Influence of ganglioside GM3 and high density lipoprotein on the cohesion of mouse brain tumor cells

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Abstract Previous findings with various murine tumor cell lines suggest an association between ganglioside GM3 and cell cohesive properties. The influence of GM3 on cohesion was studied in two mouse brain tumor cell lines: ependymoblastoma (EPEN) and CT-2A. In culture, the EPEN cells grow as islands and contain GM3 as the only ganglioside, whereas the CT-2A cells grow as a fusiform cell monolayer and contain GM2, GM1, and GD1a as major gangliosides and low amounts of GM3. To examine the role of GM3 in cohesion, both cell lines were treated with *1*) *C. perfringens* neuraminidase, *2*) anti-GM3 monoclonal antibody (mAb DH2), or *3*) were grown in serum-free medium. All three treatments caused a significant increase in the number of non-cohesive and protoplasmic process-bearing cells for the EPEN, but had no effect on the morphology of the CT-2A cells. The neuraminidase treatment removed GM3 from both cell lines and caused a significant accumulation of GM1 in the CT-2A cells. EPEN cell cohesion and GM3 content returned to control levels after removal of neuraminidase. EPEN cell cohesion was restored in serum-free medium with added high density lipoprotein (HDL). The HDL effect on the EPEN cell cohesion was dose-dependent and was not seen with other lipoproteins. We suggest that EPEN cell cohesion could involve an interaction between extracellular HDL, acting as a bridge, and GM3 molecules on opposing cell surfaces.—**Bai, H., and T. N. Seyfried.** Influence of ganglioside GM3 and high density lipoprotein on the cohesion of mouse brain tumor cells. *J. Lipid Res.* 1997. **38:** 160–172.

Supplementary key words cell cohesion • apolipoproteins • cell culture • brain tumor • ependymoblastoma cells • CT-2A cells • neuraminidase

Gangliosides are sialic acid-containing glycosphingolipids (GSLs) that are enriched in the outer leaflet of plasma membranes. The GSLs are anchored through the ceramide moiety and the attached carbohydrate moiety extends from the membrane surface (1, 2). Although the functions of GSLs in cell membranes have not been clearly established, previous studies suggest an association between changes in GSL composition and changes in oncogenesis, morphogenesis, and differentiation (3–7).

GM3, a structurally simple monosialoganglioside (**Fig. 1**), has been studied for its possible role in cell growth, differentiation, and adhesion. GM3 can modulate the function of several growth factor receptors including those for fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and the integrin receptor (8–13). Enhanced differentiation was observed in both monocytic cells and oligodendrocytes after treatment with GM3 (14, 15). Although a homophilic GM3–GM3 interaction was not found, GM3 could participate in cell adhesion through specific heterophilic interactions with various neutral GSLs including gangliotriaosylceramide (Gg3Cer), lactosylceramide (LacCer), globoside (Gb4Cer), and gangliotetraosylceramide (Gg4Cer) (16–19).

In addition to a role in cell growth, differentiation, and adhesion, previous studies also suggest an association between GM3 and cell cohesive properties, i.e., the ability of one cell to stick or cohere to another cell. In general, transformed or malignant cells that contain GM3 as the major ganglioside grow as clumps or islands in culture, whereas cells containing GM3 as a minor ganglioside grow as a fusiform monolayer. This phenomenon was observed with rat ascites hepatoma cells (20, 21), transformed rat embryo fibroblasts (22), and

Abbreviations: EPEN, ependymoblastoma; Nase, neuraminidase; mAb DH2, anti-GM3 monoclonal antibody; SSM, serum-supplemented medium; SFM, serum-free medium; GSLs, glycosphingolipids; GlcCer, glucosylceramide; GalCer, galactosylceramide; LacCer, lactosylceramide; Gb3Cer, globotriaosylceramide (Gb0se3Cer); Gb4Cer, globotetraosylceramide (globoside, Gb0se4Cer); Gg3Cer, gangliotriaosylceramide, GA2; Gg4Cer, gangliotetraosylceramide, GA1; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein.

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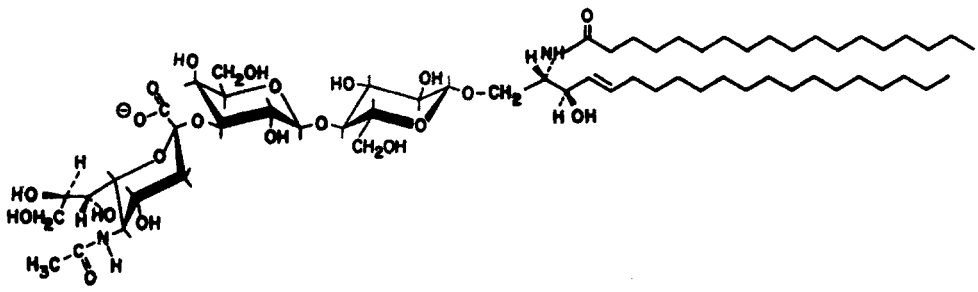

Fig. 1. Structure of ganglioside GM3 (NeuAcα2→3Galβ1→4Glcβ1→1'Ceramide).

experimental mouse brain tumor cells (23, 24). Furthermore, the GM3-rich mouse brain tumor cells that grow as islands in culture grow as cohesive tumors in vivo (24). In contrast, GM3-poor brain tumor cells that grow as a fusiform monolayer in culture grow as a soft non-cohesive tumor in vivo. These findings suggest that GM3 may influence the cohesive properties of cells grown both in vitro and in vivo.

Although much is known concerning the role of high density lipoprotein (HDL) in coronary heart disease and cholesterol transport, little is known about the role of HDL in cellular cohesion. HDL may influence the expression of endothelial adhesion molecules (25), and serum lipoproteins including HDL can interact with both neutral GSLs and gangliosides (26–28). Kivatinitz, Grabois, and Quiroga (29) recently showed that HDL was a heat-stable factor that could inhibit GM2 synthase activity and could block neuritogenesis in chicken cerebral cells. In the present study we show that cell-surface GM3 and extracellular HDL participate in the cohesive properties of a mouse brain tumor cell line. A preliminary report of these findings has appeared (30).

MATERIALS AND METHODS

The following materials were purchased from the Sigma Chemical Co. (St. Louis, MO): *C. perfringens* neuraminidase (type V), GM3 ganglioside, HDL (bovine or human plasma), LDL (human plasma), glutathione, non-immune mouse IgG, anti-mouse IgG-agarose, and peroxidase-conjugated anti-mouse IgG. Rhodamine-conjugated goat anti-mouse IgG was purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). Mouse and bovine brain gangliosides were purified in our laboratory. The IgG mAb DH2 culture supernatant was kindly provided by Dr. S. Hakomori (The Biomembrane Institute, Seattle, WA). VLDL, apoE3, and apoE4 were kindly provided by Dr. B. P. Nathan (The Cardiovscular Research Institute, University of California, San Francisco, CA). Reconstituted HDL was kindly provided by Dr. D. M. Levine (The Rogosin Institute, New York, NY). [^{14}C]galactose was purchased from New England Nuclear (Boston, MA). Neutral GSL standards were purchased from Matreya Inc. (Pleasant Gap, PA).

Cell lines and cell culture

The EPEN and the CT-2A cell lines were established from mouse brain tumors as previously described (24). These tumor cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with either 5% or 10% heat-inactivated fetal bovine serum (FBS). The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C and the medium was changed every other day.

Analysis of cohesion in the EPEN cells

As the cultured EPEN cells grow normally as islands with few protoplasmic processes, cell cohesion was measured as the percentage of free cells or cells with protoplasmic processes. A free cell was defined as any cell with no part of the cell body touching another cell, whereas a protoplasmic process was defined as a process extending from a cell that was at least as long as the average diameter of a cell body. An inverted microscope equipped with an ocular grid was used for cell counting. Only cells with nuclei falling on the vertical grid lines were scored. About 20 cells were scored in each microscopic field and 30 to 40 randomly selected fields were analyzed from three independent experiments.

Treatment of cells with *C. perfringens* neuraminidase (Nase), anti-GM3 monoclonal antibody (mAb DH2), and serum-free medium (SFM)

The cells were cultured as described in **Fig. 2** and were treated with either Nase, mAb DH2, or SFM. Nase (10 U) was dissolved in 1 ml of 0.1 M sodium acetate buffer, pH7. The Nase solution was filtered through a 0.22-μm sterile filter before addition to the cell culture medium. The cells were treated with Nase in the pres-

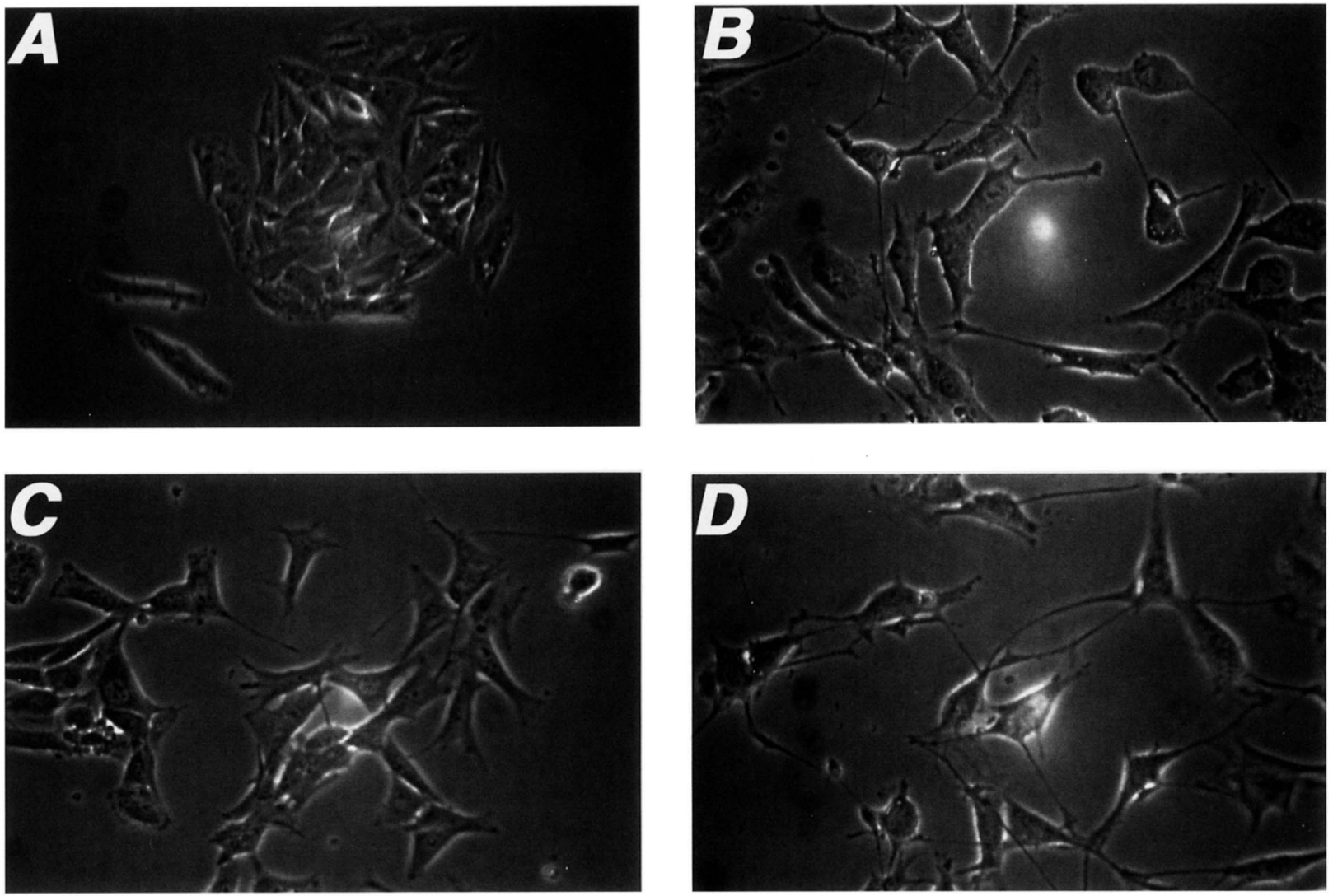

Fig. 2. Influence of Nase, mAb DH2, and SFM on the morphology of the EPEN cells. Approximately 4×10^3 cells were seeded into each well of a 24-well culture plate and were cultured for 6 days in DMEM supplemented with 5% fetal bovine serum. The cells were either untreated (A), or were treated with Nase (B) for 24 h (*C. perfringens,* 0.5 U/ml culture medium), with mAb DH2 (C) for 48 h (100 μg/ml culture medium), or with SFM (D) for 24 h (see Methods) (385 ×).

ence of 5% FBS for 24 h. Control cell cultures were treated with either inactivated Nase (boiling for 15 min) or with sodium acetate buffer only.

The IgG monoclonal antibody (mAb) DH2 hybridoma culture supernatant was described previously (31) and was concentrated according to the procedures of Hockfield et al. (32). The antibody solution was centrifuged for 15 min at 2000 rpm prior to application to cell cultures. The cells were treated with this antibody solution (100 μg/ml culture medium) in the presence of 5% FBS for 48 h. The control cell cultures were either untreated or were treated with phosphate-buffered saline (PBS) alone or with non-immune mouse IgG (100 μg/ml).

After the cells were cultured in DMEM supplemented with 5% FBS for 6 days, the serum-containing medium was removed. The cells were washed twice with SFM that contained DMEM supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, and 25 ng/ml epidermal growth factor. The cells were then grown in this SFM for 24 h.

The trypan blue exclusion procedure (33) was used to assess the viability of the cells after 24 h (Nase and SFM) or after 48 h (mAb DH2). The number of the dead cells that incorporated trypan blue were counted using a hemacytometer.

Treatment of cells with lipoproteins and glutathione

The EPEN cells were seeded and cultured for 6 days as described above. After washing the cells twice with SFM, the cells were cultured for 24 h in SFM supplemented with either HDL (80 μg protein/ml), LDL (80 μg protein/ml), VLDL (40 μg protein/ml), apoE3 (30 μg/ml), apoE4 (30 μg/ml), or apoE3 or E4 together with VLDL (40 μg protein/ml), respectively. For glutathione treatment, the concentrations of 80, 100, 160, 200 μg/ml were used. The glutathione at different concentrations was added to SFM together with HDL (120 μg/ml). To study the dosage-related effects of HDL on the EPEN cells, the cells were grown in SFM in the presence of 40, 80, or 120 μg/ml of HDL protein.

Analysis of gangliosides and neutral GSLs

The EPEN or the CT-2A tumor cells were seeded at approximately 2×10^4 cells/flask into 75-cm^2 culture flasks. The cells were cultured in DMEM supplemented with 10% FBS for 6 days. The cells were labeled with [^{14}C]galactose as described previously (34). After 24 h in culture, Nase, boiled Nase, or buffer was added. The culture medium was removed 24 h later and the flasks were washed three times with cold PBS. The cells were treated with 0.1% trypsin and harvested by centrifugation.

The isolation and purification of gangliosides and neutral GSLs from the lyophilized cell pellets and their qualitative analysis by high performance thin-layer chromatography (HPTLC) was as we described previously (23, 35, 36). The solvent systems used for the development of gangliosides and neutral GSLs are described in the figure legends. The ganglioside or neutral GSL bands were visualized by spraying the dried plates with the resorcinol–HCI reagent or the orcinol–H_2SO_4 reagent, respectively, and by baking the plates at 95–100°C for 30 min (37). The procedure of Warren (38) was used to estimate free sialic acid content.

Autoradiography of either the gangliosides or neutral GSLs was performed by exposing the radiolabeled TLC plate to Hyperfilm-^{3}H (Amersham, Arlington Heights, IL) for 7 days. Exposed films were developed with Kodak GBX developer (5 min), fixed with Kodak GBX fixer (3 min), and photographed. A Molecular Dynamics PhosphorImager (model SF) was also used to examine the distribution of the radiolabeled GSLs.

GSL biosynthesis in EPEN cells grown in serum-free medium

GSL biosynthesis was analyzed by estimating the incorporation of [^{14}C]galactose into gangliosides and neutral GSLs. The EPEN cells were seeded at approximately 2×10^4 cells/flask into the 75-cm^2 culture flasks and were cultured in DMEM supplemented with 10% FBS for 2 days. The medium was changed and the cells were cultured for an additional 6 days in either SSM or SFM. [^{14}C]galactose (0.15 μCi/ml) was then added to the cell culture medium. After incubation for 24 h, the cells were harvested and ^{14}C-labeled GSLs were isolated and purified as described above. Duplicate aliquots from either the ganglioside or neutral GSL samples were added to Ecoscint scintillation cocktail (National Diagnostics, Manville, NJ). The GSL synthesis was obtained by scintillation counting (Pharmacia LKB Liquid Scintillation counter) and was expressed as dpm [^{14}C]galactose incorporated into GSL/10^5 cells. The distribution of radiolabeled GSLs on HPTLC was performed as described above.

RESULTS

The gross morphology, histology, and ganglioside composition of the EPEN and CT-2A tumors grown in vivo and in vitro were described previously (23, 24, 34). Briefly, the EPEN tumor grows as a firm, cohesive, nonhemorrhagic mass in vivo and as cell islands in vitro. The CT-2A tumor, on the other hand, grows as a soft, non-cohesive, hemorrhagic mass in vivo and as a fusiform monolayer in vitro. The EPEN cells synthesize GM3 as the only ganglioside, whereas the CT-2A cells

synthesize GM2, GM1, and GD1a as major gangliosides, and GM3 as a minor ganglioside.

Influence of Nase on tumor cell cohesive properties

Nase treatment disrupted EPEN cell islands (reduced cohesion) and increased the number of free (non-cohesive) cells and cells bearing protoplasmic processes (Fig. 2A and B). Most of the free, non-cohesive Nase-treated cells expressed protoplasmic processes. In contrast to the EPEN cells, Nase treatment had no noticeable effect on the morphology of the CT-2A cells (**Fig. 3A and B**). The quantitative effects of Nase on EPEN cell cohesion and morphology are shown in **Fig. 4A and B**. Both the percentage of free cells and cells bearing protoplasmic processes were increased significantly by Nase. Nase treatment also reduced the growth rate of both cell lines (data not shown), but had no significant effect on cell viability. Greater than 95% of the EPEN and CT-2A cells survived the Nase treatment as assayed by trypan blue exclusion.

Influence of Nase on tumor cell GSL composition

It is important to mention that the gangliosides and neutral GSLs in each cell line migrated as double bands on the HPTLC plates (**Fig. 5** and **Fig. 6**). The double bands arise from structural heterogeneity in the ceramide portion of the molecule as previously described (23, 39, 40). Nase removes terminal sialic acids from glycoconjugates including GM3 and converts gangliosides such as GD1a to GM1 (7, 22). In the EPEN cells, Nase treatment converted most of the GM3 to LacCer, i.e., the asialo core structure of GM3 (Figs. 5A and 6). In the CT-2A cells, Nase converted most of the GM3 and GD1a to LacCer and GM1, respectively, but had little effect on the distribution of GM2, which is resistant to Nase digestion (Figs. 5B and 6). The Nase-induced increase in LacCer in the EPEN and CT-2A cells was also proportional to the level of GM3 in these cells. Besides producing the expected GSL reaction products, the Nase-induced hydrolysis of sialic acids from cell-surface glycoconjugates also increased the level of free sialic acids in the culture medium of both cell lines (data not shown).

Association between GM3 content and EPEN cell cohesive properties

The data in **Fig. 7** show that Nase treatment did not cause permanent changes in the morphological and biochemical properties of the EPEN cells. Cell cohesion (expressed as a reduction in the percentage of process-bearing cells) and GM3 content (dpm/10^5 cells) returned to control pre-treatment levels after the suspension of Nase treatment. As most of the free, non-cohesive cells also expressed protoplasmic processes (see Fig. 2B), these results show a positive association between GM3 content and the cohesive behavior of the EPEN cells, i.e., cells with high GM3 levels (days 7 and 12) were mostly cohesive (few processes), whereas cells with low GM3 levels (day 8) were mostly non-cohesive (many processes).

Influence of Nase-treated and boiled FBS on EPEN cell cohesive properties

To determine whether the Nase-induced changes in EPEN cell morphology might involve an effect of Nase on a serum factor, we treated the FBS for 15 h with Nase and then boiled the serum to stop further Nase activity. The EPEN cells grown in this Nase-treated and boiled FBS formed islands similar to those seen in Fig. 2A. These results suggested that Nase influenced EPEN cell cohesion and morphology from an effect on the cells rather than from an effect on factors in the FBS.

Influence of mAb DH2 on tumor cell cohesive properties

Treatment of the EPEN cells with mAb DH2 produced an effect similar to that seen with Nase treatment, i.e., a disruption of cell islands (reduced cohesion) and an increase in the number of free (non-cohesive) cells and cells bearing protoplasmic processes (Fig. 2A and C; Fig. 4A and B). No effect was seen with the addition of non-immune mouse IgG (100 μg/ml) as a control. Also similar to the Nase effect, mAb DH2 treatment reduced cell growth, but had little effect on cell viability (data not shown). In contrast to the EPEN cells, mAb DH2 had no noticeable effect on the morphology of the CT-2A cells (Fig. 3A and C). The binding specificity of mAb DH2 was also examined using immunostaining on HPTLC plates. mAb DH2 was specific for GM3 and did not cross-react with other gangliosides found in either bovine brain or in the solid EPEN tumor growing in vivo (data not shown). These results agree with the previous findings of others (16, 31).

Influence of SFM on tumor cell cohesive properties and GSL composition

SFM produced an effect on EPEN cell morphology similar to that seen after treatment with Nase and mAB DH2, i.e., a disruption of cell islands (reduced cohesion) and an increase in the number of free (non-cohesive) cells and cells bearing protoplasmic processes (Fig. 2A and D). Also similar to the effects of Nase and mAb DH2, SFM treatment reduced cell growth rate, but had little effect on cell viability. In contrast to the EPEN cells, SFM had no noticeable effect on the morphology of the CT-2A cells (Fig. 3A and D).

In order to determine whether SFM influenced the expression of GM3 or neutral GSLs, the EPEN cells

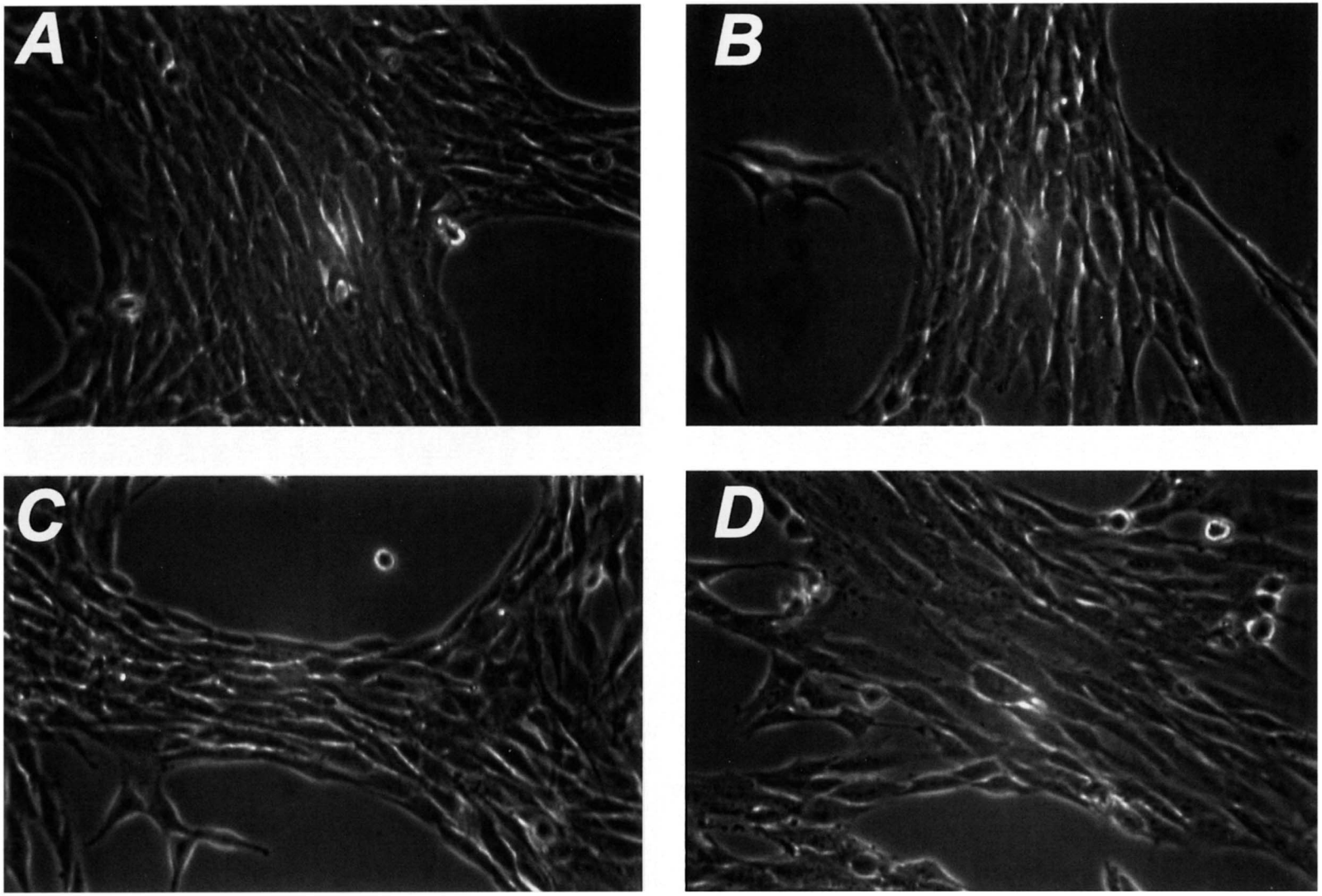

Fig. 3. Influence of Nase, mAb DH2, and SFM on the morphology of CT-2A cells. The treatment conditions for A–D were the same as those shown in Fig. 2 (385 ×).

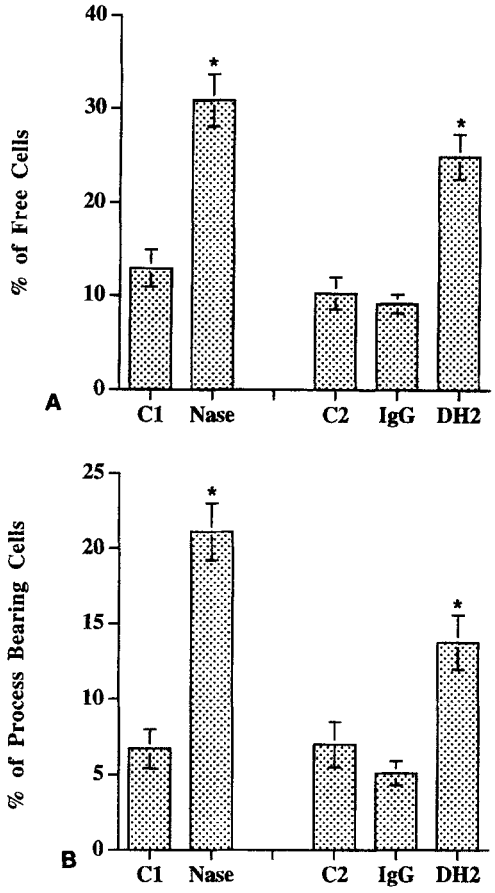

Fig. 4. Influence of Nase and mAb DH2 on the percentage of free cells (A) and protoplasmic process formation (B) of the EPEN cells. The cells were cultured and treated with either Nase or mAb DH2 as described in Fig. 2. The values were obtained from three independent experiments as described in Methods and are expressed as means ± SEM. Asterisks indicate that the values for the Nase and mAb DH2 treatments differed from those of the controls at $P < 0.01$ (two-tailed *t*-test). C1 and Nase represent treatment with sodium acetate buffer or Nase, respectively. C2, IgG, and DH2 represent treatment with PBS buffer, non-immune mouse IgG (100 μg/ml culture medium), and mAb DH2, respectively.

were cultured in the presence or absence of fetal bovine serum. SFM enhanced the content of GM3, but had no noticeable effect on the content or distribution of neutral GSLs. The content of GM3 (expressed as dpm/10^5 cells) was 1840 and 2090 dpm (two independent experiments) in serum-containing medium, and was 3550 and 3990 dpm in serum-free medium. The pattern of radiolabeled neutral GSLs (GlcCer, LacCer, and Gb3Cer) was the same as that shown in Fig. 6 lane C, for EPEN cells grown in serum-containing medium. These results suggest that the GM3 influence on EPEN cell cohesion was unlikely to involve direct homophilic (GM3–GM3) or heterophilic (GM3–neutral GSLs) interactions as SFM disrupted cell islands, but did not reduce GM3 or alter the distribution neutral GSLs. Based on these findings, we surmised that EPEN cell cohesion might involve an extracellular factor that was missing from the SFM. We therefore examined the fetal bovine serum for this factor.

Influence of HDL on EPEN cell cohesive properties

Our preliminary experiments on the cohesion of EPEN cells excluded an involvement of several serum factors including albumin, epidermal growth factor, fibroblast growth factor, and platelet-derived growth factor. Recent findings from Kivatinitz et al. (29) showed that HDL was a heat-stable factor that could also block protoplasmic process formation in chick cerebral cells. From our Nase experiments, we knew that a serum factor should be both heat-stable and Nase-resistant. To test the possible involvement of HDL in the EPEN cell cohesion phenomenon, we cultured the EPEN cells with SFM in the absence (control) or presence of bovine HDL. An HDL protein concentration of 80 μg/ml was used initially because this is the concentration that we estimated in the culture medium supplemented with 10% FBS based on the previous findings of Savion et al. (41).

Addition of HDL to EPEN cells growing in SFM caused a significant reduction in the number of free cells and process-bearing cells compared to the controls (**Fig. 8**). Indeed, the morphology of the HDL-treated EPEN cells was similar to that observed for EPEN cells grown in serum-supplemented medium (Fig. 2A). These results show that the cohesion and morphology of the EPEN cells was restored after addition of HDL to the SFM. Treatment of the bovine HDL with Nase and boiling had no influence on the HDL effect, thus fulfilling the two criteria of the serum factor. Moreover, the effect of bovine HDL on EPEN cell cohesion was also observed with human HDL and with reconstituted human HDL (42). The HDL effect on the percentage of free cells and protoplasmic processes was dosage-dependent and observed with a maximal effect at 120 μg HDL protein/ml (**Fig. 9**).

To determine the specificity of the HDL effect on EPEN cell cohesion, we examined the influence of other serum lipoproteins including LDL, VLDL, apoE3, apoE4, VLDL plus apoE3, and VLDL plus apoE4. Previous studies suggested that VLDL, apoE3, apoE4, and various combinations of these could influence neuritogenesis in cultured cells (43). In contrast to the effect of HDL, none of these lipoproteins or apolipoproteins influenced the morphology or cohesive properties of the EPEN cells grown in SFM. As oxidized lipids in HDL caused a growth-inhibiting effect on glioblastoma cells (44), we added the antioxidant glutathione, at different concentrations, to the SFM together with bovine HDL

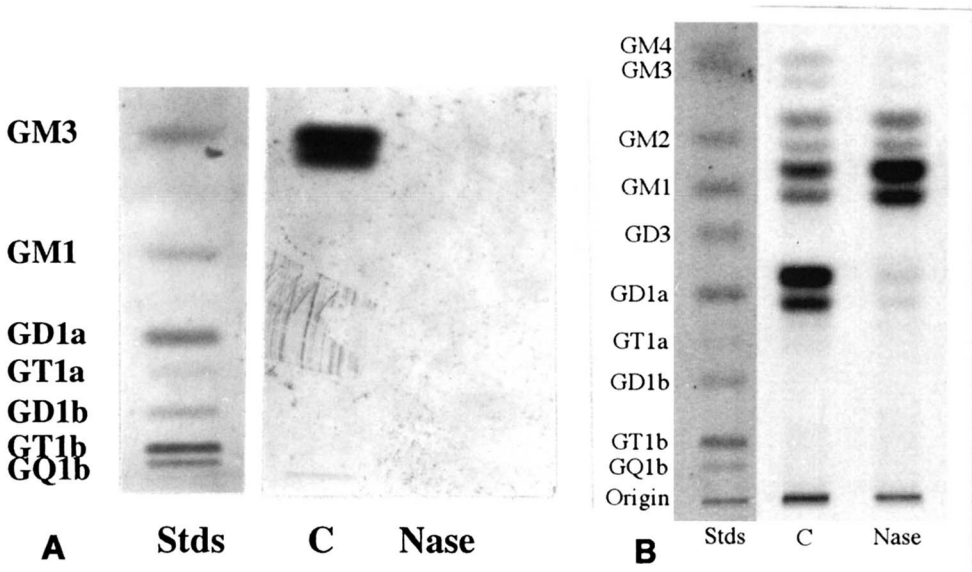

Fig. 5. Autoradiogram (A) and phosphorimage (B) of HPTLC plates showing the influence of Nase treatment on ganglioside distribution in the EPEN (A) and CT-2A (B) cells. The cells were grown for 24 h in medium containing 0.15 μCi/ml of [^{14}C]galactose. The cells were then treated with Nase (as described in the legend to Fig. 1) for 24 h in the presence of [^{14}C]galactose. For A, an amount of dpm equivalent to 1×10^5 cells (lanes C and Nase) were spotted. For B, 1500 dpm (lanes C and Nase) were spotted. Approximately 1.5 μg of ganglioside NeuAc standards (Stds) was spotted for both A and B. The plates were developed by one ascending elution with chloroform–methanol–water 50:45:10 (v/v) containing 0.02% $CaCl_2 \cdot 2H_2O$. After autoradiography (A) or phosphorimaging (B), the plates were sprayed with the resorcinol reagent to identify ganglioside standards. Lanes C and Nase indicate that the cells were treated with sodium acetate buffer or Nase, respectively.

(see Methods). This treatment also had no influence on the HDL effect.

DISCUSSION

The molecular mechanisms by which one cell coheres to another cell are likely varied and complex. Our results suggest that cell surface GM3 and extracellular HDL participate in the cohesion of cultured EPEN cells. Evidence for this comes from finding *a*), that EPEN cell cohesion was disrupted after the enzymatic removal (Nase) or immunological masking (mAb DH2) of GM3, or from growth in serum-free medium (absence of HDL) and *b*), that EPEN cell cohesion returned after the resynthesis of GM3 or after addition of HDL to the serum-free medium. Although both GM3 and HDL were necessary participants in the cohesion phenomenon, our findings also showed that neither molecule by itself was sufficient for EPEN cell cohesion.

It is unlikely that non-specific effects of the Nase, mAb DH2, or SFM treatments disrupted EPEN cell morphology or cohesion, as similar treatments had no influence on the morphology or cohesive properties of the CT-2A tumor cells. In contrast to mouse melanoma cells (3), mAb DH2 showed no cytotoxicity toward the EPEN or CT-2A cells. We cannot exclude the possibility that disrupted EPEN cell cohesion resulted in part from a treatment-induced reduction in growth rate. However, the Nase treatment also reduced the growth rate of the CT-2A cells, but did not alter their morphology. Schubert et al. (45) also found that morphological changes induced from low serum were not coupled to changes in cell division or DNA synthesis in C1300 mouse neuroblastoma cells. Although different cell lines may not always respond to the same treatment in the same way, it is unlikely that reduced growth rate alone can account for the differences in cohesive properties between the EPEN and CT-2A cells. On the contrary, our data suggest that the differential effects of the Nase, mAb DH2, or SFM treatments on EPEN and CT-2A morphology result from the differences in GM3 content in the two lines. GM3 is the major ganglioside in the EPEN cells, but is a minor ganglioside (about 5% of the total distribution) in the CT-2A cells.

Previous studies showed that Nase treatment could release cell-surface sialic acid and disrupt the aggregation of chick embryonic muscle cells (33, 46). Although Nase can remove terminal sialic acids from both glycoproteins as well as gangliosides (GM3 in the case of the EPEN cells), the anti-GM3 antibody, mAb DH2, is specific for GM3 as it does not cross-react with glycoproteins or other GSLs (16). Chatterjee, Chakraborty and

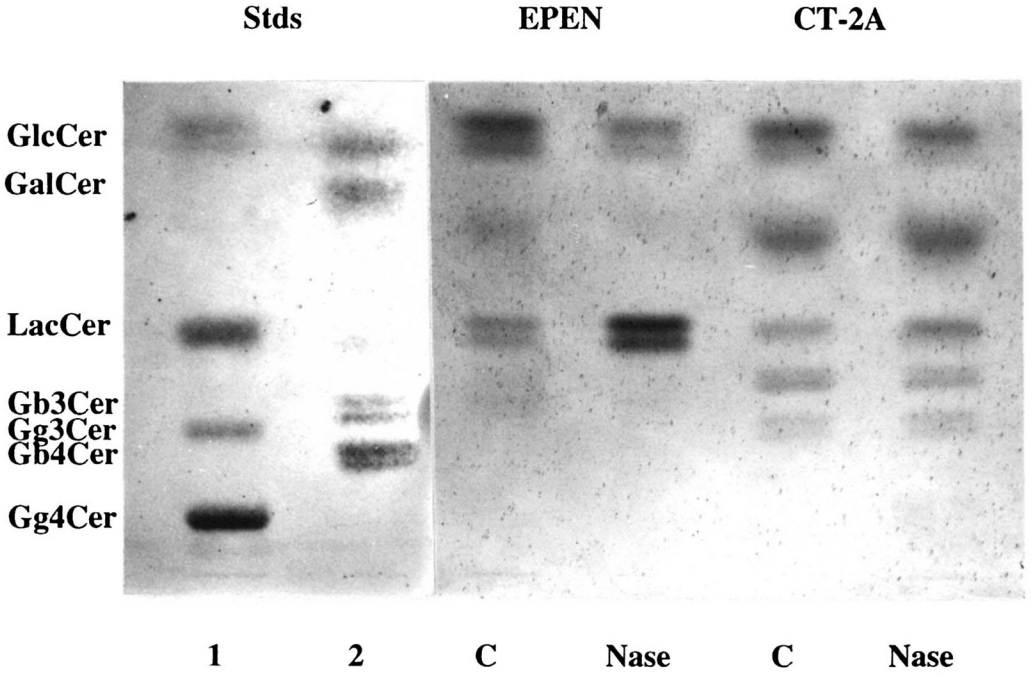

Fig. 6. Autoradiogram of an HPTLC plate showing the influence of the Nase treatment on neutral GSL distribution in the EPEN and CT-2A cells. The cells were labeled with [^{14}C]galactose and were treated with sodium acetate buffer (C) or Nase (see Methods). Approximately 1500 dpm of radiolabeled neutral GSL was spotted for the C and Nase lanes. Neutral GSL standards were spotted in lane 1 (GlcCer, LacCer, Gg3Cer, and Gg4Cer) and lane 2 (GlcCer, GalCer, LacCer, Gb3Cer and Gb4Cer). The plate was developed in a solvent system containing chloroform–methanol–2.5 M NH_4OH 65:35:8 (v/v) with 0.02% $CaCl_2{\cdot}2H_2O$. After autoradiography, the plate was sprayed with the orcinol reagent to identify neutral GSL standards. The radiolabeled band migrating between GlcCer and LacCer represents a phospholipid contaminant.

Anderson (47) also showed that an interaction between cell-surface GM3 and an anti-GM3 monoclonal antibody could induce protoplasmic processes in Neuro-2A cells. Our results with the EPEN cells are consistent with these findings and further suggest that GM3 may be involved with the observed morphological changes.

We suggest that the content of GM3, relative to that of other gangliosides, may also be important for its participation in the cohesion phenomenon. This comes from our present findings that the low level of GM3 in the CT-2A cells was associated with a fusiform non-island morphology, and from our previous findings that island formation occurred in rat fibroblasts where the content of GM3 (as a percentage of lipid bound sialic acid) comprised 57%, but did not occur in fibroblasts where the content of GM3 was 33% or lower (22). Further studies will be necessary to determine whether a critical content or threshold of GM3 is necessary for island formation.

Previous studies indicated that GM1 might play a role in neuritogenesis as the Nase-induced conversion of complex gangliosides to GM1 was associated with neurite outgrowth in cultured neural tumor cells (7). We found that disrupted cohesion was associated with the appearance of neurite-like protoplasmic processes in the treated EPEN cells. These processes, however, appeared in either the absence (Nase treatment) or presence (growth in SFM) of gangliosides. Furthermore, HDL could abolish protoplasmic processes in the EPEN cells grown in SFM. These findings are consistent with those of Kivatinitz et al. (29), who recently showed that HDL could also block neuritogenesis in chicken cerebral cells. On the other hand, no protoplasmic processes appeared in the CT-2A cells despite the Nase-induced conversion of most GD1a to GM1. We suggest that neurite formation in cultured cells is a complex phenomenon that likely involves the participation of numerous cellular and extracellular molecules.

Although the participation of GM3 in cell adhesion is well documented, little is known about the role of HDL in cell adhesion or cohesion. The HDL effect on EPEN cell cohesion was specific as no effect was found with other serum lipoproteins, i.e., LDL and VLDL or with apolipoproteins, i.e., apoE3 and apoE4, which were found to influence neuritogenesis in cultured cells (43). Furthermore, the HDL effect was observed with bovine and human HDL as well as with reconstituted human HDL (42). As the antioxidant glutathione did

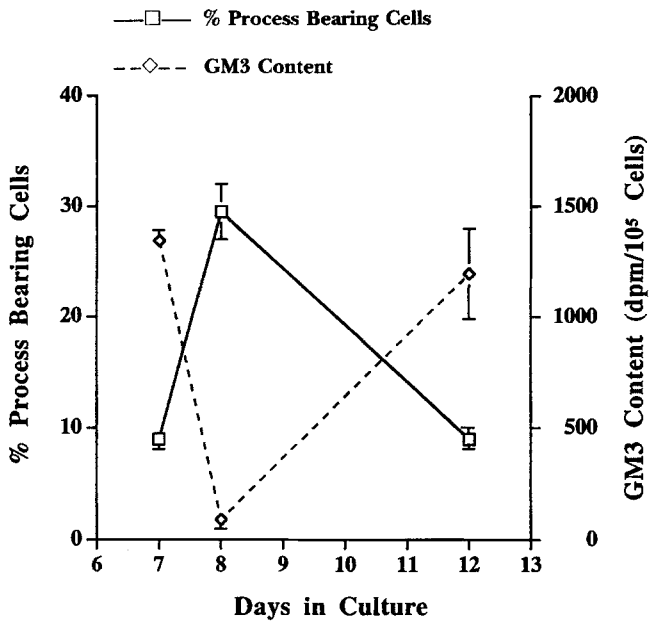

Fig. 7. Association between GM3 content and protoplasmic process formation in the EPEN cells. Approximately 2×10^4 cells/flask were seeded into 75-cm^2 culture flasks and the cells were cultured in DMEM containing 10% FBS for 6 days. [^{14}C]galactose (0.15 μCi/ml) was added to the culture medium on the sixth day. Nase was added on day 7 as in Fig. 2. After the cells were treated with Nase for 24 h, the Nase-containing medium was removed (on day 8) and the cells were cultured for another 4 days in the absence of Nase. The radiolabeled gangliosides were isolated and purified as described in Methods. For the studies of protoplasmic process formation, about 20 cells were analyzed in each field, and at least five randomly selected fields were examined for each experiment. The values represent the means and the bars the range of two independent experiments.

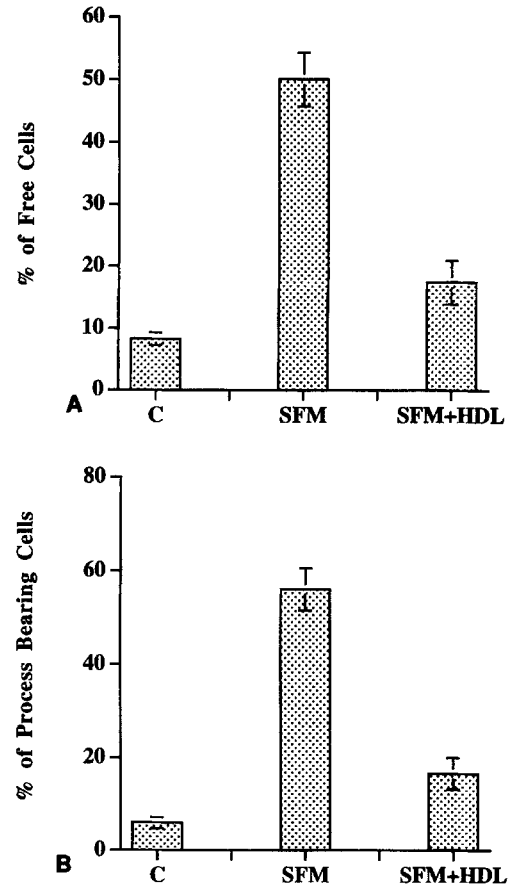

Fig. 8. Influence of HDL on the percentage of free cells (A) and protoplasmic process formation (B) of the EPEN cells. The cells were cultured in serum supplemented medium (C) for 6 days as in Fig. 2. The SSM was replaced and the cells were cultured in serum-free medium for 24 h (SFM). HDL (80 μg protein/ml) was added to the culture medium and the cells were then cultured for an additional 24 h (SFM + HDL). The values represent means ± SEM of three independent experiments as described in Methods.

not block the HDL effect, it is unlikely that the effect arose as an artifact of oxidized lipids in the HDL preparations (44). Furthermore, the HDL effect on EPEN cell cohesion was dosage-dependent and was observed at HDL protein concentrations similar to those normally present in culture medium supplemented with 10% fetal bovine serum. Besides a widely recognized role in cholesterol transport, our findings suggest a possible role for HDL in the cohesion and morphological properties of certain cell types grown in culture.

It is not known whether the participation of GM3 and HDL in EPEN cell cohesion is unique to the in vitro culture environment or might also occur in vivo. The EPEN and other experimental tumors that contain GM3 as the major ganglioside grow in the brain as firm cohesive masses, whereas the CT-2A and other tumors that contain low levels of GM3 grow as soft non-cohesive masses (24). HDL could access the growing brain tumors from either the cerebral spinal fluid (48) or from uptake through the blood–brain barrier (49). Although HDL could have similar access to the EPEN and CT-2A tumors, the low level of GM3 in CT-2A may account for its in vivo growth as a soft non-cohesive mass. It will be interesting to determine whether the participation of GM3 and HDL is similar in in vitro and in vivo growth environments.

The mechanism by which GM3 and HDL participate in EPEN cell cohesion is not known. It is unlikely that the mechanism involves a direct interaction between GM3 and HDL as rat C6 glioma cells, which also express GM3 as the major ganglioside, do not grow as islands in serum-containing medium (50). Rather than a direct GM3-HDL interaction, we speculate that the interaction may be indirect and mediated through an HDL receptor. HDL could participate as an extracellular bridge, linking apposing EPEN cells by binding to a cell-surface HDL receptor. GM3, on the other hand, could participate by modulating the function of the HDL receptor. Our notion is consistent with previous findings that

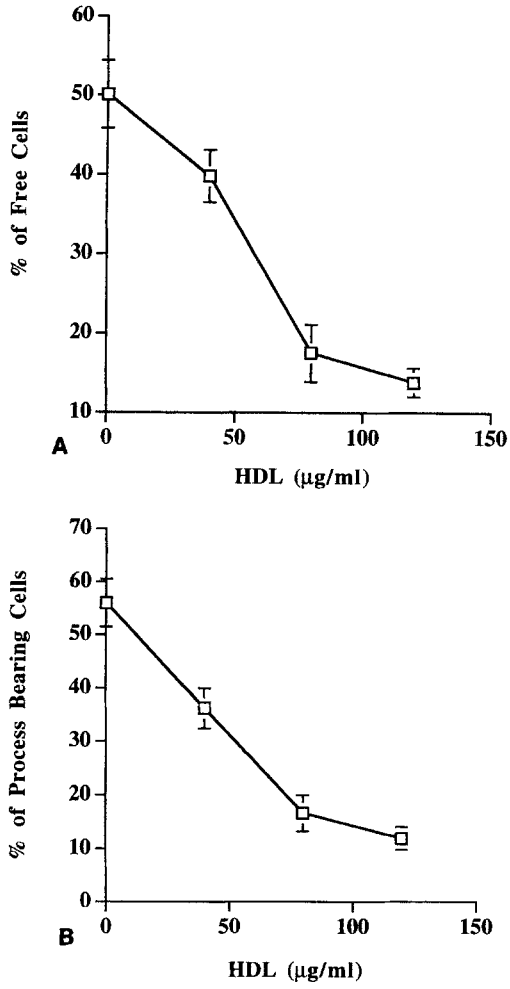

Fig. 9. Dose–response curve for influence of HDL on the percentage free cells (A) and protoplasmic process formation (B) of the EPEN cells. The cells were seeded and cultured for 6 days in SSM (see Methods). The SSM was replaced with SFM together with HDL (40, 80, and 120 μg protein/ml). After 24 h, the cells were examined (see Methods). The values represent means ± SEM of three independent experiments.

GM3 can modulate the function of several kinds of cell-surface receptors (8–13, 51–55), and that HDL can bind to a specific receptor at the cell surface (56–58). The removal of either GM3 or HDL could therefore disrupt EPEN cell cohesion as our data show. Although we suggest that EPEN cell cohesion may involve an indirect interaction between GM3 and HDL, we do not exclude other possible mechanisms. Further studies are needed to better define the mechanism by which GM3 and HDL participate in EPEN cell cohesion.

We thank Dr. W. Brunken for his generosity of time and equipment and Drs. W. T. Chiles, D. Chou, C. O'Connor, R. K. Yu, C. L. Schengrund, and S. Palay for their helpful suggestions. We thank Drs. S. Hakomori, M. Zheng, J. Brigande, and J. Ecsedy, B. P. Nathan and D. M. Levine for their material or technical assistance. We thank S. Vedder for photographic services. This work was submitted in partial fulfillment for the Ph.D. degree (H. W. B.) and was supportd by NIH grant NS 33640 and Boston College REG.

Manuscript received 3 August 1996 and in revised form 12 November 1996.

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