Porphyromonas gingivalis induces penetration of lipopolysaccharide and peptidoglycan through the gingival epithelium via degradation of coxsackievirus and adenovirus receptor

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Short title: CXADR prevents LPS and PGN penetration through gingival epithelium

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Abstract

*Porphyromonas gingivalis* is a major pathogen in severe and chronic manifestations of periodontal disease, which is one of the most common infections of humans. A central feature of *P. gingivalis* pathogenicity is dysregulation of innate immunity at the gingival epithelial interface. We previously showed that junctional adhesion molecule 1 (JAM1) was specifically degraded by *P. gingivalis*, leading to epithelial barrier breakdown in gingival tissues. Whereas, the involvement of the other JAM family protein(s) in the epithelial barrier dysregulation by *P. gingivalis* remains unknown. Here we show that Arg-specific or Lys-specific cysteine proteases named gingipains produced by *P. gingivalis* specifically degrade coxsackievirus and adenovirus receptor (CXADR), a tight junction associated protein, at R145 and K235 in gingival epithelial cells. A *P. gingivalis* strain lacking gingipains was impaired in degradation of CXADR. Knockdown of CXADR in a three-dimensional multilayered tissue model increased permeability to 40 kDa dextran, lipopolysaccharide, and proteoglycan. Inversely, overexpression of CXADR in a gingival epithelial tissue model prevented penetration by these agents following *P. gingivalis* infection. Our findings strongly suggest that *P. gingivalis* gingipains disrupt barrier function of stratified squamous epithelium via degradation of CXADR as well as JAM1, efficiently allowing bacterial virulence factors to penetrate into subepithelial tissues.
Main

Introduction

Periodontal diseases are chronic infectious diseases caused by complex actions of periodontal bacteria in oral biofilm, and one of the most common infectious diseases of humans [1]. In periodontal tissues, the subgingival epithelium responds to microbial infection and harmonizing innate immunity [2]. Lipopolysaccharide (LPS: endotoxins of gram-negative bacteria) and peptidoglycan (PGN: mesh-like patterns outside the plasma membrane of most bacteria) are prototypal classes of pathogen-associated molecular patterns (PAMPs) that are recognized by the innate immunity [3].

The epithelium lining gingival sulcus is stratified squamous. Gingival epithelial cells express molecular complexes regulating cell-to-cell adhesion [4]. Human gingival epithelial cells express junctional adhesion molecules (JAMs), members of an immunoglobulin superfamily, that seal the paracellular pathway and prevent solutes passing through the paracellular space [4,5]. Homodimerization of JAMs is important for regulating epithelial barrier function [6]. However, barrier function of the sole JAM family proteins in human epithelium remains largely unknown.

Porphyromonas gingivalis, a keystone periodontal pathogen, can impair host immune defense and promote inflammation [7]. P. gingivalis secretes Arg-specific and Lys-specific cysteine proteases, termed Arg-gingipains (RgpA and RgpB) and Lys-gingipain (Kgp), respectively, which possess abundant proteolytic activities [8,9]. Hence, host proteins targeted and degraded by gingipains could be considered crucial factors influencing pathogenesis of periodontitis. We previously identified JAM1 as a gingival epithelial barrier protein, which is specifically degraded by P. gingivalis gingipains, leading to epithelial barrier breakdown by allowing increased epithelial permeability to...
PAMPs [10]. Whereas, the possibility that other JAM family proteins are targeted by *P. gingivalis* has not been verified. In this study, we screened the JAM family proteins involved in gingival epithelial barrier dysfunction by *P. gingivalis* using a three-dimensional multilayered tissue model (3D-tissue model). We consequently identified CXADR as an additional JAM family protein targeted specifically by the pathogen. Gingipains degrades CXADR at R145 and K235, thereby inducing gingival epithelial permeability and allowing subsequent transmission of LPS and PGN. This work provides insights into process by which gingival epithelial barrier is subverted during periodontal pathogenesis.

**Results**

*P. gingivalis* gingipains degrade CXADR in gingival epithelial cells

To elucidate the JAM1-independent mechanism of gingival epithelial barrier function, we screened eight JAM family proteins [6] for their expression in immortalized human gingival epithelial (IHGE) cells. For this purpose, we used reverse transcriptional polymerase chain reaction (RT-PCR), and found that JAM2, CLMP, and CXADR were expressed in IHGE cells in addition to JAM1 (Fig. 1a). While, the negligible expression of JAM3, JAM4, CD2, and ESAM was observed in IHGE cells. Next, we investigated whether gingipains degrade these JAM family proteins cell at the endogenous protein level. We infected IHGE cells with *P. gingivalis* ATCC33277 wild type (WT) or its isogenic mutant KDP136 (Δkgp ΔrgpA ΔrgpB) for 1 h at a multiplicity of infection (MOI) of 100. We detected the decreased levels of JAM1, CXADR, and CLMP in IHGE cells were decreased by *P. gingivalis* WT, while the Δkgp ΔrgpA ΔrgpB mutant had a negligible effect (Fig. 1b). By contrast, *P. gingivalis* did not degrade JAM2 at 1 h after infection.
These results suggest that gingipains specifically degrade JAM1, CXADR, and CLMP.

To assess the contribution of CXADR or CLMP expression to the permeability of gingival epithelial cells, we generated IHGE cell lines stably expressing small hairpin RNA (shRNA) against JAM1 (shJAM1 #110 and shJAM1 #508), CXADR (shCXADR #38 and shCXADR #317), and CLMP (shCLMP #661 and shCLMP #771), respectively. We then performed permeability assays using a small-molecule fluorescent probe (Fig. 2a). Knockdown of JAM1, CXADR, and CLMP in each cell line was confirmed by immunoblot (Fig. 2b). As shown in Figure 2c, IHGE monolayers expressing shRNA against CXADR as well as JAM1 were significantly more permeable to 40 kDa FITC-dextran, FITC-labeled *P. gingivalis* LPS, and FITC-labelled *P. gingivalis* PGN than control cells expressing shRNA against firefly luciferase (shLuc). By contrast, knockdown of CLMP had a negligible effect on the permeability to these tracers.

We examined the effects of secreted gingipains on CXADR in the same way that we previously studied the impact of gingipains on JAM1 [10]. The bacterial culture supernatant was collected and administered to IHGE cells for 1 h. We detected the decreased levels of CXADR in IHGE cells by the culture supernatant of *P. gingivalis* WT. By contrast, the Δkgp ΔrgpA ΔrgpB mutant showed a negligible effect (Fig. S1). These results indicate that CXADR are degraded by secreted gingipains.

We next examined the association of *P. gingivalis* with CXADR in IHGE cells using a laser confocal microscope. At 1 h after infection of *P. gingivalis* WT the signal intensity of CXADR was reduced in the surface area of IHGE cells (Fig. 3a). By contrast, the Δkgp ΔrgpA ΔrgpB mutant failed to reduce the CXADR signal, consistent with the results in Figure 1b and 1c. In order to examine the effects of gingipains on CXADR degradation in deeper epithelium, we created a 3D-tissue model of IHGE cells.
by the cell-accumulation technique (Fig. 3b) [11]. We infected the 3D-tissue models with *P. gingivalis* WT or Δkgp ΔrgpA ΔrgpB, and analyzed the CXADR degradation in deeper epithelium using confocal microscopy. At 2 h after infection, *P. gingivalis* WT is capable of reducing the CXADR signal even in the tissues 3–4 layers below the surface (Fig 3b). By contrast, the Δkgp ΔrgpA ΔrgpB mutant failed to reduce the CXADR signal. These results suggest that gingipains deeply invade human gingival epithelial tissues and degrade CXADR.

CXADR is localized in the plasma membrane following cleavage of N-terminal signal peptide

We hypothesized that *P. gingivalis* gingipains degrades CXADR localizes on the plasma membrane, but not efficiently in intracellular space. CXADR has a predicted signal peptide followed by immunoglobulin (IG) domains and a transmembrane domain [12,13]. In order to test this hypothesis, we visualized the intracellular localization of CXADR before and after cleavage of its signal peptide. We created a plasmid encoding Myc–tagged human influenza hemagglutinin (HA)–inserted CXADR (Fig. 4a) and examined the localization of the chimeric protein in IHGE cells to determine by confocal microscopy. To monitor the CXADR localization, we stained IHGE cells expressing HA-tagged enhanced green fluorescent protein (EGFP) (Fig. S2a) or Myc–tagged HA-inserted CXADR (Fig. S2b) using anti-HA antibody, with or without permeabilization. We were able to label HA-EGFP protein with anti-HA antibody in the permeabilized IHGE cells, but not in the non-permeabilized cells, suggesting that the cytosolic proteins are not stained without permeabilization. By contrast, we were able to label HA-inserted CXADR protein with anti-HA antibody even without permeabilization,
suggesting that the ectopic protein of HA-inserted CXADR is properly transported to plasma membrane.

In order to trace the pathway(s) of CXADR, we next analyzed IHGE cells expressing Myc–tagged HA–inserted CXADR protein and each organelle marker: EGFP-SEC61β (marker for endoplasmic reticulum membrane protein); TOMM20 (marker for outer mitochondrial membrane protein; not included in endomembrane system) by confocal microscopy. We detected co-localization of the anti-Myc and anti-HA signals with SEC61β (Fig. 4b), but not TOMM20 (Fig. S3), suggesting that CXADR is transported via an endomembrane pathway. Additionally, phalloidin-stained actin in the peripheral area of IHGE cells co-localized with anti–HA-labeled CXADR, but not the Myc signal (Fig. 4c). These results suggest that the signal peptide of CXADR is cleaved at the endoplasmic reticulum and transported to the plasma membrane, and that gingipains are capable of targeting mature form of CXADR at cell surface.

**P. gingivalis, but not S. gordonii or F. nucleatum, degrades CXADR**

In order to examine whether *P. gingivalis* degrades the immature and mature CXADR, we infected IHGE cells expressing Myc–tagged HA–inserted CXADR, and the kinetics of Myc- and HA-tagged CXADR were analyzed. Considering the substrate specifically of a gingipains property, we utilized the HA tag as a marker of mature CXADR because K or R residues are not included in HA amino acid sequence (YPYDVPDYA). In addition, we distinguished immature and mature CXADR in immunoblots by the N-terminal Myc tag. As shown in Figure 5a, *P. gingivalis* infection decreased the amount of mature CXADR labeled with anti-HA, but failed to decrease on the level of immature CXADR labeled with anti-Myc. These results suggest that mature form of CXADR are
targeted by *P. gingivalis*. In order to examine the effects of another *P. gingivalis* strain, we infected IHGE cells with *P. gingivalis* TDC60 isolated from a severe periodontal lesion [14]. At 1 and 2 h after infection, *P. gingivalis* TDC60 markedly reduced mature from of CXADR (Fig 5b), indicating that other *P. gingivalis* strain is capable of degrading CXADR.

Human oral bacteria including *Fusobacterium nucleatum* and *Streptococcus gordonii* can establish mixed-species communities leading to periodontopathic biofilm formation [15]. In order to examine the effects of *S. gordonii* or *F. nucleatum* on CXADR, we infected IHGE cells expressing Myc–tagged HA–inserted CXADR with these species.

As shown in Figure 5c and 5d, *S. gordonii* and *F. nucleatum* fail to reduce the level of HA–inserted CXADR at 2 h after infection, suggesting that CXADR cannot be degraded by *S. gordonii* and *F. nucleatum*.

**CXADR R145 and K235 are responsible for degradation by *P. gingivalis***

In order to examine which residue(s) are responsible for degradation by *P. gingivalis* gingipains, we constructed plasmids encoding deleted and mutated CXADR and performed a structural analysis (Fig. 6a). We transfected these plasmids into IHGE cells and then infected cells with *P. gingivalis*. To detect the residue(s) targeted by gingipains, we replaced the R and K residues with H, a basic amino acid, in point mutation constructs.

As shown in Figure 6b, degradation was observed in CXADR full length, CXADR Δ(1–234), but not JAM1 Δ(1-234) K235H, indicating that K235 is responsible for bacterial degradation. Additionally, the level of HA-tagged CXADR Δ(1–225) K235H and CXADR Δ(1–225) R226H K235H were not decreased by the infection, indicating that R226 is not responsible for bacterial degradation. Furthermore, the level of HA-tagged
CXADR Δ(1–144) K235H was reduced following infection with *P. gingivalis*, but CXADR Δ(1–144) R145H K235H was not. These results illustrate that R145 and K235 are the residues targeted for degradation by gingipains.

**CXADR prevents penetration of LPS and PGN through gingival epithelium**

It is previously reported that CXADR mediates homotypic cell adhesion in Chinese hamster ovary (CHO) cells [16]. However, the barrier function of CXADR in human squamous epithelium remains unknown. To assess the contribution of CXADR expression to the permeability of gingival deeper epithelium, we generated a 3D-tissue model stably expressing shLuc or shCXADR using the cell accumulation technique, and then performed permeability assays using a small-molecule fluorescent probe (Fig. 7a). Effective suppression of CXADR expression was confirmed by confocal microscopy (Fig. 7b). As shown in Figure 7c, IHGE monolayer expressing shRNA against CXADR was significantly more permeable to 40 kDa FITC-dextran than control cells expressing shLuc. This result suggests that CXADR is involved in the kinetics of flux of molecules with 40 kDa between gingival epithelial cell layers.

We previously showed that JAM1 prevented permeation to dissociated LPS and PGN monomer (N-acetylglucosamine-N-acetylmuramic acid) in the 3D-tissues of gingival epithelium [10]. This result prompted us to examine whether knockdown of CXADR allows permeation of LPS and PGN. Thirty minutes after administration, we detected the increased permeability to FITC-*P. gingivalis* LPS (Fig. 7d) and FITC-*P. gingivalis* PGN (Fig. 7e) by knockdown of CXADR. In order to confirm the generality of permeation of LPS and PGN following knockdown of CXADR, we also performed the permeability assay of *Escherichia coli* LPS and *Staphylococcus aureus* PGN through
gingival epithelial tissues. In tissues expressing shCXADR, we detected the increased permeability to FITC–E. coli LPS (Fig. 7d) and FITC–S. aureus PGN (Fig. 7e) at 30 min after administration, suggesting that CXADR affects the permeability of gingival epithelium to LPS and PGN.

**Epithelial barrier function is regulated by CXADR independently of JAM1**

As CXADR and JAM1 are of JAM family protein [6], these two proteins may interact with each other to mediate its localization in surface of gingival epithelial cells. To assess the interdependence of CXADR and JAM1, we determined the localization of CXADR and JAM1 in IHGE cells expressing shCXADR #317 or shJAM1 #508 by confocal microscopy. As shown in Figure S4a, the localization of JAM1 in IHGE monolayer expressing shJAM1 was negligible, but was not disturbed in IHGE cells expressing shCXADR. In the same trend, the expression level of CXADR in IHGE monolayer expressing shCXADR was negligible, but was not disturbed in IHGE cells expressing shJAM1 (Fig. S4b). These results suggest that CXADR and JAM1 are not interdependent on localization in gingival epithelial cells.

To assess the interdependence of CXADR and JAM1 on each localization in gingival epithelium, we generated the 3D-tissue models of IHGE cells expressing shCXADR and shJAM1 and the localization of deeply seated CXADR and JAM1 was analyzed using confocal microscopy. The expression levels of IHGE cells expressing shCXADR and shJAM1 were confirmed by immunoblots (Fig. 8a). As shown in Figure 8b, shJAM1 caused JAM1, but not CXADR, to disappear even in the tissues 3–4 layers below the surface. In the same trend, shCXADR caused CXADR, but not JAM1, to disappear in the tissues below the surface. Finally, double knockdown of shCXADR and shJAM1
caused these two proteins to disappear in the tissues. These results suggest that the localization of CXADR and JAM1 are not interdependent even in gingival epithelium model. As shown in Figure 8c, depletion of either CXADR or JAM1 enhances permeability of gingival epithelium to 40 kDa dextran, and depletion of both CXADR and JAM1 showed an additional effect. These results show that CXADR mediates barrier function of gingival epithelium independently of JAM1.

**CXADR degradation is involved in penetration of gingipains through gingival epithelial barrier**

We previously showed that JAM1 degradation by gingipains is required for gingipains penetration in gingival epithelial cells [10]. In order to examine the CXADR-dependent mechanism of gingipains penetration, we employed a two-layered cell culture model, by which we can detect penetration of gingipains from the upper to the lower space (Fig. 9a). Six hours after administration of bacterial culture media, we confirmed the decreased level of HA-inserted CXADR, but not Myc-tagged CXADR, in the cells of the lower layer by *P. gingivalis* WT to a greater extent than by the Δkpg ΔrgpA ΔrgpB mutant (Fig. 9b). Next, we examined whether overexpression of CXADR, the expression level of which compensates CXADR degradation by *P. gingivalis*, blocks the loss of CXADR in gingival epithelial cells. To test this idea, we employed two-layered culture of IHGE cells overexpressing CXADR, and the localization of CXADR in cells of the lower layer were monitored following *P. gingivalis* infection. When IHGE cells overexpressing CXADR were treated with bacterial culture media of *P. gingivalis* for 30 min, we detected the remaining CXADR proteins at almost the same level as in non-treated IHGE cells (Fig. S5), suggesting that degradation can be compensated by overexpression of CXADR.
in this system. In line with this result, 6 h after administration, we detected the increased levels of HA-inserted CXADR in the lower layer when the cells in the upper layer overexpressed CXADR (Fig. 9b). These results indicate that degradation of CXADR by gingipains is important for penetration of the proteases through gingival epithelial cells.

**Degradation of CXADR by *P. gingivalis* causes penetration of LPS and PGN**

Next, we generated 3D-tissue model using IHGE cells overexpressing CXADR (Fig. 10a). We confirmed that marked amounts of CXADR were remained even in tissues infected with *P. gingivalis* (Fig. 10b). In order to examine the permeability, the tissues were then treated with 40 kDa FITC-dextran, FITC–*P. gingivalis* LPS, or FITC–*P. gingivalis*-PGN. Thirty minutes after administration, we detected the decreased permeability to 40 kDa FITC-dextran (Fig. 10c), FITC–*P. gingivalis* LPS (Fig. 10d), FITC–*P. gingivalis* PGN (Fig. 10e), FITC–*E. coli* LPS (Fig. 10f), and FITC–*S. aureus* PGN (Fig. 10g) in gingival epithelial tissues overexpressing CXADR. These results indicate that CXADR degradation by *P. gingivalis* is key for allowing the penetration of LPS and PGN in gingival epithelium.

**Discussion**

Our findings clearly suggest a molecular basis for the dysfunction of gingival epithelial barrier by *P. gingivalis*. Based on our results, we postulate the following model (Fig. 11). As CXADR as well as JAM1 localizes in a phalloidin-stained plasma membrane region in the 3D-tissue (Fig. 8b), CXADR and JAM1 function together as gingival epithelial barrier. By degrading not only JAM1 but also CXADR, *P. gingivalis* efficiently allows penetration of LPS, PGN, and gingipains derived from itself and other
bacteria through the gingival epithelium and into deeper periodontal tissues. In this manner, *P. gingivalis* is probably able to destroy the epithelial barrier, which remotely causes alveolar bone loss.

In this study, we employed a 3D-tissue model using the cell-accumulation technique by a single cell coating with fibronectin/gelatin nanofilms [11]. We were able to generate gingival epithelium even with knockdown of CXADR and JAM1 (Fig. 8b), indicating that CXADR and JAM1 are not necessary for reconstruction of gingival epithelium. Hence, the cell-accumulation technique is likely useful for defining function of JAMs in the other types of epithelium. We also generated HA-inserted CXADR for use as a probe in gingival epithelial cells. Using an HA-tagged protein, we were able to detect the responsible amino acid residues to bacterial degradation. *P. gingivalis* gingipains degrade CXADR at K235 (Fig. 6), and JAM1 at R234 [10]. The hinge region between the C-terminal IG-like domain and the transmembrane domain, possessing CXADR K235 and JAM1 R234, does not contain a consensus sequence for N-glycosylation or secondary structure including helix, β-sheet, and turn (Uniplot, JAM1: https://www.uniprot.org/uniprot/Q9Y624; CXADR: https://www.uniprot.org/uniprot/P78310). Considering that the N-terminal IG-like domain of CXADR and JAM1 is responsible for homodimerization [21,22], gingipains effectively separate the N-terminal region away from the transmembrane domain. We confirmed that single knockdown of CXADR or JAM1 increases permeability of gingival epithelial tissues to 40 kDa dextran almost at the same level (Fig. 8c). Additionally, double knockdown of CXADR and JAM1 in gingival epithelial tissues showed further increase of permeability compared to single knockdown (Fig. 8c). Upregulated CXADR or JAM1 messenger RNA expression is inhibited by the AP-1 or
the NF-κB inhibitor, respectively [23,24]. These multiple transcription pathways of CXADR and JAM1 may provide gingival epithelium a robust defense against external bacterial stress. Gingival epithelial function to control the barrier permeability is a perspective that should be further investigated in the etiology of periodontal disease.

Based on the results of this study, there remain at least two questions that we should consider in the future. One is whether other major pathogens could specifically degrade CXADR and JAM1. The increase in the amount of the “red complex” species (P. gingivalis, Treponema denticola, and Tannerella forsythia) in subgingival biofilms has been shown to be highly associated with the initiation and progression of periodontitis [17]. T. denticola expresses the serine protease, dentilisin and oligopeptidase B [18, 19]. T. forsythia expresses the cysteine protease, forsythia detachment factor [20]. The effects of these proteases on CXADR and JAM1 will be further examined.

The other one is the relationship between protein structure and the degradation. We showed that CXADR and JAM1, but not Claudin (CLDN) 1 and CLDN 4, were degraded by P. gingivalis in gingival epithelial cells [10]. JAM family proteins possess a single transmembrane domain, while CLDN family proteins are of a tetra spans transmembrane protein and possess two extracellular loops [5]. Accordingly, it is necessary to study other gingipains-degradative proteins which potentially regulates gingival epithelial barrier without an extracellular loop.
Methods (<1500 words)

Bacteria and cell culture

*P. gingivalis* ATCC 33277 (purchased from the American Type Culture Collection), TDC60 (kindly provided by Kazuyuki Ishihara, Tokyo Dental College), and KDP136 (Δkgp ΔrgpA ΔrgpB, kindly provided by Koji Nakayama, Nagasaki University) [25] were maintained anaerobically on blood agar plates (BD) and grown in trypticase soy broth (BD), supplemented with hemin (5 μg mL\(^{-1}\); WAKO), and menadione (1 μg mL\(^{-1}\); Sigma-Aldrich). *S. gordonii* ATCC 35105 were grown aerobically at 37 °C in Todd-Hewitt broth. *F. nucleatum* subsp. *nucleatum* ATCC 25586 were grown anaerobically at 37°C on blood agar plates (BD). IHGE cells (kindly provided by Shinya Murakami, Osaka University) were maintained in Humedia KG-2 (Kurabo), as described previously [26]. For the preparation of the bacterial culture supernatant of *P. gingivalis*, the bacterial culture at a stationary growth phase was centrifuged at 3,300 g for 3 min, and the supernatant was collected and administered into the culture media of IHGE cells at a ratio of 1:50.

Reverse transcriptional PCR

Total RNA was extracted from IHGE cells using TRIzol (Thermo Fisher Scientific). Complementary DNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). Reverse transcriptional PCR was performed with the primers as follows: JAM1 forward, 5’-GTGCCTTCAGCAACTCTTCC-3’; reverse, 5’-ACCAGATGCCCCAAACCAAG-3’. JAM2 forward, 5’-TGCTCTGAGTGAACTGTGG-3’; reverse, 5’-CACCTGCGATATCCAACAGA-3’. JAM3 forward, 5’-CAGACAAGTGACCCCAGGAT-3’; reverse, 5’-CAGCGATAAAGGGCTGAGTC-3’.
Cell accumulation technique

Three-dimensional culture of IHGE cells was performed as described previously [10]. IHGE cells collected by centrifugation after trypsinization were alternatively incubated for 1 min with 0.2 mg mL\(^{-1}\) fibronectin (Sigma-Aldrich) and 0.1% (w/v) gelatin (Nacalai Tesque) dissolved in phosphate-buffered saline (PBS). After each procedure, cells were washed with PBS (pH = 7.4) by centrifugation at 180 g for 2 min to remove unabsorbed polymers. After nine steps of immersion, fibronectin/gelatin nano-films were coated onto single-cell surfaces. A total of 2 × 10\(^6\) cells coated with fibronectin/gelatin nano-films were seeded on coverslips coated with 0.2 mg mL\(^{-1}\) fibronectin dissolved in PBS in 24-well plates (Iwaki). After 36 h of incubation, tissues were subjected to experiments, fixed, and analyzed on a confocal microscope (TCS SP8; Leica Microsystems). The number of \textit{P. gingivalis} cells in culture media were adjusted to an optical density at 600 nm of 0.12 to infect gingival epithelial tissues.

Antibodies and reagents
Mouse monoclonal anti–c-Myc (M4439), mouse monoclonal anti-HA (H3663), mouse monoclonal anti-JAM1 (SAB4200468), and mouse monoclonal β-ACTIN (A2228) antibodies were from Sigma-Aldrich; rabbit monoclonal anti-CXADR (10799-R271) was from Sino Biological; rabbit polyclonal anti-JAM2 (12972-1-AP) and rabbit polyclonal anti-CLMP (16127-1-AP) was from Proteintech; rabbit monoclonal anti-HA antibody (3724) was from Cell Signaling Technology; rabbit monoclonal anti-TOMM20 (ab78547) was from Abcam. FITC–conjugated secondary (goat anti–mouse IgG, A-11001) antibody (MBL), and Alexa Fluor 488–conjugated secondary (goat anti–rabbit IgG, A11008), Alexa Fluor 568–conjugated secondary (goat anti–mouse IgG, A-11004), and Alexa Fluor 635–conjugated secondary (goat anti–rabbit IgG, A-31576) antibodies (Invitrogen) were used for fluorescence microscopy. Goat anti-mouse (7076) and anti-rabbit (7074) antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) were used for immunoblotting. 4',6-diamidino-2-phenylindole (DAPI) (D1306, Invitrogen), Alexa Fluor 568– and Alexa Fluor 633–conjugated phalloidin (A12380 and A22284, respectively, Invitrogen) were used to stain IHGE cells.

**Plasmids**

The plasmid encoding Myc–tagged HA–inserted CXADR was constructed by cloning PCR products amplified from IHGE cells into pCMV-Myc (Clontech), using exogenously added EcoRI and KpnI sites. To produce HA–inserted CXADR, DNA sequence of HA-tag was inserted into CXADR with fusion PCR. Plasmids encoding HA–tagged CXADR deletion mutants and point mutations were constructed by PCR. The plasmid encoding EGFP-SEC61β was constructed by cloning PCR products amplified from IHGE cells into pEGFP-C1 (Clontech). PCR amplification was performed with KOD Plus Neo
PCR products were ligated into plasmids with T4 DNA ligase (New England Biolabs). Transfection of IHGE cells was performed using FuGENE 6 Transfection Reagent (Promega).

**Immunoblotting**

Immunoblotting was performed as described previously [10]. IHGE cells were lysed, and the lysates were cleared by centrifugation. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (0.2 μm, Bio-Rad). Membranes were blocked for 1 h at room temperature with PBST (PBS and 0.1% (v/v) Tween 20) containing 3% (w/v) skim milk, and then incubated for 1 h at room temperature with primary antibodies diluted in PBST. Next, the membranes were washed three times with PBST and incubated for 1 h at room temperature with 1:5000 dilutions of HRP-conjugated secondary antibodies in PBST. Immunoreactive bands were detected using the Pierce ECL Western Blotting Substrate (Thermo Scientific) and ChemiDoc XRS (Bio-Rad). Images were acquired using the Quantity One software (Bio-Rad).

**Immunocytochemistry**

Immunocytochemistry was performed as described previously [10]. IHGE cells were fixed with 4% (v/v) paraformaldehyde in PBS (Wako) overnight at room temperature, permeabilized with 0.1% (v/v) Triton X-100 (Wako) in PBS for 5 min at room temperature, and blocked with 0.1% (w/v) gelatin (Wako) in PBS for 20 min at room temperature. Samples were incubated for 1 h at room temperature with the indicated primary antibodies, washed four times in PBS, incubated for 1 h at room temperature with the Alexa Fluor–conjugated secondary antibodies (Invitrogen), and again washed four
times in PBS. All antibodies were diluted 1:400 in PBS. Cells were mounted onto glass slides with Vectashield Mounting medium (Vector Laboratories). Images were acquired with a confocal laser microscope (TCS SP8; Leica Microsystems) using a 64× oil-immersion object lens with a numerical aperture of 1.4. Acquired images were analyzed using the Application Suite X software (Leica Microsystems).

**Generation of cell line stably expressing CXADR**

Plasmid encoding CXADR was constructed by cloning PCR products amplified from the pCMV plasmid into pMRX-IRES-Puro (kindly provided by Nobumichi Furuta, Osaka University). Plasmids pMRX-IRES-Puro-CXADR was used for overexpression of the cDNA in IHGE cells. IHGE cells were transfected with the overexpression plasmid using FuGENE 6 (Promega). Seventy-two hours after transfection, cells stably overexpressing the cDNA were selected with puromycin (2 μg mL⁻¹; InvivoGen).

**RNA interference**

Plasmid encoding shRNA was constructed by ligation of linear DNA (Sigma-Aldrich) into pSIREN-RetroQ (631526, Clontech) or pSINsi-hU6 (3661, Takara). Plasmids pSIREN-RetroQ-shJAM1 #110 (target sequences: 5’-AAGTCAGAATTCTGAGAATAAT-3’) and #508 (target sequences: 5’-GGGATAGTGATGCCTACGAATCC-3’), pSINsi-hU6-shJAM1 #508 (Fig. 9), shCXADR #38 (target sequences: 5’-TAGTGGATTTCGCCAGAAGTTTG-3’) and #317 (target sequences: 5’-ATGTAACGAATTTACAACTGTCA-3’), and shCLMP #661 (target sequences: 5’-GTGCGAGTAACTGTACAGTATGT-3’) and #771 (target sequences: 5’-AAGGAAAGACAAAGAAAGATATG-3’)) were used for generation of
the siRNA duplex in cells. Plasmid pSIREN-RetroQ-shLuc was produced as described previously [10]. IHGE cells were transfected with the shRNA-encoding plasmid using FuGENE 6 (Promega). Seventy-two hours after transfection, cells stably expressing the shRNA were selected with puromycin (2 μg mL⁻¹) and neomycin (200 μg mL⁻¹; Invivogen).

**Preparation of FITC-labeled tracer**

Preparation of FITC-labeled tracer was performed as described previously [10]. *P. gingivalis* LPS (14F18-MM) was purchased from Invivogen. *P. gingivalis* PGN was prepared as described previously [27]. FITC–*E. coli* LPS (L7018) and *S. aureus* PGN (77140) were purchased from Sigma-Aldrich. Bacterial LPS and PGN were labeled with FITC using Fluorescein Labeling Kit-NH₂ (LK-01, Dojindo). To generate dissociated LPS and PGN, FITC-labeled LPS was diluted by 10% with water and incubated with 10 mM citrate (Wako) and 0.05% (v/v) Tween-20 (Calbiochem) for 45 min at 37°C as described previously [28], and FITC-labeled PGN was diluted by 10% with water and incubated with 0.5 mg mL⁻¹ lysozyme (Nacalai Tesque) for 45 min at 37°C to make a suspension. 40 kDa FITC-dextran (FD40, Sigma-Aldrich) were diluted by 2% with water for the permeability assay.

**Epithelial barrier function assay**

Epithelial barrier function assay was performed as described previously [10]. The in vitro epithelial permeability assay to assess barrier function was performed with 12-well cell culture inserts (353180; Corning). When IHGE cells in the upper compartment reached 100% confluence, 20 μL of FITC-dextran, FITC-LPS, or FITC-PGN was added.
to the upper compartment of the insert. After a 30-min incubation, the medium was collected from the lower compartment, and the fluorescence intensity was measured using 1420 ARVO X (PerkinElmer). Data were acquired using the WorkOut Plus software (PerkinElmer).

**Statistical analysis**

p-values were determined by t test in Excel (Microsoft); p<0.05 was considered significant.

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**Author Contributions**

H.T. and A.A. conceived and designed the experiments. H.T. and S.Y. performed the experiments. H.T. and A.A. analyzed the data. H.T., N.S., M.K., and M.M. contributed reagents, materials, and analytical tools. H.T. and A.A. wrote the paper.

**Competing Interests**
The authors have no competing financial interests to declare.
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**Figure 1.** *P. gingivalis* gingipains degrade CXADR in IHGE cells.

(a) Expression of *JAM1, JAM2, JAM3, JAM4, CD2, CLMP, ESAM,* and *CXADR* by IHGE cells analyzed by RT-PCR. (b) IHGE cells were infected with *P. gingivalis* WT or Δkgp ΔrgpA ΔrgpB mutant at an MOI of 100 for 1 h. The cells were then analyzed by immunoblotting with the indicated antibodies. β-ACTIN was used as a loading control. RT, reverse transcription. IB, immunoblot.
Figure 2. Loss of CXADR increased the epithelial barrier permeability.
(a) Schematic image of the culture insert system. Monolayer of IHGE cells stably expressing shLuc, shJAM1 #110, shJAM1 #508, shCXADR #38, shCXADR #317, shCLMP #661, and shCLMP #771 were cultured in culture inserts. FITC-labeled tracer was added to culture media in the upper compartment. Following 30 min of incubation, the transmission of tracer from the upper compartment to the lower compartment was analyzed by spectrometry. (b-d) IHGE cells stably expressing shLuc, shJAM1, shCXADR, or shCLMP were analyzed by immunoblotting with the indicated antibodies. (e-f) Permeability to 40 kDa FITC-dextran (e), FITC–P. gingivalis LPS (f), FITC–P. gingivalis PGN (g) in IHGE cells expressing indicated shRNA. Results are expressed as fold change relative to cells expressing shLuc and are the means ± SD of eight technical replicates.
Figure 3. Degradation of CXADR in IHGE cells infected with *P. gingivalis* gingipains

(a) IHGE cells were infected with *P. gingivalis* WT or Δkgp ΔrgpA ΔrgpB mutant at an MOI of 100 for 1 h. The cells were then fixed, stained with DAPI (cyan) and anti-
CXADR (yellow), and analyzed by confocal microscopy. Scale bars, 10 μm. (b)

Gingival epithelial tissues on coverslips were infected with *P. gingivalis* WT or Δkgp ΔrgpA ΔrgpB mutant for 2 h. The tissues were then fixed, stained with anti-CXADR (green) and Alexa Fluor 568–conjugated phalloidin (magenta), and analyzed by confocal microscopy. Scale bars, 30 μm.
Figure 4. Localization of CXADR in IHGE cells.

(a) Schematic view of the structure of Myc–tagged HA–inserted CXADR. Immature form of Myc–tagged HA–inserted CXADR was used as an internal control to monitor degradation flux of mature form of HA-inserted CXADR. The Myc tag is indicated by
the magenta box, and the HA-tag is indicated by the green box. The signal peptide is indicated by the blue box, and IG-LIKE or TMD domains are indicated by gray boxes or an orange box, respectively. (b,c) IHGE cells were transiently transfected with plasmids encoding Myc–tagged HA–inserted CXADR; in (b), the cells were also transfected with EGFP-SEC61β (green). Following 48 h of incubation, cells were fixed, stained with anti-Myc (b, magenta; c, green) or anti-HA (b, magenta; c, green): in (c), the cells were also stained with Alexa Fluor 633–conjugated phalloidin (magenta). The cells were then analyzed by immunofluorescence microscopy. Higher magnification of the areas indicated by white boxes in the upper panels are shown in the right side. Scale bars, 5 μm.
Figure 5. *P. gingivalis*, but not *S. gordonii* or *F. nucleatum*, degrades CXADR in IHGE cells.

(a–d) IHGE cells were transiently transfected with Myc–tagged HA–inserted CXADR plasmid. Following 48 h of incubation, the cells were infected with *P. gingivalis* ATCC 33277 (a), *P. gingivalis* TDC60 (b), *S. gordonii* DL-1 (c), or *F. nucleatum* ATCC 25586 (d) at an MOI of 100 for the indicated times. The cells were then analyzed by immunoblotting with the indicated antibodies.
Figure 6. The R145 and K235 residues are targeted for degradation of CXADR by *P. gingivalis* in IHGE cells

(a) Schematic view of the CXADR structure and derivatives. SP, IG-LIKE, or TMD
domains are indicated by gray boxes. HA-tag is shown in green. The point mutations R145H and K235H are shown in magenta. (b) IHGE cells were transiently transfected with plasmid encoding HA–inserted CXADR or the indicated CXADR mutants. Following 48 h of incubation, the cells were infected with *P. gingivalis* at an MOI of 100 for 1 h, and then analyzed by immunoblotting using the indicated antibodies.
Figure 7. CXADR is required for epithelial barrier function of gingival epithelial tissues.

(a,b) Schematic illustration (a) and confocal microscopic cross-sectional images (b) of 3D-tissue model expressing shLuc or shCXADR. Gingival epithelial tissues were fixed,
stained with anti-CXADR (green) and Alexa Fluor 568–conjugated phalloidin (magenta), and analyzed by confocal microscopy. Scale bars, 30 μm. (c–g) Permeability to 40 kDa FITC–dextran (c), FITC–P. gingivalis LPS (d), FITC–P. gingivalis PGN (e), FITC–E. coli LPS (f), and FITC–S. aureus PGN (g) in gingival epithelial tissues expressing shLuc and shCXADR. Results are expressed as fold change relative to epithelium expressing shLuc and are the means ± SD of seven technical replicates. *, p<0.05, one-tailed t test (c–e).
Figure 8. Barrier function in gingival epithelial tissues is regulated by CXADR independently of JAM1.

(a) IHGE cells stably expressing shLuc, shJAM1 #508, shCXADR #317, or both shJAM1 #508 and shCXADR #317 were analyzed by immunoblotting with the indicated antibodies. (b) Gingival epithelial tissues stably expressing shLuc, shJAM1 #508, shCXADR #317, or both shJAM1 #508 and shCXADR #317 on coverslips were fixed, stained with anti-JAM1 (green), anti-CXADR (cyan) and Alexa Fluor 568–conjugated phalloidin (magenta), and analyzed by confocal microscopy. Scale bars, 30 μm. (c) Permeability to 40 kDa FITC–dextran in gingival epithelial tissues expressing shLuc,
shJAM1 #508, shCXADR #317, or both shJAM1 #508 and shCXADR #317. Results are expressed as fold change relative to epithelium expressing shLuc and are the means ± SD of eight technical replicates. *, p<0.05, two-tailed t test.
Figure 9. *P. gingivalis* gingipains penetrate the epithelial barrier of IHGE cells.

(a, b) Schematic image of the culture insert system (a). Gingival epithelial cells WT or stably expressing Myc-tagged HA-inserted CXADR were cultured in the upper compartment and IHGE cells stably expressing Myc-tagged HA-inserted CXADR on a coverslip in the lower compartment. The bacterial culture supernatant of *P. gingivalis* WT or or Δ*kgp ΔrgpA ΔrgpB* mutant was administered to cells. Following 6 h of incubation, cells in the lower compartment were fixed, stained with anti-Myc (green), and anti-HA (magenta), and analyzed by confocal microscopy (b). Scale bars, 10 μm.
Figure 10. *P. gingivalis* degrades CXADR of gingival epithelium, causing penetration of LPS and PGN.

(a,b) Schematic illustration of the three-dimensional culture (a) and confocal microscopic
cross-sectional images (b) of the three-dimensional culture of IHGE cells. Gingival epithelial tissues (WT or overexpressing CXADR) were infected with *P. gingivalis* for 30 min. Tissues were then fixed, stained with anti-CXADR (green) and Alexa Fluor 568–conjugated phalloidin (magenta), and analyzed by confocal microscopy. Scale bars, 30 μm. (c–g) Permeability to 40 kDa FITC-dextran (c), FITC–*P. gingivalis* LPS (d), FITC–*P. gingivalis* PGN (e), FITC–*E. coli* LPS (f), and FITC–*S. aureus* PGN (g) of gingival epithelial tissues (WT or overexpressing CXADR) infected with *P. gingivalis*. Three-dimensional tissues on culture inserts were infected with *P. gingivalis* and FITC-labeled tracer in the upper compartment. Following 30 min of incubation, the transmission of tracer from the upper compartment to the lower compartment was analyzed by spectrometry. Results are expressed as fold change relative to uninfected WT cells and are the means ± SD of seven technical replicates. *, p<0.05, one-tailed *t* test (closed testing procedure).
Figure 11. Proposed model of how *P. gingivalis* gingipains send bacterial virulence factors through the gingival epithelium.

In gingival epithelial tissues CXADR (magenta) and JAM1 (cyan) are not interdependent on the permeability. *P. gingivalis* gingipains degrade CXADR and JAM1, which increases the permeability of gingival epithelium to gingipains and other factors. Subsequently, gingipains are transferred to the deeper epithelium to further degrade CXADR and JAM1, which allows LPS and PGN to penetrate the gingival epithelium and reach subepithelial tissues. Finally, gingipains, LPS, and PGN induce inflammation in gingival tissues.