The neurite-initiating effect of microbial extracellular glycolipids in PC12 cells

Hiroko Isoda^{1,*}, Hiroshi Shinmoto², Masatoshi Matsumura³ & Tadaatsu Nakahara³

¹ Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki, 305, Japan

(* Author for all correspondence, e-mail: isoda@biol.tsukuba.ac.jp)

² National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, 305, Japan

³ Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki, 305, Japan

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Abstract

The effects of several kinds of microbial extracellular glycolipids on neurite initiation in PC12 cells were examined. Addition of mannosylerythritol lipid-A (MEL-A), MEL-B, and sophorose lipid (SL) to PC12 cells caused significant neurite outgrowth. Other glycolipids, such as polyol lipid (PL), rhamnolipid (RL), succinoyl trehalose lipid-A (STL-A) and STL-B caused no neurite-initiation. MEL-A increased acetylcholine esterase (AChE) activity to an extent similar to nerve growth factor (NGF). However, MEL-A induced one or two long neurites from the cell body, while NGF induced many neurites. In addition, MEL-A-induced differentiation was transient, and after 48 h, percentage of cells with neurites started to decrease in contrast to neurons induced by NGF, which occurred in a time-dependent manner. MEL-A could induce neurite outgrowth after treatment of PC12 cells with an anti-NGF receptor antibody that obstructed NGF action. These results indicate that MEL-A and NGF induce differentiation of PC12 cells through different mechanisms.

Abbreviations: AChE, acetylcholine esterase; bFGF, basic fibroblast growth factor; dBcAMP, dibutyl cyclic adenosine mono phosphate; MEL, mannosylerythritol lipid; NGF, nerve growth factor; PL, polyol lipid; RL, rhamnolipid; SL, sophorose lipid; STL, succinoyl trehalose lipid

Introduction

Biosurfactants are naturally produced by plants, animals, and many microorganisms (Zalic et al., 1976), and they are typical amphiphilic compounds containing both lipophilic and hydrophilic moieties. Glycolipid-type biosurfactants are known as microbial extracellular or cell-associated biosurfactants, and it is commonly isolated with a high productivity. 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 (Kitamoto et al., 1990). MEL-A and MEL-B were identified as 4-*O*-(di-*O*-acetyl-di-*O*-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 (Kurosawa et al., 1994). It 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-dihydroxy-decanoic and 5-hydroxy-2-decenoic acids as identified by their lactones, (+)-3-hydroxydecan-5-olide and (R)-(-)-2-decene-5-olide, that is, R-(-)-massoilactone, respectively. Rhamnolipid was reported as a microbial growth stimulant (Hisatsuka et al., 1971), having surface activity and emulsifying capability, produced in the culture broth by a hydrocarbon-using bacterium, *Pseudomonas aeruginosa* S7B1, which consisted of rhamnose and β -hydroxydecanoic acid. Sophorose

Figure 1a. Effect of microbial extracellular glycolipids on neurite outgrowth. PC12 cells were treated with 5.0 μ M MEL-A, 5.0 μ M MEL-B, 12.5 μ g/ml PL, 25.0 μ M RL, 6.3 μ M SL, 15.0 μ M STL-A, 3.8 μ M STL-B in the presence or absence of 40 ng/ml NGF. A) Morphological changes of PC12 cells.

Figure 1b. B) The percentage of cells with neurites. 300–600 cells were scored from randomly chosen fields. w/o NGF means without NGF, w/NGF means with NGF. Values are the means of three determinations.

lipid was produced by *Torulopsis bombicola* ATCC 22214 in a mixture of glucose and safflower oil with a production of 70 g per liter. It contains the dimeric sugar sophorose and a long-chain carboxylic acid with a hydroxyl function on the penultimate or terminal carbon (Cooper et al., 1984). Two succinoyl trehalose lipids, STL-1 (2,3,4,2'-di-*O*-succinoyl-di-*O*-alkanoyl- α - α -trehalose) and STL-3 (2,3,4,2'-monosuccinoyl-trialkanoyl-trehalose) were by *Rhodococcus erythropolis* SD-74 (Uchida et al., 1989) 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, various approaches to understanding the specific mechanisms induced have included use of exogenous gangliosides as neurotrophic agents in culture (Ledden et al., 1989). Mammalian glycolipid gangliosides are ubiquitous membrane components, that are reported to modulate cell growth, adhesion, and differentiation (Hakomori et al., 1992). Although obvious differences exist, there are some similarities of amphiphilic structures between mammalian glycolipids and microbial extracellular glycolipids. Therefore, we assessed whether above-mentioned microbial extracellular glycolipids induce

neurite initiation of PC12 cells. The PC12 cell line, derived from a rat pheochromocytoma (Greene and Tischler, 1976), provides a relatively simple, and homogenous system for studying various aspects of neuronal differentiation (Greene and Tischler, 1982), because PC12 cells can survive and proliferate without requiring the presence of neurotrophic factors. Application of NGF causes PC12 cells to differentiate into a neuronal like phenotype characterized by numerous morphological and physiological changes (Greene and Tischler, 1982). In PC12 cells, nerve growth factor (NGF) interacts with two distinct plasma membrane receptor proteins: p75NGFR, a cysteine-rich glycoprotein having a relatively low affinity for NGF (Chao et al., 1992), and p140trk (Hempstead et al., 1992; Kaplan et al., 1991; Klein et al., 1991), a receptor tyrosine kinase which bind NGF with a high affinity, resulting in the rapid tyrosine autophosphorylation of the receptor and activation of signal-transducing proteins (Kim et al., 1991; Maher et al., 1988; Soltoff et al., 1992; Thomas et al., 1991, 1992; Vetter et al., 1991; Wood et al., 1992).

In this report, the neurite-initiating effect in PC12 cells by microbial extracellular glycolipids is shown.

Figure 2. Time course for the appearance of neurites of PC12 cells following treatment with MEL-A in the presence or absence of NGF. Cultures ($2 \times 10^4/\text{cm}^2$) were maintained with DMEM-ITES medium and scored at intervals for percentage of neurite-bearing cells. 300–600 cells were scored each time from randomly chosen fields. Values are the means of three determinations.

Figure 3. Distribution of PC12 cells according to neurite number. PC12 cells were cultured for 48 h with $5.0 \mu\text{M}$ MEL in the presence or absence of 40 ng/ml NGF. Cells that had one neurite were defined as monopolar, those with two as bipolar, and those with more than two neurites as multipolar type cells. Values are the means of three determinations.

Figure 4. Effects of NGF and MEL-A on AChE activity in PC12 cells. Cells were cultured for 48 h with 5.0 μ M MEL-A in the presence or absence of 40 ng/ml NGF. Values are the means of two determinations.

Materials and methods

Materials

Nerve growth factor (NGF, 2.5S), basic fibroblast growth factor (bFGF), dibutyl cyclic adenosine mono phosphate (dBcAMP) were purchased from Funakoshi, Tokyo, Japan. The anti low affinity p75^{NGFR} antibody was purchased from Cosmo Bio, Tokyo, Japan.

Production of microbial extracellular glycolipids

MEL-A and MEL-B were produced by the method of Kitamoto et al. (1990). PL was produced by the method of Kurosawa et al. (1994). RL was produced by the method of Hisatsuka et al. (1971). SL was produced by the method of Cooper et al. (1984). Both STL-1 and STL-3 (Y. Uchida et al., unpublished results) were produced by the method of Uchida et al. (1989).

Cells and cell culture

PC12 cell line was obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan) and routinely grown in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., LTD, Tokyo) supplemented with 5% fetal bovine serum (Sanko Junyaku, Tokyo), 10% horse serum (Sanko Junyaku, Tokyo), 100 μ g/ml streptomycin (GIBCO BRL, U.S.A.), and 100 units/ml penicillin (GIBCO BRL, U.S.A.) in tissue culture flasks at 37 °C humidified 5% CO₂ incubator. For the neurite outgrowth assay, the PC12 cells were washed and plated at 2×10^4 cells/cm² in serum free RPMI 1640 supplemented 5 μ g/ml insulin, 10 μ g/ml iron-free human transferrin, 25 mM ethanolamine and 25 nM selenite (RD-1; Kyokuto Pharmaceutical Kogyo Co., Tokyo, Japan) onto fresh collagen-coated dishes (IWAKI GLASS Co., Tokyo, Japan) with various glycolipids and/or 40 ng/ml of NGF.

Figure 5. Effect of antibodies against p75^{NGFR} (anti NGFR Ab) on neurite extension of PC12 cells treated with NGF or MEL-A. Cells were treated with MEL-A or NGF after pretreatment of 10 μ g/ml antibody against p75^{NGFR} and scored the percentage of neurite-bearing cells. Values are the means of three determinations.

Determination of neurite differentiation of PC12 cell

We classified the cells morphologically as to the number of neurites by the methods of Saito et al. (1988). At least 200 random neurites were counted for morphological classification. The differences in percentage of cells with neurite processes were analyzed for significance by Student's *t*-test. For acetylcholinesterase (AChE) activity, cultures were harvested and assayed as described previously (Rieger et al., 1980). Specific activity was calculated based on protein concentration as determined by the method of Lowry et al. (1951). Acetylcholinesterase activity was expressed as n mol of thiocholine released/min per mg protein (nmol/min per mg).

Results and discussion

We tested several microbial extracellular glycolipids including MEL-A, MEL-B, PL, RL, SL, STL-1, and STL-3. We found that MEL-A, MEL-B, and SL induced neurite initiation during 24 h treatment (Figure 1a). The potentiations of MEL-A, MEL-B, and SL were with a maximal effect at a concentration of 5.0 μ M, 5.0 μ M, and 6.3 μ M, respectively. We

also examined the effect of glycolipids on the neurite outgrowth of PC12 cells by the treatment with NGF (40 ng/ml), and found that MEL-A, MEL-B, PL, and SL were effective (Figure 1b).

Figure 2 shows the time course of the changes in percent of neurite-bearing cells. About 10% of cells produced the extended neurites at 12 h after treatment with MEL-A. A maximal response of the neurite outgrowth was observed at 48 h after treatment; about 30% of the PC12 cells becoming neurite-bearing. At a MEL-A concentration of 5.0 μ M, the decrease cell number did not exceed 8–10% as compared with control culture (RPMI-ITES medium only), and therefore the neuritogenic activity of MEL-A did not appear to have resulted from its cytotoxic action. However, the percentage of cells bearing neurites was gradually decreased after 48 h exposure of MEL-A, and this morphological change was not permanent. The neurite-bearing cells became round-shaped neurite and finally died. There is no sign of neurite stabilization even if MEL-A was re-added into the culture (data not shown). It is unclear whether this reduction of cell numbers is due to the apoptosis or the necrosis. When MEL-A and NGF were present together, neither neurite-degeneration nor cell death was apparent. In contrast, when cells were stimulated with

NGF alone, the percentage of neurite-bearing cells has increased progressively, and the neurite increased greatly in length and exhibited the complex branching patterns. Thus, in comparison with the effect of NGF, the morphological differentiation of PC12 cells induced by MEL-A was fast and mostly transitory, and MEL-A appears to act as a neurite initiator rather than a survival factor like NGF.

After culture for 48 h with MEL-A, the numbers of mono-, bi-, and multipolar cells were 14, 77, and 3%, respectively (Figure 3). On the other hand, in cultures with NGF alone, the numbers of mono-, bi-, and multipolar cells were 34, 16 and 47%, respectively. Thus, the neurites induced by MEL-A had a smaller proportion of the multipolar type and a larger proportion of mono- and bipolar types than neurites induced by NGF. In culture with both 40 ng/ml NGF and 5.0 μ M MEL-A, the distribution of neurites per cells were altered, with about 90% of total cells were bi- and multipolar types.

In addition to morphological changes, it is reported that NGF induced cholinergic responsiveness in PC12 cells by regulating acetylcholinesterase (AChE) and cholineacetyltransferase activities (Greene and Rein, 1977). Thus, the induction of AChE activity by neurotrophic factors is a biochemical marker for neuronal differentiation in PC12 cells. Figure 4 shows the specific activity of AChE in culture of PC12 cells incubated with NGF, MEL-A, and both. NGF induces a 1.6 fold increase in AChE activity. MEL-A stimulated AChE activity 1.8 fold in PC12 cells, of which extent of induction was similar to that of NGF after 48 h of exposure. This indicates that MEL induced cholinergic responsiveness in PC12 cells as same as that of NGF. When MEL-A and NGF were added at the same time, there was not an additive effect on AChE activity after 48 h. This means that the pathway for the increase in AChE activity by MEL-A is probably the same as that by NGF.

To examine whether the intracellular mechanism to initiate neurite outgrowth by MEL-A is different from NGF or not, after treatment with anti-low affinity p75^{NGFR} antibody, NGF or MEL-A were treated on PC12 cells. The numbers of cells with neurites were decreased in NGF treated cells (38 to 8%), while slightly decreased in MEL-A treatment (28 to 25%). Treatment with antibody alone, there was hardly effective on neurite outgrowth (Figure 5). From these results, MEL-A could induce neurite outgrowth after treated PC12 cells with anti-low affinity p75^{NGFR} antibody, which obstructed NGF action, suggesting that

MEL-A and NGF elicit neurite initiation by different mechanisms.

Compared with neurites induced by other known molecules (NGF, bFGF, dBcAMP), MEL-A-induced neurites were unique with respect to morphology, and time course appearance and disappearance (data not shown). The transitory neurite outgrowth induced by MEL-A suggests that MEL-A may play a role as a neurite initiation factor, whereas NGF is responsible for the stabilization and long-term maintenance of the differentiated state. MEL-A caused the growth of one or two long neurites per cell, and this restricted neurite number could not be caused by NGF, bFGF or dBcAMP.

To clarify the physiological significance of fewer neurites, it is necessary to examine in more detail the morphological, biochemical and functional changes caused by MEL-A, and to investigate the type of intracellular signaling. Tsuji et al. (1988) reported that addition of ganglioside (GQ1b) to neuroblastoma cells induced one long neurite, and that some protein kinase systems on the plasma membrane (ecto-type) may play an important role in GQ1b-induced neurite outgrowth. That is, there is possibility that MEL-A activated mechanisms included an ecto-type kinase and consequently make PC12 cells grow one or two long neurites. In the previous study, we have found that MEL-A induced granulocytic differentiation of human promyelocytic leukemia cell line HL60, and exhibited a significant decrease of protein kinase C activity (Isoda et al., 1997). We also reported that MEL-A inhibited the tyrosine phosphorylation while granulocytic differentiation of human myelogenous leukemia cell line K562 (Isoda et al., 1997). Although it is in process, treatment of PC12 cells with MEL-A caused decreasing in their tyrosine kinase activities (data not shown).

MEL-A is amphiphilic moiety; therefore, MEL-A has both hydrophobic and hydrophilic moieties. As the effective concentration of MEL-A (5.0 μ M) is above critical micelle concentration, MEL-A is assumed to form micelle, which could interact more on the cell components such as cell membrane, cytoplasm, nuclear, etc. Further studies on cellular and molecular mechanisms, linear signal transduction of triggering neuronal differentiation by microbial extracellular glycolipids are needed.

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