thematic review

Thematic Review Series: Sphingolipids

Ganglioside GM3 suppresses the proangiogenic effects of vascular endothelial growth factor and ganglioside GD1a

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Abstract **Gangliosides are sialic acid-containing glycosphingolipids that have long been associated with tumor malignancy and metastasis. Mounting evidence suggests that gangliosides also modulate tumor angiogenesis. Tumor cells shed gangliosides into the microenvironment, which produces both autocrine and paracrine effects on tumor cells and tumor-associated host cells. In this study, we show that the simple monosialoganglioside GM3 counteracts the proangiogenic effects of vascular endothelial growth factor (VEGF) and of the complex disialoganglioside GD1a. GM3 suppressed the action of VEGF and GD1a on the proliferation of human umbilical vein endothelial cells (HUVECs) and inhibited the migration of HUVECs toward VEGF as a chemoattractant. Enrichment of added GM3 in the HUVEC membrane also reduced the phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR-2) and downstream Akt. Moreover, GM3 reduced the proangiogenic effects of GD1a and growth factors in the in vivo Matrigel plug assay. Inhibition of GM3 biosynthesis with the glucosyl transferase inhibitor, *N*-butyldeoxynojirimycin (*N*B-DNJ), increased HUVEC proliferation and the phosphorylation of VEGFR-2 and Akt. The effects of *N*B-DNJ on HUVECs were reversed with the addition of GM3. We conclude that GM3 has antiangiogenic action and may possess therapeutic potential for reducing tumor angiogenesis.**—Mukherjee, P., A. C. Faber, L. M. Shelton, R. C. Baek, T. C. Chiles, and T. N. Seyfried. **Ganglioside GM3 suppresses the proangiogenic effects of vascular endothelial growth factor and ganglioside GD1a. *J. Lipid Res.* 2008.** 49: **929–938.**

Supplementary key words glycosphingolipid • human umbilical endothelial cell migration • matrigel plug assay • growth factor receptor • phosphorylated Akt

Gangliosides are a family of sialic acid-containing glycosphingolipids that are enriched in the outer surface of plasma membranes and have long been associated with tumor malignancy and metastasis (1–3). These molecules contain an oligosaccharide head group that is attached to a lipophilic ceramide, consisting of a sphingosine base and a long-chain fatty acid. Gangliosides can be shed from the surface of tumor cells into the microenvironment, where they can influence tumor host cell interactions to include angiogenesis (1, 4–13). Ganglioside GM3, a simple monosialoganglioside (NeuAca2→3Galb1→4Glcb1→1′-ceramide), modulates cell adhesion, proliferation, and differentiation (2, 5, 12, 14). The antiproliferative and proapoptotic effects of GM3 were observed in glioma cells grown both in vivo and in vitro (15–17). In contrast to GM3, complex gangliosides like GM2, GM1, GD1a, GD1b, GT1b, and GD3, which contain longer oligosaccharide chains than that of GM3, enhance tumor cell proliferation, invasion, and metastasis (1, 3, 14, 18, 19). Increased tumorigenic effects of complex gangliosides were observed in a variety of tumor cells, including bladder, lymphoma, glioma, neuroblastoma, and melanoma (7, 11, 14, 20–22). Specific inhibitors of ganglioside biosynthesis also reduced tumor growth (23–25), whereas gene-linked shifts in ganglioside distribution changed tumor growth and angiogenesis in vivo (4, 8, 12).

Endothelial cell signaling is important in cancer-associated vascularity (angiogenesis). The proliferation and migration of endothelial cells in response to growth factors is one of the major determinants of tumor growth and progression. Dysregulation of the balance between proangiogenic and antiangiogenic factors contributes to

Abbreviations: bFGF, basic fibroblast growth factor; EBM, endothelial basal medium; EGFR, epidermal growth factor receptor; EGM-2, endothelial growth medium; HPTLC, high-performance thin-layer chromatography; HUVEC, human umbilical vein endothelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *N*B-DNJ, *N*-butyldeoxynojirimycin; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor 2.

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the abnormal vasculature in tumors. The targeting of tumor endothelial cells, therefore, is considered important for managing tumor growth (1, 8, 26).

Vascular endothelial cells are responsive to a number of proangiogenic growth factors, including basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which promote endothelial cell survival, growth, and migration (18, 27, 28). Interestingly, complex gangliosides enhance the response of endothelial cells to the proangiogenic action of bFGF and VEGF (4, 13, 18, 29). GD1a enrichment of endothelial cells enhanced VEGF receptor dimerization, autophosphorylation, and downstream signaling pathways for endothelial cell proliferation and migration (13). The involvement of gangliosides in angiogenesis is dependent on the intact molecules, as neither asialo species nor sialic acid alone influences angiogenesis (30). In contrast to the enhancing effects of complex gangliosides on angiogenesis, GM3 reduces endothelial cell proliferation and migration (5, 8, 12, 31). Little is known, however, about the molecular mechanism by which GM3 inhibits angiogenesis.

The interactions of gangliosides with cell surface receptor molecules in tumor cells as well as in endothelial cells may be critical for the tumor-induced progression of the microenvironment (8, 13, 14). The inhibitory effects of GM3 on the epidermal growth factor receptor (EGFR) tyrosine kinase are well studied (32–34). Yoon et al. (34) showed that GM3 inhibits the EGFR tyrosine kinase through interactions with *N*-acetylglucosamine residues on the glycan units of the receptor. In contrast to the information available on the influence of GM3 on the EGFR, little is known about the influence of GM3 on other growth factor receptors, including the vascular endothelial growth factor receptor (VEGFR). VEGFR-2 or KDR is phosphorylated upon VEGF stimulation, which induces the phosphoinositide-3 kinase/Akt pathway, resulting in enhanced endothelial proliferation and migration (35, 36).

In this study, we examined the influence of the exogenous addition of GM3 on the proliferation of human umbilical vein endothelial cells (HUVECs) in the presence of VEGF and the VEGF enhancer, GD1a. We show that GM3 suppresses angiogenesis both in vitro and in vivo. We also found that GM3 enrichment of HUVECs inhibits migration toward VEGF as a chemoattractant. Additionally, we found that GM3 reduced VEGFR-2 phosphorylation and downstream Akt signaling in HUVECs, suggesting a mechanism by which GM3 reduces endothelial cell proliferation and migration. Moreover, pharmacological depletion of endogenous GM3 significantly increased HUVEC proliferation and VEGFR-2 and Akt phosphorylation.

MATERIALS AND METHODS

Reagents

Purified disialoganglioside GD1a (bovine brain), recombinant human vascular endothelial growth factor ($VEGF_{165}$), and BSA were purchased from Sigma (St. Louis, MO). Highly purified monosialoganglioside GM3 was purchased from Matreya (Pleasant Gap, PA). The commercial ganglioside preparations were the same as those used by other groups (13, 14) but were not checked for purity. Matrigel™ basement membrane matrix was purchased from BD Biosciences (Bedford, MA). The CellTiter 96 nonradioactive cell proliferation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was from Promega (Madison, WI). Endothelial growth medium (EGM-2), BulletKit medium, and supplement-free endothelial basal medium (EBM) were purchased from Cambrex (Charles City, IA). Ganglioside GD1a and VEGF were dissolved in EBM and sonicated, and aliquots stored at −20°C. Ganglioside GM3 was prepared in the same manner but was first dissolved in chloroform-ethanol (1:1, v/v) and was then evaporated and dissolved in EBM. Antibodies against total and phosphorylated VEGFR-2 and Akt were purchased from Cell Signaling (Beverly, MA). FITC-labeled Isolectin B4 was purchased from Vector Laboratories (Burlingame, CA). Calcein was purchased from Invitrogen (San Diego, CA). Anti-GM3 DH2 antibody was a gift from Dr. S. I. Hakomori (University of Washington, Seattle). Cy5-labeled anti-mouse antibody was purchased from Jackson ImmunoResearch (West Grove, PA). *N*-butyldeoxynojirimycin (*N*B-DNJ; molecular weight, 219.3) was obtained as a gift from Oxford Glycosciences (Abigdon, UK). [^{14}C]galactose was obtained from Sigma.

Cell culture

HUVECs (pooled) were purchased from Cambrex and were maintained at 37°C in EGM-2 in humidified air containing 5% CO_2. All experiments with HUVECs were conducted at passages two to six.

Animals

BALBc/J-SCID mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were used for the Matrigel plug study. All animal experiments were carried out with ethical committee approval in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the institutional care committee.

Proliferation assay

In vitro HUVEC proliferation was analyzed using the CellTiter 96 nonradioactive cell proliferation assay according to the manufacturer's protocol. Briefly, 5×10^3 HUVECs in EGM-2 were seeded in each well of a 96-well plate. After 24 h, the HUVECs were washed and treated with 20 μM GD1a and GM3 in EBM for 24 h. After this incubation, the ganglioside-containing medium was removed. The HUVECs were then washed with EBM and were stimulated with 4 ng/ml VEGF in EBM for 24 h. For the *N*B-DNJ study, cells were treated with 200 μM *N*B-DNJ in the absence and presence of GM3 for 48 h and were then stimulated with 4 ng/ml VEGF in EBM for an additional 24 h. Tetrazolium salt dye solution (15 μl) was added to each well, and the plate was returned to the incubator for 4 h. Stop solution (100 μl) was then added to solubilize the metabolite and to lyse the HUVECs. The plate was incubated for 24 h. The plate was shaken for 30 s after incubation and was read in a microplate reader (SpectraMax M5; Molecular Devices) at 595 nm. This procedure was performed in triplicate.

Flow cytometry

HUVECs were treated with 0, 0.5, 2.5, or 5.0 μM GM3 in EBM for 24 h at 37°C in 5% CO_2-humidified conditions. Confluent cells were detached with trypsin/EDTA and were washed with PBS. HUVECs (2×10^5) were incubated with anti-GM3 DH2 antibody (1:50) for 1 h on ice, washed with PBS, and incubated

with 1:500 Cy5 anti-mouse secondary antibody for 30 min on ice in darkness. Cells were then washed twice with PBS, resuspended, and analyzed with FACSDiva software (Beckman Coulter). Control experiments included HUVECs treated with only secondary antibody.

Migration assay

Confluent HUVECs in T75 flasks were rinsed and treated with 20 μM GM3 in EBM containing 0.1% BSA for a minimum of 4–5 h at 37°C in 5% CO_2-humidified conditions. A control flask of HUVECs was treated with EBM containing 0.1% BSA. Cells were washed twice in EBM and were harvested in EBM containing 0.1% BSA. According to the manufacturer's protocol, equal numbers of control and treated cells (4×10^5 cells/ml) in 250 μl of EBM were seeded on the upper insert of a 24-well migration chamber (BD Falcon Fluoroblok Insert system; 3.0 μm pore size). EBM (750 μl) containing 100 ng/ml VEGF was then placed in the lower chamber according to the protocol. This VEGF concentration was recommended and necessary for an effective migration of HUVECs. Serum-containing medium was used as a positive control for these experiments. The plates were incubated for 24 h under the humidified conditions as above. HUVECs that migrated to the underside of the membrane were washed with HBSS, and the inserts were transferred in the companion wells containing calcein (4–5 μg/ml) solution. HUVECs were incubated in calcein for 1 h and washed, and the fluorescently labeled cells were photographed using a fluorescence microscope. Fluorescence was measured using a SpectraMax M5 microplate reader (Molecular Devices).

In vivo Matrigel plug assay

Angiogenesis was analyzed using the in vivo Matrigel plug assay as we described previously (12, 37). Briefly, Matrigel (200 μl) and EBM (100 μl) containing GD1a and/or GM3 (at 20 or 40 μM) were thoroughly mixed at 4°C. Control plugs contained only the Matrigel/EBM solution. Male BALBc/SCID mice were anesthetized with Avertin (0.1 ml/10 g body weight) and then injected with Matrigel with or without gangliosides subcutaneously in the dorsal midline using a prechilled tuberculin syringe (27 gauge needle). Seven days after implantation and 30 min before necropsy, mice were injected intravenously with 100 μl of FITC-conjugated *Griffonia simplicifolia* Isolectin B4 (0.25 mg/ml) to stain vascular endothelial cells (38). Matrigel plugs with the surrounding skin were removed as described previously, and vascularity was photographed (37). Lectin-FITC was extracted from plugs by homogenizing in 500 μl of radioimmunoprecipitation buffer. The homogenate was centrifuged at 1,000 *g*, and fluorescence was measured at 490 nm using a SpectraMax M5 microplate reader as above.

Ganglioside biosynthesis in HUVECs

Synthesized gangliosides were isolated from control and *N*B-DNJ-treated HUVECs as described previously (2). Briefly, HUVECs were grown for 72 h in EGM-2 containing 5 μCi of [^{14}C]galactose and 200 μM *N*B-DNJ. The radiolabeled cells were removed from the flask with a cell scraper in PBS and were collected as a pellet. Before ganglioside isolation, unlabeled mouse ependymoblastoma gangliosides, containing GM3, were added as unlabeled carrier (2). Total lipids were extracted from the radiolabeled cells in chloroform-methanol (2:1, v/v), and dH_2O was added (20%, v/v). The radiolabeled gangliosides were separated from the total lipids by Folch partitioning into an upper aqueous phase as described (2). The upper phase gangliosides were dried under nitrogen, resuspended in chloroform-methanol (2:1, v/v), and spotted on a high-performance thin-layer chromatography (HPTLC) plate. The concentration of radiolabeled gangliosides spotted on the HPTLC plate was determined by scintillation counting. The amount of disintegrations per minute equivalent to 10,000 dpm was spotted per lane on the HPTLC plate. The HPTLC plate was developed in one ascending elution with C/M/H_2O (50:45:10, v/v) containing 0.02% $CaCl_2 \cdot dH_2O$. After autoradiography, the plates were sprayed with the resorcinol reagent to identify ganglioside standards. The amount of radiolabel incorporated into GM3 was determined from Bioscan analysis as we described previously (39).

Immunoblot analysis of HUVEC lysates

HUVECs were seeded in EGM-2 at 1×10^5 cells/well on six-well plates. When confluent, the HUVECs were washed with EBM and then incubated with 80 nM (100 ng/ml) GM3 in serum-free EBM for 24 h. The HUVECs were then washed and stimulated with 100 ng/ml VEGF in EBM for 5 min at 37°C. For the *N*B-DNJ study, cells were treated with 200 μM *N*B-DNJ in the presence and absence of GM3 for 48 h and then stimulated with VEGF for 5 min at 37°C. The HUVECs were next washed twice with PBS and treated with lysis buffer (100 μl/well) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM $NaPP_i$, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsufonyl fluoride. The lysates were centrifuged at 12,000 *g* for 20 min at 4°C. Supernatants were collected, and protein concentrations were estimated using the Bio-Rad DC protein assay. Approximately 50–100 μg of total protein from each sample was loaded onto a 12% sodium dodecyl polyacrylamide gel (Invitrogen) and analyzed by electrophoresis. Proteins were transferred to a polyvinylidene difluoride Immobilon TM-P membrane (Millipore). The membrane was blocked in 5% nonfat powdered milk in Tris-buffered saline with Tween 20 (pH 7.6) for 1 h at room temperature. Blots were then probed with primary antibody against phosphorylated VEGFR-2 (tyrosine-1175) and reprobed with primary antibody against mouse monoclonal Akt (serine-473) overnight at 4°C. The membrane was probed again with total VEGFR-2 and Akt under optimal conditions.

Statistical analysis

All data are presented as means ± SEM. Significance of differences between groups was evaluated with one-way ANOVA.

RESULTS

GM3 suppresses VEGF- and GD1a-induced HUVEC proliferation

To examine the influence of GM3 on angiogenesis, we studied the proliferation of HUVECs in culture. We evaluated the influence of GM3 on HUVEC proliferation either alone or in combination with GD1a in the presence of VEGF stimulation (**Fig. 1**). We found that GD1a (20 μM) significantly increased VEGF-induced HUVEC proliferation. In contrast, GM3 (20 μM) significantly reduced VEGF- or GD1a-induced HUVEC proliferation by ~50%. A control study was done to confirm that GM3 did not alter the uptake of MTT into HUVECs. The inhibitory effect of GM3 on HUVEC proliferation was also observed at a lower GM3 concentration of 80 nM.

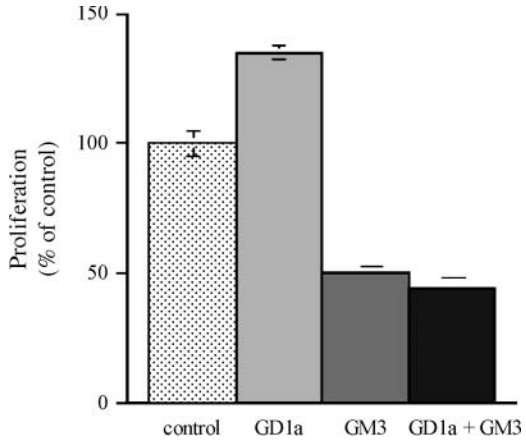

Fig. 1. GM3 inhibits vascular endothelial growth factor (VEGF)- and GD1a-induced human umbilical vein endothelial cell (HUVEC) proliferation. HUVECs were pretreated for 24 h in endothelial basal medium (EBM) in the absence (control) or in the presence of GD1a (20 μM) and/or GM3 (20 μM) and then incubated for an additional 24 h in the presence of VEGF (4 ng/ml). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure HUVEC proliferation as described in Materials and Methods. Proliferation is expressed as a percentage of the untreated, VEGF-stimulated controls. GD1a pretreatment significantly enhanced HUVEC proliferation ($P < 0.01$). GM3 pretreatment significantly suppressed VEGF- and GD1a-induced proliferation ($P < 0.001$). Values are expressed as means ± SEM (n = 3 independent experiments).

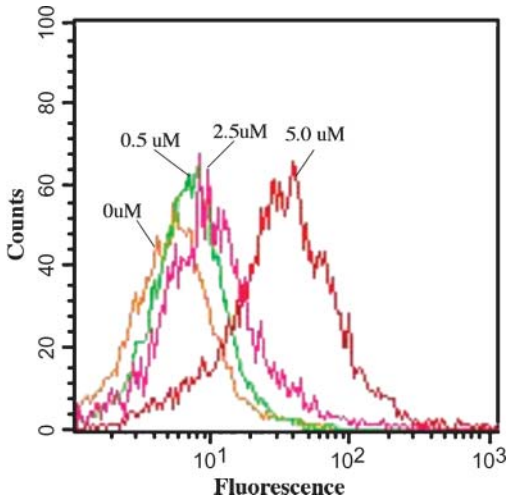

Fig. 2. Detection of added GM3 on HUVEC membranes. HUVECs were incubated with GM3 for 24 h in EBM. Flow cytometry using anti-GM3 DH2 and Cy5-labeled anti-mouse IgG was used to evaluate GM3 on the membranes of HUVECs. The concentration of added GM3 is shown above each peak: orange, 0.0 μM; green, 0.5 μM; pink, 2.5 μM; brown, 5.0 μM. The 0 μM concentration peak was the same as that for the secondary Cy5 antibody without DH2. These experiments were done in triplicate.

Detection of GM3 on HUVEC membranes

To determine whether the suppressive effects of GM3 on HUVEC proliferation were associated with the expression of GM3 on the membrane, we added different amounts of GM3 to the incubation medium and analyzed the HUVECs using flow cytometry with DH2 anti-GM3 antibody (**Fig. 2**). No DH2 binding occurred in the untreated HUVECs, indicating that DH2 does not detect the endogenous GM3 epitope. Incubation of HUVECs with GM3 caused a dose-dependent increase in DH2 binding. Because GM3 pretreatment produced no apparent cell death or toxicity, it is likely that GM3, located on the HUVEC membrane, inhibits proliferation and reduces the stimulatory effects of VEGF and/or GD1a. These findings indicate that GM3 modulates the response of HUVECs to the proangiogenic effects of VEGF and GD1a.

GM3 reduces HUVEC migration toward VEGF as a chemoattractant

We also examined whether GM3 could influence HUVEC migration toward VEGF as a chemoattractant. HUVECs, which migrated from the upper chamber to the lower chamber of the migration assay and were labeled with the fluorescent dye calcein-AM, were photographed (**Fig. 3A**) and the amount of fluorescence was quantified (Fig. 3B). HUVEC migration was significantly less in the absence than in the presence of VEGF, indicating that VEGF is a chemoattractant for HUVECs in this assay. Migration was significantly less for GM3-treated HUVECs than for non-treated HUVECs. These findings show that GM3 reduced HUVEC migration in response to VEGF.

GM3 inhibits GD1a-induced vascularization in Matrigel in vivo

Because GM3 suppressed the GD1a- and/or VEGF-induced HUVEC proliferation and migration in culture, it was of interest to examine the influence of GM3 on angiogenesis in the in vivo Matrigel plug assay. The in vivo Matrigel angiogenesis model represents early events of angiogenesis and tumor progression and is dependent on the activation and infiltration of host stromal cells, which include monocytes, macrophages, and endothelial cell precursors (12, 27, 37). Matrigel contains growth factors, which induce the infiltration of blood vessels as seen in the control plugs (**Fig. 4A**). The addition of GD1a to the Matrigel enhanced blood vessel formation into the plugs. The number and size of vessels was also greater at a GD1a concentration of 40 μM than at 20 μM, consistent with the known angiogenesis-enhancing effects of GD1a (13). The addition of equal amounts of GM3 and GD1a (40 μM) markedly reduced blood vessel formation in the plugs. Indeed, the appearance of blood vessels was noticeably less in the plugs containing GM3 and GD1a than in the control plugs, which contained no added gangliosides (Fig. 4A). Fluorescently labeled vessels were noticeably less in the GM3 + GD1a-containing plugs than in the plugs containing GD1a alone (Fig. 4B). The ratio of FITC-labeled vessels to arbitrary units was also significantly lower in the GM3 + GD1a-containing plugs than in the plugs containing GD1a alone (Fig. 4C). These results indicate that GM3 suppressed the proangiogenic effects of GD1a in an in vivo environment.

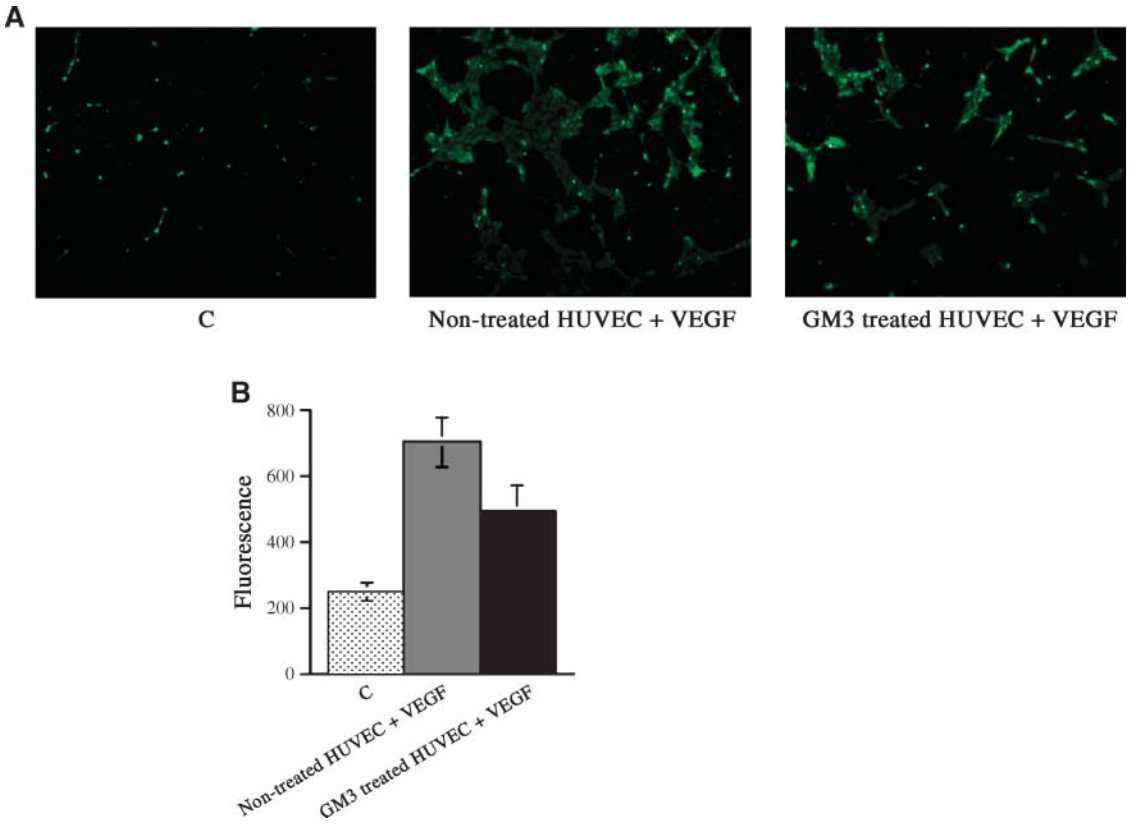

Fig. 3. GM3 inhibits VEGF-induced HUVEC migration. A: Calcein-labeled HUVECs were photographed under fluorescence microscopy (200×). B: The amount of fluorescence incorporated was measured. The HUVECs were either untreated or treated with GM3 (20 μM) and were seeded in EBM onto the upper chamber of the fibronectin-coated insert. VEGF (100 ng) in EBM was placed in the lower chamber. After 24 h, HUVECs that migrated through the filter were stained with calcein. VEGF significantly increased HUVEC migration compared with VEGF-untreated control (C) cells at $P < 0.01$. GM3 significantly reduced VEGF-induced migration at $P < 0.01$. Values are expressed as means ± SEM (n = 3 independent experiments, three wells per group per experiment).

GM3 inhibits VEGFR-2 and Akt phosphorylation

VEGF stimulates HUVEC proliferation through downstream phosphoinositide-3 kinase/Akt signaling by inducing the phosphorylation of VEGFR-2 (KDR, Flk-1) (35, 36). We used a 100 ng/ml VEGF concentration for these experiments to ensure robust VEGFR-2 phosphorylation (ratio of pVEGFR-2 to total). VEGFR-2 phosphorylation was 6-fold greater and Akt phosphorylation at serine-473 (ratio of pAkt to total) was ~2-fold greater in the presence than in the absence of VEGF after 5 min of stimulation in EBM (**Fig. 5A, B**). No VEGF phosphorylation was observed for HUVECs grown in the basal medium (EBM), which contained no serum or growth factors. In contrast, phosphorylation of VEGFR-2 and Akt was reduced significantly by 60% and 75%, respectively, in HUVECs incubated with GM3 (80 nM or 100 ng/ml) for 24 h before VEGF stimulation (Fig. 5A, B). We used the 100 ng/100 ng GM3/VEGF concentration ratio in these experiments to remain consistent with the 1:1 GM3/bFGF concentration ratio used previously in the rabbit cornea model (31). This GM3 concentration was more effective at reducing phosphorylation than was the higher concentration (20 μM) of GM3 used in the in vivo assay (data not shown). Collectively, these findings indicate that low concentrations of GM3 inhibit the VEGF-induced phosphorylation of VEGFR-2 and downstream Akt in cultured HUVECs.

Influence of *N*B-DNJ on GM3 synthesis, HUVEC proliferation, and VEGFR-2 and Akt phosphorylation

*N*B-DNJ is a nontoxic competitive inhibitor of the ceramide-specific glucosyltransferase that catalyzes the first step in ganglioside biosynthesis (23). Treatment of HUVECs with *N*B-DNJ (200 μM) reduced GM3 synthesis, as revealed by the incorporation of ^{14}C-labeled galactose into newly synthesized GM3 (**Fig. 6A**). The ganglioside pattern of HUVECs was similar to that described previously with GM3 as the predominant species (13, 40). *N*B-DNJ treatment significantly increased VEGF-induced proliferation over untreated control cells (Fig. 6B). In parallel, *N*B-DNJ significantly increased the VEGFR-2 and Akt phosphorylation (Fig. 6C, D). Because *N*B-DNJ reduces not only GM3 synthesis but also that of most other glycosphingolipids in HUVECs, we evaluated the effects of the exogenous addition of GM3 in *N*B-DNJ-treated cells. We found that GM3 addition reversed the *N*B-DNJ-induced increases in HUVEC proliferation and VEGFR-2 and Akt phosphorylation (Fig. 6B–D). These findings fur-

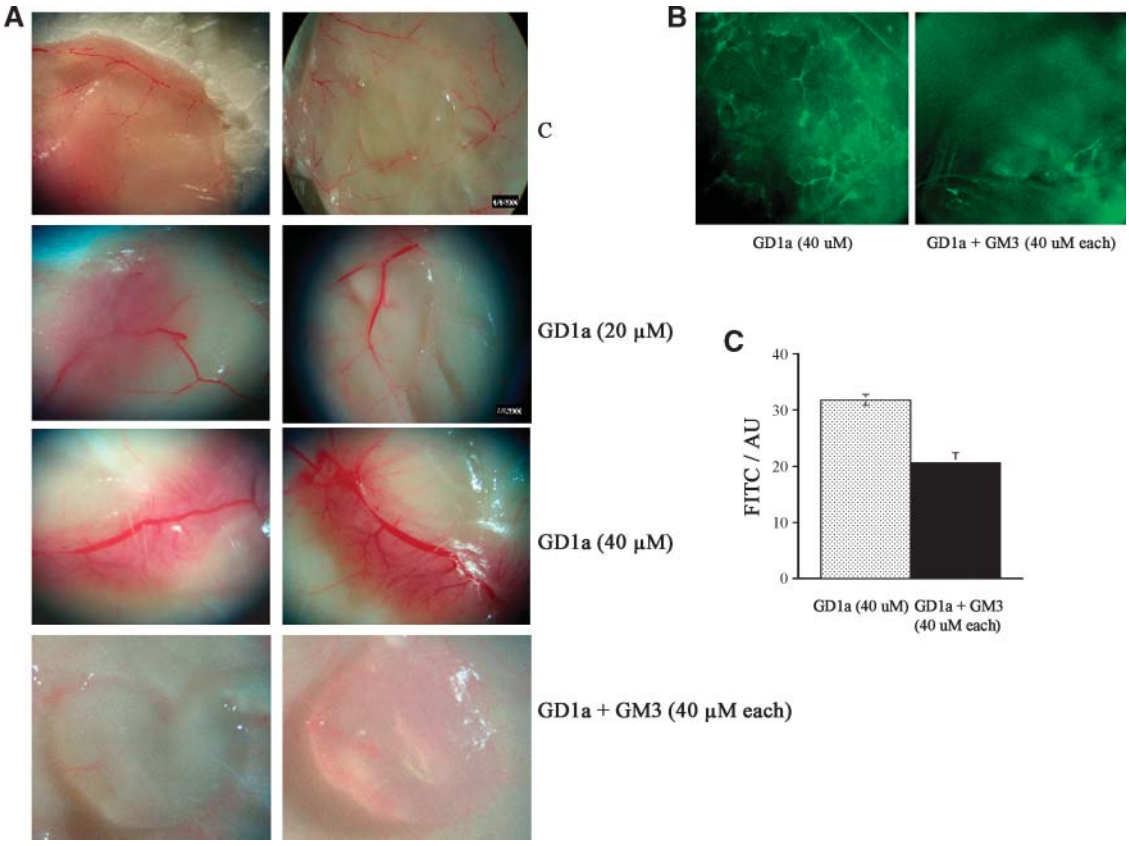

Fig. 4. GM3 inhibits the proangiogenic effects of GD1a in the in vivo Matrigel plug assay. Matrigel alone (control) or containing GD1a or GD1a with GM3 was injected subcutaneously in SCID mice as described in Materials and Methods. A: Plugs were photographed (12.5×) on day 7 after Matrigel injection to evaluate blood vessels. B: The plug vasculature was also evaluated under fluorescence microscopy (200×) in mice injected intravenously at 30 min before necropsy with FITC-labeled Isolectin B4. C: The amount of fluorescence in the plugs was measured by fluorimetry as described in Materials and Methods. Six mice per group were evaluated, and the values are expressed as means ± SEM. Vascularization as detected by fluorescence was significantly less in the plugs with GD1a and GM3 than in the plugs with only GD1a at $P < 0.01$. AU, arbitrary units.

ther support the specific role of GM3 in HUVEC proliferation and signaling.

DISCUSSION

The objective of this study was to revisit the role of ganglioside GM3 in angiogenesis in relationship to HUVEC proliferation and migration in vitro and to blood vessel formation in vivo. Gullino and coworkers (5, 6, 30, 41) first showed that changes in the relative concentrations of GM3 to complex gangliosides could stimulate or suppress angiogenesis in vitro or in vivo. Using the rabbit cornea model of angiogenesis, they found that GM3 reduced the growth and motility of microvascular endothelium while repressing the proangiogenic effects of prostaglandin E1 and bFGF (6, 30, 31). Moreover, ganglioside GD3 and other complex gangliosides (GM1 and GD1a) reduced the antiangiogenic effects of GM3. No further reports have appeared addressing the mechanism by which GM3 might inhibit angiogenesis.

We previously showed that gene-linked changes in the distribution of GM3 to GD1a significantly influenced tumor growth and angiogenesis in mouse brain tumors (8, 12). Specifically, reductions in the GM3/GD1a ratio enhanced angiogenesis in an experimental ependymoblastoma, whereas increases in the ratio decreased angiogenesis in the highly vascularized CT-2A astrocytoma. Zeng et al. (4) reported similar findings in F-11 neuroblastoma tumors with respect to the ratio of GM3 to GD3. It was not clear from these studies, however, whether it was the increase of GM3 or the reduction of GD1a or GD3 that altered angiogenesis in these tumors. We now show for the first time that GM3 binds to cultured HUVECs and reduces the phosphorylation of VEGFR-2 and Akt in the downstream signaling pathway, suggesting that GM3 by itself can suppress angiogenesis. These findings provide insight on the mechanism by which GM3, shed from tumor cells into the microenvironment, suppresses angiogenesis.

In contrast to the few reports on the antiangiogenic role of GM3, several reports have described the proangiogenic effects of complex gangliosides (4, 6, 12, 29, 42). Ladisch and coworkers (13, 42) recently found that GD1a and other complex gangliosides (GM1 and GD3) could sensitize fibroblasts or HUVECs to low concentrations of pro-

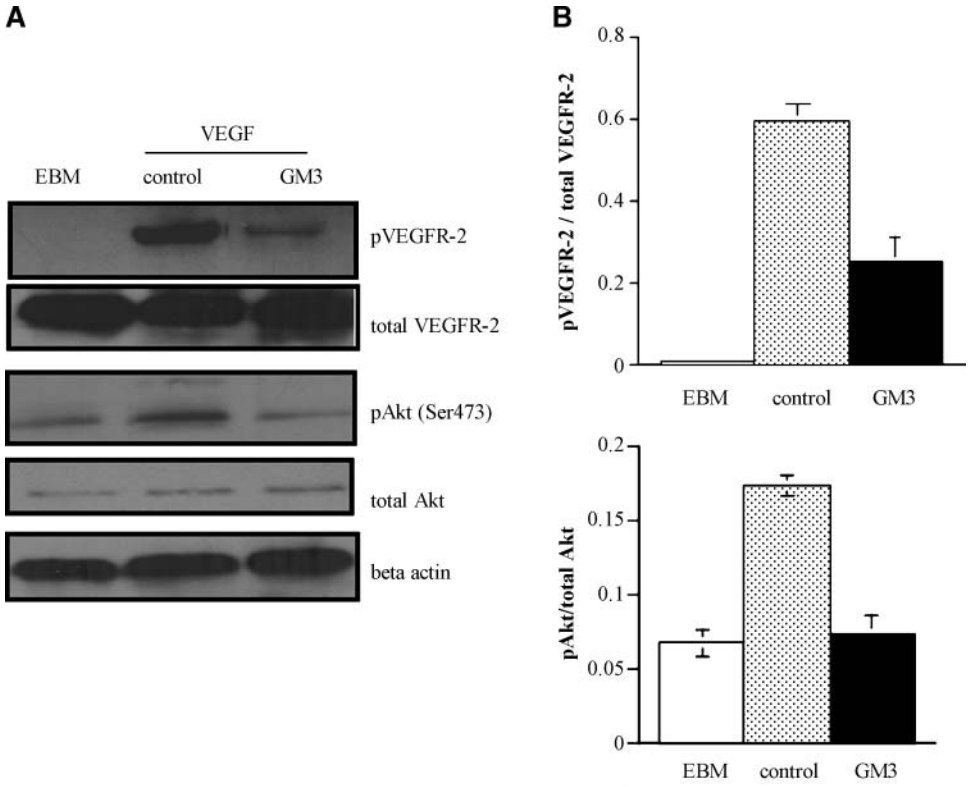

Fig. 5. GM3 inhibits vascular endothelial growth factor receptor 2 (VEGFR-2) and Akt phosphorylation in HUVECs. HUVECs were incubated with GM3 (80 nM) in EBM for 24 h and then stimulated with VEGF (100 ng/ml) for 5 min as described in Materials and Methods. Cell lysates were prepared and phosphorylation was measured. A: Detection of phosphorylated VEGFR-2, total VEGFR-2, pAkt, and total Akt by Western blot. B: Quantitation of VEGFR-2 and Akt phosphorylation over total. VEGFR-2 and Akt phosphorylation was significantly lower in GM3-treated HUVECs than in control HUVECs ($P < 0.001$). Values are expressed as means ± SEM (n = 3 independent experiments).

angiogenic growth factors. GM3, in contrast to the more complex gangliosides, did not sensitize VEGFR-2 phosphorylation (13). The effects of gangliosides on growth factor-induced angiogenesis are dependent on the type of microenvironment (in vitro or in vivo) and on the concentrations of both gangliosides and growth factors in the microenvironment (1, 13, 30, 31, 34, 43). In the present study, we found that 20–40 μM GM3 was effective at suppressing angiogenesis in the in vivo Matrigel assay and in the functional migration assay. Furthermore, GM3 was effective at suppressing HUVEC proliferation at both higher (20 μM) and lower (80 nM) concentrations, but it was most effective at suppressing VEGFR-2 phosphorylation and Akt signaling at the lower concentration. Our results also support the findings of Ziche et al. (31) in the corneal angiogenesis assay, showing that a GM3/growth factor concentration ratio of 1:1 could arrest the proangiogenic effects of angiogenesis promoters. Numerous factors can influence the incorporation and the rate of transfer of exogenously added gangliosides into cell membranes. Under some conditions, gangliosides added at lower concentrations could be more effective at entering membranes than when added at higher concentrations, as a result of micelle formation at higher concentrations (44). Collectively, these findings indicate that the influence of gangliosides on angiogenesis and cell signaling events is dependent to a large extent on the type of microenvironment and on the concentrations of gangliosides and growth factors in the microenvironment.

In response to VEGF, VEGFR-2 undergoes dimerization and tyrosine phosphorylation, which alter endothelial cell proliferation, chemotaxis, and survival (45). The localization of VEGFR-2 in HUVEC caveolae is involved in VEGF-induced downstream phosphorylation events (46). Receptors for epidermal growth factor and platelet-derived growth factor are also associated with "caveolar membranes" or glycolipid-enriched microdomains (47–49). GM3 modulates EGFR function in epithelial cells through carbohydrate-carbohydrate interaction with N-acetylglucosamine terminal residues of *N*-linked glycans located on the EGFR itself (34). It is also interesting that heparan sulfate proteoglycans, which contain *N*-linked oligosaccharides, can also enhance the response of endothelial VEGFR-2 to the VEGF165 isoform (50). Although little is known about the glycosylation of VEGFR-2, the VEGFR-2 coreceptor, neuropilin-1, is glycosylated (51). Moreover, GM3 reduces neuropilin-1 expression in CT-2A astrocytoma cells (8). It is possible, therefore, that GM3 could influence angiogenesis through interactions with carbohydrate residues on specific proteoglycans, on the

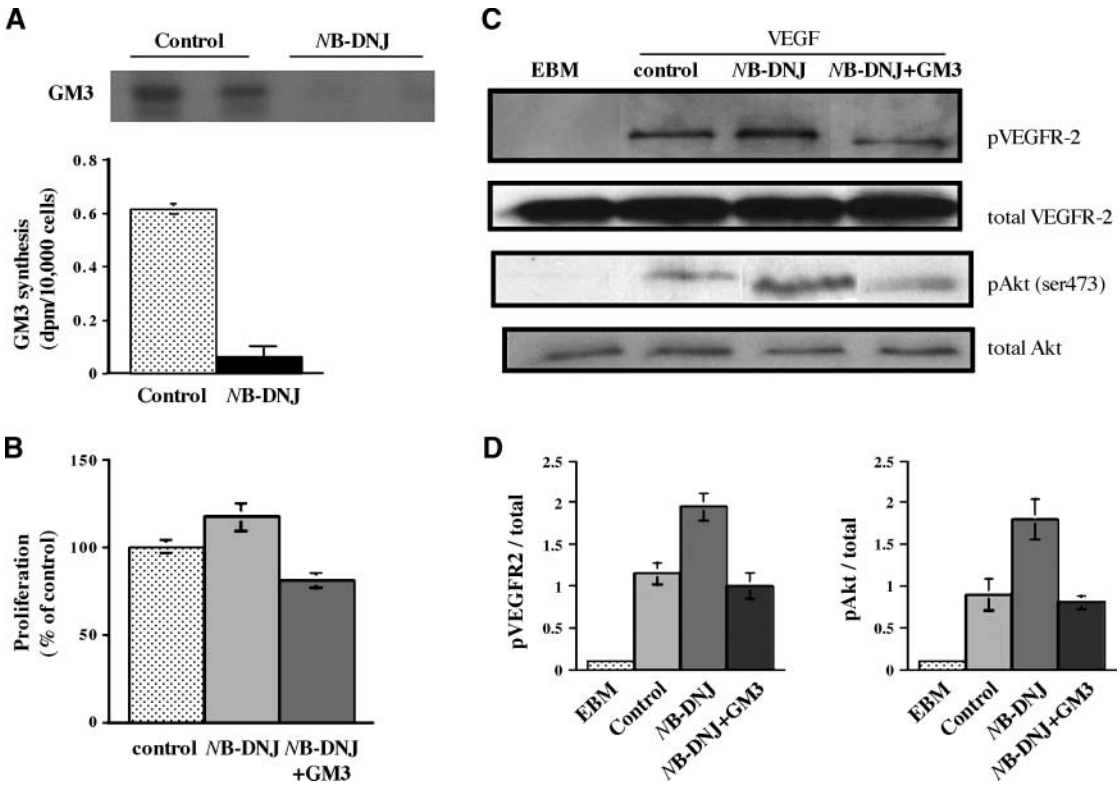

Fig. 6. Influence of *N*-butyldeoxynojirimycin (*N*B-DNJ) on HUVEC GM3 synthesis, proliferation, VEGFR-2 phosphorylation, and Akt phosphorylation. A: Top, high-performance thin-layer chromatography (HPTLC) analysis of GM3 biosynthesis. HUVECs were labeled with [^{14}C]galactose in endothelial growth medium for 72 h in the presence or absence of *N*B-DNJ (200 μM). Synthesized GM3 appeared as a double band on the HPTLC plate and was identified and quantified as described in Materials and Methods. Bottom, GM3 synthesis is expressed as dpm GM3/10^4 cells, and values are means ± interquartile ranges for two independent samples. B: MTT analysis of HUVEC proliferation. Cells were incubated with *N*B-DNJ in the presence or absence of GM3 (20 μM) for 48 h and then incubated for an additional 24 h in the presence of VEGF (4 ng/ml) as described in Materials and Methods. Proliferation is expressed as a percentage of control (nontreated) HUVECs. HUVEC proliferation was significantly faster in the *N*B-DNJ treatment group than in the control group at $P < 0.01$. HUVEC proliferation was significantly slower in the *N*B-DNJ + GM3 group than in the *N*B-DNJ group ($P < 0.001$). Values are expressed as means ± SEM (n = 3 independent experiments). C: Western blot analysis of phosphorylated VEGFR-2, total VEGFR-2, pAkt, and total Akt in HUVECs. HUVECs were incubated with *N*B-DNJ in the presence and absence of GM3 (80 nM) for 48 h and then stimulated with VEGF for 5 min as described in Materials and Methods. D: Quantitation of VEGFR-2 and Akt phosphorylation as a ratio of total phosphorylation in HUVECs. Other conditions are as described for Fig. 5 and in Materials and Methods. The ratio of VEGFR-2/total and Akt/total phosphorylation was significantly greater in *N*B-DNJ-treated cells than in untreated control cells ($P < 0.001$). The ratio of VEGFR-2/total and Akt/total phosphorylation was significantly lower in *N*B-DNJ + GM3-treated cells than untreated control cells ($P < 0.001$). Values are expressed as means ± SEM.

VEGFR-2, or on neuropilin-1 in a manner similar to that described for the EGFR. Further studies will be needed to explore these possibilities.

GM3 is the major ganglioside constituting ~90% of the whole ganglioside fraction in mammalian endothelial cells, including HUVECs (13, 40, 52). It was clear from our flow cytometry analysis using the anti-GM3 DH2 antibody that incubation of HUVECs with GM3 significantly increased GM3 levels in the HUVEC membrane in a manner similar to that seen in other cell types (14). However, DH2 did not bind to untreated HUVECs, illustrating the crypticity of endogenous GM3 to DH2. This is likely attributable to cell confluence, as described previously (53). Previous studies also showed that GM3 depletion in human fibroblasts enhances Akt/mitogen-activated protein kinase activity (54). Interestingly, *N*B-DNJ inhibition of GM3 synthesis also significantly increased HUVEC proliferation and the phosphorylation of VEGFR-2 and Akt. Our findings indicate that this is likely attributable to the specific action of GM3, because incubation of the *N*B-DNJ-treated HUVECs with GM3 reversed the effects of *N*B-DNJ. The high expression of GM3 in HUVECs could explain in part the nonproliferative or quiescent behavior of HUVECs in the absence of VEGF. Furthermore, it is possible that both endogenous and exogenous GM3 influence HUVEC proliferation through similar mechanisms.

Several previous studies showed that GM3 could inhibit tumor cell proliferation, migration, and metastasis through complex interactions with matrix molecules of the microenvironment and with the cell surface (14–17, 55). Choi et al. (56) showed that GM3 treatment inhibits the phosphatase and tensin homologue deleted on chromosome ten-mediated phosphoinositide-3 kinase/Akt/MDM2 survival signal in colon cancer cells. This signaling

pathway is also considered a target for the control of brain tumor angiogenesis (57). Together, these findings are consistent with our observations that GM3 inhibits endothelial cell migration and proliferation in vitro and in vivo through growth factor receptor inactivation and inhibition of Akt signaling. We suggest that GM3 may have broad therapeutic potential for targeting cell-signaling events in both tumor cells and tumor-associated host cells in relationship with tumor progression, metastasis, and angiogenesis. Preclinical trials are warranted to assess the therapeutic potential of GM3 as an antiangiogenic agent.

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