

Phytosphingosine promotes megakaryocytic differentiation of myeloid leukemia cells

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We report that phytosphingosine, a sphingolipid found in many organisms and implicated in cellular signaling, promotes megakaryocytic differentiation of myeloid leukemia cells. Specifically, phytosphingosine induced several hallmark changes associated with megakaryopoiesis from K562 and HEL cells including cell cycle arrest, cell size increase and polyploidization. We also confirmed that cell type specific markers of megakaryocytes, CD41a and CD42b are induced by phytosphingosine. Phospholipids with highly similar structures were unable to induce similar changes, indicating that the activity of phytosphingosine is highly specific. Although phytosphingosine is known to activate p38 mitogen-activated protein kinase (MAPK)-mediated apoptosis, the signaling mechanisms involved in megakaryopoiesis appear to be distinct. In sum, we present another model for dissecting molecular details of megakaryocytic differentiation which in large part remains obscure. [BMB Reports 2015; 48(12): 691-695]

INTRODUCTION

Megakaryocytes arise from hematopoietic stem cells and after terminal differentiation undergo apoptosis releasing platelets which are indispensable for hemostasis and thrombosis (1, 2). Megakaryocytic differentiation features stereotypical changes including cellular enlargement and polyploidization (1, 3). Expression of cell type specific surface markers including CD41a, CD61 and CD42b, is also a well-known phenomenon associated with the differentiation (3).

Although megakaryopoiesis through progressively more com-

mitted precursors is well-defined at the cellular level, molecular mechanisms including signaling pathways and regulatory genes are far from clear. Cell signaling mediated by thrombopoietin (TPO) and c-mpl clearly plays a key role in induction of megakaryocytic differentiation and platelet genesis (4). However, down-stream signaling has not been completely characterized yet. The slow progress is in large part due to the lack of a robust *in vitro* system to study the process. The primary hematopoietic stem cells are limited in supply as they cannot be renewed or expanded *in vitro* effectively.

Cell lines derived from myeloid leukemia including K562 and HEL have been useful in that they partly recapitulate the megakaryocyte differentiation in response to various signaling molecules (5, 6). For example, phorbol 12-myristate 13-acetate (PMA) can activate mitogen-activated protein kinase-extracellular signal-regulated kinase (MEK-ERK) pathway and induce CD41a expression in response to AP1 activity from K562 cells (6). We have also reported that another molecule, 2-(Trimethylammonium) ethyl (R)-3-methoxy-3-oxo-2-stearamidopropyl phosphate ((R)-TEMOSPho) also induces megakaryocytic differentiation from K562 cells and primary human bone marrow-derived CD34⁺ cells (7).

Here, we present phytosphingosine as a novel differentiation inducer for megakaryocytes using K562 and HEL cells. Hallmark events including cell size increase, polyploidization and expression of CD41a and CD42b are confirmed. Importantly, although phytosphingosine is known to activate p38 MAPK signaling cascade-dependent apoptosis in myeloma cells including K562 cells (8), we provide evidences indicating that megakaryocytic differentiation is likely mediated by an alternate unknown pathway.

RESULTS AND DISCUSSION

We have previously reported that a phospholipid, (R)-TEMOSPho, induces megakaryocytic differentiation from K562 cells and primary CD34⁺ hematopoietic progenitor cells (7). We additionally screened diverse commercially available phospholipids (Fig. S1) to identify molecules with similar activities and identified phytosphingosine as a candidate based on induction of CD41a expression (Fig. 1A). Phytosphingosine was slightly but

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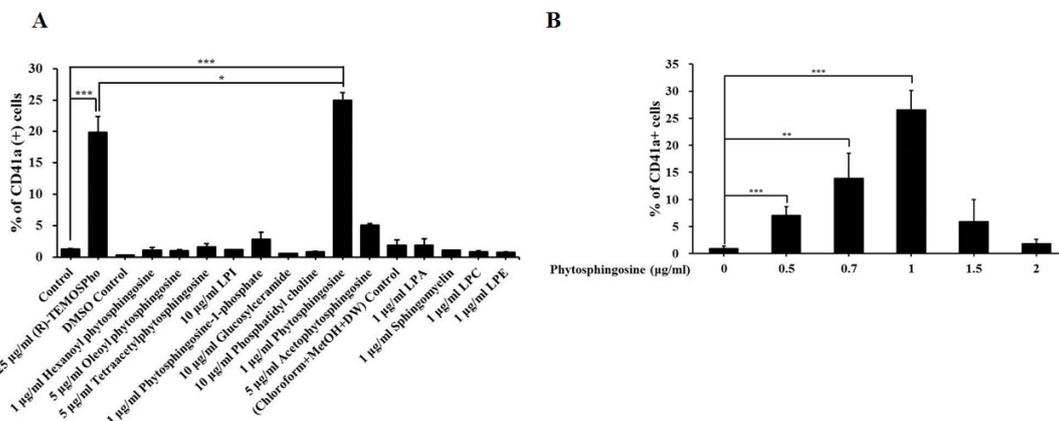


Fig. 1. Identification of phytosphingosine as a megakaryocytic differentiation inducing agent. (A) Induction of CD41a expression from K562 cells after 4 days of culture by phospholipids and sphingolipids at the indicated concentrations. Only phytosphingosine showed comparable activity to (R)-TEMOSPho. Results are averages \pm standard deviations of three independent assays. Statistical significance, tested by the Student's t-test is indicated. Typically, 10^4 events were analyzed. (B) The induction of CD41a expression in K562 cells at different concentrations of phytosphingosine. Titration of phytosphingosine shows that induction of CD41a expression peaks at 1 μ g/ml of phytosphingosine. Results are averages \pm standard deviations of four independent assays. Statistical significance, tested by the Student's t-test is indicated (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$).

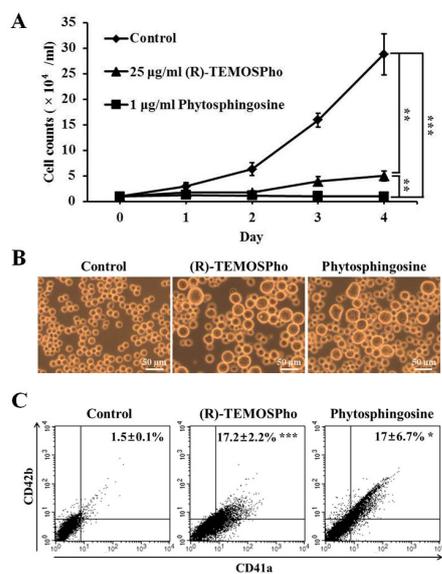


Fig. 2. Phytosphingosine-induced megakaryocytic differentiation of K562 cells. (A) Cell counts following treatment with 25 μ g/ml (R)-TEMOSPho or 1 μ g/ml phytosphingosine. (B) Cell size increase after 4 days of treatment with (R)-TEMOSPho or phytosphingosine. Cells were visually examined and photographed by phase-contrast microscopy. Scale bars represent 50 μ m. (C) Cell surface marker expression following treatment with (R)-TEMOSPho or Phytosphingosine for 6 days. The cells were labelled with monoclonal antibodies specific for the megakaryocyte cell surface markers CD41a and CD42b and analyzed by flow cytometry. Results are averages \pm standard deviations of three independent assays. Statistical significance, tested by the Student's t-test is indicated (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$).

reproducibly better than (R)-TEMOSPho in induction of CD41a expression. The inductive activity peaked at around 1 μ g/ml of phytosphingosine (Fig. 1B), and apoptosis was evident beyond that level (Fig. S2) as has been reported for K562 cells (8).

Phytosphingosine treatment led to cell cycle arrest (Fig. 2A) (11) and concomitant enlargement (Fig. 2B), consistent with megakaryocytic differentiation. Furthermore, CD41a and CD42b were co-expressed in differentiating cells (Fig. 2C). Phytosphingosine showed more potent activity than (R)-TEMOSPho in inducing cell cycle arrest, but the two reagents showed comparable activity in inducing megakaryocytic differentiation (7).

We also examined polyploidization of K562 cells which accompanies typical megakaryopoiesis. Consistently, phytosphingosine treatment led to a significant rise in the cells with increased chromosomal contents (Fig. 3A). Specifically, proportions of cells with chromosomal content of 8N increased at the cost of 2N cells with (R)-TEMOSPho and phytosphingosine treatments. At the cellular level, polyploidy cells were identified by DAPI staining. Enlarged cells with multiple nuclei within single cell boundary were readily observed among cells treated with (R)-TEMOSPho and phytosphingosine but not among control cells (Fig. 3B).

The induction of megakaryocytic differentiation by phytosphingosine was also examined using another myelocytic leukemic cell line. Application of phytosphingosine to HEL cells led to limited but qualitatively similar changes in cell size, nuclear content and expression of megakaryocyte specific markers, indicating that the differentiation effect of phytosphingosine is not restricted to K562 cells (Fig. S3).

Next, we examined the signaling pathway which phytosphingosine activates during differentiation. It has been pre-

viously reported that phytosphingosine induces apoptosis of K562 cells via activation of p38 MAPK pathway (8). In the study by Park and coworkers, 3 $\mu\text{g/ml}$ of phytosphingosine was typically used to induce apoptosis. This is the level at which we also saw apoptosis (Fig. S2) while maximal differ-

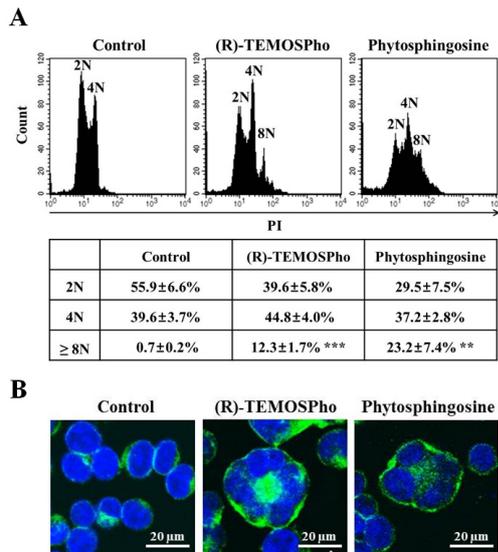


Fig. 3. Phytosphingosine-induced polyploidy of K562 cells. (A) Flow cytometric analyses for DNA contents following treatment with (R)-TEMOSPho or phytosphingosine for 4 days. Representative histograms are shown. The results from three independent assays are tabulated below. Statistical significance, tested by the Student's t-test is indicated (** $P < 0.005$, *** $P < 0.0005$). (B) K562 cells following treatment with (R)-TEMOSPho or phytosphingosine were DAPI-stained (blue) for nuclei and F-actin-stained (green) with phalloidin-Alexa Fluor 488 to highlight the boundary of single cells.

entiation was seen at 1 $\mu\text{g/ml}$. We examined activation of phosphatidylinositol 3-kinase (PI3K)-Akt, p38, and ERK signaling (2). Only AKT showed enhanced phosphorylation at 1 $\mu\text{g/ml}$ phytosphingosine treatment (Fig. 4A). Next, we attempted to block PI3K-AKT signaling with the specific inhibitor LY294002 which at 1 μM brought down phytosphingosine-mediated AKT activation near the basal level (Fig. 4B). Upon application of 5 μM LY294002, the phosphorylation of AKT was virtually abrogated (Fig. 4B). Interestingly, induction of CD41a by phytosphingosine is not affected by LY294002 even at 5 μM concentration (Fig. 4C). These data indicate that a pathway or pathways other than those examined mediate the induction of megakaryocytes by phytosphingosine.

In sum, we have demonstrated for the first time that phytosphingosine can induce differentiation of megakaryocytes from myeloid leukemia cells. Most notably, well-established changes associated with megakaryocytic differentiation including cell size increase, polyploidization and megakaryocyte specific marker expression were all demonstrated in K562 and HEL cell models. The effects on differentiation is comparable to that of (R)-TEMOSPho at least for K562 cells. Importantly, phytosphingosine is found as natural component of cells which implies that it may in fact be used as signaling molecule for megakaryocytic differentiation although this would require further investigations to demonstrate. Of note, it is likely that these two chemicals induce megakaryocytic differentiation via different mechanisms. Although PI3K-AKT pathway has been proposed to mediate the megakaryocytic induction by (R)-TEMOSPho in K562 cells (7), we were not able to observe inhibition of phytosphingosine-mediated megakaryopoiesis by applying LY294002, a PI3K-AKT inhibitor. Of note, c-MPL, the TPO receptor, is not expressed in K562 cells (9). Taken together, phytosphingosine likely activates a novel pathway for the induction of megakaryocytic differentiation. It should be noted

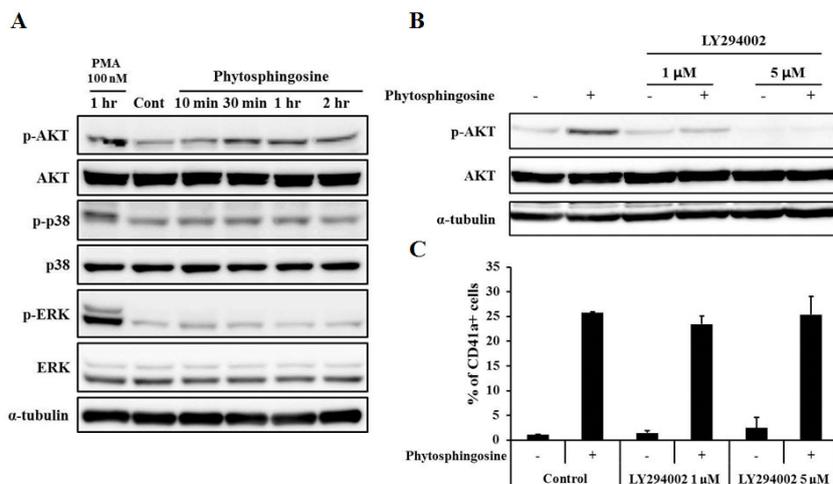


Fig. 4. Phytosphingosine-induced AKT phosphorylation is not involved in the megakaryocytic differentiation of K562 cells. (A) Immunoblot analyses were carried out using K562 cells treated with 1 $\mu\text{g/ml}$ Phytosphingosine for the indicated times. PMA treatment was used as the positive control. Note the time dependent induction of AKT phosphorylation in K562 cells. (B) Inhibition of AKT activation by 1 hour pretreatment with LY294002 at the indicated concentrations. (C) K562 cells were differentiated with 1 $\mu\text{g/ml}$ phytosphingosine in the absence or presence of LY294002. Note that % of CD41a+ cells is not altered by LY294002.

that even in TPO or c-MPL knockout mice, low level megakaryocytes and platelets are found which implies that TPO-c-MPL independent signaling pathway or pathways exist perhaps as back-up systems (3). Further molecular dissections, including determination of the down-stream signaling cascade, should lead to further understanding not only of the role of phytosphingosine but also of the mechanism of megakaryocytic differentiation.

MATERIALS AND METHODS

Cell culture

Routine K562 cell culture was carried out in RPMI 1640 medium containing 10% FBS. HEL cells were cultured in RPMI 1640 medium with 4.5 g/l glucose, 10 mM HEPES, and 1 mM sodium pyruvate, supplemented with 10% FBS.

K562 and HEL cells were obtained from the American Type Culture Collection (Manassas, VA). RPMI 1640 medium and fetal bovine serum were purchased from Hyclone Laboratories Inc. (Logan, UT).

Megakaryocyte differentiation

Phospholipids and sphingolipids were dissolved in DMSO or alcohol-containing solution prior to application to cell culture media. For megakaryocytic differentiation, 3×10^4 cells were seeded in 6-well plate with 3 ml of culture media which contained indicated levels of phytosphingosine or (R)-TEMOSPho. On subsequent days, 1 ml of fresh media containing phytosphingosine or (R)-TEMOSPho was added to the culture media.

2-(Trimethylammonium) ethyl (R)-3-methoxy-3-oxo-2-stearamidopropyl phosphate ((R)-TEMOSPho) was synthesized following previously reported methods with modifications (10). Sphingomyelin was purchased from Sigma Aldrich (St. Louis, MO), and Oleoyl phytosphingosine, Tetraacetyl phytosphingosine, Hexanoyl phytosphingosine, phytosphingosine, Acetyl phytosphingosine, Glucosyl ceramide, Phosphatidyl choline were supplied by Doosan Biotech (Seoul, Korea). Phytosphingosine-1-phosphate, Lysophosphatidyl ethanolamine, Lysophosphatidyl choline, Lysophosphatidic acid, Lysophosphatidyl inositol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Flow cytometry

Cells were stained with 10 μ l (1X) FITC conjugated anti-CD41a antibody and/or PE-Cy5-conjugated anti-CD42b antibody and were analyzed by flow cytometry in a FACS Calibur BD Biosciences (San Jose, CA) using the BD Cell-Quest™ Pro version 6.0 software BD Biosciences (San Jose, CA). For DNA content and apoptosis analyses (12), cells were incubated for 25 minutes on ice in staining solution (25 μ g/ml PI, 0.1% saponin, 30 μ g/ml RNase A in PBS pH 7.4) and analyzed by FACS Calibur.

FITC-conjugated anti-CD41a antibody and PE-Cy5-conjugated anti-CD42b antibody were purchased from BD Biosciences

(San Jose, CA, USA). Propidium iodide (PI) was from Sigma-Aldrich (St. Louis, MO). RNase A was purchased from USB (Cleveland, OH).

Phalloidin staining

Cells were fixed and permeabilized with 1% paraformaldehyde in PBS containing 0.1% Triton X-100 for 1 hour at room temperature (RT). Subsequently, cells were blocked with 1% BSA in PBS containing Tween 20 (0.1%) for 1 hour at RT and then stained for F-actin with phalloidin-Alexa Fluor 488 (1:200, Invitrogen, Carlsbad, CA) at RT for 1 hour. Cells were counterstained with 4'-6-Diamidino-2-phenylindole (DAPI) and visualized using a Zeiss laser scanning microscope (LSM 510 META, Zeiss, Germany) using a 40 \times /1.2 W apochromat objective (Zeiss, Germany).

Alexa 488-conjugated phalloidin was purchased from Invitrogen (Carlsbad, CA). 4'-6-Diamidino-2-phenylindole (DAPI) was from Sigma-Aldrich (St. Louis, MO).

Western blotting

Whole-cell lysates (50 μ g) from K562 cells were subjected to 10% SDS-PAGE, transferred to nitrocellulose transfer membrane and incubated with primary antibodies overnight at 4°C. Anti-p-AKT, anti-AKT, anti-p-Erk-1,2, anti-Erk-1,2, anti-p-p38 and anti-p38 antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA) and used at a concentration of 1:1,000. After incubation with the secondary antibodies, blots were developed using an ECL immunodetection kit.

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