The Hemoglobin Receptor Protein of *Porphyromonas gingivalis* Inhibits Receptor Activator NF- κ B Ligand-Induced Osteoclastogenesis from Bone Marrow Macrophages

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Extracellular proteinaceous factors of *Porphyromonas gingivalis*, a periodontal pathogen, that influence receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL)-induced osteoclastogenesis from bone marrow macrophages were investigated. The culture supernatant of *P. gingivalis* had the ability to inhibit RANKL-induced in vitro osteoclastogenesis. A major protein of the culture supernatant, hemoglobin receptor protein (HbR), suppressed RANKL-induced osteoclastogenesis in a dose-dependent fashion. HbR markedly inhibited RANKL-induced osteoclastogenesis when present in the culture for the first 24 h after addition of RANKL, whereas no significant inhibition was observed when HbR was added after 24 h or later, implying that HbR might interfere with only the initial stage of RANKL-mediated differentiation. HbR tightly bound to bone marrow macrophages and had the ability to induce phosphorylation of ERK, p38, NF- κ B, and Akt. RANKL-induced phosphorylation of ERK, p38, and NF- κ B was not suppressed by HbR, but that of Akt was markedly suppressed. HbR inhibited RANKL-mediated induction of c-Fos and NFATc1. HbR could induce beta interferon (IFN- β) from bone marrow macrophages, but the induction level of IFN- β might not be sufficient to suppress RANKL-mediated osteoclastogenesis, implying presence of an IFN- β -independent pathway in HbR-mediated inhibition of osteoclastogenesis. Since rapid and extensive destruction of the alveolar bone causes tooth loss, resulting in loss of the gingival crevice that is an anatomical niche for periodontal pathogens such as *P. gingivalis*, the suppressive effect of HbR on osteoclastogenesis may help the microorganism exist long in the niche.

Periodontal diseases are chronic inflammatory conditions of supporting tissues of teeth affecting the majority of adult human population (32, 34). Alveolar bone resorption is a major clinical characteristic of periodontitis including chronic adult periodontitis (44). Osteoclasts, which are responsible for bone resorption, are derived from hematopoietic stem cells. Two molecules, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL), are essential for differentiation to osteoclasts (18, 20, 48, 55). M-CSF, which is indispensable for macrophage maturation, binds to its receptor in early osteoclast precursors, thereby providing signals required for their survival, proliferation, and differentiation to osteoclasts. RANKL, belonging to the tumor necrosis factor alpha (TNF- α) family, binds to its receptor, receptor activator of NF- κ B (RANK), and activates several intracellular signaling pathways, leading to osteoclastic differentiation and activation.

Evidence has revealed that the initiation and progression of periodontitis are closely associated with quantitative and qualitative changes of microorganisms in the subgingival biofilm. Among them, the gram-negative anaerobic bacterium *Porphy-*

romonas gingivalis has been implicated to be one of the major etiological agents of chronic adult periodontitis. *P. gingivalis* colonizes in periodontal pockets and spreads into deeper tissues, including connective and bone tissues (11, 14, 21, 22, 25, 40, 47). The bacterium possesses a number of virulence factors, such as fimbriae, lipopolysaccharide (LPS), and cysteine proteinases named gingipains (11, 21). In general, LPS displays multiple biological and immunological activation through Toll-like receptors (TLRs) and also has been reported to potently stimulate bone resorption in both in vitro and in vivo studies (7, 10, 35, 43); however, *P. gingivalis* LPS has a weak ability to activate inflammation (38, 57). Fimbriae mediate bacterial adhesion to human epithelial cells and have the ability to induce proinflammatory responses through TLRs (1, 9). Gingipains, which include arginine-specific gingipains (Arg-gingipain-A [RgpA] and Arg-gingipain-B [RgpB]) and lysine-specific gingipain (Lys-gingipain [Kgp]), degrade a number of proteins including extracellular matrix proteins, cytokines, complements, antibodies, and proteinase inhibitors (4, 15, 37). In a previous study, it was found that infection with viable *P. gingivalis* wild-type strain induced RANKL expression in osteoblasts, whereas infection with an Rgp/Kgp-null (*rgpA rgpB kgp*) mutant did not (33).

In the present study, we investigated effects of *P. gingivalis* on osteoclast differentiation and find that the culture supernatant of *P. gingivalis* inhibited RANKL-induced in vitro osteo-

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clast formation from bone marrow macrophages and that the hemoglobin receptor protein (HbR), one of the major components of the culture supernatant, contributes to the inhibition.

MATERIALS AND METHODS

Animals and reagents. Five-week-old male ddY mice (17, 19, 28, 42) were obtained from Japan SLC, Inc. (Shizuoka, Japan). All animal experiments were done in accordance with the 1989 Guidelines for Animal Experimentation Nagasaki University. Alpha minimal essential medium, L-glutamine, and penicillin-streptomycin solution, fast red violet LB salt, naphthol AS-MX phosphoric acid, and anti- β -actin antibody were purchased from Sigma (St. Louis, MO). Fetal bovine serum was from Sanko Junyaku Co., Ltd. (Tokyo, Japan). Recombinant human M-CSF was from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). *N,N*-Dimethyl formamide, sodium (+)-tartrate dehydrate, Triton X-114, and recombinant murine soluble RANKL were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-p38, anti-phospho-p38 (Thr¹⁸⁰/Tyr¹⁸²), anti-phospho-NF- κ B p65 (Ser⁵³⁶), anti-Akt1/2/3, and anti-phospho-Akt (Ser⁴⁷³) antibodies were from Cell Signaling Technology (Beverly, MA). Anti-ERK2, anti-phospho ERK1/2 (Tyr²⁰⁴), anti-NFATc1, and anti-TRAF6 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-c-Fos antibody was from Oncogene Research Products (Cambridge, MA). Re-Blot Plus Strong Ab Stripping Solution and rabbit anti-mouse beta interferon (IFN- β) antibody were from CHEMICON International, Inc. (Temecula, CA). Mouse IFN- β was from PBL Biomedical Laboratories (Piscataway, NJ).

Preparation of protein extracts of *P. gingivalis* culture supernatants. *P. gingivalis* strain ATCC 33277 was grown anaerobically (10% CO₂, 10% H₂, 80% N₂) in enriched brain heart infusion broth (30) for 7 days. A supernatant was withdrawn from the culture by centrifugation at 10,000 $\times$ g for 30 min. Ammonium sulfate was added to this at 70% saturation. After centrifugation at 10,000 $\times$ g for 20 min, the precipitate was dissolved in phosphate-buffered saline (PBS) and dialyzed against PBS. LPS was removed from the sample by phase separation with Triton X-114 as previously described (24). The protein extracts were used for experiments for in vitro osteoclastogenesis at the concentrations of 0.1, 1, and 10 μ g/ml.

Two-dimensional gel electrophoresis. A protein sample (20 μ g, 25 μ l) was mixed with 75 μ l of the sample buffer (40 mM Tris-HCl [pH 8.0], 9.5 M urea, 1% NP-40, 5% β -mercaptoethanol). Proteins were separated by isoelectric focusing in an 11-cm Immobiline DryStrip (Amersham Biosciences, Piscataway, NJ) in the pH range from 4.0 to 7.0 and then by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in an 8 to 18% ExcelGel SDS gradient gel (Amersham Biosciences) using the Multiphor II system (Amersham Biosciences) according to the manufacturer's instructions. The gel was stained with Coomassie brilliant blue.

Purification of HbR from HbR-overexpressing *Escherichia coli*. Recombinant HbR was purified essentially according to the method of Nakayama et al. (31). Briefly, *E. coli* BL21(DE3) harboring pKD349 and pLysS was grown to an optical density (540 nm) of 0.5. HbR was induced with addition of IPTG (isopropyl- β -D-thiogalactopyranoside; 1 mM). After cell lysis by sonication, ammonium sulfate was added to the cell lysate to give 35% saturation. The precipitated proteins were dialyzed and applied to a column (1.6 by 25 cm) of DEAE-Sepharose (Sepharose CL-6B; Pharmacia, Uppsala, Sweden). Proteins were eluted with a 0 to 1 M linear NaCl gradient. The fraction (400 mM NaCl) showing the prominent protein peak was used in the present study. Purity of HbR in the fraction was ca. 98% as determined by SDS-PAGE. Removal of LPS was done as described above.

In vitro osteoclastogenesis. Bone marrow cells obtained from tibias and femurs of 5-week-old mice were suspended in alpha minimal essential medium containing 10% heat-inactivated fetal bovine serum with 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μ g of streptomycin/ml (culture medium) and then incubated in culture medium supplemented with M-CSF (100 ng/ml) at 10⁷ cells per 10 ml in a 10-cm culture dish. After 3 days of culture, cells were washed with culture medium to remove nonadherent cells. Adherent cells were then harvested by mild flushing with culture medium supplemented with M-CSF using a pipette and seeded at 10⁶ cells per 10 ml in a 10-cm dish. After an additional 3-day culture, adherent cells were obtained by the same procedure as described above. We used these cells as bone marrow macrophages in the present study. For in vitro osteoclastogenesis, bone marrow macrophages (10⁴ cells) were cultured in 200 μ l of culture medium supplemented with M-CSF (50 ng/ml) and RANKL (50 ng/ml) using a 96-well plate. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. M-CSF and RANKL were used at 50 ng/ml in all of the experiments of the present study.

TRAP staining. Osteoclast formation was evaluated by tartrate-resistant acid phosphatase (TRAP) staining since TRAP was one of marker enzymes for osteoclasts. Cultured cells were fixed with 4% paraformaldehyde for 30 min and then 0.2% Triton X-100 for 5 min at room temperature and were incubated in acetate buffer (pH 5.0) containing fast red violet LB salt, naphthol AS-MX phosphate, and 50 mM sodium tartrate.

Measurement of TRAP intensity. Following TRAP staining, the plates were scanned by a transparent light scanner, and the red color image was extracted from the scanned image by using PhotoShop (Adobe Systems, Inc., San Jose, CA). The intensity of the red color image was measured by using National Institutes of Health image analysis and is represented here as TRAP intensity.

Cell viability assay. Cell viability was determined by using the Cell Counting Kit (Dojindo Laboratories) according to the manufacturer's instructions. This kit, similar to the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide assay, measures intracellular mitochondrial dehydrogenase activity in living but not in dead cells by forming water-soluble formazan dye with the tetrazolium compound, WST-1, a sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate.

Immunoblotting. Bone marrow macrophages were stimulated with M-CSF, RANKL, and/or HbR and incubated for the indicated time. After the incubation, the cells in the 24-well plate were rinsed twice with ice-cold PBS containing 2 mM sodium orthovanadate, followed by addition of 50 μ l of SDS-sample buffer containing 2 mM sodium orthovanadate–100 mM dithiothreitol. The whole-cell lysate was then subjected to sonication (15-s pulse twice with 60-s interval; Bioruptor UDC-200T; Cosmo Bio, Tokyo, Japan) in ice-cold water. After boiling for 5 min, 5 μ l of the lysate (20 μ g of protein) was applied to SDS-PAGE (10% gel), and the proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated in 5% skim milk, 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 (TBST) at room temperature for 1 h and then incubated with an antibody at a dilution of 1:200 to 1:10,000 in TBST at room temperature for 1 h. After being washed five times with TBST for 10 min, the membrane was incubated with a horseradish peroxidase-conjugated second antibody (Dako, Glostrup, Denmark) at a dilution of 1:1,000 to 1:10,000 in TBST at room temperature for 1 h. The membrane was washed five times with TBST for 10 min. Signals were detected by using an ECL plus kit (Amersham Biosciences). In some experiments for probing the membrane was treated with stripping solution at room temperature for 15 min, followed by three washes with TBST.

Immunofluorescence microscopy. Bone marrow macrophages were cultured with M-CSF and RANKL in the presence or absence of HbR for 24 h. The cells were fixed with 4% paraformaldehyde, blocked with 5% skim milk in PBS at room temperature for 1 h, and then incubated with anti-HbR antibody (31) at a dilution of 1:500 in 1% skim milk in PBS at room temperature for 1 h. After being washed five times with 1% skim milk in PBS for 10 min, the cells were incubated with Alexa 488-conjugated anti-rabbit immunoglobulin G antibody (Molecular Probes, Inc., Eugene, OR) at a dilution of 1:500 in 1% skim milk in PBS at room temperature for 1 h. After being washed five times with 1% skim milk in PBS for 10 min, the cells were subjected to fluorescence microscopy (Axiovert 200M; Zeiss, Göttingen, Germany).

Quantification of IFN- β in supernatants of bone marrow macrophage cultures. Bone marrow macrophages were cultured for 24 or 48 h in the culture medium with M-CSF alone or with either RANKL alone, HbR (10 μ g/ml) alone, or combinations of RANKL and HbR. IFN- β in the culture supernatants was quantified by using a mouse IFN- β enzyme-linked immunosorbent assay (ELISA) kit (PBL Biomedical), according to the manufacturer's protocol.

Statistical analysis. Statistical analysis of the data was performed by using the Student *t* test.

RESULTS

Inhibitory effect of the culture supernatant of *P. gingivalis* on RANKL-induced in vitro osteoclastogenesis from bone marrow macrophages. We examined the effect of the culture supernatant of *P. gingivalis* on RANKL-induced in vitro osteoclastogenesis from bone marrow macrophages (Fig. 1). TRAP-positive multinuclear cells appeared after culturing bone marrow macrophages in the presence of 50 ng of RANKL and 50 ng of M-CSF/ml for 5 days, whereas no TRAP-positive cells were detected in the culture in the presence of M-CSF alone. When the *P. gingivalis* culture supernatant was added to the cell culture in

FIG. 1. Inhibitory effect of *P. gingivalis* culture supernatant on RANKL-induced osteoclastogenesis from bone marrow macrophages. Bone marrow macrophages (10^4 cells) were incubated in the culture medium containing M-CSF and RANKL with or without *P. gingivalis* culture supernatants (0.1, 1, and 10 µg/ml) or LPS (1 ng/ml) in the presence or absence of polymyxin B (0.1 µg/ml) for 5 days. Cells were fixed and stained for TRAP, and the TRAP-positive multinuclear cells were counted. The results are from one experiment and are representative of three separate experiments. *, $P < 0.05$; **, $P < 0.01$ (compared to the experiment with RANKL and M-CSF).

the presence of RANKL and M-CSF, TRAP-positive multinuclear cells were markedly decreased, indicating an inhibitory effect of the culture supernatant on osteoclast formation. LPS in the *P. gingivalis* culture supernatant was less than 10 pg/µg protein. Polymyxin B (100 ng/ml), which completely canceled LPS (1 ng/ml)-mediated inhibition of osteoclast formation, failed to cancel inhibition of osteoclast formation by the culture supernatant (Fig. 1). The culture supernatant contained a few proteins as revealed by two-dimensional gel electrophoresis (Fig. 2). A major protein was identified as the HbR by immunoblot analysis with anti-HbR antibody (data not shown).

HbR-mediated inhibition of RANKL-induced osteoclast formation from bone marrow macrophages. HbR was purified from *Escherichia coli* overexpressing the intragenic HbR domain region as previously described (Fig. 3A) (31). Purity of HbR was more than 98% and LPS contamination was less than 10 pg/µg protein. To examine the effect of HbR on RANKL-induced osteoclast formation from bone marrow macrophages, various concentrations of HbR were added to the culture of bone marrow macrophages with RANKL and M-CSF (Fig. 3B). HbR markedly inhibited formation of TRAP-positive multinuclear cells in a dose-dependent fashion (Fig. 3C). TRAP intensity was also decreased in a dose-dependent fashion (Fig. 3D). These results indicated that HbR had an inhibitory effect on RANKL-induced osteoclast formation from bone marrow macrophages.

HbR-mediated inhibition of osteoclast formation is not caused by cell death. To determine whether HbR-mediated inhibition of RANKL-induced osteoclast formation was caused

FIG. 2. Two-dimensional gel analysis of *P. gingivalis* culture supernatant. Crude protein extracts of the supernatant of *P. gingivalis* 33277 culture for 7 days were subjected to two-dimensional gel analysis. The arrows indicate HbR.

by cell death, we determined viability of bone marrow macrophages that had been treated with various concentrations of HbR in the presence of RANKL and M-CSF for 5 days (Fig. 3E). None of the concentrations of HbR in the range of 0.01 to 10 µg/ml caused decrease of cell viability, or rather they caused increase of cell viability, demonstrating that HbR-mediated inhibition of RANKL-induced osteoclast formation was not due to cell death.

HbR-mediated inhibition in the initial stage of osteoclast differentiation. We examined whether HbR affected RANKL-induced osteoclastogenesis when it was present in bone marrow macrophage culture at different time periods after the addition of RANKL (Fig. 4). When HbR was present for 24 h or longer from the start of the culture, osteoclastogenesis was markedly suppressed. In contrast, no significant inhibition was observed when HbR was added 24 h or later after the start of the culture. These results indicated that HbR affected osteoclast precursors in the initial stage of differentiation.

Adherence of HbR to the cell surface of bone marrow macrophages. To determine whether HbR binds to bone marrow macrophages, bone marrow macrophages were mixed with HbR, washed and subjected to immunostaining with anti-HbR and fluorescence microscopy (Fig. 5). Morphological change of the bone marrow macrophages was not observed 24 h after the start of incubation with HbR, but fluorescence was observed on the cell surface, indicating that HbR had the ability to bind to the surface of bone marrow macrophages.

Effects of HbR on known signaling pathways induced by RANKL. The key osteoclastogenic cytokine RANKL, upon binding to its unique receptor, RANK, activates three major intracellular signals. Major signaling pathways induced through RANKL/RANK binding are the mitogen-activated protein kinase member pathways, the NF-κB pathway, and the phosphatidylinositol 3-kinase/Akt pathway (2, 8, 50). To determine the effect of HbR on RANKL-mediated signal transduction, we examined the phosphorylation of ERK, p38, NF-κB, and Akt of bone marrow macrophages treated with RANKL in the presence or absence of HbR (Fig. 6A). Phosphorylation of ERK, p38, NF-κB, and Akt was observed within 30 min after addition of

FIG. 3. Inhibitory effect of HbR on RANKL-induced osteoclastogenesis from bone marrow macrophages and no inhibitory effect of HbR on the viability of bone marrow macrophages. (A) HbR was purified from HbR-overexpressing *E. coli* by DEAE-Sepharose column chromatography. Lanes: 1, before IPTG induction; 2, after IPTG induction; 3, precipitates with ammonium sulfate; 4, after DEAE-Sepharose. (B to E) Bone marrow macrophages were incubated in the culture medium with M-CSF and RANKL in the presence of the indicated concentrations of HbR for 5 days. Cells were stained for TRAP (B), the numbers of TRAP-positive multinuclear cells were counted (C), and the TRAP intensity was determined (D). Cell viability was determined by using a cell counting kit (E). The results are from one experiment and are representative of three separate experiments. **, $P < 0.01$ (compared to the experiment with RANKL and M-CSF).

RANKL in the absence of HbR. Interestingly, HbR itself had the ability to activate ERK, p38, NF- κ B, and Akt. Treatment with both HbR and RANKL failed to activate Akt within 30 min, whereas it activated ERK, p38, and NF- κ B earlier and more strongly than did treatment with HbR or RANKL alone. These results suggested that HbR might interfere with the phosphatidylinositol 3-kinase/Akt pathway or that it might overactivate ERK, p38 and NF- κ B pathways, leading to the inhibition of RANKL-induced osteoclastogenesis.

Expression of c-Fos and NFATc1 was induced 8 to 24 h and 1 to 2 days after addition of RANKL, respectively (Fig. 6B). The induction of these proteins was completely suppressed by HbR. On the other hand, the expression of TRAF6 of bone

marrow macrophages cultured with M-CSF and RANKL was not affected by HbR.

Induction of IFN- β from bone marrow macrophages by treatment with HbR. It is known that IFN- β has the potential to inhibit osteoclast formation by suppressing induction of c-Fos (51). We examined whether IFN- β was a candidate for the putative humoral factor for HbR-mediated inhibition of osteoclastogenesis. Bone marrow macrophages stimulated with RANKL for 24 or 48 h were found not to produce IFN- β , whereas bone marrow macrophages stimulated with HbR in the presence or absence of RANKL for 24 and 48 h were found to produce IFN- β at the concentration of ca. 0.5 U/ml (20 pg/ml) (Fig. 7A). To examine what concentration of IFN- β

FIG. 4. HbR-mediated inhibition in the initial stage of osteoclast differentiation. Bone marrow macrophages were incubated in the culture medium with M-CSF, RANKL, and HbR (10 μ g/ml). After 1, 2, 3, or 4 days of incubation, the culture medium was changed to that with M-CSF and RANKL to remove HbR. On the other hand, bone marrow macrophages were incubated in the culture medium with M-CSF and RANKL. After 1, 2, 3, or 4 days of incubation, HbR (10 μ g/ml) was added to the culture, and the incubation was continued for 4 days, 3 days, 2 days, or 1 day, respectively. Cells were stained for TRAP (A), the numbers of TRAP-positive multinuclear cells were counted (B), and the TRAP intensity was determined (C). The results are from one experiment and are representative of three separate experiments.

can inhibit RANKL-induced osteoclast formation from bone marrow macrophages, various concentrations of recombinant mouse IFN- β were added to the culture of bone marrow macrophages with M-CSF and RANKL (Fig. 7B). IFN- β partially and completely inhibited osteoclast formation from bone marrow macrophages at 10 and 100 U/ml, respectively. Next, a

neutralizing anti-IFN- β antibody was added to the culture medium simultaneously with IFN- β . IFN- β (100 U/ml)-mediated inhibition of osteoclast formation was completely suppressed by anti-IFN- β antibody (1,000 U/ml). Anti-IFN- β antibody (1,000 U/ml), however, showed no effect on HbR-mediated inhibition of osteoclast formation (Fig. 7B). These results indicated that HbR-mediated inhibition of osteoclast formation might not be caused by IFN- β that was induced by HbR.

DISCUSSION

In previous studies of ours and other researchers, HbR was found to have the ability to bind hemoglobin and act as a high-affinity hemophore at the *P. gingivalis* cell surface to capture porphyrin from hemoglobin (6, 31, 36). HbR, also called Hgp15 or HA2, is intragenically encoded by *rgpA*, *kpg*, and *hagA* (4, 15, 37). Hemagglutinin domain proteins such as Hgp44 are also encoded by these genes, suggesting the putative mechanism of acquisition of heme from erythrocytes (31). HbR is located at the surface of *P. gingivalis* cells with Rgp, Kgp, and hemagglutinin domain proteins and also released from the bacterial cells by secretion and autolysis as one of the major extracellular proteins in prolonged incubation (16, 46), suggesting that released HbR may influence function and differentiation of host cells. DeCarlo et al. (5) reported that anti-HbR antibodies are found in the sera of patients with periodontal diseases, and the serum antibody titers specific for the HbR domain vary inversely with periodontal disease severity, suggesting that HbR produced by *P. gingivalis* cells is ex-

FIG. 5. Fluorescence microscopy. Bone marrow macrophages were treated with (B and D) or without (A and C) HbR (10 μ g/ml) for 24 h. HbR was then visualized by immunostaining with anti-HbR and Alexa 488-conjugated anti-rabbit IgG antibodies. Cells were observed by light microscopy (A and B) and fluorescence microscopy (C and D).

FIG. 6. Effects of HbR on RANKL-induced signaling. (A) Bone marrow macrophages were treated with RANKL alone, HbR (10 μ g/ml) alone, or RANKL plus HbR for the indicated times. Proteins in the cells were separated by SDS-PAGE, transferred to PVDF membranes, and then immunostained with anti-phospho-ERK1/2 (Tyr²⁰⁴), anti-ERK2, anti-phospho-p38 (Thr¹⁸⁰/Tyr¹⁸²), anti-p38, anti-phospho-NF- κ B p65 (Ser⁵³⁶), anti- β -actin, anti-phospho-Akt (Ser⁴⁷³), and anti-Akt1/2/3. (B) Bone marrow macrophages were incubated in the culture medium with M-CSF alone, M-CSF plus RANKL, or a combination of M-CSF, RANKL, and HbR (10 μ g/ml) for the indicated times. Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and then immunostained with anti-NFATc1, anti-TRAF6, anti-c-Fos, and anti- β -actin.

posed to host immune systems. In the present study, we found that HbR had the ability to tightly bind to the surface of bone marrow macrophages and induce the phosphorylation of ERK, p38, NF- κ B, and Akt, suggesting that the binding of HbR to the cell surface elicited signal transduction. The activation of NF- κ B and mitogen-activated protein kinases such as ERK and p38 is known to be associated with upregulation of pro-inflammatory cytokine gene expression. In this context, Sharp et al. (45) reported that lipid A-associated proteins of *P. gingivalis* have the ability to induce interleukin-6 (IL-6) in human monocytes and that the active component in the lipid A-associated proteins is HbR. These findings indicate that HbR may induce various cytokines from bone marrow macrophages.

Previous studies have shown that *P. gingivalis* infection stimulates RANKL expression of osteoblasts and mononuclear leu-

FIG. 7. HbR-mediated induction of IFN- β and the effect of anti-IFN- β antibody on HbR- or IFN- β -mediated inhibition of osteoclastogenesis. (A) HbR-mediated induction of IFN- β . Bone marrow macrophages were incubated in the culture medium with M-CSF alone, M-CSF plus RANKL, M-CSF plus HbR (10 μ g/ml), or a combination of M-CSF, RANKL, and HbR for 24 or 48 h. The amounts of IFN- β in culture supernatants were determined. (B) Effect of anti-IFN- β antibody on HbR- or IFN- β -mediated inhibition of osteoclastogenesis. Bone marrow macrophages were incubated in the culture medium with M-CSF, RANKL, and anti-IFN- β antibody (0, 10, 100, or 1,000 U/ml) in the presence of IFN- β (0, 1, 10, or 100 U/ml) or HbR (10 μ g/ml) for 5 days. The TRAP-positive multinuclear cells were counted. " α IFN- β " indicates anti-IFN- β antibody. The results are from one experiment and are representative of three separate experiments. **, $P < 0.01$ (compared to the experiment with RANKL and M-CSF).

kocytes, leading to osteoclastogenesis (33). However, Choi et al. (3) found that in case of *P. gingivalis*, although its ability to induce RANKL expression and suppress osteoprotegerin expression is stronger than those abilities of other periodontal pathogens, *Treponema denticola* and *Treponema socranskii*, its ability to induce osteoclastogenesis is not stronger than those of *T. denticola* and *T. socranskii*, and these authors suggested

that *P. gingivalis* might have a component to inhibit osteoclast formation and/or it may induce inhibitory cytokines of osteoclast formation. In the present study, we found that HbR, one of the major components of the *P. gingivalis* culture supernatant, had the ability to inhibit RANKL-induced in vitro osteoclast formation from bone marrow macrophages. Since LPS has the ability to inhibit RANKL-induced osteoclastogenesis from bone marrow macrophages (49, 58), it is notable that the amount of LPS in the HbR sample used in the present study was too small to inhibit osteoclast formation and that polymyxin B failed to suppress HbR-mediated inhibition of osteoclastogenesis (data not shown).

HbR suppressed RANKL-mediated induction of c-Fos and NFATc1 expression. The necessity of c-Fos in osteoclast differentiation is revealed by the severe osteopetrotic phenotype of c-Fos-deficient (*Fos*^{-/-}) mice (13, 54). Recently, Matsuo et al. (26) found that the expression of NFATc1 is induced in *Fos*^{+/-} osteoclast precursor cells but not in *Fos*^{-/-} cells during osteoclast differentiation and that c-Fos directly regulates the promoter of the *NFATc1* gene of osteoclast precursor cells. Therefore, suppression of the NFATc1 expression by HbR can be explained by the inhibition of c-Fos induction. The observation that RANKL-mediated induction of c-Fos precedes the induction of NFATc1 may be consistent with that idea. Takayanagi et al. (51) found that IFN- β , which is transcriptionally induced by c-Fos, negatively regulates osteoclastogenesis by downregulating c-Fos at the protein level. In the present study, we did not find RANKL-mediated induction of IFN- β , whereas IFN- β was induced by HbR in both the presence and the absence of RANKL. However, the concentration of the induced IFN- β appeared not to be sufficient to suppress osteoclastogenesis, as revealed by ELISA analysis. Further, anti-IFN- β , the concentration of which effectively suppressed the inhibitory effect of IFN- β on osteoclastogenesis, failed to suppress the inhibitory effect of HbR on osteoclastogenesis. These findings suggest that HbR-mediated induction of IFN- β may not account for the inhibitory effect of HbR on osteoclastogenesis. Since HbR treatment is likely to elicit the production of various cytokines, cytokines other than IFN- β may be involved in the inhibitory effect of HbR on osteoclastogenesis. IL-4, IL-7, IL-10, IL-12, IL-18, IFN- γ , IFN- β , 4-1BB, and granulocyte-macrophage colony-stimulating factor have been found to have the potential to inhibit osteoclast formation (12, 23, 27, 29, 39, 41, 51–53). Alternatively, signaling induced by HbR may interfere with RANKL-mediated c-Fos induction since the signaling appears to suppress Akt phosphorylation that takes place 10 to 15 min after the addition of RANKL. Interestingly, HbR alone induced Akt phosphorylation after 30 min. In this context, *P. gingivalis* cells were found to induce Akt phosphorylation during a 24-h infection of primary gingival epithelial cells, contributing to the survival of the infected cells (56). Further experiments are needed to elucidate the mechanism of HbR-mediated inhibition of RANKL-induced in vitro osteoclast formation from bone marrow macrophages.

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