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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* 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.

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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 ($C \leq 8$), 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-1β-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 counterstained 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