

Inhibition of Cytokinesis by a Lipid Metabolite, Psychosine

Takayuki Kanazawa,* Sachiko Nakamura,* Michiko Momoi,[‡] Toshiyuki Yamaji,* Hiromu Takematsu,[‡] Hajime Yano,[§] Hisataka Sabe,[§] Akitsugu Yamamoto,^{||} Toshiyuki Kawasaki,* and Yasunori Kozutsumi[‡]

*Department of Biological Chemistry, Graduate School of Pharmaceutical Sciences, and [‡]Laboratory of Membrane Biochemistry and Biophysics, Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan; [§]Department of Molecular Biology, Osaka Bioscience Institute, Suita 565-0874, Japan; and ^{||}Department of Physiology and Liver Research Center, Kansai Medical University, Moriguchi 570-0074, Japan

Abstract. Although a number of cellular components of cytokinesis have been identified, little is known about the detailed mechanisms underlying this process. Here, we report that the lipid metabolite psychosine (galactosylsphingosine), derived from galactosylceramide, induced formation of multinuclear cells from a variety of nonadherent and adherent cells due to inhibition of cytokinesis. When psychosine was added to the human myelomonocyte cell line U937, which was the most sensitive among the cell lines tested, cleavage furrow formed either incompletely or almost completely. However, abnormal contractile movement was detected in which the cellular contents of one of the hemispheres of the contracting cell were transferred into its

counterpart. Finally, the cleavage furrow disappeared and cytokinesis was reversed. Psychosine treatment also induced giant clots of actin filaments in the cells that probably consisted of small vacuoles with filamentous structures, suggesting that psychosine affected actin reorganization. These observations could account for the formation of multinuclear globoid cells in the brains of patients with globoid cell leukodystrophy, a neurological disorder characterized by the accumulation of psychosine due to galactosylceramidase deficiency.

Key words: cytokinesis • multinuclear cell • globoid cell leukodystrophy • psychosine • actin filament

Introduction

Cytokinesis is a multistage process consisting of cleavage furrow formation, contraction, and cell division. Lines of evidence suggest that Rho signaling pathways are involved in the regulation of cytokinesis. Rho-associated kinase and citron kinase, which is a partner of the active form of Rho, play a role in cytokinesis (Goto et al., 1998; Madaule et al., 1998). The dominant-negative form of ECT2, an exchange factor for Rho GTPase, inhibits cytokinesis (Tatsumoto et al., 1999). The Rho family itself is also known to function in cytokinesis (Mabuchi et al., 1993; Kishi et al., 1993; O'Connell et al., 1999). Although a number of proteins are involved in cytokinesis, the regulation of cytokinesis is not fully understood. Analysis of physiologically or pathologically occurring inhibition of cytokinesis and the resultant formation of multinuclear cells would be useful for better understanding of the mechanism of cytokinesis.

Globoid cell leukodystrophy (GLD)¹ or Krabbe's dis-

ease is a neurological disorder characterized by severe myelin loss and mental and motor deterioration in infants (Krabbe, 1916; Matsushima et al., 1994; Suzuki, 1998). Another characteristic of GLD is the formation of globoid cells, from which the name GLD is derived. Globoid cells are multinuclear globular giant cells, and the white matter of GLD patients contains many globoid cells (Krabbe, 1916; Matsushima et al., 1994; Suzuki, 1998). Based on the results of morphological and biochemical analyses, globoid cells are thought to be derived from microglia and/or macrophages (Suzuki, 1984; Ohno et al., 1993; Matsushima et al., 1994). Immune response antigen (Ia) expression on microglia and/or macrophages has been found to be a trigger of globoid cell formation using Ia-transgenic mice and twitcher mice, a murine model of GLD (Duchen et al., 1980; Matsushima et al., 1994). However, the mechanism of globoid cell formation, including the multiple nuclei and the increased cell size, has not been clarified.

The primary defect of GLD is the deficiency of galactosylceramidase, a lysosomal enzyme that catalyzes the hy-

antigen; Psy, psychosine; SPC, sphingosylphosphorylcholine; TPA, 12-O-tetradecanoylphorbol acetate.

Address correspondence to Dr. Y. Kozutsumi, Laboratory of Membrane Biochemistry and Biophysics, Graduate School of Biostudies, Kyoto University, Yoshida Shimoadachi, Sakyo-ku, Kyoto 606-8501, Japan. Tel.: 81-75-753-4562. Fax: 81-75-753-4605. E-mail: yasu@pharm.kyoto-u.ac.jp

¹Abbreviations used in this paper: GalCer, galactosylceramide; GlcPsy, glucopsychosine; GLD, globoid cell leukodystrophy; Ia, immune response

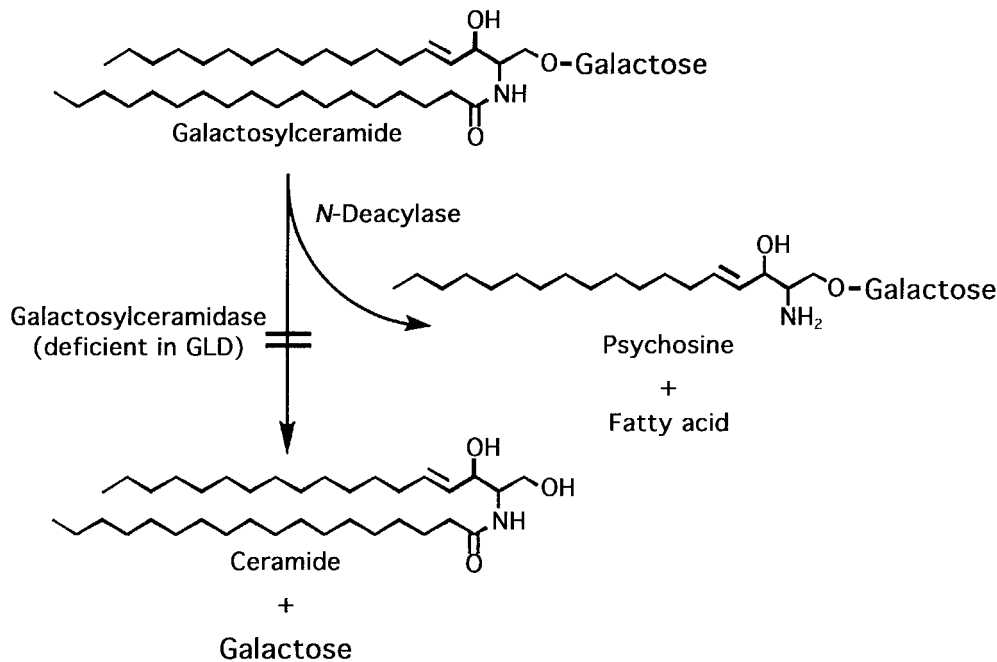


Figure 1. Metabolic pathway of GalCer. Characteristics of GLD.

drolisis of galactosylceramide (GalCer) to galactose and ceramide (Kobayashi et al., 1985; Mitsuo et al., 1989) (Fig. 1). GalCer is virtually absent in the mammalian brain before myelination and increases rapidly during myelination (Suzuki and Suzuki, 1989). GalCer is formed from ceramide and UDP-galactose catalyzed by GalCer synthase, and knock-out mice deficient in this enzyme show abnormal myelin function (Coetzee et al., 1996), suggesting that GalCer is essential for the maintenance of myelinating oligodendroglia. GLD leads to the accumulation of GalCer and its metabolic intermediate psychosine (Psy) due to galactosylceramidase deficiency (Svennerholm et al., 1980; Suzuki, 1998). Usually, GalCer is predominantly metabolized to galactose and ceramide by galactosylceramidase. However, in galactosylceramidase deficiency, GalCer is significantly converted to Psy by deacylation (Svennerholm et al., 1980) (Fig. 1). Psy accumulated in the oligodendroglia, and Schwann cells may result in the destruction of these cells concomitant with severe myelin loss (Nagara et al., 1986; Ida et al., 1990; Suzuki, 1998).

In this study, we showed that Psy inhibited cytokinesis, particularly the process of contraction, and induced formation of multinuclear giant cells, which could account for the formation of globoid cells in GLD patients.

Materials and Methods

Materials

N-Acetylpsychosine and lysosulfatide were purchased from Matreya, Inc. All other lipids were from Sigma Chemical Co. Lipids were dissolved in ethanol and stored at -20°C . Rhodamine-phalloidin was from Molecular Probes. FITC-phalloidin was from Sigma Chemical Co. 12-*O*-tetradecanoylphorbol acetate (TPA) was from Wako Chemicals.

Cells and Cell Culture

U937 human leukemia cells and HeLa human cervix adenocarcinoma cells were obtained from Japanese Cancer Research Resources. Other cell

lines were from the American Type Culture Collection. U937 cells were maintained in RPMI 1640 containing 10% FCS. HeLa cells were maintained in MEM containing 10% calf serum.

Flow Cytometry

Cells were cultured as indicated, harvested, and then fixed with 70% ethanol. Then, the cells were treated with RNase A (0.1 mg/ml in PBS) at 37°C for 30 min, stained with propidium iodide (50 $\mu\text{g}/\text{ml}$ in PBS), and then subjected to flow cytometry with a FACScan™ flow cytometer (Becton Dickinson) for measurement of the DNA content.

Confocal Microscopy

U937 cells and HeLa cells were incubated with or without Psy. For detection of multinuclear cell formation, cells were fixed with 4% paraformaldehyde for 15 min, and then digested with RNase A (0.1 mg/ml in PBS containing 0.1% Triton X-100) for 30 min at 37°C . After these treatments, the cells were stained with propidium iodide for 10 min. To stain actin filaments, paraformaldehyde-fixed cells were treated with FITC- or rhodamine-phalloidin dissolved in PBS containing 0.1% Triton X-100 and 1% BSA. The stained cells were observed by confocal laser microscopy (FLUOVIEW™; Olympus).

EM

Cells were incubated with Psy (2 μM) or vehicle alone (ethanol) for 72 h. The cells were then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h, washed three times in the same buffer for 10 min, and postfixed in 1% OsO_4 in the same buffer for 1 h. After washing in distilled water, the cells were incubated with 50% ethanol for 10 min, and were block stained with 2% uranyl acetate in 70% ethanol for 2 h. The cells were further dehydrated with a graded series of ethanol and embedded in epoxy resin. Ultra-thin sections were double stained with uranyl acetate and lead citrate, and were observed under a Hitachi H7000 electron microscope (Hitachi).

Time-lapse Video Microscopy

Aphidicolin-synchronized U937 cells (2.0×10^6) were incubated with or without 5 μM Psy in tissue culture flasks for 7.5 h. Psy was used at this concentration to shorten the induction time. After gassing with 5% CO_2 and 95% air, the flasks were placed on a heated stage of an Olympus LX70 inverted microscope equipped with a camera (MCD-350; Olympus) and time-lapse video recorder (LVR-300 AN/OL; Sony-Olympus).

Psy Determination

Liquid chromatography/ion-spray ionization tandem mass spectrometry was performed essentially as described (Mano et al., 1997). The multiple reaction monitoring mode was used, with ions monitored at m/z 462 (precursor ion)–282 (daughter ion) for Psy. U937 cells were incubated with Psy (1 μ M) for 48 h and harvested. The incorporated Psy was extracted, separated by a Waters 625 LC system (Millipore) equipped with a Develosil ODS HG-5 reversed-phase column (35 \times 2.0 mm i.d., 5 μ m; Nomura Chemical Co.), and analyzed by an API III plus mass spectrometer (Perkin-Elmer Sciex Instruments).

Measurement of Phagocytotic Activity

U937 cells (1.0×10^6) were treated with TPA (0.16 μ M) (Gidlund et al., 1981) and the phagocytotic activity was measured by incubation with FITC-labeled and opsonized yeast cells (2.0×10^7) for 3 h (Ragsdale and Grasso, 1989). Psy (2 μ M) was added to the cells 24 h before TPA treatment and remained in the medium during TPA treatment and the measurement of phagocytotic activity. The phagocytotic cells containing the FITC-labeled yeast were visualized by confocal laser microscopy and counted.

Results

Induction of Globoid-like Cells from U937 Cells

To test the hypothesis that Psy induces the formation of globoid cells in GLD, we constructed a tissue culture model for globoid cell formation. Based on the results of morphological and biochemical analyses, the globoid cells in GLD are thought to be derived from microglia and/or macrophages (Suzuki, 1984; Ohno et al., 1993; Matsushima et al., 1994). The human myelomonocyte cell line U937 was therefore used as a model cell line for the formation of GLD globoid cells. When U937 cells were incubated with Psy (2 μ M), multinuclear globular giant cells were formed

that were similar to the globoid cells in GLD (Fig. 2, a and b). The cells were \sim 20–40 μ m in diameter after a 96-h incubation with Psy, which was not inconsistent with the average size (50 μ m) of globoid cells (Duchen et al., 1980). The nuclear contents of these giant cells were 2N, 4N, 8N, 16N, 32N, and 64N with hypodiploid peaks after 96 h of Psy treatment (Fig. 2, e and f), suggesting that multinuclear cell formation is not due to cell–cell fusion but to cell cycle progression without cytokinesis. Hypodiploid peak formation was probably due to apoptosis of a portion of the cells, consistent with the Psy function reported previously (Cho et al., 1997). On the other hand, treatment with GalCer, which also accumulates in the brains of patients with GLD, did not lead to globoid-like cell formation (data not shown). The Psy incorporated into U937 cells from the medium was determined by means of liquid chromatography/ion-spray ionization tandem mass spectrometry (Mano et al., 1997) after extraction of Psy from the cells. The amount of accumulated Psy was calculated as 5.24 nmol/mg protein, which corresponded to 7.4% of that added. The Psy accumulation did not induce a significant increase in GalCer (data not shown), supporting the suggestion that GalCer does not induce globoid-like cell formation. Furthermore, the Psy-induced formation of globoid-like cells was not due to the degradation of Psy to sphingosine, because sphingosine treatment induces apoptosis of U937 cells but not multinuclear cell formation (Sweeney et al., 1996). Thus, the accumulated Psy directly induced globoid-like cell formation.

Cell Specificity for the Formation of Multinuclear Globoid-like Cells Induced by Psy

The cell specificity for globoid-like cell formation was ex-

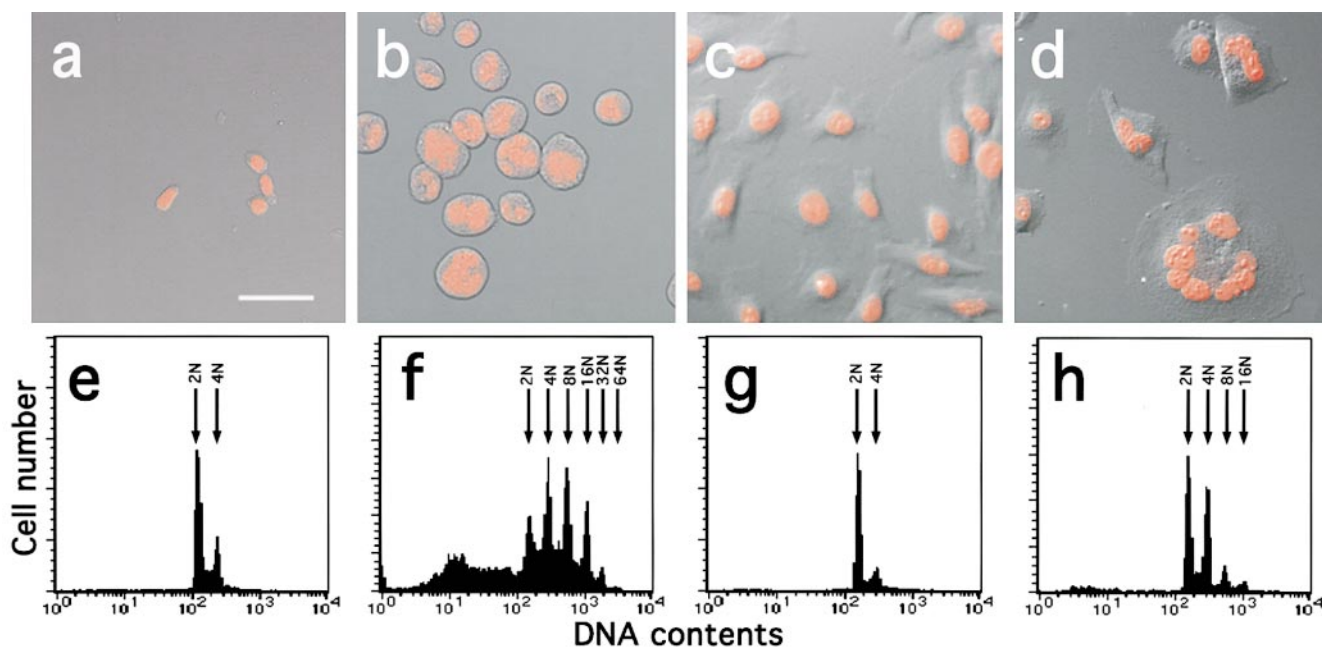


Figure 2. Psy-induced formation of multinuclear cells from U937 and HeLa cells. U937 cells were incubated with vehicle alone (ethanol) as a control (a and e) or 2 μ M Psy (b and f) for 96 h. HeLa cells were incubated with vehicle alone (c and g) or 35 μ M Psy (d and h) for 77 h. Nuclei in the cells were stained with propidium iodide, and then the stained cells were observed by confocal laser microscopy (a–d). The DNA contents of these cells were determined by flow cytometry (e–h). Bar, 50 μ m.

aminated using several cell lines. Among the cell lines tested, U937 cells, HL-60 human promyelocytic leukemia cells, CTLL-2 mouse cytotoxic T cells, Hep G2 human hepatoma cells, and HeLa human cervix adenocarcinoma cells formed multinuclear giant cells on incubation with Psy, suggesting that Psy-dependent multinuclear cell formation is a common phenomenon in many cell types. However, the amount of Psy required differed between cell lines, with U937 cells being the most sensitive. The minimum concentration of Psy that induced the formation of globoid-like cells was 1.0 μM , and the induction was dose-dependent (data not shown). On the other hand, HeLa cells required the highest amount of Psy for multinuclear giant cell formation; 35 μM Psy was needed for the minimum induction (Fig. 2, c and d). However, the shape of the giant multinuclear cells derived from HeLa cells was not globoid-like, because the cells still adhered to the culture dishes even after treatment with Psy. On the other hand, Psy was extremely cytotoxic to Neuro2A mouse neuroblastoma cells and P19 embryonal carcinoma cells.

These cells, therefore, died on Psy treatment (data not shown).

Psy Inhibits Cytokinesis

To determine whether Psy-induced multinuclear cell formation is due to inhibition of cytokinesis, cytokinesis was monitored in U937 cells by time-lapse video microscopy. In the absence of Psy, the cleavage furrow formed within minutes after anaphase onset and then the midbody, an intercellular bridge, emerged (Fig. 3 a). The cells divided into daughter cells within ~ 90 min after formation of the cleavage furrow. On the other hand, Psy-treated cells also had cleavage furrows, but they were shallow in about half of the cells (data not shown) and deep in the remaining half (Fig. 3 b). However, abnormal contractile movement was observed after cleavage furrow formation. As shown in Fig. 3 b, the contents of one of the hemispheres of the contracting cells were transferred into its counterpart. Finally the cleavage furrow disappeared and Psy-treated

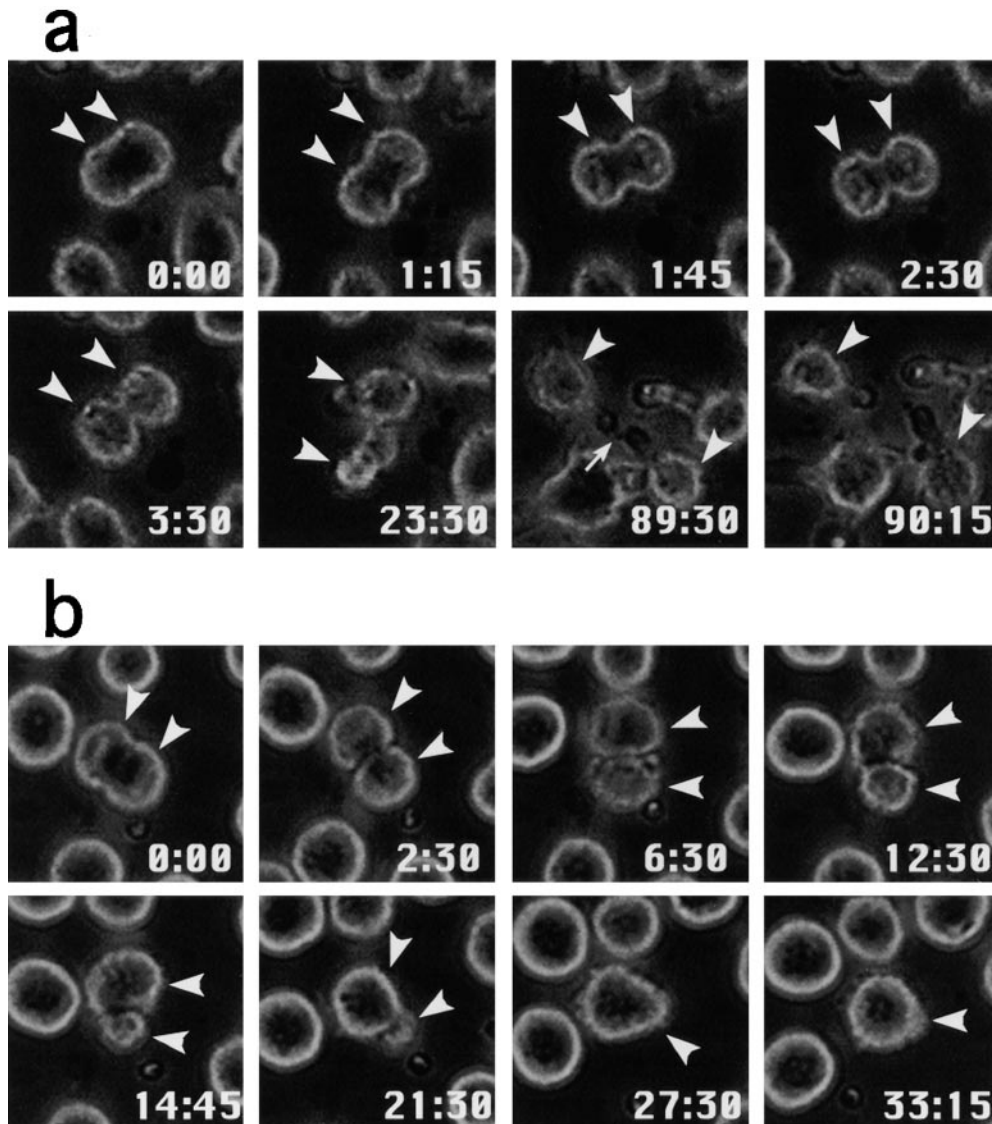


Figure 3. Video microscopy of Psy-treated U937 cells. Aphidicolin-synchronized U937 cells were incubated with vehicle (a) or 5 μM Psy (b). Cells were monitored by time-lapse phase contrast video microscopy. Images are shown at selected time points (min:sec). Each first image (0:00) shows the anaphase. Arrowheads indicate the prospective daughter cells (a and b) or those cells in which cytokinesis was reversed (b). The arrow indicates the midbody (a).

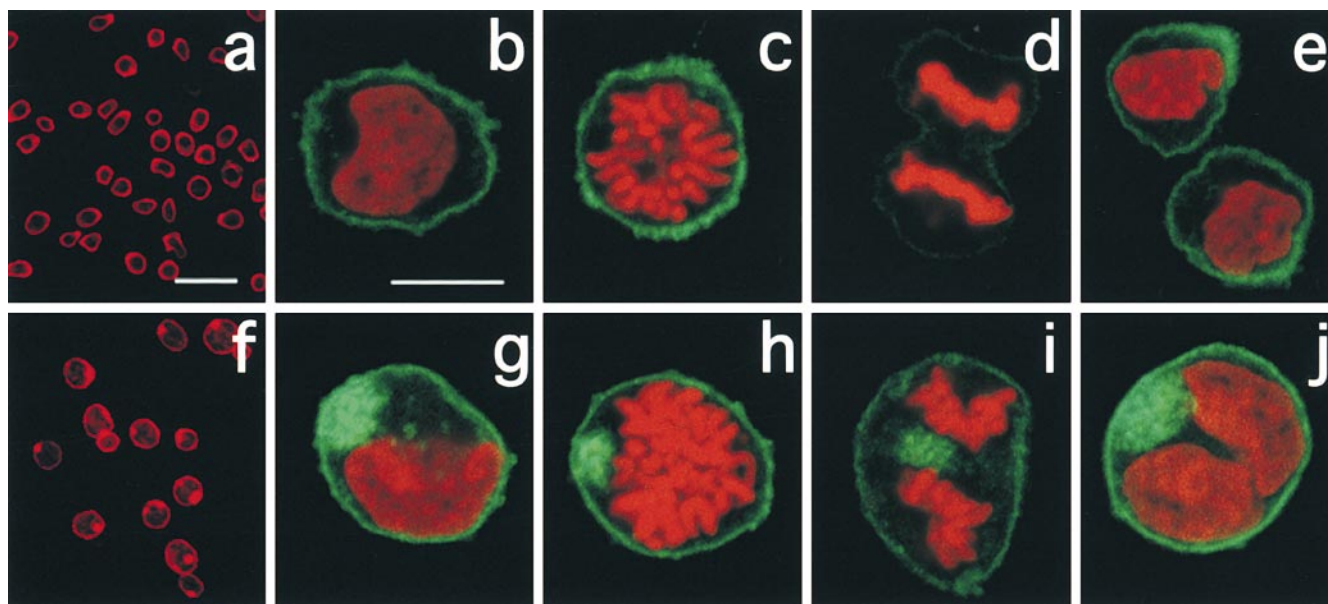


Figure 4. Giant actin clots induced in U937 cells. In a and f, U937 cells were incubated with vehicle alone (a) or 2 μ M Psy (f) for 48 h and actin filaments were stained with rhodamine-conjugated phalloidin (red). In b–e and g–j, aphidicolin-synchronized U937 cells were incubated with vehicle (b–e) or 2 μ M Psy (g–j) for 9 h. At this time point, most cells were in the mitotic phase. Cells were then treated with FITC-phalloidin (green) for actin filament staining and with propidium iodide (red) for nuclear staining. b and g, prophase cells; c and h, prometaphase cells; d and i, telophase cells; and e and j, cells that had passed through the mitotic phase. Bars: 50 μ m (a and f); and 10 μ m (b–e and g–j).

cells became round. Therefore, the midbody could not be detected in the Psy-treated cells. These observations indicated that Psy inhibits cytokinesis and induces formation of multinuclear cells.

Psy Induces the Formation of Giant Clots of Actin Filaments

To elucidate the mechanism of the inhibition of cytokinesis, actin filaments of Psy-treated U937 cells were visualized with FITC- or rhodamine-phalloidin. As shown in Fig. 4, a and f, abnormal actin localization was detected in the Psy-treated cells. The giant actin clots were detected after incubation with Psy for 5 h and continued to be detected even during M phase (Fig. 4, g–j).

Psy Induces the Assembly of Small Vacuoles

To study the structure of the actin clots, the Psy-treated cells were subjected to EM. In the Psy-treated cells, assembly of small vacuoles (0.3–0.8 μ m in diameter) was observed along with the plasma membrane, where the actin clots were detected (Fig. 5 b). However, no such vacuoles were observed in the control cells (Fig. 5 a). Most vacuoles had low-density contents. Furthermore, filamentous structures were observed at higher magnification on EM (Fig. 5 c). These structures were seen around vacuoles, and some of the filaments were associated with the vacuole membrane. The average length of these structures was 0.5 μ m. These observations suggested that the giant clots of actin filaments could contain small vacuoles with these filamentous structures.

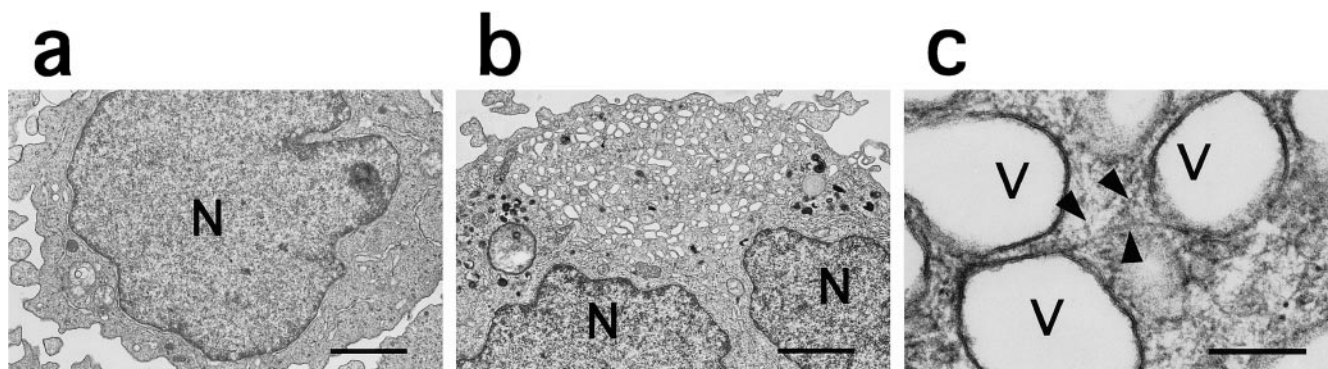


Figure 5. EM of psycosine-treated U937 cells. Cells were incubated with vehicle (a) or 2 μ M Psy (b and c). Ultra-thin sections were stained and observed under an electron microscope. Bars: 2 μ m (a and b); and 0.2 μ m (c). Arrowheads indicate filamentous structures (c). N and V indicate nucleus and vacuole, respectively.

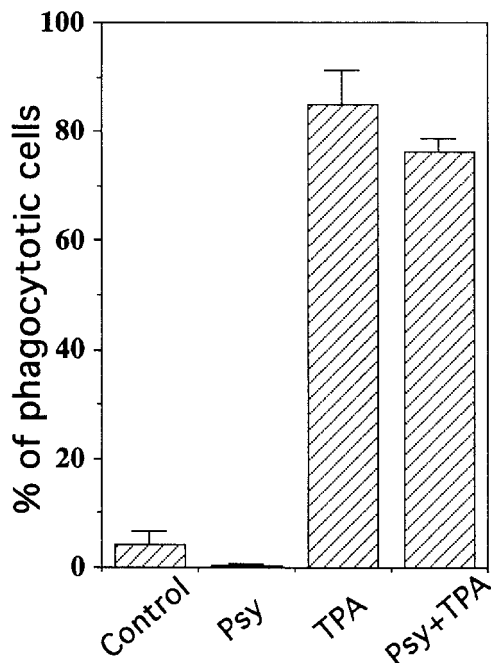


Figure 6. Effects of psychosine on phagocytotic activity of TPA-treated U937 cells. Cells were treated with or without TPA and the phagocytotic activity was measured. The TPA treatment and the measurement of phagocytotic activity was performed in the presence of 2 μ M Psy or vehicle alone (ethanol). Values are means of three experiments.

Effects of Psy on Phagocytotic Activity

To examine the general effects of Psy on the processes involving the membrane cytoskeleton, the effects of Psy on the phagocytotic activity of U937 cells was examined. U937 cells were differentiated to phagocytotic cells by TPA (Gidlund et al., 1981) and the phagocytotic activity was measured. Even in the presence of Psy, >90% of the activity was detected in TPA-treated cells (Fig. 6). On the other hand, Psy strongly inhibited the phagocytotic activity in TPA-untreated cells, although the phagocytotic activity of the untreated cells was very low.

Lipid Specificity for the Induction of the Multinuclear Globoid-like Cells

To determine the lipid specificity for the induction of these multinuclear cells, the effects of several lipids related to Psy were examined. As shown in Fig. 7, glucopsychosine (GlcPsy) exhibited the highest level of globoid-like cell formation among the lipids examined, followed by Psy, sphingosylphosphorylcholine (SPC), and lysosulfatide, in that order. These observations indicated that the free amino group of the sphingosine portions of these sphingolipids is indispensable for the induction. Indeed, *N*-acyl derivatives of these sphingolipids, including *N*-acetylpsychosine, GalCer, glucosylceramide, sulfatide, and sphingomyelin, showed no activity (data not shown).

Discussion

Our results showed that Psy inhibits cytokinesis and in-

duces the formation of multinuclear globular giant cells. Time-lapse video microscopy revealed that Psy-induced formation of multinuclear cells was not due to cell-cell fusion but to inhibition of cytokinesis. The DNA contents of multinuclear cells also indicated the inhibition of cytokinesis. Psy-treated cells showed abnormal contractile movement after cleavage furrow formation. The contents of one hemisphere of the cells moved into its counterpart and the cleavage furrow disappeared. Similar abnormal contractile movement was reported in the cells overexpressing citron kinase mutant. Citron kinase is one of the splice variants of citron, which is a partner of the active form of Rho and contains a protein kinase domain (Madaule et al., 1998). Overexpression of a kinase-active citron kinase mutant induced such transfer of the cellular contents into the other hemisphere. The similarity between the two suggested that Psy might interfere with citron kinase and/or Rho. However, during the abnormal contractile movement, the transferred materials were pushed back again into the original hemisphere, and these push-pull movements occurred repeatedly in the citron kinase mutant, but no such repeated movement was detected in the Psy-treated cells. The detailed mechanism of action of Psy, therefore, remains to be determined.

Citron is a target protein of Rho, which controls the formation of actin structures (Machesky and Hall, 1996). Indeed, Psy induced abnormal giant clots of actin filaments near the plasma membrane in the cells. Electron micrographs indicated the assembly of small vacuoles along the plasma membrane where the actin clots were observed. Furthermore, filamentous structures were associated with these vacuoles, suggesting that the abnormal giant clots of actin filaments contained the small vacuoles, although it is not clear whether these filamentous structures were derived from actin filaments. The origin of the small vacuoles is not known, but on the basis of their shapes, the vacuoles may be derived from endosomes or lysosomes. The relationship between inhibition of cytokinesis and formation of actin clots is unclear, but these observations strongly suggested that Psy affects actin reorganization in the cells. However, Psy did not inhibit all the processes involving the membrane cytoskeleton, because phagocytosis by the TPA-treated U937 cells was not significantly inhibited by Psy, suggesting that inhibition of cytokinesis and formation of actin clots were not due to nonspecific effects of the membrane integrity.

Recent studies indicated that glycosphingolipids and sphingomyelin are localized in membrane microdomains or rafts, which represent subcompartments of the plasma membrane (Deckert et al., 1996; Simons and Ikonen, 1997; Harder and Simons, 1999). These microdomains provide sites of interaction with the actin-based cytoskeleton. Psy is an abnormal glycosphingolipid lacking its acyl chain, and therefore accumulation of Psy may disturb the lipid microdomains and lead to the induction of giant clots of actin filaments. Alternatively, Psy is known to inhibit protein kinase C (Hannun and Bell, 1987; Sugama et al., 1991; Yamada et al., 1996) and mitochondrial cytochrome c oxidase (Igisu et al., 1988; Cooper et al., 1993), and to induce cell death (Ida et al., 1990; Cho et al., 1997) and the calcium spike response (Okajima and Kondo, 1995; Lai et al.,

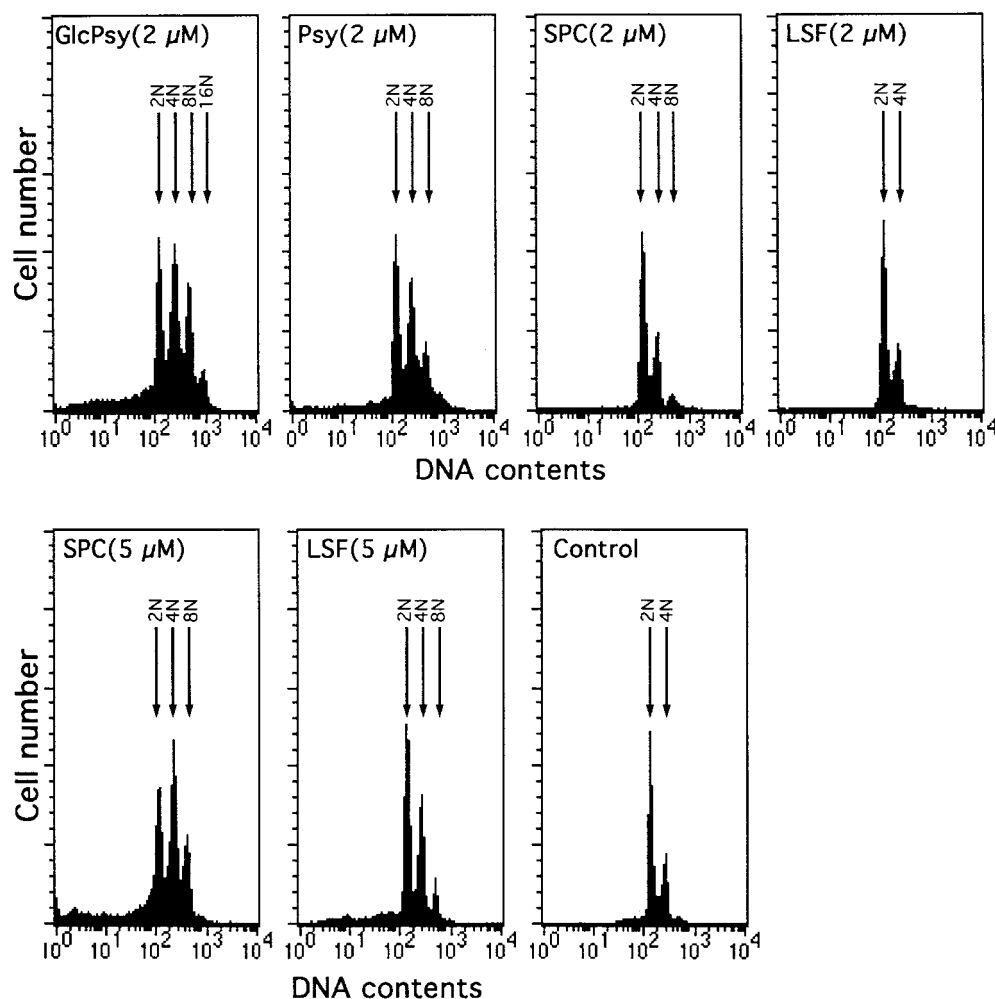


Figure 7. Multinuclear cell formation by U937 cells with various sphingolipids. U937 cells were incubated with GlcPsy (2 μ M), Psy (2 μ M), SPC (2 or 5 μ M), lysosulfatide (LSF) (2 or 5 μ M), or vehicle alone (ethanol) as a control for 48 h, harvested, and then subjected to flow cytometry after staining of nuclei.

1997; Gonda et al., 1999). These biological actions may be involved in inhibition of cytokinesis.

Psy-induced multinuclear cells were similar to the globoid cells detected in the brains of GLD patients. Psy is primarily accumulated in oligodendroglia, which contain abundant GalCer, a precursor of Psy. The accumulated Psy is cytotoxic (Cho et al., 1997), and these cells were therefore injured or died on Psy accumulation, probably due to apoptosis. The resultant oligodendroglia could be engulfed by microglia and/or macrophages. The secondary accumulation of Psy in microglia and/or macrophages probably induces the formation of globoid cells. The toxicity of Psy to the oligodendroglia would not be the direct trigger of demyelination, but phagocytosis by microglia and/or macrophages could be the trigger, because Ia transgenic twitter mice, in which Psy accumulates in oligodendroglia but phagocytosis by microglia and/or macrophages is impaired, do not show severe demyelination (Matsushima et al., 1994).

Among the lysosphingolipids examined, Psy and GlcPsy potently induced the formation of multinuclear globoid-like cells. On the other hand, lysosulfatide and SPC showed weak but significant activity. Thus, lysosphingolipids, including GlcPsy, Psy, lysosulfatide, and SPC, have the ability to inhibit cytokinesis and to induce the formation of multinuclear cells in many types of cells.

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