

# Membrane Ganglioside Enrichment Lowers the Threshold for Vascular Endothelial Cell Angiogenic Signaling

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## Abstract

**Malignant tumor progression depends on angiogenesis, requiring vascular endothelial cell migration, and proliferation, triggered by tumor-derived vascular endothelial cell growth factor (VEGF). We show that gangliosides, which are actively shed by tumor cells and bind to normal cells in the tumor microenvironment, have the potential to sensitize vascular endothelial cells to respond to subthreshold levels of VEGF: Ganglioside enrichment of human umbilical vein vascular endothelial cells (HUVEC) caused very low, normally barely stimulatory, VEGF concentrations to trigger robust VEGF receptor dimerization and autophosphorylation, as well as activation of downstream signaling pathways, and cell proliferation and migration. Thus, by dramatically lowering the threshold for growth factor activation of contiguous normal stromal cells, shed tumor gangliosides may promote tumor progression by causing these normal cells to become increasingly autonomous from growth factor requirements by a process that we term tumor-induced progression of the microenvironment.** (Cancer Res 2006; 66(21): 10408-14)

## Introduction

Malignant transformation is a multistep process consisting of both genetic and epigenetic factors. Collectively, these changes lead to increasing autonomy of malignant tumor cells from host control, a process originally recognized by Foulds (1). Of particular importance is the increasing independence of the tumor cell from the multiple normal host control mechanisms for regulating cell growth, including loss of normal cell cycle checkpoint controls, resistance to apoptosis, increased expression of autocrine growth factors or receptors, and activating mutations/truncations of specific growth factor receptors (2). In addition to these progression-associated phenotypic changes within tumor cells, there is increasing awareness that alterations in the tumor microenvironment are also important for malignancy. Changes in the cellular composition, in growth factor production, and in the integrity/synthesis of extracellular matrix components are among the described tumor microenvironmental changes associated with malignant progression (3, 4). Here, we show that increasingly autonomous tumor cells can also cause normal cells within this microenvironment to become more autonomous.

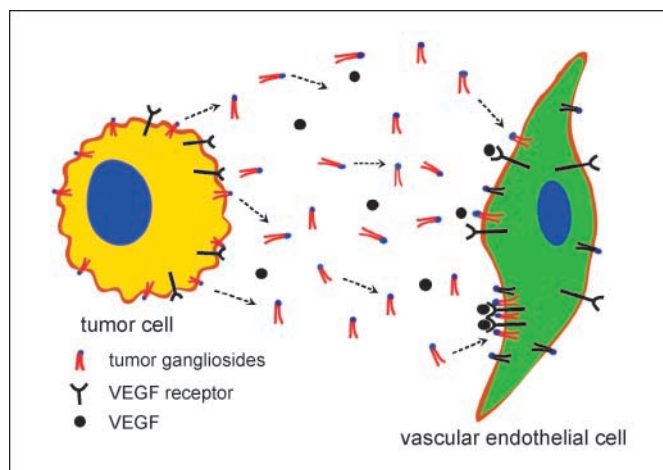
Taking the example of angiogenesis (5), tumors release signals to develop the adequate vascular system needed to expand beyond a

certain minimal size (6, 7). Soluble tumor-derived angiogenic/growth factors, such as vascular endothelial cell growth factor (VEGF), facilitate this, in part by stimulating the migration and proliferation of normal vascular endothelial cells. Because the production of these growth factors by tumor cells is rate limiting for the development of new vessels to supply the expanding neoplasm (8), the extent of the angiogenic response by endothelial cells in the tumor microenvironment will depend on the magnitude of growth factor release by the tumor cells, which may fall short of achieving optimal VEGF concentrations in the microenvironment. Furthermore, tumors that are initially expanding may not be able to produce adequate growth factor to stimulate this proliferation/activation, whereas VEGF-dependent signaling in general may be subject to modulation (positive or negative) by other molecules that interact with the cell (9). Such interactions in the tumor microenvironment may be critical in supporting angiogenesis associated with malignant progression. We propose and provide evidence for a new concept, that one such critical interaction is tumor-induced reduction of the dependence of normal (stromal) cells upon the growth factors normally required for angiogenesis. We term this process tumor-induced progression of the microenvironment, in which the tumor cells themselves cause surrounding normal, nontransformed cells to respond to very low levels of VEGF and thereby escape normal regulatory control mechanisms. Specifically, we implicate the sialic acid-containing cell surface glycosphingolipids, gangliosides, in this process. Gangliosides are shed in substantial quantities by many different tumor cells into the tumor microenvironment (10, 11). They are taken up and bind efficiently to host cells that are found in this microenvironment, such as fibroblasts (12), antigen-presenting cells (13) and, as shown here, vascular endothelial cells. *In vivo*, ganglioside shedding can have an important permissive effect on tumor progression (11). For example, addition of purified tumor gangliosides to a ganglioside-deficient, poorly tumorigenic AKR lymphoma cell line in a syngeneic mouse model markedly increased their tumorigenicity (11); progression of human neuroblastoma is highly correlated with shedding of gangliosides by this tumor (14); and certain gangliosides (G<sub>M1</sub>, G<sub>T1b</sub>) enhance angiogenesis in the rabbit cornea model (15). Conversely, pharmacologic inhibition of ganglioside synthesis and shedding has resulted in markedly reduced experimental tumor formation (16) or progression (17) of melanoma.

This and other prior evidence led us to explore the possibility that one mechanism by which tumor-derived gangliosides enhance malignant progression is to enhance the angiogenic response. We hypothesized that tumor cell ganglioside shedding followed by uptake of tumor-shed gangliosides by normal vascular endothelial cells (Fig. 1) would lower the threshold and increase the efficiency of their response to angiogenic factors, such as VEGF, so that very low VEGF concentrations that normally do not trigger VEGF receptor (VEGFR) activation and downstream signaling could do so.

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**Figure 1.** Proposed role of tumor gangliosides in the tumor microenvironment. Gangliosides are actively synthesized and released, by shedding from tumor cells, and bind to and functionally alter normal cells in the tumor microenvironment.

This lowered response threshold would make vascular endothelial cells less dependent on VEGF and its concentration in the microenvironment, and consequently more autonomous from host control mechanisms. Here, we provide evidence for this concept by demonstrating that the enrichment of HUVEC membranes with purified GD1a ganglioside result in enhanced activation, signaling, and migration and proliferation of these cells. We suggest that this phenotypic change in endothelial cells induced by shed tumor gangliosides is part of a larger process, tumor-induced progression of the microenvironment, exemplified by enhanced responsiveness of ganglioside-enriched stromal cells that leads to decreased requirements for high levels of growth factors.

## Materials and Methods

**Reagents.** Highly purified  $G_{D1a}$ ,  $G_{M3}$ ,  $G_{M1}$ ,  $G_{D3}$ , and lactosylceramide (bovine brain) were purchased from Matreya (Pleasant Gap, PA). Recombinant human VEGF<sub>165</sub> was purchased from Biosource (Camarillo, CA). Gangliosides and VEGF were dissolved in supplement-free endothelial basal medium (EBM, BioWhittaker, Inc., Walkersville, MD) and stored at  $-20^{\circ}\text{C}$  until use. Anti-VEGF-R2 antibody (N-931, rabbit polyclonal IgG) and phospho-Tyr antibody (PY99, mouse monoclonal IgG2b) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-protein kinase C $\epsilon$  (PKC $\epsilon$ ; rabbit polyclonal IgG), anti-phospho-PKC $\epsilon$  (Ser<sup>719</sup>, rabbit polyclonal IgG), anti-phosphatidylinositol 3-kinase (PI3K) p85 (rabbit antiserum), anti-mouse IgG [horseradish peroxidase (HRP) conjugate], and anti-rabbit IgG (HRP conjugate) were obtained from Upstate Biotechnology (Lake Placid, NY). Phosphorylated P44/42 mitogen-activated protein kinase (MAPK; Thr<sup>202</sup>/Tyr<sup>204</sup>) antibody (rabbit polyclonal IgG) and P44/42 MAPK antibody (rabbit polyclonal IgG) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). [<sup>125</sup>I]VEGF (human recombinant, 1,000-2,500 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ), tritiated thymidine (methyl-<sup>3</sup>H-thymidine 6.70 Ci/mmol) from New England Nuclear (Boston, MA), and bis(sulfosuccinimidylpropionate)suberate, BS<sup>3</sup>, from Pierce (Rockford, IL).

**Ganglioside purification.** Total lipid extracts of cell pellets were obtained by extracting the lyophilized pellets twice with chloroform/methanol (1:1, by volume) for 18 hours at  $4^{\circ}\text{C}$ . Gangliosides were isolated by partitioning the total lipid extract in di-isopropyl ether/1-butanol/17 mmol/L aqueous NaCl (6:4:5, by volume) as described (18). Gangliosides, in the lower aqueous phase, were further purified by Sephadex G-50 gel filtration. High-performance TLC (HPTLC) analysis of gangliosides used  $10 \times 20$  cm

precoated silica gel 60 HPTLC plates (Merck, Darmstadt, Germany), which were developed in chloroform/methanol/0.25% aqueous  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (60:40:9, by volume) to separate gangliosides, stained as purple bands with resorcinol reagent, and quantified by densitometry compared with brain ganglioside standards.

**Cell culture.** Human umbilical vein endothelial cells (HUVEC), purchased from Clonetics (BioWhittaker), were cultured in endothelial cell growth medium (EGM) in humidified air containing 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . EGM consists of EBM plus 2% fetal bovine serum (FBS), 10 ng/mL human recombinant epidermal growth factor (hEGF), 1  $\mu\text{g}/\text{mL}$  hydrocortisone, 1  $\mu\text{g}/\text{mL}$  gentamicin, 1  $\mu\text{g}/\text{mL}$  amphotericin-B, and 2 mL bovine brain extract. The culture medium was changed every 2 days, cell viability was determined by trypan blue dye exclusion, and cell passages 3 to 7 were used in all studies.

**HUVEC proliferation assay.** HUVECs were seeded in 96-well culture plates (Costar, Corning, NY) at  $0.5 \times 10^4$  to  $4 \times 10^4$  cells in 200  $\mu\text{L}$  EGM per well and cultured for 24 hours, and then starved in EBM  $\pm 5$  to 10  $\mu\text{mol}/\text{L}$  GD1a for 6 hours. Then, as indicated in the figure legends, they were exposed for 3 or 24 hours to 0 to 2 ng VEGF/mL, and to 0.5  $\mu\text{Ci}$  [<sup>3</sup>H]thymidine per well during the last 3 hours. Thymidine uptake was quantified by  $\beta$ -scintillation counting.

**VEGF stimulation and preparation of cell lysates.** HUVEC cells were seeded in EGM at  $2 \times 10^5$  per dish ( $100 \times 20$  mm; area = 55  $\text{cm}^2$ ) or  $1.5 \times 10^5$  per well (area = 9.4  $\text{cm}^2$ ). When confluent, the cells were incubated with gangliosides in EBM for 6 hours, washed twice with EBM, and exposed to 0.1 to 5 ng/mL VEGF in EBM for 5 minutes at  $37^{\circ}\text{C}$ . Then, cells were immediately washed again twice with ice-cold PBS and lysed with lysis buffer (1 mL per 100 mm dish or 300  $\mu\text{L}$  per well). Lysis buffer contains 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium PPI, 1 mmol/L  $\beta$ -glycerolphosphate, 1 mmol/L  $\text{Na}_2\text{VO}_4$ , 1  $\mu\text{g}/\text{mL}$  leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. The lysate was transferred to microcentrifuge tubes, sonicated briefly on ice, and centrifuged at  $10,000 \times g$  for 10 minutes at  $4^{\circ}\text{C}$ . Proteins were quantified by the Lowry method using bovine albumin as the standard.

**Immunoprecipitation and immunoblot analysis of HUVEC lysates.** In the VEGFR-2 autophosphorylation and PI3K phosphorylation assays, for immunoprecipitation, 100  $\mu\text{g}$  of total protein were mixed with 100  $\mu\text{L}$  washed Protein G-Sepharose agarose bead slurry (50  $\mu\text{L}$  packed beads) and stirred for 2 hours at  $4^{\circ}\text{C}$  to preclear nonspecific binding. After microcentrifugation at  $14,000 \times g$  for 5 seconds, the supernatant was transferred to a new microcentrifuge tube and mixed with 4  $\mu\text{g}$  anti-VEGFR-2 (Flk-1) antibody (N-931, rabbit polyclonal IgG) for VEGFR-2, and with 10  $\mu\text{L}$  anti-PI3K p85 (rabbit antiserum) for PI3K. The mixture was incubated at  $4^{\circ}\text{C}$  overnight with stirring. The immune complexes were recovered by adding 50  $\mu\text{L}$  washed Protein G-Sepharose agarose bead slurry and gently rocking the mixture for 2 hours at  $4^{\circ}\text{C}$ . After microcentrifugation at  $14,000 \times g$  for 5 seconds and removal of the supernatant, the beads were washed thrice with ice-cold lysis buffer, resuspended in 20  $\mu\text{L}$  3 $\times$  SDS sample buffer, boiled for 5 minutes, and recentrifuged. The supernatants were loaded onto 7.5% (for VEGFR-2) or 10% (for PI3K) SDS-polyacrylamide gels. Autophosphorylation of VEGFR-2 and phosphorylated PI3K was detected by Western blot analysis using an antiphosphotyrosine antibody, p-Tyr (PY99, 1:1,000), with gentle agitation overnight at  $4^{\circ}\text{C}$ , and HRP-conjugated second antibody (1:2,000) for 1 hour at room temperature. Total VEGFR and PI3K were detected by anti-VEGFR antibody and anti-PI3K p85 antibody (1:1,000) under optimal conditions. VEGFR autophosphorylation plateaued between 4 and 16 ng/mL VEGF, and peaked within 5 to 10 minutes of VEGF exposure. Ten micrograms cell lysate were used to quantify phosphorylated MAPK and 20  $\mu\text{g}$  for phospho-PKC $\epsilon$  and loaded onto a 12% SDS-polyacrylamide gel. Phosphorylated P44/42 MAPK antibody (1:1,000) was used to detect phosphorylated MAPK, and anti-MAPK antibody (1:1,000) was used to detect total MAPK. PKC $\epsilon$  phosphorylation was measured with anti-phospho-PKC $\epsilon$  (Ser<sup>719</sup>) antibody (1:1,000) and total PKC $\epsilon$  with anti-PKC $\epsilon$  antibody (1:1,000). HRP-conjugated second antibody (1:2,000) was used as above.

**[<sup>125</sup>I]VEGF binding assay.** Binding of [<sup>125</sup>I]VEGF to whole cells was assessed using modifications of methods for Scatchard analysis. Three

parallel sets of  $1 \times 10^4$  HUVEC cells were grown in 96-well plates in EGM for 24 hours, washed twice with EBM twice, and incubated with 0 to 20  $\mu\text{mol/L}$   $\text{G}_{\text{D1a}}$  in EBM for 6 hours. Then, one set of cells was washed and incubated at  $4^\circ\text{C}$  for 2 hours with 0.063 to 50 ng/mL  $^{125}\text{I}$ -labeled human recombinant VEGF in EBM to determine total binding. The second set was treated as above, but with the addition of 300 ng/mL unlabeled VEGF to test for nonspecific binding. After washing, 50  $\mu\text{L}$  lysis buffer were added to each well on ice for 30 minutes, and then 20  $\mu\text{L}$  lysate were used to quantify [ $^{125}\text{I}$ ]VEGF binding. Specific binding was calculated by subtraction of nonspecific binding of [ $^{125}\text{I}$ ]VEGF from total binding.

**VEGFR dimerization assay.** HUVECs were cultured in  $100 \times 20$  mm culture dishes to preconfluence, washed twice with 5 mL serum-free EBM, preincubated with  $\text{G}_{\text{D1a}}$  in EBM for 6 hours, and exposed to VEGF (5 ng/mL) in EBM for 5 minutes. The cells were then washed twice with ice-cold PBS, incubated with 3 mL PBS containing 1 mg/mL  $\text{BS}^3$  on ice for 30 minutes, washed twice with ice cold PBS, and lysed in 1 mL lysis buffer for 20 minutes on ice. Total VEGFR-2 (Flk-1) was immunoprecipitated with anti-VEGFR-2 antibody (Flk-1; 1:1,000). About 500  $\mu\text{g}$  lysate protein was loaded onto a 7.5% SDS-PAGE gel. Dimerization of VEGFR-2 was detected by Western blot using an anti-VEGFR-2 (Flk-1) antibody.

**Scratch wound assay.** Early passage HUVEC cells ( $5 \times 10^4$ ), seeded in EGM in collagen I-coated six-well plates, were cultured to confluence. The cells were then starved in serum-free EBM with or without 20  $\mu\text{mol/L}$   $\text{G}_{\text{D1a}}$  for 6 hours, washed with fresh EBM, and the cell monolayers were scratched with a 1,000  $\mu\text{L}$  pipette tip and incubated for 18 hours with or without 0.1 ng/mL VEGF in EGM with a reduced (1%) FBS concentration and not containing EGF. The scratched area was photographed at the beginning and at the end of the assay using an inverted phase-contrast ZEISS Axiovert 135 microscope ( $5\times/0.12$  object). The number of cells in six representative scratch fields in each well was quantified.

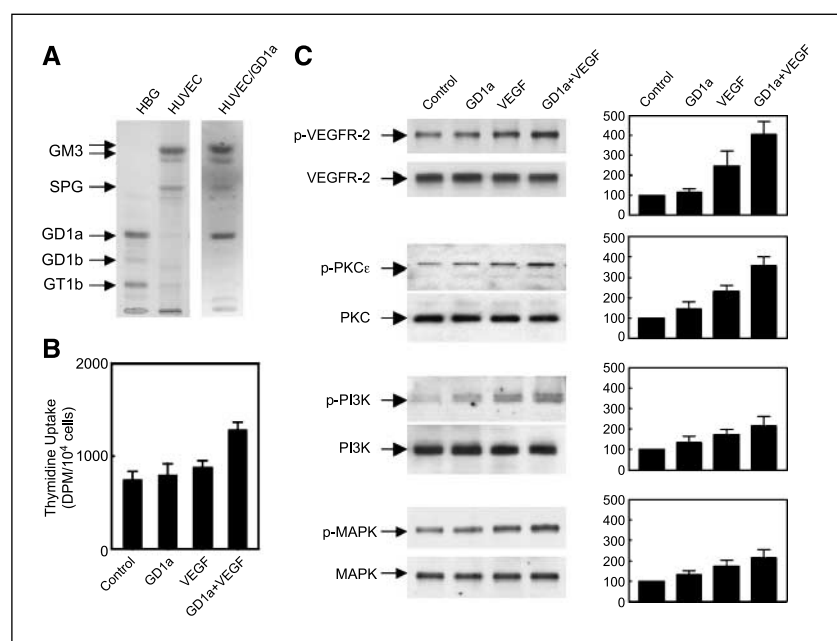
**Statistical analysis.** Results are the mean  $\pm$  SD of three to five independent experiments. Statistical significance was evaluated by two-way ANOVA in all experiments except Figs. 3 and 4 in which Graphpad PRISM 3.03 software and the group  $t$  test were used to analyze the binding, receptor dimerization, and VEGFR phosphorylation data.

## Results

**Ganglioside enrichment, signaling, and HUVEC proliferation.** We first assessed the responsiveness of ganglioside-enriched vascular endothelial cells to VEGF, in experiments that modeled

the likely *in vivo* circumstance, in which gangliosides shed by tumor cells will bind to surrounding normal cells that are in close proximity to the tumor cells (12). We exposed HUVEC to a highly purified ganglioside,  $\text{G}_{\text{D1a}}$ , washed away unincorporated molecules, and then observed the effects of membrane enrichment with exogenous ganglioside molecules. Exposure of HUVEC to 20  $\mu\text{mol/L}$   $\text{G}_{\text{D1a}}$  resulted in significant incorporation (25 nmol  $\text{G}_{\text{D1a}}$ /10<sup>8</sup> cells, which raised the total ganglioside content by 35%, to 71 nmol gangliosides/10<sup>8</sup> cells; Fig. 2A). We then incubated ganglioside-enriched HUVEC with 1 ng VEGF/mL in a short-term (3 hours) proliferation assay. Ganglioside-enriched HUVEC showed a highly significant 71% increase in DNA synthesis, compared with an only 18% increase in control cells exposed to this same concentration of VEGF ( $P < 0.001$ ; Fig. 2B), extending a previous observation that ganglioside enrichment increased HUVEC proliferation in serum-containing culture medium (19).

Signaling through VEGFR-2 (KDR, Flk-1), triggered by VEGF and leading to HUVEC proliferation, is well described; VEGF binding is followed by VEGFR dimerization and autophosphorylation (20, 21). Several downstream pathways are subsequently triggered, including the VEGFR/PKC/MAPK and VEGFR/PI3K/MAPK pathways (22). We quantified these VEGF-induced signaling pathways using a VEGF concentration (5 ng/mL) that yields optimal responses in control cells *in vitro*. VEGFR activation (autophosphorylation) in HUVEC preincubated with 20  $\mu\text{mol/L}$   $\text{G}_{\text{D1a}}$  for 6 hours in serum-free medium was twice that of nonenriched HUVEC ( $P < 0.01$ ; Fig. 2C), and downstream signaling molecules [PKC ( $P < 0.01$ ), PI3K, and MAPK] were also more highly phosphorylated in ganglioside-enriched cells (Fig. 2C), although the effect on PI3K and MAPK was not statistically significant. Pretreatment with inhibitors of VEGFR-2, PKC, and PI3K activation each completely blocked VEGF-induced and ganglioside-enhanced phosphorylation of the respective substrate, as well as downstream MAPK phosphorylation (not shown), demonstrating the specific involvement of each of these pathways in VEGF signaling, and preincubation with PD98059 (an inhibitor of MAPK/extracellular signal-regulated kinase 1 activity) completely blocked VEGF-induced MAPK



**Figure 2.** Ganglioside  $\text{G}_{\text{D1a}}$  enrichment enhances VEGF-induced HUVEC proliferation and signaling. **A**, ganglioside pattern and  $\text{G}_{\text{D1a}}$  incorporation in HUVEC. Total cellular gangliosides were purified from HUVEC, which had been exposed to 20  $\mu\text{mol/L}$   $\text{G}_{\text{D1a}}$  in EBM for 6 hours, and resolved by HPTLC. *HBG*, human brain ganglioside standards.  $\text{G}_{\text{D1a}}$  incorporation is representative of two separate experiments. Normal HUVEC contain 46 nmol gangliosides ( $\sim 4.3 \times 10^8$  molecules per cell), consisting primarily of GM3 and SPG (32). **B**, HUVECs were starved for 6 hours in serum-free EBM with 5  $\mu\text{mol/L}$   $\text{G}_{\text{D1a}}$ , washed, and stimulated with 1 ng/mL VEGF in EGM for 24 hours. [ $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci}$ ) was added during the last 3 hours and thymidine incorporation was quantified. Enhancement of DNA synthesis by  $\text{G}_{\text{D1a}}$ -enriched cells was significant ( $P < 0.001$ ); mean results of three separate experiments. **C**, HUVECs were incubated with 20  $\mu\text{mol/L}$   $\text{G}_{\text{D1a}}$  in EBM for 6 hours, washed, exposed to 5 ng/mL VEGF for 5 minutes at  $37^\circ\text{C}$ , and phosphorylation was assessed. The graphs at the right of the Western blots are the composite of three separate experiments.  $\text{G}_{\text{D1a}}$  preincubation significantly enhanced VEGF-induced VEGFR-2 and PKC $\epsilon$  phosphorylation ( $P < 0.01$ ); PI3K and MAPK phosphorylation were visibly but not statistically significantly increased.



activation (not shown), further confirming the specificity of the association of these pathway intermediates with ganglioside-enhanced VEGF signaling.

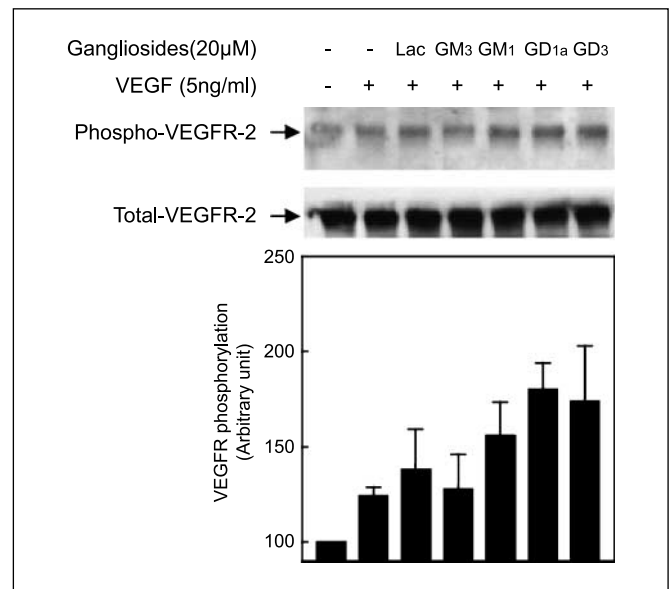
#### Ganglioside structure and effects on VEGFR activation.

Because members of the ganglioside family of glycolipids vary in carbohydrate structure, we wondered whether enhancement of signaling was unique to GD1a ganglioside. We therefore compared VEGF-induced VEGFR-2 autophosphorylation in HUVEC treated with each of four individual and highly purified gangliosides—two monosialogangliosides—GM1, GM3—and two disialogangliosides—GD1a, GD3—as well as lactosylceramide, which is a neutral glycosphingolipid (lacks sialic acid). Membrane enrichment in three gangliosides substantially enhanced VEGF-induced autophosphorylation over that of control cells (GM1,  $P = 0.02$ ; GD1a,  $P = 0.006$ ; and GD3,  $P = 0.04$ ), whereas GM3 and lactosylceramide caused no significant change ( $P > 0.05$ ; Fig. 3). That multiple ganglioside molecular species can facilitate growth factor responsiveness is important, because each may be found in the complex mixtures of gangliosides shed by tumor cells.

**VEGF binding by ganglioside-enriched HUVEC.** Ganglioside enrichment of the cell membrane enhanced VEGF-induced VEGFR-2 autophosphorylation and downstream signaling, suggesting that the proximal effect of membrane enrichment could be enhanced cellular binding of VEGF. In fact, GD1a enrichment caused a substantial increase in [ $^{125}$ I]VEGF binding to HUVEC (Fig. 4A), already seen at very low VEGF concentrations (0.06–0.25 ng/mL; Fig. 4A, *inset*). The calculated maximal binding after 20  $\mu$ mol/L GD1a preincubation of [ $^{125}$ I]VEGF to HUVEC was also greatly increased, from  $5.9 \pm 0.4$  (control) to  $15.2 \pm 1.9$  pmol/ $10^8$  cells (Table 1). Thus, GD1a enrichment of the membrane resulted in a significant increase in the number of receptors accessible to ligand binding, without stimulating an increase in total receptor protein (Fig. 2C), supporting the concept that gangliosides shed by tumor cells and subsequently taken up by endothelial cells in the immediate tumor microenvironment could enhance VEGF binding, resulting in a lowered VEGF requirement for responsiveness by endothelial cells *in vivo*.

#### HUVEC stimulation by suboptimal VEGF concentrations.

The full effect of membrane ganglioside enrichment on VEGF activation of HUVEC was discovered by assessing in detail the initial steps—VEGFR dimerization (Fig. 4B) and autophosphorylation (Fig. 4C). Throughout the entire wide range of VEGF concentrations studied, from the optimal *in vitro* concentration of 4 to 5 ng/mL down to the highly suboptimal concentrations of 0.1 to 0.25 ng/mL, both VEGFR dimerization and autophosphorylation were enhanced by membrane ganglioside enrichment. Dimerization increased by  $\sim 50\%$  at optimal *in vitro* VEGF concentrations (Fig. 4B). Importantly, this effect was proportionately greater at very low VEGF concentrations (0.25 ng/mL) with dimerization doubling in ganglioside-enriched cells ( $P = 0.04$ ). VEGFR autophosphorylation followed a similar pattern—increased at optimal and greatly magnified at very low VEGF concentrations (Fig. 4C). Thus, the higher binding at very low VEGF concentrations (Fig. 4A, *inset*) translates into increased formation of VEGFR dimers (Fig. 4B), which are high-affinity receptors ( $K_d < 50$  pmol/L) having a 100-fold higher VEGF binding ability than monomers (20). Dimerization of VEGFR subunits is responsible for initiating VEGFR autophosphorylation (Fig. 4C) and signaling (23). In fact, VEGFR autophosphorylation in control cells in response to an optimal VEGF concentration (2–4 ng/mL) was fully reproduced in ganglioside-enriched cells by an almost 10-fold lower VEGF concen-



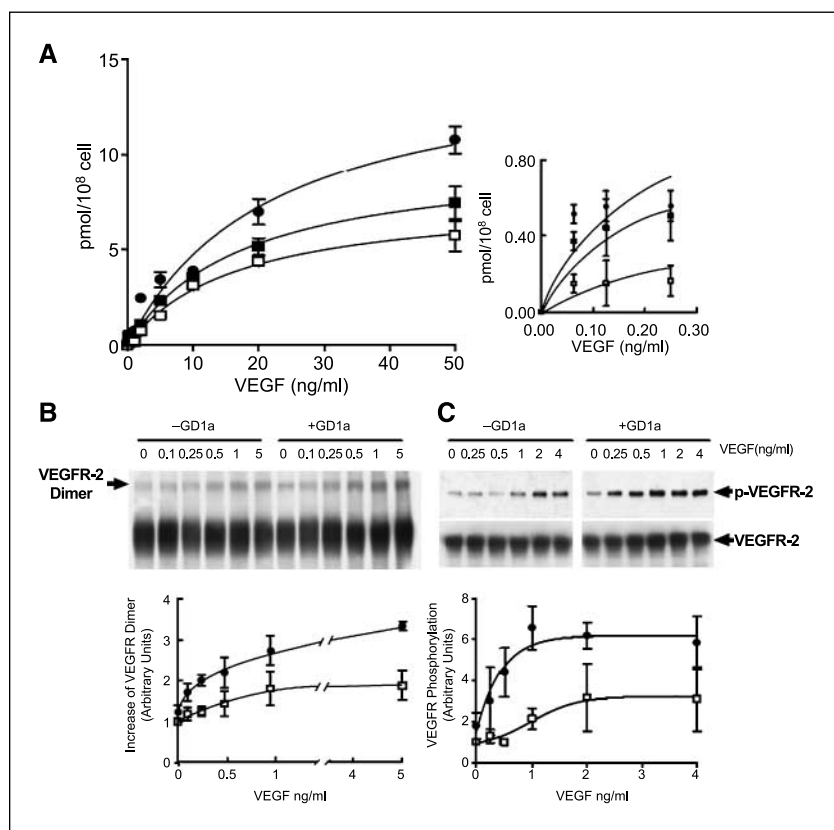
**Figure 3.** Ganglioside structure and enhancement of HUVEC VEGFR-2 phosphorylation. HUVECs were incubated with 20  $\mu$ mol/L of the individual gangliosides and lactosylceramide in EBM for 6 hours, washed, and exposed to 5 ng/mL VEGF in EBM for 5 minutes at 37°C. Detection of phosphorylated VEGFR-2 and total VEGFR-2 were as in Fig. 2C. A representative Western blot and the combined results of four separate experiments determining VEGFR-2 autophosphorylation are shown. Membrane enrichment in three gangliosides, GM1, GD1a, and GD3, substantially enhanced VEGF-induced autophosphorylation over that of control cells ( $P < 0.05$ ), whereas GM3 and lactosylceramide (*Lac*) caused no significant change.

tration (0.25 ng/mL;  $P = 0.004$ ), showing that ganglioside GD1a enrichment enhanced the sensitivity and activity of VEGFR-2, dramatically lowering the threshold for receptor responsiveness.

These findings led us to probe VEGFR activation and signaling in ganglioside-enriched HUVEC at the even lower VEGF concentration of 0.1 ng/mL, or  $\sim 2\%$  of the optimum concentration *in vitro*. This concentration initiates only minimal VEGFR autophosphorylation and is nonstimulatory to VEGFR signaling through PI3K and PKC $\epsilon$  in control, normal HUVEC (Fig. 5). By contrast, in ganglioside-enriched HUVEC, this subthreshold VEGF concentration (0.1 ng/mL; right-hand columns in Fig. 5A) caused increased VEGFR autophosphorylation and activation of PI3K, PKC $\epsilon$ , and MAPK signaling pathways. VEGFR autophosphorylation, for example, was stimulated to a level that was 60% greater in ganglioside-enriched endothelial cells compared with untreated controls. Similarly, whereas this very low concentration of VEGF was insufficient to activate the downstream PI3K and PKC $\epsilon$  pathways in control cells, striking phosphorylation of PI3K and PKC $\epsilon$ , and of MAPK, was observed in ganglioside-enriched cells ( $P < 0.01$ ; Fig. 5A).

#### Proliferation and migration of ganglioside-enriched HUVEC.

The completely unexpected robust activation of signal transduction pathways in ganglioside-enriched HUVEC by very low, suboptimal concentrations of VEGF described above was accompanied by a magnified, rapid onset of HUVEC DNA synthesis (Fig. 5B). As little as 0.25 ng VEGF/mL initiated a striking increase in DNA synthesis in ganglioside-enriched cells, whereas control untreated HUVECs were essentially unresponsive to this concentration of VEGF (Fig. 5B). Finally, this activation and signaling of ganglioside-enriched HUVEC by very low VEGF concentrations translated into a marked combined effect on cell proliferation and



**Figure 4.** GD1a enrichment increases [ $^{125}$ I]VEGF binding and VEGFR-2 dimerization and autophosphorylation in HUVEC. **A**, HUVECs were incubated with 20  $\mu$ mol/L GD1a in EBM for 6 hours, washed and incubated at 4  $^{\circ}$ C for 2 hours with 0.063 to 50 ng/mL [ $^{125}$ I]-labeled human recombinant VEGF in EBM. Nonspecific binding was assessed under the same conditions, with the addition of 300 ng unlabeled VEGF/mL. Specific binding was calculated by subtraction of the nonspecific binding of [ $^{125}$ I]VEGF from the total counts bound. Combined data of three separate experiments, analyzed using GraphPad Prism 3.03 software. *Inset*, enlarged binding curves at low (0-0.5 ng/mL) [ $^{125}$ I]VEGF concentrations. **B**, HUVEC preincubated with GD1a in EBM for 6 hours were exposed to 0.1 to 5 ng VEGF/mL in EBM for 5 minutes. Dimerization was assessed by cross-linking using 1 mg/mL BS $^3$  in PBS, immunoprecipitation of total VEGFR-2 (Flk-1), and detection of dimers by Western blot using an anti-VEGFR-2 antibody. **C**, HUVEC treatment and phosphorylation analysis were as in Fig. 2C, but using lower VEGF concentrations (0.25-4 ng/mL). *Bottom*, means of three experiments.  $\square$ , control;  $\blacksquare$ , 10  $\mu$ mol/L GD1a;  $\bullet$ , 20  $\mu$ mol/L GD1a.

migration, which can be assessed in the scratch wound assay (24, 25). The composite results of five separate experiments (representative experiment shown in Fig. 6) revealed that ganglioside-enriched HUVEC migrated substantially more than did nonenriched HUVEC, in response to 0.1 ng/mL VEGF ( $351 \pm 74$  versus  $210 \pm 61$  cells migrated into the scratch, per high power field, at 18 hours;  $P < 0.001$ ). Also, the small VEGF-independent selective stimulating effect of ganglioside enrichment on phosphorylation of PI3K and PKC $\epsilon$  (but not VEGFR or MAPK; Fig. 5A, *left-hand columns*), which may be due to ligand-independent Src activation caused by membrane ganglioside enrichment as reported in fibroblasts (26) and other cells (27), did not translate into an effect on migration, in that only ganglioside enrichment of HUVEC (without exposure to VEGF) did not significantly increase

migration over that of control untreated HUVEC ( $179 \pm 71$  versus  $153 \pm 41$  cells,  $P > 0.05$ ). Nor was the effect of 0.1 ng/mL VEGF alone on control HUVEC migration significant ( $P > 0.08$ ). A contribution of proliferation was also noted in this assay, as previously described (24), in that complete inhibition of HUVEC proliferation by exposure of cells to actinomycin D reduced the total number of cell found in the scratch after 18 hours, but this did not abrogate VEGF-induced migration of the ganglioside-enriched HUVEC (not shown). Overall, these findings point specifically to a positive interactive effect between cell membrane ganglioside enrichment and VEGF signaling resulting in vascular endothelial cell proliferation and migration, especially when exposure of the cells is only to a very low concentration of VEGF, as might be predicted to occur *in vivo*.

**Table 1.** Modulation of [ $^{125}$ I]VEGF binding to HUVEC by membrane ganglioside enrichment

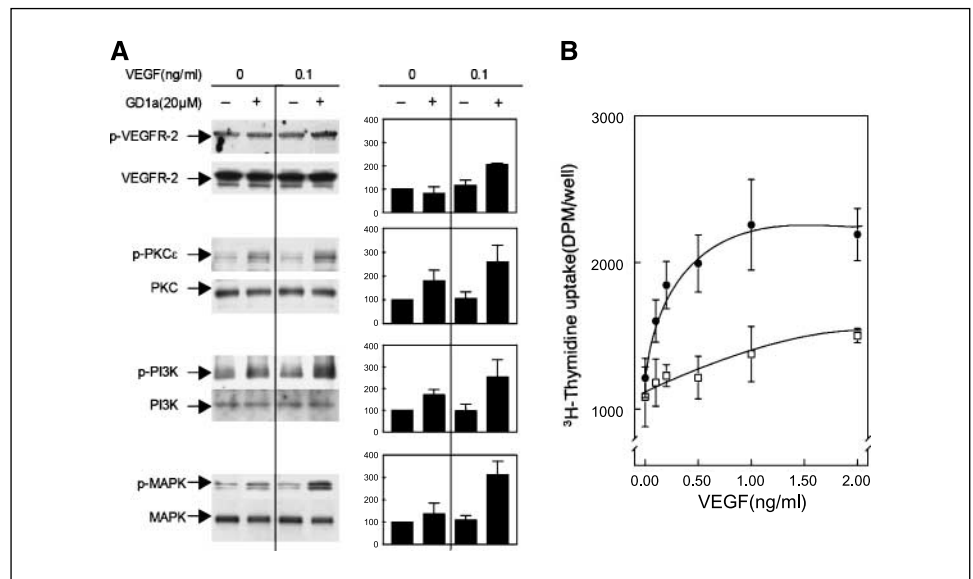
	Maximal binding (pmol/ $10^8$ cells $\pm$ SD)	Receptor number per cell ( $\pm$ SD) $\times 10^4$	$K_D$ (nmol/L $\pm$ SD)
Control	$5.9 \pm 0.37$	$3.6 \pm 0.22$	$0.27 \pm 0.044$
10 $\mu$ mol/L GD1a	$9.9 \pm 0.56^*$	$6.0 \pm 0.34^*$	$0.41 \pm 0.054^*$
20 $\mu$ mol/L GD1a	$15.2 \pm 1.85^{*,\dagger}$	$9.2 \pm 1.12^{*,\dagger}$	$0.52 \pm 0.137^*$

NOTE: HUVECs were cultured to subconfluence, starved in serum-free medium with or without GD1a for 6 hours, and binding of [ $^{125}$ I]VEGF was quantified. The total number of VEGFRs was markedly increased after GD1a preincubation (e.g., from  $3.6 \pm 0.22$  to  $9.2 \pm 1.12 \times 10^4$  per cell), whereas binding affinity was slightly reduced ( $0.27 \pm 0.04$  to  $0.52 \pm 0.14$  nmol/L). Thus, ganglioside GD1a preincubation increased the amount of [ $^{125}$ I]VEGF bound to HUVEC and also increased the effective total number of VEGFR on the membrane.

\* $P < 0.05$  (compared with control).

$\dagger P < 0.05$  (compared with 10  $\mu$ mol/L GD1a).

**Figure 5.** GD1a enrichment lowers the threshold for VEGF signaling and DNA synthesis in HUVEC. **A**, HUVEC treatment and phosphorylation analysis were identical to that in Fig. 2C, but using the very low VEGF concentration of 0.1 ng/mL. The composite is the result of three separate experiments. GD1a preincubation enhanced VEGF-induced phosphorylation of VEGFR-2 ( $P < 0.001$ ), PKC $\epsilon$  ( $P < 0.001$ ), PI3K ( $P < 0.01$ ), and MAPK ( $P < 0.01$ ). **B**, HUVECs were preincubated for 6 hours in 20  $\mu$ mol/L GD1a and exposed to 0 to 2 ng/mL VEGF and 0.5  $\mu$ Ci/well [ $^3$ H]thymidine. Three hours later, the cells were harvested and thymidine uptake was quantified. Points, mean of three separate experiments; bars, SD.  $\square$ , control;  $\bullet$ , 20  $\mu$ mol/LGD1a.



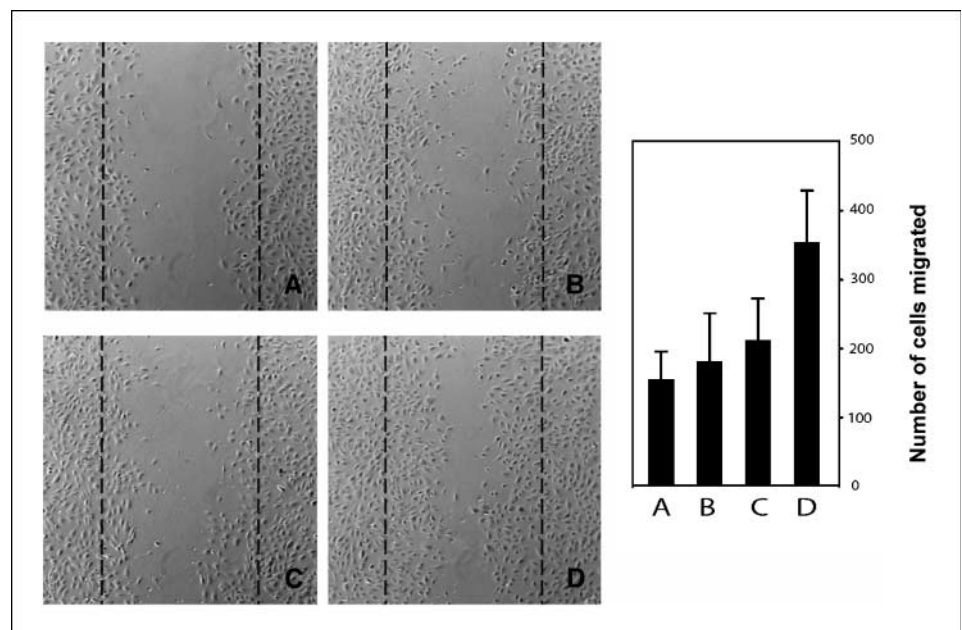
## Discussion

These findings strongly support the concept that ganglioside enrichment modifies the growth factor responsiveness of normal cells in the tumor microenvironment along a continuum toward autonomy. This increasingly autonomous state is characterized by transformation from completely growth factor-dependent normal cells to cells capable of vigorously responding to almost trace growth factor concentrations that are hardly able to stimulate a proliferative and migratory response in normal cells that have not been enriched with gangliosides. Because ganglioside enrichment of normal cell membranes by tumor cell shedding is a pathophysiologic process that reduces the requirement for growth factor, it can be viewed as the tumor "preparing its own bed." That is, tumor-shed, ganglioside-mediated conditioning of the tumor microenvironment causes phenotypic changes in normal cells, leading to decreased requirement for growth factors such as VEGF

(Fig. 1). This enhanced growth factor responsiveness of ganglioside-enriched vascular endothelial cells could be an important contributing factor to the formation and maintenance of a new tumor-associated vasculature *in vivo*.

Our findings identify exogenous gangliosides, which in the context of malignancy are shed into the local microenvironment by tumor cells, as potent enhancers of VEGF signaling and responses by HUVEC. An increase in sensitivity of the target cell, caused by a change in membrane ganglioside composition, can enhance a growth factor response, which can ultimately facilitate vessel formation. In tumor systems, this may be very potent, because ganglioside shedding (with the specific gangliosides shed reflecting the ganglioside composition of the tumor) is a very active process resulting in up to micromolar concentrations of tumor gangliosides, even in the peripheral circulation (14). Particularly highly efficient binding of tumor-shed gangliosides

**Figure 6.** GD1a enrichment triggers HUVEC migration and proliferation at subthreshold VEGF concentrations. Monolayer of confluent HUVECs were preincubated with or without 20  $\mu$ mol/L GD1a, scratched, and then incubated with or without 0.1 ng/mL VEGF in EGM with reduced (1%) FBS. The migration of cells into the scratch was evaluated 18 hours later. Six photographic fields of each of five separate experiments were quantified for the number of cells that had migrated. Columns, mean; bars, SD. A representative field is shown for each condition: medium control, i.e., without GD1a preincubation and no VEGF added (A), GD1a preincubation (B), 0.1 ng/mL VEGF (C), and GD1a preincubation and 0.1 ng/mL VEGF (D). Comparing VEGF and GD1a/VEGF,  $P < 0.001$ .





to target cells (12) and shared biological properties of structurally diverse gangliosides further underscore the potential effect of these molecules and their dynamic property of being actively shed by tumor and certain other proliferating cells. This mechanism of ganglioside-enhanced responsiveness to growth factors could also have a significant negative effect on therapies that target VEGF or VEGFR, because ganglioside-enriched endothelial cells would be predicted to more effectively survive such treatments, given their enhanced ability to activate signaling pathways with very low levels of stimulus.

How gangliosides of the cell membrane amplify growth factor responsiveness and lower the response threshold is unknown. One possibility is that gangliosides and other glycosphingolipids interact with other plasma membrane constituents to form signal transduction complexes (termed glycosynapses) on the plasma cell membrane (28). Another is that gangliosides may modulate growth factor receptors (29) or act as coreceptors (30). They clearly increase EGF-induced cell signaling (31), the number of receptors available for binding, and dimerization of this receptor tyrosine kinase (26). More generally, ganglioside insertion into the plasma cell membrane could alter the biophysical properties of lipid rafts, which harbor receptor tyrosine kinases in the plasma membrane.

The ability of gangliosides to lower the response threshold to growth factors could be important for both abnormal and normal

physiologic processes, such as enhancing growth factor responsiveness during neurodevelopment and generally facilitating high proliferative activity in other physiologic conditions. Targeting ganglioside production by tumor cells, or the shedding of these molecules into the tumor microenvironment, might help to restore a higher, more stringent threshold of responsiveness of adjacent cells (e.g., vascular endothelial cells), thereby serving to limit malignant tumor progression. Additionally, such strategies to limit ganglioside production/shedding may be very important as adjunct therapies, because treatments that target growth factors or their receptors in the microenvironment may be more effective if target cells have a greater requirement for these growth factors. Conversely, patients that exhibit defective wound healing as a result of diabetes or aging may benefit clinically by the topical addition of gangliosides to enhance cellular responsiveness to wound-healing stimuli.

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