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Serine dipeptide lipids of *Porphyromonas gingivalis* inhibit osteoblast differentiation: Relationship to Toll-like receptor 2

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Abstract

Porphyromonas gingivalis is a periodontal pathogen strongly associated with loss of attachment and supporting bone for teeth. We have previously shown that the total lipid extract of *P. gingivalis* inhibits osteoblast differentiation through engagement of Toll-like receptor 2 (TLR2) and that serine dipeptide lipids of *P. gingivalis* engage both mouse and human TLR2. The purpose of the present investigation was to determine whether these serine lipids inhibit osteoblast differentiation in vitro and in vivo and whether TLR2 engagement is involved. Osteoblasts were obtained from calvaria of wild type or TLR2 knockout mouse pups that also express the Col2.3GFP transgene. Two classes of serine dipeptide lipids, termed Lipid 654 and Lipid 430, were tested. Osteoblast differentiation was monitored by cell GFP fluorescence and osteoblast gene expression and osteoblast function was monitored as von Kossa stained mineral deposits. Osteoblast differentiation and function were evaluated in calvarial cell cultures maintained for 21 days. Lipid 654 significantly inhibited GFP expression, osteoblast gene expression and mineral nodule formation and this inhibition was dependent on TLR2 engagement. Lipid 430 also significantly inhibited GFP expression, osteoblast gene expression and mineral nodule formation but these effects were only partially attributed to engagement of TLR2. More importantly, Lipid

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430 stimulated TNF- α and RANKL gene expression in wild type cells but not in TLR2 knockout cells. Finally, osteoblast cultures were observed to hydrolyze Lipid 654 to Lipid 430 and this likely occurs through elevated PLA2 activity in the cultured cells. In conclusion, our results show that serine dipeptide lipids of *P. gingivalis* inhibit osteoblast differentiation and function at least in part through engagement of TLR2. The Lipid 430 serine class also increased the expression of genes that could increase osteoclast activity. We conclude that Lipid 654 and Lipid 430 have the potential to promote TLR2-dependent bone loss as is reported in experimental periodontitis following oral infection with *P. gingivalis*. These results also support the conclusion that serine dipeptide lipids are involved in alveolar bone loss in chronic periodontitis.

Keywords

Porphyromonas gingivalis; osteoblasts; serine lipids; Toll-like receptor 2

Introduction

1.1 *Porphyromonas gingivalis* is a periodontal pathogen strongly associated with the expression of destructive periodontal disease in adults (chronic periodontitis) whereby alveolar bone and connective tissue attachment are destroyed around affected teeth. Oral infection of experimental animals with *P. gingivalis* is associated with bone loss mediated at least in part by engagement of Toll-like receptor 2 (TLR2) [1–3]. We have determined that the total lipid extract of *P. gingivalis* inhibits osteoblast differentiation in vivo and in vitro and this process is mediated through engagement of TLR2 [4]. Although many factors of *P. gingivalis* have been implicated as potential TLR2 ligands, we have recently determined that the serine dipeptide lipids of *P. gingivalis* that are prevalent in *P. gingivalis* total lipid extracts as well as other *Bacteroidetes* recovered in subgingival plaque, engage both human and mouse TLR2 [5].

1.2 Serine dipeptide lipids of *P. gingivalis* are comprised of two major classes [5]. The most abundant class produced by *P. gingivalis*, termed Lipid 654, consists of three lipid species with negative ion masses of 653.5, 639.5 and 625.5 m/z in the order of decreasing abundance. These three lipid species share a serine-glycine dipeptide moiety where glycine is amide-linked primarily to 3-OH isobranched (iso) C_{17:0}. The beta hydroxyl of 3-OH isoC_{17:0} is substituted with a fatty acid, most frequently isoC_{15:0} that is held in ester linkage. The second class of serine lipids, termed Lipid 430, is composed of three species with negative ion masses of 429.3, 415.3 and 401.3 m/z representing the de-esterified core lipid structures of the Lipid 654 class. Both of these lipid classes can be isolated from *P. gingivalis* but Lipid 654 predominates by at least two orders of magnitude over Lipid 430 in lipid extracts of this organism. While both lipid classes have been reported to engage TLR2, the effects of both lipid classes on bone cells and the dependence on TLR2 engagement remains to be established.

1.3 We recently observed that Lipid 654 is converted to Lipid 430 through the action of both honey bee venom, porcine pancreatic or recombinant human secretory phospholipase A2 (PLA2) (data not shown). These enzymes are members of the secretory PLA2 family [6].

PLA2 is elevated in chronically inflamed tissues such as those associated with rheumatoid arthritis and atherosclerosis. PLA2 represents an enzyme class comprised of many forms but cytosolic and secretory PLA2 forms appear to be most important in chronic inflammatory reactions such as those associated with atherosclerosis development [6]. Few reports have documented PLA2 enzyme levels and their relationship to diseased periodontal tissues. However, secretory PLA2 is known to be secreted from activated macrophages [7, 8] and both cytosolic and secretory PLA2 is released from neutrophils [9]. Because prostaglandin levels are elevated in diseased periodontal tissues [10, 11], it is assumed that PLA2 enzymes are elevated in these diseased tissues. The purpose of the present investigation was to evaluate both unhydrolyzed Lipid 654 and PLA2-generated Lipid 430 preparations for their capacity to affect osteoblast differentiation in vitro as a potential mechanism for promotion of alveolar bone loss in periodontal disease. Furthermore, this investigation evaluated the relationship between prostaglandin secretion from osteoblasts and conversion of Lipid 654 to Lipid 430 in cultures of osteoblasts.

Materials and Methods

2.1 Reagents

BBL biosate peptone, trypticase peptone, yeast extract and brain heart infusion, and high performance liquid chromatography (HPLC) solvents were obtained from Fisher Scientific. Lipoteichoic acid was obtained from InvivoGen. Porcine pancreatic phospholipase A2 (PP PLA2, 600 units/mg protein) was obtained from Sigma. Collagenase P (*Clostridium histolyticum* 1.8 U/mg protein) was obtained from Roche. LPS (*Salmonella typhimurium*) and trypsin (0.25%) in EDTA (1X) were obtained from Gibco. D₄-Prostaglandin E₂ was obtained from Cayman Chemical.

2.2 Bacterial growth

Bacteria were grown in broth culture as previously described. *P. gingivalis* (ATCC #33277, type strain) was inoculated into basal (peptone, trypticase and yeast extract) medium supplemented with hemin and menadione (Sigma) and brain heart infusion (BHI) [12]. Culture purity was verified by Gram stain, lack of growth in aerobic culture and formation of uniform colonies when inoculated on brain heart infusion agar plates and grown under anaerobic conditions. The suspension cultures were incubated for four days in an anaerobic chamber flushed with N₂ (80%), CO₂ (10%) and H₂ (10%) at 37°C and the bacteria were harvested by centrifugation (2000 × g for 2 hour).

2.3 Lipid extraction, fractionation and characterization

Lipids were extracted from lyophilized bacterial pellets. Generally 2 to 4g of bacterial pellet was extracted for each semipreparative fractionation. The bacterial samples were weighed and dissolved in chloroform:methanol:water (1.33:2.67,1, v/v/v, 2g of bacterial pellet in a total of 20 ml of solvent) as previously described [12]. The mixture was vortexed at 15 min intervals for 2 hr and the mixture was supplemented with 6 ml of chloroform and 6 ml of (2N KCl + 0.5N K₂HPO₄). The mixture was vortexed and centrifuged (2000 × g) at 20°C for 4 h. The lower organic phase was removed and dried under nitrogen. The dried extract was reconstituted in HPLC solvent (hexane:isopropanol:water, 6:8:0.75, v/v/v, 24 ml) and

vortexed [12]. The sample was centrifuged at $2500 \times g$ for 10 minutes and the supernatant removed for HPLC analysis. Semipreparative HPLC fractionation was accomplished using a Shimadzu HPLC system equipped with dual pumps (LC-10ADvp), automated controller (SCL-10Avp) and in line UV detector (SPD-10Avp). Lipids were fractionated using normal phase isocratic separation (Ascentis®Si, 25 cm $\times$ 10 mm, 5 μ m, Supelco Analytical) with a solvent flow of 1.8 ml/min and 1 min fractions. HPLC solvent was composed of hexane:isopropanol:water (6:8:0.75, v/v/v). The effluent was monitored at 205 nm. Replicate fractionations were pooled and dried under nitrogen. The dried samples were reconstituted in HPLC solvent for mass spectrometric analysis as described below. Fractions containing Lipid 654 were pooled and dried before additional HPLC fractionation.

2.4 Acidic HPLC fractionation of Lipid 654 and Lipid 430-

Lipid 654 fractions were further purified by elution over the same HPLC column at 1.8 ml/min but using HPLC solvent supplemented with 0.1% acetic acid. Purity of Lipid 654 was verified by electrospray-mass spectrometry (ESI-MS or MS/MS) as described below. Approximately 1.5 mg of highly purified Lipid 654 was divided into 6 aliquots and each aliquot was sonicated (20 sec) in hydrolysis buffer (1 ml of 10 mM Tris, pH 7.5, 2.5 mM calcium chloride and 150 mM NaCl). Each aliquot was supplemented with 100 U of PP PLA2 and stirred at room temperature. After 4 days, the hydrolyzates were acidified with 25 μ l of acetic acid and each aliquot was extracted three times with 1 ml of chloroform. The chloroform extracts were combined and dried under nitrogen. The hydrolyzed lipid sample was then dissolved in 2 ml of acidic HPLC solvent and fractionated using the same normal phase column and elution conditions described above for purification of Lipid 654. Fractions eluting from 14–16 minutes were pooled and were shown to contain highly enriched Lipid 430. Lipid 430 mass spectral characteristics were verified using ESI-MS and ESI-quadrupole time of flight (QTOF)-MS/MS (AB Sciex QStar instrument).

2.5.1 Mass spectrometry—Lipid samples in HPLC fractions from either semi-preparative neutral or acidic fractionations, were dissolved in the neutral HPLC solvent described above and were injected over a normal phase column (Ascentis®Si, 3 cm $\times$ 2.1 mm, 5 μ m, Supelco Analytical) interfaced with an API4000 Qtrap instrument from Sciex. Neutral HPLC solvent was delivered under isocratic conditions with a Shimadzu LC-10ADvp pump at a flow rate of 100–120 μ l/min. Total ion chromatograms were acquired using negative ion mode and a mass range of 100 to 1800 amu, and MS/MS acquisitions used parameters optimized for specific lipid products. Collision energies for negative ion products were typically between –30 and –55 V depending on the precursor ion under investigation. Negative ion ESI was carried out at –4,500 V, with a declustering potential of –90 V, focusing potential of –350 V, and entrance potential of –10 V. Multiple reaction monitoring (MRM) negative ion transitions for Lipid 654 were 653.5/131.1, 653.5/306.2, 653.5/349.3 and 653.5/381.4 m/z and transitions for Lipid 430 were 430.2/140.9, 430.3/173.1 and 430.3/382.3 m/z . Lipid 430/Lipid 654 ratios were calculated using the area of extracted ion chromatograms (XIC) only for the 430.3/382.3 and 653.5/381.4 m/z transitions.

2.5.2 For determination of prostaglandin levels in osteoblast medium samples, 1.7 ml of medium was supplemented with 10 ng of D₄-prostaglandin E₂ and vortexed. The samples were acidified to pH 3.0 and extracted with CHCl₃ (3 × 1 ml). The extracts were pooled and dried under nitrogen. At the time of prostaglandin analysis, samples were infused into the API4000 Qtrap instrument (see above) and were analyzed by MRM for both deuterated and non-labeled PGE₂ using negative ion transitions of 355.6/337.2 and 351.6/333.3 *m/z*, respectively. XIC peaks for the above transitions were electronically integrated and the internal standard recovery was used to calculate authentic PGE₂ levels in medium samples.

2.6.1 Mice—*Tlr2*−/− (TLR2 KO) mice, bred onto a C57BL/6 background, were a generous gift of Dr. S. Akira (Osaka University, Japan) [13]. TLR2 −/− mice were backcrossed 9 times and verified by genotyping to be homologous for TLR2 −/− status. All mice were maintained and bred in accordance with University of Connecticut Center for Laboratory Animal Care regulations. TLR2 knockout mice were cross bred with transgenic mice harboring the type I collagen promoter driven transgene, Col2.3GFP, which is expressed in differentiated osteoblasts. Col2.3GFP mice were established on a C57BL/6 background. After establishing *Tlr2*−/− status by genotyping in GFP positive F2 offspring, mice were bred for evaluation of serine lipid effects in primary cultures of osteoblasts. Mouse pups (4–7 day old) derived from either Col2.3GFP wild type or TLR2 KO:Col2.3GFP breeding pairs were used to prepare primary cultures of osteoblasts. Cells were prepared by digesting calvaria in normal phosphate buffered saline containing 1.5 U/ml collagenase P with 0.05% trypsin in 0.2 mM EDTA. Calvaria were subjected to four fifteen minute digestions and the first digest was discarded. Cells from the last three fifteen minute digestions were pooled, centrifuged and dispersed in DMEM medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µM non-essential amino acids and 10% fetal calf serum. Cells were plated at 1.5×10^4 cells/cm² (1.5×10^5 cells per well) in 6 × 35 mm culture plates. Cells became confluent around day 5–6 and at day 7, the medium was changed to differentiation medium consisting of α-MEM containing 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml of fungizone, 50 µg/ml ascorbic acid, 4 mM β-glycerophosphate and 10% fetal calf serum. Cells were cultured for three and Axiovision software (Zeiss). Fluorescence was detected using the following filter sets (Chroma Technology: ET500/5x, ET535/30mm, T525LP for EGFP). Mineral deposits were quantified using von Kossa silver stained cultures weeks with medium changes at 2–3 day intervals. Lipid preparations were sonicated (30 sec, 3 watts) in PBS immediately prior to supplementing cell cultures. GFP fluorescence was determined in cells in culture using a Zeiss Observer Z-1 inverted microscope and images were captured using an AxioCam MRc digital camera that were scanned with an Epson V600 scanner. GFP expression and von Kossa stained nodules were quantified from digitally acquired images using ImageJ [14]. Predetermined thresholds for GFP and silver stained granules were consistently applied for all images acquired within specific experiments. For the von Kossa silver nitrate staining method, cultures were fixed in 10% formalin for 10–15 min. After rinsing, the fixed plates were incubated with 5% silver nitrate solution under UV light using 2 cycles of auto-crosslink (1200 mJoules × 100) in a UV Stratalinker (Stratagene, La Jolla, CA).

2.6.2 For the in vivo evaluation of bacterial lipid effects on osteoblast differentiation in mice calvaria, lipids were sonicated (30 sec, 3 watts) in PBS. Six to eight week old Col2.3GFP wild type or TLR2 KO:Col2.3GFP mice were lightly anesthetized with isofluorane. Phosphate buffered saline (PBS control) was administered subcutaneously to the left side of each mouse calvarium and Lipid 430 (1 µg in 50 µl) or Lipid 654 (5 µg in 50 µl) was administered subcutaneously to the parietal bone surface on the right side. The solutions were administered from anterior to posterior as the syringe needle was withdrawn over the surface of the parietal bone from the posterior region of the orbit to just anterior to the ear. Mice were euthanized after 7 days and calvaria were harvested. Calvaria were cryosectioned and photographed for Col2.3GFP expression by fluorescence microscopy.

2.6.3 RNA purification from osteoblast cultures was carried out using Nucleospin RNA kit (Macherey-Nagel) according to the manufacturer's instructions. cDNA was prepared from 1µg of sample RNA using the SuperScript II Reverse Transcriptase (ThermoFisher Scientific). QPCR was carried out using TaqMan Universal PCR Master Mix (ThermoFisher Scientific) in an ABI 7900HT Fast Real-Time PCR System (ThermoFisher Scientific). PCR primer sequences for gene expression analyses were designed by ThermoFisher Scientific and are listed in the table below.

Gene Abbreviation	Assay ID	Species
Alp	Mm00475831_m1	Mouse
Ibsp	Mm00492555_m1	Mouse
Osteocalcin	Mm03413826_mH	Mouse/Human
Dmp1	Mm00803833_g1	Mouse
Runx2	Mm00501580_m1	Mouse
Tnfa	Mm00443258_m1	Mouse
Opg (Tnfrsf11b)	Mm00435452_m1	Mouse
Rankl (Tnfsf11)	Mm00441908_m1	Mouse
Col1a2	Mm00483888_m1	Mouse
Gapdh	Mm99999915_g1	Mouse
Actb	Mm02619580_g1	Mouse
18S rRNA	4319413E	Mouse/Human

RNA samples were recovered from osteoblast cultures treated with control medium, 0.25 µg/ml Lipid 430 or 1 µg/ml Lipid 654, and the expression of three housekeeping genes was quantified in parallel with either osteocalcin or bone sialoprotein gene expression. The three housekeeping genes included 18S, Gapdh and Actb (β-Actin). For 18S gene expression, two different batches of primers were compared. Each trial was run in triplicate. One factor ANOVA demonstrated no significant difference between 18S expression, regardless of

primer batch, and Gapdh expression. Actb expression was significantly different from 18S expression for two comparisons (Fisher LSD, $p < 0.05$). These results indicate that 18S and Gapdh are equivalent housekeeping genes for monitoring osteoblast differentiation gene expression in osteoblasts in culture. However, Actb is not consistent with 18S and Gapdh as a housekeeping gene for monitoring osteoblast differentiation gene expression.

2.7 Statistical Analysis

Data are expressed as the mean $\pm$ standard error for data sets showing normal distribution characteristics. Statistical testing included Analysis of Variance (ANOVA) with pairwise comparisons using the Fisher LSD test or the student t-test for simple group mean comparisons. A p value of 0.05 was considered to be significant. StatPlus:mac® software was used for statistical testing.

Results

3.1 Calvaria from Col2.3GFP transgenic mouse pups (4–7 day old) on either a wild type background or a TLR2 knockout background (TLR2 KO) were digested with collagenase and osteoblasts were recovered as described in the Materials and Methods. The Col2.3GFP transgene indicating the expression of the collagen reporter (Col2.3GFP), is maximally expressed in osteoblasts during 18–21 days in culture. *P. gingivalis* Lipid 654 at 1 $\mu\text{g/ml}$ inhibited osteoblast differentiation (Col2.3GFP expression) and function (von Kossa stained mineral deposits) in wild type cells (Figure 1A) but not in TLR2 KO osteoblasts (Figure 1B). However, Lipid 430 (0.25 $\mu\text{g/ml}$) inhibited Col2.3GFP expression and von Kossa stained mineral deposits in TLR2 KO osteoblasts (Figure 1C) and wild type cells (data not shown). Col2.3GFP expression and von Kossa stained mineral deposits in TLR2 KO cells without lipid treatment are shown in Figure 1D. These results indicate that Lipid 654 inhibits osteoblast differentiation and function through engagement of TLR2 but Lipid 430 produced by PLA2 hydrolysis of Lipid 654 inhibits osteoblast function at considerably lower doses than Lipid 654 yet does not engage TLR2.

3.2 Additional in vitro trials ($n=3$) were carried out using replicate cell isolates of osteoblasts derived from Col2.3GFP wild type or TLR2 KO:Col2.3GFP mouse pups. GFP expression and mineral deposit formation were quantified from digitally acquired images using the ImageJ software [14]. The summarized results in Figure 2A and 2B show significant inhibition of osteoblast differentiation and function in wild type Col2.3GFP cells but not in TLR2 KO:Col2.3GFP cells treated with Lipid 654 of *P. gingivalis*. For comparison, cells were treated with either *P. gingivalis* total lipid extract (Pg TL, 0.5 $\mu\text{g/ml}$) or lipoteichoic acid (LTA, 0.1 $\mu\text{g/ml}$, TLR2 positive control). Note that Lipid 430 inhibited osteoblast differentiation and function equally for cells derived from both wild type and TLR2 KO cells. Lipid 430 inhibited osteoblasts to a similar extent as Lipid 654 but at dose that was four to ten fold less than Lipid 654. On a molar basis, Lipid 430 is between 2.5 and 6.5 times more potent than Lipid 654. This composite analysis confirmed that lipid 654 inhibits osteoblast differentiation through engagement of TLR2 and that lipid 430 does not appear to engage TLR2 but still markedly inhibits osteoblasts.

3.3 In addition to the in vitro cultures, we also examined the effects of Lipid 430 and Lipid 654 in vivo. Lipid 430 (1 µg in 50 µl of PBS) or Lipid 654 (5 µg in 50 µl of PBS) was injected subcutaneously to the calvaria of mice (Figure 3). Both Lipid 430 and Lipid 654 inhibited in vivo osteoblast differentiation in Col2.3GFP wild type mice calvaria. However, Lipid 430 inhibited Col2.3GFP expression in TLR2 KO calvaria but Lipid 654 did not substantially inhibit Col2.3GFP expression in TLR2 KO mice. These results indicate that the inhibitory action of Lipid 654 on osteoblasts, but not Lipid 430, is mediated in vivo through engagement of TLR2.

3.4 These lipid effects were further analyzed by examining osteoblast gene expression by RTqPCR (Figure 4). Osteoblast gene expression included osteoprotegerin (OPG), osteocalcin, alkaline phosphatase (ALP), bone sialoprotein (iBSP) and dentin matrix protein 1 (DMP1). All doses of Lipid 654 significantly inhibited osteoblast gene expression as did *P. gingivalis* total lipid (PG TL), Lipid 430 and LTA (A). In contrast, TNF-α, RANKL, Col1a2 and RUNX2 gene expression was not significantly affected by Lipid 654 treatment. Therefore, inhibition of osteoblast differentiation genes in these cultures is not paralleled by similar changes in other genes associated either with osteoclast activation (TNF-α, RANKL) or osteoblast development (Col1a2 and RUNX2). Increased TNF-α gene expression could reflect the activity of cells other than osteoblasts in these cultures.

3.5 Next we evaluated Lipid 654 dose responses for osteocalcin, iBSP and DMP1 gene expression in either wild type or TLR2 KO osteoblasts as shown in Figure 5. Osteoblast gene expression in lipid treated cultures was normalized to the control gene expression (arbitrarily set to 1) and subsequently was normalized to 18S RNA gene expression. Negative values indicate the fold inhibition of gene expression relative to control cultures whereas positive values indicate stimulation of gene expression over control osteoblasts. This analysis revealed that Lipid 654 inhibits osteoblast gene expression in wild type cells in a dose-dependent manner but does not in TLR2 KO cells. Furthermore, lipopolysaccharide (LPS from *Salmonella typhimurium*, 1 µg/ml) inhibited osteoblast gene expression equally in both wild type and TLR2 KO osteoblasts. LPS is known to act through TLR4 and therefore produces the same responses in wild type and TLR2 KO osteoblasts.

3.6 Furthermore, we evaluated the effect of Lipid 430 on osteoblast differentiation using the same target genes evaluated with Lipid 654 treatment. Wild type osteoblasts were exposed to increasing doses of Lipid 430 and osteoblast differentiation genes were significantly inhibited relative to controls by Lipid 430 (Figure 6A). However, Lipid 430 significantly inhibited gene expression in TLR2 KO cells with all doses of Lipid 430 except for osteocalcin expression with 0.05 µg/ml of Lipid 430. Lipid 430 inhibited osteoblast gene expression to a greater extent in wild type cells when compared with TLR2 KO cells. These results show that Lipid 430 inhibits osteoblast gene expression partially through TLR2 although it should be emphasized that Lipid 430 from *P. gingivalis* contains less than 10% Lipid 654 as a contaminant (Nemati et al., submitted). This evidence shows that other cell activation processes beyond TLR2 engagement must be involved with the Lipid 430 inhibition of osteoblast function.

3.7 Other osteoblast genes of importance include those listed in Figure 6B. Lipid 430 significantly simulated RANKL expression in wild type cells with all doses. TNF- α expression was also significantly elevated in wild type cells but only at lower doses of Lipid 430. Col1a2 and RUNX2 gene expression were unaffected by Lipid 430 for both wild type and TLR2 KO cells. In contrast to osteoblast gene expression shown on Figure 4A and 4B, Lipid 430 significantly stimulated RANKL and TNF- α expression. Although the TLR2 KO cells showed elevated RANKL expression, this finding is inconsistent with RANKL expression in Figure 4B and is therefore assumed to be a spurious result.

3.8 Since PLA2 releases arachidonic acid from phospholipids as a requisite step for prostaglandin synthesis, we investigated the levels of prostaglandin (PG) E₂ and the ratio of Lipid 430 to Lipid 654 in media samples from calvarial cell cultures. Media samples from osteoblast cultures were evaluated by LC-MS for PGE₂ synthesis as shown in Figure 7A. PGE₂ levels in control media samples increased with longer time in culture. Indomethacin (10⁻⁵ M) prevented the increase in PGE₂ levels as expected given its capacity to inhibit cyclooxygenase (COX) 1 and COX-2. Lipid 654 treatment significantly increased the levels of PGE₂ in osteoblast medium relative to controls but only for the 18–21 day time intervals. However, Lipid 430 treatment had no significant effect on PGE₂ release. Because PGE₂ release increased from 14 to 21 days of culture, cellular PLA2 is presumed to be elevated for arachidonic acid release and subsequent conversion to PGE₂. In order to detect Lipid 430 levels in culture media samples it was necessary to pool media samples by treatment category from four replicate experiments. Figure 7B shows the change in Lipid 430 to Lipid 654 ratio as determined using ESI-MRM-MS mass spectrometry. The highest ratios were observed with cells exposed to Lipid 654 during the 16–18 day interval or the 18–21 day interval depending on the dose of Lipid 654 used to treat the cells. These results suggest that increased PLA2 activity in calvarial cultures associated with osteoblast differentiation and increased PGE₂ release, is also associated with increased conversion of Lipid 654 to Lipid 430.

Discussion

4.1 Alveolar bone loss around teeth afflicted with chronic periodontitis is thought to occur through both activation of osteoclast mediated bone resorption as well as inhibition of bone formation [15]. The present study is primarily directed toward the issue of inhibition of bone formation although the lipid classes described here increase TNF- α secretion from mouse macrophages (see below). Chronic periodontitis, in contrast to gingival health or gingivitis, is associated with significantly elevated accumulation of bacterial lipids in gingival tissues that contain 3-OH isobranched (iso) C_{17:0} [16]. Over the past twenty years, the complex lipids of *P. gingivalis* that contain 3-OH iso C_{17:0} have been identified and evaluated for biological responses [5, 12, 17, 18]. More recently, other investigators have demonstrated that oral infection with *P. gingivalis* in experimental animals mediates bone loss around teeth through engagement of TLR2 and not TLR4 [1–3]. Lack of TLR4 involvement in periodontal bone loss following oral infection with *P. gingivalis* suggested that LPS from *P. gingivalis* or other phylogenetically related periodontal organisms is not involved in alveolar bone loss following oral infection with *P. gingivalis*. Evaluation of HPLC-separated lipid fractions from *P. gingivalis* in HEK cells stably transfected to express TLR2 revealed that

two serine dipeptide lipids, including Lipid 654 and Lipid 430 classes, engage both human and mouse TLR2 [5]. However, the present work shows that Lipid 430, generated from Lipid 654 by treatment with phospholipase A2, will inhibit osteoblast differentiation just as is observed with Lipid 654 but only with partial engagement of TLR2. Importantly, Lipid 430 produces effects on osteoblasts at mass doses that are typically four to ten fold lower than Lipid 654. Although Lipid 430 produced with PLA2 hydrolysis of Lipid 654 has identical MS/MS spectra to Lipid 430 isolated from *P. gingivalis* and synthetic lipid 430 standard (unpublished data) the PLA2-generated Lipid 430 contained substantially less contaminating Lipid 654 when compared with Lipid 430 isolated from *P. gingivalis*. The Lipid 654 contamination of PLA2-generated Lipid 430 is a likely reason for the minimal but measurable engagement of TLR2 by enzymatically generated Lipid 430 but this will require additional work to establish.

4.2 *P. gingivalis*, a primary periodontal pathogen, produces a variety of virulence factors that could promote periodontal bone loss either through increased osteoclast activity or inhibition of bone formation, or both. *P. gingivalis* virulence factors that are reported to be TLR2 agonists include LPS [19], fimbriae [20], phosphoethanolamine dihydroceramides [21], lipoprotein [22] and serine dipeptide lipids [5]. A recent report revealed that fimA (the major fimbrial subunit in *P. gingivalis*), lipid A and LPS of *P. gingivalis* are not significant TLR2 agonists [23] and in the case of LPS, another report indicated that a lipoprotein contaminant of *P. gingivalis* LPS accounts for the observed TLR2 engagement [24]. Phosphoethanolamine dihydroceramides (PE DHC) have been discounted as TLR2 agonists because PE DHC lipids previously reported to engage TLR2 [21] have subsequently been shown to contain significant levels of Lipid 654. A recent report has also shown that lipid A prepared from the LPS of *P. gingivalis* is contaminated with Lipid 654 [5] and this could account for the TLR2 activity previously attributed to *P. gingivalis* LPS [19] or lipid A [25]. Our previous work supports the conclusion that lipids containing 3-OH iso C_{17:0} recovered in gingival tissue samples directly correlate with the presence of chronic periodontitis in humans [16]. Synthetic lipopeptide of *P. gingivalis* does not contain 3-OH iso C_{17:0} yet this product engages TLR2 [26] and there are no reports demonstrating elevated levels of *P. gingivalis* lipoprotein in gingival tissues from chronic periodontitis sites. Since the dominant form of Lipid 654 produced by *P. gingivalis* contains 3-OH iso C_{17:0}, the serine dipeptide lipids of *P. gingivalis* appear to be critical virulence factors in gingival tissues that could promote TLR2-dependent bone loss at chronic periodontitis sites.

4.3 The present study compared the capacity of two serine dipeptide lipid preparations of *P. gingivalis*, including Lipid 654 and Lipid 430, to affect osteoblast differentiation and function in vitro and in vivo. Lipid 654, which is the dominant form of serine lipid produced by *P. gingivalis*, was shown to inhibit osteoblast differentiation and function in vitro at doses of 0.5 to 1 µg/ml in vitro when dispersed as liposomes and delivered to adherent calvarial cells in culture. Lipid 654 also inhibited osteoblasts through engagement of TLR2. Our previous report demonstrating TLR2 dependent inhibition of osteoblast differentiation in culture by the total lipid extract of *P. gingivalis* is likely accounted for by Lipid 654 since no other major lipid classes of *P. gingivalis* have been shown to engage TLR2. Lipid 654 is a prevalent lipid class of *P. gingivalis* and virtually all *Bacteroidetes* recovered from the

gingival crevice produce Lipid 654 [5]. Therefore many genera of organisms of the human periodontal microbiome are capable of producing Lipid 654 that could participate in the progression of periodontal disease. We also demonstrate here that the de-esterified form of Lipid 654, called Lipid 430, produced through the action of phospholipase A2 (PLA2) is also a potent inhibitor of osteoblasts. Lipid 430 is at least four times more potent than Lipid 654 on a mass basis and is at least 2.5 times more potent than Lipid 654 on a molar basis. However, Lipid 430 is relatively weak in engaging TLR2. In the present report, we observed conversion of Lipid 654 to Lipid 430 in calvarial cells. This finding suggests that osteoblasts could affect the relative engagement of TLR2 by metabolically converting Lipid 654 to Lipid 430. We have previously observed that either porcine pancreatic, honey bee venom or human recombinant secretory PLA2 will convert Lipid 654 to Lipid 430 (data not shown). Activated macrophages are also known to produce secretory PLA2 [7, 8] and resident macrophages associated with calvarial osteoblast cultures could promote the conversion of Lipid 654 to Lipid 430. Furthermore, inflammatory cells including macrophages and neutrophils [9] associated with chronic periodontal disease sites could contribute to increased Lipid 430 levels through their expression of secretory or cytosolic PLA2, respectively. Identifying the specific form of PLA2, of which there are over a dozen different forms, that mediates conversion of Lipid 654 to Lipid 430 will be the subject of future investigation. We have also observed that Lipid 654 and Lipid 430 both increase TNF- α secretion from RAW 264.7 mouse macrophage cells but neither lipid class directly promotes osteoclastogenesis in M-CSF primed bone marrow cells (data not shown). These results suggest that Lipid 654 and Lipid 430 can mediate increased osteoclast-mediated bone resorption indirectly by stimulating TNF- α secretion from macrophages. This issue is not the focus of this report but will be the subject of future investigation.

4.4 It should be noted that serine dipeptide lipids are recovered in human blood [27] and carotid atheromas [5], and all *Bacteroidetes* of the gastrointestinal tract evaluated to date produce these lipids [5]. Therefore, long-term systemic exposure to low levels of these serine dipeptide lipids could affect the general homeostasis of bone throughout the skeleton. It is also important to note that these serine lipids accumulate on tooth surfaces and in bacterial calculus attached to teeth at periodontal disease sites indicating their capacity to adsorb to mineralized surfaces [5]. Of note, the relatively poorly understood sudanophilic zone reported to be a mucopolysaccharide zone surrounding mineralizing bone [28, 29] could be an important site for these bacterial lipids to accumulate and alter behavior of bone cells later in life. Finally, ovariectomy-induced bone loss is reported to be attenuated in TLR2 KO mice [30] suggesting a potential role of TLR2 in postmenopausal osteoporosis. Because of the established presence of Lipid 654 in blood of humans [27], the question of Lipid 654 presence in bone, particularly in pathologically altered bone in osteopenia and osteoporosis, should be considered.

4.5 RUNX2 and Col1a2 gene expression in calvarial cells was unchanged by Lipid 654 and Lipid 430 indicating that the function of these genes in osteoblast development is largely unaffected by Lipid 430 and Lipid 654. This evidence suggests that Lipid 654 and Lipid 430 inhibit osteoblast gene expression downstream of RUNX2 gene expression which could involve post-translational modification RUNX2 protein and subsequent alteration in

osteoblast gene expression [31]. This will be the subject of future investigation. Of the osteoblast differentiation genes inhibited by Lipid 654 and Lipid 430, our results show consistent inhibition of osteocalcin, bone sialoprotein and dentin matrix protein gene expression following exposure to either bacterial serine lipid class. Of note, Lipid 430 but not Lipid 654 significantly increased both TNF- α and RANKL gene expression in wild type calvarial cells but not in TLR2 KO cells. Only low doses of Lipid 430 significantly stimulated TNF- α gene expression. The possibility exists that cell types other than osteoblasts account for these changes in TNF- α gene expression. Inhibition of osteoblast differentiation by the serine lipid classes of *P. gingivalis* is consistent with changes in gene expression but the effects of these lipid classes on osteoclast activation factors is primarily attributed to Lipid 430 and needs further evaluation.

Conclusions

5.1 In summary, this investigation has shown that a new group of virulence factors of *P. gingivalis* are capable of inhibiting the osteoblast phenotype characteristics and gene expression in culture and in the case of Lipid 654, inhibition of osteoblast differentiation is mediated strongly through TLR2. Evidence is presented that Lipid 430 inhibits osteoblast differentiation in part through engagement of TLR2 but can also stimulate gene expression of factors known to activate osteoclasts and this effect is dependent on the expression of TLR2 in calvarial cell cultures. Because these serine dipeptide lipids are produced by known periodontal pathogens, we conclude that serine dipeptide lipids likely participate in bone loss associated with chronic periodontitis.

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Abbreviations used

(TLR2)	Toll-like receptor 2
(PLA2)	phospholipase A2
(ESI-MS or MS/MS)	electrospray-mass spectrometry
(QTOF)	ESI-quadrupole time of flight
(TLR2 KO)	TLR2 knockout
(ANOVA)	Analysis of Variance
(LTA)	lipoteichoic acid
(OPG)	osteoprotegerin
(ALP)	alkaline phosphatase
(iBSP)	bone sialoprotein
(DMP1)	dentin matrix protein 1

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Highlights

- This manuscript shows that bacterial serine dipeptide lipids inhibit osteoblast differentiation and function.
- One class of the serine dipeptide lipids produces these effects on osteoblasts through engagement of Toll-like receptor 2 (TLR2).
- Another related serine dipeptide lipid class only partially engages TLR2.
- The bacterial serine dipeptide lipids are likely to influence the alveolar bone loss associated with destructive periodontal disease.

Figure 1.

Effects of *P. gingivalis* Lipid 654 and Lipid 430 on Col2.3GFP expression and mineral deposit formation in wild type and TLR2 KO calvarial cells. Calvarial cells were isolated as described in the Materials and Methods. Lipid preparations were sonicated in PBS and added to culture wells at the indicated concentrations. GFP fluorescence indicates differentiated osteoblasts expressing the Col2.3GFP reporter. The mineral deposits are visualized as von Kossa silver stained nodules. Wild type cells treated with Lipid 654 (1 $\mu\text{g/ml}$) are shown in A, TLR2 KO cells treated with Lipid 654 (1 $\mu\text{g/ml}$) are shown in B, TLR2 KO cells treated with Lipid 430 (0.25 $\mu\text{g/ml}$) are shown in C and untreated control cells are shown D. TLR2 KO control cells appear as the TLR2 KO cells treated with Lipid 654.

Figure 2.

Effects of *P. gingivalis* Lipid 654, Lipid 430 and total lipids on Col2.3GFP expression and mineral deposit formation in wild type and TLR2 KO calvarial cells. The results are depicted as the percent GFP or VK stained areas for the treated culture normalized to the untreated culture area (control) in the same culture dish. GFP and VK stained areas were acquired using the ImageJ program and are averaged for three experiments. One factor ANOVA with pairwise comparisons (Fisher LSD) revealed significant differences between wild type (WT) and TLR2 KO calvarial cells for cultures treated with Lipid 654 (either 0.5 $\mu\text{g/ml}$ or 1.0 $\mu\text{g/ml}$) or *P. gingivalis* total lipids (Pg TL 0.5 $\mu\text{g/ml}$). Lipid 430 (0.1 or 0.25 $\mu\text{g/ml}$) substantially inhibited both Col2.3GFP expression (Frame A) and von Kossa stained nodules (Frame B) for both wild type and TLR2 KO cells. For cultures treated with Lipid 654 or *P. gingivalis* total lipids, the brackets indicate significant differences between wild type and TLR2 KO responses for either GFP expression and von Kossa mineral deposit formation (by one factor ANOVA with pairwise comparisons using Fisher LSD test, $p < 0.05$, denoted by brackets).

Figure 3.

Effect of *P. gingivalis* Lipid 654 and Lipid 430 on Col2.3GFP expression in vivo. Lipids were sonicated in PBS (30 sec, 3 watts). Col2.3GFP wild type or TLR2 KO:Col2.3GFP mice were lightly anesthetized with isoflurane and phosphate buffered saline (PBS control), Lipid 654 (5 μ g/50 μ l) or Lipid 430 (1 μ g/50 μ l) was administered subcutaneously to the calvarial surface with a Pressure Lok® syringe. The solution was administered from anterior to posterior as the syringe needle was withdrawn over the surface of the parietal bone. One week after administration of lipids, animals were euthanized and calvaria harvested. Calvaria were cryosectioned and evaluated by fluorescence microscopy for Col2.3 GFP expression (green). The frontal section of the calvaria (upper frame) shows the location of the subcutaneous administration of either vehicle control (PBS) or serine lipids. The middle frames show the effect of Lipid 654 and Lipid 430 on Col2.3GFP expression in wild type animals or TLR2 KO animals. The frame at right shows the GFP expression evaluated using serial sections (n=3) of each calvaria specimen and GFP fluorescence area was quantified using ImageJ. The surveyed area for each calvaria section was consistently of the same size and captured only the midportion of the outer cortical layer of each section. One factor ANOVA revealed significant differences with pairwise comparisons (using Fisher LSD test, $p < 0.05$, denoted by brackets).

Figure 4.

Effects of Lipid 654 and Lipid 430 on calvarial cell gene expression. Calvarial cells were obtained from wild type mice and were cultured for 21 days. Cellular RNA was extracted and after reverse transcription, was evaluated by RTqPCR for the indicated genes. Gene expression for osteoprotegerin (OPG), osteocalcin, alkaline phosphatase (ALP), bone sialoprotein (iBSP), and dentin matrix protein (DMP1) were significantly inhibited relative to controls (one factor ANOVA with pairwise comparisons using Fisher LSD, $p < 0.05$) with exposure to Lipid 654 at all doses, Lipid 430 (0.5 $\mu\text{g/ml}$), *P. gingivalis* total lipid and lipoteichoic acid (LTA) (Figure 4A). However, TNF- α , RANKL, Col1a2 and RUNX2 gene expression was not significantly affected by the same culture treatments (Figure 4B).

Figure 5.

Osteoblast gene expression in either wild type or TLR2 KO cells exposed to increasing levels of Lipid 654. Cells were obtained from wild type or TLR2 KO mice as described in the Materials and Methods. Cells were treated with the indicated levels of Lipid 654 as described and gene expression was determined in RNA extracts from day 21 cultures. Gene expression in control cultures and lipid treated cultures were individually normalized to 18S RNA and the lipid treated gene expression levels were divided by the respective control gene expression levels. Negative numbers on the Y axis indicate the fold inhibition of gene expression whereas positive numbers indicate the fold stimulation of gene expression. Lipid 654 showed a dose response inhibition of osteocalcin, bone sialoprotein and dentin matrix protein in wild type cells but not TLR2 KO cells. Lipopolysaccharide (LPS, *Salmonella typhimurium*, 1 μ g/ml) was the control (TLR4 agonist) and showed equal inhibition of osteoblast gene expression in wild type and TLR2 KO cells.

Figure 6.

Effect of Lipid 430 on calvarial cell gene expression. Cells were obtained from wild type mice and were cultured for 21 days. Cells were treated with the indicated levels of Lipid 430 as described in the Materials and Methods and gene expression was determined in RNA extracts from day 21 cultures. Cellular RNA was extracted and evaluated by RTqPCR for the indicated genes. Osteoprotegrin (OPG), bone sialoprotein (iBSP), and dentin matrix protein (DMP1) expression was significantly lower than controls in wild type cells (one factor ANOVA with pairwise comparisons using Fisher LSD, $p < 0.05$) with exposure to all doses of Lipid 430 and *P. gingivalis* total lipid (Figure 6A). TLR2 KO cells showed less inhibition by Lipid 430 but all genes were significantly inhibited relative to controls except for osteocalcin at 0.05 $\mu\text{g/ml}$ (one factor ANOVA with pairwise comparisons using Fisher LSD, $p < 0.05$). TNF- α and RANKL expression in wild type cells was significantly stimulated by Lipid 430; in the case of RANKL, all doses of Lipid 430 significantly stimulated RANKL expression whereas only 0.5 $\mu\text{g/ml}$ of Lipid 430 significantly stimulated TNF- α expression (Figure 6B). However, RUNX2 and Col1a2 expression in wild type cells was not affected by treatment with Lipid 430 (Figure 6B). In contrast, TNF- α , RANKL, RUNX2 and Col1a2 expression was unaffected by increasing doses of Lipid 430 in TLR2 KO cells.

Figure 7.

Prostaglandin E₂ and Lipid 654 hydrolysis to Lipid 430 in calvarial cell cultures. Wild type cells were isolated and cultured for 21 days. Cells were treated with *P. gingivalis* total lipid, Lipid 654, Lipid 430, indomethacin and lipoteichoic acid for the indicated time intervals. At 21 days the media samples from each well were collected for analysis. Media samples were supplemented with D₄-PGE₂ (10 ng/ml) and were processed as described in the Materials and Methods. PGE₂, Lipid 654 and Lipid 430 were quantified by ESI-MRM analysis as described. PGE₂ levels in media samples (n=4 for each treatment) were significantly elevated in 18–21 day cultures compared to 14–16 day cultures and only in cultures treated with 0.5 µg/ml and 1.0 µg/ml of Lipid 654 but not Lipid 430 (Figure 7A, two factor ANOVA with pairwise comparisons using Fisher LSD test, p<0.05, denoted by brackets). Because Lipid 430 levels were low in individual samples, the culture media samples for each treatment group were pooled and re-evaluated for Lipid 430 and Lipid 654 using ESI-MRM. The results in Figure 7B show increased Lipid 430/Lipid 654 ratios with 16–18 day media samples or the 18–21 day media sample depending on the dose of Lipid 654 administered to the calvarial cell cultures. Only one trial is depicted for each of the disease of Lipid 654.