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Special Issue

Mannosylerythritol lipid induces granulocytic differentiation and inhibits the tyrosine phosphorylation of human myelogenous leukemia cell line K562

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Abstract

Mannosylerythritol lipid (MEL), which induced granulocytic differentiation of human promyelocytic leukemia cell line HL60, also induced differentiation of human myelogenous leukemia cell line K562. MEL inhibited insulin-dependent cell proliferation and induced leukocyte esterase activity of K562 cells. MEL markedly increased the differentiation-associated characteristics in granulocytes, such as nitroblue tetrazolium (NBT) reducing ability, expression of Fc receptors, and phagocytic activity of K562 cells. The tyrosine phosphorylation in K562 cells inhibited by MEL. These results suggest that MEL directly down-regulates the tyrosine kinase activities in K562 cells to inhibit the cell proliferation and to induce the differentiation.

Abbreviations: GLSs, glycosphingolipids; MEL, mannosylerythritol lipid; NAE, α-naphtyl acetate esterase; NCAE, naphthol AS-D-chloroacetate esterase; NBT, nitroblue tetrazolium; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; TGF, transforming growth factor.

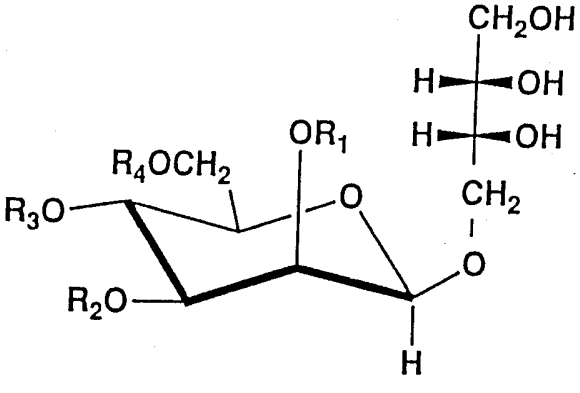

Figure 1. Structure of mannosylerythritol lipid (MEL) produced by *Candida antarctica* T-34 from soybean oil. R^{1-4} = 2x acetyl + 2x fatty acid.

Introduction

In the previous study, we have found that microbial extracellular glycolipids, Succinoyl trehalose lipid (STL) induced monocytoic differentiation while Mannosylerythritol lipid (MEL) induced granulocytic differentiation of human promyelocytic leukemia cell line HL60, and both STL and MEL at the concentration which induced differentiation of HL60 cells exhibited a significant decrease of protein kinase C activity (Isoda *et al.*, 1997). In this paper, we examined the effect of such glycolipids on human myelogenous leukemia cell line K562 and found that MEL also induced the differentiation into granulocyte. MEL was produced by *Candida antarctica* T-34 from soybean oil at concentrations of about 40g per liter of culture broth (Kitamoto *et al.*, 1990). MEL were identified as 4-*O*-(di-*O*-acetyl-di-*O*-alkanoyl-β-D-mannopyranosyl)-erythritol (Figure 1). K562 was established by Lozzio and Lozzio (1975) from a patient with chronic myeloid leukemia in blast cell transformation, and this line has been used to study erythroid differentiation. Erythroid differentiation of the cells is induced by various compounds, including hemin,

sodium butyrate, d-amino-levulinic acid, ara-C, adriamycin, and actinomycin D (Rowley *et al*., 1981; Hozumi, 1983; Anderson *et al*., 1979). We found that MEL increased the differention associated characteristics of the K562 cells such as NBT reducing ability, Fc receptor expression and phagocytic activities. Furthermore, we examined the tyrosine phosphorylation regulation in the cells by the method of the western blot analysis, because specific types of ganglioside are reported to inhibit receptor-associated tyrosine kinase activity, and therefore gangliosides in the lipid bilayer surrounding the receptors are considered to have an important role in modulation of cell proliferation through direct or indirect interaction with receptor-associated tyrosine kinase (Bremer *et al*., 1984; 1986, Hanai *et al*., 1988; Igarashi *et al*; Zeng *et al*., 1995). Ubiquitous membrane components such as gangliosides and glycosphingolipids are known to modulate cell growth, cell adhesion, and transmembrane signaling. Dramatic changes in GSLs composition and metabolism have been observed during oncogenesis, differentiation, and oncognenic transformation. However many obvious differences exist between mammalian glycolipids and MEL. In this study, we show the granulocytic differentiation of K562 cell line by MEL.

Materials and methods

Preparation of MEL

MEL was prepared by the method of Kitamoto *et al*. (1993). The long fatty acid composition of MEL were octanoate (17.5%), decanoate (10.1%) and tetradecanoate (1.1%). Found: C, 59.95; H, 8.60%. Calcd. for $C_{34}H_{60}O_{13}$ (2 acetyl, 2 decanoyl): C, 60.33; H, 8.94%.

Cells and cell growth

The human myelogenous leukemia cell line K562 was obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan). The cells were maintained in RPMI medium containing 10% FBS at 37 °C in humidified 5% CO_2/95% air. For MEL treatment, the cells were cultured in a serum-free ERDF, (Kyokuto Pharmaceutical Kogyo Co. Tokyo, Japan) -ITES medium, in which ITES contains 5 μg/ml insulin, 10 μg/ml iron-free human transferrin, 25 μM ethanolamine and 25 mM selenite, (RD-1; Kyokuto Pharmaceutical Kogyo Co. Tokyo, Japan). The cells were counted using a hemacytometer, and the viability was estimated by the trypan blue dye exclusion method. MEL dissolved in PBS were sonicated and sterilized by passage through a MILLEX-GV 0.22 μm filter unit (Millipore Products Division, Bedford, USA). They were added to the culture medium at the desired concentration.

Determination of cell differentiation

For cytochemical assessment of cell differentiation, specific and nonspecific leukocyte esterase activities (Li *et al*., 1973) were determined cytochemically by the esterase double-staining method for NAE and NCAE using reagents for determination of esterase (SIGMA CHEMICAL Co. Ltd. St. Louis, USA). Specific and nonspecific esterase activities were examined on days 3.

To assess the granulocytic differentiation of K562, differentiation-associated characteristics in granulocytes such as NBT reducing ability, expression of Fc receptors and phagocytic activities were investigates. The NBT-reducing ability was determined by the modified method of Takeda *et al*. (1988) (Isoda *et al*., 1996). The expression of Fc receptors on the cell surface of K562 was assayed by erythrocyte rosette formation (Takeda *et al*., 1982). The phagocytic activity of K562 cells was measured by counting the percentage of the cells that phagocytosed yeast particles. The yeast-phagocytic activity was investigated with opsonized yeast (Takeda *et al*., 1988).

Western blot analysis of the cells treated with MEL

K562 (2×10^6 cells) were incubated with 5.0 μM of MEL. The incubation was stopped by adding 100 μl of cell suspension to the same volume of 2X Laemmli's sample buffer (1X is 62.5 mM Tris-HCl, pH 6.8, 4% SDS, 5% b-mercaptoethanol, 8.5% glycerol, 2.5 mM orthovanadate, 10 mM paranitrophenylphosphate, 12 μg/ml leupeptin, 12 μg/ml aprotinin, 1.25 mM phenilmethanesulfonyl fluoride, 0.025% bromophenol blue) preheated at 95 °C. The samples were boiled for 5 min to completely denature the proteins and were then loaded onto 12.5% SDS-polyacrylamide gel electrophoresis (Laemmli *et al*., 1970).

Proteins on the gels were transferred to Immobilon PDVF membranes (milipore Corporation). Nonspecific sites were blocked using 1X Block Ace (Dainihon Pharmaceutical Industry, Japan). The anti-phosphotyrosine (mono, X200, BioMakor, Israel)

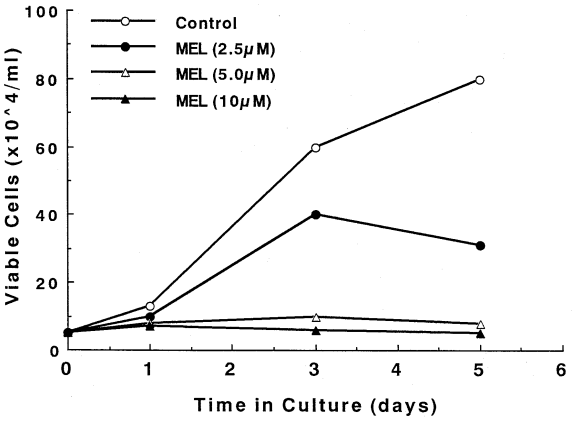

Figure 2. Effect of MEL on growth of K562. K562 cells were seeded at a initial concentration of 5.0×10^4 cells per ml and grown in the ERDE-ITES medium with or without MEL. Cells were counted with a hemacytometer, and viability was estimated by trypan blue dye exclusion. Each data point represents the mean of three measurements. Standard deviation were <10%.

were then incubated with the membranes for 2h at room temperature in fresh blocking solution. The membranes were washed three times at room temperature in TBS-Tween and then incubated with horseradish peroxidase-labeled sheep anti-mouse IgG (Cosmo Bio, Japan) for 1h at room temperature at a final dilution of 1/1500 in 10X Block Ace. The membranes were washed three times with TBS-Tween and the phosphotyrosine bands were revealed using Konica Immunostein HRP-1000 (Konica, Japan).

Results

Effect of MEL on insulin-dependent growth of K562 cells

The effect of MEL on the insulin-dependent growth of K562 cells, studied by adding MEL to the cells cultures. In the presence of MEL, the cell growth was inhibited in a dose-dependent manner (Figure 2). At 5.0 μM of MEL, K562 cell growth was completely inhibited. On the second day of cultivation, morphological changes of K562 were observed. The cells exhibited drastic morphological changes were found adhered to the bottom of the flak (Figure 3-B).

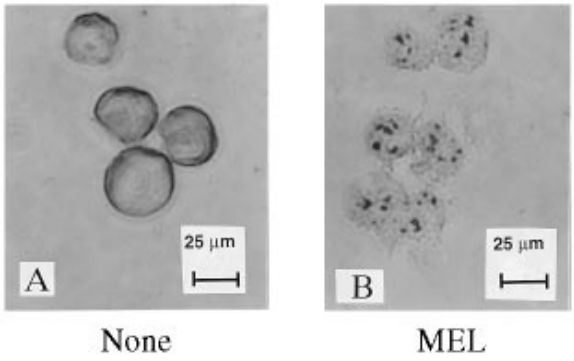

Figure 3. Giemsa staining of K562 cells cultured in the ERDF-ITES medium with 5.0 μM of MEL for 2 days. (A) Untreated K562 cells, (B) 5.0 μM of MEL treated K562 cells.

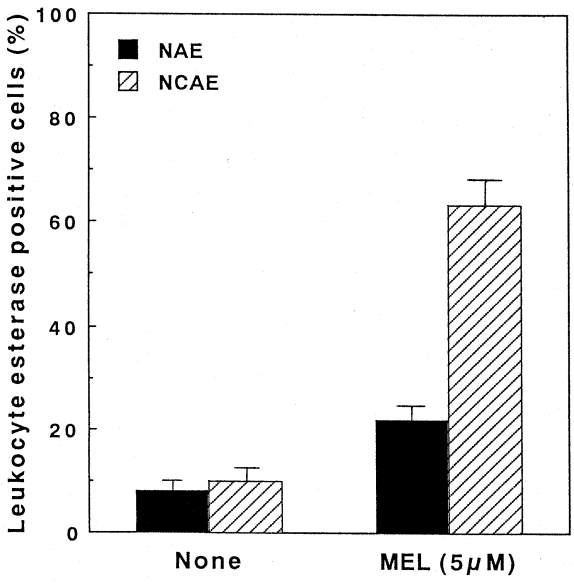

Figure 4. α-Naphthyl acetate esterase (NAE) activity and Naphthol AS-D-chloroacetate esterase (NCAE) activity of 5.0 μM of MEL treated cells. K562 cells were cultured in the ERDF-ITES medium with 5.0 μM of MEL for 3 days. Vales are mean of three determinations. Standard deviation were <10%.

Differention of K562 cells associated with insulin-dependent growth inhibition by MEL

K562 cells treated with 5.0 μM of MEL expressed NCAE-positive cells (from 10 to 63%) and NAE-positive cells (from 8 to 21%) which correspond to indicate specificity for the granulocytic lineage and the monocyticlineage, respectively (Figure 4). As shown in Figure 5, MEL induced the occurrence of NBT reducing ability (from 6 to 43%), expression of Fc receptors (from 12 to 38%), and phagocytic activities

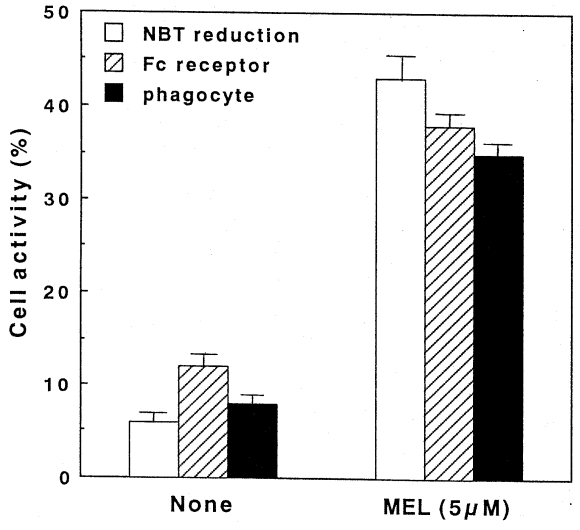

Figure 5. Differentiation-associated characteristic properties of 5.0 μM of MEL treated cells. NBT-reducing activity was determined by colorimetric assay. Assay of the appearance of Fc receptors was performed by standard techniques for erythrocyte-antibody (EA) rosette formation. Phagocytic activity is expressed as the eprcentage of cells ingesting yeast particvles. Values are mean of three determinations. Standard deviation were <10%.

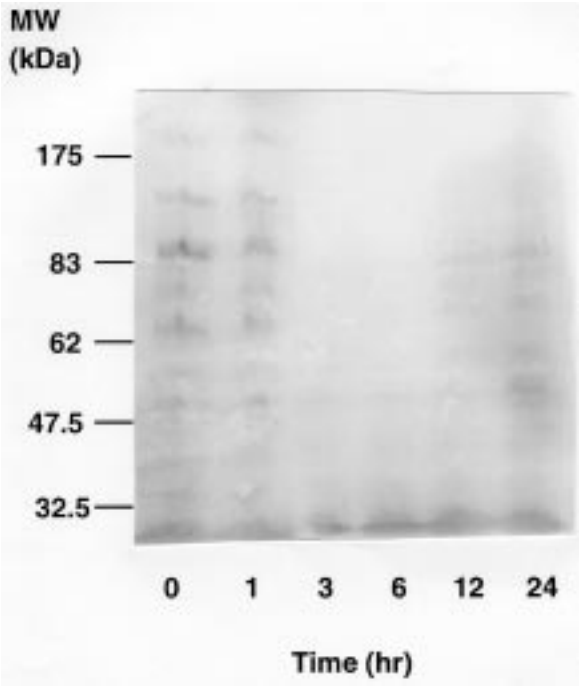

Figure 6. Time course of tyrosine phosphorylation pattern in MEL treated K562 cells. Immunoblotting and revelation of phosphotyrosine was conducted, as described in *Materials and Methods.*

(from 8 to 34%) in K562 cells. These results showed that MEL treatment induce the differentiation of K562 cells.

Time course of tyrosine phosphorylation in K562 cells treated with MEL

Time course of tyrosine phosphoylation in K562 cells treated with 5 μM of MEL was monitored. As shown in Figure 6, tyrosine phosphorylation of 55-, 65-, 95-, and 135-kDa proteins were detected in non treated cells, whereas a decrease in the level of phosphoproteins of such protein was observed in response to 5 μM of MEL at 3 hr. It was returned after 12 to 24 hr in K562 cells treated with 5 μM of MEL, and especially, tyrosine phosporylation of a 55-kDa protein was enhanced.

Discussion

The differentiation-induction ability of MEL on human myelogenous leukemia cell line K562 was examined. MEL inhibited the insulin-depent proliferation of K562 cells and enhanced differentiation associated characteristics in monocyte and granulocyte. The induction of specific and non specific leukocyte esterase showed that MEL induced granuulocytic differentiation. To confirm whether MEL regulated protein kineases in K562 cells, the tyrosine phosphorylation patterns were examined by the western blot analysis. The tyrosine phosphorylated proteins observed in non-treated cells were clearly inhibited by 5μM of MEL at 3 hr.

Insulin is a common factor required for metabolism of essentially all types of animal cells (Sinclair *et al*., 1988) and its receptor is well-characterized. The insulin receptor consists of two insulin-binding subunits (135-kDa) and two 95-kDa subunits showing tyrosine kinase activity. When insulin binds to the receptor, tyrosine phosphorylation at the 95-kDa subunits occurs (Nojiri *et al*., 1991). In this study, tyrosine phosphorylation of 95- and 135-kDa proteins which shows insulin-dependent receptor phosphorylation was observed in non-treated K562 cells grown in a serum-free ERDF-ITES medium, and the inhibitory effect of MEL on it was demonstrated. These results suggest that MEL may have interacted on the cell membrane and inhibited insulin receptor tyrosine kinase activity in cell membrane, inhibited cell proliferation, then induced differentiation of K562 cells.

In our previous study MEL induced granulocytic differentiation of HL60 that has been induced to differentiate into mature granulocytes (Collins *et al*., 1977; Brietman *et al*., 1980) and macrophage-like cells (Rovera *et al*., 1979, McCarthy *et al*., 1983) and consists of stem-like cells that are multipotent with respect to myeloid or macrophage differentiation. In this study, MEL also induced granulocytic differentiation of K562 that has been consists of stem-like cells that are multipotent with respect to granulocytes, monocyte, megakaryocyte or erythrocyte differentiation. Further studies on the mechanism of granulocytic differentiation induction by MEL compared to a wide variety of compounds including dimethyl sulfoxide (DMSO) and retinoic acid (RA) (Breitman *et al*., 1983) are needed.

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