Ganglioside GM3 Suppresses the Pro-Angiogenic Effects of Vascular Endothelial Growth Factor and Ganglioside GD1a

Purna Mukherjee, Anthony C. Faber, Laura M Shelton, Rena C Baek, Thomas C. Chiles, and Thomas N. Seyfried

Department of Biology, Boston College, Chestnut Hill, MA 02467

Corresponding Author: Thomas N. Seyfried, Department of Biology, Boston College, Higgins hall, 140 Commonwealth avenue, Chestnut Hill, MA 02467, Tel. 617-552-3563; Fax. 617-552-2011; email: (thomas.seyfried@bc.edu)

Abbreviations used: HUVEC - human umbilical vein endothelial cells, VEGF- vascular endothelial endothelial growth factor, VEGFR-2- vascular endothelial growth factor 2, EGFR- endothelial growth factor receptor, PI-3kinase- Phosphoinositide-3 kinase, NB-DNJ- N-butyldeoxynojirimycin, NP-1- neuropilin1, EBM- endothelial basal media, EGM - endothelial growth medium, MTT- [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], FITC- fluorescein isothiocyanate, i.v.- intravenous, BSA - bovine serum albumin, bFGF - basic fibroblast growth factor, PBS - phosphate buffer saline, HBSS-Hank’s balanced salt solution, RIPA buffer-radioimmunoprecipitation buffer, EDTA - ethylene diamine tetra acetic acid, HPTLC- high performance thin layer chromatography, GluNAc - N-acetylglucosamine.
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 monosialo-ganglioside GM3 counteracts the pro-angiogenic effects of vascular endothelial growth factor (VEGF) and of the complex disialo-ganglioside GD1a. GM3 suppressed the action of VEGF and GD1a on the proliferation of human umbilical vein endothelial cells (HUVEC), and inhibited the migration of HUVEC towards VEGF as a chemoattractant. Enrichment of added GM3 in the HUVEC membrane also reduced phosphorylation of VEGF receptor 2 (VEGFR-2) and downstream Akt. Moreover, GM3 reduced the pro-angiogenic effects of GD1a and growth factors in the in vivo Matrigel plug assay. Inhibition of GM3 biosynthesis with the glucosyl transferase inhibitor, N-butyldexyranojirimycin (NB-DNJ), increased HUVEC proliferation and phosphorylation of VEGFR-2 and Akt. The effects of NB-DNJ on HUVEC were reversed with addition of GM3. We conclude that GM3 has anti-angiogenic action and may possess therapeutic potential for reducing tumor angiogenesis.
Introduction

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 monosialo ganglioside (NeuAca2->3Galb1->4Glcβ1->1' Ceramide), modulates cell adhesion, proliferation and differentiation (2, 5, 12, 14). The anti-proliferative and pro-apoptotic 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). 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 pro-angiogenic and anti-angiogenic factors contributes to the abnormal vasculature in tumors. The targeting of tumor endothelial cells is therefore considered important for managing tumor growth (1, 8, 26).
Vascular endothelial cells are responsive to a number of pro-angiogenic growth factors to include fibroblast growth factor beta (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 pro-angiogenic action of bFGF and VEGF (4, 13, 18, 29). GD1a enrichment of endothelial cells enhanced VEGF receptor dimerization, autophosphorylation, and enhanced 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 alone influence 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 have been well studied (32-34). Yoon et al showed that GM3 inhibits the EGFR tyrosine kinase through interactions with GluNAc residues on the glycan units of the receptor (34). 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 to include the VEGFR. VEGFR-2 or KDR is phosphorylated upon VEGF stimulation, which induces the PI-3 kinase-Akt pathway, resulting in enhanced endothelial proliferation and migration (35, 36).

In this study we examined the influence of exogenous addition of GM3 on the proliferation of HUVEC 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 HUVEC inhibits migration towards VEGF as a chemoattractant. Additionally we found that
GM3 reduced VEGFR-2 phosphorylation and downstream Akt signaling in HUVEC 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\textsubscript{165}), and bovine serum albumin (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\textsuperscript{TM} basement membrane matrix was purchased from BD Biosciences (Bedford, MA). CellTiter 96 Non-Radioactive cell proliferation 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, were sonicated, and aliquots stored at \(-20^\circ\text{C}\). 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 (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, Washington). Cy5-labeled anti-mouse antibody was purchased from Jackson ImmunoResearch (Westgrove, PA). N-butyldeoxyxojirimycin NB-DNJ (219.3, MW) was obtained as a gift from Oxford Glycosciences, Abigdon, UK. \textsuperscript{14}C galactose was obtained from Sigma (St. Louis, MO).
Cell Culture: Human umbilical vein endothelial cells (HUVEC), pooled, was purchased from Cambrex (Charles City, IA) and were maintained at 37°C in EGM2 in humidified air containing 5% CO₂. All experiments with HUVEC were conducted at passage 2-6.

Animals: BALB c/J-severe combined immunodeficient (SCID) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and were used for matrigel plug study. All animal experiments were carried out with the 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 Non-Radioactive cell proliferation assay following the manufacturer’s protocol. Briefly, 5 x 10³ HUVEC in EGM-2 were seeded in each well of a 96 well plate. After 24 hours, the HUVEC were washed and treated with 20 μM of GD1a and GM3 in EBM for 24 hours. After this incubation, the ganglioside containing medium was removed. The HUVEC were then washed with EBM and were stimulated with 4 ng/ml of VEGF in EBM for 24 hours. For the NB-DNJ study, cells were treated with 200μM of NB-DNJ in the absence and presence of GM3 for 48 hours and were then stimulated with 4 ng/ml of VEGF in EBM for additional 24 hours. Tetrazolium salt dye solution (15 μl) was added to each well and the plate was returned to the incubator for 4 hours. Stop solution (100μl) was then added to solubilize the metabolite and to lyse the HUVEC. The plate was incubated for 24 hours. The plate was shaken for 30 seconds after incubation and was read in a microplate reader SpectraMax, M5 (Molecular Device, CA) at 595 nm. This procedure was performed in triplicate.

Flow Cytometry: HUVEC were treated with 0, 0.5, 2.5, 5.0 μM of GM3 in EBM for 24 hours at 37°C in 5% CO₂ humidified condition. Confluent cells were detached with trypsin/EDTA and
were washed with PBS. HUVEC (2 x10^5) were incubated with anti-GM3 DH2 antibody (1:50) for 1 hr on ice, washed with PBS, incubated with 1:500 Cy5 anti-mouse secondary for 30 min on ice in darkness. Cells were then washed twice with PBS, resuspended, and were analyzed by FACSDiva software (Beckman coulter, CA). Control experiments included HUVEC treated with only secondary antibody.

Migration assay: Confluent HUVEC in T75 flasks were rinsed and treated with 20 μM of GM3 in EBM containing 0.1% BSA for a minimum of 4 -5 hours at 37°C in 5% CO2 humidified condition. A control flask of HUVEC 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 x 10^5 cells/ml) in 250 μl 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 of VEGF was then placed in the lower chamber according to the protocol. This VEGF concentration was recommended and necessary for an effective migration of HUVEC. Serum containing media was used as a positive control for these experiments. The plates were incubated for 24 hours under the humidified condition as above. HUVEC that migrated to the underside of the membrane were washed with Hanks balanced salt solution (HBSS) and the inserts were transferred in the companion wells containing calcein (4-5 μg/ml) solution. HUVEC were incubated in calcein for 1 hr, were washed, and the fluorescently labeled cells were photographed using a fluorescence microscope. Fluorescence was measured using a SpectraMax, M5 (Molecular Device, CA).

In vivo Matrigel plug assay: Angiogenesis was analyzed using the in vivo Matrigel plug assay as we previously described (12, 37). Briefly, Matrigel (200 μl) and EBM (100 μl) containing GD1a and/or GM3 (at either 20 μM 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/10g of body weight) and were then injected with Matrigel with or without gangliosides subcutaneously (s.c.) in the dorsal midline using a pre-chilled tuberculin syringe (27-gauge needle). Seven days after implantation and 30 minutes before necropsy, mice were injected i.v. with 100 ul 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 previously described and vascularity was photographed (37). Lectin-FITC was extracted from plugs by homogenizing in 500 ul of radioimmunoprecipitation (RIPA) buffer. The homogenate was centrifuged at 1,000 x g and the fluorescence was measured at 490 nm using a SpectraMax, M5 as above.

**Ganglioside biosynthesis in HUVEC:** Synthesized gangliosides were isolated from control and NB-DNJ treated HUVEC as previously described (2). Briefly, HUVEC were grown for 72 h in EGM-2 containing 5 μCi of 14C-galactose and 200 μM NB-DNJ. The radiolabeled cells were removed from the flask with a cell scraper in PBS and were collected as a pellet. Prior to ganglioside isolation, unlabelled mouse ependymoblastoma gangliosides, containing GM3, were added as unlabeled carrier (2). Total lipids were extracted from the radiolabeled cells in chloroform: methanol (2: 1 by vol), and dH2O was added (20% by vol). The radiolabeled gangliosides were separated from the total lipids by Folch partitioning into an upper aqueous phase as we described (2). The upper phase gangliosides were dried under nitrogen, were resuspended in chloroform: methanol (2:1 by vol), and were spotted on a high-performance thin-layer chromatography (HPTLC) plate. The concentration of radiolabeled gangliosides spotted on the HPTLC was determined by scintillation counting. The amount of disintegrations per minute (dpm) equivalent to 10,000 cells was spotted/lane on the HPTLC. The HPTLC plate was developed in one ascending elution with C:M:H2O (50:45:10 by v/v) containing 0.02 % CaCl2-dH2O. 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 previously described (39).

**Immunoblot analysis of HUVEC lysates:** HUVEC were seeded in EGM-2 at $1 \times 10^5$ cells/well in 6 well plates. When confluent, the HUVEC were washed with EBM and were then incubated with 80 nM (100 ng/ml) of GM3 in serum free EBM for 24 hr. The HUVEC were then washed and stimulated with 100 ng/ml of VEGF in EBM for 5 minutes at 37°C. For the NB-DNJ study, cells were treated with 200 μM of NB-DNJ in the presence and absence of GM3 for 48 hours and then stimulated with VEGF for 5 minutes at 37°C. The HUVEC 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$_2$EDTA, 1 mM EGTA, 1% Triton, 2.5 mM NaPP, 1 mM -glycerophosphate, 1 mM Na$_3$VO, 1 μg/ml leupeptin, and 1 mM phenylmethylsufonyl fluoride. The lysates were centrifuged at 12, 000 x g for 20 min at 4°C. Supernatants were collected, and protein concentrations were estimated using the BioRad DC protein assay (BioRad, CA). Approximately 50-100 μg total protein from each sample were loaded onto a 12% sodium dodecyl polyacrylamide gel (Invitrogen, CA) and were analyzed by electrophoresis. Proteins were transferred to a PVDF immobilon TM-P membrane (Millipore, MA). The membrane was blocked in 5% non-fat powdered milk in Tris-buffered saline with Tween 20 (pH 7.6) for 1 hr at room temperature. Blots were then probed with primary antibody against phosphorylated VEGFR-2 (Tyr1175) and were reprobed with that of against mouse monoclonal Akt (Ser473) 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 mean ± standard error. Significance of differences between groups was evaluated with one-way analysis of variance.
Results

GM3 suppresses VEGF and GD1α induced HUVEC proliferation: To examine the influence of GM3 on angiogenesis, we studied the proliferation of HUVEC in culture. We evaluated the influence of GM3 on HUVEC proliferation either alone or in combination with GD1α in the presence of VEGF stimulation (Fig. 1). We found that GD1α (20 μM) significantly increased the VEGF induced HUVEC proliferation. In contrast, GM3 (20 μM) significantly reduced VEGF or GD1α induced HUVEC proliferation by about 50%. A control study was done to confirm that GM3 did not alter uptake of MTT into HUVEC. The inhibitory effect of GM3 on HUVEC proliferation was also observed at a lower GM3 concentration of 80 nM.

Detection of GM3 on HUVEC membrane: To determine whether the suppressive effects of GM3 on HUVEC proliferation were associated with expression of GM3 on the membrane, we added different amounts of GM3 to the incubation medium and analyzed the HUVEC using flow cytometry with DH2 anti-GM3 antibody (Fig. 2). No DH2 binding occurred in the untreated HUVEC indicating that DH2 does not detect the endogenous GM3 epitope. Incubation of HUVEC with GM3 caused a dose-dependent increase in DH2 binding. Since GM3 pre-treatment 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 GD1α. These findings indicate that GM3 modulates the response of HUVEC to the pro-angiogenic effects of VEGF and GD1α.

GM3 reduces HUVEC migration towards VEGF as a chemoattractant: We also examined whether GM3 could influence HUVEC migration towards VEGF as a chemoattractant. HUVEC, 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 HUVEC in this assay. Migration was significantly less for GM3-treated HUVEC than for non-treated HUVEC. 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 activation and infiltration of host stromal cells, which include monocytes, macrophages, and endothelial cell precursors (12, 27, 37). Matrigel contains growth factors, which induce infiltration of blood vessels as seen in the control (C) 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 angiogenic 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 (AU) was also significantly lower in the in the GM3 + GD1a-containing plugs than in the plugs containing GD1a alone (Fig. 4C). These results indicate that GM3 suppressed the pro-angiogenic effects of GD1a in an in vivo environment.

**GM3 inhibits VEGFR-2 and Akt phosphorylation:** VEGF stimulates HUVEC proliferation through downstream PI-3 kinase-Akt signaling by inducing phosphorylation of the VEGF receptor 2 (VEGFR-2, KDR, Flk-1) (35, 36). We used a 100 ng/ml VEGF concentration for
these experiments to insure robust VEGFR-2 phosphorylation (ratio of pVEGFR-2 to total). VEGFR-2 phosphorylation was 6-fold greater and Akt phosphorylation at Ser473 (ratio of pAkt to total) was about 2-fold greater in the presence than in the absence of VEGF after 5 min of stimulation in EBM (Fig. 5A-C). No VEGF phosphorylation was observed for HUVEC grown in the basal medium (EBM), which contained no serum or growth factors. In contrast, phosphorylation of VEGFR-2 and Akt was significantly reduced by 60% and 75%, respectively, in HUVEC incubated with GM3 (80 nM or 100 ng/ml) for 24 hr prior to VEGF stimulation (Fig. 5A-C). 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 in 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 VEGF-induced phosphorylation of VEGFR-2 and downstream Akt in cultured HUVEC.

Influence of NBDNJ on GM3 synthesis, HUVEC proliferation, and VEGFR-2 and Akt phosphorylation: NB-DNJ is a non-toxic competitive inhibitor of the ceramide-specific glucosyltransferase that catalyzes the first step in ganglioside biosynthesis (23). Treatment of HUVEC with NB-DNJ (200 μM) reduced GM3 synthesis as revealed by the incorporation of 14C labeled galactose into newly synthesized GM3 (Fig. 6A). The ganglioside pattern of HUVEC was similar to that previously described with GM3 as the predominant species (13, 40). NB-DNJ treatment significantly increased VEGF induced proliferation over untreated control cells (Fig. 6B). In parallel, NB-DNJ significantly increased the VEGFR-2 and Akt phosphorylation (Fig 6C & D). Since NB-DNJ reduces not only GM3 synthesis, but also that of most other glycosphingolipids in HUVEC, we evaluated the effects of exogenous addition of GM3 in NB-DNJ treated cells. We found that GM3 addition reversed the NB-DNJ-induced increases in
HUVEC proliferation and VEGFR-2 and Akt phosphorylation (Fig. 6 B-D). These findings further 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 co-workers first showed that changes in the relative concentrations of GM3 to complex gangliosides could stimulate or suppress angiogenesis in vitro or in vivo (5, 6, 30, 41). Using the rabbit cornea model of angiogenesis, they found that GM3 reduced the growth and motility of microvascular endothelium while repressing the pro-angiogenic effects of prostaglandin E1 and basic fibroblast growth factor (b-FGF) (6, 30, 31). Moreover, ganglioside GD3 and other complex gangliosides (GM1 and GD1a) reduced the anti-angiogenic 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 elevations in the ratio decreased angiogenesis in the highly vascularized CT-2A astrocytoma. Zeng et al reported similar findings in F-11 neuroblastoma tumors with respect to the ratio of GM3/GD3 (4). It was not clear from these studies, however, whether it was the elevation of GM3 or the reductions of GD1a or GD3 that altered angiogenesis in these tumors. We now show for the first time that GM3 binds to cultured HUVEC and reduces 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 anti-angiogenic role of GM3, several reports have described the pro-angiogenic effects of complex gangliosides (4, 6, 12, 29, 42). Ladisch and co-workers recently found that GD1a and other complex gangliosides (GM1 and GD3) could sensitize fibroblasts or HUVEC to low concentrations of pro-angiogenic growth factors (13, 42). 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 of GM3 was effective in suppressing angiogenesis in the in vivo Matrigel assay and in the functional migration assay. Furthermore, GM3 was effective in suppressing HUVEC proliferation at either higher (20 μM) or lower (80 nM) concentrations, but was most effective in suppressing VEGFR-2 phosphorylation and Akt signaling at the lower concentration. Our results also support the findings of Ziche et al in the corneal angiogenesis assay showing that a GM3: growth factor concentration of 1:1 could arrest the pro-angiogenic effects of angiogenesis promoters (31). 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 in entering membranes than when added at higher concentrations due to 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 that alters 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 (GEM) (47-49). GM3 modulates EGFR function in epithelial cells through carbohydrate-carbohydrate interaction with GlcNAc 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 glycosylation of VEGFR-2, the VEGFR-2 co-receptor, neuropilin-1 (NP1), is glycosylated (51). Moreover, GM3 reduces NP1 expression in CT-2A astrocytoma cells (8). It is therefore possible that GM3 could influence angiogenesis through interactions with carbohydrate residues on specific proteoglycans, on the VEGFR-2, or on NP1 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 about 90% of the whole ganglioside fraction in mammalian endothelial cells including HUVEC (13, 40, 52). It was clear from our flow cytometry analysis using the anti-GM3 DH2 antibody that incubation of HUVEC 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 HUVEC illustrating the crypticity of endogenous GM3 to DH2. This is likely due to cell confluency as previously described (53). Previous studies also showed that GM3 depletion in human fibroblasts enhances Akt/mitogen-activated protein kinase activity (54). Interestingly, NB-DNJ-inhibition of GM3 synthesis also significantly increased HUVEC proliferation and phosphorylation of VEGFR-2 and Akt. Our findings indicate that this is likely due the specific action of GM3 since incubation of the NB-DNJ-treated HUVEC with GM3 reversed the effects of NB-DNJ. The high expression of GM3 in HUVEC could explain in part the non-proliferative or quiescent behavior of HUVEC 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 showed that GM3 treatment inhibits the PTEN mediated PI-3kinase/Akt/MDM2 survival signal in colon cancer cells (56). This signaling pathway is also considered a target for controlling brain tumor angiogenesis (57). Viewed 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 in tumor-associated host cells in relationship to tumor progression, metastasis, and angiogenesis. Preclinical trials are warranted to assess the therapeutic potential of GM3 as an anti-angiogenic agent.

Acknowledgements: We thank Hong-Wei Bai, Daniel Kirschner, Weihua Zhou, and Jeremy Marsh for technical assistance. The research was supported from NIH grants (NS 055195 and CA102135), and the Boston College research expense fund.
Legends to Figures

**Figure 1.** GM3 inhibits VEGF- and GD1a-induced HUVEC proliferation. HUVEC were pretreated for 24 hrs in EBM in the absence (control) or in the presence of GD1a (20 μM) and/or GM3 (20 μM), and were then incubated for an additional 24 hr in the presence of VEGF (4 ng/ml). The MTT assay was used to measure HUVEC proliferation as described in methods. Proliferation is expressed as % 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).

**Figure 2.** Detection of added GM3 on HUVEC membrane. HUVEC were incubated with GM3 for 24 hr in EBM. Flow cytometry using anti-GM3 DH2 and Cy5 labeled anti-mouse IgG was used to evaluate GM3 on the membrane of HUVEC. 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. The experiments were done in triplicate.

**Figure 3.** GM3 inhibits VEGF-induced HUVEC migration. A) Calcein-labeled HUVEC were photographed under fluorescence microscopy (200x), and B) the amount of fluorescence incorporated was measured. The HUVEC 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 lower chamber. After 24 hours HUVEC cells that migrated through the filter were stained with calcein. VEGF significantly increased HUVEC migration compared
to 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, 3 wells/group/experiment).

Figure 4. GM3 inhibits the pro-angiogenic effects of GD1α in the in vivo Matrigel plug assay. Matrigel alone (control) or containing GD1α or GD1α with GM3 was injected subcutaneously (s.c.) in SCID mice as described in methods. A) Plugs were photographed (12.5 x) on day 7 after Matrigel injection to evaluate blood vessels. B) The plug vasculature was also evaluated under fluorescence microscopy (200 x) in mice injected i.v. 30 min before necropsy with FITC–isolectin-B4. C) The amount of fluorescence in the plugs was measured by fluorimetry as described in methods. Six mice/group were evaluated and the values are expressed means ± SEM. Vascularization as detected by fluorescence was significantly less in the plugs with GD1α and GM3 than in the plugs with only GD1α at p < 0.01.

Figure 5. GM3 inhibits VEGFR-2 and Akt phosphorylation in HUVEC. HUVEC were incubated with GM3 (80 nM ) in EBM for 24 hours and were then stimulated with VEGF (100 ng/ml) for 5 minutes as in 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 HUVEC than in control HUVEC (p < 0.001). Values are expressed as means ± SEM (n = 3 independent experiments).
Figure 6. Influence of NB-DNJ on HUVEC GM3 synthesis, proliferation, VEGFR-2 phosphorylation, and Akt phosphorylation. A, top) HPTLC analysis of GM3 biosynthesis. HUVEC were labeled with $^{14}$C galactose in EGM-2 for 72 hrs in the presence or absence of NB-DNJ (200 μM). Synthesized GM3 appeared as a double band on the HPTLC plate and was identified and quantified as described in Methods. A, bottom) GM3 synthesis is expressed as dpm of GM3/10⁴ cells and values are the mean ± interquartile range for 2 independent samples. B) MTT analysis of HUVEC proliferation. Cells were incubated with NB-DNJ in the presence or absence of GM3 (20 μM) for 48 hours and were then incubated for an additional 24 hours in the presence of VEGF (4 ng/ml) as described in Methods. Proliferation is expressed as % of control (non-treated HUVEC). HUVEC proliferation was significantly faster in the NB-DNJ treatment group than in the control group at p < 0.01. HUVEC proliferation was significantly slower in the NB-DNJ + GM3 group than in the NB-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 HUVEC. HUVEC were incubated with NB-DNJ in the presence and absence of GM3 (80 nM) for 48 hrs and were then stimulated with VEGF for 5 minutes as in Methods. D) Quantitation of VEGFR-2 and Akt phosphorylation as a ratio of total phosphorylation in HUVEC. Other conditions as described in Figure 5 and in Methods. The ratio of VEGFR-2/total and Akt/total phosphorylation was significantly greater in NB-DNJ treated cells than untreated control cells (p < 0.001), respectively. The ratio of VEGFR-2/total and Akt/total phosphorylation was significantly lower in NB-DNJ + GM3 treated cells than untreated control cells (p < 0.001).
References


Figure 1

Proliferation (% of control)

- Control
- GD1a
- GM3
- GD1a + GM3
Figure 3
Figure 4
Figure 5

A) 

<table>
<thead>
<tr>
<th></th>
<th>EBM</th>
<th>control</th>
<th>GM3</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pVEGFR-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total VEGFR-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAkt (Ser473)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total Akt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>beta actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B) 

- **pVEGFR-2 / total VEGFR-2**
- **pAkt / total Akt**

Bar graphs comparing EBM, control, and GM3 conditions for pVEGFR-2 and pAkt levels.
Figure 6