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Porphyromonas gingivalis Gingipains-Mediated Degradation of Plasminogen Activator Inhibitor-1 Leads to Delayed Wound Healing Responses in Human Endothelial Cells

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Keywords
Bacterial infection · Endothelial cells · Pathogenesis · Wound healing · Periodontitis

Abstract
Plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor, is constitutively produced by endothelial cells and plays a vital role in maintaining vascular homeostasis. Chronic periodontitis is an inflammatory disease characterized by bleeding of periodontal tissues that support the tooth. In this study, we aimed to determine the role of PAI-1 produced by endothelial cells in response to infections caused by the primary periodontal pathogen *Porphyromonas gingivalis*. We demonstrated that *P. gingivalis* infection resulted in significantly reduced PAI-1 levels in human endothelial cells. This reduction in PAI-1 levels could be attributed to the proteolysis of PAI-1 by *P. gingivalis* proteinases, especially lysine-specific gingipain-K (Kgp). We demonstrated the roles of these degradative enzymes in the endothelial cells using a Kgp-specific inhibitor and *P. gingivalis* gingipain-null mutants, in which the lack of the proteinases resulted in the absence of PAI-1 degradation. The degradation of PAI-1 by *P. gingivalis* induced a delayed wound healing response in endothelial cell layers via the low-density lipoprotein receptor-related protein. Our results collectively suggested that the proteolysis of PAI-1 in endothelial cells by gingipains of *P. gingivalis* might lead to the deregulation of endothelial homeostasis, thereby contributing to the permeabilization and dysfunction of the vascular endothelial barrier.

Introduction

Chronic periodontitis is characterized by a cumulative inflammatory burden induced by persistent infection caused by periodontopathic bacteria, leading to bleeding,

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fibrinolysis, and inflammation. The pathogenesis of chronic periodontitis positively correlates with the presence of the keystone pathogen *Porphyromonas gingivalis* in the periodontal pockets and atherosclerotic plaques [1–3]. *P. gingivalis* is most frequently identified in the blood vessels of patients with chronic periodontitis [4], where it induces adherence of the bacteria to endothelial cells in blood vessels, and causes events that are implicated in the induction of vascular inflammation [5]. The bacteria utilize diverse virulence factors that manipulate the immune responses of the host, thereby inducing chronic inflammation and bone loss. *P. gingivalis* synthesizes two types of cysteine proteinases, arginine-specific gingipains (RgpA and RgpB) and lysine-specific gingipain (Kgp) [6, 7], which induce the production of proinflammatory cytokines, leading to chronic inflammation and destruction of periodontal tissues, including alveolar bone and tooth loss [7]. However, gingipains also proteolytically degrade host molecules including those in complement system, CD14, and intercellular adhesion molecule-1 and cytokines such as interleukin (IL)-1β, IL-6, IL-8, interferon-γ, and tumor necrosis factor-α, leading to the escape of *P. gingivalis* from the innate immune responses and resulting in persistent inflammation in the periodontal tissues [8].

The plasminogen activation system regulates vascular homeostasis via plasmin, an active form of serine protease, to degrade fibrin into fibrin degradation products. The conversion of plasminogen into plasmin occurs via the binding of plasminogen to the plasminogen activators (PAs), that is, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Plasminogen activator inhibitor type 1 (PAI-1) is a serine proteinase inhibitor that primarily inhibits PAs. PAI-1 is expressed on platelets and endothelial cells in the blood vessels and fibroblasts, platelets, macrophages, hepatocytes, adipocytes, and the mesothelial cells of the inflamed tissues [9, 10]. PAI-1 produced by endothelial cells supports their maintenance by regulating vascular remodeling and wound healing by inducing cell migration, angiogenesis, and inflammation in the vascular endothelium [10–14]. Furthermore, congenital PAI-1 deficiency, an autosomal recessive disease, is characterized by a bleeding tendency with minor wounds and impaired wound healing because of the lack of PAI-1 functions [15–17]. However, excessive PAI-1 levels are associated with pathophysiological phenomena, such as cardiovascular disease, tissue fibrosis, and cancer [18].

Most oral bacteria have been identified in the blood cultures of patients after tooth brushing or during dental procedures [19]. *P. gingivalis* and gingipains play a vital role in exacerbating the pathogenesis of systemic conditions involving blood vessel dysfunction [1, 2, 20]. It has been reported that intensive periodontal treatment of patients with chronic periodontitis ameliorates systemic inflammation and vascular endothelial function [21]. *P. gingivalis* has also been detected in the brain tissues or aneurysmal tissues of patients with Alzheimer's disease or atherosclerosis, respectively [20, 22]. The *P. gingivalis* gingipains colonizing the brain in Alzheimer's disease were neurotoxic [20]. Coculturing of human platelets with *P. gingivalis* resulted in decreased Pam_3CSK_4-induced PAI-1 production [23]. These findings suggest that decreased PAI-1 production in response to *P. gingivalis* possibly plays a role in the pathogenesis of systemic diseases associated with blood vessel barrier dysfunction.

In the present study, we focused on the impact of *P. gingivalis* on the production of PAI-1 by endothelial cells. We also investigated the role of PAI-1 in modulating the endothelial barrier. Our results indicated that *P. gingivalis* gingipains proteolytically degraded PAI-1 produced by human endothelial cells, resulting in a delayed wound healing response. The results from this study suggest that gingipain-mediated degradation of PAI-1 in endothelial cells may promote periodontal tissue bleeding in periodontal disease, induce the destruction of periodontal tissues, and lead to the spread of *P. gingivalis* infection from these tissues to the systemic organs via the bloodstream.

Materials and Methods

Reagents

Gingipain inhibitors KYT-1 and KYT-36 were obtained from the Peptide Institute (Osaka, Japan). KYT-1 is an Rgp-specific inhibitor and KYT-36 is a Kgp-specific inhibitor [24]. The PAI-1 antagonist PAI-039 tiplaxtinin was obtained from MedChemExpress (Monmouth Junction, NJ, USA). Recombinant human receptor-associated protein (RAP) and recombinant human PAI-1 (rhPAI-1) mutant were obtained from Sigma-Aldrich (St. Louis, MO, USA). RAP is an antagonist of the low-density lipoprotein receptor-related protein (LRP). It is known that wild-type PAI-1 is not stable and gets converted into an inactive latent form, with a half-life ranging between 1 and 2 h [25]. The rhPAI-1 mutant is an altered form of human PAI-1 containing 4 mutated amino acids that markedly improved functional stability, because of its inability to convert to the latent form. The residue numbers for the four mutations present in rhPAI-1 mutant are N150H, K154T, Q319L, and M354I [25]. As a consequence, the mutant protein exhibits markedly increased thermal stability of the active form relative to active wild-type PAI-1. All other reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Cells and Culture Conditions

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD, USA). HUVECs were cultured in endothelial cell growth medium-2 (EGM-2) (Lonza), composed of endothelial cell growth basal medium-2 (EBM-2) supplemented with 2% heat-inactivated fetal bovine serum (FBS), 5 ng/mL hEGF, 0.5 ng/mL hVEGF, 20 ng/mL R^3-IGF-1, 1 μg/mL ascorbic acid, 0.2 μg/mL hydrocortisone, 10 ng/mL hFGF-β, 22.5 μg/mL heparin, and 0.1% gentamicin/amphotericin-B (Lonza) using collagen type I-coated 100-mm dishes (IWAKI®; AGC TECHNO GLASS, Shizuoka, Japan). Experiments with *P. gingivalis* live bacteria were performed using EGM-2 without antibiotics. Confluent monolayer cells at passages 5 through 7 were used for the experiments. The viability of the HUVECs after infection with *P. gingivalis* was measured based on the amount of NanoLuc® substrate diffused from the viable cells using the RealTime-Glo MT Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The assay involves adding NanoLuc® luciferase and a cell-permeable substrate to cells in culture. The substrate is reduced to NanoLuc® substrate by metabolically active cells, which diffuses from the cells into the culture medium. The luminescence of the NanoLuc® substrate in the culture supernatants was determined using a GloMax® Discover Microplate Reader (Promega). The proliferation of *P. gingivalis*-infected HUVECs was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The number of viable cells was calculated based on the absorbance using SoftMax Pro, a data analysis software (Molecular Devices, Menlo Park, CA, USA).

Purification and Activation of Gingipains

Kgp and RgpA were purified from the culture supernatants of *P. gingivalis* HG66, as previously described [6]. Gingipains are stable between room temperature and 37°C in the absence of cysteine, which is the most effective reducing agent for activation of the enzyme [6]. The enzymes were diluted to activate gingipains at 10 μM in 0.2 M HEPES, 5 mM $CaCl_2$, and 10 mML cysteine at pH 8 and incubated at 37°C for 10 min. After activation, they were diluted with complete medium or 0.1 M Tris buffer for the experiments with HUVECs or rhPAI-1, respectively.

Culture of P. gingivalis and Preparation of Whole-Cell Lysates

In this study, we used two wild-type strains and five gingipain mutant strains of *P. gingivalis* to investigate the *P. gingivalis* gingipains-mediated reduction in PAI-1 levels. The strains W83, ATCC 33277, KDP129 (Δ*kgp*), KDP131 (Δ*rgpA*), KDP132 (Δ*rgpB*), KDP133 (Δ*rgpA* Δ*rgpB*), and KDP136 (Δ*rgpA* Δ*rgpB* Δ*kgp*) were cultured anaerobically at 37°C for 60 h in enriched tryptic soy broth containing 1% tryptone, 3% tryptic soy, 2.5% yeast extract, 0.1% L-cysteine, 5 μg/mL hemin, and 0.5 μg/mL of menadione. After 60 h of incubation, log-phase bacterial culture was centrifuged for 15 min at 8,500 *g* and washed three times with phosphate-buffered saline (PBS). All bacterial strains were cultured on tryptic soy agar plates at 37°C under anaerobic conditions. The CFU/mL was measured after 72 h of culture. After being washed three times with PBS, whole bacterial cells in the logarithmic growth phase were resuspended in PBS, lyophilized by centrifugation in vacuo, and then resuspended in the same volume of distilled water. The bacterial cultures were sonicated for 1 min and then centrifuged for 20 min at 10,000 *g* at 4°C to separate bacterial cells from the culture medium and collect outer membrane vesicles (OMVs). Subsequently, the supernatants were ultracentrifuged for 1 h at 150,000 *g* at 4°C, and the pellet enriched in OMVs was resuspended in 5 mM $MgCl_2$ prepared in PBS. Protein concentrations in the OMV fractions were measured using a bicinchoninic acid (BCA) protein assay.

Measurement of Cytokines Production Using an Enzyme-Linked Immunosorbent Assay

The concentrations of PAI-1 in the culture supernatants were measured using enzyme-linked immunsorbent assay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The concentrations of the cytokines were calculated from the absorbance using a data analysis program (SoftMax Pro; Molecular Devices).

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Isolation of total RNA, reverse transcription, and real-time quantitative polymerase chain reaction (PCR) assays were performed. Total cellular RNA was extracted using an RNeasy® kit (Qiagen Inc., Valencia, CA, USA) with DNase treatment (RNase-free DNase set; Qiagen) according to the manufacturer's instructions, and 500 ng of total RNA were reverse-transcribed into 500 ng of cDNA using a Transcriptor First Strand cDNA Synthesis Kit® (Roche Diagnostic Co., Indianapolis, IN, USA). Polymerase chain reactions were performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) using 50 ng of cDNA and the primers in an Applied Biosystems 7500 System® (Thermo Fisher Scientific, Waltham, MA, USA) under the following amplification conditions: 40 cycles of 95°C for 15 s and 60°C for 60 s. Primers were designed using LightCycler probe design software® (Roche Diagnostics Co., Mannheim, Germany). The following are the sequences of the primer used in the study (forward/reverse): human PAI-1 (5′-TCATAGTCTCAGCCCGC-3′; 5′-CCTTCAGAAAGAGTCATTAACAC-3′); and human GAPDH (5′-TGAACCATGAGAAGTATGACAACA-3′; 5′-TCTTCTGGGTGGCAGTG-3′). The relative induction of PAI-1 mRNA expression was determined after normalization to the GAPDH reference gene, whose expression level was defined as 1.

Detection of PAI-1 by Western Blotting and Silver Staining

The cell lysates were prepared using Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA) after infecting the HUVECs with *P. gingivalis*. Proteins (4 μg) from the cell lysates were separated by 12% SDS-PAGE under reducing conditions to detect the intracellular expression of PAI-1 in HUVECs. The gel was then transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan). The blots were then blocked using the Bullet Blocking One (Nacalai Tesque, Kyoto, Japan) for 5 min at room temperature (RT), before being incubated overnight at 4°C with mouse anti-PAI-1 mAb (Santa Cruz Biotechnology, Dallas, TX, USA) at 0.4 μg/mL (1:500), and horseradish peroxidase-conjugated horse anti-mouse IgG (Cell Signaling) at 1:2,000 for 1 h at RT.

For the detection of enzymatic digestion of rhPAI-1, 0.2 μg (454 nM) of rhPAI-1 (BioLegend, San Diego, CA, USA) was treated with 0.095 μg (45.4 nM) of purified Kgp or 0.086 μg (45.4 nM) of RgpA, at an enzyme:substrate (E:S) molar ratio of 1:10 in 0.1 M Tris-HCl, pH 7.6, containing 150 mM NaCl, 5 mM $CaCl_2$, and 0.5% bovine serum albumin (BSA), incubated at 37°C for 15 min, then solubilized with Cell Lysis Buffer (Cell Signaling), and boiled at 95°C for 5 min. The aliquots (0.05 μg) of rhPAI-1 were separated by 12%

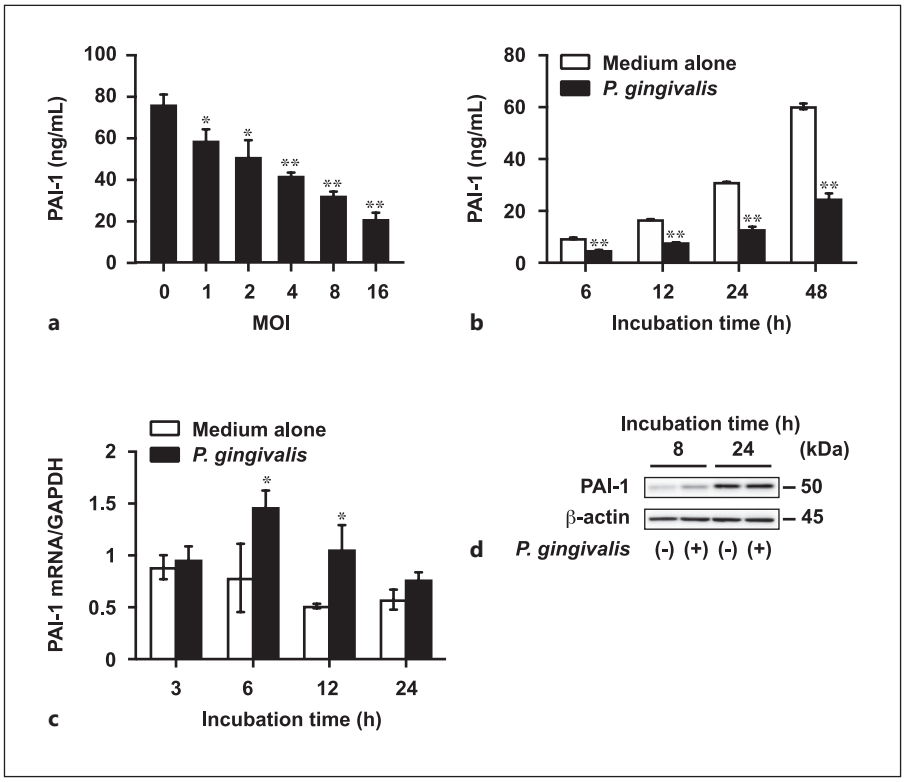


Fig. 1. *P. gingivalis* infection reduces PAI-1 production by human endothelial cells. HUVECs were infected with *P. gingivalis* W83 live bacteria at the indicated MOI (**a**) for 24 h or at an MOI of 16 for the indicated times (**b**). **a**, **b** Bar plots quantifying the amount of PAI-1 in the culture supernatants as measured by ELISA. **c**, **d** HUVECs were infected with *P. gingivalis* W83 live bacteria at an MOI of 16 for the indicated times. **c** Total RNA was extracted, converted to cDNA, and RT-qPCR quantified PAI-1 transcripts in control versus infected cells. **d** Total cell lysates were extracted and analyzed by Western blotting with anti-PAI-1 mAb. Data are representative of three independent experiments and are shown as means ± SD. Statistically significant differences are indicated as follows: $^{**}p < 0.01$; $^{*}p < 0.05$ compared with the respective untreated control. PAI-1, plasminogen activator inhibitor-1; HUVECs, human umbilical vein endothelial cells; MOI, multiplicity of infection; ELISA, enzyme-linked immunosorbent assay.

SDS-PAGE under reducing conditions. The gel was then transferred to a polyvinylidene difluoride membrane (ATTO). The blots were then blocked using the Bullet Blocking One (Nacalai Tesque) for 5 min at RT, before being incubated overnight at 4°C with mouse anti-His mAb (Fujifilm Wako, Osaka, Japan) at 0.01 μg/mL (1:5,000), and horseradish peroxidase-conjugated horse anti-mouse IgG (Cell Signaling) at 1:3,000 for 1 h at RT.

Finally, the blots were treated with the Chemi-Lumi One Super detection reagent (Nacalai Tesque). The chemiluminescent signal was detected using a FUSION FX luminescent image analyzer (Vilber Smart Imaging, Collegien, France). The intensity of the signals was quantified via densitometry scanning using ImageJ software (https://imagej.nih.gov/ij/), and the results are shown as relative values of the expression compared with that of the untreated control, which was converted to 1.

Enzyme Activity Assay

The amidolytic activities of gingipains from live bacterial cells, lyophilized whole cells, and OMVs of *P. gingivalis* W83 or ATCC 33277 were assayed at 37°C using 0.5 mM N-α-benzoyl-L-arginine-*p*-nitroanilide (BA-pNA) for Rgp and 0.5 mM Z-His-Glu-Lys-4-methylcoumaryl-7-amide for Kgp in 1.0 mL of 0.2 M Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, and 10 mM cysteine (pH 7.6). The absorbance of *p*-nitroaniline or 7-amino-4-methylcoumarin released from the substrate was measured at 405 nm and 370 nm, respectively.

Assessment of Cell Migration of Endothelial Cells by in vitro Scratch Assay

An in vitro scratch assay was performed as described previously [26], with some minor modifications to assess the effect of *P. gingivalis* on the migration of endothelial cells. Briefly, HUVECs were cultured to create a confluent monolayer in a collagen type I-coated 24-well plate (IWAKI®; AGC TECHNO GLASS). The HUVEC cell monolayer was scraped in a straight line to create a scratch with a p200 pipette tip. After scratching, the edge of the scratch was smoothened, and the debris was removed by washing twice with PBS, replaced with 1 mL of EGM-2, and observed by microscopy. The percentage of the closure area of the scratch was measured using ImageJ software until the scratch was closed entirely.

Statistical Analysis

All experiments were performed in triplicate to ensure reproducibility, and the data shown are representative results. Experimental values are presented as the mean ± standard deviation. The significance of the differences between the control and treated samples was evaluated using a Student's *t* test. Statistical significance was set at $p < 0.05$.

Results

P. gingivalis Infection Reduces PAI-1 Production in Human Endothelial Cells

PAI-1 produced by endothelial cells of the vascular endothelium regulates physiological functions such as hemostasis, wound healing, and inflammation [11, 27, 28]. In this study, we infected HUVECs – that constitutively produce high levels of PAI-1 under steady-state conditions (Fig. 1a)

– with live *P. gingivalis* and measured the PAI-1 levels in the culture supernatant to determine whether *P. gingivalis* affects PAI-1 production. Infection of live *P. gingivalis* W83 in HUVECs for 24 h resulted in a dose-dependent reduction in PAI-1 production (Fig. 1a), a phenomenon that continued until 48 h after infection (Fig. 1b). We did not observe any reduction in viability upon infecting the HUVECs with live *P. gingivalis* W83 at a multiplicity of infection (MOI) of 16 for 24 h, which led us to conclude that infection-mediated cytotoxicity did not play a role in the reduction of PAI-1 production (online suppl. Fig. 2a; see www.karger.com/doi/10.1159/000519737 for all online suppl. material). However, the expression of PAI-1 mRNA (Fig. 1c) and PAI-1 protein levels (Fig. 1d) did not decrease until 24 h after *P. gingivalis* W83 infection (MOI of 16), indicating that the *P. gingivalis*-induced reduction in PAI-1 levels did not involve the downregulation of de novo protein synthesis in the endothelial cells. We then stimulated the HUVECs with lyophilized whole bacterial cells or *P. gingivalis*-derived OMVs to identify which components of *P. gingivalis* reduced PAI-1 production in the endothelial cells. Stimulation of HUVECs with lyophilized whole *P. gingivalis* W83 cells (Fig. 2a) or OMVs (Fig. 2b) resulted in significantly reduced PAI-1 production in a dose-dependent manner. Cytotoxicity was not responsible for reduced PAI-1 production upon stimulation with lyophilized whole cells or OMVs because the viability of HUVECs did not change (online suppl. Fig. 2b, c). Our investigation revealed that the PAI-1 production by HUVECs reduced significantly upon coculturing them with live *P. gingivalis* W83 cells even when cell culture inserts (0.4 μm pore size) were used to separate the bacteria from HUVECs physically (Fig. 2c). However, the lyophilized *P. gingivalis* W83 whole cells failed to reduce PAI-1 production by HUVECs in the presence of the filter (Fig. 2d). These results collectively indicated the involvement of secreted factors by live *P. gingivalis* and membrane-bound factors expressed on live *P. gingivalis*, lyophilized whole cells, and OMVs of *P. gingivalis*, in reducing PAI-1 production by endothelial cells. Furthermore, the expression levels of PAI-1 mRNA did not change significantly in the absence or presence of the filter (Fig. 2e, f). These results suggest that the induction of PAI-1 mRNA expression in endothelial cells may require recognizing bacterial cell wall components.

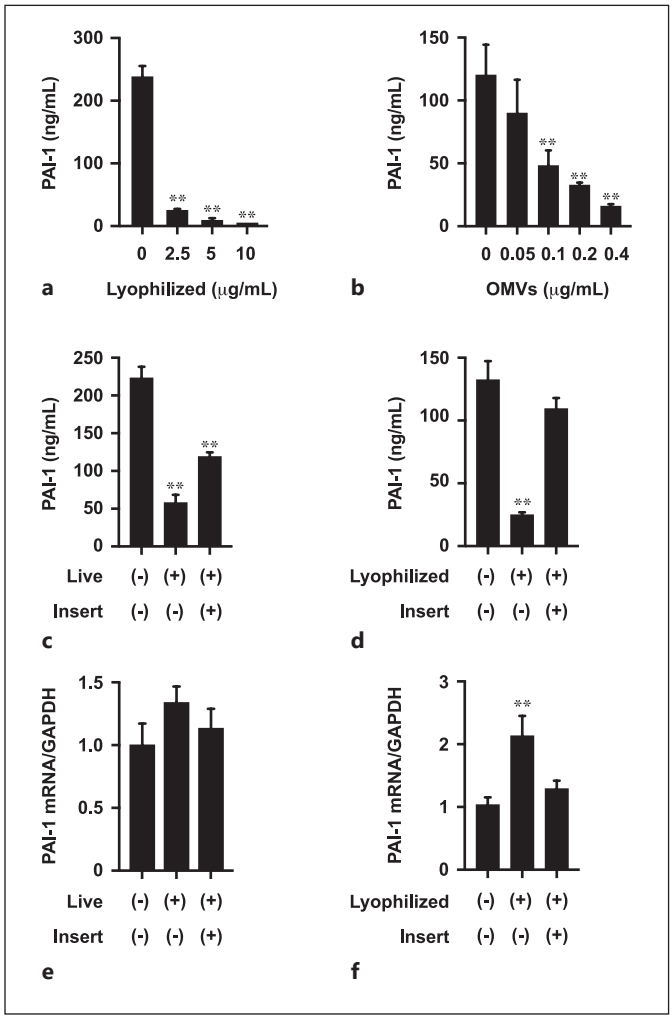
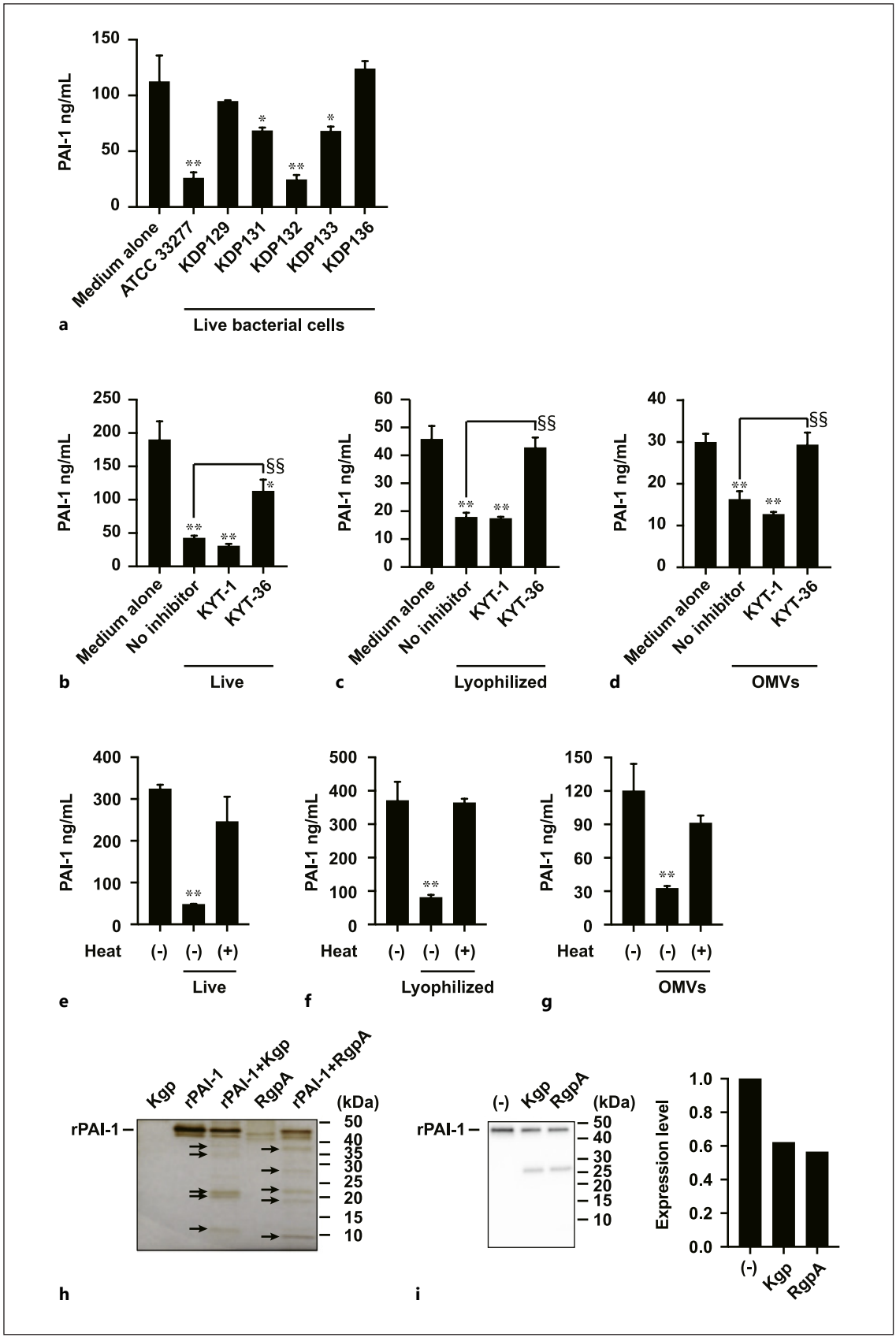


Fig. 2. Bacterial components derived from *P. gingivalis* reduce PAI-1 production in human endothelial cells. **a**, **b** HUVECs were stimulated with the indicated concentrations of lyophilized whole cells (**a**) or OMVs (**b**) derived from *P. gingivalis* W83 for 24 h. **c–f** *P. gingivalis* W83 live bacteria at an MOI of 16 (**c**, **e**) or lyophilized whole cells at 5 μg/mL (**d**, **f**) were added to the upper chamber, whereas HUVECs were cultured in the lower chamber. **a–d** The level of PAI-1 in the supernatants was analyzed using ELISA. **e**, **f** Total cellular RNA was extracted, and PAI-1 transcripts were quantified by RT-qPCR. Data are representative of three independent experiments and are shown as means ± SD. Statistically significant differences are indicated as follows: $^{**}p < 0.01$ compared with the respective untreated control. PAI-1, plasminogen activator inhibitor-1; HUVECs, human umbilical vein endothelial cells; MOI, multiplicity of infection; ELISA, enzyme-linked immunosorbent assay; OMVs, outer membrane vesicles.

P. gingivalis Gingipains Reduce PAI-1 Production in Human Endothelial Cells

P. gingivalis possesses two types of cysteine proteinases called gingipains, Rgps (RgpA and RgpB), and Kgp [6, 7]. We next examined whether gingipains degraded PAI-1, thereby reducing its levels by using *P. gingivalis* gingipain-deficient mutants. We used *P. gingivalis* strains lacking various combinations of gingipains to infect HUVECs and measured the resulting PAI-1 production (Fig. 3a).



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We used live bacterial cells for this experiment (Fig. 3a) because Kgp and RgpA, but not RgpB, harbor the hemagglutinin/adhesin domains that enable these proteinases to be expressed on the *P. gingivalis* cell membrane [6]. Thus, the lyophilized whole bacterial cells did not express RgpB on the cell membrane. *P. gingivalis* ATCC 33277 is the wild-type parent strain of *P. gingivalis* mutants. We found that the live *P. gingivalis* W83 and ATCC 33277 strains were equally efficient at reducing PAI-1 production by HUVECs (data not shown). Moreover, *P. gingivalis* ATCC 33277 and KDP132 (Δ*rgpB*) equally and significantly reduced PAI-1 production by HUVECs (Fig. 3a). Infection with *P. gingivalis* KDP131 (Δ*rgpA*) or KDP133 (Δ*rgpA* Δ*rgpB*) partially, but significantly, reduced PAI-1 production by HUVECs, similar to ATCC 33277 (Fig. 3a). In contrast, *P. gingivalis* KDP129 (Δ*kgp*) or KDP136 (Δ*rgpA* Δ*rgpB* Δ*kgp*) gingipain-null mutants did not significantly reduce PAI-1 production (Fig. 3a). These results suggest that Kgp is essential for the degradation of PAI-1 and that RgpA is partially involved in PAI-1 degradation. We found that the reduction in PAI-1 production by HUVECs infected with live *P. gingivalis* W83 cells was also partially inhibited upon pretreatment with KYT-36 (Kgp inhibitor); however, the PAI-1 production in the cells infected with KYT-36-pretreated *P. gingivalis* significantly recovered compared to those infected with *P. gingivalis* without the inhibitor pretreatment (Fig. 3b). In contrast, HUVECs infected with *P. gingivalis* pretreated with KYT-1 (Rgp inhibitor), the PAI-1 production did not recover compared with that in live *P. gingivalis* W83 cells (Fig. 3b). These results collectively indicated that the reduction in PAI-1 production by HUVECs in response to infection with live *P. gingivalis* was mediated by Kgp. Furthermore, the reduction in PAI-1 levels upon exposure to lyophilized whole cells or OMVs of *P. gingivalis* W83 was inhibited entirely upon exposure to lyophilized whole cells or OMVs pretreated with KYT-36, but not with KYT-1 (Fig. 3c, d). PAI-1 production by HUVECs was not significantly different from that observed in the unstimulated control as it was from the infection conditions with the live bacteria, lyophilized whole cells, and OMVs of *P. gingivalis* W83, which were preheated at 70°C for 1 h to eliminate enzyme activity (Fig. 3e–g). We confirmed that the enzyme activities of Rgps and Kgp were intact in lyophilized whole cells (online suppl. Fig. 1a, b) or OMVs (online suppl. Fig. 1e, f) derived from *P. gingivalis* W83. Moreover, the activities of Rgp and Kgp in lyophilized whole *P. gingivalis* W83 were entirely inhibited by KYT-1 and KYT-36, respectively (online suppl. Fig. 1c, d). These results indicate an association between Kgp activity and the reduction in PAI-1 production induced by lyophilized whole cells or OMVs from *P. gingivalis*. We also treated recombinant human PAI-1 (rhPAI-1) (44 kDa) with purified Kgp or RgpA, and the cleaved protein fragments of rhPAI-1 were detected using silver staining to demonstrate the proteolytic degradation of PAI-1 by gingipains. Our investigation did not reveal the presence of any cleaved protein fragments in the untreated rhPAI-1 control in the Tris buffer containing 0.5% BSA (Fig. 3h). However, five bands with molecular weights corresponding to 12, 21, 23, 36, and 48 kDa were detected upon treating 454 nM of rhPAI-1 with 45.4 nM of Kgp (Fig. 3h). Likewise, bands with molecular weights corresponding to 10, 19, 24, 28, and 38 kDa were detected upon treating 454 nM of rhPAI-1 with 45.4 nM of RgpA (Fig. 3h). Furthermore, we found that the amount of rhPAI-1 decreased within 15 min after treatment with Kgp and RgpA (Fig. 3i). One band with a molecular weight of about 26 kDa cleaved fragment by the treatment with Kgp and RgpA may be contained His-tag (Fig. 3i). However, this fragment could not be observed by silver staining because the amount of protein may be too small to observe (Fig. 3h). It has been

Fig. 3. Involvement of gingipains to attenuate PAI-1 production by human endothelial cells. **a** HUVECs were infected with live *P. gingivalis* ATCC 33277 (wild-type), *P. gingivalis* KDP129 (Δ*kgp*), *P. gingivalis* KDP131 (Δ*rgpA*), *P. gingivalis* KDP132 (Δ*rgpB*), *P. gingivalis* KDP133 (Δ*rgpA* Δ*rgpB*), or *P. gingivalis* KDP136 (Δ*rgpA* Δ*rgpB* Δ*kgp*) gingipain-null mutant bacteria at an MOI of 16 for 24 h. **b–d** HUVECs were stimulated for 24 h with live bacteria (MOI of 16) (**b**), lyophilized whole cells (5 μg/mL) (**c**), or OMVs (200 ng/mL) (**d**) of *P. gingivalis* W83, or those pretreated with 1 μM of KYT-1 (Rgps inhibitor) or 1 μM of KYT-36 (Kgp inhibitor) for 15 min at 37°C. **e–g** PAI-1 production by HUVECs stimulated for 24 h with *P. gingivalis* W83 live bacteria (MOI of 16) (**e**), lyophilized whole cells (5 μg/mL) (**f**), and OMVs (200 ng/mL) (**g**), or preheated (70°C for 1 h) of those. **a–g** The amount of PAI-1 in the culture supernatants was then analyzed by ELISA. **h**, **i** Recombinant human PAI-1 containing a His-tag was treated by purified Kgp or RgpA at 37°C for 15 min, separated by SDS-PAGE, and then visualized by silver staining (**h**) or analyzed by Western blotting with an anti-His mAb (**i**). Arrow indicates the cleaved PAI-1 fragments. Data are representative of three (**a–g**) or two (**h**, **i**) independent experiments and are shown as means ± SD. Statistically significant differences are indicated as follows: $^{**}p < 0.01$; $^{*}p < 0.05$ compared with the respective untreated control. $^{§§}p < 0.01$ compared with the respective control that was not treated with the gingipain inhibitor. PAI-1, plasminogen activator inhibitor-1; HUVECs, human umbilical vein endothelial cells; MOI, multiplicity of infection; ELISA, enzyme-linked immunosorbent assay; OMVs, outer membrane vesicles.

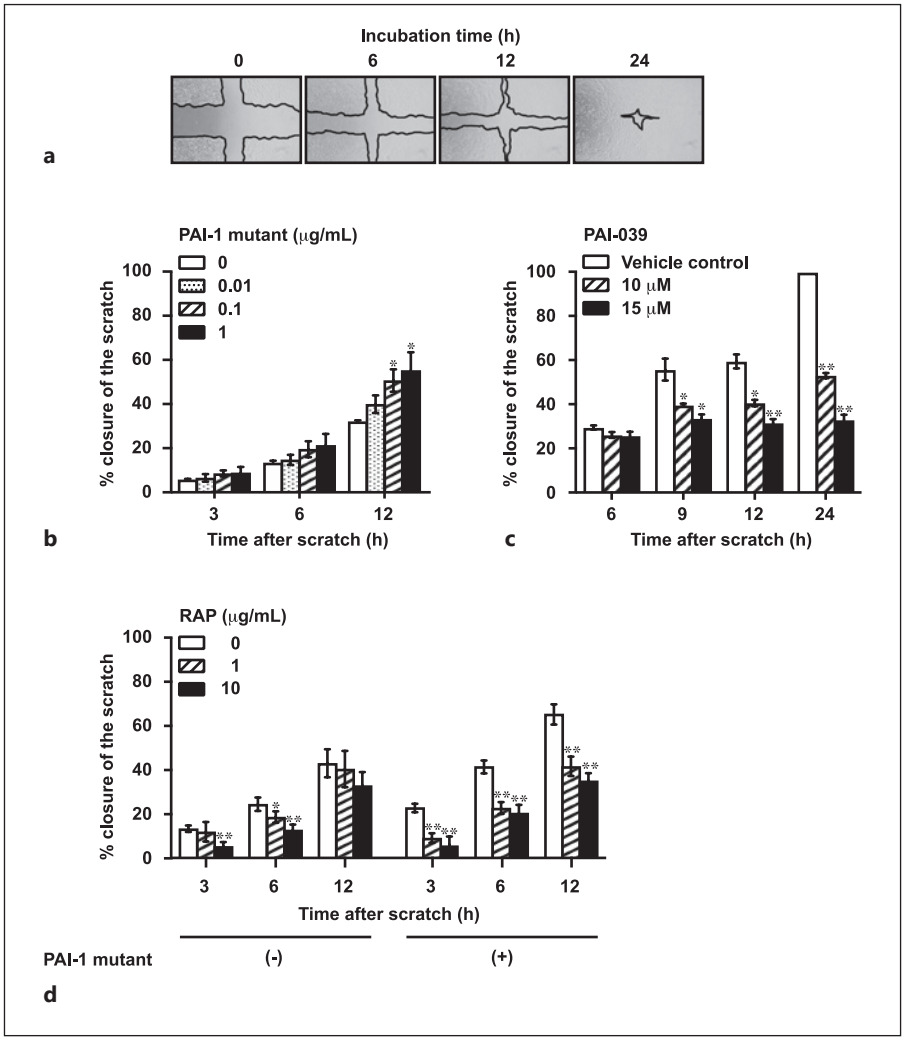

Fig. 4. Roles for PAI-1 in wound healing of endothelial cells. HUVECs grown in a cell monolayer were scratched, and then the cell migration after the injury was assessed by measuring the scratched area of the cells. **a** The closure of the scratched region of the untreated cells. Lines indicate margins of the scratched areas. **b–d** HUVECs were treated with the indicated concentrations of rhPAI-1 mutant (**b**), PAI-039 (PAI-1 inhibitor) (**c**), or rhPAI-1 mutant (0.5 μg/mL) ± the indicated concentrations of RAP (**d**) for the indicated times. The percentage of closure of the scratched area. Data are representative of three independent experiments and are shown as means ± SD. Statistically significant differences are indicated as follows: $^{**}p < 0.01$; $^{*}p < 0.05$ compared with the respective untreated control. PAI-1, plasminogen activator inhibitor-1; HUVECs, human umbilical vein endothelial cells; RAP, receptor-associated protein; rhPAI-1, recombinant human PAI-1.

reported that gingipains alter antigenicity by degrading the epitope of the antigens expressed on the cell surface [8]. Therefore, to eliminate the possibility that gingipains alter the binding of anti-human PAI-1 antibody to recombinant human PAI-1, we next examined whether gingipains degraded PAI-1 by Western blotting using a recombinant human PAI-1 containing a His-tag. On the other hand, the cleaved fragments observed by silver staining (Fig. 3h) could not be observed in Western blotting (Fig. 3i), which may be intermediate fragments that do not contain the His-tag degraded by gingipains. These results showed that both Kgp and RgpA were equally capable of proteolytically cleaving rhPAI-1. Furthermore, Kgp efficiently reduced the production of PAI-1 by endothelial cells, indicating that rhPAI-1 is a substrate of gingipains and that the gingipain-digested fragments of PAI-1 may lose their function.

Role of PAI-1 in Regulating HUVEC Migration during Wound Healing

It is known that PAI-1 contributes to wound healing by promoting cell migration [26, 28]. Endothelial cell-produced PAI-1 induces cell migration during the wound healing process in various pathological conditions of periodontitis [27, 29]. We further examined whether the *P. gingivalis*-induced reduction in PAI-1 production affects endothelial cell migration using an in vitro scratch assay [26]. We found that when HUVECs cultured as a confluent monolayer were scraped, the scratched region almost completely closed after 24 h (Fig. 4a) [25]. We treated the HUVECs with recombinant human PAI-1 mutant to confirm whether the closure of the scratched region could be attributed to the endothelial cell-derived PAI-1-induced cell migration (Fig. 4b). We also observed that the closure of the scratched region of the HUVEC

monolayer was significantly promoted in a dose-dependent manner (0.1–1 μg/mL), 12 h after stimulation with the rhPAI-1 mutant (Fig. 4b; online suppl. Fig. 4a). Furthermore, our investigations revealed that the PAI-1-induced wound repair could not be attributed to promoting the cell proliferation induced by the PAI-1 mutant, as cell proliferation remained unchanged upon the treatment (online suppl. Fig. 4b). Moreover, the closure of the scratched region was inhibited by PAI-039, a PAI-1 inhibitor, to similar extent as the no inhibitor control (Fig. 4c; online suppl. Fig. 4c). The inhibition of wound repair could be attributed to reduced cell migration in the presence of PAI-039 as the cell proliferation of HUVECs was not affected by PAI-039 (online suppl. Fig. 4d). The closure of the scratched region in the HUVEC monolayer was also delayed by mitomycin C (online suppl. Fig. 3a, b) that inhibited cell migration and proliferation (online suppl. Fig. 3c) [30]. It has been reported that PAI-1 binds to the LRP on endothelial cells, thereby triggering the Janus kinase (JAK)/signal transducer and activator of transcription (STAT)-1-mediated signaling to promote endothelial cell migration [29]. Our data showed that the closure of the scratched region in the HUVEC monolayer was inhibited upon treatment with RAP, an LRP antagonist, 3 and 6 h after scratching (Fig. 4d; online suppl. Fig. 4e). In addition, RAP also inhibited the promotion of wound repair by the PAI-1 mutant (Fig. 4d; online suppl. Fig. 4e). These data indicated that the wound healing response of HUVECs promoted the PAI-1-induced activation of LRP signaling. Therefore, it is suggested that the wound healing response of blood vessels requires the PAI-1-induced migration of endothelial cells, a signal transmitted via LRP in an autocrine manner.

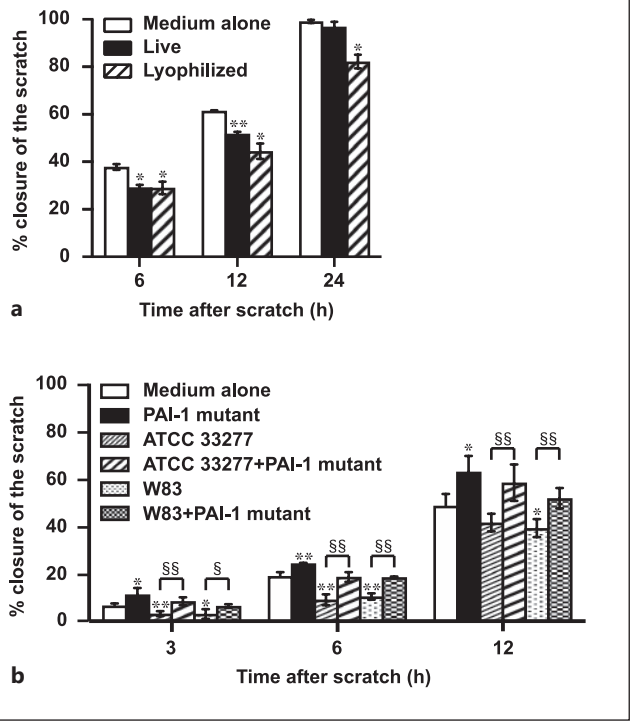

Fig. 5. *P. gingivalis* delays wound healing of endothelial cells in a PAI-1-dependent manner. HUVECs in a cell monolayer were scratched, then cell migration after the injury was assessed by measuring the scratched area of the cells. HUVECs were stimulated with live bacteria (MOI of 16) or lyophilized whole cells (5 μg/mL) of *P. gingivalis* W83 (**a**), or lyophilized whole cells (5 μg/mL) of *P. gingivalis* W83 or ATCC 33277 ± rhPAI-1 mutant (0.5 μg/mL) (**b**) for the indicated times. The percentage of closure of the scratched area. Data are representative of three independent experiments and are shown as means ± SD. Statistically significant differences are indicated as follows: $^{**}p < 0.01$; $^{*}p < 0.05$ compared with medium alone. $^{§§}p < 0.01$; $^{§}p < 0.05$ compared with the respective control stimulated with *P. gingivalis* but not treated with the rhPAI-1 mutant. PAI-1, plasminogen activator inhibitor-1; HUVECs, human umbilical vein endothelial cells; MOI, multiplicity of infection; rhPAI-1, recombinant human PAI-1.

P. gingivalis Gingipains Induces a Delay in Human Endothelial Cell Wound Healing

We next investigated whether *P. gingivalis* attenuates the wound healing response of human endothelial cells using an in vitro scratch assay. A confluent HUVEC monolayer was scraped, and the closure of the scratched regions in the absence or presence of *P. gingivalis* was observed. The culture of HUVECs with live or lyophilized whole *P. gingivalis* W83 significantly delayed the closure of the scratched regions (Fig. 5a; online suppl. Fig. 5a). Our investigation confirmed that the delays in wound repair were not caused by the *P. gingivalis*-induced inhibition of HUVEC proliferation (online suppl. Fig. 5b). Moreover, we observed that the delays in wound repair of the HUVEC monolayers by the lyophilized whole *P. gingivalis* W83 and ATCC 33277 were significantly corrected by the PAI-1 mutant (Fig. 5b; online suppl. Fig. 5c). These findings collectively indicated that *P. gingivalis* disturbed the PAI-1-dependent migration of endothelial cells, resulting in the attenuation of the wound healing response of the endothelial cell layer.

Next, we investigated whether the *P. gingivalis*-induced delayed wound healing response was mediated by gingipains. As expected, the wound closure was delayed in the cells infected with live *P. gingivalis* ATCC 33277, but not in cells infected with live *P. gingivalis* KDP136 or heat-inactivated *P. gingivalis* ATCC 33277 (Fig. 6a; online suppl. Fig. 6a). HUVEC proliferation was also not altered

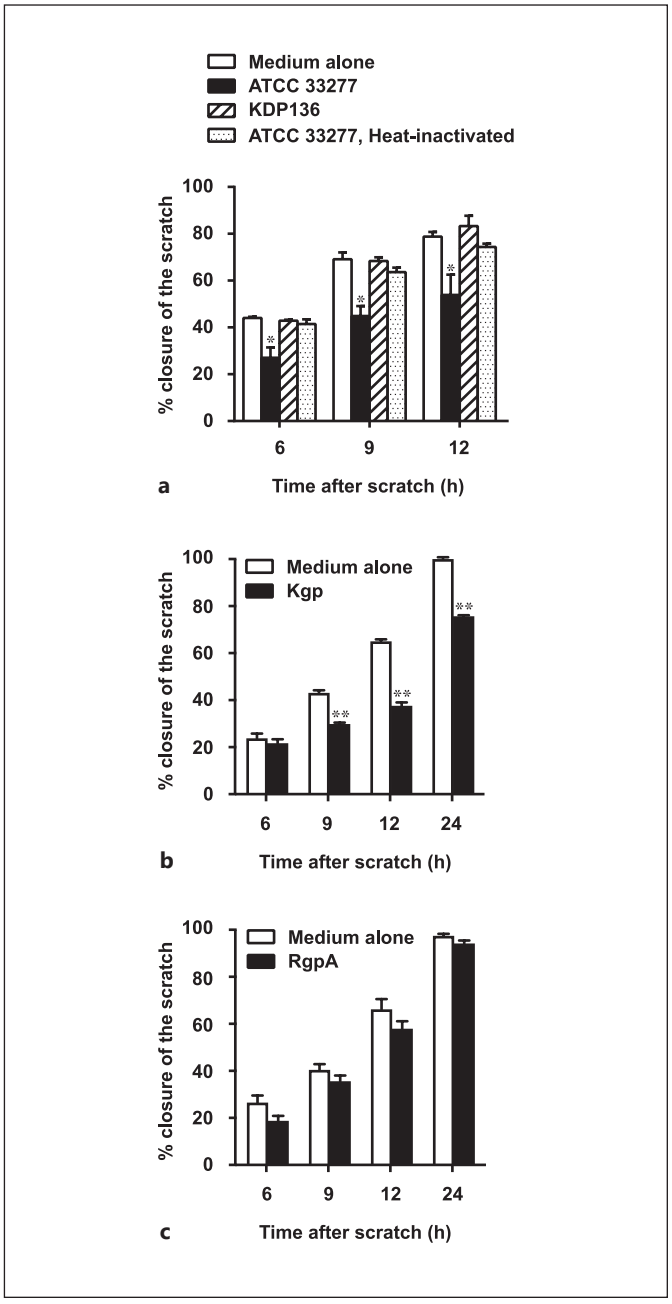

Fig. 6. Kgp preferentially induces the delay in wound healing of endothelial cells. HUVECs in a cell monolayer were scratched, and the cell migration after the injury was assessed by measuring the scratched area of the cells. **a** HUVECs were stimulated with live bacteria (MOI of 16) of *P. gingivalis* ATCC 33277, *P. gingivalis* KDP136, or heat-pretreated (70°C for 1 h) *P. gingivalis* ATCC 33277 for the indicated times. HUVECs were treated with purified Kgp (0.07 μM) (**b**) or RgpA (0.07 μM) (**c**) for the indicated times. Data are representative of three (**a**) or two (**b**, **c**) independent experiments and are shown as means ± SD. Statistically significant differences are indicated as follows: $^{**}p < 0.01$; $^{*}p < 0.05$ compared with the respective untreated control. HUVECs, human umbilical vein endothelial cells; MOI, multiplicity of infection.

upon infection with *P. gingivalis* ATCC 33277 or KDP136, suggesting the involvement of gingipains in inhibiting endothelial cell migration (online suppl. Fig. 6b). Depending on our findings so far, we further investigated the role of gingipains in the *P. gingivalis*-induced delayed wound healing response. The closure of the scratched regions of the HUVEC monolayer was significantly delayed upon treatment with Kgp (Fig. 6b; online suppl. Fig. 6c). However, there was no change in the closure of the scratched regions in RgpA-treated cell monolayers (Fig. 6c; online suppl. Fig. 6d). These results indicated that the degradation of PAI-1 by Kgp played a critical role in the delayed wound healing response of human endothelial cells. These findings suggest that *P. gingivalis* induces Kgp-mediated degradation of PAI-1, a phenomenon that attenuates LRP-mediated cell migration, resulting in the delayed wound healing response of the endothelial cell layer.

Discussion

In this study, we utilized multiple approaches to demonstrate that *P. gingivalis* infection reduces PAI-1 production in human endothelial cells using HUVECs. Gingival endothelial cells isolated from periodontally healthy individuals exhibit the production of PAI-1 constitutively [31]. The reduction of PAI-1 production by endothelial cells resulted from the proteolytic cleavage of PAI-1 by bacterial proteinases, gingipains. A reduction in PAI-1 production attenuates the migration of endothelial cells, leading to a delay in their wound healing responses, which involved in the activation of LRP-mediated signaling by PAI-1 in an autocrine manner. Based on these results, we have provided evidence that the degradation of PAI-1 by purified gingipains delays the wound healing responses of the endothelium cell layer, which might induce the dysfunction of vascular endothelium and the bleeding of periodontal tissues, resulting in the invasion of oral bacteria into the blood vessels. These findings suggest that *P. gingivalis* may manipulate the innate immune system of the blood vessels by using bacterial proteinases to degrade PAI-1 produced by endothelial cells, thereby spreading the bacterial infection from periodontal tissues.

PAI-1 localizes in the gingival epithelium and connective tissue, and its expression levels are almost comparable in the gingival tissues of healthy individuals and patients with chronic periodontitis [32]. Furthermore, PAI-1 expression was not elevated in inflamed gingiva in experimental periodontitis [33]. Periodontitis is a mixed

infection and the periodontopathogenic bacteria form biofilms in the subgingival environment. Activation of plasminogen-bound *Fusobacterium nucleatum*, a periodontopathogenic bacteria, by uPA significantly induced the degradation of fibronectin or the tissue inhibitor of metalloproteinase-1, thereby promoting host tissue destruction [34]. These findings suggest that the decrease in PAI-1 levels due to the gingipain-induced degradation of PAI-1 may cause the destruction of periodontal tissues via the activation of uPA. In contrast to PAI-1 expression in periodontal tissues, PAI-1 levels are elevated in the plasma of patients with severe chronic periodontitis and are associated with the degree of inflammatory responses in periodontal tissues [35]. The expression of PAI-1 mRNA is induced by bacterial lipoprotein via TLR2 expressed on endothelial cells [36], and *P. gingivalis* has been reported to invade endothelial cells [37–40]. Invaded endothelial cells have been shown to produce PAI-1 upon infection with large numbers of *P. gingivalis* at an MOI of 100, but not upon infection with a noninvasive strain of *P. gingivalis* that lacks fimbriae [41]. In blood vessels, a clearance of fibrin is carried out by plasmin, which is converted from plasminogen through the binding of tPA, which has been shown to exacerbate atherosclerosis [42]. Since endothelial cells produce both PAI-1 and tPA, increased PAI-1 production leads to the inactivation of PAs [43]. These findings suggest that *P. gingivalis* may induce an inflammatory response in endothelial cells and blood vessels, exacerbating systemic diseases such as atherosclerosis. In fact, *P. gingivalis* induces PAI-1 expression in human endothelial cells and human platelets [23, 41]. The results of our study corroborate the findings from such studies by highlighting how PAI-1 is degraded by gingipains from *P. gingivalis* in the periodontal tissues. Taking these findings, together with our results, into consideration, the function of PAI-1 in periodontitis may have two implications: (a) the decreased PAI-1 level in periodontal tissues may cause the delayed wound repair of the endothelium, and the bleeding and destruction of periodontal tissues by the activation of PAs, and (b) the increased PAI-1 level in blood vessels may cause the attenuation of plasminogen activation system by the inactivation of PAs.

Our study revealed how PAI-1 production in HUVECs was markedly reduced by stimulating lyophilized whole cells expressing Kgp and RgpA on the cell membrane (Fig. 2a). This PAI-1 reduction was abolished when the lyophilized whole cells were preheated (1 h at 70°C) to inactivate the enzyme activity (Fig. 3f). Furthermore, the PAI-1 reduction in response to exposure to the lyophilized whole cells was abolished in the coculture system using cell culture inserts (Fig. 2d), suggesting the role of Kgp and RgpA in the reduction of PAI-1 production by endothelial cells. Moreover, a previous report has demonstrated how the production of PAI-1 in human platelets induced by Pam_3CSK_4, a TLR2 ligand, is attenuated by *P. gingivalis*, but not by the Kgp-deficient strain [23]. PAI-1 production in HUVECs was not reduced upon infection with *P. gingivalis* KDP129 (Δ*kgp*) and KDP136 (Δ*kgp* Δ*rgpA* Δ*rgpB*) (Fig. 3a). However, the reduced PAI-1 production in HUVECs partially recovered upon infection with *P. gingivalis* KDP131 (Δ*rgpA*) and KDP133 (Δ*rgpA* Δ*rgpB*), but not upon infection with *P. gingivalis* KDP132 (Δ*rgpB*) (Fig. 3a). These results collectively indicated that the efficiency of gingipains to degrade PAI-1 is more dependent on Kgp than on RgpA. Moreover, RgpB also did not exhibit PAI-1-reducing activity. Our experiments to determine the reaction of rhPAI-1 with purified gingipains revealed that Kgp and RgpA had almost equal efficiency with respect to promoting gingipain-mediated degradation of rhPAI-1 (Fig. 3i). We hypothesized three possibilities that could explain the higher efficiency of Kgp to degrade PAI-1 than the Rgps. First, Kgp and RgpA bind to fibrinogen, fibronectin, and laminin because hemagglutinin/adhesin domains augment the cleavage of fibrinogen by the catalytic domain of gingipains [44], indicating that the hemagglutinin/adhesin domain may effectively bind to PAI-1. Second, it has been reported that RgpA and Kgp form membrane-bound complexes, outer membrane vesicles, and soluble forms and exert major pathogenic factors [44, 45]. Fleetwood et al. [46] reported that the RgpA-Kgp complex efficiently cleaves pro-uPA, a precursor of uPA, and plasminogen, yielding active uPA and plasmin, respectively. Third, proteinase inhibitors may inhibit Rgps activity. It has been reported that Kgp digests fibrinogen more effectively than Rgp in the presence of the plasma [47, 48]. Kgp is the most potent fibrinogen/fibrin-degrading enzyme of the three gingipains in the presence of the human plasma [47]. The enzyme activities of Rgps are inhibited by the plasma proteinase inhibitor α2-macroglobulin, while Kgp is resistant [49]. Moreover, α2-macroglobulin has also been found in FBS [50]. In this study, recombinant PAI-1 was treated with purified gingipains using a buffer in the presence of 0.5% BSA (Fig. 3i) as PAI-1 is stabilized by interaction with plasma components, such as vitronectin [29]. PAI-1 is synthesized in the active conformation but spontaneously converts to a latent form with a relatively short active half-life of 1–2 h [25, 29]. Importantly, as our experiments aimed at measuring

PAI-1 produced by HUVECs stimulated with *P. gingivalis* used a medium containing 2% FBS, proteinase inhibitors in the serum may inhibit the degradation of PAI-1 by Rgps.

Endothelial cells are the main cellular constituents of blood vessels and separate blood from the underlying tissues. It is known that PAI-1 plays a vital role in the migration of immune cells at the site of inflammation [51, 52]. In this study, the delays in wound repair by the HUVEC monolayers caused by *P. gingivalis* were significantly rescued by adding PAI-1 mutant (Fig. 5b; online suppl. Fig. 5c). These results suggest that the degradation of PAI-1 by *P. gingivalis* caused the delay in wound healing. Furthermore, the wound closure was delayed in the cells cocultured with *P. gingivalis* ATCC 333277, but not in cells cocultured with live *P. gingivalis* KDP136 or heat-inactivated *P. gingivalis* ATCC 33277 (Fig. 6a; online suppl. Fig. 6a). In addition, the wound healing responses of the HUVEC monolayers depended on PAI-1 produced by the cells because 15 μM of PAI-039 completely inhibited the wound closure (Fig. 4b; online suppl. Fig. 4c), but not the cell proliferation (online suppl. Fig. 4d). These results suggested that the recovery of the delay in wound healing by *P. gingivalis* KDP136 or heat-inactivated *P. gingivalis* was probably responsible for protecting the degradation of PAI-1. PAI-1 expressed in smooth muscle cells regulates cell migration by activating the LRP and the downstream JAK/STAT signaling pathway [29]. Furthermore, PAI-039 inhibits the migration of smooth muscle cells in an STAT1-dependent manner [53]. In this study, the wound healing of the HUVEC monolayers was inhibited by PAI-039 and RAP, and this effect was mediated through PAI-1-induced autocrine activation of LRP signaling (Fig. 4c, d; online suppl. Fig. 4c, e). The delayed wound healing responses were probably due to the degradation of PAI-1 in HUVECs by gingipains restored by adding the PAI-1 mutant (Fig. 5b; online suppl. Fig. 5c). PAI-1 produced by endothelial cells plays a role in tissue remodeling during wound repair in the vascular endothelium of periodontal tissues [28].

In chronic periodontitis, bleeding from periodontal pockets is beneficial for *P. gingivalis* because the bacterium requires hemin, which comes from erythrocytes, for the growth of the bacterium [54, 55]. Kgp plays an indispensable role in the release of hemin from hemoglobin [56, 57]. In this study, wound repair of the HUVEC monolayers was also delayed upon treatment with purified Kgp (Fig. 6b; online suppl. Fig. 6c). The decreasing level of PAI-1 leads to excessive fibrinolysis due to the processing of plasminogen to plasmin, resulting in bleeding caused by insufficient wound healing responses [58]. Taken together, the Kgp-mediated decrease in PAI-1 levels is associated with promoting fibrinolysis and contributing to the bleeding tendency in periodontal tissues [59]. In summary, we hypothesized that the degradation of endothelial cell-derived PAI-1 by *P. gingivalis* gingipains delayed endothelial wound repair, resulted in increased bleeding risks, promotion of periodontal tissue destruction, and spreading of the *P. gingivalis* infection from the periodontal tissue to various other systemic organs via the bloodstream.

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Statement of Ethics

No ethical approval was sought as this research did not require animal or human involvement.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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Author Contributions

H. Tada and L.-T. Song conceived and designed the study, performed data acquisition and analysis, contributed to the interpretation of the data, prepared the figures, and drafted the manuscript. T. Nishioka, E. Nemoto, and T. Imamura provided technical supports. J. Potempa, C.-Y. Li, K. Matsushita, and S. Sugawara contributed to the interpretation of the data. All authors reviewed, revised, and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary files. Further inquiries can be directed to the corresponding author upon reasonable request.

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