Ganglioside \( G_M3 \) Inhibits the Proliferation of Cultured Keratinocytes

Amy S. Paller,*† Sheryl L. Arnsmeyer,* Maira Alvarez-Franco,* and Eric G. Bremer‡

Departments of *Pediatrics and †Dermatology, Northwestern University Medical School; and ‡Department of Immunology/Microbiology, Rush Medical School, Chicago, Illinois, U.S.A.

Ganglioside \( G_M3 \) is the predominant ganglioside of keratinocyte membranes. It has been proposed in other cell types that \( G_M3 \) may participate in the regulation of cell proliferation. To examine the role of \( G_M3 \) in keratinocyte proliferation, purified \( G_M3 \) was added to cultured keratinocytes from normal foreskin, from lesional skin of patients with psoriasis and ichthyosis, and to cutaneous squamous carcinoma cell lines. Supplemenal \( G_M3 \) inhibited the growth of all cultured keratinocytes in a dose-dependent manner at concentrations of 10–100 \( \mu \)M. Keratinocytes from patients with psoriasis and ichthyosis were most sensitive to the inhibitory effects of \( G_M3 \), and confluent undifferentiated keratinocytes were least sensitive. No change in differentiation was noted after addition of \( G_M3 \), \( G_D3 \), 9-0-acetyl-\( G_D3 \), and \( G_D1b \) also inhibited keratinocyte proliferation. Gangliosides \( G_M1 \) and \( G_D1a \) and sialic acid had little effect. Addition of 50 \( \mu \)M \(^{3}H\)-\( G_M3 \) to cultured keratinocytes resulted in 1.7 times the amount of cellular \( G_M3 \). These data suggest that hematoside (\( G_M3 \)) and “b” pathway gangliosides (\( G_D3 \), \( G_D1b \)), generated by the preferential activation of sialyltransferase II versus N-acetylgalactosaminyItransferase, may be involved in control of keratinocyte growth but not of differentiation. J Invest Dermatol 100:841–845, 1993

Gangliosides are sialylated glycosphingolipids that are predominantly located in the plasma membrane. Alterations in ganglioside metabolism have been observed during density-dependent growth inhibition and oncogenic transformation [1,2] suggesting that gangliosides may participate in the regulation of cell proliferation. In fact, sphingolipids, including ganglioside \( G_M3 \), have been shown to be potent pharmacologic regulators of cell proliferation and differentiation [2,3] probably through direct interaction with growth factor receptors [3–5].

Gangliosides comprise 0.1% of the lipids of epidermis, and 65% of the ganglioside of keratinocyte membranes in vitro is \( G_M3 \) [6]. Monoclonal antibodies directed against \( G_M3 \) bind strongly to the stratum corneum of normal epidermis, but binding is decreased or absent in many disorders of epidermal hyperproliferation, including psoriasis, squamous cell carcinoma (SCC), and the hyperproliferative forms of ichthyosis [7]. Furthermore, the ganglioside content of tissue from both nodular and sclerosing basal cell carcinomas differs from that of normal keratinocytes in vivo, with a markedly increased overall ganglioside content and a significant concentration of 9-0-acetyl-\( G_D3 \) [6]. These observations suggest that gangliosides, particularly \( G_M1 \) and 9-0-acetyl-\( G_D3 \), may play a role in the regulation of keratinocyte proliferation. The purpose of the experiments presented here was to examine the pharmacologic effect of gangliosides on keratinocyte proliferation and differentiation.

MATERIALS AND METHODS

Materials High-performance thin-layer chromatography (HPTLC) plates were from Merck (Darmstadt, Germany); rabbit polyclonal antibodies were provided as follows: anti-desmoplinin antibody by Dr. K. Greene (Chicago, IL) and anti-invulcin antibody from Biomedical Technologies, Inc. (Stoughton, MA). Ganglioside standards for HPTLC plates were used without purification and purchased as follows: \( G_M2 \) from Boehringer-Mannheim (Indiana, IN); \( G_D3 \) from Genzyme (Boston, MA); \( G_D1b \), \( G_D1a \), \( G_D3b \), \( G_D3b \), and sialic acid from Sigma (St. Louis, MO). For addition to cultures, \( G_M1 \), \( G_M3 \), \( G_D3 \), \( G_D1a \), and 9-0-acetyl-\( G_D3 \) were prepared and purified as fully as described below; these gangliosides were used as standards without full purification. \( G_D1b \) (Sigma, St. Louis, MO) was further purified by HPLC before addition to cultures.

Cells and Culture of Keratinocytes Keratinocytes from discarded normal foreskin and lesional skin from patients with psoriasis and ichthyosis, biopsied after informed consent, were seeded in complete serum-free keratinocyte growth medium (KGM, Clonetech Corp., San Diego, CA) [8], with low calcium (0.07 mM or 0.15 mM) or high calcium (1.5 mM) concentration. Cutaneous squamous cell carcinoma (SCC) lines SCC-12-B2, SCC-12-F2, and SCC-13 (courtesy of Dr. James Rheinwald, Boston, MA) [9] were initiated in 75% Dulbecco’s minimum essential medium with 25% Ham’s F-12 and 20% fetal calf serum and subsequently grown in KGM as for keratinocytes.

Purification of Gangliosides for Treatment of Cultured Cells Gangliosides \( G_M3 \), \( G_M1 \), and \( G_D1a \), \( G_D3 \), and 9-0-acetyl-\( G_D3 \) were extracted from dog erythrocytes [7,10], bovine brain [11], SK-MEL-28 melanoma cells [American Type Culture Collection], and trout liver [12], respectively, and prepared from the total lipid extracts with chloroform:methanol as previously described [7]. Gangliosides were quantitated by dry weight and colorimetric resorcinol assays [13].

Abbreviations: GalNAcT, N-acetylgalactosaminyItransferase; HC, 1.5 mM Ca\(^{2+}\) KGM; HPTLC, high-performance thin-layer chromatography; KGM, keratinocyte growth medium; LC, 0.15 mM Ca\(^{2+}\) KGM; NANA, N-acetyllactosaminic acid; ST-II, sialyltransferase II.

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Reprint requests to: Dr. Amy S. Paller, Division of Dermatology 107, Children’s Memorial Hospital, 2300 Children’s Plaza, Chicago, IL 60614.

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Addition of Ganglioside to Cultured Keratinocytes

Normal, psoriatic, and ichthyotic keratinocytes were seeded at a density of 5 × 10^3 cells/ml in 0.15 mM Ca^{++} and 5 × 10^4 for growth in 1.5 mM Ca^{++} KGM. SCC lines were seeded at a density of 5 × 10^6 cells/well for growth in 0.15 mM Ca^{++} and at 2.5 × 10^4 cells/well for growth in 1.5 mM Ca^{++} KGM. The G_{M3}, G_{M1}, G_{D1a}, G_{D1b}, G_{D3}, 9-0-acetyl-G_{D3}, and sialic acid were added as described by Bremer et al [3] to keratinocytes in second or third passage or to the SCC lines in 24-well flat-bottomed plates at final concentrations of 1–100 μM, with at least triplicate cultures at each concentration. Gangliosides were added on day 2 and subsequently every other day with feeding until the cultured cells were harvested (day 6 for pre-confluent and day 12 for post-confluent cells). KGM medium without gangliosides was added to control cultures whenever ganglioside was added to treated cultures. The time course of the effect of gangliosides was determined by assessment of cell proliferation daily during the course of study. To study the reversibility of inhibition of proliferation by G_{M3}, keratinocytes were incubated with or without G_{M3} for 72 h; cells treated with G_{M3} were subsequently washed and grown in KGM medium without G_{M3} for 12–72 h. All experiments were repeated at least three times. Keratinocytes from 10 patients with plaque-type psoriasis were studied; the ichthyotic keratinocytes were from patients with non-bullous congenital ichthyosiform erythroderma (n = 2) and bullous congenital ichthyosiform erythroderma (n = 1).

Proliferation Assays

For preconfluent cultures, 1 μCi/ml ³H-thymidine (specific activity 25 Ci/mmol, Amersham Corp., Arlington Heights, IL) was added and incubated with cells for 24 h before harvesting [14]. Radioactivity was counted in a scintillation counter (TmAnalytic model 6892). For studies of post-confluent cells, 4 μCi/ml of methyl-³H-thymidine was added and keratinocytes were harvested 2 h later, owing to the catabolism of thymidine by post-confluent keratinocytes [15]. Cellular proliferation was also assessed by counting cells with a hemacytometer. Counting by hemacytometer correlated well with counting by ³H-thymidine labeling. Cell viability was determined by trypan blue exclusion of harvested cells, as well as of non-adherent cells collected before feeding and harvesting. Counting was performed in triplicate.

Detection of Differentiation

The effect of supplemental gangliosides on cellular differentiation was assessed by cornified envelope formation, and cell morphology by light microscopy and the alteration in immunostaining with antibodies against desmoplakin and involucrin. The number of cornified envelopes was measured by resistance to lysis by detergent in the presence of reducing agent, as described by Rice and Green [16]. Measurements of cornified...
envelope formation were performed in triplicate. Immunofluorescent studies with rabbit anti-human desmoplakin antibody [17] and rabbit anti-human involucrin antibody [18] followed routine immunofluorescent techniques [19], with keratinocytes grown to pre-confluence in KGM medium with 0.07 mM or 1.5 mM calcium.

**Radiolabeling of GM₃** Purified GM₃ was labeled at the ceramic moiety with ³H-NaBH₄ (specific activity 60 Ci/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO) and palladium by the method of Schwarztann [20] to provide a product that is stable to the in vivo action of neuraminidase. The ³H-labeled GM₃ was analyzed by HPTLC, resorcinol detection of ganglioside bands and development by autoradiography. Specific radioactivity of known amounts of GM₃ was determined in duplicate in a liquid scintillation counter (Beckman LS3801).

**Addition of Radiolabeled GM₃** Fifty micromoles ³H-GM₃ was added to cultures on days 2 and 4 as above. At the time of harvesting for cell counting, keratinocyte gangliosides were extracted as described previously [6] and separated on HPTLC plates. Control plates were run to identify gangliosides with resorcinol spraying and gangliosides GM₁, GM₂, GM₃, and GD₃ were applied as standards. The bands of GM₃ were scraped after exposure to the x-ray film, dissolved in chloroform: methanol 2:1, and centrifuged. Supernatants with ganglioside and collected culture media were analyzed for radioactivity by scintillation counting.

**RESULTS**

**Effect of Ganglioside Addition on Proliferation** Previous experiments have demonstrated that GM₃ is the major ganglioside of keratinocytes in vivo [6]; GM₁, GM₂, GD₁, and GD₃a are also present in smaller amounts. Recently, 9-O-acetylated GD₃ was shown to be a significant ganglioside of basal carcinoma cells [6]. As a result of these findings, these gangliosides as well as sialic acid were selected to be added to cultured keratinocytes. Owing to the very limited quantities of purified GD₃ and 9-O-acetyl-GM₃, we were only able to study the effects of these gangliosides in undifferentiated keratinocytes.

GM₃, GD₃, 9-O-acetyl-GD₃, and GD₃b inhibited proliferation of keratinocytes grown in 0.15 mM Ca²⁺ in the range of 10–100 μM (Fig 1a), with inhibition by 9-O-acetyl-GD₃ > GM₃ > GD₃ > GD₃b. In contrast, GM₁, GD₄a, and sialic acid were not significantly inhibitory although, of these, GD₄a showed the greatest inhibitory effect. Time-course studies showed maximal inhibition 2 d after the addition of GM₃. When cells that had been inhibited by treatment with GM₃ for 72 h were washed and incubated without GM₃, recovery through proliferation was noted within 72 h (Table I).

GM₃ also inhibited proliferation of keratinocytes grown in 1.5 mM Ca²⁺ (Fig 1b), the SCC-12P2 cell line (Fig 1c; inhibition of proliferation of the SCC-12B2 cell line was similar), and the SCC-13 cell line (Fig 1d). In studies on all of these cells, GM₃ was only slightly inhibitory at 50 and 100 μM concentrations, although greater inhibition was noted in the SCC-13 line. Ichtchyo- and psoriatic keratinocytes were slightly more sensitive than normal keratinocytes to the inhibitory effects (38.5 ± 9.41% of control and 37.07 ± 5.29% of control at 50 μM GM₃, respectively; control numbers of cells from patients with psoriasis; N = 10, were 8.94 X 10⁶ ± 0.66 X 10⁶ cells). Confluent keratinocytes were less sensitive than keratinocytes grown to a pre-confluent state (59.65 ± 6.49% of control at 50 μM GM₃ versus 42.5 ± 6.69% of control in pre-confluent KCs). The inhibition of confluent cells may reflect cell toxicity, because confluent cells are not proliferating; however, the number of viable keratinocytes following treatment with all gangliosides and with sialic acid was always at least 75–90% of control viability, with no relationship noted between concentration and toxicity.

**Effect of Added Ganglioside on Differentiation** Light microscopic examination of cells treated with GM₃ did not reveal gross morphologic changes of differentiation. However, with concentrations of GM₃ of 50 μM and 100 μM increased dendritcity and elongation of 3–5% of cells was noted. These elongated cells excluded trypan blue dye. The percentage of cells with cornified envelopes was not altered by treatment with GM₃. In keratinocytes cultured in KGM with 0.07 mM Ca²⁺, anti-desmoplakin antibody bound to the perinuclear region in a spotty pattern without deposition at areas of cell-cell contact, regardless of whether cells were exposed to GM₃. Likewise, in keratinocytes grown in KGM with 1.5 mM Ca²⁺, the antibodies bound intensely along the periphery of cells at sites of cell-cell contact, regardless of whether cultured in the presence of GM₃. Keratinocytes treated and untreated with 50 μM GM₃ exhibited no difference in the staining patterns with anti-involucrin antibody, showing increased numbers of cells with intense cytoplasmic and peripheral staining with anti-involucrin antibodies in 1.5 mM Ca²⁺ KGM.

**Ganglioside Incorporation into Keratinocytes** Following treatment of keratinocytes in 0.15 mM Ca²⁺ with ³H-GM₃ (189,000 cpm/μmol), incorporation into extracted membrane was only noted in the ganglioside band that migrated with the GM₃ standard. No radioactivity was detected in non-ganglioside fractions. 0.585 ± 0.015% of the labeled GM₃ (153.2 μg sialic acid/10⁶ cells) incorporated into the membrane. Since the total amount of ganglioside in undifferentiated keratinocytes is approximately 150 μg sialic acid/10⁶ cells, of which 60% is GM₃ (90 μg sialic acid/10⁶ cells), the content of GM₃ is less than 1.7 times that of untreated cells when 50 μM GM₃ is added (Table II); membrane flux with replacement of unlabeled by labeled GM₃ could further lower the amount of increase in GM₃ content following supplementation. The amount of the other major ganglioside keratinocytes remained virtually unchanged after GM₃ addition.

**Table I. Keratinocyte Recovery Following 72 h of Treatment with 50 μM GM₃**

<table>
<thead>
<tr>
<th>Treatment of Cells</th>
<th>Number of Cells ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No GM₃</td>
<td>2.57 ± 0.29 X 10⁶</td>
</tr>
<tr>
<td>No recovery</td>
<td>1.63 ± 0.20 X 10⁶</td>
</tr>
<tr>
<td>12 h recovery</td>
<td>1.78 ± 0.19 X 10⁶</td>
</tr>
<tr>
<td>24 h recovery</td>
<td>2.02 ± 0.37 X 10⁶</td>
</tr>
<tr>
<td>48 h recovery</td>
<td>2.21 ± 0.43 X 10⁶</td>
</tr>
<tr>
<td>72 h recovery</td>
<td>2.85 ± 0.26 X 10⁶</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Modulation of cell proliferation by glycolipids has been addressed by the addition of purified glycolipids to cultured cells. Exogenously added GM₃ has been shown to inhibit proliferation in culture [3,21–24] of a variety of normal cells and cell lines, suggesting that GM₃ may function in vivo as a modulator of cellular proliferation. Furthermore, modified forms of GM₃ may inhibit or stimulate cell proliferation. Lyso-GM₃ (without the fatty acyl chain) also inhibits
growth of A431 cells [25,26], whereas an analogue of G\textsubscript{M3} (de-N-acetyl-G\textsubscript{M3}) may encourage proliferation [26].

We have previously shown that G\textsubscript{M3} is the predominant ganglioside of human epidermis [6]. The overall content of G\textsubscript{M3}, however, is only 0.065% of the total lipid of epidermis, an amount that would be more consistent with a role as a regulatory membrane component than as a structural one. Anti-G\textsubscript{M3} antibody binding to human stratum corneum is strong in normal skin, but deficient or absent in a variety of hyperproliferative disorders, including psoriasis, hyperproliferative forms of ichthyosis, squamous cell carcinoma, and the cornoid lamellae of porokeratosis [7]. Furthermore, alterations in the total content of ganglioside and also of 9-O-acetyl-G\textsubscript{M3} in basal cell carcinomas [6] further suggest that G\textsubscript{M3} and other related gangliosides may participate in regulation of cell growth.

In this study, we cultured keratinocytes in defined KGM medium without serum and without a feeder layer of fibroblasts, because we have found significant amounts of ganglioside in fetal calf serum (1.4 \( \mu \)g G\textsubscript{M3}/ml serum) versus in KGM with bovine pituitary extract (0.00625 \( \mu \)g G\textsubscript{M3}/ml, unpublished data), and because G\textsubscript{M3} is the predominant ganglioside of cultured fibroblasts [27]. In this report, we have found that supplemental G\textsubscript{M3} inhibits proliferation of cultured normal keratinocytes, keratinocytes from patients with hyperproliferative disorders of epidermis, and cutaneous SCC lines. The inhibition is dose dependent and is not associated with significant cell toxicity as determined by trypan blue exclusion. Our studies on cell recovery further suggest that G\textsubscript{M3} inhibits keratinocyte proliferation in a reversible manner, although we cannot distinguish from these studies if all cells or merely a subpopulation of cells are recovering.

Previous studies have suggested a correlation of increased G\textsubscript{M3} cell and cell differentiation. Undifferentiated "crypt" cells of intestinal epithelia have lactosylceramide (the astalo form of G\textsubscript{M3}), but no G\textsubscript{M3} or G\textsubscript{M3} synthetase [28]. With transformation to villus cells, high levels of G\textsubscript{M3} and G\textsubscript{M3} synthetase are found, with a low content of lactosylceramide. Supplemented gangliosides have been shown to induce neurite outgrowth in neuroblastoma cells, a phenomenon associated with neuronal differentiation [29,31]. In addition, G\textsubscript{M3} has previously been shown to induce "differentiation" into specific differentiation pathways of human leukemic cell lines [23,32].

Keratinocyte differentiation was not induced by adding G\textsubscript{M3}, as demonstrated by similarities in cornified envelope production and in expression of desmoplakin and involucrin in treated and untreated cells. Although growth arrest of keratinocytes often occurs as a consequence of keratinocyte differentiation, decreased proliferation does not necessarily result in the onset of differentiation. Isolucine starvation or ethionine supplementation, for example, results in reversible inhibition of proliferation in the G\textsubscript{1} phase of the cell cycle without associated increased keratinocyte differentiation [33-35]. Recently TGF-\( \beta \) has also been shown to reversibly inhibit keratinocyte growth without increasing involucrin expression or cell envelope formation [34,36].

Ganglioside synthesis follows two major pathways, with G\textsubscript{M3} as a substrate [6,37]. In one pathway, G\textsubscript{M3} may be sialylated by sialyltransferase-II (ST-II) to form G\textsubscript{D3}, which may be reversibly acetylated at the 9-0-acetyl position to form 9-0-acetyl-G\textsubscript{D3}. G\textsubscript{D3} may also be conjugated with N-acetylgalactosamine and subsequently with galactose to form GD\textsubscript{H} ("b" pathway). Alternatively, G\textsubscript{M3} may be conjugated with N-acetylgalactosaminyltransferase (GalNAcT) and subsequently with galactose to form G\textsubscript{M1}, which may be further sialylated to form GD\textsubscript{H} ("a" pathway). It is intriguing to note that the inhibitory gangliosides all derive from the "b" pathway, whereas the gangliosides without effect are part of the "a" pathway. The shift from synthesis of "b" pathway to "a" pathway gangliosides has been shown to depend directly on the activities of ST-II and GalNAcT enzymes [38-40]. Our results suggest that the enzymes that synthesize and metabolize keratinocyte gangliosides and thus regulate the relative concentrations of membrane gangliosides may participate in regulating proliferation by balancing the amounts of G\textsubscript{M3}, G\textsubscript{D3}, 9-0-acetyl-G\textsubscript{D3}, and G\textsubscript{M1}. Similarly, an altered content of gangliosides has been associated with decreased proliferation in intestinal mucosal cells [28] and in neuronal cells [41]. In addition, shifts in synthesis from ganglioside to lacto- to globo-series glycolipids has been noted with myeloid cell differentiation [42].

To effect the inhibition, G\textsubscript{M3} concentrations of 10-100 \( \mu \)M are required. Although the pharmacologic effect is quite significant, the amount of ganglioside in the medium when 50 \( \mu \)M G\textsubscript{M3} is added, is, by calculation, more than 300 times that in the keratinocyte cell membrane. Other investigators have proposed that supplemental gangliosides modulate proliferation by incorporation into the cell membrane, the lipid moiety being inserted into the lipid layer [43,44]. We have demonstrated that 50 \( \mu \)M \( ^{3} \text{H}-\text{G} \text{M3} \) incorporates into cells as G\textsubscript{M3} at an amount that is only 1.7 times that of the normal cellular content of G\textsubscript{M3}, without consideration of replacement of unlabeled G\textsubscript{M3} by radiolabeled G\textsubscript{M3}. In our studies, supplemental \( ^{3} \text{H}-\text{G} \text{M3} \) was not detectably metabolized during the time course of the experiments, suggesting that the inhibitory effect is most likely due directly to "b" pathway ganglioside inhibition.

The mechanism of inhibition of keratinocyte proliferation by gangliosides is unknown, although inhibition of phosphorylation of growth factor receptors, especially epidermal growth factor and fibroblast growth factor receptors, has resulted from the addition of G\textsubscript{M3} to other cells [4,21,45]. Studies of the effect of keratinocyte gangliosides on proliferation and of the interaction of gangliosides with keratinocyte growth factors and receptors may promote better understanding of the regulation of keratinocyte proliferation. Recently, administration anti-ganglioside antibodies has resulted in tumor regression in vivo of melanomas (anti-G\textsubscript{D3} [46,47] and anti-G\textsubscript{M1} lactone [48]) and of neuroblastomas (anti-G\textsubscript{M1} [49]). Gangliosides and anti-ganglioside antibodies may prove to be new means of therapy for hyperproliferative and neoplastic cutaneous disorders.

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