ABSTRACT

Ganglioside GD3 is overexpressed in many types of tumors and may be associated with tumor progression and the development of metastatic potential. In our previous study (G. Zeng et al., Biochemistry, 38: 8762-8769, 1999), we established a subclone of the rat dorsal root ganglion-derived F-11 cells in which the expression of ganglioside GD3 was inhibited by stable transfection of the antisense vector against CMP-NeuAc:GM3 α2–8 sialyltransferase (GD3-synthase) gene. This cell line exhibits markedly reduced rate of tumor growth in vivo. Here, we further characterized the antisense-transfected cell line, and the results showed that these cells formed small, minimally vascularized tumors exhibiting extensive necrosis. In vivo Matrigel assay revealed reduced vascularization and low hemoglobin content in the antisense xenografts. Significantly fewer new vessels were found on the antisense xenografts and the skin around them than those on/around the xenografts formed by the sense-transfected and untransfected F-11 cells. The hemoglobin content of the antisense xenografts was much lower than that of the xenografts formed by the control cells. The reduced angiogenesis in the antisense xenografts was correlated with a decrease in vascular endothelial growth factor (VEGF) production. The expression of VEGF was suppressed in the antisense xenografts and the conditioned culture media of the antisense-transfected F-11 cells as determined by Western blotting analysis. This was further confirmed by immunohistochemistry of the tumors using antibodies against VEGF and platelet/endothelial cell adhesion molecule (PECAM-1). Therefore, our results demonstrate that reduced tumor growth in nude mice by suppression of GD3-synthase expression in F-11 cells results from minimal angiogenesis of the tumors through down-regulation of the VEGF expression, which indicates an important role for GD3 in tumor angiogenesis.

INTRODUCTION

One of the most remarkable advances in our understanding of cancer pathogenesis is that the progression of solid tumors depends on tumor angiogenesis. It is reported that tumor growth beyond a few cubic millimeters strictly depends on tumor angiogenesis (1). Consistent with this notion, studies also showed that tumors with a luxuriant vasculature have a higher fraction of dividing cells and lower necrosis rates than tumors with a poorly developed vasculature (1, 2). Therefore, neovascularization is a critical requirement for tumor growth. Numerous angiogenic factors that regulate this process have been identified (3, 4). Among them is VEGF,4 which has been implicated in the neovascularization of a wide variety of tumors (5–7). VEGF, also known as vascular permeability factor, is a M₆ 36,000–45,000 dimeric glycoprotein that may be expressed in several isoforms (121, 165, 189, and 205 amino acids) resulting from alternative splicing of a single primary transcript (8). VEGF has been identified in the conditioned media from numerous cell lines and is expressed in many tumors (5–7, 9, 10). The gene for this angiogenic factor has ~20% homology to platelet-derived growth factor and ~50% homology to placenta growth factor (11, 12). Many studies suggest that VEGF is the angiogenic factor most closely associated with induction and maintenance of the neovascularization (1, 9, 13).

The regulation of VEGF expression by human tumor cells has been implicated as a key factor in human tumorigenic and metastatic potential (6, 14). It is clear that VEGF expression is tightly regulated by both transcriptional and posttranscriptional mechanisms (14, 15); however, the signal transduction pathways that regulate these mechanisms remain largely unknown. VEGF expression has been shown to be controlled by environmental factors such as the limited availability of oxygen or glucose (14). Alternatively, gangliosides and other growth factors like platelet-derived growth factor and transforming growth factor family members also have been shown to stimulate VEGF production (14, 16–18). Gangliosides are sialic acid-containing glycosphingolipids and participate in various cellular processes (19, 20). There is considerable evidence for their role in tumorigenesis, e.g., the composition of gangliosides can undergo marked changes during oncogenic transformation (21, 22). The potential importance of gangliosides in tumor cell growth has been suggested by demonstrating reduction of experimental tumor angiogenesis and growth through the regulation of VEGF expression (16–18). The secretion of VEGF from tumor cells into culture media is stimulated by the addition of exogenous ganglioside GD3 (16). Formation of new vessels induced by angiogenic factors can be stimulated or repressed in the cornea by reduction or enhancement of the GM3:GD3 ratio of tissue gangliosides, respectively (17). Synthesis of complex gangliosides GM2, GM1, and GD1a in a mouse tumor cell line that synthesizes only GM3, enhances VEGF expression and stimulates vascularization in vivo (18).

The F-11 cell line is a fusion product of embryonic rat dorsal root ganglion (DRG) cells with a mouse neuroblastoma cell line N18TG-2. These cells represent the transformed DRG neurons as they express sensory neuronal antigens (23) and high levels of ganglioside GD3 and OAc-GD3 (24), and exhibit strong tumorigenicity and invasive ability (25). A subclone of the F-11 cells in which the expression of ganglioside GD3 and OAc-GD3 was inhibited by stable transfection of the antisense vector against GD3-synthase gene showed that the growth rate of the tumor cells in nude mice was remarkably reduced (25). We also showed that suppressed tumor growth resulted from the decreased level of GD3, rather than that of OAc-GD3 (25). This observation provides evidence that ganglioside GD3 plays important roles in the regulation of tumor growth in vivo. In the present study, we further characterized the antisense-transfected F-11 cells in an in vivo Matrigel model and reported that the reduced growth of the GD3-suppressed xenografts resulted from the suppression of angiogenesis in tumors, most likely through the down-regulation of the VEGF production.
**MATERIALS AND METHODS**

**Cell Culture and Cell Lines.** The F-11 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 95% air and 5% CO₂ at 37°C as described previously (25). Stock cultures were passed weekly and supplied with fresh medium every 3 days. The stably transfected cells were maintained in the media described above supplemented with 500 µg/ml Genetecin. Three cell lines were used in this study. These were untransfected F-11 cells and the sense-transfected F-11 cells and antisense-transfected F-11 cells that we had established previously by stable transfection with the sense and antisense vectors containing the entire GD3-synacthase cDNA, respectively (25).

**Tumor Growth in Nude Mice.** Female athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN) were housed under specific pathogen-free conditions and used at 6 weeks of age. Cells from exponential cultures of un transfected F-11 cells, the sense-transfected F-11 cells, and the antisense-transfected F-11 cells were tripynized, washed, and resuspended in sterile PBS. Mice were injected s.c. behind the anterior forelimb with 5 × 10⁵ cells in 0.25 ml of PBS per site. In vivo Matrigel model of angiogenesis was carried out as described by Passanti et al. (26). Cell suspension (5 × 10⁵ cells/ml) was mixed with four volumes of growth factor-reduced Matrigel (Becton Dickinson Labware, Bedford, MA), and 0.5 ml of Matrigel cell suspension (containing 5 × 10⁵ cells per site) was injected s.c. into nude mice. The mice that received s.c. injections were killed after 12 days, and the xenografts were removed, washed in PBS and kept at −70°C for additional determinations.

**Analysis of Gangliosides.** The ganglioside isolation procedures have been described previously (25). Briefly, the xenografts (at least three xenografts from different mice) were homogenized, and the total lipids were extracted with chloroform:methanol (1:1, v/v) and chloroform:methanol:water (30:60:8, v/v/v; Solvent A), successively. The combined extracts were adjusted into the ratio of Solvent A and applied to a DEAE-Sephadex-A-25 column (acetaate form). The column was eluted with Solvent A to remove neutral lipids. The ganglioside fraction was then eluted with 15 ml of chloroform:methanol:0.8 M sodium acetate (30:60:8, v/v/v; Solvent B). After drying, the sample was resuspended in 0.5 ml of Solvent A and desalted. The ganglioside fractions were analyzed by HPTLC using plates of Silica Gel 60. The plates were developed with chloroform:methanol:2% aqueous CaCl₂:2H₂O (50:45:10, v/v/v; Solvent C). Gangliosides were visualized by spraying the plate with the resorcinol hydrochloride reagent, followed by heating the covered plate at 100°C for 30 min.

**Determination of Hemoglobin Content.** Neovessels were quantitated by the hemoglobin content of the Matrigel-xenografts. Hemoglobin content was determined using the plasma hemoglobin kit (Sigma, St. Louis, MO) as described by the manufacturer based on the procedure described by Lijana and Williams (27) and Standerer and Vanderjagt (28). Samples were from at least three individual mice. Gels formed by Matrigel without cells in nude mice were used as negative control. The concentration of hemoglobin in Matrigel was calculated from a known amount of hemoglobin (Sigma) assayed in parallel. Protein content of the samples was determined by the Bradford method (29) using the Bio-Rad protein assay reagent.

**Western Blotting Analysis.** Xenografts obtained by s.c. injection of F-11 cells into nude mice with or without Matrigel were homogenized in a lysis buffer [9.1 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, 150 mM NaCl (pH 7.4), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 30 µg/ml aprotinin] and kept on ice for 30 min. A supernatant was collected by centrifuging the homogenate at 10,000 × g for 10 min at 4°C and stored at −70°C. Protein concentrations of the extracts were measured by the Lowry method (30) using BSA as a standard. Samples of conditioned cell media were prepared from cultures of F-11 cells. The same number of the three types of cells were cultured in 35-mm dishes in DMEM containing 10% FBS and grew to 80–90% confluence. Then, the media were changed to 2 ml of serum-free DMEM per dish and incubated at 37°C for 24 h. Conditioned media were collected and concentrated by centrifugation using Eppendorf centrifuge filter tubes (M, 10,000 cutoff). Protein contents of the conditioned media, before and after concentration, were determined by the Bradford method (29). Western blotting was performed as described previously (31). Fifty µg of protein of each sample was loaded on the gel. Primary antibodies were mouse anti-VEGF antibody (C-1; working concentration, 0.067 µg/ml) and goat anti-PECAM-1 antibody (M-20; working concentration, 0.067 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). Mouse anti-β-actin antibody (CA-15; working concentration, 0.11 µg/ml; Sigma) was used as an internal control. Protein-antibody complexes were visualized by the chemiluminescent reagents (Pharimacia Biotech, Piscataway, NY) and exposed to Kodak X-Omat film.

**Histology and Immunohistochemistry.** Tumors obtained from nude mice by s.c. injection of F-11 cells were fixed in 4% paraformaldehyde-PBS and processed and embedded in paraffin for H&E staining by the routine procedure. Immunoperoxidase staining was performed at room temperature using ImmunoCruz Staining System (Santa Cruz Biotechnology) as described by the manufacturer. The antibodies used for immunohistochemistry were mouse anti-VEGF antibody and goat anti-PECAM-1 antibody as used for Western blotting. In brief, deparaffinized sections were treated with peroxidase block solution for 5 min to reduce endogenous peroxidase activity and were washed in PBS. Sections were incubated with serum block solution for 20 min, followed by incubation with one of the diluted primary antibodies for >2 h and were washed in PBS. The specimens were incubated with corresponding biotinylated secondary antibodies for 30 min and then HRP-streptavidin complex for 30 min. Color (brown) was developed with HRP substrate for 10 min. After immunostaining, sections were stained with hematoxylin.

**RT-PCR.** Total RNA was prepared from cultured F-11 cells or xenografts obtained by s.c. injection of F-11 cells into nude mice with or without Matrigel, using Trizol reagent (Life Technologies, Rockville, MD) as described by the manufacturer. The specific primers for rat VEGF were selected from the published sequence (GenBank accession no. L20913; Ref. 32), involved in an alternative splicing of several exons: sense, 5′-TCAACCGCCTCCTGTGTCG; and antisense, 5′-ATCGTGTCCTCCGTAGAGGG. Primers for rat β-actin were designed based on the published sequence (GenBank accession no. X103672; Ref. 33), located on exon2 and exon4: sense, 5′-CCTTCCTCAATGAC-3′; antisense, 5′-ACGTCACACTTCTAG-3′; DNA was synthesized from total RNA by RT in a 20-µl reaction containing 0.5 µg of random primers, 200 units of SuperScript RNAse H reverse transcriptase (Life Technologies), 1.0 µg of total RNA, 4 µl of 5× RT buffer [375 mM KCl, 250 mM Tris-HCl (pH 8.3), and 15 mM MgCl₂], 5 mM DTT, 0.1 mM each dNTP, 20 units of RNasin (Life Technologies, Rockville, MD). Each mixture was incubated at 37°C for 1 h and then quick-chilled on ice. PCR was performed using a 50-µl reaction mixture containing 5 µl of RT reaction mixture, 1× PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 1% Triton X-100], 0.2 mM each dNTP, 1.5 mM MgCl₂, 2.5 units of Taq polymerase, 50 pmol of VEGF primers, and 50 pmol of β-actin primers. After an initial denaturation at 94°C for 3 min, PCR was carried out for 30 cycles at 94°C for 50 s, 57°C for 1 min, and 72°C for 1 min. The PCR products were then electrophoresed on a 1.2% agarose gel, were stained with 0.5 µg/ml ethidium bromide, and were photographed.

**Quantification by Densitometry.** Relative amounts of gangliosides, mRNA, and proteins determined by HPTLC, RT-PCR and Western blotting analysis, respectively, were quantified by scanning the area of each band using a Shimadzu CS-9000 scanner densitometer according to the manufacturer’s instruction. β-Actin was used as a normalization control for VEGF mRNA and protein.

**RESULTS**

**Histology and Ganglioside Composition of Antisense-transfected F-11 Cells Growing as Xenografts in Nude Mice.** When we inoculated s.c. the untransfected F-11 cells, the sense-transfected F-11 cells and the antisense-transfected F-11 cells into athymic nude mice, the average volume of the tumors formed by the antisense-transfected F-11 cells was one-third smaller than those formed by the untransfected F-11 cells or the sense-transfected F-11 cells at day 13 (25). In addition to the difference in size, xenografts showed different histological patterns (Fig. 1). The untransfected F-11 cells and the sense-transfected F-11 cells formed large tumors with little or no necrosis (Fig. 1, A and B). In contrast, the antisense-transfected F-11 cells the GD3 expression of which was largely suppressed formed small tumors with large amounts of central necrosis surrounded by only a thin rim of viable tumor cells (Fig. 1C). Necrosis of the antisense xenografts represented 60–80% of tumor volume. These data indicated...
that the GD3-suppressed tumor cells formed small tumors exhibiting extensive necrosis in vivo. Moreover, the ganglioside composition of the antisense xenografts was essentially the same as that of the antisense-transfected F-11 cells, showing dramatic decreases in GD3 and OAc-GD3 concentrations accompanying with an accumulation of the precursor GM3 (Table 1; Ref.25).

Reduced Vascularity in the Matrigel-Xenografts Formed by the Antisense-transfected F-11 Cells. Matrigel model is a simple, rapid, and quantitative assay for angiogenesis to assess inducers and/or inhibitors. A solution of Matrigel is mixed with angiogenic or angiostatic agents and injected s.c. in mice where it forms a gel. Sprouts from vessels of the adjacent tissue grow into the gel. Angiogenesis can be quantified by image analysis of vessels and by measuring the hemoglobin content with the gel (26). The results in Fig. 1 showed large amounts of central necrosis in the antisense xenografts. This high necrosis rate could be attributable to a poorly developed vasculature (1, 2). To test directly the effect of GD3-suppression in the F-11 cells on angiogenesis in vivo, we modified the angiogenesis model described previously (26), involving s.c. injection of athymic nude mice with growth factor-reduced Matrigel that contained stably transfected F-11 cells. Although the same number of the tumor cells (5 × 10⁶) mixed with the same volume of Matrigel (0.5 ml) per site was injected s.c. into nude mice, the Matrigel-xenografts formed by the antisense-transfected F-11 cells were much smaller than those formed by untransfected F-11 cells and the sense-transfected F-11 cells. These observations are consistent with our previous results obtained by injection of the cells without Matrigel (25), which indicates that Matrigel did not affect the growth properties of the tumor cells. In this in vivo Matrigel model, untransfected F-11 cells and the sense-transfected F-11 cells provoked a strong angiogenic response as seen on the surface of the Matrigel-xenografts (data not shown) and the skin flaps overlying the Matrigel-xenografts (Fig. 2, A–C). The neovessels induced by the antisense-transfected F-11 cells were much smaller, in both number and diameter, than those induced by the control F-11 cells. To quantitate angiogenesis provoked by the tumor cells, the content of hemoglobin in the xenografts was determined (Table 2). The hemoglobin content in the antisense xenografts was less than 20% of that in the control xenografts as determined in equal amounts of total proteins. Therefore, the GD3-suppressed F-11 cells were much less angiogenic than the parental F-11 cells and the sense-transfected F-11 cells.

Synthesis of VEGF Transcripts in Cultured Tumor Cells and Xenografts. To determine whether the reduced angiogenesis that resulted from the decrease of the GD3 concentration is attributable to an alteration in the expression of angiogenic factors, VEGF mRNA in cultured F-11 cells and xenografts obtained by growing the cells with or without Matrigel in the s.c. of immunodeficient nude mice was determined by RT-PCR (Fig. 3). RT-PCR that used the designed primers revealed two bands of the most abundant splice variants of VEGF in F-11 cells, which are the secreted 121- and 165-amino acid isoforms of VEGF. The results shown in Fig. 3 and Table 3 indicated that the cultured F-11 cells synthesized VEGF mRNA at high levels as compared with the β-actin transcripts. There was little difference in the synthesis of VEGF mRNA between the antisense-transfected F-11 cells and control sense-transfected and untransfected F-11 cells (Fig. 3A; Table 3). However, the VEGF mRNA level in the antisense xenografts with or without Matrigel was markedly decreased, compared with that in the xenografts formed by the control F-11 cells (Fig. 3, B and C; Table 3), which indicated that the expression of VEGF in vivo might be correlated with the level of GD3 concentration. That the synthesis of VEGF mRNA in vivo was not affected by the altered GD3 expression could be attributable to altered expression or unknown effects by the transfection processes and remains to be elucidated.

Determination of VEGF in Conditioned Media and Xenografts from Antisense-transfected F-11 Cells. The expression of VEGF was determined at the protein level by Western blotting using the antibody against rat VEGF. Results showed that the concentration of the VEGF protein in the xenografts obtained by growing the antisense-transfected F-11 cells with or without Matrigel in nude mice was greatly reduced (Fig. 4, A and C; Table 3), which was consistent with the reduced expression of VEGF in the transcriptional level (Fig. 4, B and C; Table 3). Growth factor-reduced Matrigel showed slight reaction with anti-β-actin antibody, and no VEGF was detected under the same conditions (Fig. 4C). Although suppression of GD3 expression did not change the level of transcription of VEGF gene in the cultured antisense-transfected F-11 cells (Fig. 3A; Table 3), secretion

Table 1  Ganglioside composition of xenografts

The ganglioside composition was analyzed by HPTLC and subjected to densitometry as described in “Materials and Methods.” Values are presented as percentages (mean ± SD) of three separate determinations and, therefore, refer to the relative amounts of each ganglioside in the sample. Total amount of gangliosides in each sample was designated as 100%.

<table>
<thead>
<tr>
<th>Ganglioside</th>
<th>F-11 cells</th>
<th>Sense-transfected F-11 cells</th>
<th>Antisense-transfected F-11 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM3</td>
<td>19.1 ± 1.7</td>
<td>28.5 ± 3.9</td>
<td>45.1 ± 4.0</td>
</tr>
<tr>
<td>GM1</td>
<td>13.2 ± 2.3</td>
<td>12.6 ± 2.0</td>
<td>20.4 ± 3.5</td>
</tr>
<tr>
<td>OAc-GD3</td>
<td>19.5 ± 0.2</td>
<td>18.9 ± 2.3</td>
<td>35.5 ± 0.4</td>
</tr>
<tr>
<td>GD3</td>
<td>20.6 ± 0.9</td>
<td>21.1 ± 3.5</td>
<td>39 ± 0.9</td>
</tr>
<tr>
<td>GD1a</td>
<td>13.5 ± 2.0</td>
<td>4.8 ± 0.9</td>
<td>10.5 ± 1.4</td>
</tr>
<tr>
<td>GD1b</td>
<td>8.1 ± 2.1</td>
<td>6.5 ± 2.4</td>
<td>8.7 ± 3.1</td>
</tr>
<tr>
<td>GT1b</td>
<td>6.0 ± 0.5</td>
<td>7.8 ± 1.4</td>
<td>6.8 ± 1.2</td>
</tr>
</tbody>
</table>
was detected in the sections of xenografts formed by untransfected F-11 cells and the sense-transfected F-11 cells (Fig. 5, A and B). Cytoplasmic staining of VEGF was revealed in the control tumor cells (brown color) and strong staining was present in the adjacent endothelium of blood microvessels (arrow). In contrast, the peripheral rim of viable tumor from the antisense-transfected F-11 cells had no staining with the antibody to VEGF, and little microvessels could be seen (Fig. 5C). These results were further confirmed by the antibody against PECAM-1. PECAM-1 (CD31) is a transmembrane glycoprotein that is expressed on continuous endothelia in all tissues, predominantly on the surface of platelets, and is concentrated at the junctions between endothelial cells (34–36). Immunostaining of PECAM-1 did not show staining in the section of xenografts from the antisense-transfected F-11 cells (Fig. 5F), whereas positive staining was present in the sections of xenografts from the control F-11 cells, particularly around the microvessels (Fig. 5, D and E). The production of PECAM-1 protein was reduced in the antisense xenografts as detected by Western blotting (Fig. 4A). Therefore, our results showed a dramatic decrease in the production of the angiogenic factor VEGF, as well as endothelial cell-related antigen PECAM-1, in the antisense xenografts.

**DISCUSSION**

Our previous studies showed that the specific inhibition of GD3-synthase gene expression in the antisense-transfected F-11 cells decreased cell proliferation in culture moderately, whereas it greatly reduced the rate of growing the tumor cells in nude mice (25). We also found that down-regulation of GD3-synthase expression in the antisense-transfected F-11 cells correlated with the reduction in cell migration and invasion in vitro and tumor growth and metastasis in vivo (37). In addition, two separate clones of the antisense-transfected F-11 cells obtained from experiments using different vectors that contained different lengths of the antisense fragments gave the same results (25). Therefore, the effects attributable to the selection pro-

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**Table 2** Hemoglobin content of Matrigel-xenografts

<table>
<thead>
<tr>
<th>Xenografts formed by</th>
<th>Hemoglobin (mg/g total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransfected F-11 cells</td>
<td>20.83 ± 4.03</td>
</tr>
<tr>
<td>Sense-transfected F-11 cells</td>
<td>25.79 ± 4.55</td>
</tr>
<tr>
<td>Antisense-transfected F-11 cells</td>
<td>4.11 ± 1.35</td>
</tr>
</tbody>
</table>

of VEGF protein into serum-free medium by the antisense-transfected F-11 cells was greatly inhibited (Fig. 4B; Table 3). However, we were unable to detect the VEGF protein in the cell extracts prepared from the three cell lines by Western blotting under the same conditions (data not shown), probably because of the rapid secretion of the protein from the F-11 cells into media. This result indicated that there was no accumulation of VEGF protein inside the antisense-transfected F-11 cells. Addition of exogenous ganglioside GD3 in cultures of the antisense-transfected F-11 cells, as well as in the cultures of the control F-11 cells, did not change the amounts of secreted VEGF in the conditioned media (data not shown). These data suggest that there may be different mechanisms involving the effect of GD3 on VEGF production between in vivo and in vitro.

**VEGF and PECAM-1 Immunostaining of Tumor Xenografts.** Immunohistochemistry was also used to assess VEGF protein in tumor xenografts. Sections of the three types of tumor xenografts were immunostained with the rat anti-VEGF antibody. Abundant VEGF

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**Fig. 2** Formation of neovessels on skin overlying Matrigel-xenografts. Matrigel-xenografts were removed and the skin flaps overlying the xenografts formed by untransfected F-11 cells (A), untransfected F-11 cells with Matrigel (B), sense-transfected F-11 cells (C), antisense-transfected F-11 cells with Matrigel (D), and Matrigel without cells (E) were photographed. Bar, 1 cm.

**Fig. 3** Analysis of VEGF gene expression in vivo and in vitro by RT-PCR. Total RNA was prepared from the cultured tumor cells (A) and the xenografts formed by growing tumor cells in nude mice without Matrigel (B) or with Matrigel (C) as described in “Materials and Methods”; and VEGF mRNA was determined by RT-PCR analysis. The levels of β-actin mRNA were used as internal control. Two bands of VEGF were amplified by the primers representing the 121- and 165-amino acid isoforms of VEGF, the most abundant splice variants of VEGF. Lane M, molecular markers: 100-bp DNA ladder; Lane U, untransfected F-11 cells or xenografts; Lane S, the sense-transfected F-11 cells or sense xenografts; and Lane A, the antisense-transfected F-11 cells or antisense xenografts.
cesses and other variabilities of creating stable transfectants should be limited in the studies using these clones. In the present study, we investigated the mechanism that involved the altered gangliosides GD3 in tumor growth. (Although both GD3 and OAc-GD3 were suppressed in the antisense-transfected F-11 cells, OAc-GD3 was ruled out of this action as discussed below). The role of ganglioside GD3 in tumorigenesis was suggested mainly by the fact that the composition of ganglioside GD3 changes during cellular transformation (22, 23). It has been shown that some growth factors and their receptors appear to be regulated by ganglioside GD3, and GD3 may promote angiogenesis (16, 17, 38–40). This was evident by using exogenous gangliosides. Addition of exogenous GD3 in culture media promotes angiogenesis (16, 17, 38–40). This was evident by using exogenous GD3 in culture media stimulates VEGF release from glioma cell lines (16), and an increase in the ratio of GD3:GM3 by adding exogenous GD3 or GM3 in rabbit cornea stimulates the formation of neovessels (17). Here, we showed that suppression of GD3-synthase gene expression in the F-11 cells formed small, minimally vascularized tumors that exhibited extensive necrosis, and that the reduced angiogenesis in the antisense xenografts was correlated with a remarkable decrease in the production of angiogenic factor VEGF. Therefore, we provided evidence for the first time that suppression of GD3-synthase gene expression reduced angiogenesis when the F-11 cells grew in vivo.

The F-11 cells express high levels of hemato-series gangliosides, especially GD3 and its downstream product OAc-GD3, reflecting the fact that they originated from neuroectodermal cells (24). GD3 is a structurally simple ganglioside and plays an important role in cell growth, proliferation, and cellular transformation (41). OAc-GD3 was first described in primitive neuroectodermal cells, and indirect evidence showed that OAc-GD3 could be involved in cell differentiation (42). Besides, GD3 is overexpressed in many tumor cells of neuroectodermal or epithelial origin (43–45), whereas the expression of OAc-GD3 is restricted to particular cancers such as melanoma and breast cancer (42, 43, 46, 47). Therefore, these two gangliosides may function differently. In our experiments, both GD3 and OAc-GD3 were selectively down-regulated by antisense inhibition of the GD3-synthase gene expression in the antisense-transfected F-11 cells (25), but the involvement of OAc-GD3 in tumorigenicity and/or angiogenesis was ruled out for the following reasons. First, we have found that inoculation in nude mice of the OAc-GD3-depleted F-11 subclone in which ganglioside OAc-GD3 was reduced to 30% whereas GD3 was increased to 150% (24) did not show a reduced tumor growth rate (unpublished data, Ref.25). Thus, the reduced tumor growth rate of the antisense-transfected F-11 cells was most likely attributable to a specific reduction in GD3 (25). Second, in this study, we found that there was no difference in the expression of VEGF between the xenografts formed by the OAc-GD3-depleted F-11 cells and their parental F-11 cells as determined by Western blotting (Fig. 4D). Therefore, we conclude that the decrease in OAc-GD3 concentration did not contribute to the reduced angiogenesis in in vivo tumor growth.

In vivo growth of tumors depends on tumor angiogenesis. We reported here that reduced tumor growth in vivo by the suppression of GD3-synthase gene expression in the F-11 cells is attributable to minimal angiogenesis of the tumors through down-regulation of the VEGF expression. However, the mechanism that links the interactions between specific gangliosides and angiogenesis and/or VEGF expression is unknown. The F-11 cells express three major gangliosides: GM3, GD3, and OAc-GD3, which contribute to 37, 23, and 18%, respectively, of the total gangliosides (24). Specific inhibition of GD3-synthase gene expression in the antisense-transfected F-11 cells or xenografts reduced the concentration of GD3 to 5–10 times less than that of the parental cells or xenografts (Ref. 25; Table 1). Therefore, the reduced angiogenesis and VEGF production can be primarily accounted for by the reduction of the concentration of GD3. However, it should be noted that there was a significant accumulation of GM3, the precursor of GD3, in the antisense-transfected F-11 cells and xenografts when GD3 synthesis was inhibited (Ref. 25; Table 1). GM3 has been described as a modulator of growth factor receptors such as those of fibroblast and epidermal growth factors (40, 48), which in turn may have effects on angiogenesis. Therefore, whether there are secondary effects on angiogenesis from the inhibition of the sialyltransferase in F-11 cells remains to be elucidated.

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**Table 3: Expression of VEGF in vitro and in vivo**

<table>
<thead>
<tr>
<th>VEGF</th>
<th>mRNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protein&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Untransfected</strong></td>
<td><strong>Sense-transfected</strong></td>
<td><strong>Antisense-transfected</strong></td>
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<tr>
<td>F-11 cells</td>
<td>F-11 cells</td>
<td>F-11 cells</td>
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<tr>
<td>F-11 cells</td>
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<td>F-11 cells</td>
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<tr>
<td>F-11 cells</td>
<td>F-11 cells</td>
<td>F-11 cells</td>
</tr>
<tr>
<td>mRNAs</td>
<td>164 ± 31%</td>
<td>175 ± 33%</td>
</tr>
<tr>
<td>Protein</td>
<td>100 ± 11.6%</td>
<td>94.9 ± 4.6%</td>
</tr>
<tr>
<td>mRNA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>212 ± 57%</td>
<td>223 ± 54%</td>
</tr>
<tr>
<td>Protein&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(195 ± 49%)</td>
<td>(157 ± 14%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data were obtained by densitometry from three separate determinations and are expressed as the percentages of mean ± SD.

<sup>b</sup> VEGF mRNA detected by RT-PCR as shown in Fig. 3 is presented as the percentage of that of β-actin, which was designated as 100%. Data obtained from Matrigel-xenografts as shown in Fig. 3C are in parentheses.

<sup>c</sup> The amount of VEGF protein in xenografts as shown in Fig. 4A are expressed as the percentage of that of β-actin, which was designated as 100%. Data obtained from Matrigel-xenografts as shown in Fig. 4C are in parentheses.
Koochekpour et al. (16) showed that the addition of exogenous GD3 in culture media stimulated VEGF release from glioma cells. In our case, the addition of exogenous GD3 in the culture media did not enhance the production of VEGF in the conditioned media of parental F-11 cells and the sense-transfected F-11 cells nor complement the suppressed secretion of VEGF of the antisense-transfected F-11 cells (data not shown). We also found previously that changes in morphology and the growth rate of the antisense-transfected F-11 cells (25) could not be complemented by the addition of ganglioside GD3 into culture media.5 Our data indicate that there must be different responses to exogenous gangliosides between cell lines, e.g., glioma cells and dorsal root ganglia cells, attributable to the cell-type specificity. The different responses may also reflect different actions on angiogenesis between endogenous and exogenous GD3 in F-11 cells. Another possibility could be attributable to certain unknown effects from the inhibition of the sialyltransferase. As mentioned above, an accumulation of the precursor GM3 might contribute, at least in part, to the reduction of angiogenesis, and, thus, the addition of exogenous GD3 could not reverse these effects. On the other hand, the addition of anti-GD3 antibody to cultured F-11 cells did not result in the changes observed in the antisense-transfected F-11 cells (data not shown). The reasons could be that the effect of GD3 on VEGF production may be indirect and complicated, and, thus, the temporary blocking of GD3 on the cell surface by the antibody may not represent an effect from the permanent suppression of endogenous GD3.

VEGF has an important role in facilitating tumor growth in vivo by stimulating tumor angiogenesis. Most of tumorigenic cell lines express high levels of VEGF mRNA both in vivo and in vitro (Fig. 3; Table 3). Inhibition of GD3-synthase expression in the antisense-transfected cells down-regulated the expression of VEGF in vivo at both mRNA and protein levels, whereas transcription of the VEGF gene in the cultured antisense-transfected F-11 cells was not affected by the decreased level of GD3 in the cells, although the secretion of VEGF from the cells into media was almost completely inhibited (Figs. 3 and 4; Table 3). A similar result was recently reported by Manfredi et al. (18) that the induction of the biosynthesis of the “a” series gangliosides GM2, GM1, and GD1a in an ependymoblastoma cell line by transfection of the cells with the GM2-synthase cDNA increased the VEGF mRNA level only when the tumor cells were growing in vivo but not when they were growing in cultures. How changes in the ganglioside composition affect the expression of VEGF remains to be elucidated. It is possible that, in the in vivo case, the effect of the ganglioside(s) may be through the interaction between tumor cells and host cells as suggested previously (18). In other words, the VEGF mRNA detected in vivo may include the VEGF mRNA that is synthesized by the host cells in which transcription of VEGF gene may be greatly stimulated by tumor cells.

Manipulation of gene expression by antisense technology is a powerful tool to mimic changes in ganglioside composition that occur during cell differentiation, proliferation, and transformation (18, 24, 25, 50, 51). Using this strategy, it is possible to define the biological roles of individual ganglioside(s). On the other hand, in the clinical treatment of cancer patients, monoclonal antibodies against tumor gangliosides have shown partial or complete response but the effectiveness was usually only transient, and inadequate response rates were often observed (52–54). Antisense strategy can be a promising therapy as an alternative to antibody strategy for cancer treatment. In the present study, we provided indirect evidence that the decrease in the concentration of endogenous GD3 down-regulated the expression of angiogenic factor
VEGF, which resulted in remarkably reduced tumor growth in vivo. In addition to elucidating the biological function of GD3 in tumor growth, the synergetic effects between tumor gangliosides and angiogenic factors on tumorigenesis should facilitate the use of combined gene therapy in future.

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Suppression of Ganglioside GD3 Expression in a Rat F-11 Tumor Cell Line Reduces Tumor Growth, Angiogenesis, and Vascular Endothelial Growth Factor Production

Guichao Zeng, Luoyi Gao, Stéphane Birklé, et al.


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