Osteoprotegerin protects endothelial cells against apoptotic cell death induced by *Porphyromonas gingivalis* cysteine proteinases

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Abstract

Osteoprotegerin (OPG) is a key regulator of osteoclastogenesis during the progression of periodontitis. Recent reports suggest that osteoprotegerin may also prevent arterial calcification and contribute to endothelial cell survival. To determine whether the vascular functions of osteoprotegerin are involved in periodontitis, we examined whether osteoprotegerin contributed to the survival of endothelial cells damaged by *Porphyromonas gingivalis* cysteine proteinases (gingipains). Gingipain proteinases cleave a broad range of host proteins, and are important virulence factors of *P. gingivalis*, a major causative bacterium of adult periodontitis. Human microvascular endothelial cells (HMVEC) were exposed to activated gingipain extracts from *P. gingivalis* 381, with and without pretreatment with osteoprotegerin. Cell viability was quantified by the tetrazolium (WST-8) reduction assay, and apoptosis was examined using Hoechst 33342 nuclear staining. After 16 h of treatment with activated gingipain extracts, HMVEC showed near-complete detachment from the tissue culture dish, and apoptosis was evident by 24 h. Pretreatment of HMVEC with osteoprotegerin reduced the extent of both cellular detachment and apoptotic cell death. Our results indicated that osteoprotegerin pretreatment protected HMVEC against detachment and apoptotic cell death induced by gingipain-active bacterial cell extracts. These results also suggest that osteoprotegerin may function as a survival factor for endothelial cells during periodontitis.

Introduction

Porphyromonas gingivalis, a gram-negative anaerobe, has been implicated as a major etiological agent of periodontitis, based on the frequency at which it is isolated from periodontal lesions, and the potency of its virulence factors (Mayrand & Holt, 1988; Socransky & Haffajee, 1992). Gingipains are cysteine proteinases released by the bacterium, and consist of two types: those that hydrolyze peptide bonds specifically after an arginine residue (gingipain-R; RgpA or RgpB), or a lysine (gingipain-K; Kgp) residue (Ciborowski *et al.*, 1994; Pike *et al.*, 1994). Gingipains have come under scrutiny recently due to their ability to activate and/or degrade a broad range of host proteins and cytokines (Imamura *et al.*, 1994; Sugawara *et al.*, 2000; Katz *et al.*, 2002). Recently, it has been reported that gingipains can cleave cell surface adhesion molecules and induce apoptosis in endothelial cells (Sheets *et al.*, 2005). However, the mechanism by which gingipains induce apoptosis of the endothelium is yet to be elucidated.

Recently, we demonstrated that osteoprotegerin is produced by human microvascular endothelial cells (HMVEC) in response to *P. gingivalis* challenge (Kobayashi-Sakamoto *et al.*, 2004). Osteoprotegerin is a member of the tumor necrosis factor (TNF) receptor superfamily. Osteoprotegerin binds to the receptor activator of the nuclear factor-κB (NF-κB) ligand (RANKL), thereby neutralizing its function, and negatively regulating osteoclast differentiation and survival (Simonet *et al.*, 1997; Yasuda *et al.*, 1998). In addition, recent reports have shown that administration of osteoprotegerin prevents the calcification of blood vessels by warfarin and vitamin D, and protects endothelial cells from apoptosis in response to serum deprivation (Malyankar *et al.*, 2000; Price *et al.*, 2001; Pritzker *et al.*, 2004). These findings suggest that osteoprotegerin may have a protective role in the vasculature.

Although osteoprotegerin has been implicated as a mediator of endothelial cell survival, and *P. gingivalis* induces osteoprotegerin release from the endothelium, the role of osteoprotegerin in endothelial cell function during periodontitis is poorly understood. During the progression of periodontal disease, the periodontal vasculature is profoundly affected (Pober *et al.*, 1990; Collin-Osdoby *et al.*, 2001). It has also been shown that endothelial cell injury and dysfunction are induced by gingipains (Kobayashi-Sakamoto *et al.*, 2003; Sheets *et al.*, 2005). In the current study, we were interested in the effect of osteoprotegerin on gingipain-induced endothelial cell damage. By looking specifically at the role of osteoprotegerin in gingipain-induced cell detachment and apoptosis in cultured HMVEC, we demonstrated that osteoprotegerin may protect endothelial cells from damage and possibly apoptosis in response to gingipains.

Materials and methods

Reagents

Leupeptin, *N*α-tosyl-L-lysine chloromethyl ketone (TLCK), *N*α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA), *p*-nitroaniline, and cysteine were purchased from Sigma co. (St Louis, MO). 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), Cell Counting Kit-8, and Hoechst dye 33342 were purchased from Dojindo Laboratories (Kumamoto, Japan). Phosphate-buffered saline (PBS) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan), and the Vectastain Elite ABC kit and biotinylated antigoat IgG were purchased from Vector Lab Inc. (Burlingame, CA). Acetyl-lysine-*p*-nitroanilide (Ac-Lys-pNa), Phe-Pro-Arg-chloromethyl ketone (FPR-cmk), and benzyloxycarbonyl-Phe-Lys-chloromethyl ketone (Z-FK-cmk) were obtained from Bachem Bioscience (King of Prussia, PA). Recombinant human osteoprotegerin and human osteoprotegerin-specific polyclonal antibody were purchased from R&D Systems (Minneapolis, MN).

Gingipain-active extract preparation and protease assay

Porphyromonas gingivalis strain 381 was grown anaerobically (70% N_2, 15% H_2, and 15% CO_2) at 37 °C for 24 h in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) containing hemin (5 μg mL^{-1}) and menadione (1 μg mL^{-1}). Bacterial cultures (1000 mL) were centrifuged (12 000 ***g***, 45 min, 4 °C) to remove cells, and extracellular proteins in the culture supernatant were precipitated in a saturated ammonium sulfate solution (85%) at 4 °C for 12 h. Precipitated proteins were collected by centrifugation (10 000 ***g***, 60 min). The resulting pellet was dissolved in 20 mL of 50 mM sodium acetate buffer, pH 5.3, containing 0.5% CHAPS and applied to a Sephacryl S-200 (Amersham Biosciences, Buckinghamshire, UK) column as described by Pike *et al.* (1994). Fractions were collected and pooled based on their Rgp- or Kgp-specific activity. Pooled fractions were combined, concentrated, and extensively dialyzed against 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5, for 24 h at 4 °C. The protein preparation was aliquoted, and stored at −80 °C until use. Protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). To determine gingipain activity, aliquots (10 μL) of the preparations were incubated in 96-well microplates at 37 °C with 190 μL of 100 mM Tris-HCl, pH 7.6, containing either 1 mM BAPNA (to detect Rgp A and B activity) or 1 mM Ac-Lys-*p*NA (to detect Kgp activity), and 10 mM cysteine. Absorbance at 405 nm was determined using a microplate reader (Plate Analyzer ETY-300, TOYO, Tokyo, Japan). Gingipain activity was defined as pmol of *p*-nitroaniline per min released.

Endothelial cell culture

HMVEC were purchased from Clonetics (San Diego, CA) and cultured in endothelial cell medium (EGM-2MV, Clonetics) according to the manufacturer's instructions. Cells were grown in 25-cm^2 flasks (Corning Costar Co., Cambridge, MA), and passaged every 3 or 4 days. Cells from passage four through seven were used for all experiments.

Activation of gingipains in *P. gingivalis* extracts for HMVEC treatment

Immediately before use, *P. gingivalis* gingipain extracts were activated by incubation with 10 mM cysteine for 10 min at room temperature. Osteoprotegerin is very sensitive to reducing agents, so cysteine was removed using a Zeba Desalt Spin column (Pierce Rockford, IL) before the activated extracts were applied to HMVEC. To inhibit gingipain activity, activated extracts were pretreated with 10 mM TLCK for 5 min at room temperature, and then applied to a Zeba Desalt Spin column before being applied to HMVEC.

Assessment of cell viability and apoptosis

HMVEC were grown in EGM-2MV media, and cell suspensions were obtained enzymatically according to the manufacturer's instructions. Osteoprotegerin is constitutively produced and released by HMVEC grown in EGM-2MV. Therefore, before every experiment, cells were washed twice in human endothelial serum-free medium (H-SFM; Invitrogen, Carlsbad, CA), resuspended in H-SFM, plated at 4×10^4 cells well^{-1} (100 μL well^{-1}) in 96-well plates (Corning), in the absence or presence of the indicated concentrations of recombinant osteoprotegerin, and allowed to adhere overnight. The following day, 100 μL of H-SFM containing activated gingipain extract was added to the cells (final

concentration of activated extract 3.5 or 7 μg mL^{-1}) and the cells were incubated for an additional 24 h. HMVEC were monitored by phase contrast microscopy using an Olympus microscope. Cell viability was determined using Cell Counting Kit-8, in which 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) is used as a substrate. WST-8 is reduced by intracellular dehydrogenases to generate WST-8-formazan, which has a maximal absorbance at 450 nm. Briefly, after incubation for 24 h in the presence or absence of activated extracts, 10 μL of the kit reagent was added to each well, and the cells were incubated for an additional 3 h. Cell viability was measured by scanning the plate with a microplate reader at 450 nm. Each experiment was carried out in triplicate wells. Cell death was expressed as the percentage loss of viability. To identify apoptotic cells, fluorescence microscopy was used to examine for staining patterns indicative of apoptosis among cells incubated with Hoechst dye 33342, such as punctuate nuclear staining and rounded nuclei. To further characterize apoptosis, and distinguish between necrotic and apoptotic cell death, we analyzed single-strand DNA (ssDNA) fragments in nuclei using an enzyme-linked immunosorbent assay (ELISA) detection kit and a mouse monoclonal antibody to ssDNA (Chemicon International, Temecula, CA). This antibody does not recognize DNA in the double-stranded conformation and is a specific measure of apoptosis (Frankfurt & Krishan, 2001). As a negative control, cells were treated with S1 nuclease, according to the manufacturer's instructions.

Assessment of osteoprotegerin degradation by gingipain-active extracts

Gingipain extracts were preincubated with cysteine for 10 min at room temperature (10 mM final concentration), and cysteine was removed from the activated extracts using a Zeba Desalt Spin column. Activated, purified *P. gingivalis* extracts (7 μg mL^{-1} final concentration) were then incubated for various times with recombinant osteoprotegerin (2 μg mL^{-1} final concentration) in 5 mM Tris-HCl (pH 7.6). Reactions were stopped by the addition of TLCK (2 mM final concentration). To inhibit gingipain activity, activated and desalted *P. gingivalis* extracts were preincubated for 5 min at room temperature with either TLCK, FPR-cmk, leupeptin, or Z-FK-cmk, and incubated with recombinant osteoprotegerin in 5 mM Tris-HCl (pH 7.6) at 37 °C for 90 min. Samples were stored frozen at −20 °C until analyzed by Western blotting.

Electrophoresis and Western blotting

Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% polyacrylamide gels, and then transferred onto a nitrocellulose membrane (Trans-Blot transfer medium, Bio-Rad Laboratories). The membrane was incubated at room temperature for 60 min with goat antihuman recombinant osteoprotegerin polyclonal antibody, diluted 1 : 1000 in PBS containing 0.1% Tween 20. The membranes were washed three times for 10 min each in PBS/0.1% Tween 20, and then incubated with secondary antibody (antigoat IgG) for 30 min at room temperature. After three washes, the membrane was incubated with ABC reagent conjugated to peroxidase for 30 min at room temperature, and proteins were visualized using diaminobenzidine/H_2O_2 in 0.1 M Tris-HCl, pH 7.5.

Data analysis

All of the experiments in this study were conducted at least three times, in triplicate. Unless otherwise stated, only the results obtained of one representative experiment are shown. Experimental values are the means ± SD of triplicate wells. Statistical analysis was performed using Student's *t*-test, and the significance level was set as $P < 0.05$.

Results

Gingipain-active extracts induce apoptosis in HMVEC followed by cell detachment

Activated gingipain extracts isolated from cultures of *P. gingivalis* contained 1174 U of Rgp activity μg^{-1} of protein and 74.8 U of Kgp activity μg^{-1} of protein. When HMVEC were exposed to activated gingipain extracts, near-complete cell detachment was observed after 16 h (Fig. 1a, left and middle panels). Apoptotic cell death was assessed using Hoechst 33342 nuclear staining. Untreated HMVEC had a normal nuclear morphology (Fig. 1b, left panel). In contrast, after 24 h of treatment with activated gingipain extract, we observed condensed chromatin and the presence of apoptotic bodies in a substantial proportion of HMVEC, indicating that these cells were undergoing apoptotic cell death (Fig. 1b, right panel). The induction of apoptosis was further confirmed by ELISA, using a specific monoclonal antibody to ssDNA, a specific and sensitive marker of apoptosis. As shown in Fig. 1c, there was a significant increase in the OD of HMVEC cultures that were exposed to activated gingipain extracts, as compared with unexposed cultures. As expected, cultures treated with S1 nuclease did not show any increase in OD. We next exposed HMVEC to gingipain extracts that had been pretreated with TLCK, an inhibitor of serine and cysteine proteinases. Under these conditions, cells displayed no morphological changes or loss of cell adhesion (Fig. 1a, right panel). As shown in Fig. 1d, cell death occurred in 53.8% of the cells exposed to activated gingipain extracts, and TLCK pretreatment of the extracts reduced cell death to 10.8%. These results suggested that gingipain

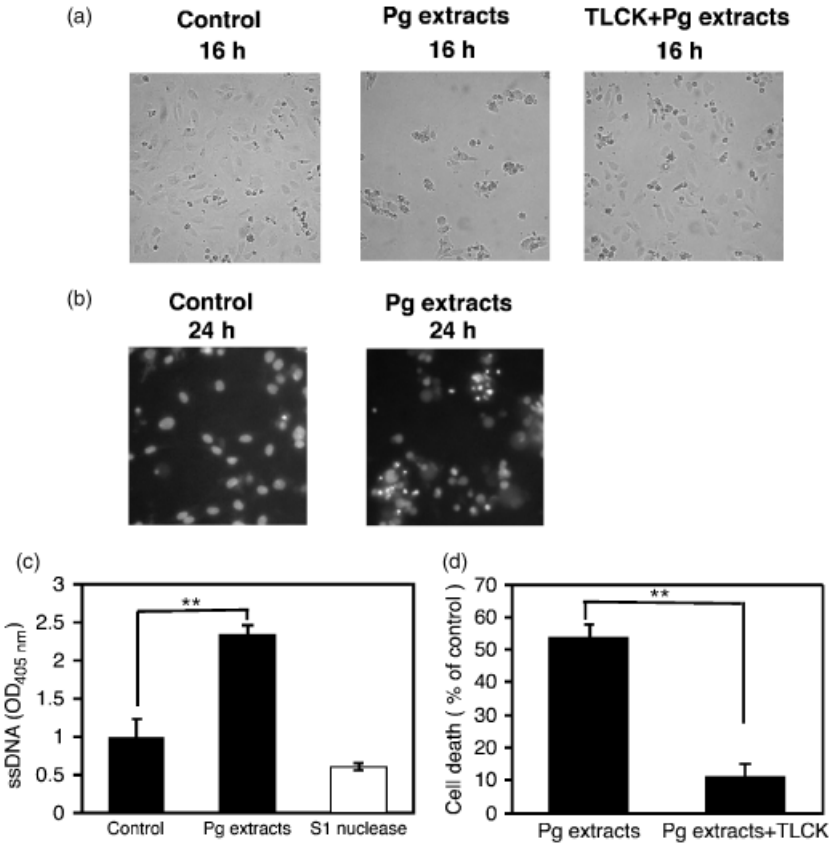

Fig. 1. Loss of adhesion and apoptosis in HMVEC exposed to activated gingipain extracts. HMVEC were cultured in the absence (control) or presence (Pg extracts) of 7 μg mL^{-1} of activated gingipain extracts, or activated gingipain extracts that had been pretreated with 10 mM TLCK (TLCK+Pg extracts), as described in Materials and methods. Control HMVEC were incubated in H-SFM alone. The treatment time is indicated above each panel. (a) Representative phase-contrast images of HMVEC. Magnification: × 100. (b) Cells were treated with activated gingipain extracts, or incubated in H-SFM alone for 24 h, then stained with Hoechst 33342, and nuclear morphology was assessed by fluorescence microscopy. Magnification: × 200. (c) HMVEC were treated as described above for 24 h. ss-DNA ELISA using a monoclonal antibody to detect apoptosis induced by activated gingipain extracts. Cells treated with nuclease S1 were used as a negative control for apoptosis. Data are expressed as OD (at 405 nm) and represent the means ± SD of triplicate walls. (d) HMVEC were treated as described above for 24 h, and the cell viability was analyzed using a Cell Counting Kit 8. Cell death is expressed as the percentage loss of viability compared with the untreated control. Data represent the means ± SD of triplicate walls. $^{**}P < 0.005$.

activity was responsible for the observed endothelial cell detachment and apoptosis.

Osteoprotegerin pretreatment protects cells against cell death induced by activated gingipain extracts

To examine the effect of osteoprotegerin on gingipain-induced endothelial cell death, we pretreated HMVEC with recombinant osteoprotegerin, and then exposed them to activated gingipain extracts. As shown in Fig. 2a and b, recombinant osteoprotegerin pretreatment appeared to suppress the extent of cell detachment and cell death in response to activated gingipain extracts, albeit rather weakly. Next, we exposed the cells to half-concentrated activated gingipain extracts. As shown in Fig. 2c, we found that osteoprotegerin suppressed significantly gingipain-induced endothelial cell death in a dose-dependent manner.

Osteoprotegerin degradation by gingipain-active extracts

Osteoprotegerin pretreatment suppressed gingipain-induced endothelial cell death, albeit rather weakly at high gingipain extract concentrations. However, at lower concentrations of gingipain extract, the protective effect of osteoprotegerin was greater (compare Fig. 2b and c). To begin to understand the suppressive activity of osteoprotegerin, we examined whether gingipain extracts were acting directly on osteoprotegerin to reduce its ability to suppress cell death. As shown in Fig. 3a, recombinant osteoprotegerin was degraded by activated gingipain extracts in a time-dependent manner. As shown in Fig. 3b, pretreatment of gingipain extracts with TLCK or FPR-cmk efficiently inhibited degradation of recombinant osteoprotegerin. FPR-cmk is an effective inhibitor of both Rgps and Kgp (Potempa *et al.*, 1997). Pretreatment with leupeptin, an inhibitor of Rgps, or Z-FK-cmk, an inhibitor of Kgp, did not prevent degradation of recombinant osteoprotegerin. However, addition of both leupeptin and Z-FK-cmk inhibited degradation of recombinant osteoprotegerin. These results indicated that both Rgps and Kgp contributed to the degradation of osteoprotegerin.

Discussion

In this study, we demonstrated that activated gingipain extracts induced cell detachment and apoptosis in cultures of HMVEC. These results are consistent with previous reports that gingipain-induced apoptosis in several cell types, including endothelial cells (Chen *et al.*, 2001; Sheets

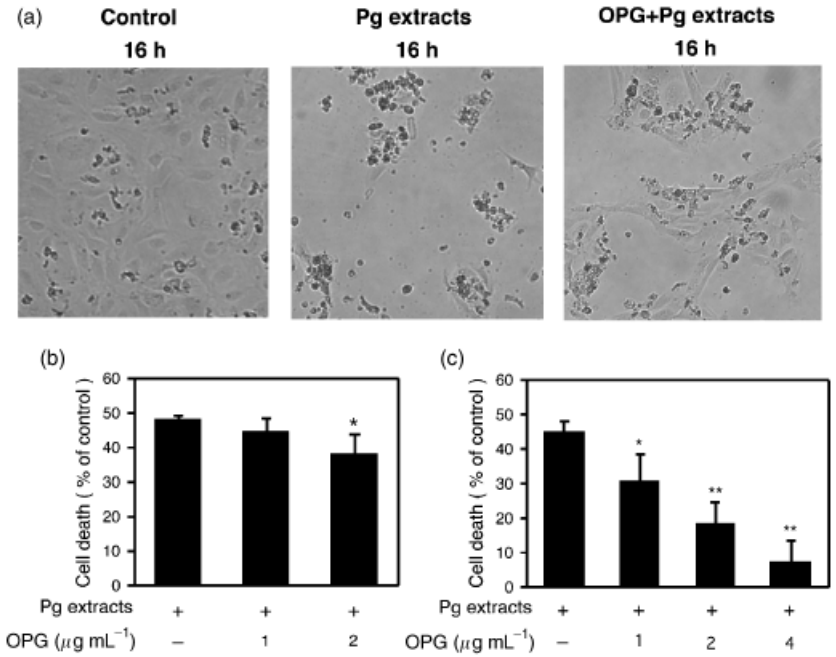

Fig. 2. Osteoprotegerin pretreatment protects HMVEC from gingipain-induced cell death. (a) HMVEC were plated overnight in H-SFM in the absence or presence of 2 μg mL^{-1} recombinant osteoprotegerin. Cells were then treated for 16 h with 7 μg mL^{-1} activated gingipain extracts as described in Materials and methods. Control, no OPG, no Pg extract; Pg extracts, no OPG, +Pg extract; OPG+Pg extracts, +OPG and Pg extract. Control cells were incubated in H-SFM alone. Representative phase-contrast images are shown. Magnification: × 100. (b) HMVEC were pretreated as described for A with 1 or 2 μg mL^{-1} recombinant osteoprotegerin and then exposed to 7 μg mL^{-1} activated gingipain extract for 24 h. Cell viability was assessed using a Cell Counting Kit 8. Cell death was expressed as the percentage loss of viability compared with the untreated control. (c) HMVEC were pretreated with 1, 2, or 4 μg mL^{-1} recombinant osteoprotegerin and then exposed to 3.5 μg mL^{-1} activated gingipain extract for 24 h. Cell viability was assessed using a Cell Counting Kit 8. Cell death was expressed as the percentage loss of viability compared with the untreated control. Data represent the means ± SD of triplicate walls. $^{*}P < 0.05$ and $^{**}P < 0.005$ vs. HMVEC treated with Pg extracts in the absence of recombinant osteoprotegerin.

et al., 2005). It is likely that cell detachment induced by gingipains is not a result of cell death, but a result of cleavage of membrane-bound adhesion molecules (Sheets *et al.*, 2005). Therefore, our results suggest that loss of attachment in response to activated gingipain induces apoptosis in HMVEC.

The role of osteoprotegerin in inhibiting osteoclastogenesis is well documented (Simonet *et al.*, 1997; Yasuda *et al.*, 1998). Several recent studies have shown that osteoprotegerin has additional roles in endothelial cell survival and the prevention of arterial calcification (Bucay *et al.*, 1998; Pritzker *et al.*, 2004). With regard to periodontitis, it is generally accepted that periodontal pathogens stimulate inflammation-induced osteoclast formation by acting through the RANKL/OPG system. For example, *P. gingivalis* infection induces RANKL expression in osteoblasts (Okahashi *et al.*, 2004). Inhibition of RANKL function *in vivo* by osteoprotegerin reduces alveolar bone destruction and the number of periodontal osteoclasts after microbial challenge (Teng *et al.*, 2000). However, it is not known what, if any, role the vascular functions of osteoprotegerin play in periodontitis. In this study, we demonstrated that osteoprotegerin protects endothelial cells from gingipain-induced apoptosis. This is the first demonstration that osteoprotegerin protects endothelial cells under pathologic conditions, and strongly supports the existence of a modulatory role for osteoprotegerin in periodontitis.

Several mechanisms for osteoprotegerin-mediated endothelial cell protection can be proposed. First, osteoprotegerin may protect endothelial cells by blocking the action of TNF-related apoptosis-inducing ligand (TRAIL). It is generally accepted that osteoprotegerin protects many cells by blocking TRAIL-mediated signaling (Holen *et al.*, 2002; Pritzker *et al.*, 2004). However, to date, there is no evidence linking TRAIL and detachment-induced apoptosis in the endothelium. Alternatively, osteoprotegerin may act in a TRAIL-independent manner to induce integrin expression, specifically integrin $\alpha_v\beta_3$, and enhance integrin-mediated endothelial cell adhesion. Osteoprotegerin has been identified as one of the integrin $\alpha_v\beta_3$-related antiapoptotic factors (Malyankar *et al.*, 2000). It is interesting to speculate that osteoprotegerin enhances cell surface integrin expression, resulting in extended survival and delayed apoptosis when the cells are exposed to gingipains.

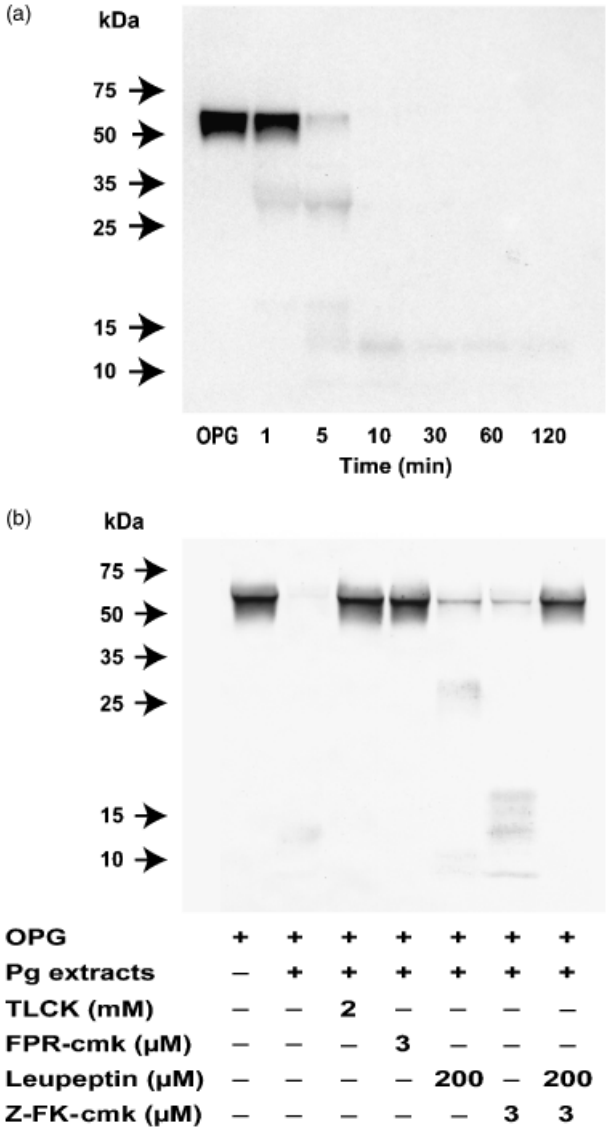

Fig. 3. Cleavage of osteoprotegerin by activated gingipain extracts. (a) Activated gingipain extracts (7 μg mL^{-1} final concentration) were mixed with recombinant osteoprotegerin (2 μg mL^{-1} final concentration in each reaction) and incubated at 37 °C. Reactions were stopped at the indicated time points by addition of TLCK (final concentration 2 mM). Samples were analyzed by Western blot using polyclonal anti-OPG antibody, as described in Materials and methods. Control (lane OPG) samples were incubated without activated gingipain extracts. (b) Activated gingipain extracts were preincubated with the indicated proteinase inhibitors and combinations of inhibitors, as described in Materials and methods. Extracts were then combined with recombinant osteoprotegerin (2 μg mL^{-1} final concentration) and incubated for 90 min. Western blot analysis was performed as described in Materials and methods. Data are representative of three independent experiments.

The suppressive effect of osteoprotegerin on endothelial cell death depended on the concentration of gingipain extract. We speculate that this was due at least in part to the degradation of osteoprotegerin by the gingipain extracts. A previous report showed that the mean value of gingipain activity in gingival crevicular fluids from periodontitis patients with attachment loss was 40 000–90 000 U mL^{-1}, as determined by Z-Val-Lys-Lys-Arg-AFC (Eley & Cox, 1996). According to our estimation of osteoprotegerin degradation activity of activated gingipain extracts using *N*-a benzyloxycarbonyl-L-Arg-*p*-nitroanilide, 7 μg mL^{-1} of extracts contained 8218 activity units mL^{-1}. It is conceivable that the local concentration of gingipains around *P. gingivalis* was much higher than in gingival crevicular fluids. Therefore, osteoprotegerin degradation by gingipains most likely occurs *in vivo*. In a previous study, we demonstrated that *P. gingivalis* cell-free supernatants degraded osteoprotegerin. However, intact, live *P. gingivalis* induces osteoprotegerin production in HMVEC, as seen by the ability of *P. gingivalis* lipopolysaccharide to induce osteoprotegerin production (Kobayashi-Sakamoto *et al.*, 2004). On the other hand, immunostaining analysis showed that osteoprotegerin localized to endothelial cells in gingival tissues from patients without periodontitis. In addition, the levels of osteoprotegerin were significantly lower in gingival tissues from patients with severe chronic periodontitis (Crotti *et al.*, 2003). These results indicate that endothelial cells are the main source of osteoprotegerin in gingival tissue, and that the production of osteoprotegerin in endothelial cells is downregulated in periodontitis tissue. These findings suggest that although osteoprotegerin released from endothelial cells contributes to cell survival at an early stage of infection, gingipains continuously degrade osteoprotegerin, allowing *P. gingivalis* to gradually introduce dysfunction in endothelial cells because the host is no longer able to produce sufficient levels of osteoprotegerin in periodontitis tissue. Additional studies are needed to confirm how gingipains interact with osteoprotegerin to regulate endothelial cell function *in vivo*.

In the current study, we demonstrated that Kgp and Rgps degrade osteoprotegerin. However, it remains to be determined whether both gingipains induce endothelial cell apoptosis, and whether osteoprotegerin pretreatment interferes with Kgp- or Rgp-induced cell death by the same mechanism. Activated gingipain extracts from RGP- and KGP-deficient mutants would be a suitable experiment to elucidate this point.

Osteoprotegerin is one of the main regulatory molecules of bone metabolism, and appears to also play a role in vascular function. As such, degradation of osteoprotegerin by gingipains could strongly affect periodontitis progression. The results of our current study indicate that osteoprotegerin is also a potent endothelial cell survival factor. Therefore, degradation of osteoprotegerin may also accelerate detachment-induced endothelial cell apoptosis. Apoptosis induced by loss of cell adhesion is linked to angiogenesis, inflammation, and vascular remodeling, and

is also involved in pathological remodeling of cardiovascular tissues (Michel, 2003). Recent evidence from epidemiological studies suggests that there is a link between periodontal infection and an increased risk of atherosclerosis (Beck *et al*., 1996; Chiu, 1999). Thus, gingipains may contribute to the pathogenesis of cardiovascular diseases by mediating osteoprotegerin degradation, and subsequent endothelial cell apoptosis.

In conclusion, we have presented evidence that osteoprotegerin protects endothelial cells from gingipain-induced apoptosis, and that osteoprotegerin is at least partly degraded by both Rgps and Kgp. Our current findings, taken together with those of previous studies, indicate that during periodontitis progression, osteoprotegerin functions as a potential regulator of vascular homeostasis, in addition to its role in bone remodeling.

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