Gingipains of Porphyromonas gingivalis Modulate Leukocyte Adhesion Molecule Expression Induced in Human Endothelial Cells by Ligation of CD99

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Porphyromonas gingivalis has been implicated as a key etiologic agent in the pathogenesis of destructive chronic periodontitis. Among virulence factors of this organism are cysteine proteinases, or gingipains, that have the capacity to modulate host inflammatory defenses. Intercellular adhesion molecule expression by vascular endothelium represents a crucial process for leukocyte transendothelial migration into inflamed tissue. Ligation of CD99 on endothelial cells was shown to induce expression of endothelial leukocyte adhesion molecule 1, vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and major histocompatibility complex class II molecules and to increase adhesion of leukocytes. CD99 ligation was also found to induce nuclear translocation of NF-κB. These results indicate that endothelial cell activation by CD99 ligation may lead to the up-regulation of adhesion molecule expression via NF-κB activation. However, pretreatment of endothelial cells with gingipains caused a dose-dependent reduction of adhesion molecule expression and leukocyte adhesion induced by ligation of CD99 on endothelial cells. The data provide evidence that the gingipains can reduce the functional expression of CD99 on endothelial cells, leading indirectly to the disruption of adhesion molecule expression and of leukocyte recruitment to inflammatory foci.

Periodontal diseases are chronic inflammatory diseases affecting the well-vascularized connective tissues that comprise the supporting tissues of the teeth (12). Among periodontal pathogens, most evidence points to a pathogenic role for Porphyromonas gingivalis, which is more frequently detected in active lesions of periodontitis than in healthy or gingivitis sites (37, 38). Previous studies have shown that P. gingivalis may penetrate the epithelial barrier surrounding the gingival sulcus and invade endothelial cells (3, 17, 34). Virulence of P. gingivalis is associated with the proteolytic enzymes expressed by this gram-negative anaerobic bacterium (21, 24). These cysteine proteinases, referred to as Arg-gingipain (two genes code for RgpA and RgpB, respectively) and Lys-gingipain (one gene codes for Kgp), can degrade key components of the immune system (32, 33, 48). Gingipains have also been shown to down-regulate endothelial intercellular junctional cadherin (36) and platelet endothelial cell adhesion molecule 1 (PECAM-1) expression in association with increased vascular permeability (47). With localized assault on the periodontal tissues by P. gingivalis and its virulence factors, as well as transient bacteremia and systemic translocation following dental care and treatment (2, 23), endothelial cells throughout the vasculature are potential targets for P. gingivalis.

In relation to periodontal lesions, an extensive local inflammatory reaction, with tissue edema, has been observed (26, 29). When endothelial cells undergo inflammatory activation by proinflammatory mediators including cytokines such as tumor necrosis factor alpha, the increased expression of cell adhesion molecules, e.g., endothelial leukocyte adhesion molecule 1 (ELAM-1) and vascular cell adhesion molecule 1 (VCAM-1), promotes the adherence of specific types of circulating leukocytes (19). ELAM-1 may be transiently induced on endothelium and is expressed in postcapillary venules at sites of acute and chronic inflammation (10, 16). It mediates the adhesion of neutrophils and memory T lymphocytes to endothelial cells (39). VCAM-1, although not normally expressed on noninflamed endothelium, is suggested to play a critical role in regulating mononuclear cell accumulation at sites of inflammation (11). Intercellular adhesion molecule 1 (ICAM-1), comparatively, is constitutively expressed on endothelial cells, and it is critical to the adhesion and migration of lymphocytes through blood vessel walls (18). ICAM-1 deficiency has been demonstrated to increase the severity of alveolar bone loss after P. gingivalis infection (5).

A number of endothelial cell-associated adhesion molecules expressed at cell junctions such as PECAM-1, members of the junctional adhesion molecule family, and CD99 have also been implicated in leukocyte transvascular migration (30, 35). The CD99 (MIC2) gene encodes a 32-kDa glycosylated transmembrane glycoprotein that is expressed on many cell types (20). Although the functional role of CD99 is not yet fully understood, it has been implicated in multifactorial cellular events including homotypic cell adhesion (8) and apoptosis of immature thymocytes and Ewing's sarcoma cell lines (7). CD99 appears to function distally to the point at which PECAM-1 plays its role in diapedesis (35).

Despite the fact that the identity of the ligand for CD99 is not yet known, engagement of CD99 with agonistic antibody has been shown to induce marked effects that include up-regulation...
of surface lymphocyte function-associated antigen 1 (LFA-1)/ICAM-1-mediated adhesion of lymphocytes (22), up-regulation of surface lymphocyte function-associated antigen 1 (LFA-1)/ICAM-1-mediated cell adhesion molecule expression on endothelial cells, characterized. Moreover, little is known about the role of augments T-cell receptor-mediated activation of mitogen-activated protein kinases and c-Jun N-terminal kinase (46). However, the CD99-mediated response in endothelial cells has not been characterized. Moreover, little is known about the role of P. gingivalis gingipains in the modulation of functional expression of CD99 on endothelial cells.

In this paper we demonstrate that CD99 localized at the endothelial cell junctions is sensitive to proteolysis by gingipains. To elucidate the functional role of CD99 degradation by gingipains, we investigated cell surface protein changes related to CD99 function by incubating cells with anti-CD99 monoclonal antibody (MAb). We show here that CD99 ligation induces rapid expression of ELAM-1, VCAM-1, ICAM-1, and MHC class II molecules on endothelial cells that was associated with translocation of NF-κB-dependent activity and leukocyte adhesion. Gingipains are capable of causing degradation and a decrease of CD99-mediated cell adhesion molecule expression on endothelial cells, as well as decreased adhesion of leukocytes.

MATERIALS AND METHODS

Chemicals and reagents. Bovine serum albumin, goat serum, HEPES, l-cysteine, NP-40, paraformaldehyde, protease inhibitor cocktails (for mammalian tissues), saponin, sodium azide (NaN₃), sodium dodecyl sulfate (SDS), Nα-toy-4-1-β-seryl chloride methyl ester (TLCK), Trizma base, Tris-hydrochloride (Tris-HCl), Triton X-100, trypsin, and Tween 20 were purchased from Sigma (St. Louis, Missouri). Fetal cell serum, M199, 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 Phosphate-buffered saline (PBS) and Trypticase soy broth were purchased from Bio-Rad (Richmond, Calif.). All reagents for electrophoresis and Western blotting were from Bio-Rad (Richmond, Calif.).

Recombinant cytokines and antibodies. Human recombinant gamma interferon was obtained from R&D Systems (Minneapolis, Minn.). Mouse monoclonal antibodies specific for human CD5, CD45 (ICAM-1), CD62E (ELAM-1), CD99 (MIC-2), CD106 (VCAM-1), and NF-κB p65 subunit were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Monoclonal antibody specific for human MHC class II was purchased from Dako (Glostrup, Denmark).

Bacterial strain and proteinase purification. Porphyromonas gingivalis (ATCC 33277) was grown in enriched Trypticase soy broth under anaerobic conditions for 48 h. Arg-gingipain and Lys-gingipain proteinase-adhesin complexes were purified according to the method previously described (48).

Evaluation of cell adhesion molecule expression in HUVECs. Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase treatment of the vessels as described previously (25). Confluent HUVECs grown on 12-well plates (Corning Costar, Cambridge, Mass.) were incubated with medium alone or stimulated with the indicated concentrations of anti-CD99 MAb or an isotype-matched MAb for up to 2 h. In functional studies of CD99 molecules, HUVECs were pretreated with or without 5 mM cys-teine-activated RgpA or Kgp at 70 nM for 1 h under serum-free conditions. Cells were then washed gently with medium and stimulated with 5 μg/ml of anti-CD99 MAb for 1 h. Expression of ELAM-1, VCAM-1, ICAM-1, and HLA-DR was detected by flow cytometry and immunoblot analysis. For flow cytometric analysis, cells were harvested by scraping and washed twice with fluorescence-activated cell sorting (FACS) washing buffer (0.1% bovine serum albumin and 0.01% NaN₃ in cold PBS), followed by incubation with corresponding primary mouse anti-human MABs (1:50) or with matched isotype control antibodies at 4°C for 50 min. After being washed with FACS washing buffer, cells were labeled with fluorescein isothiocyanate-conjugated rabbit anti-mouse Abs (1:100) for 50 min. The fluorescence of cells was analyzed using a FACScan flow cytometer (BD Biosciences).

Immunofluorescence staining. HUVECs for immunofluorescence assay were grown on 1% gelatin-coated eight-well chamber culture well slides (Lab-Tek, San Diego, CA) until a confluent monolayer was achieved. For CD99 or CD99 staining, endothelial cells were cultured with medium alone or 25 nM of activated RgpA for various times at 37°C in the absence of serum. HUVECs were also treated with 2 mM TLCK-inhibited RgpA for 1 h without serum. After exposure to experimental conditions, cells were washed in PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. For NF-κB protein localization, HUVECs were stimulated with 5 μg/ml of anti-CD99 MAb or control isotype-matched MAb for the indicated times. Cells were also treated with 70 nM of activated RgpA or TLCK-inhibited RgpA for 1 h. After the incubation, cells were permeabilized in 0.1% saponin in PBS for 20 min after the fixation with paraformaldehyde. After being washed with PBS, the cells were blocked with 5% goat serum for 30 min to prevent nonspecific staining. The slides were then stained with primary antibody against anti-CD99 MAB or anti-CD9 MAB or anti-human NF-κB MAB (1:50 each) for 50 min at room temperature. After unbound primary antibodies were washed away with PBS, Alexa Fluor 488-labeled rabbit anti-mouse immunoglobulin G (heavy plus light chain)-conjugated secondary antibody (1:200) was added for another 50 min. After extensive washing, the slides were covered with a Vectashield antifading mounting medium from Vector Laboratories (Burlingame, CA) and were visualized using a Zeiss fluorescence microscope (Thornwood, NY) connected to a Nikon digital camera (Tokyo, Japan).

Quantitative fluorescence intensity of CD99 or CD9 expression in endothelial monolayers. Fluorescence intensity was assessed using IQ Studio Imaging system (version 1.1; Media Cybernetics, Silver Spring, MD). Briefly, using the line tool in IQ Studio, a 10- by 1,000-pixel region (2.5 pixels = 1 μm; 4 × 400 μm) of interest was selected in each of the images of CD99 or CD. The intensity was measured with the x axis depicting distance in pixels and the y axis depicting the fluorescence intensity.

HUVEC NF-κB p65 subunit detection. To quantify active NF-κB in nuclear extracts of HUVECs in response to 5 μg/ml of anti-CD99 MAB, or 70 nM of activated RgpA, nuclear protein from endothelial cells was prepared in a cold hypotonic lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 2 mM dithiothreitol, 0.1% Triton X-100, protease inhibitor cocktail) according to the supplier's nuclear extraction protocol (Chemicon International, Australia). Nuclear protein lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis with a monoclonal antibody to the p65 subunit of NF-κB.

PMN adhesion assay. Human polymorphonuclear leukocytes (PMNs) separated from peripheral blood of healthy volunteers were isolated by Ficoll gradient separation (Robbins Scientific, Sunnyvale, CA) followed by hypotonic red cell lysis to visualize the adherent cells. PMNs were washed three times and resuspended in a membrane-associated dye, calcein-AM (10 μM) (31) (Molecular Probes, Eugene, Ore.), 0.5 μg/ml of anti-CD99 MAb, or control MAb for 1 h. After incubation, the cells were washed and calcein-AM-labeled PMNs were added. The PMN-HUVEC preparation was covered with foil and incubated at 37°C with occasional agitation for 1 h. After the incubation, the nonadherent neutrophils were removed by careful aspiration and washed twice with culture medium at 37°C. HUVECs were grown in 12-well plates to confluence and were incubated in the presence or absence of activated RgpA at 70 nM, or with 2 mM TLCK-treated RgpA for 1 h. Monolayers were then washed gently in culture medium three times and stimulated with 5 μg/ml of anti-CD99 MAb or control MAb for 1 h. After incubation, the cells were washed and calcein-AM-labeled PMNs were added. The PMN-HUVEC preparation was covered with foil and incubated at 37°C with occasional agitation for 1 h. After the incubation, the nonadherent neutrophils were removed by careful aspiration and washed twice with culture medium. The plate was read on a fluorescence reader (Baxter Scientific Products, Philadelphia, Pa.) with excitation at 494 nm and emission at 510 nm.

Immunoblot analysis. After the experimental conditions, endothelial cells were washed twice with 5 mM Tris-Cl buffer (pH 7.5) and mixed with 20% (vol/vol) protease inhibitor cocktail (Sigma). HUVECs were lysed in SDS reducing sample buffer by three cycles of heat (100°C for 3 min), whole-cell extracts were electrophoresed by 10% SDS-polyacrylamide gel electrophoresis (27), and proteins were transferred onto polyvinylidene difluoride membranes (42). Immunoblot detection was performed using the primary anti-human Abs (1:500) and the corresponding alkaline phosphatase-conjugated secondary Abs (1:5,000). 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-tolidine salt (0.8 mg) in 10 ml of 100 mM Tris-Cl (pH 9.5).
Statistical analysis. All data were expressed as means ± standard errors of the means (SEM). Differences between groups were examined for statistical significance using Student’s t test for unpaired data and the paired t test for paired data. A P value of <0.05 denoted the presence of a statistically significant difference.

RESULTS

Down-regulation of CD99 on HUVECs by gingipains. CD99 molecules are expressed on unstimulated endothelial cells (35). The expression of CD99 on HUVECs after RgpA and Kgp treat-
FIG. 2. CD99 distribution on HUVECs in the presence of RgpA. HUVECs were grown to confluence in an eight-well chamber culture slide and incubated with 23 nM RgpA for different times or with control medium alone. HUVECs were also treated with 2 mM TLCK-treated RgpA (RgpTL) for 60 min. Cells were fixed with 4% paraformaldehyde and subsequently stained with anti-CD99 MAb (A) or anti-CD9 MAb (B) followed by Alexa Fluor-conjugated secondary antibody. Cells were imaged using conventional fluorescence microscopy and digital photography. Images of HUVEC monolayers stained for surface antigen shown above were assessed for surface immunofluorescence using IQ Studio software as detailed in Materials and Methods. The fluorescence intensity of a region (10 × 1,000 pixels) was marked by arrowheads on each panel and then plotted as a histogram with the y axis representing the fluorescence intensity and the x axis representing distance (pixels). The peaks in fluorescence intensity for CD99 or CD9 represent the regions of intercellular junctions, and the ditches show the nonjunctural areas of staining. Each plot is representative of several separate measurements from two different experiments.
ment under serum-free conditions was determined by flow cyto-
metric analysis. Both RgpA and Kgp efficiently reduced CD99
expression on HUVECs in a time- and dose-dependent manner
(Fig. 1A and B). Treatment of the HUVECs with 70 nM RgpA or
Kgp for 15 min reduced detection of CD99 from 80% to approx-
imately 13% (RgpA) and 20% (Kgp) of positive cells. Figures 1C
and 1D demonstrate the FACS profiles of CD99 expression on
HUVECs after the addition of indicated levels of gingipains for
1 h. We next investigated the effect of gingipains on CD9 that is
constitutively expressed at intercellular junctions of endothelial
cells (45) under serum-free conditions. Expression of the trans-
membrane molecule CD9, which belongs to a family of tetraspan-
ins (28), was not affected by treatment with 70 nM RgpA for 1 h
(Fig. 1E). The results indicate that gingipains degrade CD99
specifically.

To further identify CD99 expression on HUVECs after gingipain treatment, a time course study was followed by
Western blotting. Using an anti-CD99 monoclonal antibody, a
32-kDa band corresponding to intact CD99 was detected in the
cell lysate of untreated HUVECs (Fig. 1F). The 32-kDa CD99
band disappeared after a 15-min treatment of cells with RgpA or
Kgp, respectively. To confirm that the gingipains are responsible
for the degradation of CD99 on HUVECs, the proteinase inhib-
itor TLCK was incubated with gingipains before addition to
HUVECs. Hydrolysis of the 32-kDa CD99 band was markedly

**FIG. 3. Influence of gingipains on CD99 MAb-induced expression of cell adhesion molecules on HUVECs.** (A) Confluent HUVECs were
stimulated with the indicated concentrations of anti-CD99 MAb alone or an isotype-matched (Isot.) MAb. ELAM-1 expression was analyzed by
flow cytometry after a 2-h culture. (B) HUVECs were incubated with anti-CD99 (A-CD99) MAb or isotype-matched (Isot.) MAb at the indicated
times and concentrations. After incubation, cells were treated with specific antibody for VCAM-1 and measured by flow cytometry. (C and D)
HUVECs were pretreated in the presence of various concentrations of activated RgpA or Kgp for 1 h without serum. Cells were then washed gently
with medium and stimulated with 5 μg/ml of anti-CD99 or isotype-matched (Isot.) MAb for 1 h. Surface expression of ELAM-1 was then evaluated
as described in Materials and Methods. Error bars indicate the means ± SEM. The data shown are from three independent experiments that
yielded similar results. *, *P < 0.05; **, P < 0.01; and ***, P < 0.001, compared with corresponding controls.
inhibited after a 60-min treatment with either TLCK-treated RgpA or TLCK-treated Kgp. The results verified that reduction of CD99 expression on HUVECs was due to proteolysis by gingipains. Figure 1G confirms that gingipains preferentially cleave CD99 on HUVECs, as the expression of CD9 was unchanged after 1 h of incubation.

Reduced dispersal of CD99 at cell junctions following RgpA treatment. Immunofluorescence microscopy demonstrated that CD99 was localized to intercellular junctions of HUVECs (Fig. 2A). After a 5-min incubation with RgpA, CD99 was not able to bind CD99 Ab in a homophilic manner and the distribution of CD99 was diffuse in nature. A subsequent 15-min incubation of HUVECs with RgpA induced prominent contraction and progressive intercellular gap formation. Intensity of CD99 staining was significantly reduced on cell surfaces after 15 to 30 min of incubation with RgpA. HUVECs treated with RgpA inhibited by TLCK showed a pattern of reactivity that was similar to that of unstimulated cells, with no obvious changes in the staining and distribution of CD99. These results support the contention that CD99 localized on HUVECs is sensitive to proteolysis by gingipains. To address the possibility of the toxic effect of gingipains, cellular viability of HUVECs after gingipain treatment at 70 nM was performed. The results indicate that more than 90% of HUVECs are viable at 1 h, as assessed by a 0.4% trypan blue exclusion test, demonstrating that gingipains have only a slight cytotoxicity. We also observed that CD9 staining at the disrupted endothelial cell-cell junctions was minimally altered after 1 h of gingipain treatment (Fig. 2B). To confirm the fluorescence microscopy analysis results, the total surface expression of these junctional proteins was quantified using image analysis software. The peaks of fluorescence intensity for CD99 correspond to lateral junction regions, whereas the valleys correspond to nonjunc-
versely, anti-CD99 MAb at 5 μg/ml induced a 50% increase in ELAM-1 expression above that seen with isotype control MAb-treated HUVECs and near-maximal effect at 5 μg/ml (Fig. 3A). The initial stimulation with 5 μg/ml of anti-CD99 MAb for 10 min increased by 40% the number of HUVECs expressing VCAM-1 (Fig. 3B), and expression reached maximal levels within 1 h to 2 h. CD99-induced ELAM-1 expression reached its maximum after 2 h of stimulation, similar to the kinetics of VCAM-1 up-regulation observed in parallel experiments (data not shown). Similarly, ICAM-1 expressed by endothelial cells was up-regulated by 5 μg/ml of CD99 MAb after 1 h (Fig. 4C).

Analytical flow cytometry revealed that preliminary treatment of HUVECs by gingipains down-regulated the expression of ELAM-1, VCAM-1, and ICAM-1 induced by anti-CD99 MAb (Fig. 3 and 4). The reduction of cell adhesion molecule expression by gingipain pretreatment in CD99-stimulated HUVECs was also concentration dependent. Increasing concentrations of the gingipains attenuated the induction of ELAM-1 by anti-CD99 MAb, with inhibition greatest at 70 nM (P < 0.001) (Fig. 3C and D). The induction of VCAM-1 and ICAM-1 expression was similarly inhibited by gingipains in CD99-stimulated HUVECs (Fig. 4). In general, the effect of RgpA on the reduction of anti-CD99 MAb-induced cell adhesion molecule expression was more efficient than that for Kgp. Inhibition of CD99-induced expression of E-selectin, VCAM-1, and ICAM-1 was blocked by TLCK-treated RgpA or Kgp (Fig. 4D), indicating that proteolysis of CD99 was a requirement for this effect, supporting evidence demonstrated in Fig. 1. Endothelial E-selectin, VCAM-1, and ICAM-1 expression after gingipain pretreatment and anti-CD99 MAb ligation was also evaluated by Western blot analysis. Intact but quantitatively fewer surface molecules were observed, and these proteins were not directly degraded by the gingipains (Fig. 5). The results correlated well with the flow cytometric analysis.

Inhibition of CD99-induced MHC class II expression by gingipains in HUVECs. In light of the increase in cell adhesion molecules observed in HUVECs, the effect of CD99 ligation on MHC class II antigen expression on HUVECs was studied. Unstimulated HUVECs did not express surface HLA-DR. Conversely, anti-CD99 MAb at 5 μg/ml induced rapid up-regulation of HLA-DR expression on HUVECs in a time-dependent manner (Fig. 6A). Expression of HLA-DR peaked at 2 h. The effect was anti-CD99 MAb specific, since treatment of isotype-matched control Ab did not up-regulate MHC class II antigen expression on HUVECs. The kinetics of endothelial cell HLA-DR induction by CD99 ligation are more rapid than those induced by gamma interferon with minimal endothelial expression of HLA-DR after 1 h of incubation with this cytokine (data not shown). To evaluate the effect of gingipains on CD99-induced HLA-DR expression, HUVECs were pretreated with gingipains before CD99 ligation. Pretreated HUVECs exhibited large variations in the level of HLA-DR expression in response to anti-CD99 MAb, while isotype-matched control Ab did not induce the same response (Fig. 6B and C). The optimum concentration of gingipains required to achieve complete reduction of CD99-induced HLA-DR activation was 70 nM. Proteolytic activity of gingipains was required for the inhibitory activity, as RgpA and Kgp pretreated with TLCK before addition to HUVECs did not decrease the CD99-induced HLA-DR expression. Endothelial HLA-DR expression after gingipain pretreatment and anti-CD99 MAb ligation was also evaluated by immunoblot analysis. Quantitatively fewer surface molecules were detected, and the proteins were not cleaved by the gingipains. The results correlated well with flow cytometric analysis (Fig. 6D).

**CD99 cross-linking on HUVECs increases leukocyte adhesion.** Endothelial-leukocyte adhesion was examined using phase-contrast photomicrography. PMNs demonstrated relatively low levels of adhesion to untreated HUVECs. Pretreatment of endothelial monolayers with CD99 MAb for 1 h up-regulated the expression of cell adhesion molecules (Fig. 3 and 4). Increased numbers of leukocytes appeared to be attached relatively uniformly across the surface of the treated endothelial monolayer (Fig. 7A). The adhesion was also evaluated using calcein-labeled PMNs. As shown...
in Fig. 7B, CD99 MAb pretreatment (at 1.25 μg/ml) of passaged endothelial monolayers resulted in an ~55% increase in labeled PMN adhesion over basal levels (P < 0.05), and a doubling in cell adhesion was obtained with 5 μg/ml of CD99 MAb (P < 0.001). In RgpA-treated HUVECs, PMN adhesion levels were similar to those of untreated control cells but significantly lower (by 67%) than the level stimulated by CD99 MAb pretreatment (P < 0.05) (Fig. 7C). RgpA inhibited with the proteinase inhibitor TLCK was ineffective.

Gingipains decrease nuclear NF-κB translocation in CD99 MAb-stimulated HUVECs. Recent reports have provided support for the significance of the transcription factor NF-κB in regulating the expression of ELAM-1 and ICAM-1 (15). Hence, we analyzed whether CD99 induced cell adhesion molecule expression on endothelial cells through NF-κB activation. Using immunofluorescence, NF-κB was localized to the cytoplasm of untreated HUVECs (Fig. 8A), while heavy nuclear staining was detected in cells exposed to 5 μg/ml CD99 MAb for 15 min to 30 min (Fig. 8A), indicative of nuclear localization of activated NF-κB at the single-cell level. Further, treatment of HUVECs with isotype control MAb did not induce NF-κB translocation (data not shown). Gingipain-treated
cells showed diminished staining for cytoplasmic NF-κB whereas this remained elevated when endothelial cells were treated with TLCK-inhibited RgpA. To quantify the endothelial NF-κB translocation after CD99 MAb treatment, Western blot analysis of the nuclear p65 subunit of NF-κB was performed. NF-κB p65 band density was significantly increased in CD99 MAb-induced HUVECs after 15 to 30 min compared with unstimulated cells (Fig. 8B and C). In contrast, the NF-κB
p65 band density was similar to that of untreated cells when HUVECs were treated with 70 nM gingipains or TLCK-inhibited RgpA (RgpTL) as indicated. Images were obtained using fluorescence microscopy. NF-κB localization was visualized by binding with an Alexa Fluor-conjugated secondary antibody. Cells displaying activated NF-κB are indicated by arrows. (B) Nuclear protein lysates of HUVECs were resolved by SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis with a monoclonal antibody to the p65 subunit of NF-κB. NF-κB p65 band density was increased in response to anti-CD99 MAb at 15 (lane 2) and 30 (lane 3) min compared with unstimulated HUVECs (lane 1). In contrast, NF-κB p65 band density was unchanged when HUVECs were treated with 70 nM Kgp (lane 4) or RgpA (lane 5) or TLCK-inhibited RgpA (RgpTL) (lane 6) for 30 min. This panel is representative of three separate experiments. The mean integrated optical band density determined by the TotalLab software (version 1.1; Greenwoods Corner, Auckland, New Zealand) is shown in panel C. Error bars indicate the means ± SEM. *, *P < 0.05, and **, *P < 0.01, compared with untreated HUVECs.

DISCUSSION

The migration of leukocytes from the vascular lumen into the surrounding extravascular tissue is a characteristic feature of an inflammatory response. The mechanism of leukocyte penetration through the endothelial barrier is unclear although recent findings have demonstrated a role for PECAM-1 and the integrin α5β1 in leukocyte migration through the perivascular basement membrane in vivo (41). Also, a recent study has demonstrated that other adhesive proteins, such as CD99, play a critical role in the migration of leukocytes through endothelial junctions (35). We previously showed that gingipains induce intercellular junctional PECAM-1 expression accompanied by enhanced transfer of neutrophils across the monolayer (47). The present study indicates that the gingipains can also readily reduce functional CD99 expression on endothelial cells. These studies implicate P. gingivalis gingipains, through their ability to disrupt endothelial cell junctional complexes, in modulation of the process of leukocyte migration through the vessel wall.

CD99 is a 32-kDa molecule that is extensively O glycosylated, with carbohydrate chains representing 14 kDa (44%) of the apparent molecular size (4). Although CD99 has not been fully characterized, the sialylated membrane form of CD99
is possibly related to other sialomucin-type glycoproteins, which represent a group of signal-transducing surface molecules involved in cellular adhesion processes. It is noteworthy that human CD99 contains five Arg-X and 12 Lys-X bonds, which are possible sites of cleavage by RgpA and Kgp, respectively. We also present evidence that the tetrahedral protein CD99 localized at endothelial intercellular junctions was unchanged after gingipain treatment. However, it remains undetermined whether the gingipains might affect vascular permeability and regulation of leukocyte extravasation potentially controlled by CD99.

Cell adhesion and diapedesis, mediated by specific cell surface molecules, play a central role in inflammation. The recruitment of leukocytes from the circulation into the extravascular space involves several steps. Among these, interactions of leukocyte sialosyl-LeX with ELAM-1, of integrins LFA-1 and Mac-1 with ICAM-1, and of VLA-4 with VCAM-1 are crucial steps in forming firm adhesion (9), whereas transendothelial migration of leukocytes through endothelial junctions is regulated sequentially by two distinct molecules, PECAM-1 and CD99 (30, 35). Based on the conditions established for this study, it was noted that a threshold concentration of CD99 ligation is necessary for maximal induction of adhesion molecules on endothelial cells. We demonstrated a strong correlation between ELAM-1, ICAM-1, and VCAM-1 up-regulation induced by CD99 engagement and the ability of human leukocytes to adhere to HUVECs, and all these factors may accelerate the recruitment of leukocytes to the inflammatory site.

This study also suggests that gingipains can modulate leukocyte adherence to endothelial cells via proteolysis of the CD99 molecule. The decreased endothelial expression of E-selectin, VCAM-1, and ICAM-1 in response to anti-CD99 MAb was related to hydrolysis of junctional CD99 molecules by gingipains. The fact that reduction of adhesion molecule expression could be eliminated by pretreating the gingipains with a cysteine proteinase inhibitor, TLCK, suggests that the hydrolysis of the CD99 proteins correlated with the loss of CD99 ligation-induced activities. While leukocyte recruitment can be enhanced by CD99 engagement during leukocyte-endothelial cell interactions, the ability of the gingipains to inactivate CD99 may lead to the lack of cell adhesion molecule expression and down-regulation of inflammatory responses during the early stages of lesion development.

MHC class II molecules essentially present antigenic peptides to the TCR of CD4+ T cells (1), and previous studies has revealed that fewer macrophages express detectable MHC class II molecules in advanced periodontitis lesions (13). We report here that CD99 engagement can enhance the capacity of endothelial cells to express class II MHC molecules, and thus, degradation of CD99 by gingipains potentially reduces the effectiveness of antigen presentation to CD4+ T cells. Subsequently, reduced numbers of activated T cells would result in decreased migration and proliferation of memory CD4+ T lymphocytes into gingival tissues. Indeed, reduced CD4+/CD8+ T-cell ratio has been detected in gingival tissues of patients with periodontitis (40).

During the inflammatory response, circulating cytokines interact with the vascular endothelium, resulting in the activation and nuclear translocation of NF-κB (15). In the present report, ligation of CD99 on endothelial cells was shown to stimulate translocation of the p65 subunit of NF-κB to the nucleus. Our findings demonstrate that the NF-κB signaling cascade is an associated component in the induction of adhesion molecule expression in the CD99-mediated adhesion response. While different strains of P. gingivalis have been shown to induce NF-κB translocation in endothelial cells (44), our data suggest that gingipains may interfere with the NF-κB signaling cascade, to subsequently decrease the inducible regulation of cellular inflammatory molecules.

To conclude, the observations indicate that ligation of CD99/E2 molecules can transmit a positive activation signal and act on vascular endothelium to alter the functional surface properties related to leukocyte adhesion. The data support the view that the NF-κB signaling cascade is a component in the CD99-mediated adhesion response. In recent years gingipains have been associated with the ability to destroy epithelial barriers and infiltrate vascularized periodontal tissues (3, 17, 34). In line with these findings, the results of the present study support a role for gingipains in down-regulation of CD99 expressed on endothelial junctions. While we previously reported that gingipains can affect leukocyte transmigration in an in vitro model (47), the present study showed that gingipains can reduce CD99-mediated cell adhesion molecule expression and the antigen-presenting capability of endothelial cells. In total, P. gingivalis and gingipains can act on endothelium and limit the junctional CD99-mediated adhesivity for circulating leukocytes and the inflammatory response; this may contribute to the successful persistent colonization of the organism in the periodontal pocket.

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