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Phosphoglycerol dihydroceramide, a distinctive ceramide produced by *Porphyromonas gingivalis*, promotes RANKL-induced osteoclastogenesis by acting on non-muscle myosin II-A (Myh9), an osteoclast cell fusion regulatory factor

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Abstract

Among several virulence factors produced by the periodontal pathogen *Porphyromonas gingivalis* (*Pg*), a recently identified novel class of dihydroceramide lipids that contains a long acyl-chain has the potential to play a pathogenic role in periodontitis because of its higher level of tissue penetration compared to other lipid classes produced by *Pg*. However, the possible impact of *Pg*

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Conflicts of Interest

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ceramides on osteoclastogenesis is largely unknown. In the present study, we report that the phosphoglycerol dihydroceramide (PGDHC) isolated from *Pg* enhanced osteoclastogenesis *in vitro* and *in vivo*. Using RAW264.7 cells, *in vitro* assays indicated that PGDHC can promote RANKL-induced osteoclastogenesis by generating remarkably larger TRAP⁺ multinuclear osteoclasts compared to *Pg* LPS in a TLR2/4-independent manner. According to fluorescent confocal microscopy, co-localization of non-muscle myosin II-A (Myh9) and PGDHC was observed in the cytoplasm of osteoclasts, indicating the membrane-permeability of PGDHC. Loss- and gain-of-function assays using RNAi-based Myh9 gene silencing, as well as overexpression of the Myh9 gene, in RAW264.7 cells showed that interaction of PGDHC with Myh9 enhances RANKL-induced osteoclastogenesis. It was also demonstrated that PGDHC can upregulate the expression of dendritic cell-specific transmembrane protein (DC-STAMP), an important osteoclast fusogen, through signaling that involves Rac1, suggesting that interaction of PGDHC with Myh9 can elicit the cell signal that promotes osteoclast cell fusion. Taken together, our data indicated that PGDHC is a *Pg*-derived, cell-permeable ceramide that possesses a unique property of promoting osteoclastogenesis via interaction with Myh9 which, in turn, activates a Rac1/DC-STAMP pathway for upregulation of osteoclast cell fusion.

Keywords

RANKL-induced osteoclastogenesis; Non-muscle myosin II-A; Phosphoglycerol dihydroceramide; Rac1 small GTPase; DC-STAMP; *Porphyromonas gingivalis*

1. Introduction

Our understanding of the molecular mechanisms underlying pathogenic bone resorption induced in periodontal diseases (PD), a polymicrobial infectious disease [1], has been advanced in the last decade by the finding that RANKL produced by host immune cells and fibroblasts in the periodontitis lesion is prominently engaged in osteoclastogenesis [2–6]. In the periodontitis lesion, it appears likely that both osteoclast differentiation and resorption of bone are pathogenically promoted by the effects of factors produced by bacteria in the dental plaque. Nonetheless, the bacteria-derived molecule(s) that cause(s) such dysregulation remain(s) elusive.

Among the >500 species of microorganisms that exist in the oral cavity, *Porphyromonas gingivalis* (*Pg*) plays a critical role in promoting alveolar bone resorption in the course of PD progression [7–9]. Dysbiosis at the periodontal site is known to result from *P. gingivalis* interference with host immune response [10,11]. Lipopolysaccharides are found in the outer membrane of Gram-negative bacteria and elicit strong immune responses, but LPS produced by *P. gingivalis* is structurally distinct from other Gram-negative oral bacteria and elicits unique host innate immune and inflammatory responses [12]. *P. gingivalis* LPS promotes inflammatory response via its ligation with both Toll-like receptor-4 (TLR4) and TLR2 [12], while an altered form of Lipid A in *P. gingivalis* LPS appears to interrupt TLR4 activation [13]. Nonetheless, it was reported that LPS derived from *P. gingivalis* can hardly be detected in the diseased periodontal tissues of humans [14,15]. Generally, in contrast to cell-permeable ceramides that contain a short acyl chain (C 8), ceramides with a long acyl

chain ($C > 8$) as well as dihydroceramides with all different lengths of side chains do not penetrate into cells [16–19]. However, a complex of sphingolipids isolated from *P. gingivalis*, including phosphoethanolamine dihydroceramide (PEDHC) and phosphoglycerol dihydroceramide (PGDHC), was identified in relatively large amounts in inflamed human periodontal tissues [20], irrespective of having long acyl chains ($C > 16$). Furthermore, it was reported that PGDHC promotes IL-1 β -mediated release of PGE2 from primary cultures of gingival fibroblasts [21] and causes apoptosis in chondrocytes [22], suggesting that PGDHC may upregulate inflammation and interfere with new bone formation. Nevertheless, to the best of our knowledge, no study has ever addressed the effects of PGDHC on bone resorption processes mediated by osteoclastogenesis.

It has been demonstrated that TLR2 and TLR4 are associated with periodontal bone loss caused in a mouse model of *P. gingivalis*-induced periodontitis [23–25]. While LPS produced by *P. gingivalis* can promote osteoclastogenesis through binding to TLR4 [26], recent studies revealed that serine dipeptide lipids produced by *P. gingivalis* can act on TLR2 which, in turn, inhibits osteoblastogenesis [27,28]. On the other hand, lipid A derived from *P. gingivalis* is contaminated with phosphorylated dihydroceramide lipids which can also stimulate TLR2 [29]. These lines of evidence suggest that *P. gingivalis* can release both TLR2- and TLR4-ligands that can affect bone remodeling processes. Therefore, in anticipation that PGDHC might also react with TLR2/4, TLR2/4 double knockout (DKO) mice were employed in this study to determine if the effects of PGDHC on osteoclastogenesis are TLR2/4-dependent or -independent. Contrary to our expectation, results from the osteoclastogenesis assay using bone marrow cells isolated from TLR2/4 DKO mice showed that PGDHC can promote RANKL-mediated osteoclastogenesis in a manner independent of TLR2/4. Interestingly, instead of binding to TLR2/4 expressed on the cell surface, PGDHC interacted with a cytoskeletal protein localized to cytoplasm. Specifically, non-muscle myosin IIA (Myh9) elicited a cell signal involving Rac1 to upregulate the expression of DC-STAMP, a key osteoclast fusogen responsible for the cell fusion process during osteoclastogenesis.

2. Material and Methods

2.1. Phosphoglycerol dihydroceramide lipids preparation

PGDHC was isolated from *Porphyromonas gingivalis* (ATTC strain #33277) as previously described [21,22]. Purity of this lipid isolate was confirmed by liquid chromatography-mass spectrometry (LC-MS) and structural verification using electrospray ionization (ESI) MS/MS. For biological experiments, PGDHC was dissolved in 70% ethanol. An equal amount of ethanol was used as a control for all studies.

2.2. Animals

TLR2/4 DKO mice, as well as their wild-type (WT) (C57BL/6 J) mice, were used in this study (6- to 8-week-old). To generate TLR2/4 DKO mice, TLR2 KO mice (B6.129-Tlr2tm1Kir/J; Jackson Laboratory) and TLR4 KO mice (a generous gift from Dr. Shizuo Akira, Osaka University, Osaka, Japan) were intercrossed. Animals were kept in conventional animal housing with a 12-h light-dark cycle at constant temperature. The

experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Forsyth Institute.

2.3. A murine calvarial injection model

To evaluate the effects of PGDHC on *in vivo* osteoclastogenesis, a mouse model of calvarial injection was utilized following a published protocol with some modifications [30]. Under anesthesia with ketamine (80 mg/kg) and xylazine (10 mg/kg), WT or TLR2/4 DKO mice (6- to 8-week-old; 5 mice/group) received a calvarial injection of the following solutions: 1) 0.1% ethanol in PBS (control); 2) 10 µg/ml of murine recombinant RANKL (rRANKL) dissolved in PBS containing 0.1% ethanol; 3) a mixture of 10 µg/ml of murine rRANKL and 10 µg/ml of PGDHC dissolved in PBS containing 0.1% ethanol. More specifically, each solution at the volume of 150 µl was injected into the site between calvarial bone and periosteum membrane. The animals were given each injection every other day for 5 days, and then mice were sacrificed at Day-10.

2.4. Histology and immunohistochemistry of calvarial tissues

Calvarial bone samples fixed in 4% formaldehyde were decalcified in 10% EDTA (Thermo Fisher Scientific) for 2 weeks at 4 °C. Subsequently, the decalcified samples were dehydrated in graded alcohol and embedded in paraffin. Frontal calvarial sections, including sagittal suture area (thickness at 6-µm), were prepared for histological analysis. To stain tartrate-resistant acid phosphatase (TRAP)-positive (TRAP+) OCs, sections were first incubated in 0.2 M acetate buffer containing 50 mM L-(+)-Tartaric acid (Sigma, St. Louis, MO) at room temperature and then in TRAP staining solution (0.2 M acetate buffer, 50 mM L-(+)-Tartaric acid, 0.5 mg/ml Naphthol AS-MX phosphate, 1.1 mg/ml Fast Red ASTR salt; Sigma) at 37 °C. In some experiments, sections were additionally stained for Myh9 non-muscle myosin using a rabbit anti-Myh9 polyclonal Ab (clone ab154509) at 1:750 (Abcam). Next, using a complex formed by horseradish peroxidase-conjugated avidin-biotin, Vectastain Elite ABC and DAB Peroxidase (HRP) substrate kits (Vector Laboratories) were used to resolve the staining according to the manufacturer's recommendation. Finally, the sections were counter-stained with either hematoxylin or 0.1% fast green solutions (Sigma) at room temperature.

2.5. Osteoclastogenesis assay using RAW264.7 cells in vitro

Murine RAW264.7 monocytes (ATCC, Rockville, MD) were cultured in a humidified incubator (5% CO₂ in air) at 37 °C and maintained on 9-cm diameter uncoated plastic dishes in α-MEM containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA). For osteoclastogenesis experiments, 1×10^3 cells were seeded on a 96-well tissue culture plate with 1 µg/ml of PGDHC, synthetic cell membrane-permeable C6 ceramide (Avanti Polar Lipids, Alabaster, AL), or non-permeable inert C6 dihydroceramide (Avanti Polar Lipids), in the presence or absence of rRANKL (50 ng/ml, ProSpec, East Brunswick, NJ). In some experiments, a specific Rac1 inhibitor, NSC23766 (1 µM; Cayman Chemical, Ann Arbor, MI), was applied. Five days later, cells were stained for TRAP using a leukocyte acid phosphatase kit (Sigma). TRAP+ cells with more than three nuclei were considered as osteoclasts. TRAP+ multinuclear cells were counted, and the results were expressed as numbers per well.

2.6. RANKL-induced osteoclastogenesis assay using primary cultures of bone marrow-derived cells and peritoneal macrophages

To obtain bone marrow-derived monocytes or peritoneal macrophages, mononuclear cells were isolated from femur and tibiae [31] or from peritoneal lavage [32], respectively, from WT or TLR 2/4 DKO mice. Then, isolated respective cells were preincubated with recombinant M-CSF (rM-CSF; 50 ng/ml) in α -MEM containing 10% of FBS for 3 days. After preincubation, the cells were incubated with rM-CSF (30 ng/ml, BioLegend) and rRANKL (50 ng/ml) in the presence or absence of PGDHC lipid (1 μ g/ml). Osteoclastogenesis was evaluated by TRAP staining as described above.

2.7. Preparation of protein lysate

Protein lysate of RAW 264.7 cells was prepared using lysis buffer containing KCl (2.7 mM), KH₂PO₄ (1.5 mM), NaCl (137 mM), NaH₂PO₄ (8.1 mM), EDTA (5 mM pH 8.0), Glycerol (10% v/w), Triton X-100 (1% v/w), SDS (0.1% w/w), and NP-40 (1% v/w). Briefly, cells were homogenized in the lysis buffer using a needle and syringe. The protein concentration of homogenates was measured with the Micro BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL). For Western blot, protein concentrations of different samples were adjusted to equal level.

2.8. Preparation of PGDHC-bound glass beads

A published protocol was used to bind PGDHC onto glass beads [33]. Briefly, glass beads were washed with benzene 3 times and resuspended in benzene. Then, PGDHC dissolved in ethanol was added to glass beads in benzene. To prepare unbound intact glass beads, the same volume of ethanol alone was added to the glass beads after they were washed with benzene 3 times. Glass beads were then incubated at 50 °C with occasional shaking overnight to evaporate benzene and ethanol, followed by washing again in PBS. PGDHC-bound and unbound glass beads were extensively washed with the protein lysis buffer mentioned above.

2.9. Immunoprecipitation of PGDHC ligand

Protein lysate of RAW 264.7 cells was pre-cleared with intact unbound glass beads 6 times. Briefly, unbound glass beads were added into protein lysate and incubated at 4 °C with shaking for 4 h. After incubation, glass beads were separated using a centrifuge tube filter (Corning, Corning, NY). Then, PGDHC-bound glass beads were added in pre-cleared cell lysate and incubated at 4 °C with shaking overnight. After incubation, PGDHC-bound glass beads were separated using a centrifuge tube filter. Proteins bound onto control unbound glass beads or PGDHC-bound glass beads were eluted with SDS sample buffer by brief boiling. The proteins present in each SDS-treated sample were separated by SDS-PAGE using the NuPAGE Novex Bis-Tris Gel system (Invitrogen, Carlsbad, CA), followed by visualization of proteins by silver staining [34].

2.10. Mass spectrometry analysis of ligand for PGDHC

Silver-stained protein bands indicating the putative ligands for PGDHC were excised from gel, followed by destaining. Mass spectrometry analysis was performed at Tufts University

Core Facility. Using mass spectrometry, the original proteins for the detected molecular fingerprints were identified by the NCBI non-redundant protein database (<ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr>).

2.11. Myh9 RNA interference

Validated siRNAs specific for mouse Myh9 were synthesized by Invitrogen (Carlsbad, CA). *Myh9*-specific siRNAs or non-silencing siRNAs were transfected into RAW 264.7 cells using Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

2.12. Overexpression of Myh9

Expression plasmids that encode human *Myh9*, CMV-GFP-NMHC II-A (Addgene plasmid 11,347) or pTRE-GFP-NMHC II-A (Addgene plasmid 10,844), and its control isoform *Myh10*, NMHC II-B (Addgene plasmid 11,348 and Addgene plasmid 10,845), were obtained from Dr. Robert Adelstein through Addgene, a nonprofit plasmid repository (<http://www.addgene.org/>) [35,36]. Expression plasmids were transfected into RAW 264.7 cells using Lipofectamine® LTX Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. In some cases, gene expression was induced by addition of Doxycycline (1 µg/ml) for the expression plasmids utilizing the Tet-on system (pTRE-GFP-NMHC II-A).

2.13. RNA extraction and Real-time PCR

RNA of RAW 264.7 cells was extracted using the E.Z.N.A. total RNA kit (Omega Bio-tek, Norcross, GA) according to the manufacturer's instructions. Altogether, 1 µg of RNA was reverse transcribed with qScript (Quanta, Gaithersburg, MD). Real-time PCR was performed in a final volume of 25 µl of Phire Hot Start II DNA Polymerase mixture (New England Biolabs, Ipswich, MA) containing EvaGreen dye (Phenix Research, Candler, NC), using an iCycler instrument (Bio-Rad Laboratories, Benicia, CA). Fold changes of the gene of interest were calculated as 2^{-Ct} using glyceraldehyde 3-phosphate dehydrogenase (GAPDH; for mouse transcripts) as a reference gene. The primer sequences for GAPDH, TRAP, matrix metalloproteinase (MMP9), DC-STAMP and Myh9 are presented in Table 1.

2.14. Western blot analysis for Myh9 knockdown and overexpression

Equal amounts of protein lysate of RAW 264.7 cells were subjected to SDS-PAGE in NuPAGE Novex Bis-Tris gel, followed by transblotting to a nitrocellulose membrane. Primary antibodies (rabbit polyclonal to anti-Myh9 [clone ab154509] or anti-β-actin [clone ab8227] Abs, both from Abcam) and an HRP-conjugated anti-rabbit IgG secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) were used following the standard protocol established in the laboratory. Small GTPases, including Rac1, RhoA and Cdc42, were identified using a Rho-GTPase antibody sampler kit (Cell Signaling Technology, Danvers, MA). Specific protein bands detected by the respective antibody were visualized by ECL reagent (Millipore, Billerica, MA).

2.15. Biotinylation of PGDHC

For biotinylation of PGDHC, vicinal hydroxyl groups in PGDHC were oxidized using sodium periodate (NaIO₃) to generate an aldehyde. After dialysis of PGDHC reacted with NaIO₃, biotinylation of aldehyde moieties in PGDHC molecule was carried out with the EZ-Link® Biotin Hydrazides kit (Thermo Scientific, Rockford, IL) according to the manufacturer's recommendations. Finally, biotinylation of PGDHC was confirmed by a colorimetric reaction using streptavidin-HRP (Roche, Indianapolis, IN) and TMB (Sigma) detection system.

2.16. Observation of intracellular colocalization of PGDHC and Myh9

RAW 264.7 cells were incubated with murine rRANKL (50 ng/ml) on Millicell® EZ slides (EMD Millipore) for 4 days. After incubation, cells were fixed with 4% paraformaldehyde in PBS, permeabilized by 1% Tri-ton X-100, and blocked by BlockAce (AbD Serotec, Raleigh, NC). Biotinylated PGDHC and/or rabbit IgG anti-Myh9 antibody were reacted with the fixed RAW264.7 cells overnight and then thoroughly washed. Streptavidin-Texas red (EMD Chemicals) and/or anti-rabbit IgG-Dylight488 (Jackson ImmunoResearch) was/were then reacted. Immunofluorescence was observed using FSX100 (Olympus America, Center Valley, PA).

2.17. Statistical Analysis

Multiple comparisons were performed by a one-way analysis of variance (ANOVA) with Tukey post hoc test. A $p < 0.05$ was considered statistically significant. All data are presented as mean $\pm$ standard deviation (SD). All data are representative of at least three independent experiments.

3. Results

3.1. PGDHC lipid upregulated RANKL-induced osteoclastogenesis in vitro and in vivo

We first evaluated the effects of PGDHC stimulation on RANKL-induced osteoclastogenesis using the RAW 264.7 cell line as osteoclast precursors (OCPs). The effects of PGDHC, in comparison to *Pg* LPS, as well as cell-permeable C6-ceramide and non-cell-permeable C6-dihydroceramide [19,37], on RANKL-induced osteoclastogenesis *in vitro* were evaluated by TRAP staining (Fig. 1A). The addition of PGDHC to RANKL-stimulated RAW 264.7 cells significantly enhanced the number of large TRAP⁺ multinucleated cells compared to that stimulated with the combination of RANKL and *Pg* LPS, C6-ceramide, and C6-dihydroceramide, or RANKL alone, *in vitro* (Fig. 1 B). While *Pg* LPS also promoted RANKL-induced osteoclastogenesis, the TRAP⁺ osteoclasts induced by RANKL in the presence of either *Pg* LPS or C6-dihydroceramide were smaller in size than those stimulated with RANKL with PGDHC or C6-ceramide (Fig. 1A). Interestingly, PGDHC, as well as C6-ceramide alone, but not *Pg* LPS or C6-dihydroceramide, induced the generation of TRAP-negative multinuclear cells (Fig. 1A). These results indicated that PGDHC promotes the cell fusion event during RANKL-induced osteoclastogenesis in a manner similar to that of cell-permeable C6-ceramide, but different from that caused by non-cell-permeable C6-dihydroceramide or *Pg* LPS.

Next we tested the effect(s) of PGDHC on the expression of mRNA for DC-STAMP, MMP-9, and TRAP, all of which play critical roles in osteoclast cell fusion (DC-STAMP) and bone resorption caused by mature osteoclasts (MMP-9 and TRAP). Addition of PGDHC to RANKL-stimulated RAW 264.7 cells significantly enhanced the expression of mRNAs for DC-STAMP, MMP-9 and TRAP (Fig. 2). Further, stimulation of RAW 264.7 cells with PGDHC alone induced the expression of mRNAs for DC-STAMP, but not MMP-9 or TRAP (Fig. 2). Since DC-STAMP plays a pivotal role in the induction of cell fusion during RANKL-induced osteoclastogenesis [38,39], elevated DC-STAMP mRNA expression by PGDHC stimulation alone appeared to support the finding from the TRAP staining assay where PGDHC alone induced the generation of TRAP-negative multinuclear cells (Fig. 1A&B).

Finally, we evaluated the effects of PGDHC on osteoclastogenesis *in vivo* using a mouse calvarial model. Local injection of a PGDHC and RANKL mixture over calvaria significantly enhanced the emergence of TRAP⁺ osteoclasts compared to calvaria that received either PGDHC or RANKL alone or control saline injection (Fig. 3 A–C), suggesting that PGDHC can promote RANKL-induced osteoclastogenesis *in vivo*. In sum, these data indicated that PGDHC isolated from *P. gingivalis* can enhance RANKL-induced osteoclastogenesis *in vitro*, as well as *in vivo*.

3.2. PGDHC promoted RANKL-induced osteoclastogenesis in a manner independent of TLR-2 or TLR-4

Since it was reported earlier that serine lipids of *Pg* (not PGDHC) inhibit osteoblast functions in a TLR2-dependent manner [40], we examined whether TLR2 signaling is engaged in PGDHC-mediated augmentation of osteoclastogenesis using macrophages isolated from TLR2/4 DKO mice.

After isolation from peritoneal exudates and bone marrow of TLR2/4 DKO mice, macrophages were stimulated with rM-CSF/rRANKL in the presence or absence of PGDHC. Similar to macrophages isolated from their wild-type mice, both bone marrow macrophages (Fig. 4A&B), as well as peritoneal macrophages (Fig. 4C&D), derived from TLR2/4 DKO mice showed an increase in the number of TRAP⁺ osteoclasts in response to PGDHC. These results indicated that PGDHC enhances RANKL-induced osteoclastogenesis in a manner independent of TLR2/4.

3.3. PGDHC is a cell-permeable ceramide and colocalizes with non-muscle myosin IIA (Myh9) in RANKL-stimulated osteoclasts

Based on the results shown in Fig. 4 indicating that PGDHC may act on a molecule different from TLR2/4, we hypothesized that PGDHC could act on a molecule expressed distinctively on osteoclasts, compared to its osteoblast activity via TLR2. To identify a putative ligand for PGDHC expressed in OCPs, a pull-down assay was performed by reacting RAW 264.7 cell lysate with PGDHC-coupled beads. SDS-PAGE gel electrophoresis revealed 42 and 230 kDa major bands which were identified as β -actin and Myh9, respectively, by mass spectrometry analysis (Fig. 5A; Suppl. Fig. 1). Interestingly, it was reported earlier that Myh9, an actin-binding cytoskeletal protein, plays pivotal roles in the control of cell adhesion, cell migration

and cytoskeletal architecture [41], but plays a downregulatory role in the fusion of OCPs [42].

Since those reported biological properties of Myh9 corresponded to our findings shown above (Figs. 1–3), we next investigated the possible interaction between Myh9 and PGDHC during osteoclastogenesis. RANKL-stimulated RAW 264.7 cells were incubated with biotinylated PGDHC and anti-Myh9 antibody, followed by reaction with streptavidin-Texas red and Dylight 488-labeled secondary antibody. Myh9 was detected in the podosomes of multinucleated osteoclasts as well as in their nuclei (Fig. 5 B). PGDHC was found to be colocalized with Myh9 in the multinucleated osteoclasts (Fig. 5 B, C), providing evidence that PGDHC is a cell-permeable ceramide of bacterial origin and that its interaction with Myh9 promotes osteoclast cell fusion. It is noteworthy that PGDHC was also detected in the nuclei of osteoclasts indicating its ability to penetrate through the nucleus membrane (Fig. 5 B).

3.4. PGDHC enhances RANKL-mediated osteoclastogenesis via binding to Myh9

To evaluate whether binding of PGDHC to Myh9 enhances osteoclastogenesis, we tested the roles of Myh9 in mediating the action of PGDHC via loss- and gain-of-function approaches.

The effect of Myh9 loss-of-function on PGDHC-mediated promotion of RANKL-induced osteoclastogenesis was performed using siRNA for Myh9. Treatment of RAW264.7 cells with siRNA for Myh9 significantly reduced the expression of Myh9 mRNA (Fig. 6A). As a consequence, the amount of Myh9 protein in RAW264.7 cells was also remarkably decreased (Fig. 6B). Consistent with a previous report showing that the diminished expression of Myh9 can promote osteoclast cell fusion [42], stimulation of siRNA-Myh9-treated RAW264.7 cells demonstrated a significantly elevated number of TRAP⁺ osteoclasts in response to stimulation with RANKL. Moreover, compared to siRNA-Myh9-treated RAW264.7 cells stimulated with RANKL alone, the number of TRAP⁺ multinucleated cells was further increased when PGDHC was added to siRNA-Myh9-treated RAW264.7 cells stimulated with RANKL (Fig. 6C&D). It is noteworthy that the size of TRAP⁺ multinuclear osteoclasts was remarkably larger in PGDHC-treated siRNA-Myh9 RAW264.7 cells in the presence of RANKL compared to the other groups tested (Fig. 6C). These results indicated that Myh9 expressed in RANKL-stimulated osteoclasts downregulates osteoclastogenesis, but that the binding of PGDHC with Myh9 can abrogate such downregulation mediated by Myh9. In sum, these observations indicated that ligation of non-muscle myosin II-A with PGDHC stimulates RANKL-induced fusion of OCPs *in vitro*.

Since it was reported that regulated temporal degradation of Myh9 (non-muscle myosin IIA) during RANKL-mediated osteoclastogenesis results in the formation of large osteoclasts [42], we evaluated the effects of PGDHC on RANKL-stimulated RAW264.7 cells stably overexpressing *Myh9* gene. Using *Myh9* transfection plasmid, the enhanced expression of Myh9 was confirmed in RAW264.7 cells using qPCR and W-blot (Fig. 7 A&B). As a consequence of transfection with *Myh9*-overexpression plasmid, the size and number of TRAP⁺ multinuclear osteoclasts differentiated from RANKL-stimulated RAW264.7 cells were significantly reduced compared to control RAW 264.7 cells transfected with mock plasmid (Fig. 7D). On the other hand, RAW264.7 cells that received a *Myh10*-

overexpression plasmid did not alter the size and number of TRAP+ multinuclear osteoclasts differentiated from RANKL-stimulated RAW264.7 cells compared to the control transfection with mock plasmid (Fig. 7 E).

However, when PGDHC was added to above noted *in vitro* assay of RANKL-induced osteoclastogenesis, the total number of RANKL-induced TRAP+ multinuclear cells increased in RAW264.7 cells overexpressing Myh9, suggesting that PGDHC acts on Myh9 overexpressed in RANKL-stimulated RAW264.7 cells and promotes osteoclastogenesis (Fig. 7 C&D). In contrast to the PGDHC's upregulation effects on the osteoclastogenesis in RAW264.7 cells overexpressing Myh9, no significant change in the number of total TRAP+ multinuclear cells was observed in RANKL-stimulated RAW264.7 cells overexpressing Myh10 in the presence or absence of PGDHC (Fig. 7E). In sum, these results suggest that the PGDHC-mediated upregulation of osteoclastogenesis is associated with Myh9, but not Myh10.

3.5. Cell-permeable PGDHC elevates DC-STAMP expression via Rac1 signaling pathway

It was demonstrated earlier that inhibition of Myh9 activity by a cell-permeable blebbistatin or siRNA leads to activation of the Rac1 pathway in various cell lines *in vitro* [43,44]. The known small GTPases include Rac1, RhoA, and Cdc42. Among these, only Rac1 appears to play a role in osteoclast cell fusion [45], and cell-permeable C6-ceramide can activate the Rac1 signaling pathway [46]. Therefore, we next tested the impact of cell-permeable PGDHC on Rac1 GTPase activation in RAW 264.7 cells *in vitro*. As expected, both cell-permeable ceramides, i.e., PGDHC and C6-ceramide, enhanced the expression of Rac1 in RANKL-stimulated RAW 264.7 cells, whereas non-cell-permeable C6-dihydroceramide did not affect the expression of Rac1 (Fig. 8 A). Importantly, Rac1 inhibitor (NSC23766) significantly downregulated PGDHC-induced increase of DC-STAMP mRNA expression in RANKL-stimulated RAW 264.7 cells (Fig. 8B). NSC23766 also abrogated the effect of C6 ceramide on DC-STAMP mRNA expression induced in RANKL-stimulated RAW264.7 cells. However, no significant effect of NSC23766 on DC-STAMP mRNA expression was observed in the RANKL-stimulated RAW264.7 cells exposed to C6-dihydroceramide. Finally, in accordance with the expression patterns of DC-STAMP mRNA (Fig. 8B), NSC23766 suppressed the TRAP + osteoclasts induced by RANKL in either the presence or absence of PGDHC or C6 ceramide (data not shown), suggesting that Rac1 is engaged in constitutional RANKL-induced osteoclastogenesis and that PGDHC promoted osteoclast fusion via a mechanism that upregulates DC-STAMP expression by Rac1 signaling.

4. Discussion

Ceramides, a class of sphingolipids, are important structural components of the host membrane [47]. Accumulated evidence supports the conclusion that host-derived biological ceramides also act as second messengers in various signaling pathways, including cell growth, survival and inflammatory responses [48]. Possible engagement of host-derived ceramides in the inflammatory processes of periodontitis was also implicated [49,50]. However, since bacteria possess distinct membrane structures that do not contain ceramide, the production of ceramides by bacteria was thought to be a rare occurrence. For this reason,

finding a unique lipid complex containing phosphoglycerol dihydroceramide, or PGDHC, produced by *P. gingivalis* in the human periodontitis lesion brought to light a novel pathological property of this periodontal pathogen [20,21]. Indeed, PGDHC promotes IL-1b-mediated release of PGE2 and induces apoptosis in gingival fibroblasts [21], suggesting the pathogenic engagement of PGDHC in inflammatory responses in the context of periodontitis. However, the possible effects of PGDHC on alveolar bone destruction in periodontitis have been unknown. Thus, the present study, for the first time, demonstrated that PGDHC, a unique lipid produced by *P. gingivalis*, can promote RANKL-mediated osteoclastogenesis, especially in a manner independent of TLR2, thereby gaining additional insight into the pathogenesis of *P. gingivalis* in the context of periodontitis.

To the best of our knowledge, this is the first study reporting that long chain ceramides, i.e., PGDHC, which is derived from the bacterium *Pg*, can penetrate the cell membrane of OCPs and modulate osteoclastogenesis by acting on a cytoplasmic molecule engaged in regulating cell fusion. It is well demonstrated that host-derived long chain ceramides are not cell-permeable [17] and that C1P ceramide (C14/C16), one such long chain ceramide, can activate macrophages via binding to putative C1P receptor expressed on the cell surface [51]. We identified that PGDHC could promote RANKL-mediated osteoclastogenesis by acting on Myh9, a unique cytoskeletal regulatory molecule for osteoclast cell fusion, whereas PGDHC alone did not induce the formation of functional TRAP+ osteoclasts in the absence of RANKL. It was also shown that PGDHC could upregulate RANKL-induced osteoclastogenesis *in vitro* from various types of OCPs, including RAW264.7 cells, primary culture of peritoneal macrophages, as well as bone marrow-derived osteoclast precursors, indicating that PGDHC-mediated upregulation of osteoclastogenesis may occur in a variety of contexts, including homeostatic bone remodeling, as well as pathogenic bone resorption in bone lytic diseases (Figs. 1 & 4). Most importantly, PGDHC induced the upregulation of RANKL-mediated osteoclastogenesis by PGDHC in a manner independent of TLR2/4 pathways (Fig. 4). Therefore, the finding that PGDHC promotes osteoclastogenesis in a manner independent of TLR2/4 pathways suggested that 1) no components or moieties that bind TLR2/4 are present in PGDHC as confounding factors and 2) PGDHC acts on a unique molecular target present in OCPs, i.e., Myh9, in a manner different from its suppressive activity on osteoblastogenesis through TLR2 activation [40].

That PGDHC induced osteoclastogenesis independent of TLR2/4 activation was unexpected. Therefore, we next sought a possible PGDHC ligand in osteoclasts using a pull-down assay combined with mass spectroscopy. This resulted in the identification of a novel interaction between a non-muscle myosin IIA (Myh9) and PGDHC which in turn actively promotes osteoclastogenesis. In support of this finding, McMichael et al. reported that Myh9 is a downregulator in the cell fusion event in osteoclasts [42]. More specifically, although Myh9 is constitutively expressed in mononuclear immature osteoclasts, transiently increased level of cathepsin B enzyme during the cell fusion stage of RANKL-mediated osteoclastogenesis causes the degradation of Myh9 which, in turn, allows osteoclast cell fusion [42]. The role of Myh9 as a downregulator in osteoclast cell fusion is further proven by Myh9 gene knockout using RNAi [42]. Based on this report [42], we performed experiments to further confirm the direct biological relationship between PGDHC and Myh9.

Both loss- and gain-of-function assays were performed using RNAi-based Myh9 gene silencing, as well as Myh9-overexpression plasmid in OCPs (RAW264.7), which together showed that ligation of PGDHC with Myh9 enhances RANKL-induced osteoclastogenesis. Especially, the overexpression of Myh9 in RAW264.7 cells suppressed RANKL-mediated development of TRAP⁺ multinuclear osteoclasts, whereas addition of PGDHC to Myh9-overexpressing RAW264.7 cells reversed the effects of overexpressed Myh9 and allowed the emergence of TRAP⁺ multinuclear osteoclasts. In addition, fluorescent confocal microscopy revealed a co-localization of PGDHC and Myh9 was also observed in the osteoclast cytoplasm as well as nuclei (Fig. 5B), indicating the PGDHC is able to penetrate through not only cell membrane but also nuclear membrane. Although the Myh9 (non-muscle myosin II) was originally found as a cytoskeletal protein present in the cytoplasm [52], it was also found to be present in nucleus [53]. Because it is well known that only short chain ceramides, but not long chain ceramides, are able to pass across the cell membrane barrier [19], identification of PGDHC, a long chain dihydroceramide, in the osteoclast's cytoplasm as well as nuclei is an interesting finding of this study. To the best of our knowledge, PGDHC is the very first exogenous cell membrane permeable dihydroceramide derived from bacteria that binds Myh9 and affects its activity.

Finally, in the pull-down assay to identify the target molecules for PGDHC expressed in osteoclasts, β -actin was found among the proteins pulled down by PGDHC, along with Myh9. It is well established that Myh9 plays an important role in cell shape and cytoskeletal organization [35,54]. Since Myh9 is an actin-binding protein that has an actin crosslinking site and actin contractile properties [41], it is plausible that β -actin could be co-precipitated with Myh9 by PGDHC-conjugated beads. Based on our loss- and gain-of-function assays, as described above, it is conceivable that PGDHC directly binds to Myh9, as a consequence of penetration into cytoplasm, which, in turn, results in the formation of a complex with actin in the cytoplasm. Since the β -actin-binding compounds Cytochalasin D and Latrunculin B produced by fungi and sponges, respectively, suppress RANKL-induced osteoclastogenesis [55–57], it is likely that the binding of an exogenous molecule with cytoplasmic β -actin could inhibit osteoclastogenesis. Therefore, if PGDHC directly binds β -actin, such binding should inhibit osteoclastogenesis, rather than promote it, as we found in this study. For this reason, PGDHC-mediated promotion of osteoclastogenesis excludes the possibility of direct binding between PGDHC and β -actin as an underlying mechanism. On the other hand, it is important to note that various molecules that regulate actin, including TKS5 and dynamin, have been suggested as contributors to OCP fusion [58,59]. Among various small GTPases, only Rac1 is reported to upregulate the cell fusion event through the reorganization of actin cytoskeleton [45]. In the immortalized mouse monocyte cell line system, Rac1 null cells, but not Rac2 null or their primary cell counterparts, display a severe defect in osteoclastogenesis and function [60]. In the current study, it was clearly demonstrated that both cell-permeable bacterial PGDHC and host C6-ceramides significantly upregulate Rac 1 expression in RAW 264.7 cells *in vitro* compared to C6-dihydroceramide that cannot penetrate host cells (Fig. 8 A). Our results correlate well with those from an earlier study which found that cell-permeable C6-ceramide upregulates Rac 1 activity in various cell lines *in vitro* [46]. We also demonstrated that inhibition of Rac1 activity using NSC23766 significantly downregulated the expression of DC-STAMP, an important osteoclast fusogen, in response to stimulation

with PGDHC *in vitro*. Among various mediators that are reported to be engaged in osteoclast fusion, including CD9, DC-STAMP, Atp6v0d2 and OC-STAMP [61], DC-STAMP plays a pivotal role in osteoclast cell fusion because DC-STAMP-KO mice show severe osteopetrotic phenotype and OCPs isolated from DC-STAMP-KO mice are unable to generate multinuclear TRAP⁺ cells *in vitro* [62,63]. Collectively, therefore, the findings from this study, along with these published lines of evidence, indicate that cell-permeable PGDHC promotes fusion of OCPs mediated, in part, by the Myh9/Rac1/DC-STAMP pathway.

5. Conclusions

When taken together, these results strongly suggest that PGDHC, a novel class of cell-permeable long chain dihydroceramide derived from the periodontal pathogen, *Pg*, can promote RANKL-induced osteoclastogenesis via interaction with Myh9, an osteoclast cell fusion regulatory cytoskeletal protein, in a manner dependent on Rac1/DC-STAMP, not TLR2/4. Further studies will be carried out to evaluate the molecular mechanism that allows PGDHC to penetrate the cytoplasm of osteoclasts and the pathophysiological effects of PGDHC in the context of periodontal lesions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Effects of PGDHC on *in vitro* RANKL-induced osteoclastogenesis using RAW 264.7 cells
A) TRAP staining of RAW 264.7 cells incubated with or without RANKL in the presence or absence of *P. gingivalis* LPS (*Pg* LPS), PGDHC, host cell-permeable C6 (hC6-C) and host non-cell-permeable C6-dihydroceramide (hC6-DHC) for 5 days. **B)** Number of TRAP+ osteoclasts per well formed in the culture of RAW 264.7 cells stimulated with RANKL in the presence or absence of *Pg* LPS, PGDHC, hC6-C, and hC6-DHC for 5 days. **C)** Number of TRAP- multinucleated cells per well formed in the culture of RAW 264.7 cells stimulated with *Pg* LPS, PGDHC, hC6-C, and hC6-DHC for 5 days. For TRAP staining, the cells were fixed and stained with TRAP kit (Sigma). TRAP+ cells with more than three nuclei were considered as osteoclasts. Arrowheads show multinucleated TRAP-negative cells. Data are expressed as mean $\pm$ SD, and representative of three independent experiments is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 2. Relative mRNA expressions of DC-STAMP, matrix metalloprotease-9 (MMP-9), and TRAP in RAW 264.7 cells stimulated with RANKL in the presence or absence of PGDHC
RAW 264.7 cells were stimulated with RANKL (50 ng/ml) in the presence or absence of PGDHC (1 μ g/ml) for 4 days, and RNA was extracted. After reverse transcription, the expression of some osteoclast marker genes was examined by real-time PCR. Fold changes of the gene of interest were calculated as $2^{-\Delta\Delta C_t}$ using GAPDH as a reference gene. Data are expressed as mean $\pm$ SD, and representative of three independent experiments is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 3. Effects of PGDHC on RANKL-mediated osteoclastogenesis induced in mouse calvaria *in vivo*

A) and **B)** Histological images of TRAP+ osteoclasts at lower and higher magnification, respectively, that emerged in decalcified sections of WT mouse calvaria that received control PBS alone, PGDHC (alone), RANKL (in PBS), and RANKL + PGDHC (in PBS); **C)** Measurement of TRAP+ osteoclasts (the area occupied by cytoplasm of TRAP+ cells) revealed that calvarial injection with RANKL/PGDHC induced a significantly elevated level of osteoclasts in the calvarial sagittal sutures when compared to that induced by injection with RANKL alone. Data are expressed as mean $\pm$ SD, and representative of two independent experiments (5 mice/group) is shown. Scale bar – 10 μ m. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 4. PGDHC lipid promoted RANKL-induced osteoclastogenesis in a TLR2- and TLR4-independent manner. **A)** Images of TRAP staining and **B)** number of TRAP+ osteoclasts formed in the culture well of bone marrow-derived macrophages of WT or TLR2/4 DKO mice stimulated with or without RANKL in the presence or absence of PGDHC lipid, respectively; **C)** and **D)** the same experiments as **A)** and **B)** were performed using peritoneal macrophages isolated from WT or TLR2/4 DKO mice. Arrowheads indicate TRAP+ multinucleated cells. Data are expressed as mean $\pm$ SD, and representative of three independent experiments is shown. * $p < 0.05$, significantly higher compared to RANKL alone.

Fig. 5. Co-localization of non-muscle myosin II-A (Myh9) and cell-permeable PGDHC in multinucleated osteoclasts. **A)** Silver-stained proteins co-precipitated with PGDHC-coupled beads in pull-down assay from RAW 264.7 cell lysate were separated by SDS-PAGE electrophoresis. Two major bands of eluate from PGDHC-bound beads are highlighted with blue and red squares which corresponded to 42 kDa and 230 kDa molecules, respectively. Mass spectrometry analysis revealed that the 42 kDa and 230 kDa bands corresponded to β -actin and Myh9, with sequence coverage of 42 and 30.1%, respectively (Suppl. Fig. 1). **B)** Representative immunofluorescent images of a multinucleated and mononuclear osteoclast showing staining for Myh9 (green), PGDHC (red) and nuclei (blue). **C)** Fluorescent intensities of Dylight 488 and Texas Red emissions in the cross-sectional plane (yellow line in Myh9 and PGDHC images, respectively) show colocalization of Myh9 and PGDHC in the large multinuclear osteoclast. Data are expressed as representative of three independent experiments.

Fig. 6.

PGDHC promoted RANKL-induced osteoclastogenesis, even after the suppression of Myh9 expression, using RNA interference (RNAi) for Myh9 mRNA *in vitro*. **A**) Gene expression of Myh9 mRNA in RAW264.7 cells treated with non-silencing siRNA (NS siRNA) and siRNA specific for Myh9 mRNA; **B**) Western blot analysis showed nearly complete suppression of Myh9 protein expression by siRNA treatment compared to NS-siRNA treatment, both of which were performed for RANKL-stimulated RAW 264.7 cells; **C**) and **D**) TRAP staining and number of TRAP+ osteoclasts emerging in RANKL- or RANKL/PGDHC-stimulated RAW 264.7 cells reacted with NS-siRNA or siRNA-myh9, respectively. Data are expressed as mean $\pm$ SD, and representative of three independent experiments is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 7. Effects of PGDHC on RANKL-induced osteoclastogenesis, using Myh9- overexpressed RAW264.7 cells *in vitro*

A) Myh9 gene expression in RAW 264.7 cells was induced by transfection with CMV-NMHC II-A (Myh9) plasmid; B) Western blot analysis showed increased Myh9 protein expression in CMV-NMHC II-A plasmid-transfected RAW 264.7 cells; C) TRAP staining of RANKL-stimulated RAW264.7 cells transfected with either Myh9- or Myh10-expression plasmid in the presence or absence of PGDHC; D) and E) Number of TRAP+ osteoclasts per well formed in RANKL- or RANKL/PGDHC-stimulated RAW 264.7 cells transfected with NMHC II-A (Myh9)- or NMHC II-B (Myh10)-expression plasmid, respectively. Data are expressed as mean $\pm$ SD, and representative of three independent experiments is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 8. Cell-permeable PGDHC upregulates expression of DC-STAMP, an important osteoclast fusogen, in a Rac1-dependent manner

A) Expression levels of three major GTPases, Rac1, RhoA, and Cdc42, by RANKL-stimulated RAW264.7 cells in response to ceramides and dihydroceramides: RANKL-stimulated RAW264.7 cells were incubated in the presence or absence of PGDHC, host cell-permeable C6-ceramide (hC6-C), or non-cell-permeable host C6-dihydroceramide (hC6-DHC) for 3 days, followed by the analysis of each GTPase expression using Western blot (left panel). The band intensity of each GTPase detected by W-blot was monitored using a densitometry, and expressed in the histograms (right graphs). **B)** PGDHC and hC6-C significantly upregulated DC-STAMP mRNA expression in RANKL-stimulated RAW 264.7 cells via a Rac1-dependent pathway: RANKL-stimulated RAW264.7 cells were incubated in the presence or absence of PGDHC, hC6-C, or hC6-DHC with or without Rac1 inhibitor (NSC23766) for 3 days. Expressions of DC-STAMP mRNA were evaluated by qPCR. Data are expressed as mean $\pm$ SD, and representative of three independent experiments is shown.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 1

Primer sets for real-time PCR.

Gene name (gene ID)	Direction of sequence	Reference or primer bank ID
mouse GAPDH (14433)	F: 5'-CTCCCACTCTTCCACCTTCG-3' R: 5'-TTGCTGTAGCCGTATTCAAT-3'	[64]
mouse TRAP (11433)	F: TACCTGTGTGGACATGACC R: CAGATCCATAGTGAAACCGC	[64]
mouse MMP-9 (17395)	F: TCCAGTACCAAGACAAAG R: TTGCACTGCACGGTTGAA	[64]
mouse DC-STAMP (75766)	F: 5'-TGGAAGTTCACCTTGAAACTACGTG-3' R: 5'-CTCGGTTTCCCGTCAGCCTCTCTC-3'	[65]
mouse Myh9 (17886)	F: 5'-AGGATGTGGATCGGATCATTGG-3' R: 5'-GGGTTGGTATTCTCAACGTG-3'	20137006a3
Human Myh9 (4627)	F: 5'-AGGACCAGAACTGCAAGCTG-3' R: 5'-GCGCTCTTCCAAGTCAGTGA-3'	12667788a3