Forum Minireview

Current Topics in Pharmacological Research on Bone Metabolism: Osteoclast Differentiation Regulated by Glycosphingolipids

Satoshi Fukumoto1,*, Tsutomu Iwamoto2, Eiko Sakai2, Kenji Yuasa1, Emiko Fukumoto3, Aya Yamada1, Tomokazu Hasegawa1, Kazuaki Nonaka1, and Yuzo Kato2

1Section of Pediatric Dentistry, Division of Oral Health, Growth and Development, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
2Division of Oral Molecular Pharmacology, Department of Developmental and Reconstructive Medicine, and 3Division of Oral Health Services Research, Department of Public Health, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan

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Abstract. Glycosphingolipids are thought to play important roles in the development and function of several tissues, although the function of glycolipids in osteoclastogenesis has not been clearly demonstrated. In the present study, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), a glucosylceramide synthase inhibitor, completely inhibited osteoclastogenesis induced by macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). Following treatment with D-PDMP, nearly all glycosphingolipid expression was dramatically reduced on the surface of bone marrow cells, which suggests that glycosphingolipids are necessary for osteoclastogenesis. To determine which kinds of glycolipids are important for osteoclastogenesis, we added several types of purified glycolipids to D-PDMP treated bone marrow cells, as the precursor of osteoclasts is known to express glucosylceramide (GlcCer) and lactosylceramide (LacCer). Following treatment with RANKL, ganglioside GM3 and GM1 were increased in the treated bone marrow cells, whereas other types were not detected using thin layer chromatography analysis. In cells cultured with those glycolipids, exogenously added LacCer rescued osteoclastogenesis blocking by D-PDMP. Furthermore, receptor activator of nuclear factor κB (RANK) induced the recruitment of tumor necrosis factor (TNF)-associated factors 2 and 6 (TRAF2 and 6, respectively) to the cytoplasmic tail of RANKL with activated IκB kinase and IκB phosphorylation, while D-PDMP treatment inhibited RANKL and induced IκB phosphorylation, and that inhibition was recovered by LacCer. In addition, RANK, TRAF2, TRAF6, and LacCer were found localized in lipid rafts on the cell surfaces. These results suggest that glycosphingolipids, especially LacCer, are important for the initial step of RANKL-induced osteoclastogenesis via lipid rafts.

Keywords: glycosphingolipid, lipid raft, lactosylceramide, osteoclast, RANKL (receptor activator of nuclear factor κB ligand)

Introduction

Glycosphingolipids (GSLs) are ubiquitously expressed in embryonal and adult tissues in mammals. In particular, they are enriched in nervous tissues, and their carbohydrate moiety structures are strictly regulated according to developmental stage and tissue differentiation (1). The biological roles of gangliosides have been investigated in many studies, with various functions reported such as receptors for bacterial toxins (2) and some viruses (3), and modulators for Ca2+ ions (4), and adhesion molecules (5 – 7), and growth factor receptors (8). These functions can be classified into two major groups, recognition molecules for exogenous soluble molecules and modulators of cis-acting receptor mole-
cules for various growth/differentiation factors (8). GSLs are composed of lipid rafts, which are glycolipid-enriched microdomains located on the cell surface, with the majority clustered and associated closely with single or multiple signal transducer molecules. For example, GM3 ganglioside is organized with c-Src, Rho, focal adhesion kinase (FAK), and Ras in B16 melanoma cells (9, 10). However, the expression and functions of GSLs in osteoclastogenesis have not clearly been demonstrated. In this study, we review the role of GSLs, especially lactosylceramide (LacCer), in osteoclastogenesis induced by receptor activator of nuclear factor κB ligand (RANKL).

**Bioynthesis of glycolipids**

Recent progress in the molecular cloning of glycosyltransferase genes responsible for the synthesis of GSLs has enabled analysis of the regulatory mechanisms involved with GSL expression and biological functions, by manipulation of genes to modify GSL profiles in cultured cells and experimental animals (11 – 13). For example, LacCer is synthesized by two glycosyltransferases, glucosyltransferase and galactosyltransferase, to transfer glucose and galactose to ceramide (Fig. 1). Further, GM3 synthase (α2,3-sialyltransferase) transfers sialic acid (neuraminic acid) to LacCer (14).

D-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) inhibits glucosylceramide (GlcCer) synthase, which leads to extensive depletion of glycosphingolipids derived from GlcCer (Fig. 1). That process has been shown to be a useful tool for studying the various functional roles of endogenous GSLs in living cells (15). Furthermore, several in vivo studies have demonstrated that D-PDMP treatment inhibits tumor growth and metastasis as well as renal hypertrophy associated with diabetes mellitus.

**Expression of glycolipids during osteoclastogenesis**

Bone marrow cells were isolated using a Sephadex G-10 column and cultured with macrophage-colony stimulating factor (M-CSF), after which neutral and acidic glycosphingolipids were purified, and then analyzed using thin layer chromatography (TLC). In these cells, GlcCer and LacCer were detected in the neutral fraction, while GSLs, gangliosides containing sialic acid, known as gangliosides, were not detected in the acidic fraction (Fig. 2) (16). Next, purified bone marrow cells were differentiated into tartrate-resistant acid phosphatase (TRAP) positive mononuclear and multinuclear cells and used as osteoclast-like cells. Three days after stimulation with RANKL and M-CSF, ganglioside GM3 and GM1 were detected in the acidic fraction.
fraction of these cells, while no gangliosides that contained GM3 and GM1 were observed without stimulation by M-CSF and RANKL (Fig. 2). Furthermore, the expression levels of lipid components, including ceramide, sphingosine, and sphinganine, were not changed by stimulation with M-CSF and RANKL. These results suggest that the majority of GSLs in cultured osteoclasts is composed of GlcCer, LacCer, GM3, and GM1. These GSLs are mostly localized in the lipid rafts of TRAP positive cells. In addition, GM3 and GM1 expression induced by RANKL in purified bone marrow cells was inhibited by D-PDMP, while those of GlcCer and LacCer were reduced (Fig. 3A). These results indicate that D-PDMP inhibits the biosynthesis of GSLs in bone marrow cells, which is similar to the findings of previous reports (16).

Glycosyltransferase inhibitor D-PDMP inhibits RANKL-induced osteoclastogenesis

GSLs and cholesterol are components of lipid rafts that have been implicated in signal transduction. A
number of studies have examined the effects of cholesterol on signaling using methyl-β-cyclohextrin (MβCD) and filipin (17, 18). Both of those agents bind cholesterol and thereby impede the formation of lipid rafts. A recent study that utilized MβCD and filipin showed that disruption of lipid rafts blocked Akt activation and actin ring formation and induced apoptosis in osteoclasts (19). However, the mechanisms of GSLs on lipid rafts during osteoclastogenesis have not been demonstrated. We examined the effects of GSLs toward osteoclastogenesis using a non-apoptotic dose of d-PDMP to avoid inhibiting cell growth. Mature multinucleated osteoclasts are formed by the fusion of TRAP-positive mononuclear osteoclasts. In the present study, d-PDMP inhibited TRAP-positive mononuclear cell formation after 3 days and multinuclear cell formation after 5 days following stimulation of purified bone marrow cells with M-CSF and RANKL (Fig. 3B) (16).

A 50% reduction in the number of TRAP-positive mononuclear cells was observed among cells treated with 2.5 μM of d-PDMP along with an approximately 70% reduction of TRAP-positive multinuclear cell formation. With 20 μM of d-PDMP, TRAP-positive mononuclear and multinuclear cells did not appear (Fig. 3B). These results suggest that GSLs are necessary for RANKL-induced osteoclastogenesis.

RANKL induced the phosphorylation of IκB and mitogen-activated protein (MAP) kinase, whose signaling is essential for osteoclast differentiation and survival (20 – 22). To analyze the effects of GSLs and d-PDMP on RANKL-induced phosphorylation of these molecules, we examined their phosphorylation by Western blotting. d-PDMP inhibited the phosphorylation of both IκB and MAP kinase induced by RANKL, with similar results observed for the phosphorylation of MAP kinase by M-CSF (16). Recently, lipid rafts disrupted by MβCD were shown to inhibit osteoclastogenesis and MβCD also inhibited the RANKL-induced phosphorylation of Akt, located downstream of RANK. These results indicate that glycolipid-enriched lipid rafts are important for signaling induced by RANKL (19).

RANK, receptor of RANKL, localized in lipid rafts

GSLs are believed to be integral components of plasma membrane microdomains, known as rafts, including the detergent insoluble microdomain (DIM), and glycosphingolipid enriched microdomain (GEM), which is rich in GSLs and cholesterol (23, 24). These lipid domains assemble receptors and glycosylphosphatidylinositol (GPI) anchored proteins on their external surface, and signaling molecules such as Src family tyrosine kinases, G proteins, nitric oxide synthase on their inner surface, and regulate membrane trafficking and signal activity. These clustered components can be separated into a light density membranous fraction that is detergent insoluble by density gradient centrifugation (25). Recent studies have shown that the TNF receptor family, which includes TNFR1, CD40, and RANK, is associated with membrane rafts (19, 26 – 28). Furthermore, the disruption of lipid rafts by MβCD extracted cholesterol induces apoptosis of RANKL-treated osteoclasts and reduces the amount of c-Src in lipid rafts (19). During osteoclastogenesis, the raft component flotillin, along with two important osteoclast factors TRAF2 and TRAF6 as well as c-Src, are increased after stimulation with M-CSF and RANKL (Fig. 5). RANK and flotillin are also co-localized in the detergent insoluble fraction, indicating that they exist in lipid rafts. In addition, RANK and the ganglioside GM1 have been shown to be co-localized in plasma membranes by immunofluorescence analysis. In undifferentiated osteoclasts, TRAF6 is not localized in lipid rafts, but it undergoes translocation into those rafts after treatment with RANKL. These results suggest that lipid rafts containing GSLs may be important for the translocation of TRAF6 following stimulation with RANKL and regulation of RANK signaling. In fact, MβCD and filipin, which destroy lipid rafts by binding to cholesterol, have been shown to dramatically abolish the translocation of TRAF6 and c-Src into lipid rafts following treatment with RANKL. However, the function of GSLs in lipid rafts during osteoclastogenesis is not clearly understood.

Lactosylceramide regulates RANKL signaling

RANKL-induced phosphorylation of IκB was significantly reduced in cells treated with d-PDMP, as was the phosphorylation of MAP kinase induced by RANKL (16). To determine the effects of GSLs on RANKL-induced signal transduction, exogenous GSLs were added to the culture media. Using several different kinds of glycolipids and lipid components, we found that the addition of LacCer recovered the phosphorylation of IκB and MAP kinase inhibited by d-PDMP (Fig. 4). GM3 and GM1 expression induced by M-CSF and RANKL was inhibited or totally suppressed by d-PDMP (Fig. 3A). Since d-PDMP also inhibited osteoclast formation, the expressions of GM3 and GM1 were thought to be important events for RANK-mediated signal transduction in osteoclasts, and they may have crucial roles in lipid rafts. Furthermore, the expression level of LacCer was slightly decreased by M-CSF and RANKL treatment, as compared with that following...
treatment with M-CSF alone. Since LacCer expression is observed in the early stage of osteoclastogenesis, rather than in differentiated osteoclasts, it may have a role in the early stage of osteoclast signal transduction. Intercellular adhesion molecule-1 (ICAM-1) is also important for cell-to-cell adhesion and fusion in osteoclasts, and it is highly expressed in mononuclear cell cultures in which pre-osteoclast-like mononuclear cells are forming. ICAM-1 expression also induces TNF-α which is regulated by LacCer, and D-PDMP inhibits the expression of ICAM-1 in endothelial cells (29). These results also suggest that LacCer is important for the initial differentiation of osteoclasts.

RANKL binds to its receptor RANK and then activates the downstream molecules described above. RANK expression is also induced by treatment with RANKL, but not M-CSF, while D-PDMP inhibits the expression of RANK induced by RANKL. Further, the reduction of RANK expression by D-PDMP was dramatically recovered by exogenous LacCer administration. These results suggest that LacCer may have two functions in initial osteoclastogenesis, regulation of the phosphorylation of downstream signaling by RANK and RANK expression. In our previous study, when cell surface glycolipids were altered by an over-expression of glycosyltransferase, for example, the GD3 and GM1 synthase gene, the phosphorylation of MAP kinase and dimerization of growth factor receptors were affected. Therefore, exogenously generated LacCer may modify the conformation of RANK in lipid rafts, especially its trimerization and translocation.

In summary, our results provide new insight into osteoclastogenesis regulation by GSLs, especially LacCer. These findings may be important for the phosphorylation of MAP kinase (MAPK) and IκB by RANKL is diminished by D-PDMP and recovered by LacCer. RANKL induces the phosphorylation of MAPK and IκB after 5 min. D-PDMP treatment inhibited the phosphorylation of these kinases completely. However, reduced phosphorylation of both kinases by D-PDMP was recovered with exogenous added LacCer. Similar results were observed in osteoclastogenesis induced by RANKL. D-PDMP treatment inhibited TRAP-positive cell formation. LacCer also recovered TRAP-positive cell formation inhibited by D-PDMP (data not shown). Mean values ± S.D. of five experiments are presented.

![Fig. 4. Phosphorylation of MAP kinase (MAPK) and IκB by RANKL is diminished by D-PDMP and recovered by LacCer. RANKL induces the phosphorylation of MAPK and IκB after 5 min. D-PDMP treatment inhibited the phosphorylation of these kinases completely. However, reduced phosphorylation of both kinases by D-PDMP was recovered with exogenous added LacCer. Similar results were observed in osteoclastogenesis induced by RANKL. D-PDMP treatment inhibited TRAP-positive cell formation. LacCer also recovered TRAP-positive cell formation inhibited by D-PDMP (data not shown). Mean values ± S.D. of five experiments are presented.](image1)

![Fig. 5. A model of the localization of RANK in lipid rafts. Lipid rafts have been thought to be a specialized plasma membrane. They are enriched in glycosphingolipids, cholesterol, sphingomyelin, and lipid-anchored membrane proteins; and they characterized by a light buoyant density and resistance to solubilization by Triton X-100. In particular, GM1 has been suggested to be a useful marker of lipid rafts as well as a main structural protein, caveolin. RANK, c-Src, and TRAF6 are co-localized in lipid rafts, indicating that lipid rafts are important for the signaling of RANKL during osteoclastogenesis.](image2)
development of therapeutic reagents for regulation of osteoclastogenesis, inhibition of glycosyltransferase, destruction of lipid components in lipid rafts, and as well as a strategy for administration of GSLs.

References