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MINIREVIEW

Are Sphingolipids and Serine Dipeptide Lipids underestimated Virulence Factors of *Porphyromonas gingivalis*?

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ABSTRACT

The keystone periodontal pathogen *Porphyromonas gingivalis* produces phosphorylated dihydroceramide lipids (sphingolipids) such as phosphoethanolamine dihydroceramide (PE DHC) and phosphoglycerol dihydroceramide (PG DHC) lipids. Phosphorylated DHCs (PDHCs) from *P. gingivalis* can affect a number of mammalian cellular functions such as potentiation of prostaglandin secretion from gingival fibroblasts, promotion of RANKL-induced osteoclastogenesis, promotion of apoptosis and enhancement of autoimmunity. In *P. gingivalis*, these lipids affect anchoring of surface polysaccharides, resistance to oxidative stress and presentation of surface polysaccharides (anionic polysaccharides and K-antigen capsule). In addition to phosphorylated dihydroceramide lipids, serine dipeptide lipids of *P. gingivalis* are implicated in alveolar bone loss in chronic periodontitis through interference with osteoblast differentiation and function, and promotion of osteoclast activity. As a prerequisite for designation as bacterial virulence factors, bacterial sphingolipids and serine dipeptide lipids are recovered in gingival/periodontal tissues, tooth calculus, human blood, vascular tissues and brain. In addition to *P. gingivalis*, other bacteria of the genera *Bacteroides, Parabacteroides, Porphyromonas, Tannerella* and *Prevotella* produce sphingolipids and serine dipeptide lipids. The contribution of PDHCs and serine dipeptide lipids to the pathogenesis of periodontal and extraoral diseases may be an underappreciated area for microbial-host interaction and should be more intensively investigated.

KEYWORDS: *P. gingivalis*, virulence factors, sphingolipids, ceramides, periodontitis, extraoral diseases

INTRODUCTION

Sphingolipids, as amphipathic molecules, demonstrate both hydrophobic and hydrophilic constituents and, as such, have long been considered little more than structural lipid components of eukaryotic cell membranes. More recent studies, however, have shown that mammalian sphingolipids such as ceramide (the simplest sphingolipid), sphingosine and sphingosine-1-phosphate lipids, are central to a number of important biological processes. Mammalian sphingolipids are known to make complexes with proteins and other cell membrane lipids, often sterols, to form so-called lipid rafts (1, 2). Lipid rafts are fluctuating nanoscale collections of sphingolipid, cholesterol and proteins in cell membranes, constituting platforms that operate in membrane signaling and trafficking. Of note, most bacteria of the Bacteroidetes phylum and a few bacteria of the Chlorobi phylum (3) produce sphingolipids but only members of the Bacteroidetes phylum are reported to produce novel serine dipeptide lipids (4-7). These lipid classes demonstrate amphipathic characteristics and possess important biological properties. When members of the Bacteroidetes phylum, particularly oral Bacteroidetes, contact human cells, bacterial sphingolipids and serine lipids may incorporate into the eukaryotic cell membranes thereby potentially affecting cell function. As an example, lipid raft components are used by the periodontal pathogen *Porphyromonas gingivalis* (8), for entry into host epithelial cells (9, 10).

Chronic periodontitis is manifested as progressive loss of periodontal attachment and alveolar bone, leading to pathological pocket formation around the teeth. If left untreated, it can lead to loss of teeth. Chronic periodontitis affects approximately 65 million (47 %) US adults of 30 years and older (11). Chronic periodontitis has also been associated with extraoral diseases such as cardiovascular diseases, diabetes, preterm birth, Alzheimer's disease, rheumatoid arthritis and pancreatic cancer (12-14). The keystone periodontal pathogen *P. gingivalis* produces many virulence factors of which the major classes include lipopolysaccharide (LPS), a polysaccharide-rich capsule, gingipains, fimbriae and peptidyl-arginine deiminase. *P. gingivalis* LPS is considered to be important in bone destruction in periodontitis. However, previous reports have shown that LPS of *P. gingivalis* is present only in minor amounts in diseased periodontal tissues from humans (15, 16). In contrast to LPS, *P. gingivalis* dihydroceramides (DHCs) (17, 18) and

serine dipeptide lipids (19) are exposed to diseased gingival tissues at levels capable of inducing an inflammatory response (20).

Within the diseased periodontal crevice (pocket), *P. gingivalis* is known to directly contact and attach to sulcular epithelial cells (21) (FIGURE 1), and co-culture of *P. gingivalis* with epithelial cells reveals an ultrastructural thickening of the merged membranes as *P. gingivalis* is internalized (22). This cell membrane contact could deliver bacterial lipids directly to the cell membranes of host epithelial cells. Another mechanism for bacterial lipid entry into cells is demonstrated by the uptake of total lipids of *P. gingivalis* into human gingival fibroblasts when cells are exposed to lipid films in culture (20). Therefore, *P. gingivalis* lipids are likely transferred to cells of gingival tissues either by direct contact with bacteria or by chemical diffusion from lipid contaminated surfaces of diseased teeth. Either process can lead to deposition of bacterial lipids into eukaryotic cell membranes, thereby exposing cells, including lipid rafts, to elevated levels of non-mammalian sphingolipids and serine dipeptide lipids. In contrast to periodontal Bacteroidetes, gastrointestinal *Bacteroides* are recovered primarily in the colon (3) but little is known about breakdown of these bacterial sphingolipids in the colon or their transport within this organ.

P. gingivalis produces several classes of phosphorylated dihydroceramides (PDHCs) as well as other novel complex lipids (see TABLE 1, FIGURE 2 and 3) including serine dipeptide lipids. The first to be characterized were the sphingolipids which are quite similar but not identical to mammalian sphingolipids. PDHCs can be detected in, quantitated and distinguished from eukaryotic sphingolipids using multiple reaction monitoring (MRM)-mass spectrometry (MS) (23). The sphingoid bases in *P. gingivalis* DHC lipids comprise saturated aliphatic chains of 17, 18 or 19 carbons with *iso*branching of the odd numbered carbon chains (see FIGURE 2) (17, 18). The sphingoid base is designated sphinganine (dihydrosphingosine) or dihydroceramide (DHC) when amide-linked to a fatty acid chain, which in *P. gingivalis* sphingolipids is usually 3-OH *iso*branched $C_{17:0}$ (see FIGURE 2) (18). From *P. gingivalis* lipid extracts, three major DHC species have been recognized: the free DHC, phosphoethanolamine DHC (PE DHC) and phosphoglycerol DHC (PG DHC) lipids (17, 18). The free DHC lipids are presumed to serve as precursor core structures for the synthesis of PE DHC and PG DHC lipids (see FIGURE 2). The *iso*branched aliphatic chains of phosphoglycerol dihydroceramides and

phosphatidylethanolamines (PEA) of *P. gingivalis* (see FIGURE 2) are thought to be partially responsible for the bioactivity of these lipids (24). PG DHC can be further modified with the addition of *iso*branched $C_{15:0}$ to the 3-OH group of the dihydroceramide core fatty acid chain (18). This lipid was later termed substituted (sub) PG DHC but in some prior reports, the term PG DHC was used instead of sub PG DHC. However, if PG DHC lipids are not substituted with *iso*$C_{15:0}$ or other fatty acids, this class was specifically designated as unsub PG DHC lipids in previous reports (see FIGURE 2).

Contrasted with the structures of PDHC lipids are the serine dipeptide lipids of *P. gingivalis*, including Lipid 654 and Lipid 430 (FIGURE 3). Lipid 654 of *P. gingivalis*, which is the dominant serine dipeptide lipid class of *P. gingivalis*, was first described in *Fusobacterium meningosepticum* and was termed flavolipin (7). Flavolipin was originally reported to be a Toll-like receptor 4 ligand (4) but recent work has shown that Lipid 654 is a TLR2 ligand rather than a TLR4 ligand (19, 25). Toll-like receptors are known to recognize exogenous ligands and are reported to function within lipid rafts (26). Just as with *P. gingivalis* sphingolipids, serine dipeptide lipids are recovered in diseased periodontal tissues (19) as well as blood (27) and artery walls of humans (6). Of physiological importance is that the mammalian enzyme phospholipase A2 (PLA2), typically expressed in chronic inflammatory reactions, hydrolyzed Lipid 654 of *P. gingivalis* to another serine lipid class called Lipid 430 (5, 6). Just as with Lipid 654, Lipid 430 also activated human embryonic kidney cells transfected with human TLR2 and also increased serum CCL2 (MCP-1) levels in wild type mice but not TLR2 knockout mice (19). However, mouse osteoblast function and differentiation were markedly inhibited by Lipid 430 regardless of TLR2 status (25) . In this brief review, focus is directed toward sphingolipids and serine dipeptide lipids of *P. gingivalis*, and their potential roles as virulence factors, particularly as they relate to bone loss in chronic periodontitis. Sphingolipids in both bacteria and fungi have previously been reviewed (28).

POTENTIATION OF PROSTAGLANDIN SECRETION FROM GINGIVAL FIBROBLASTS

It is reasonable to suspect that penetration of bacterial lipids into gingival tissues combined with chronic inflammation can promote destructive periodontitis. *P. gingivalis* total lipids (20), PG DHC lipids (18) and phosphatidylethanolamine lipids (PEA) (24) promoted interleukin-1β (IL-1β)-mediated secretion of inflammatory mediators (prostaglandin E_2 (PGE_2) and 6-keto prostaglandin $F_{2\alpha}$) from human gingival fibroblasts and changed their cellular morphology in culture (18, 24). The potentiation of PGE_2 synthesis and secretion from fibroblasts, possibly mediated through TLR2, could be an important mechanism for *P. gingivalis* to promote inflammatory reactions and change host responses including osteoclast mediated bone resorption, thereby promoting tissue breakdown in periodontal disease.

PROMOTION OF RANKL-INDUCED OSTEOCLASTOGENESIS

A possible effect of PG DHC on alveolar bone destruction, which is an essential feature of periodontitis, has been reported recently. PG DHC lipids were found to promote receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclastogenesis by interacting with non-muscle myosin IIA (Myh9) (29). The latter is an osteoclast cell fusion regulatory cell protein localized to the cytoplasm of host cells. Myh9 elicited a signal that made Ras-related C3 botulinum toxin substrate 1 (Rac1) upregulate the expression of dendritic cell-specific transmembrane protein (DC-STAMP). The latter is known as a key osteoclast fusogen that is responsible for the cell fusion process during osteoclastogenesis. Noteworthy, the process depended on Rac1/DC-STAMP and not on TLR2/TLR4 engagement. Thus, instead of binding to TLR2/4 expressed on the cell surface, PG DHC interacted with a cytoskeletal protein that is localized to the cytoplasm. In addition, non-muscle myosin IIA (Myh9) produced a cell signal that involved Rac1 to upregulate the expression of DC-STAMP. This key osteoclast fusogen is responsible for the process of cell fusion during osteoclastogenesis. Further, the study clearly showed that PG DHC could penetrate cell membranes of osteoclast precursors and enter the nuclei. This brought significant new insight into the process of macrophage fusion required for osteoclast formation and promotion of osteoclast-induced bone breakdown associated with the development of chronic periodontitis.

PDHC AND SERINE DIPEPTIDE LIPID INTERFERENCE WITH OSTEOBLAST DIFFERENTIATION AND OTHER CELLULAR EFFECTS

Total lipids from *P. gingivalis* and PE DHC and PG DHC lipids prepared free of lipid A were studied for their effects on primary calvarial osteoblast cultures from mice (30). This investigation revealed that osteoblast differentiation and fluorescent transgene expression for calvarial osteoblast differentiation (the transgene for the rat type I collagen promoter fragment pOBCol2.3GP) (31), were inhibited in a concentration-dependent manner. Osteoblast proliferation, viability or apoptosis were not markedly affected. Simultaneously, common osteoblast differentiation genes were downregulated (*Runx2, ALP, OC, BSP, OPG* and *DMP-1*) whereas RANKL, tumor necrosis factor alpha and *MMP-3* genes were upregulated. Mineral nodule formation *in vitro* was prevented. Total lipids and PE DHC and PG DHC lipid fractions of *P. gingivalis* also inhibited calvarial osteoblast gene expression and function *in vivo.* These lipid preparations inhibited osteoblasts through TLR2 engagement, which agrees with observations on alveolar bone loss in animals infected orally with *P. gingivalis* (32-34). They could therefore act as a microbial virulence factor in periodontitis by inhibiting osteoblast function and gene expression. More recent work isolated a serine dipeptide lipid fraction from *P. gingivalis* lipids using HPLC fractionation with an acidic solvent together with improved HPLC equipment capable of considerably improved chromatographic resolution. Though serine dipeptide lipids (6, 25) are minor lipid constituents of the total lipids of *P. gingivalis*, this approach allowed the purification of serine dipeptide lipids essentially free of contaminating phosphorylated dihydroceramides and other complex lipids. The serine dipeptide lipid class, Lipid 654, has since been shown to account for the TLR2-dependent inhibition of osteoblast differentiation and function (25). It was reported that PE DHC lipids of *P. gingivalis* also increased secretion of IL-6 from dendritic cells *in vitro* (35) and this effect was mediated through engagement of TLR2. It was later determined using improved HPLC equipment that trace amounts of Lipid 654 contaminating the PE DHC lipid preparation were likely accounting for the TLR2-mediated effect on dendritic cells (see FIGURE 3) (19). The serine dipeptide lipids may also promote extraoral inflammatory diseases, e.g., cardiovascular diseases (6). This idea is supported by the fact that dihydroceramide and serine dipeptide lipids are not only found in gingival tissues but

also in human blood, vascular tissue and brain (23). Recently, it was suggested that commensal Bacteroidetes, to which *P. gingivalis* belongs, that reside in the oral cavity and gut contribute to the pathogenesis of TLR2-dependent atherosclerosis through deposition and metabolism of serine dipeptide lipids in artery walls (6). In addition to *P. gingivalis*, the common dental pulpal pathogen *P. endodontalis*, produces the Lipid 654 class (36). The Lipid 654 preparations isolated from both *Porphyromonas* species were shown to promote osteoclast formation from RAW 264.7 cells (36) indicating that in addition to sub PG DHC lipids, osteoclast activation is promoted by serine dipeptide lipids and that bacterial source of Lipid 654 is probably not a critical issue.

Interestingly, phosphorylated dihydroceramide and serine dipeptide lipids have been reported to contaminate free lipid A isolated from *P. gingivalis* LPS (37) and may be responsible, at least to some extent for the TLR2-mediated effects of *P. gingivalis* LPS on osteoblasts (38). Regardless, the PG DHC and serine dipeptide lipids mediate important effects on bone cells and therefore should be regarded as virulence factors.

PROMOTION OF APOPTOSIS

Although *P. gingivalis* dihydroceramides promote inflammatory secretory reactions in fibroblasts, little is known about their effects on vascular cells. Zhalten et al. (39) studied the effects of extracted and purified *P. gingivalis* lipids on endothelial cells from the human umbilical vein. The PG DHC (sub PG DHC) lipid fraction but not the PE DHC lipid fraction of *P. gingivalis* initiated endothelial cell apoptosis *in vitro*, but not necrosis. PG DHC activated caspase 3, 6 and 9. Inhibition of these caspases significantly reduced PG DHC-mediated apoptosis in endothelial cells. Sub PG DHC also induced release of apoptosis-inducing factor. Pre-incubation of cells with the reactive oxygen species (ROS) scavenger N-acetylcysteine reduced *P. gingivalis*-induced endothelial apoptosis. Apoptosis was stimulated by exogenous synthetic sphingosines similar to endogenous mammalian ceramides formed in endothelial and other cells (39). In addition, *P. gingivalis*-derived lipids were able to induce apparent apoptosis in gingival fibroblasts (18) similar to apoptosis later reported in gingival fibroblasts when co-cultured with *P. gingivalis* (40) and in chondrocytes with exposure to *P. gingivalis* itself (41, 42). It was proposed that lipids from *P. gingivalis* might induce apoptosis in joints even in the absence

of live *P. gingivalis* (42), which is noteworthy. The authors also suggested that *P. gingivalis* lipids could interfere with and impair the repair process in cartilage, thereby linking *P. gingivalis* to rheumatoid arthritis (42-45).

EXPRESSION AND ANCHORING OF SURFACE POLYSACCHARIDES

Moye et al. (46) recently reported that deletion of the PG1780 gene in *P. gingivalis* strain W83 rendered this organism unable to produce dihydroceramide lipids. The PG1780 gene encodes for the putative serine palmitoyltransferase enzyme of *P. gingivalis*. Serine palmitoyltransferase is the enzyme responsible for the first step in the synthesis of long chain base precursors of sphingolipids. Moye et al. (46) showed that the ΔPG1780 mutant is devoid of dihydroceramide lipids whereas dihydroceramide synthesis was restored in the complemented mutant strain (ΔPG1780 pTCOW-1780). In addition, electron micrographic evaluation of the ΔPG1780 mutant suggested that surface glycans were diminished. Further evaluation demonstrated that the ΔPG1780 mutant expressed low levels of K-antigen but showed increased expression of anionic polysaccharide relative to the W83 parent strain. Of note, the K1 capsule null strain (ΔPG0106) showed very low expression of both K antigen and anionic polysaccharide when compared with the ΔPG1780 mutant or parent strain. These results demonstrate that sphingolipid synthesis in *P. gingivalis* is associated with the expression of specific cell surface polysaccharides although the exact role of sphingolipids in this process remains to be determined. Moye et al. (46) also demonstrated that lack of sphingolipid synthesis by *P. gingivalis* decreased the expression of cell-associated arginine (Arg) and lysine (Lys) gingipains (46), the "trypsin like" proteases expressed by this organism. Bainbridge et al. (48) found that deletion of a 77bpIR element at the 5′end of the K-antigen capsule synthesis locus of *P. gingivalis* changed the presentation of capsule, O-LPS, and A-LPS, and reduced cell-associated Arg- and Lys-gingipain activity. This evidence suggests that sphingolipid synthesis may alter cell surface gingipain expression indirectly though reduced K antigen expression. Finally, the lipid moiety responsible for covalently anchoring capsular polysaccharides to the outer membrane has been identified in only a few bacterial species (47) and includes the direct covalent attachment of capsular polysaccharides to a lyso-phosphatidylglycerol motif as reported by Willis et al. (47). It is unknown whether capsular

polysaccharides are anchored to sphingolipids in *P. gingivalis* cell membranes. Future research is expected to clarify this possibility.

RESISTANCE TO OXIDATIVE STRESS

Moye et al. (46) reported that the ΔPG1780 sphingolipid-deficient strain of *P. gingivalis* was more sensitive to oxidative stress after exposure to oxygen even for very short intervals. When the parental strain and the mutant ΔPG1780 were cultured to early exponential phase and the cultures were treated with 150-250 µM hydrogen peroxide or water as control, the parental strain survived the addition of all concentrations of hydrogen peroxide. The mutant cultures treated with 200 or 250 µM H_2O_2 were quickly killed and did not recover after 30 h. Cultures of the mutant strain exposed to the lowest concentration of hydrogen peroxide (150 µM), decreased initially in density, but recovered by the end of the experiment. This demonstrated that the ΔPG1780 mutant was much more sensitive to hydrogen peroxide than the parental strain. Accordingly, sphingolipids seem to play an important role in the resistance of *P. gingivalis* to oxidative stress and therefore to the survival of this bacterium. A report by An et al. (49) indicated that sphingolipids are important in *Bacteroides fragilis* protection from oxidative stress. However, a previous report demonstrated that *B. fragilis* does not produce PG DHC lipids (23), suggesting that PE DHC sphingolipids may be important in protection of *B. fragilis* from oxidative stress. Also, other factors can participate in the resistance of *P. gingivalis* to oxidative damage such as antioxidant enzymes, DNA binding protein (Dps), the hemin layer, enzymatic removal of deleterious products caused by ROS and response regulators (for a review see (50)).

ENHANCEMENT OF AUTOIMMUNITY

Phosphorylated dihydroceramides are derived from Bacteroidetes bacteria found in multiple sites in humans such as the oral cavity, the gastro-intestinal tract and the vagina. These bacterial lipids could be tipping factors enhancing autoimmunity in man. In a study by Nichols et al., *P. gingivalis* phosphorylated dihydroceramides, particularly PE DHC, significantly increased

experimental allergic encephalomyelitis (EAE) in a murine model of multiple sclerosis (35). EAE was used as a model for autoimmune disease in this study. The increased autoimmune disease severity resulting from administration of *P. gingivalis* lipids was mediated in a TLR2-dependent manner. Though the enhanced TLR2-dependent autoimmune response was attributed to PE DHC lipids, other minor lipid classes of *P. gingivalis*, including the recently described serine dipeptide lipids, could account for the enhanced engagement of TLR2.

COMPARATIVE EFFECTS OF SPHINGOLIPIDS IN INFLAMMATORY DISEASES

The distribution of PDHC lipid classes was different in diseased periodontal tissues compared to tissues from healthy controls (23, 51). The primary dihydroceramide lipids of *P. gingivalis* recovered from diseased gingival tissue were the unsub PG DHC lipids whereas lesser amounts of PE DHC and sub PG DHC lipids were detected. PG DHC lipids were also more abundant than PE DHC lipids on periodontally-diseased teeth (51). This suggested that progression of disease could be associated with a shift in the PDHCs species released from the microbiota associated with periodontitis or a shift in the transport or metabolic hydrolysis of specific PDHC lipids in diseased gingival tissues. PE DHC and PG DHCs also affected gingival fibroblasts differently, probably due to differences in their polar head groups (18) . As already mentioned, sub PG DHCs caused fibroblast rounding in culture and increased the production of prostaglandin E_2 with IL-1β co-treatment (18), and promoted apoptosis in endothelial cells (42) and chondrocytes (39). In contrast, the phosphatidylethanolamine (PEA) lipid fraction did not induce endothelial apoptosis *in vitro* (42) but did promote cell rounding in gingival fibroblasts (24). Because a homologous synthetic PEA lipid standard without *iso*branched fatty acids did not cause cell rounding, it was concluded that the *iso*branched aliphatic chains are also contributing to the apparent cell rounding of gingival fibroblasts (24). The difference in the biological activity of PG DHC and PE DHC has been ascribed to the different phosphorylated head group substitution and/or the addition of esterified *iso*branched $C_{15:0}$ fatty acid (18, 51). The interpretation of the relative clinical importance of dihydroceramides is additionally complicated by the fact that members of other genera in the oral and intestinal microbiota can produce PDHC lipids such as *Bacteroides*,

Parabacteroides, *Prevotella*, *Tannerella* and *Porphyromonas* (23). However, none of these bacteria, except for *T. forsythia*, are considered keystone pathogens in periodontitis.

CONCLUDING REMARKS

Sphingolipids are not only structural components of mammalian membranes but have important functions in specialized membrane domains (lipid rafts and protein complexes) and affect signaling for a number of cellular processes. Bacterial sphingolipids are similar in structure but are not identical to human sphingolipids. The importance of sphingolipids in disease has often been overshadowed by that of LPS, which in the case of *P. gingivalis* LPS can be contaminated with sphingolipids when prepared by published methods. *P. gingivalis* sphingolipids and serine dipeptide lipids produce cellular effects particularly relevant to the essential pathological features of periodontitis including promotion of osteoclastogenesis and inhibition of osteoblast function. *P. gingivalis* sphingolipids also potentiate prostaglandin secretion from gingival fibroblasts and are implicated in promoting IL-6 secretion from dendritic cells. These lipids also promote apoptosis in endothelial cells, potentially contributing to vascular lesions. The latter could be important in cardiovascular disease since *P. gingivalis* PDHC and serine dipeptide lipids have been detected in carotid atherosclerotic lesions. However, *P. gingivalis* is not the only organism responsible for production of dihydroceramide and serine dipeptide lipids since several bacterial species and genera within members of the Bacteroidetes phylum of the oral cavity, vagina and intestine may account for these lipids in blood, tissues and brain. Recovery of PDHC lipids in human brain tissues could occur through transport of PDHC lipids in blood and subsequent deposition into vascular elements of neural tissues, but could also result from penetration through the blood brain barrier. Future research will evaluate these possibilities. *P. gingivalis* is not the sole causal agent in periodontitis either, although it is regarded as the most important keystone bacterium here. Recently discovered serine dipeptide lipids of *P. gingivalis* have been implicated in alveolar bone loss in chronic periodontitis and represent a new class of TLR2 ligands with structural similarity to diacylated lipopeptides. Lipid 430 is highly unusual in that it produces strong proinflammatory responses and inhibitory effects on osteoblasts, and yet it contains only one fatty acid acyl chain. Bacterial sphingolipids might also play a role in autoimmune disease through acute and chronic activation of the immune system e.g., in rheumatoid arthritis and

multiple sclerosis. The distribution and composition of DHCs depend on the tissue site and disease status. This implies that healthy periodontal sites may contain bacterial sphingolipids but the distribution can be different from that in diseased sites. Since these bacterial sphingolipids and serine dipeptide lipids are recovered in periodontal and other human tissues and since they possess important biological properties affecting inflammation and host responses, they should be classified as virulence factors. Research on the role of these substances in human diseases, which are only beginning to be understood, should be intensified.

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505

506 **FIGURE LEGENDS**

507

508 **FIGURE 1** Model for phosphorylated dihydroceramide and serine dipeptide lipid penetration
509 into gingival tissues and relevant biological effects in the expression of chronic periodontitis. (1)
510 The serine dipeptide parent lipid, Lipid 654, is recovered in subgingival calculus and subgingival
511 plaque. Lipid 654 promotes osteoclast formation from RAW cells, inhibits osteoblast
512 differentiation and function, and is implicated in dendritic cell release of IL-6. (2) Lipid 654 can
513 be de-esterified by mammalian phospholipase A2 (PLA2) enzymes, thus producing within
514 gingival tissues another serine dipeptide lipid, Lipid 430. Lipid 430 inhibition of osteoblast
515 differentiation and function occurs at lower levels when compared with Lipid 654. (3)
516 Phosphorylated dihydroceramide lipids (PDHCs) including PE DHC, sub PG DHC and unsub PG
517 DHC lipids (see FIGURE 2), are abundant in lipid extracts of subgingival calculus, but are also
518 recovered in subgingival plaque and gingival tissues. (4) Sub PG DHC lipids promote IL-1β-
519 mediated prostaglandin production in gingival fibroblasts and cell fusion during
520 osteoclastogenesis of RAW cells. (5) Sub PG DHC lipids promote gingival fibroblast cell death
521 in culture. Penetration of sub PG DHC lipids through junctional epithelium could therefore
522 promote fibroblast cell death along the tooth root surface.

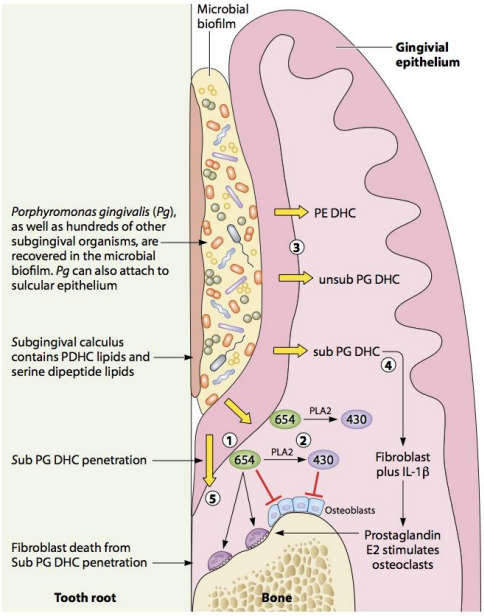
523 **FIGURE 2** Structures of novel *P. gingivalis* dihydroceramide and phospholipids ([a] adapted from
524 (18), [b] from (51), [c] from (17) and [d] from (24)). *The long chain bases vary from 17 to 19 carbons
525 in length. The 17 and 19 carbon long chain bases are *iso*branched as shown but the 18 carbon
526 long chain base exists as a straight aliphatic chain. Note the *iso*branched saturated fatty acids
527 ($C_{15:0}$ and $C_{13:0}$) in phosphatidylethanolamine (PEA) lipids.

528

529 **FIGURE 3** Structures of serine dipeptide lipids from *P. gingivalis* (adapted from (7, 19)). *Lipid
530 654 was originally described in *Flavobacterium meningosepticum* and was termed Flavolipin by
531 Shiozaki et al. (7).
532

533 **FIGURE 1.**

534



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536 **FIGURE 2.**

537

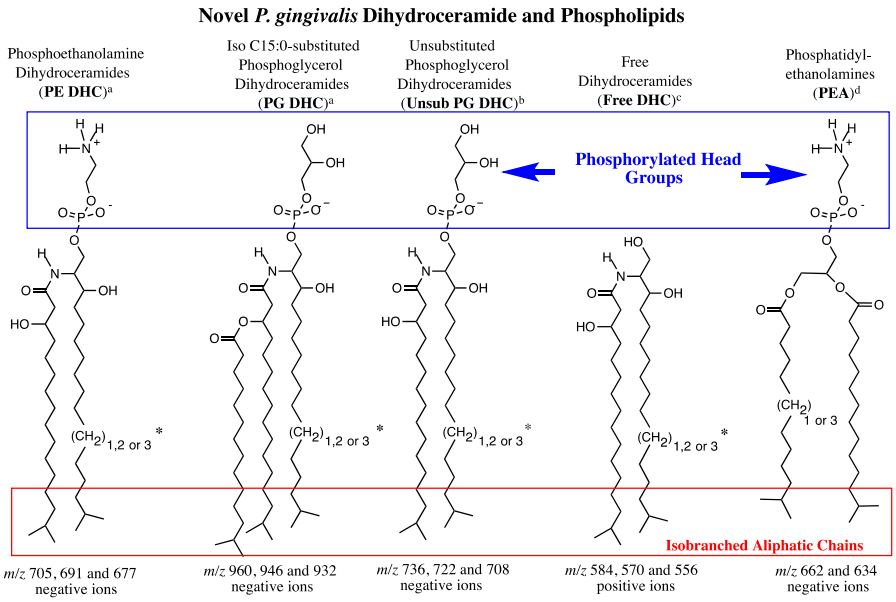

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FIGURE 3.

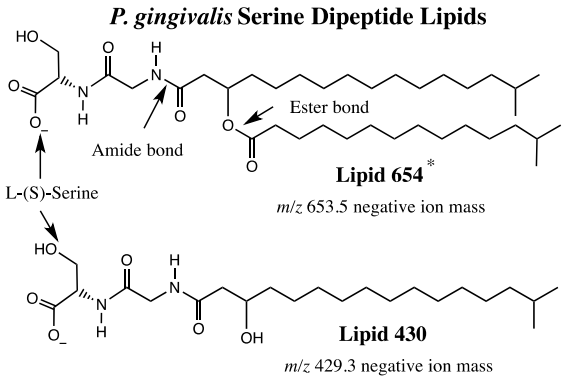

TABLE 1 Lipid abbreviations

DHC	Dihydroceramide
PE DHC	Phosphoethanolamine dihydroceramide
PG DHC	Phosphoglycerol dihydroceramide
PDHCs	Phosphorylated dihydroceramides
Sub PG DHC	Substituted phosphoglycerol dihydroceramide
Unsub PG DHC	Unsubstituted phosphoglycerol dihydroceramide
PEA	Phosphatidylethanolamine