Glycosphingolipids of animal cells are located in the plasma membrane, and play roles in a variety of cellular events, including growth and differentiation (3). Considerable evidence suggests that glycosphingolipids exogenously added to cell culture medium are incorporated into the plasma membrane, and function as well as those synthesized in situ (3, 5, 6, 7, 15). Nojiri et al. demonstrated that ganglioside GM3 characteristically increased during macrophage-like cell differentiation of human promyelocytic leukemia cell line HL-60 (11). They further proved that exogenous ganglioside GM3 was highly potent for differentiation-induction of the same cell line along a monocytic lineage (12). It is conceivable that exogenous ganglioside molecular species that specifically stimulate cell differentiation or maturation in culture system could be characteristic molecules which are synthesized in the cells and play roles in processes of cell differentiation and maturation.

The process of megakaryocyte maturation before platelet-shedding involves 1) the cytoplasmic growth with acquisition of platelet-specific proteins, such as acetylcholinesterase in rodent megakaryocytes, and 2) the organization of the platelet specific organelles into "platelet fields" by demarcation membranes. In our laboratory, potentiation of murine megakaryocytopenesis by ganglioside GD1a was demonstrated during 7-day incubation in a liquid culture system (19). Ganglioside GD1a appeared to be one of molecular species that induce megakaryocytic maturation. In the present study, the effects of ganglioside GD1a and the other ganglioside series on the terminal steps of the cell maturation of murine megakaryocytes were examined in a short-term culture system.

MATERIALS AND METHODS

Gangliosides from bovine brain (Sigma Type II), GM1, GD1a, neuraminidase from Clostridium perfringens (Sigma Type X-A) and protease from Streptomyces griseus were purchased from Sigma Chemical Company, USA, and gangliosides GM2, GM3 and GD1b from Iatron Laboratories, Inc., Japan. BDF1 female mice (6 weeks old, Shizuoka Laboratory Animal Center, Japan) were employed in all studies.

Bone marrow cell collection and megakaryocyte enrichment and cell culture were carried out essentially according to the method of Handagama et al. (4). The RPMI 1640 culture medium with 0.38% sodium citrate, 15% fetal calf serum (FCS, Flow Laboratories), 500 U of penicillin/ml and 50 μg of streptomycin/ml was used to make a colloidal Percoll (Pharmacia) solution of 1.050 g/ml density. The solution was used to flush out, as well as to suspend, the bone marrow cells for density gradient centrifugation. The suspension was placed in
polypropylene tubes. This was overlaid with the RPMI 1640 medium which contained 0.38% citric acid and 15% FCS. The tubes were centrifuged in a swingout rotor at 1,000 × g for 30 min at room temperature. The low-density band of cells at the upper interface (megakaryocyte-enriched fraction) was used.

Ten μl of the megakaryocyte-enriched fraction was placed into a chamber, which was constructed by sealing with grease a glass slide and a plastic sheet (0.2 mm thick) through which 2 holes (9 mm diameter) were cut. The glass surface of the chamber was coated with polylysine. Addition of 10 μl of the megakaryocyte-enriched cell suspension resulted in the seeding of approximately 30 to 70 megakaryocytes/chamber. Control cultures consisted of the megakaryocyte-enriched fraction diluted with an equal volume of the incubation medium, which consisted of RPMI 1640 medium with 15% FCS, 500 U of penicillin/ml and 50 μg streptomycin/ml. In test culture, various metabolic inhibitors and gangliosides were added in appropriate concentrations to the incubation medium before mixing with the megakaryocyte-enriched fraction. The chambers were covered with cover glasses and incubation was done at 37°C with 5% CO2-95% air.

Megakaryocytes were readily identified by their large size under a phase-contrast microscope. With incubation time, fragmentation of megakaryocyte cytoplasm was observed. These megakaryocytes with fragmented cytoplasm were defined as platelet-producing megakaryocytes. Megakaryocytes and the platelet-producing megakaryocytes in culture chambers were counted and the ratio of the platelet-producing megakaryocytes to total megakaryocytes was calculated.

Treatment of ganglioside preparations with neuraminidase was carried out as follows. Neuraminidase suspension (insoluble enzyme attached to beaded agarose, suspended in 2.0 M (NH₄)₂SO₄, pH 7.0) in a volume of 0.325 ml (0.125 units) was centrifuged at 7,000 rpm for 5 min (Eppendorf centrifuge 5414S) at 4°C, and the supernatant was removed. The pellet was washed twice with 0.3 ml of 0.1 M phosphate buffer (pH 6.5) by centrifugation under the same conditions as described above. To the washed pellet, 0.25 ml of a ganglioside solution (2 mg/ml 0.1 M phosphate buffer, pH 6.5) was added and the suspended mixture was incubated for 4 min at 37°C. The reaction was terminated by cooling the mixture in an ice-water bath for 2 min, and then the mixture was centrifuged at 7,000 rpm for 5 min at 4°C. The supernatant was used as neuraminidase-treated ganglioside. For treatment with protease, the protease preparation (0.125 units, insoluble enzyme attached to cross-linked beaded agarose, lyophilized powder, stabilized with lactose) was washed once with 0.1 M phosphate buffer (pH 7.0) by centrifugation, and to the pellet, a ganglioside solution (2 mg/ml, in 0.1 M phosphate buffer, pH 7.0) was added, and the suspended mixture was incubated for 4 min at 30°C. Centrifugation was carried out at 7,000 rpm. The supernatant was used as protease-treated ganglioside.

RESULTS

The density gradient procedure resulted in cell preparations containing approximately 30 megakaryocytes/1000 nucleated cells. Megakaryocyte numbers were increased by nearly 150 times from the initial suspension which contained approximately 20 megakaryocytes/10⁵ bone marrow cells. Figure 1 shows a typical image of intact megakaryocytes in immature (Fig. 1A) and mature (Fig. 1B) states, and the platelet-producing megakaryocytes with fragmented cytoplasm (Fig. 1C). Electron microscopic observation of the cell with fragmented cytoplasm similar to Fig. 1C showed the stage of full maturation with heavy granulation and formation of platelet demarcation membranes (Fig. 2).

Figure 3 shows time-course of formation of the platelet-producing megakaryocytes in the culture system. In control system, the number of total megakaryocytes decreased slightly and reached a plateau level of approximately 70% of the number at the start of incubation. The platelet-producing megakaryocytes were not seen at the start of incubation. With incubation time, the platelet-producing megakaryocytes appeared and increased (Fig. 3B). The ratio of the platelet-producing megakaryocytes to total megakaryocytes increased markedly during the initial 3 hours and then gradually,

![Fig. 1. Typical image of cultured megakaryocytes. (A) Immature megakaryocyte with large round nucleus. (B) Mature megakaryocyte with convoluted nucleus. (C) Platelet-producing megakaryocyte. Extension of short pseudopods and fragmentation of cytoplasm were observed. Incubation: 12 hours at 37°C, 5% CO₂-95% air. ×600.](image-url)
and at a later phase, maintained a plateau level during 24-hour incubation. The number of the platelet-producing megakaryocytes amounted to approximately 7% of the number of total megakaryocytes at 12-hour incubation (Fig. 3C). When bovine brain ganglioside mixture was added to the culture medium, an increase after a transient decrease in the number of total megakaryocytes was observed. The number of total megakaryocytes reached a maximum level at 12-hour incubation and then decreased (Fig. 3A). The number of the platelet-producing megakaryocytes also increased with incubation time and reached a maximum value (Fig. 3B). The ratio of the platelet-producing megakaryocytes to total megakaryocytes amounted to approximately 12%, suggesting the stimulation of platelet-producing megakaryocyte formation by the ganglioside mixture (Fig. 3C).

In the presence of KCN, NaF or monoiodoacetic acid, the ratio of the platelet-producing megakaryocytes to total megakaryocytes markedly decreased (Fig. 4B), although the number of total megakaryocytes also showed a tendency to decrease by these inhibitors (Fig. 4A). The results suggest that the formation of the platelet-producing megakaryocytes is dependent on the energy-producing systems.

In order to examine the effects of various gangliosides on the cytoplasmic maturation, the megakaryocyte-enriched bone marrow cell suspension was incubated for 12 hours in the presence of ganglioside preparations in

![Electron microscopic observation of the platelet-producing megakaryocyte. Similar cell to (C) in Fig. 1. Demarcation membrane systems were developed and platelet fields were clearly delineated (arrows). ×7,400.](image)

**Fig. 3.** Time course of the platelet-producing megakaryocytes formation and effect of ganglioside mixture on it. GSM: bovine brain ganglioside mixture, 1500 μg/ml (△). Control (○). The points represent the mean values, and the vertical bars the variation of two separate experiments. An average of 147 megakaryocytes in several chambers was counted at each incubation time in an experiment.
the culture medium. Figure 5 shows dose-response curves of the platelet-producing megakaryocyte formation to various ganglioside preparations. Ganglioside GD1a increased the number of total megakaryocytes dose-dependently (Fig. 5A), and markedly stimulated formation of the platelet-producing megakaryocytes (Fig. 5B). The ratio of the platelet-producing megakaryocytes to total megakaryocytes increased dose-dependently, and reached a plateau level at a concentration of 125 μg GD1a/ml (Fig. 5C). On the other hand, ganglioside GM1 did not affect the number of total megakaryocytes even at higher concentrations, although it stimulated formation of the platelet-producing megakaryocytes. Thus, the ratio was increased dose-dependently by GM1, markedly to a concentration of 250 μg/ml and gradually at higher concentrations. Gangliosides GM2, GM3 and GD1b had little effects on either the number of total megakaryocytes or the ratio of the platelet-producing megakaryocytes to total megakaryocytes. Ganglioside mixture showed moderate effects on both the values, as compared with other ganglioside preparations (Fig. 5).

Ganglioside GD1a treated with neuraminidase did not increase the number of total megakaryocytes during incubation, although it still stimulated formation of the platelet-producing megakaryocytes. The activity of neuraminidase-treated GD1a was similar to that of non-treated ganglioside GM1, suggesting GD1a was converted to GM1 by neuraminidase treatment. On the other hand, treatment of ganglioside GM1 with neuraminidase exerted no significant effect on the activity.

![Fig. 4. Effect of inhibitors of energy metabolism on formation of the platelet-producing megakaryocytes. KCN: 2 mM, NaF: 23.8 mM, IA (monooiodoacetic acid): 0.05 mM. The number of the cells at the start of incubation: 34–60 megakaryocytes/chamber. An average of 234 megakaryocytes was counted in several chambers for each incubation system. Incubation: 18 hours.]

![Fig. 5. Dose responsiveness of the platelet-producing megakaryocyte formation to various gangliosides. In the presence of ganglioside GM1 (●), GD1a (■), GM2 (○), GD1b (▲), GM3 (▲) and bovine brain ganglioside mixture (GSM ●), the megakaryocyte-enriched bone marrow cell suspension was incubated for 12 hours. The number of the cells at the start of incubation: 26 to 70 megakaryocytes/chamber. An average of 282 megakaryocytes was counted in several chambers for each incubation system. In GM1, GD1a, GD1b and GSM, the points represent the mean values of two separate experiments, and the vertical bars represent the variation.]

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Table 1. Effects of neuraminidase- and protease-treated ganglioside GD1a and GM1 on the platelet-producing megakaryocyte formation.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ganglioside GD1a (500 µg/ml)</th>
<th>Ganglioside GM1 (500 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>non-treated</td>
<td>neuraminidase-treated</td>
</tr>
<tr>
<td>Exp. 1a</td>
<td>8.7</td>
<td>15.5</td>
<td>18.8</td>
</tr>
<tr>
<td>b</td>
<td>36.1 ± 4.3 (7)</td>
<td>56.6 ± 3.0 (8)</td>
<td>33.3 ± 4.3 (8)</td>
</tr>
<tr>
<td>Exp. 2a</td>
<td>7.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>b</td>
<td>39.8 ± 4.0 (10)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Exp. 3a</td>
<td>8.4</td>
<td>13.8</td>
<td>—</td>
</tr>
<tr>
<td>b</td>
<td>34.0 ± 1.5 (7)</td>
<td>47.9 ± 5.8 (8)</td>
<td>—</td>
</tr>
</tbody>
</table>

a: Platelet-producing megakaryocytes (% of total megakaryocytes).
b: Total megakaryocytes/chamber (mean ±SE). The number of chambers examined is given in parentheses.
Incubation: 12 hrs at 37°C.

Protease treatment exerted no significant effect on the activities of either ganglioside GD1a or GM1 (Table 1). The results suggest that the activities of ganglioside preparations GD1a and GM1 were not due to contaminating proteins or polypeptides.

**DISCUSSION**

Platelets are thought to be delineated within the cytoplasm of megakaryocytes by the demarcation membrane systems at the terminal stage of cytoplasmic maturation of the cells. Scanning electron microscopic studies revealed that extravascularly located megakaryocytes extended long cytoplasmic processes through bone marrow sinusoids in vivo (1, 9). For the mechanism of platelet-shedding, some investigators believe that these processes subsequently develop segmental constrictions and separate into individual platelets, and thus platelet demarcation proceeds to completion within these processes (9, 10, 14, 17). Others believe that these processes are not precursors to platelet production, but platelet demarcation is completed within the central cytoplasmic mass of the megakaryocytes (2, 9, 13). On the other hand, Trowbridge et al. (18) have proposed that platelets are formed in the pulmonary circulation by physical fragmentation of the megakaryocyte cytoplasm. In an in vitro study, Levine (8) observed that platelet-shedding or demarcation were never seen in the pseudopods of guinea pig megakaryocytes, although the megakaryocytes appeared to be fully mature, with heavy granulation and development of central cytoplasmic demarcation membranes, and with pseudopods spread out over a wide area. Handagama et al. (4) also demonstrated long cytoplasmic processes by rat megakaryocytes in a culture system with a phase-contrast microscope.

In the present study, murine megakaryocytes developed short cytoplasmic processes and dispersed cytoplasmic fragments in a short-term culture. Long extend-
of demarcation membrane formation. By contrast, GM1 may be incorporated into mature megakaryocytes and stimulate the latter step, the formation of platelet demarcation membrane. The possibility cannot be ruled out that, however, ganglioside GD1a in megakaryocytes might transformed into ganglioside GM1, and then GM1 induce the latter step of the maturation. Further studies on possible incorporation of exogenous GD1a and GM1 into plasma membrane of the target cells will clarify the mechanism involved in maturation-induction by these ganglioside molecules.

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REFERENCES


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