Microbial Extracellular Glycolipid Induction of Differentiation and Inhibition of the Protein Kinase C Activity of Human Promyelocytic Leukemia Cell Line HL60

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The biological activities of 7 microbial extracellular glycolipids including mannosylerythritol lipid (MEL)-A, MEL-B, polyol lipid (PL), rhamnolipid (RL), sophorose lipid (SL), succinoyl trehalose lipid (STL)-1, and STL-3 were investigated. All glycolipids except for RL were found to induce cell differentiation instead of cell proliferation in the human promyelocytic leukemia cell line HL60. To identify the differentiation direction of the induced cells, the leukocyte esterase activities were cytologically investigated, and the results showed that MEL-A, MEL-B, and PL induced HL60 to differentiate into granulocytes, while SL, STL-1, and STL-3 induced differentiation into monocytes. The 6 effective glycolipids also increased nitroblue tetrazolium (NBT) reducing ability, which is a common differentiation-associated characteristic in monocytes and granulocytes. Furthermore, it was also observed that these 6 glycolipids inhibited the activity of phospholipid- and Ca²⁺-dependent protein kinase. Additionally, the 6 effective glycolipids also induced the human myelogenous leukemia cell line K562 and the human basophilic leukemia cell line KU812 to differentiate into monocytes, granulocytes, and megakaryocytes.

Key words: biosurfactant; glycolipid; leukemia; differentiation; PKC

Microbial glycolipids are known as extracellular or cell-associated biosurfactants, such glycolipids are typical amphiphilic molecules containing both lipophilic and hydrophilic moieties. Mannosylerythritol lipids, MEL-A and MEL-B, were produced by Candida antarctica T-34 in soybean oil with a production of 40 g per liter of culture broth.¹ MEL-A and MEL-B were identified as 4-O-(di-O-acetyl-di-0-alkanoyl-β-d-mannopyranosyl)-erythritol and 4-O-(mono-O-acetyl-di-O-alkanoyl-β-d-mannopyranosyl)erythritol, respectively. Polyol lipid was produced by Aureobasidium sp. A-21 with a productivity of about 35 g per liter of culture medium containing no CaCO₃ as a neutralizing agent,² which is a mixture of fatty acid esters of arabitol and mannitol, and the two main components of the lipophilic moiety of the lipids proved to be 3,5-dihydroxydecanoic and 5-hydroxy-2-decenoic acids as identified by their lactones, (+)-3-hydroxydecan-5-oxide and (R)-(−)-2-decen-5-oxide, that is, R-()-massoic acid, respectively. Rhamnolipid was reported as a growth stimulant,³ having surface activity and emulsifying capability, produced in the culture broth by a hydrocarbon-using bacterium, Pseudomonas aeruginosa S-B,⁴ which consisted of rhamnose and β-hydroxydecanoic acid. Sophorose lipid was produced by Torulopsis bombicola ATCC 22214 in a mixture of glucose and safflower oil with a production of 70 g per liter, which contains the dimeric sugar sophorose and a long-chain carboxylic acid with a hydroxyl function on the pentultimate or terminal carbon.⁵ Two succinoyl trehalose lipids, STL-1(2,3,4,2-di-O-succinoyl-di-O-alkanoyl-α,β-trehalose) and STL-3(2,3,4,2-monosuccinoyl-trialkanoyl-trehalose) were by Rhodococcus erythropolis SD-7⁶ and Rhodococcus sp. TB-42 (Y. Uchida et al., unpublished results) with a production of about 30 to 40 g per liter, respectively.

On the other hand, glycosphingolipids (GSLs) and gangliosides are ubiquitous membrane components, that are reported to modulate cell growth, adhesion, and transmembrane signaling, because dramatic changes in the GSLs composition and metabolism of GSLs were observed during oncogenesis, differentiation, and oncogenic transformation.⁷ Recently, it has been reported that certain gangliosides were found to induce the differentiation of leukemia cells when added to culture media,⁶ and regulate some protein kinases in cells. Glycolipids produced by microorganisms contain no components of the cell membrane, but their structures are similar to gangliosides and both of them are amphiphilic compounds. To investigate whether the various glycolipids produced by microorganisms have abilities to induce the differentiation of cells, the human acute promyelocytic leukemia cell line HL60 was used in this study. The HL60 cell line is a useful model system for testing with respect to the granulocytic or monocytic differentiation.¹⁰ It has been reported that HL60 is induced to differentiate into monocyte-like cells by incubation with a wide variety of compounds including dimethyl sulfoxide (DMSO) and retinoic acid (RA)¹¹–¹³ or into monocyte-macrophage like cells by incubation with 1α,25-dihydroxy-

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Abbreviation: GSLs, glycosphingolipids; MEL, mannosylerythritol lipid; NAE, α-naphthyl acetate esterase; NCAE, naphthol AS-D-chloroacetate esterase; NBT, nitroblue tetrazolium; PKC, protein kinase C; PL, polyol lipid; RL, rhamnolipid; SL, sophorose lipid; STL, succinoyl trehalose lipid.
Results

Effects of glycolipids on differentiation-induction of leukemia cells

The effects of glycolipids on the proliferation of HL60 were examined. After 2 days of culture, 5.0 μM of MEL-A, 5.0 μM of MEL-B, 15 μg/ml of PL, 10 μg/ml of SL, 2.5 μM of STL-1, and 5.0 μM of STL-3 but not 50 μg/ml of RL inhibited the proliferation of HL60 cells (Fig. 1). On the second day of cultivation, morphological changes of HL60 were observed in each culture treated with the 6 glycolipids. MEL-A, MEL-B, and PL caused drastic morphological changes, and cells were found to adhere to the bottom of flasks (Fig. 2B, C, D). SL, STL-1, and STL-3 caused cells to aggregate and the cells enlarged, while the ratio of nuclear to cytoplasm is decreased (Fig. 2E, F, G).

Cellular esterases are ubiquitous, apparently representing a series of different enzymes acting upon selected substrates. Under defined reaction conditions, hemopoeitic cell types can be identified with specific esterase substrates. This method provides hematologists and hematopathologists with a means of distinguishing granulocytes from monocyes. To do the test, the preparations of fixed cells were incubated with either naphthol AS-D chloroacetate (NCAE) or α-naphthyl acetate (NAE) in the presence of freshly formed diazonium salt. Enzymatic hydrolysis of ester linkages liberates free naphthol compounds that couple with the diazonium salt, and form highly colored deposits at the sites of active enzyme. HL60 cells treated with MEL-A, MEL-B, and PL highly expressed NCAE activities that corresponded to the specificity for the granulocytic lineage. On the other hand, the cells treated with SL, STL-1, and STL-3 expressed NAE activities that corresponded to the specificity for the monocytic lineage (Fig. 3). Cells treated with TPA, which is a typical inducer of monocyte-macrophage differentiation in HL60 cells, also expressed 75% of the NAE activity (data not shown).

NBT reducing ability of HL60 cells treated with various glycolipids

According to the observed growth inhibition, morphological changes, and specific and non specific esterase ac-
Activities, HL60 cells treated with glycolipids were assumed to have a tendency to differentiate into monocytes or granulocytes. To confirm this assumption, a differentiation-associated characteristic, NBT reducing ability was investigated.

NBT reducing ability is often used as a marker for neutrophiles, monocytes, macrophages and lymphocytes numerous pathogenic bacteria or to cancer cells. The toxic response is demonstrated by the cellular activity of reducing the water-soluble nitroblue tetrazolium dye to insoluble intracellular blue-black formazan due to the O$_2^-$ and H$_2$O$_2$ release upon activation. To compare the differentiation-
associated characteristics, NBT reducing ability of cells treated with various glycolipids except for RL were examined. All cells treated with glycolipids showed activities above 30% and the values were significantly higher than those of non-treated cells. Especially, the cells treated with PL and MEL-A showed higher than 40% activities (Fig. 4).

Inhibition of kinase activity of PKC

The PepTag Assay is a kit using as a substrate a brightly colorful, fluorescent peptide which is highly specific for PKC. Phosphorylation of their specific substrate by PKC alters the net charge at peptide from +1 to -1. This change in the net charge of the substrate causes the phosphorylated and unphosphorylated versions of the substrate to separate rapidly on the agarose gel at neutral pH. The phosphorylated peptide migrates toward the positive electrode while the unphosphorylated one migrates toward the negative electrode.

For the control cells, phosphorylated peptide migrated towards the anode (+), while nonphosphorylated one migrated towards the cathode (-). However, the cells treated with 6 effective glycolipids were found to have no phosphorylated peptide migrations (Fig. 5). On the other hand, the cells treated with RL showed the same result as that of the control one (data not shown). These results indicate that 6 glycolipids inhibited PKC activity at the concentration of inducing the differentiation of HL60 cells.

Effects of glycolipids on other leukemia cells

To examine whether the glycolipids can induce the differentiation of other leukemic cell lines, the human myelogenous leukemia cell line K562 and human basophilic leukemia cell line KU812 were investigated. Almost 80% of K562 cells treated with 5 μM MEL-A or 7.5 μM MEL-B expressed NCAE activities, which corresponds to the differentiation into granulocyte lineage. On the other hand, cells treated with 25 μg/mL of PL, 15 μg/mL of SL, and 5 μM of STL-3 showed high NAE (F) activities, which corresponds to the differentiation into megakaryocyte lineage. However, cells treated with 5 μM STL-1 expressed only 40% of the differentiation characteristics of monocyte lineage (Fig. 6).
With repeat to KU812 cells, 5.0 μM of MEL-A, 5.0 μM MEL-B, 25 μg/ml of PL, 10 μg/ml of SL, and 5.0 μM STL-3 treated cells expressed NCAE activity, which corresponds to the differentiation into granulocyte lineage. Only cells treated with 5.0 μM STL-1 showed NAE activity, which corresponds to the differentiation into monocyte lineage (Fig. 7).

Discussion

In this study, we examined whether microbial extracellular glycolipids have abilities to induce human leukemia cell differentiation. It was found that the 6 glycolipids, 5.0 μM MEL-A, 5.0 μM MEL-B, 15 μg/ml of PL, 10 μg/ml of SL, 2.5 μM STL-1, 5.0 μM STL-3 were found to induce the cell differentiation instead of cell proliferation in the human promyelocytic leukemia cell line HL60. These glycolipids also increased NBT reducing ability, which is a common characteristic to identify the cell differentiation into monocytes and granulocytes. Furthermore, they also inhibited PKC activities of HL60 cells. PKC comprises a family of serine/threonine protein kinases implicated in the cell regulation, differentiation, and proliferation.20,21 Inhibitors of PKC are expected to be anti-tumor agents because the tumor-promoting phorbol esters are known to activate PKC directly.22,23 Hakomori et al.60 reported that in human colonic cancers, GM3 and other GSLs and sphingolipids had strong inhibitory effects on PKC. Since the microbial extracellular glycolipids are biosurfactants having surface activities, they might interact with the cell membrane and down-regulate PKC activity like GM3. Further studies on the differentiation-inducing activity of microbial extracellular glycolipids, whether it is due to a simple detergent-like effect or a specific action on the cell membrane, are needed.

The glycolipids also induced differentiation of human myelogenous leukemia cell line K562 and human basophilic leukemia cell line KU812. The K562 cell line was established by Lozzio and Lozzio24 from a patient with chronic myeloid leukemia in blast cell transformation, and has been used for the study of erythroblast differentiation. Erythroid differentiation of the cell can be induced by various compounds, including hemin, sodium butyrate, δ-amino-levulinic acid, ara-C, adriamycin, and actinomycin D.25-27 A unique Ph positive leukemic cell line, designated KU812, having basophilic characteristics was established by Kishi et al.,28 and the cytological and immunological properties of KU812 have also been reported.29 KU812 has been suggested to have a bipotent differentiation capacity inducible towards the monocyte/macrophage lineage by PMA treatment40 and towards the basophilic lineage by growth in serum-free media.31

In our study, MEL-A and MEL-B induced HL60, K562, and KU812 to granulocytic differentiation, while STL-1 induced monocytic differentiation, but PL, SL, and STL-3 had no peculiarity on differentiation directions on three cell lines. Furthermore, STL-1 and STL-3 are different in their numbers of succinic acids and fatty acids, while MEL-A and MEL-B are only different in the numbers of acetyl groups, so the numbers of fatty acids seem essential to the differentiation direction through their affinities to the cell membranes. However, each sugar moiety seems not effective on differentiation directions.

Recently, Ishigami reported that biosurfactants showed a small critical micelle concentration (CMC) value and higher surface and interfacial tension lowering actions in spite of their bulky structure, and such functions may be attributed to their excellent molecular orientations at the interfaces.32 However, the effects on cell membranes by forming micelles of biosurfactants are still unclear. Further studies on the correlation between the chemical structures and the biological functions of glycolipid-type biosurfactants are needed.

References