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Functional Implication of the Hydrolysis of Platelet Endothelial Cell Adhesion Molecule 1 (CD31) by Gingipains of *Porphyromonas gingivalis* for the Pathology of Periodontal Disease

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Periodontitis is a response of highly vascularized tissues to the adjacent microflora of dental plaque. Progressive disease has been related to consortia of anaerobic bacteria, with the gram-negative organism *Porphyromonas gingivalis* particularly implicated. The gingipains, comprising a group of cysteine proteinases and associated hemagglutinin domains, are major virulence determinants of this organism. As vascular expression of leukocyte adhesion molecules is a critical determinant of tissue response to microbial challenge, the objective of this study was to determine the capacity of gingipains to modulate the expression and function of these receptors. Given the potential multifunctional role of platelet endothelial cell adhesion molecule 1 (PECAM-1) in the vasculature, the effect of gingipains on PECAM-1 expression by endothelial cells was examined. Activated gingipains preferentially down-regulated PECAM-1 expression on endothelial cells compared with vascular cell adhesion molecule 1 and endothelial-leukocyte adhesion molecule 1, but the reduction in PECAM-1 expression was completely inhibited in the presence of the cysteine proteinase inhibitor TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone). Endothelial monolayers treated with activated gingipains demonstrated progressive intercellular gap formation that correlated with reduced intercellular junctional PECAM-1 expression as determined by Western blotting and immunofluorescence microscopy. This was accompanied by enhanced transfer of both albumin and neutrophils across the monolayer. The results suggest that degradation of PECAM-1 by gingipains contributes to increased vascular permeability and neutrophil flux at disease sites.

Periodontal diseases are chronic inflammatory diseases affecting the well-vascularized connective tissues of the periodontium (20). Microbial challenge at the gingival sulcus leads to an inflammatory response in the adjacent soft tissues that is characterized by a migration of leukocytes from postcapillary venules into the extravascular tissue. It has been shown that the numerical density of vascular profiles in the connective tissue increases significantly subjacent to the altered epithelial lining of the periodontal pocket (11). Characteristic endothelial cell adhesion molecule expression related to leukocyte infiltration of human gingiva has been demonstrated (38, 44).

Porphyromonas gingivalis has been implicated as a major etiological agent in the onset and progression of chronic periodontitis (59). The trypsin-like cysteine proteinases or gingipains (Arg-gingipain, encoded by the two genes *rgpA* and *rgpB*, and a Lys-specific gingipain encoded by a single gene, *kgp*) have been closely linked with the virulence of this gram-negative anaerobic bacteria (28, 31, 36). The outer membrane-associated gingipains (RgpA and Kgp) extracted from *P. gingivalis* ATCC 33277 contain a catalytic domain and a hemagglutinin/adhesin domain (54, 70). Cysteine proteinase activities may affect cytokine inactivation and degradation (69, 70), acquisition of metabolically necessary iron and porphyrin from hemoglobin (14), enhancement of vascular permeability through plasma prekallikrein activation and bradykinin release (33), and degradation of epithelial cell-cell junctional complexes (37).

Leukocyte emigration from the bloodstream into tissues at sites of inflammation is controlled by sequential intercellular adhesion events with endothelial cells that line the vascular wall. The initial rolling and tethering steps are mediated by members of the selectin family including endothelial leukocyte adhesion molecule 1 (ELAM-1 or E-selectin) and L-selectin (4, 8, 60). Vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1), located on the surface of cytokine-activated endothelium, belong to the immunoglobulin supergene family and are considered to be involved in the next step of leukocyte-endothelium interaction, where a tighter adhesion takes place (5, 51). Gemmell et al. found that endothelial cells did not express ELAM-1 in chronically inflamed periodontal tissue (27). Other studies have indicated that ELAM-1, VCAM-1, and ICAM-1 can be detected on endothelial cells adjacent to the junctional epithelium early in the course of experimentally induced gingivitis (44, 50, 65), suggesting that they are involved in crucial processes which direct leukocyte migration into the tissues and toward the gingival sulcus. Further, ICAM-1 on oral epithelial cells has recently been shown to be susceptible to proteolysis by gingipains (63). The importance of cell adhesion molecules is highlighted by the rapid and severe periodontitis that characterizes leukocyte adhesion deficiency where polymorphonuclear leukocytes (PMNs) are unable to migrate through the endothelium of gingival blood vessels (22).

Leukocyte adhesion to endothelial cells is followed by leu-

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kocyte penetration at interendothelial cell junctions, thus permitting leukocyte migration into the subendothelial space (4). Platelet endothelial cell adhesion molecule 1 (PECAM-1) is one of the most abundant proteins on the endothelial cell surface, with nearly one million molecules per cell (49). PECAM-1 is expressed on the surface of platelets and leukocytes and, in particular, at intercellular junctions of endothelial cells (48). Studies have shown a role for PECAM-1 in interendothelial adhesion (16) and leukocyte-endothelial cell interactions (6). Muller et al. (45, 46) were the first to show that pretreating monocytes or neutrophils with antibodies specific for PECAM-1 inhibited their emigration across an endothelial cell monolayer in a quantitative in vitro assay of transendothelial migration. Other studies have also demonstrated an abnormal transit of polymorphonuclear leukocytes across vascular basement membranes in PECAM-1-deficient mice (19, 64).

PECAM-1 has also been implicated in vascular responses in wound healing, angiogenesis (15, 25), and the development of the early cardiovascular system (2). Investigation of cultured PECAM-1-deficient endothelial cells has provided confirmatory evidence that PECAM-1 has an important function in the maintenance of a vascular permeability barrier (24). In the lesions of gingivitis and periodontitis, the expression of PECAM-1 on mononuclear infiltrates increases significantly with increasing size of infiltrate (27). Although these studies implicate cell adhesion molecules in the pathogenesis of periodontal disease, the ability of gingipains from *P. gingivalis* to interact with these molecules has not been previously examined.

In this study we report that the gingipains adhere to human umbilical vein endothelial cells (HUVECs) and rapidly degrade PECAM-1 at endothelial cell lateral junctions to induce the formation of intercellular gaps. In contrast, tumor necrosis factor α (TNF-α)-induced VCAM-1 and ELAM-1 expression on HUVECs is minimally affected by gingipains. Evidence is presented to show that loss of PECAM-1 on HUVECs can facilitate neutrophil transmigration in vitro. Hence, gingipains of *P. gingivalis* may contribute to the vascular disruption of periodontal tissue and facilitate leukocyte extravasation into the site.

MATERIALS AND METHODS

Chemicals and reagents. Bovine serum albumin, Evans blue dye, formyl-Met-Leu-Phe (fMLP), L-cysteine, paraformaldehyde, protease inhibitor cocktails (for mammalian tissues), sodium azide (NaN_3), sodium dodecyl sulfate (SDS), *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), Trizma base, Tris-hydrochloride (Tris-HCl), trypsin, and Tween 20 were purchased from Sigma (St. Louis, Missouri). Fetal calf serum (FCS) and RPMI medium were obtained from ICN Biochemicals (Irvine, Calif.); 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from Calbiochem (La Jolla, Calif.). Phosphate-buffered saline (PBS) and Trypticase soy broth were purchased from Oxoid (Basingstoke, United Kingdom). Calcein-AM and Alexa Fluor 488 were purchased from Molecular Probes (Eugene, Oreg.). All reagents for electrophoresis and Western blotting were from Bio-Rad (Richmond, Calif.).

Recombinant cytokines and antibodies. Human recombinant TNF-α was obtained from R&D Systems (Minneapolis, Minn.). Mouse monoclonal antibodies (MAb) specific for human CD31 (PECAM-1), CD62E (ELAM-1), and CD106 or VCAM-1 (MAb 51-10C9) were purchased from Becton Dickinson Inc. (Heidelberg, Germany). A blocking antibody to PECAM-1 (clone HEC7) was also purchased from Research Diagnostics Inc. (Flanders, N.J.). MAb IIB2 was raised in mice against gingipains as previously described (14). IIB2 recognizes the hemagglutinin/adhesin domains, HA1 and HA3, of RgpA and Kgp (39 kDa) from *P. gingivalis*.

Bacterial strain and proteinase purification. *P. gingivalis* (ATCC 33277) was grown in enriched Trypticase soy broth under anaerobic conditions for 48 h. Organisms at a density of 1.5 gm/cm^3 were suspended in saline, stirred for 1 h at 4°C, washed three times with pyrogen-free water, and lyophilized. Arg-gingipain and Lys-gingipain proteinase-adhesin complexes were purified according to the method previously described (70). Purified gingipains were dialyzed against five changes of PBS with 1 mM $CaCl_2$, pH 7.5.

Binding of gingipains to endothelial cells. HUVECs were isolated by collagenase treatment of the vessels as described elsewhere (35). Endothelial cells were seeded in 1% gelatin-coated 12-well plates (Corning Costar, Cambridge, Mass.) in M199 medium supplemented with 30 μg of endothelial cell growth factor per ml and containing 20% FCS, 10 U of heparin per ml, 50 U of penicillin ml, and 50 μg of streptomycin per ml. Cells used in these experiments were confluent and at passage levels four through six. Before the experiment, FCS was withheld, and the cells were cultured in M199 medium. Confluent endothelial cell monolayers were then treated with 5 mM cysteine-activated RgpA and Kgp at concentrations of 210 to 0.9 nM (1:3 serial dilution) for 1 h at 37°C. In serum experiments, cell monolayers were incubated in the presence or absence of 10 to 40% FCS or 2 mM TLCK (dialyzed against PBS and cultured in the absence of serum) for 1 h at 37°C. HUVECs were also tested in the presence or absence of the indicated concentrations of cysteine added to the gingipains. After the incubations, cells were harvested by gentle scraping and washed three times with 2 ml of PBS, stained with mouse IIB2 MAb, followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (IgG; Dako, Sydney, Australia), and the relative labeling of the cell population was quantified in a Becton Dickinson FACScan analyzer. Isotype-matched IgG1 (Dako) was used as a control.

Evaluation of PECAM-1 expression on HUVECs. Confluent HUVECs were incubated with a 5 mM concentration of cysteine-activated RgpA or Kgp at concentrations of 70, 23, or 7.8 nM at 37°C without serum and harvested by scraping at 15-min intervals for up to 1 h. Gingipain-treated HUVECs were similarly cultured in the presence or absence of 5, 10, and 20% FCS or 2 mM TLCK for 1 h. Endothelial cells were then washed twice with cold PBS–0.1% bovine serum albumin–0.01% NaN_3 and stained with the corresponding primary mouse anti-human MAbs (1:50) against PECAM-1 or with matched isotype control antibody. Cells were then labeled with a 1:100 concentration of fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Dako) and quantitated by using a Becton Dickinson FACScan analyzer. Incubations were for 50 min at 4°C for each stage. Volume gates were set to include the entire HUVEC population. Data were collected as histograms of relative fluorescence in a logarithmic scale on the *x* axis, with cell number as a linear scale on the *y* axis. To assess the effect of fixation on PECAM-1 expression, HUVECs were fixed in 3% paraformaldehyde in PBS for 5 min at room temperature and washed twice with PBS. Fixed or unfixed endothelial cells were then incubated with activated gingipains (70 nM each) for 1 h at 37°C without serum. After the incubation, HUVECs were washed, stained for surface PECAM-1, and analyzed by flow cytometry.

Western blot analysis of PECAM-1 expression on HUVECs after gingipains treatment. Confluent HUVECs grown on 1% gelatin-coated six-well plates (Corning Costar) were cultured with activated RgpA or Kgp (70 nM each) for various times at 37°C in the absence of serum. After incubation, reactions were stopped with 2 mM TLCK, and HUVECs were isolated by gently scraping. HUVECs were also treated with 2 mM TLCK-treated gingipains for 1 h without serum. Endothelial cells were then washed twice with 5 mM Tris-HCl buffer (pH 7.5) and mixed with 20% (vol/vol) protease inhibitor cocktail (Sigma). Endothelial cells were then lysed directly in SDS reducing sample buffer by three cycles of heat (100°C for 3 min) and vigorous mixing. Aliquots were then resolved by SDS–10% polyacrylamide gel electrophoresis (39) and transferred to polyvinylidene difluoride membranes (66). The primary antibody, mouse anti-human PECAM-1 MAb, was diluted 1:500 in 5% nonfat dry milk–Tris-buffered-saline before use. Alkaline phosphatase rabbit anti-mouse (Dako), diluted 1:5,000, was used as the secondary antibody. Membranes were washed five times in Tris-buffered saline–0.1% Tween 20 between each step. Color was developed in a solution containing nitroblue tetrazolium chloride (1.65 mg) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (0.8 mg) in 10 ml of 100 mM Tris-HCl (pH 9.5).

Endothelial cell permeability assay. The permeability of HUVEC monolayers to Evans Blue albumin (EBA; 0.67 mg Evans Blue dye/ml and 4% bovine serum albumin in M199 medium), as a measure of gap junctional area, was determined as previously described (52). HUVEC monolayers were grown to confluence on tissue culture-treated transwells (3-μm pore size; 12-mm diameter) (Corning Costar) in 24-well microtiter plates. The upper transwell and lower culture plate wells were filled with M199 medium (50 and 500 μl, respectively). For treatment of the monolayers, the transwells were washed with M199 medium without serum and then treated for 30 min with 5 mM cysteine-activated or TLCK-inhibited

gingipains (70 nM concentration of RgpA or Kgp) or with the cysteine buffer control. After treatment, the transwells were again washed three times with medium; 500 μl of medium from the bottom well was collected for analysis and replaced at times 0, 10, 20, 30, and 60 min, and 50 μl of fresh EBA was replaced in the transwell at the same time intervals. All samples were diluted 1:1 in M199 medium, and mean optical density at 630 nm was evaluated by spectrophotometry. EBA standards were used to create a calibration curve of optical density versus milligrams of albumin per milliliter. The diffusion of EBA from the luminal buffer in the transwell to the abluminal buffer in the lower well was expressed as milligrams of albumin/minute/centimeter^2 of monolayer.

PMN transmigration assay. Human PMNs were isolated by Ficoll gradient separation (Robbins Scientific, Sunnyvale, Calif.), followed by hypotonic red cell lysis. For the visualization of transmigrating cells, PMNs were fluorescently labeled with the membrane-associated dye calcein-AM (10 μM) (47) (Molecular Probes) for 30 min at 37°C with occasional shaking. After two washes in complete RPMI medium, cells were adjusted to a density of 10^6 cells/ml and resuspended in culture medium at 37°C. HUVECs were plated onto 0.1% gelatin-coated transwells (3-μm pore size; 12-mm diameter) (Corning Costar) in 24-well microtiter plates for 2 to 3 days. PMN transmigration was also evaluated with TNF-α (10 ng/ml)-induced HUVECs for 4 h. Before the experiment, medium M199 in upper and lower chambers was replaced with serum-free medium. Confluent HUVECs were then incubated with 5 mM cysteine-activated gingipains (a 70 nM concentration of RgpA or Kgp) in the presence or absence of 2 mM TLCK, 10 μg of anti-CD31 MAb (clone HEC7) or isotype-matched control per ml, or with control medium for 30 min, after which the transwells were again washed gently three times with M199 medium. To create a chemotactic gradient for neutrophils, fMLP (Sigma, St. Louis, Mo.) (23) at a concentration of 1 μM was added to the lower compartments of the transwell units. Calcein-AM labeled neutrophils were added to the upper chamber at a density of 10^6 cells/well. At different time points (1 or 2 h), transmigrated PMNs were recovered from the lower compartment, and the fluorescence of the samples was measured by a Perkin Elmer LS-50B spectrofluorimeter at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

Immunofluorescence staining. HUVECs for immunofluorescence assays were grown on 1% gelatin-coated eight-well chamber culture slides (Lab-Tek, San Diego, Calif.) until a confluent monolayer was achieved. Endothelial cells were then cultured with activated RgpA (70 nM) for 15 and 30 min at 37°C in the absence of serum. HUVECs were also treated with 2 mM TLCK-treated RgpA for 30 min without serum. After exposure to experimental conditions, cells were washed in PBS and fixed with 3% paraformaldehyde for 10 min. After washing, the slide was stained with primary antibody against anti-PECAM-1 for 45 min, unbound primary antibody was washed away, and cells were labeled with Alexa Fluor 488 (Molecular Probes)-conjugated secondary antibody for another 45 min at room temperature. After washing, the slide was mounted and analyzed with a Zeiss fluorescence microscope (Thornwood, N.Y.) connected to a Nikon digital camera (Tokyo, Japan).

Statistical analysis. All data were expressed as means ± standard error of the means (SEM). Differences between groups were examined for statistical significance by using a Student's *t* test for unpaired data and a paired *t* test for paired data. A P value of <0.05 indicated the presence of a statistically significant difference.

RESULTS

Effect of serum, cysteine, and proteinase inhibitors on binding of gingipains to HUVECs. The ability of gingipains to bind to HUVECs was examined by flow cytometry after a 1-h incubation. Figure 1A shows that, under serum-free conditions, the binding of activated RgpA or Kgp to HUVECs occurred in a dose-dependent manner. Both RgpA and Kgp exhibited similar binding activity to HUVECs, with 50% binding at approximately 2.7 nM and maximal effect near 70 nM. As serum is a rich source of proteinase inhibitors (30, 67), the effect of serum on gingipain binding to HUVECs was examined. The binding of a 70 nM concentration of RgpA or Kgp to HUVECs was only slightly affected by incubation in the presence of serum up to 40% (Fig. 1B). Further, the addition of 2 mM TLCK, an inhibitor of RgpA and Kgp, did not reduce binding, demonstrating that cysteine proteinase activity was not required (Fig. 1B). Figures 1C and D demonstrate the fluorescence-activated cell sorter (FACS) profiles of 70 nM RgpA and 70 nM Kgp bound to HUVECs after 1 h of incubation in the presence of 20% FCS. As cysteine is known as an effective reducing agent for the activation of gingipains, we tested the effect of various levels of cysteine on the binding activity of 70 nM gingipains to HUVECs. The amount of cysteine had little effect on binding as ~80% of maximal gingipain binding to HUVECs was achieved without activation (Fig. 1E). The results suggest that the adhesin domains of the gingipains are able to bind to HUVECs and that proteolytic activity of the gingipains is not critical in this process.

Reduction of PECAM-1 on HUVECs by gingipains. PECAM-1 molecules are constitutively expressed on endothelial cells (49). We first investigated the ability of purified RgpA and Kgp to affect the expression of PECAM-1 by HUVECs under serum-free conditions. Both RgpA and Kgp efficiently reduced PECAM-1 expression on HUVECs in a time- and dose-dependent manner (Fig. 2A, B). Treatment of the HUVECs with a 70 nM concentration of RgpA or Kgp for 15 min reduced detection of PECAM-1 from 90% to approximately 25% (RgpA) and 40% (Kgp) of positive cells. Figures 2C and D demonstrate the FACS profiles of PECAM-1 expression on HUVECs after the addition of the indicated levels of gingipains for 1 h. To further identify PECAM-1 expression on HUVEC surfaces after gingipain treatment, a time course study was followed by Western blotting with the anti-PECAM-1 MAb (Fig. 2E). In untreated HUVECs, there was a 130-kDa band corresponding to intact PECAM-1. After a 15-min incubation with either RgpA or Kgp at 70 nM, the 130-kDa band representing the PECAM-1 was reduced. The 130-kDa PECAM-1 band disappeared after a 30- or 60-min treatment with RgpA or Kgp, respectively. To verify that the gingipains are responsible for the proteolytic cleavage of surface PECAM-1 on HUVECs, the proteinase inhibitor TLCK was incubated with gingipains before being added to the HUVECs. Under these conditions, the intensity of the 130-kDa PECAM-1 band was unaffected after a 60-min treatment with TLCK-treated RgpA or Kgp. The results correlated well with the flow cytometric analysis and confirmed that the reduction of PECAM-1 expression on HUVECs was due to proteolysis by gingipains.

Extracellular enzymatic digestion of PECAM-1 by gingipains on HUVECs. The reduction of surface PECAM molecules on HUVECs was due to the enzymatic activity of gingipains since the cysteine proteinase inhibitor TLCK (at 2 mM) blocked the effect completely (Fig. 3A). Further, gingipains effectively diminished PECAM-1 expression by ~30% in the presence of 10% FCS. The capacity of the gingipains to reduce PECAM-1 expression was fully suppressed in the presence of 20% serum.

To determine whether the reduction in PECAM-1 expression may reflect either shedding or internalization of these adhesion glycoproteins (32) rather than proteolytic cleavage by gingipains, HUVECs were fixed before gingipain treatment. As shown in Fig. 3B, both RgpA and Kgp down-regulated PECAM-1 expression on both fixed and unfixed HUVECs ($P < 0.001$). Taken together, these data demonstrated adhesin-mediated binding of gingipains to endothelial cells and signif-

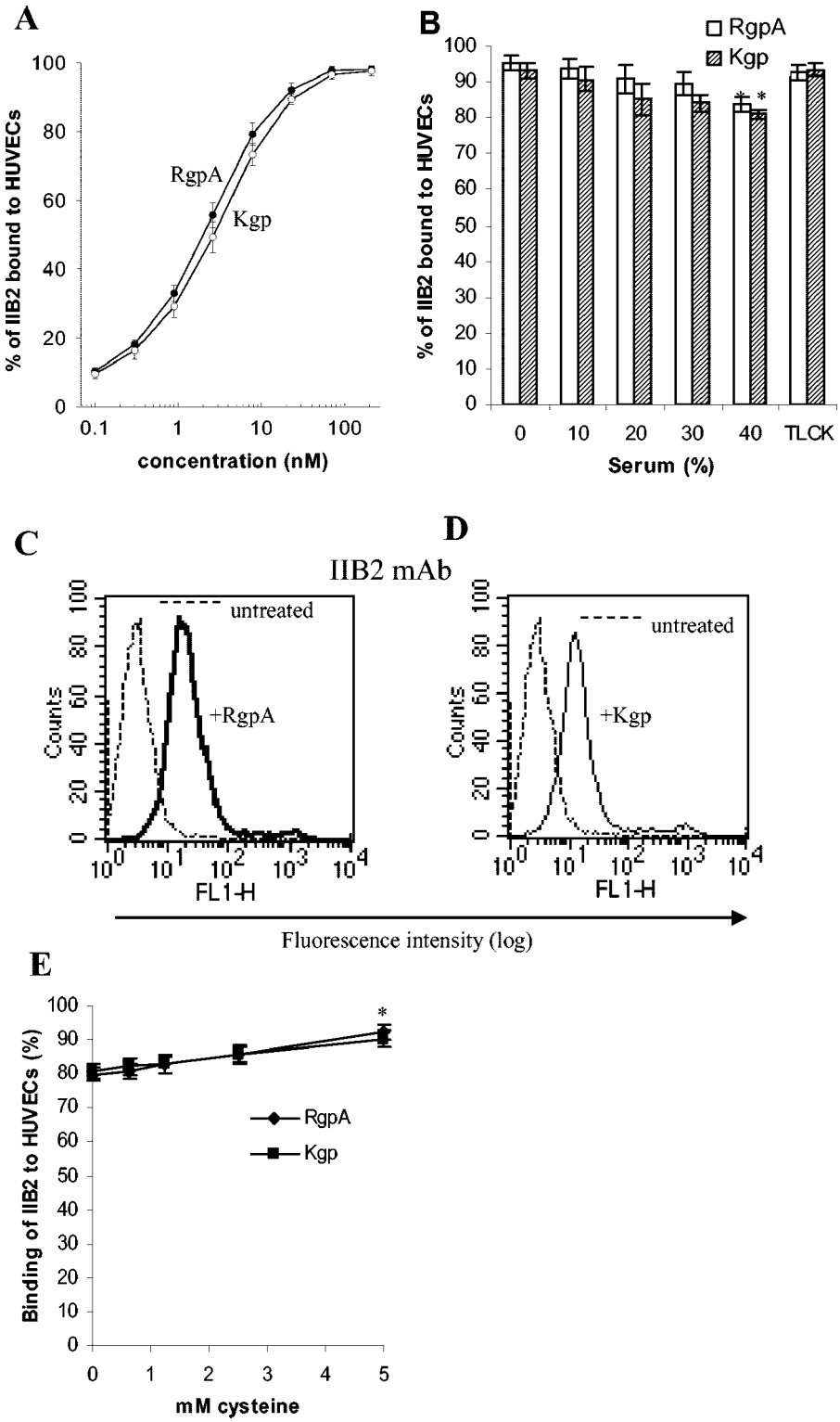

FIG. 1. Binding of gingipains to HUVECs. (A) A confluent monolayer of HUVECs was treated with serially diluted (1:3) gingipains (a 5 mM concentration of cysteine-activated RgpA or Kgp from 210 nM) for 1 h at 37°C in the absence of serum. After incubation, cells were washed with 2 ml of PBS three times, stained with IIB2 MAb, and subjected to FACS analysis as described in Materials and Methods. Isotype-matched IgG1 was used as a control. (B) The ability of activated gingipains at 70 nM to bind HUVECs in the presence or absence of various levels of FCS or 2 mM TLCK was monitored for 1 h. (C and D) Fluorescence histograms obtained by FACS of activated gingipains (70 nM) binding to HUVECs in 20% FCS for 1 h. (E) Endothelial cell monolayers were incubated with 70 nM gingipains in the presence or absence of the indicated concentrations of cysteine for 1 h. Cells were then washed, stained with IIB2 MAb, and subjected to flow cytometry as above. Results are representative of three separate experiments that yielded similar results. Error bars indicate the means ± SEM. *, $P < 0.05$ compared with untreated cells.

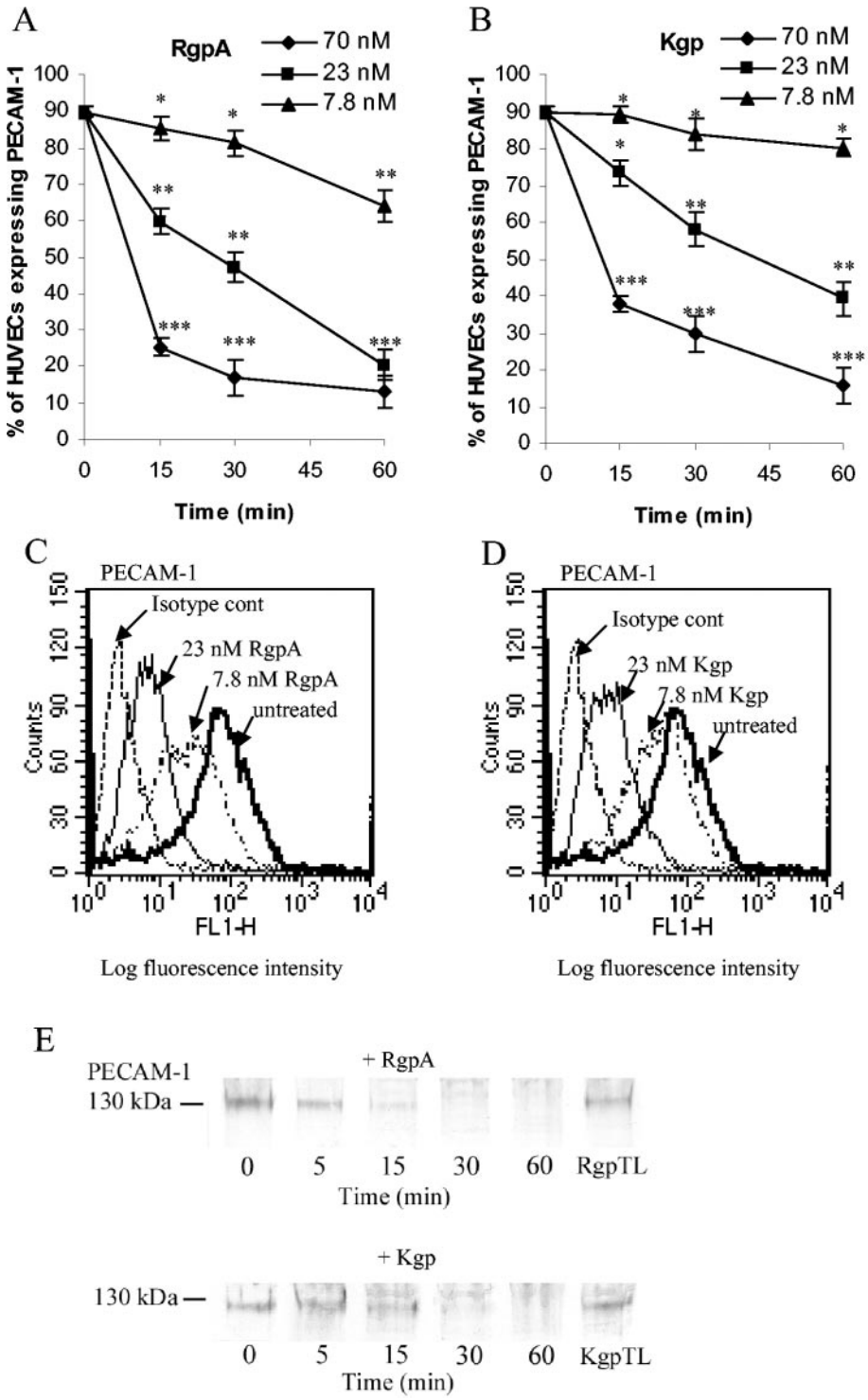

FIG. 2. Decrease of PECAM-1 expression on HUVECs following gingipain treatment. (A and B) Confluent HUVECs were incubated with a 5 mM concentration of cysteine-activated RgpA or Kgp at the indicated concentrations and times without serum for 1 h at 37°C. HUVECs were then harvested by scraping, stained with anti-PECAM-1 or isotype-matched antibody, and measured by flow cytometry. Results are representative of three separate experiments that yielded similar results. Error bars indicate the means ± SEM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with untreated HUVECs. (C and D) Representative FACS profiles of PECAM-1 expression on HUVECs after treatment in the presence or absence of gingipains at indicated concentrations for 1 h. Results are representative of three separate experiments that yielded similar results. (E) Confluent HUVECs were incubated with RgpA or Kgp at a concentration of 70 nM for various times at 37°C under serum-free conditions. Proteolysis was inhibited in aliquots by the addition of 2 mM TLCK. (Endothelial cells were then harvested by scraping, washed with PBS, and lysed directly in SDS reducing sample buffer with 20% (vol/vol) protease inhibitor cocktail. Aliquots were then subjected to SDS–10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes for Western blot analysis with antibody against PECAM-1 as described in Materials and Methods. RgpTL, TLCK-inhibited RgpA; KgpTL, TLCK-inhibited Kgp.

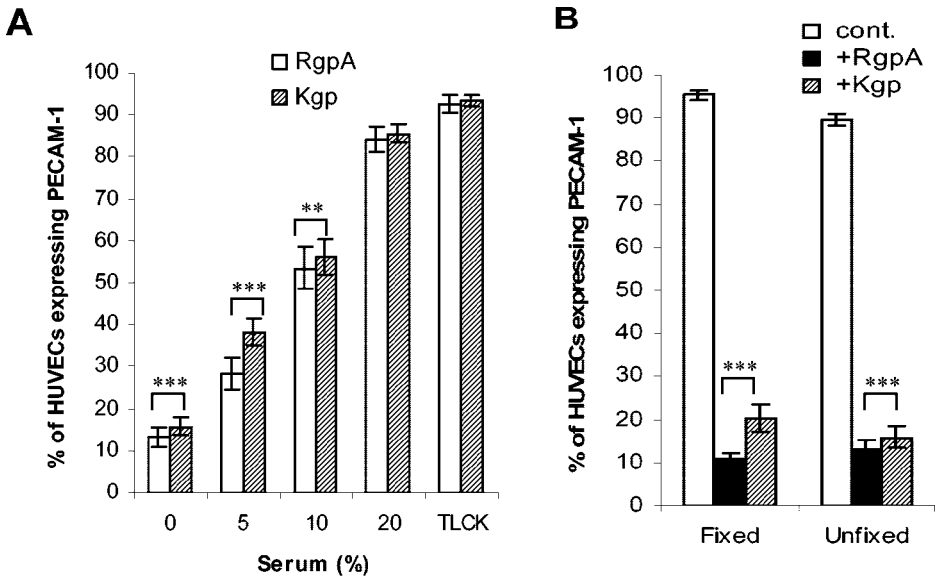

FIG. 3. Serum inhibition of proteolysis of PECAM-1 by gingipains. (A) Confluent HUVECs were cultured with activated RgpA or Kgp (70 nM each) in the presence or absence of FCS at indicated concentrations or with TLCK for 1 h at 37°C. PECAM-1 expression on HUVECs was then measured by flow cytometry. (B) HUVECs were fixed with 3% paraformaldehyde for 5 min at room temperature. After washing with PBS, fixed or unfixed cells were incubated with RgpA or Kgp (70 nM each) in the absence of serum for 1 h at 37°C. PECAM-1 expression on HUVECs was then evaluated by flow cytometry. Error bars indicate the means ± SEM. **, $P < 0.01$, and ***, $P < 0.001$ compared with corresponding controls [cont].

icant proteolysis by the gingipains of PECAM-1 on the endothelial cell surface.

TNF-α-induced VCAM-1 and ELAM-1 expression on HUVECs is not readily altered by treatment with gingipains. Adhesion molecules such as VCAM-1 or ELAM-1 are absent or minimally expressed on unactivated HUVECs but are readily induced by cytokines such as TNF-α (4). We directly compared the effects of gingipains on VCAM-1 and ELAM-1 expression to the effects observed for PECAM-1. TNF-α-activated HUVECs were incubated for 1 to 3 h in the presence or absence of 70 nM gingipain under serum-free conditions (Fig. 4A). The percentage of cells expressing VCAM-1 and ELAM-1 was slightly decreased by approximately 10 to 15% after 1 to 2 h of treatment with RgpA or Kgp compared to the untreated HUVEC cultures. VCAM-1 expression on HUVECs was decreased by a further 5 to 10% after 3 h of treatment with gingipains. Expression of ELAM-1 was significantly ($P < 0.001$) reduced by approximately 40% at 3 h in RgpA-treated HUVEC cultures compared with untreated HUVECs. However, when 20% FCS was added to the gingipain-treated HUVEC cultures for 1 to 3 h, no reduction of the VCAM-1 and ELAM-1 expression levels on HUVECs was observed, which is similar to serum effects on PECAM-1 proteolysis by gingipains (data not shown).

The reduction of PECAM-1 expression on PMNs by gingipains. As PECAM-1 is also expressed on neutrophils (48), we examined the effects of gingipain treatment on PMN PECAM-1 expression. Under serum-free conditions, treatment of neutrophils with a 70 nM concentration of RgpA or Kgp significantly ($P < 0.001$) reduced PECAM-1 surface expression by approximately 90% after a 1-h incubation (Fig. 4B). Further, the reduction of PECAM-1 expression was completely blocked by the RgpA and Kgp inhibitor TLCK.

Vascular permeability is increased following treatment with gingipains in vitro. To demonstrate the effect of gingipains on HUVEC contraction and intercellular gap formation, photomicrographs were taken, and monolayer permeability to EBA was recorded (52). Photographs taken at 0, 15, and 60 min after exposure to a 70 nM concentration of RgpA or Kgp showed a rapid and obvious contraction of endothelial cells and gradual intercellular gap formation (Fig. 5A). In contrast, a 60-min exposure to TLCK-treated RgpA or Kgp resulted in neither endothelial cell contraction nor intercellular gap formation, as seen by comparing photographs with those taken of untreated HUVECs (Fig. 5A). Gingipains also induced a prompt increase in the permeability of monolayers to EBA. Buffer treatment was used as a negative control. Figure 5B shows that the same conditions resulted in a two- to threefold increase in EBA permeability within 10 min. Endothelial monolayer permeability increased with time after the addition of gingipains, and there was no change induced by TLCK-inhibited gingipains or buffer treatment. These visual and permeability data confirm that gingipains induced intercellular gap formation.

Gingipains enhance transmigration of PMNs across HUVEC monolayers. The modification of permeability was quantified by establishing the transmigration time course of PMNs across endothelial cell monolayers seeded on porous transwell chambers. Prior to the addition of PMNs to the upper compartments of the transwell units, endothelial cell monolayers underwent treatment with gingipains at a concentration of 70 nM or with proteolytically inhibited gingipains for

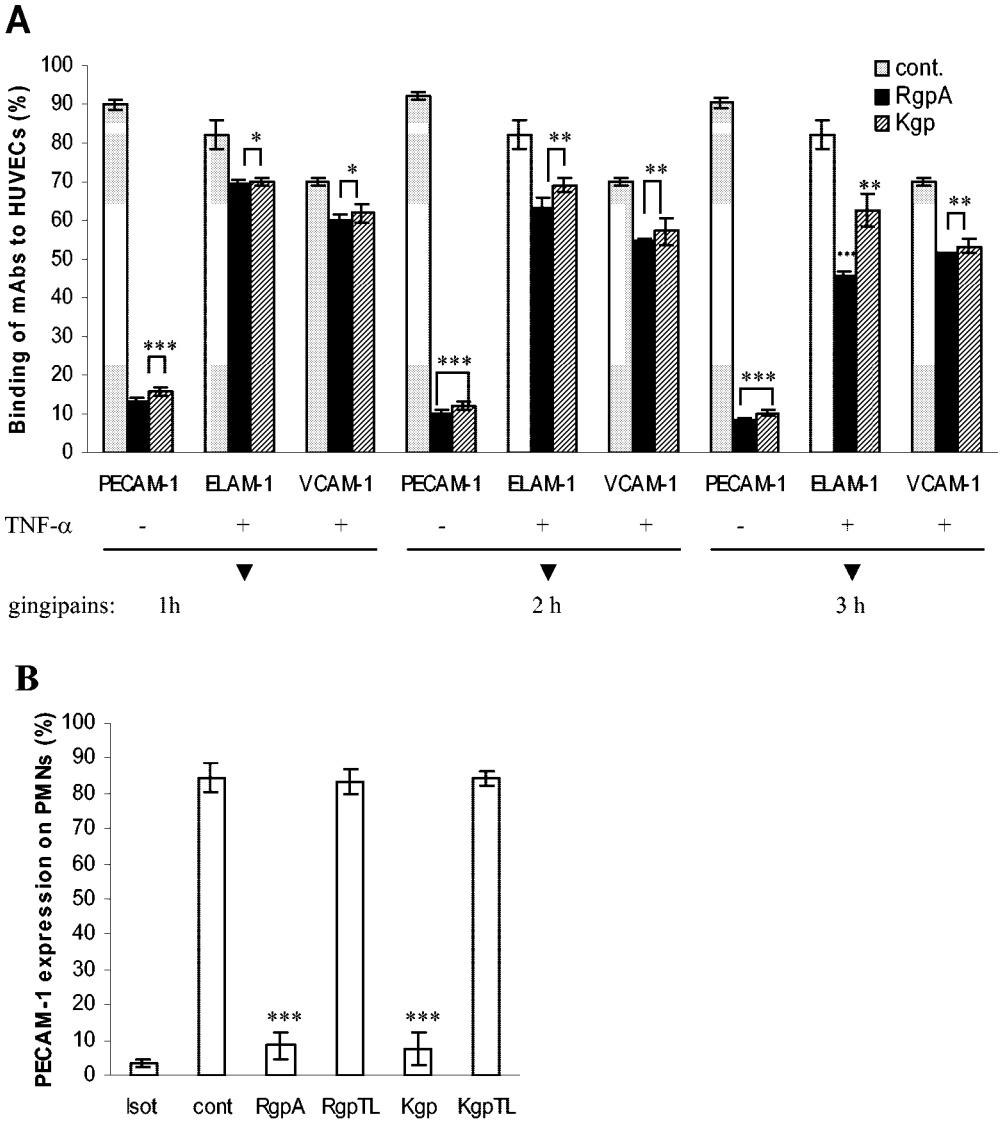

FIG. 4. Time course of the effect of gingipains on PECAM-1, VCAM-1, and ELAM-1 expression on HUVECs. (A) Confluent HUVECs were treated with activated gingipain (a 70 nM concentration of RgpA or Kgp) for 1, 2, and 3 h without serum, washed, and stained for PECAM-1 expression by flow cytometry. For the measurement of VCAM-1 and ELAM-1 expression, HUVECs were preincubated with 10 ng of TNF-α per ml for 4 h. Endothelial cells were then washed twice with M199 medium, and RgpA or Kgp (70 nM each) was added to the cultures for 1, 2, and 3 h under serum-free conditions. The data shown are from three independent experiments that yielded similar results. (B) Purified PMNs were treated with RgpA or Kgp (70 nM each) with or without TLCK (TL) for 1 h in the absence of serum. PMNs were then subjected to indirect immunofluorescence and flow cytometry. The data are representative of three separate experiments that showed the same loss in PECAM-1 expression on PMNs. Error bars indicate the means ± SEM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with untreated HUVECs. Isot, isotype; cont, control.

1 h. In response to chemoattractant in the lower compartment, the number of transmigrated neutrophils increased by ~30% after a 1-h culture period and by ~50% after a 2-h incubation in the chambers ($P < 0.001$), compared to treatment with medium alone (Fig. 6A). Similarly, in TNF-α-activated HUVEC monolayers, gingipain treatment allowed more PMNs to transmigrate (Fig. 6B). Inhibition of gingipains by TLCK significantly blocked the enhancement of PMN transmigration. The results correlated with the finding that gingipains can disrupt interendothelial cell junctions and increase vascular permeability.

PECAM-1 expression on HUVECs is diminished in response to RgpA treatment. To examine whether the transmigration of PMNs across HUVEC monolayers, facilitated by challenge with RgpA, was associated with changes in the distribution of PECAM-1 at interendothelial junctions, confluent HUVEC monolayers were incubated with either RgpA or control medium, and the distribution of PECAM-1 was analyzed by fluorescence microscopy. As shown in Fig. 7, unstimulated confluent cells were brightly stained for PECAM-1 molecules that showed a linear and homogeneous distribution at cell borders (Fig. 7). Conversely, staining for PECAM-1 was less

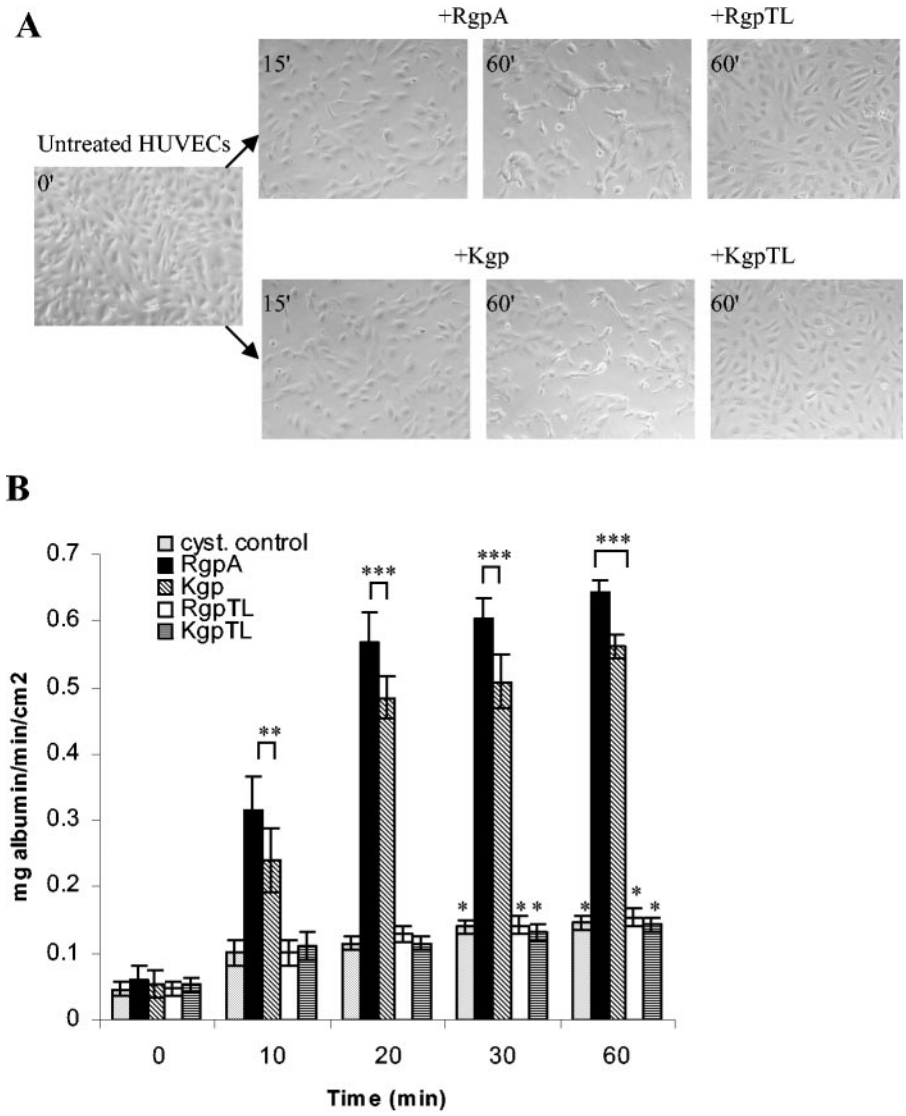

FIG. 5. Photomicrographs and EBA permeability of HUVEC monolayers after treatment with gingipain. (A) Serial photomicrographs of gingipain-treated HUVEC monolayers with or without 2 mM TLCK (TL) at the indicated times. (B) Confluent endothelial cell monolayers seeded on porous transwell filters were treated either with buffer containing 5 mM cysteine only or with activated gingipain (70 nM) in the presence or absence of 2 mM TLCK (TL) for 30 min without serum. The upper compartments of the transwell units were then washed gently, and the diffusion of EBA from the M199 medium in the transwell to the medium in the lower well was measured as milligrams of albumin/minute/centimeter^2 of monolayer. Samples were taken at several time points before and after treatment. Error bars indicate the means ± SEM. The data correspond to mean values obtained from three similarly treated independent transwell units. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with cysteine (cyst) controls.

intense in gingipain-stimulated HUVECs, with the intensity of staining significantly reduced on the cell surface after a 30-min incubation with gingipains (Fig. 7). HUVECs stimulated with TLCK-treated RgpA exhibited a pattern of reactivity that was similar to that of the control cells, with no overt changes in the staining and distribution of PECAM-1.

DISCUSSION

Previous reports have implied that *P. gingivalis* may penetrate the epithelial barrier surrounding the gingival sulcus (18). Further, *P. gingivalis* has been observed within gingival tissues in vivo (55), whereas tissue penetration and destruction by *P. gingivalis* have been demonstrated in vitro (1). These results indicated that *P. gingivalis* may invade deeper structures of the connective tissue. Likewise, it has been reported that *P. gingivalis* can adhere to and invade endothelial cells (17). Also, it is interesting that *P. gingivalis* has been detected in atherosclerotic plaques (13). The present study indicates the role of gingipains in the ability of *P. gingivalis* to localize to endothelial cells. The results suggest that gingipains are likely to adhere to endothelial cells in vivo, as serum concentrations up to 40% have little affect on binding activity. We speculate that the ability of gingipains to adhere to endothelial cells may contribute not only to localized inflammation but also, through entering the bloodstream, to the development of systemic reactions such as atherosclerosis (3). The data also suggest that adher-

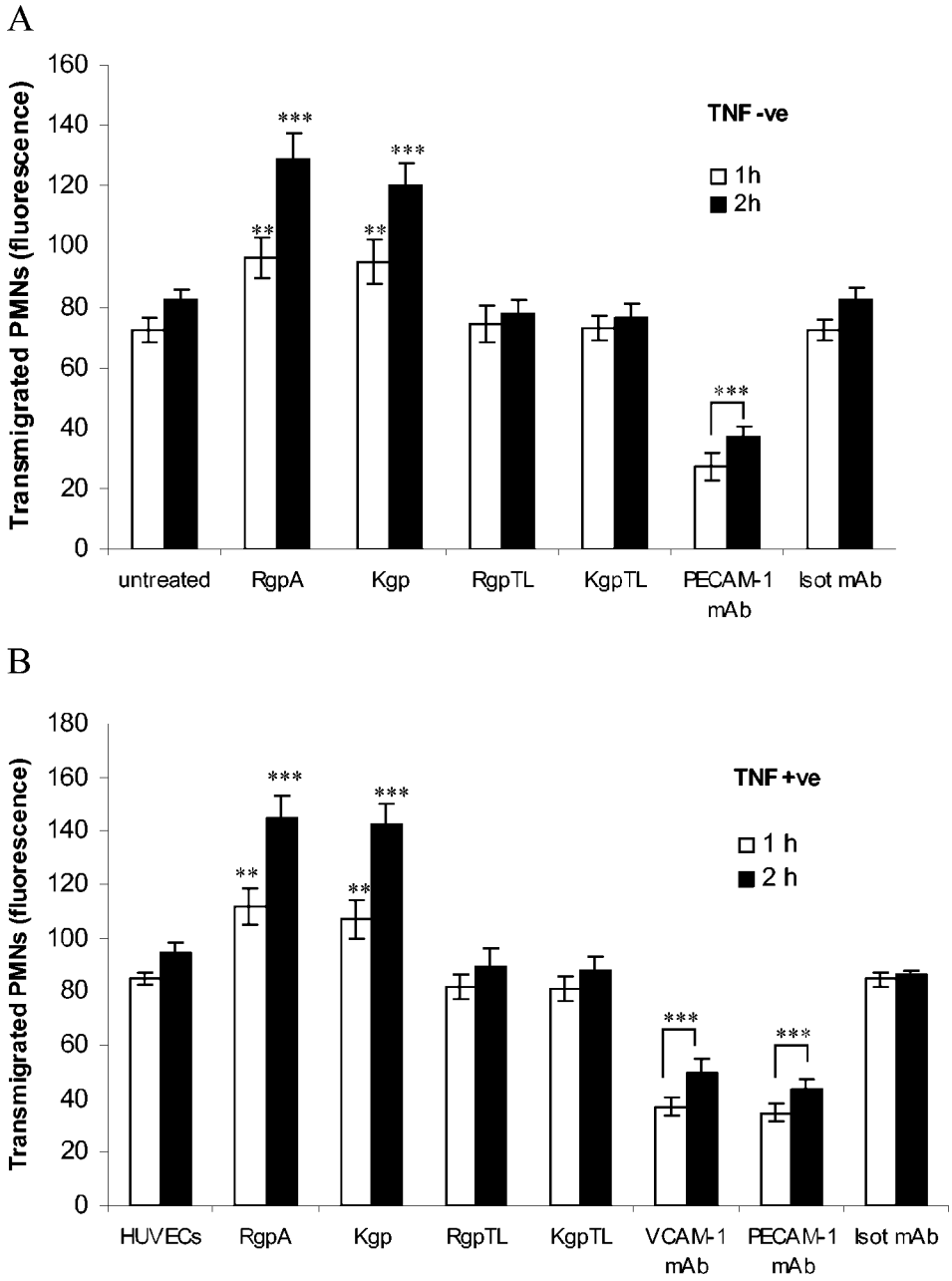

FIG. 6. Time course of neutrophil transmigration through gingipain-treated HUVEC monolayers. Confluent endothelial cell monolayers were seeded on porous transwell filters in the absence (A) or presence (B) of 10 ng of TNF-α per ml for 4 h. The wells were then rinsed twice, and untreated HUVECs were used for comparison. Endothelial cells were then treated either with activated gingipain (70 nM) in the presence or absence of 2 mM TLCK (TL), with anti-PECAM-1 antibody (clone HEC7 at a concentration of 10 μg/ml), or with isotype-matched antibody for 30 min without serum. Anti-VCAM-1 antibody (10 μg/ml) was also added as a control for the TNF-α-activated HUVECs. Monolayers (top wells) were concurrently washed gently in culture medium; 1 μM fMLP was added to the lower wells as a stimulant after the addition of calcein-AM labeled PMNs (10^6/well) to the upper compartments of the transwell units. After the addition of neutrophils for 1 to 2 h, supernatants containing PMNs from the lower wells were centrifuged and resuspended in 200 μl of RPMI medium. The fluorescence of calcein-labeled PMNs was then measured by fluorimetry. The data correspond to mean values obtained from three similarly treated independent transwell units. Error bars indicate the means ± SEM. **, $P < 0.01$, and ***, $P < 0.001$ compared with untreated HUVECs. Isot, isotype; −ve, negative; +ve, positive.

ence of the adhesin domain of gingipains to HUVECs is likely to facilitate the hydrolysis of interconnecting adherens junction PECAM-1 molecules mediated by the catalytic domain of the proteinases. The lack of PECAM-1 cleavage following pretreatment of gingipains with TLCK, a cysteine protease inhibitor that inhibits gingipain activity, strongly supports the findings.

Both RgpA and Kgp were shown to efficiently degrade PECAM-1 on endothelial cells. We observed that threshold levels of gingipains were required for degradation of

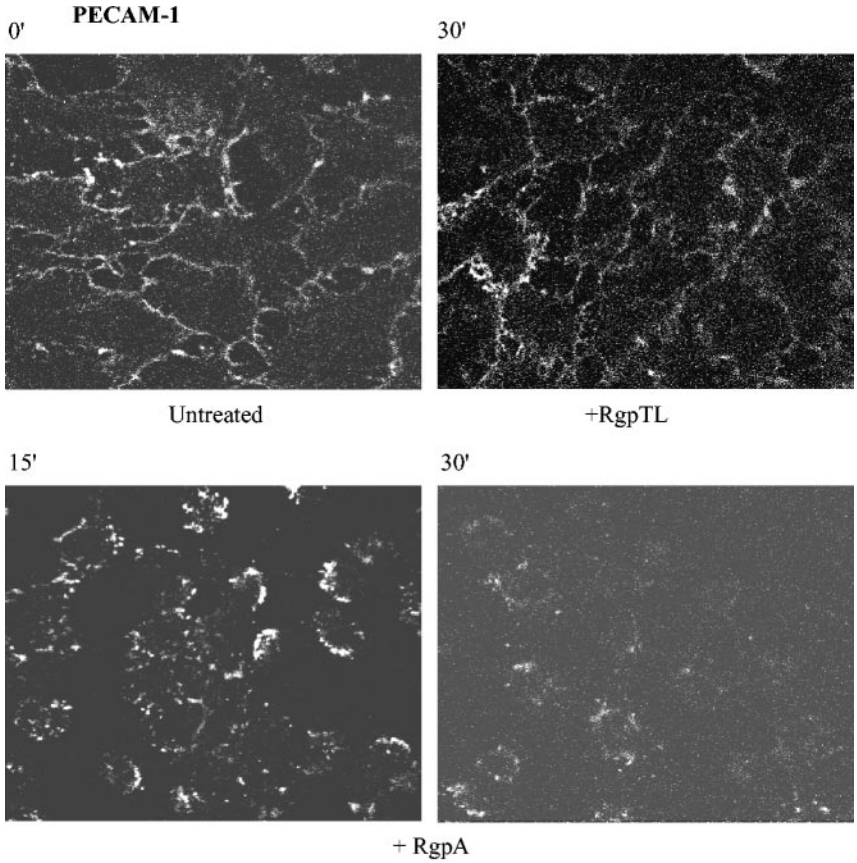

FIG. 7. Immunofluorescence showing intercellular PECAM-1 staining on HUVECs treated with gingipains. HUVECs were grown to confluence in an eight-well chamber culture slide and incubated with 70 nM RgpA for 15 and 30 min or with control medium alone. HUVECs were also treated with 2 mM TLCK-treated RgpA (RgpTL) at a concentration of 70 nM for 30 min. Cells were then stained with anti-PECAM-1 MAb followed by Alexa Fluor-conjugated secondary antibody. Cells were imaged by using conventional fluorescence microscopy and a digital camera. Note the decrease of intercellular PECAM-1 staining in the gingipain-treated HUVECs and the normal distribution of PECAM-1 in HUVECs.

PECAM-1 to occur. PECAM-1 is a single chain 130-kDa membrane glycoprotein containing six extracellular Ig-like domains of the C2 subclass, one transmembrane region, and a cytoplasmic tail (48). A recent study with chimeric constructs of human PECAM-1 revealed that homophilic binding of PECAM-1 on adjacent cells and positively charged sequences (^{599}RKAKA K^{604}) in the juxtamembrane region of the transmembrane domain of PECAM-1 are important for its localization to cell-cell borders (62). In this context, the hemagglutinin domains have been reported to regulate intracellular dispersion of gingipains taken up by epithelial cells (57), and the proteinases have specificity for arginine and lysine residues (54), with the ^{599}RK AKAK^{604} sequence a potential target.

The major proteinase inhibitors present in human serum are alpha-1-antitrypsin, alpha-1-antichymotrypsin, and alpha-2-macroglobulin (α_2M). Two strains of *P. gingivalis* (W83 and H185) have been shown to degrade most serum proteins, including the plasma proteinase inhibitors alpha-1-antitrypsin and α_2M, but not alpha-1-antichymotrypsin (10). In another study, α_2M was shown to inhibit the catalytic activity of gingipain R efficiently but not gingipain K (30). While the serum protease inhibitors may limit PECAM-1 cleavage on HUVECs, the actual effect remains undetermined in the present study. The observed effect of serum in limiting downregulation of PECAM-1 on HUVECs could be attributed to serum albumin, a known preferred target of gingipain activity (29), acting as a competitive substrate, resulting in increased retention of PECAM-1. The present report indicates that gingipains at a concentration of 70 nM effectively reduce PECAM-1 expression on HUVECs in the presence of 10% serum, whereas limited hydrolysis of PECAM-1 was observed in the presence of 20% serum. The level of gingipains in crevicular fluid at inflamed sites was estimated at 40 to 90 nM (21), comparable to the amount used in this study. Hence, it is possible that proteolytic cleavage of PECAM-1 by gingipains occurs in vivo.

The present study shows that gingipains are able to increase the permeability of endothelial monolayers. The kinetics of endothelial permeability induced by gingipains correlated with the kinetics of PECAM-1 degradation. It can therefore be presumed that treatment of endothelial monolayers with gingipains disrupts intercellular junctions, at least by degradation of the PECAM-1 involved in endothelial monolayer integrity. Gap formation by gingipains appears to be different from TNF-α-promoted intercellular gap formation, where actin peripheral bundles are converted into stress fibers and where

increased permeability to macromolecules is observed (7). Changes in endothelial cell intercellular permeability by gingipains occur in parallel with changes in the rate of leukocyte transmigration. We conclude that reduced PECAM-1 expression at the junctions after gingipains treatment may disrupt the tight assembly of endothelial cells, thus allowing leukocyte transmigration in this system.

Evidence was obtained that gingipains did not preferentially degrade ELAM-1 and VCAM-1 expressed by endothelial cells compared with PECAM-1. RgpA reduced half of the TNF-α-induced ELAM-1 expression on endothelial cells after 3 h of incubation, reflecting the limited activity of RgpA in degradation of ELAM-1. ELAM-1 binds neutrophils selectively but not the majority of lymphocytes (5). It has been suggested that gingival blood vessels might be functionally specialized to constitutively express ELAM-1 and ICAM-1 and thus facilitate leukocyte traffic into the gingival crevice (44). The present investigation indicated that prolonged exposure to gingipains produced by *P. gingivalis* might modify the rolling or tethering steps of leukocytes mediated by ELAM-1 expression on endothelial cells.

It has been reported that two regions of VCAM-1, localized to domains 4 and 5, are sensitive to proteolytic cleavage by endopeptidase Glu-C of *Staphylococcus aureus* (53). However, gingipains exhibit minimal activity against TNF-α-induced VCAM-1 expression on endothelial cells in vitro. VCAM-1 is a selective adhesion molecule for mononuclear cells (9). The resistance of VCAM-1 on endothelial cells to gingipains suggests a functional involvement of this molecule during adhesion and transendothelial migration of lymphocytes in periodontitis. Our results indicate that PMN transmigration across cytokine-activated endothelial cells can be facilitated by gingipains and may potentially contribute to leukocyte accumulation at periodontal sites.

The expression of PECAM-1 on PMNs could play an important role in the modulation of inflammatory foci by influencing responses to chemotaxis, cell adhesion, or mediators of inflammation such as cytokines and complement (46). Also, the transendothelial migration of leukocytes across the subendothelial basal lamina is mediated via the heterophilic interaction of domain 6 of leukocyte PECAM-1 with unknown component(s) of the basement membrane (41, 46). We observed that gingipains can also down-regulate PECAM-1 expression on PMNs. The data suggest the potential ability of gingipains to reduce the heterophilic interaction of leukocyte PECAM-1 with endothelial cells at sites of inflammatory gingival tissue.

Gingipain-K has potent fibrinogenolytic activity that can contribute to a bleeding tendency at periodontitis sites (34). On the other hand, a recent study has demonstrated prolonged bleeding times in PECAM-1-deficient mice (42). These studies are consistent with a role for gingipains in the acquisition of the iron or porphyrin necessary for bacterial growth and virulence (14), potentially mediated by modulating endothelial cell PECAM-1 expression and extending bleeding time.

Leukocyte transmigration through the endothelium is a multistep process. PECAM-1 is an important component at endothelial junctions. However, three additional adhesive proteins, junctional adhesion molecule (40, 43), CD99 (56) and vascular endothelial-cadherin (61), expressed at endothelial cell-cell junctions, have recently been demonstrated to be involved in leukocyte transmigration. To date, only the epithelial-cadherin and neuronal-cadherin of epithelial cells are known to be degraded by *P. gingivalis* proteases (12). Further investigation is under way to resolve a possible interaction of gingipains and these adhesion proteins.

The creation of intercellular gaps and the relative sparing of adhesion receptors important in anchoring mononuclear leukocytes is noteworthy in relation to the pathology of the lesion. *P. gingivalis* infection can cause local inflammation, which contributes to the ulceration of the gingiva and local vascular changes. The observed expression of endothelial ELAM-1 and VCAM-1 under *P. gingivalis* proteinase challenge is consistent with the initial stages of gingival inflammation characterized by the continuous migration of leukocytes (44, 50, 65). Further, the present investigation indicates that gingipains from *P. gingivalis* may contribute to the initiation of endothelial barrier destruction by cleaving PECAM-1 at the endothelial cell-cell junction. *P. gingivalis* and the gingipains may interact with endothelium of the microvasculature from the perivascular aspect, along a gradient from the plaque mass in the periodontal pocket to the adjacent connective tissues. Equally likely, gingipains may enter the vasculature through localized sites of microulceration of the pocket wall, interacting with endothelium from the lumenal aspect. Consequently, gingipains can promote microvascular permeability and could potentially contribute to gingival crevicular flooding and leukocyte accumulation at periodontal sites infected with *P. gingivalis*. Of relevance also is the observed correlation between the occurrence of *P. gingivalis* and neutrophil elastase levels in the gingival crevicular fluid at periodontitis sites (71). While the recruitment of leukocytes and participation of inflammatory mediators such as proinflammatory cytokines may amplify the magnitude of tissue and vascular damage at periodontitis sites, these inflammatory products can also enter the circulation to produce systemic effects. Significantly, *P. gingivalis* proteinases may overcome host immune responses by effectively down-regulating both afferent and efferent arms of the cell-mediated immune response (69, 70). Further, gingipains are effective in stimulating polyclonal activation of B lymphocytes (68), an observation compatible with the observed perivascular accumulation of degenerate B-cell derivatives as a characteristic feature of the lesion of periodontitis (26, 58). Conversely, mediation of vascular permeability by gingipains could contribute to the capacity of *P. gingivalis* to enter the circulatory systems. The capacity of gingipains to attach to endothelial cells as demonstrated in the present study could potentiate the damaging effects of these enzymes, thereby decreasing the integrity and strength of vessel walls, with implications for the risk of atherosclerosis and related cardiovascular events.

ACKNOWLEDGMENT

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