-1 and occludin of hCMEC/D3 cells intracellularly.

3.5. Intracellular localization of gingipains in hCMEC/D3 cells after infection with WT P. gingivalis or treatment with its culture supernatant

To address the localization of Kgp and Rgp in hCMEC/D3 cells, we

Fig. 3. PAR2, MMP9, and MMP13 were not required for the reduction in ZO-1 and occludin in hCMEC/D3 cells after infection with WT P. gingivalis and treatment with WT P. gingivalis culture supernatant. (A, C) The immunoblots show ZO-1 and occludin in hCMEC/D3 cells after infection with WT P. gingivalis (MOI: 1000) (A) and treatment with WT P. gingivalis culture supernatant for 4 h (C) in the absence or presence of 50 μg/mL PAR2 antagonist peptides, 10–100 nM MMP9 inhibitor I, or 100 μM CLR2198 (MMP13 inhibitor). GAPDH was the loading control. (B, D) The mean intensity of ZO-1 (top) and occludin (bottom), which were detected by the immunoblots shown in A (B) or C (D), were measured and normalized against the GAPDH signal values. They are shown here relative to the values in uninfected cells (B) or cells after incubation with Control medium (D). The data are presented as the mean ± SE of three independent experiments, and p values were calculated using a one-way ANOVA with a post-hoc Tukey’s test. A value of p < 0.05 was considered to indicate statistical significance.

Fig. 4. Gingipains directly degrade human recombinant ZO-1 and occludin. (A–B) The immunoblots show ZO-1 (A) and occludin (B) in the mixture of protein extract after ammonium sulfate precipitation from WT P. gingivalis culture supernatant and human recombinant ZO-1-Myc/DDK (A) or GST-occludin (B). The recombinant tight junction proteins (400 ng of each) were incubated with protein extract from WT P. gingivalis culture supernatant for 25 °C (A) or 37 °C (B) for 30 min in the absence or presence of 10⁻⁴ M KYT1 and/or KYT36. After incubation, the mixtures were subjected to immunoblotting.
conducted an immunohistological examination using the mouse monoclonal antibody 61BG1.3, which recognizes hemagglutinating proteases of WT *P. gingivalis*, including RgpA and Kgp. Immunoreactivities for RgpA and/or Kgp were detected in both the cytosol and nuclei of hCMEC/D3 cells after infection of WT *P. gingivalis* and treatment with WT *P. gingivalis* culture supernatant (Fig. 5A and B), suggesting the intracellular localization of gingipains.

3.6. Intracellular localization of OMVs and OMV-induced degradation of ZO-1 and occludin

Gram-negative bacteria like *P. gingivalis* secrete OMVs, which

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**Fig. 5.** Intracellular localization of gingipains in hCMEC/D3 cells after infection and treatment with culture supernatant from WT *P. gingivalis*. (A) The confocal microscopy images of hCMEC/D3 cells after infection (MOI: 1000) or treatment with culture supernatant from WT *P. gingivalis* for 4 h. Fluorescence images were merged with DIC images. (B) An orthogonal analysis of confocal microscopy images in hCMEC/D3 cells after infection or treatment with culture supernatant from WT *P. gingivalis*. Green and red rectangles are side views of serial confocal sections of the same area. Gingipain (green) and nuclei (blue) were visualized using antibodies recognizing the hemagglutinating proteases of WT *P. gingivalis*, including RgpA, Kgp, and Hoechst 33342. Scale bar; 20 μm.

**Fig. 6.** Intracellular localization of WT *P. gingivalis* OMVs and OMV-induced degradation of ZO-1 and occludin. (A) Fluorescent images merged with DIC images of hCMEC/D3 cells after incubation with Cy5 Mono NHS Ester-labeled OMVs (red) and gingipain staining with antibodies against the hemagglutinating proteases of WT *P. gingivalis*, including RgpA, Kgp (green) and nuclear staining with Hoechst 33342 (blue). Scale bar; 20 μm. (B) The immunoblots show ZO-1 and occludin in hCMEC/D3 cells after incubation with OMVs and OMV-free culture supernatant of WT *P. gingivalis* for 4 h. (C) The mean intensity of ZO-1 (left) and occludin (right), which were detected by the immunoblots shown in B, were measured and normalized against the GAPDH signal values. They are shown here relative to the values in cells after incubation with Control medium. The data are presented as the mean ± SE of three independent experiments, and *p* values were calculated using a one-way ANOVA with a post-hoc Tukey’s test. A value of *p* < 0.05 was considered to indicate statistical significance.
transport various virulence factors into host cells. In the WT *P. gingivalis* culture supernatant, gingipains exist at least two forms, free form and OMV-bound form. To address a possible involvement of OMVs in the degradation of tight junction proteins, we examined the cellular localization and possible effects of isolated OMVs from WT *P. gingivalis* on the degradation of ZO-1 and occludin. At 4 h and 6 h after incubation, Cy5-labeled and OMVs were visible in both the cytosol and nucleus of hCMEC/D3 cells (Fig. 6A). Almost all Cy5 signals were overlapped with signals of antibodies against hemagglutinating proteases of WT *P. gingivalis* including gingipains. Rather surprisingly, both isolated OMVs from WT *P. gingivalis* and OMV-free culture supernatant markedly degraded ZO-1 and occludin to the same extent in hCMEC/D3 cells (Fig. 6B–C). These results indicate that both free and OMV-bound gingipains can penetrate hCMEC/D3 cells to degrade ZO-1 and occludin.

4. Discussion

In the present study, we demonstrated that WT *P. gingivalis* infection as well as its culture supernatant induced gingipains-mediated degradation of ZO-1 and occludin in hCMEC/D3 cells, leading to an increase in the permeability of the hCMEC/D3 cell monolayer. Gingipains can phosphorylate ERK1/2 through the activation of PAR2 in hCMEC/D3 cells, but the PAR2 activation failed to induce the degradation of ZO-1 and occludin in hCMEC/D3 cells after infection and incubation with culture supernatant from WT *P. gingivalis*. In contrast, treatment with protein extracts from WT *P. gingivalis* culture supernatant markedly degraded both human recombinant ZO-1 and occludin, but no significant degradation was observed in the presence of KYT1 and KYT36, suggesting that gingipains can directly degrade ZO-1 and occludin. Though all experiments about occludin were consistent and indicated that Kgp are mainly responsible for the degradation of occludin, it was also noted some differences in degradation profiles of ZO-1 obtained by differential experiments. KDP129 and KDP136 infection experiments suggested that Rgp are required for degradation of ZO-1, while experiments using KDP129 and KDP136 culture supernatant suggest that Kgp are responsible for the degradation of ZO-1. In *in vitro* digestion assays in the presence of either or both KYT1 and KYT36 suggest that Rgp and Kgp cooperatively contribute to the degradation of ZO-1. Although the reason for the differential degradation profiles of ZO-1 remains unclear, it may be concluded that both Rgp and Kgp may play some important roles in the degradation of ZO-1. There is increasing evidence that chronic infection with *P. gingivalis* induces memory deficits in middle-aged mice (Ding et al., 2018; Huang et al., 2021). Therefore, the degradation of ZO-1 and occludin by gingipains after *P. gingivalis* infection is a critical step in BBB disruption and the subsequent infiltration of *P. gingivalis* itself and/or its virulence factors in the brain parenchyma and memory deficits.

Occludin is a type II transmembrane protein composed of four transmembrane domains, suggesting that Kgp cleaves lysine residues of its extracellular domain. Unfortunately, however, there are no lysine residues in the extracellular domain of occludin (Supplementary Fig. 2). ZO-1 is an intracellular scaffolding protein interacting with the intracellular domain of occludin. To address the possibility that Kgp and Rgp intracellularly degrade occludin and ZO-1, we examined the localization of gingipains in hCMEC/D3 cells after WT *P. gingivalis* infection or treatment with culture supernatant using the mouse monoclonal antibody 61BG1.3. RgpA and/or Kgp localized to the cytoplasm and nuclei of hCMEC/D3 cells after WT *P. gingivalis* infection and treatment with its culture supernatant. The intracellular localization of RgpA and/or gingipains was also detected in astrocytes, microglia, and neurons in the hippocampus of orally *P. gingivalis*-infected mice according to experiments using 61BG1.3 (Ilievski et al., 2018). In the hippocampus of AD patients, the intracellular localization of RgpB was also observed in primarily neurons as well as occasionally astrocytes but not in microglia according to experiments using the polyclonal antibody of RgpB, CAB101 (Dominy et al., 2019).

Gram-negative bacteria release OMVs that contain specific cargo molecules and have diverse functions, including the transport of virulence factors. *P. gingivalis* produces small (20–500 nm) proteo-liposomal OMVs. WT *P. gingivalis* OMVs possess various components of outer membrane constituents, including LPS, muramic acid, a capsule, fimbriae, and gingipains (Nakao et al., 2014). *P. gingivalis* OMVs are potent vehicles for transmitting virulence factors into the host cells (Furuta et al., 2009; Singhrao and Olsen, 2018; Nara et al., 2021). Therefore, WT *P. gingivalis* OMVs containing gingipains are key pathogenic factors linking periodontitis to systemic diseases, including diabetes and AD (Singhrao and Olsen, 2018; Nara et al., 2021; Zhang et al., 2020). In the present study, we detected intracellular gingipains in hCMEC/D3 cells after infection and treatment of WT *P. gingivalis* culture supernatant by antibodies that recognize the HA domain but not the catalytic domains of gingipains. WT *P. gingivalis* sequesters two forms of gingipains: free form containing only catalytic domain, and OMV-associated form containing both catalytic and adhesion domains (Kadowaki et al., 1994; Abe et al., 1998; Okamura et al., 2021). Because the HA domain is part of the adhesion domain, our results suggest that gingipains are internalized in hCMEC/D3 cells through OMV after treatment with WT *P. gingivalis* culture supernatant. We found that both isolated OMVs from WT *P. gingivalis* and OMV-free culture supernatant markedly degraded ZO-1 and occludin to the same extent in hCMEC/D3 cells. Therefore, we may hypothesize that both free and OMV-bound gingipains can penetrate hCMEC/D3 cells to degrade ZO-1 and occludin. Additional experiments are necessary to elucidate the mechanism underlying penetration of free gingipains in hCMEC/D3 cells.

Interestingly, *P. gingivalis* OMVs were translocated to the liver in mice after intraperitoneal administration (Seyama et al., 2020). In these mice, the hepatic glycogen synthesis was decreased in response to insulin, so high glucose levels were maintained in a gingipain-dependent manner, suggesting that the delivery of gingipains mediated by *P. gingivalis* OMVs elicits changes in glucose metabolism in the liver and contributes to the progression of diabetes mellitus. It was demonstrated that OMVs of the oral bacterium *Aggregatibacter actinomycetemcomitans* can systemically cross the normal C57BL6 mouse BBB after intracardiac injection in a dose-dependent manner (Han et al., 2019). Furthermore, *A. actinomycetemcomitans* OMVs were shown to be taken up into meningeal macrophages and cortical microglia after intravenous injection (Ha et al., 2020). This may expand upon our previous observations that gingipains induce cell migration and inflammatory response of microglia (Liu et al., 2017; Nonaka and Nakanishi, 2020).

In conclusion, our observations have showed that gingipains increased permeability of hCMEC/D3 cell monolayer after infection of WT *P. gingivalis* as well as incubation with its culture supernatant containing secreted gingipains through direct degradation of tight junction proteins. In addition, the close immunohistochemical examination using antibody recognizing gingipains containing the HA domain like OMV-processed gingipains are key pathogenic factors linking periodontitis to systemic diseases, including diabetes and AD (Singhrao and Olsen, 2018; Nara et al., 2021). Therefore, WT *P. gingivalis* OMVs containing gingipains are key pathogenic factors linking periodontitis to systemic diseases, including diabetes and AD (Singhrao and Olsen, 2018; Nara et al., 2021; Zhang et al., 2020). In the present study, we detected intracellular gingipains in hCMEC/D3 cells after infection and treatment of WT *P. gingivalis* culture supernatant by antibodies that recognize the HA domain but not the catalytic domains of gingipains. WT *P. gingivalis* sequesters two forms of gingipains: free form containing only catalytic domain, and OMV-associated form containing both catalytic and adhesion domains (Kadowaki et al., 1994; Abe et al., 1998; Okamura et al., 2021). Because the HA domain is part of the adhesion domain, our results suggest that gingipains are internalized in hCMEC/D3 cells through OMV after treatment with WT *P. gingivalis* culture supernatant. We found that both isolated OMVs from WT *P. gingivalis* and OMV-free culture supernatant markedly degraded ZO-1 and occludin to the same extent in hCMEC/D3 cells. Therefore, we may hypothesize that both free and OMV-bound gingipains can penetrate hCMEC/D3 cells to degrade ZO-1 and occludin. Additional experiments are necessary to elucidate the mechanism underlying penetration of free gingipains in hCMEC/D3 cells.

In conclusion, our observations have showed that gingipains increased permeability of hCMEC/D3 cell monolayer after infection of WT *P. gingivalis* as well as incubation with its culture supernatant containing secreted gingipains through direct degradation of tight junction proteins. In addition, the close immunohistochemical examination using antibody recognizing gingipains containing the HA domain like OMV-processed gingipains and Cy5-labeled WT *P. gingivalis* OMV revealed OMV bound gingipains were internalized in hCMEC/D3 cells. Given the above, it is most likely that the delivery of gingipains mediated by *P. gingivalis* OMVs into cerebral microvascular endothelial cells plays essential roles in BBB damage and the subsequent infiltration of *P. gingivalis* itself and/or its virulence factors to the brain parenchyma and cognitive decline (Fig. 7). Possible changes in the BBB permeability and memory deficits in mice after systemic administration of *P. gingivalis* OMVs are to be elucidated in future studies.

Credit author statement

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Tomoko Kadowaki: Resources, Writing - Review & Editing.

Hiroshi Nakaishi: Conceptualization, Project administration, Writing - Original Draft, Writing - Review & Editing.
Fig. 7. A schematic illustration representing the delivery of gingipains to cerebral microvascular endothelial cells by *P. gingivalis* OMVs through blood circulation. After entering in cerebral microvascular cells, gingipains degrade tight junction proteins including ZO-1 and occludin, resulting in the increased BBB permeability.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2022.105282.

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