Gangliosides Influence Angiogenesis in an Experimental Mouse Brain Tumor

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ABSTRACT

Gangliosides are sialated glycosphingolipids present on the plasma membranes of all vertebrate cells. Tumors shed gangliosides into the extracellular microenvironment, which may influence tumor-host cell interactions. We have investigated the role of gangliosides on the growth and angiogenesis of the EPEN experimental mouse brain tumor. EPEN cells express only ganglioside GM3, and the solid tumors formed in vivo are sparsely vascularized with extensive necrosis. We stably transfected the EPEN cells with the cDNA for N-acetylgalactosaminyl transferase, a key enzyme for the synthesis of complex gangliosides. In addition to GM3, the transfected cell line (EPEN-GNT) expressed complex gangliosides GM2, GM1, and GD1a. The EPEN-GNT tumor was more densely vascularized with less necrosis and grew more rapidly than the nontransfected EPEN or mock-transfected (EPEN-V) control tumors. Also, VEGF gene expression was higher in the EPEN-GNT tumor than in the control tumors. The synthesis of complex gangliosides in the EPEN-GNT tumor cells also stimulated vascularization in an in vivo Matrigel assay for angiogenesis. These results indicate that the ratio of GM3 to complex gangliosides can influence the growth and angiogenic properties of the EPEN experimental mouse brain tumor and are consistent with previous findings in other systems. We conclude that gangliosides may be important modulators of brain tumor angiogenesis.

INTRODUCTION

Angiogenesis involves the sprouting of new microvessels from existing vessels and is a prerequisite for tumor progression (1–3). Microvessel density is positively correlated with tumor malignancy and ultimately influences patient prognosis (4, 5). Brain tumors, especially glioblastomas, are highly angiogenic and are refractory to most therapies. Consequently, antiangiogenesis therapy may be an attractive treatment for brain tumors (6).

The recruitment of blood vessels into a developing tumor is initiated by factors intrinsic to the tumor cells (7, 8). The migration and proliferation of endothelial cells in the tumor microenvironment are two hallmark features of angiogenesis (9). Many factors in the tumor environment, including growth factors and extracellular matrix molecules, modulate the angiogenic response (10, 11). These factors are secreted by both the tumor cells and host cells (e.g., macrophages; Ref. 12). VEGF is an endothelial-specific mitogen that is important for endothelial cell proliferation and migration (13, 14). Experimental alterations of tumor VEGF levels can influence vessel density and tumor growth rate (15, 16). Antibodies that block endothelial cell adhesion to the extracellular matrix can also inhibit tumor angiogenesis and growth (17, 18).

Gangliosides are good candidates for modulators of tumor angiogenesis because their negatively charged oligosaccharide head group extends into the extracellular environment (19, 20). These molecules can also be shed into the extracellular environment, where they can influence tumor-host cell interactions (21). Gangliosides can also modulate receptors, such as those for growth factors and cell adhesion (22). The structurally simple ganglioside GM3 regulates the function of the epithelial growth factor and fibroblast growth factor receptors (23, 24). In addition, gangliosides modulate the function of the integrin receptors including α5β1, α2β1, and ανβ3 (25–27). Hence, gangliosides influence the activity of several molecules important in angiogenesis.

In the rabbit cornea model of angiogenesis, GM3 inhibits basic fibroblast growth factor-induced angiogenesis in a dose-dependent manner (28). However, the antiangiogenic effect of GM3 is counteracted by complex gangliosides (GM2, GM1, GD3, GD1b, and GT1b; Ref. 29). Complex gangliosides enhance the action of angiogenesis inducers. For example, ganglioside GM3 stimulates the release of VEGF from human glioma cell lines and also enhances the migration of cytokine-stimulated endothelial cells (28, 30). It is suggested that the ratio of GM3 to complex gangliosides can significantly influence angiogenesis (29).

To directly test the hypothesis that the ratio of GM3 to complex gangliosides influence tumor angiogenesis, we genetically altered ganglioside biosynthesis in a well-characterized mouse brain tumor cell line and analyzed the growth and angiogenic properties of these tumors in vivo.

MATERIALS AND METHODS

Mice and Experimental Brain Tumors. The C57BL/6J (B6) strain was obtained from The Jackson Laboratory (Bar Harbor, ME). All B6 mice used in this study were propagated in the animal facility of the Department of Biology, Boston College, using animal husbandry conditions described previously (31). Male B6 mice, ~6 weeks of age, were used as tumor recipients. The EPEN experimental mouse brain tumors and transfected tumor lines were maintained by serial transplantations s.c. in the flank or intracranially in the brain, as described previously (32). Female athymic nude mice (NCr-nu/nu), 6–8 weeks of age, were obtained through the NIH, National Research Resources. All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institution Animal Care Committee.

Cell Culture and Transfections. The EPEN cell line was established from a murine ependymoblastoma tumor as described previously (33). The cells were maintained in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma) and 0.5% penicillin/streptomycin (Life Technologies). The cells were cultured in a humidified atmosphere with 95% air and 5% CO2 at 37°C. The EPEN cells were stably transfected with the expression vector pCMV containing a 1.6-Kb GalNAc-T cDNA insert (a gift from Dr. Richard Proia, NIH) using the Lipofectamine protocol (Life Technologies). The cells were cotransfected with the pcDNA3 vector (Invitrogen, San Diego, CA), which contained a neomycin resistance gene for selection. Cells that survived 400 μg/ml G418 (Life Technologies) treatment for 2 weeks were expanded and analyzed for the expression of GalNAc-T using RT-PCR with primers that were described previously (34). Positive cells were subcloned to ensure a single clonal population. To confirm that GalNAc-T was expressed in the tumors grown in vivo, RT-PCR was used with primers (forward, 5'-CGGTTGACCTCTAAACCGC-3'; reverse, 5'-TGACACTATAAGATCATTCAAGC-3') that amplified a 1.3-kb portion of the transfected GalNAc-T mRNA only. RT-PCR was done as described previously (34).

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; GalNAc-T, N-acetylgalactosaminyl transferase; RT-PCR, reverse transcription-PCR; HPTLC, high-performance TLC.
The sections (7 tumors were fixed in 4% formaldehyde and processed for frozen sectioning. Gliosides, as described previously (41).

Digestion were conducted for preliminary structural studies of gangliosides and analyzed using HPTLC and quantified as described previously (37, 40).

Samples were vortexed and centrifuged at 800 × g for 10 min. The supernatant was transferred to another tube, and the pellets were washed with 2 ml of 1:1 chloroform:methanol (v/v) and centrifuged for 10 min at 800 × g. The resulting supernatant was added to the previous supernatant. Polar and non-polar lipids were separated by Folch partitioning (36, 37). Gangliosides were isolated and purified by modification of methods described previously (37, 38). Briefly, the Folch “upper phase” was converted to a chloroform:methanol:water ratio of 30:60:8 (v/v/v). The samples were added to a DEAE-Sephadex A-25 (Pharmacia Biotech, Uppsala, Sweden) ion exchange column that was equilibrated in 30:60:8 chloroform:methanol:water (v/v/v). Nonacidic molecules were eluted from the column with 20 ml of the same solvent. For in vitro samples, the gangliosides were eluted from the column with 20 ml of 30:60:8 chloroform:methanol:0.8 ammonium acetate (v/v/v). For in vivo samples, the gangliosides were eluted from the column with 30 ml of 30:60:8 chloroform:methanol:0.8 sodium acetate (v/v/v). The solvents were evaporated under vacuum, and then the samples were treated with base 0.5 N NaOH at 37°C for 1.5 h and desalted as described previously (32, 37).

Ganglioside Isolation. To detect gangliosides that were shed from the cultured cells, the medium from radiolabeled cells was collected as described above and passed through a 0.2 μm filter (Corning) to remove dead or fragmented cells. The medium was then frozen at −80°C and lyophilized to remove water. The dried medium was then used for the isolation of gangliosides as described above. The percentage of gangliosides shed into the culture medium over a 48-h period was estimated by dividing the dpm of supernatant gangliosides by the total dpm of gangliosides synthesized (cells plus supernatant) × 100.

Ganglioside Analysis. For radiolabeled cells, the amount of radioactive incorporation into gangliosides was determined by placing 50 μl of sample into 6 ml of Ecoscint A (National Diagnostics) scintillation solution and counting dpm on a 1219 Rackbeta Counter (LKB Wallac). The concentration of ganglioside sialic acid from solid tumors grown in vivo was quantified using a resorcinol colorimetric assay and was expressed as μg of sialic acid per 100 mg dry weight, as we described previously (39). Ganglioside distribution was analyzed using HPTLC and quantified as described previously (37, 40).

Cholera toxin B immunostaining and Clostridium perfringens neuraminidase (Sigma) digestion were conducted for preliminary structural studies of gangliosides, as described previously (41).

Immunohistochemistry and Microvessel Density. Blood vessel immunostaining and H&E staining was done on tumors grown intracranially. Briefly, tumors were fixed in 4% formaldehyde and processed for frozen sectioning. The sections (7 μm) were postfixed in 1:1 methanol:acetone (v/v) at −20°C for 10 min, and then endogenous peroxidase was inactivated by incubation in methanol containing 0.3% hydrogen peroxide for 20 min at −20°C. The sections were then blocked with goat serum and incubated with anti-type IV collagen antibody (1/400; Biodesign, Kennebunk, ME) for 2 h at room temperature to highlight blood vessel basement membrane. The primary antibody was detected using the Vectastain avidin-biotin peroxidase kit (Vector Laboratories, Burlingame, CA), according to the manufacturer’s instruction, and was visualized using 3-diaminobenzidine with metal enhancement (Pierce, Rockford, IL). Sections were counterstained with aqueous hematoxylin. Microvessel density was quantified by examining areas of vascular hot spots, as described previously by Weidner et al. (4) with some modifications. Type IV collagen-positive microvessels appeared brown/black in color. Sections were scanned at low magnification (×4 and ×10) for the localization of vascular hot spots. The 10 most vascular areas were determined and then counted for vessel number at high magnification (×40). Only darkly stained vessels, which were clearly separated from each other, were counted. For each tumor, at least two independent sections were stained with H&E and immunostained for blood vessels.

Northern Blot. RNA was isolated from tumors that were grown in the brain to approximately equal size or grown in culture to approximately equal densities as described previously (34). Total RNA (15 or 20 μg) was separated on a denaturing formaldehyde agarose gel, hybridized to a nylon membrane (Gene Screen Plus; DuPont NEN) by capillary action, and then fixed using a UV cross-linker (Stratagene, La Jolla, CA). The blot was hybridized with a 32P-labeled 654-bp probe that recognizes all mouse VEGF splice variants. The probe was a PCR fragment amplified from a cloned VEGF cDNA (by K. P. C.) using the primers (forward, 5′-TGATGCTAGAATGTTTCT-3′; reverse, 5′-GAATCAGCTCGGCTCGCTTC-3′). The blot was stripped and rehybridized with a 32P-labeled actin probe (Stratagene). Bands were visualized and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Stable Transfection of GalNAc-T cDNA Induced Neosynthesis of Complex Gangliosides. The EPEN cells do not express the gene for GalNAc-T, a key enzyme for the synthesis of complex gangliosides, and thus synthesize only ganglioside Gα3 (Fig. 1; Ref. 34). The EPEN cells stably transfected with the mouse GalNAc-T cDNA synthesized complex gangliosides (GM2, GM1, and Gα3) in addition to GM3 (Fig. 1B). The identity of these gangliosides was confirmed using neuraminidase digestion and cholera toxin B immunostaining (data not shown). The ganglioside bands in the region of GM3 and GD1a migrated slightly faster than the standards, possibly due to longer fatty acyl chains in the tumor gangliosides. The trace levels of GM2 found likely result from its rapid use as a precursor for the synthesis of GM1. The synthesized gangliosides migrated on the HPTLC as double bands because of differences in ceramide structure. Although Gα3 comprised 100% of the total radiolabeled gangliosides in the control EPEN and EPEN-V cells, it was reduced to 55% of the total in the EPEN-GNT cells. In addition, the complex gangliosides, GM1 and GD1a, comprised approximately 16 and 26%, respectively, of the total radiolabeled gangliosides in the EPEN-GNT cells (Fig. 1B). These findings indicate that the stable transfection of EPEN cells with the GalNAc-T gene induced the expression of complex gangliosides.

To determine whether the cultured EPEN-V and EPEN-GNT tumor cells shed gangliosides into the medium, we compared the content and distribution of gangliosides in the cultured cells with that in the conditioned medium. The EPEN-V and EPEN-GNT tumor cells shed...
Complex Ganglioside Synthesis Stimulated Brain Tumor Growth in Vivo but not in Vitro. Growth curves were performed to determine whether neosynthesis of complex gangliosides altered the growth of the cultured tumor cells and solid tumors. Although the EPEN, EPEN-V, and EPEN-GNT cells grew at similar rates in culture (Fig. 4A), the EPEN-GNT tumor grew significantly faster than the EPEN and EPEN-V tumors in vivo (Fig. 4B). Therefore, the neosynthesis of complex gangliosides did not affect tumor cell proliferation in the culture environment but stimulated tumor growth in the in vivo host environment.

Complex Ganglioside Synthesis Increased Vascular Density and Mitotic Index. To determine whether changes in ganglioside biosynthesis influenced angiogenesis, we examined tumor morphology and blood vessel densities using H&E staining and type IV collagen immunostaining (Figs. 5 and 6). Although the EPEN-GNT tumor had little or no areas of necrosis, the EPEN and EPEN-V tumors had multiple necrotic regions that were identified in at least two independent sections of each tumor (Fig. 5). The vascular density was 1.7 times greater in the EPEN-GNT tumor than in the control tumors (Figs. 5 and 6A). In addition, the number of thrombotic vessels was less in the EPEN-GNT tumors than in the controls (data not shown). The majority of vessels in the EPEN-GNT tumors appeared to be of typical tumor microvessel morphology. Furthermore, the number of cells undergoing mitosis was significantly greater in the EPEN-GNT tumor than in controls (Fig. 6B). Taken together, these

Fig. 1. Biosynthetic pathway and distribution of gangliosides isolated from cultured EPEN, EPEN-V, and EPEN-GNT cells and conditioned medium. A, ganglioside biosynthetic pathway showing that GalNAc-T is necessary for the synthesis of complex gangliosides. B, HPTLC of 14C-labeled gangliosides from cultured cells and gangliosides shed into the cultured medium. Approximately 3000 and 1000 dpm of radiolabeled gangliosides were spotted for cells and medium, respectively. The plate was developed by one ascending run with chloroform:methanol:water containing 0.02% CaCl2 (55:45:10, v/v/v). After visualizing radiolabeled lanes with a Phosphorimager, the plates were sprayed with resorcinol reagent and heated to 100°C to visualize the standards (STD).

Fig. 2. Detection of GalNAc-T gene expression in vivo using RT-PCR. GalNAc-T mRNA was detected in the EPEN-GNT tumor but was not detected in the EPEN and EPEN-V tumors. The GalNAc-T expression vector and EPEN-GNT cells grown in culture were used as controls for the PCR reaction. No bands were detected when reverse transcriptase was omitted from the cDNA reaction. To ensure that cDNA was present in samples, RT-PCR was performed using N-cadherin primers, where all samples had positive bands.

approximately 5.7 and 6.0% of their total gangliosides, respectively, into the medium after 48 h. The distribution of shed gangliosides was similar to that of the cellular gangliosides for both tumor cell lines. For example, the EPEN-V cells shed only GM3, whereas the EPEN-GNT cells shed GM3, GM1, and GD1a (Fig. 1B). The trace band migrating in the region of GD1a stained with cholera toxin B, suggesting that it was a GM1 structure. Interestingly, the shorter chain fatty acid species (lower band of doublet) were more prevalent in the culture medium, whereas the longer chain fatty acid species (upper band of doublet) were more prevalent in the intact cells (Fig. 1B). These data show that the EPEN-V and EPEN-GNT tumors shed significant amounts of gangliosides into the extracellular environment.

The control and EPEN-GNT cells were grown s.c. in syngeneic B6 mice, where all cell lines readily formed tumors. We confirmed the expression of the transfected GalNAc-T gene in the in vivo EPEN-GNT tumor using RT-PCR (Fig. 2). The ganglioside concentration (μg of sialic acid/100 mg dry weight) of the EPEN, EPEN-V, and EPEN-GNT tumors did not differ significantly and was 44 ± 9.3, 48 ± 3.3, and 42 ± 1.2, respectively (n = 3 independent samples for each tumor, and the values are expressed as means ± SE). However, the relative distribution of gangliosides in the control and EPEN-GNT tumors differed significantly (Fig. 3A). Although GM3, GM1, and GD1a were present in all three tumors, the ratio of complex gangliosides (GD1a) to GM3 was 4–6-fold greater in the EPEN-GNT tumor than in the control tumors (Fig. 3B). It is important to mention that the ganglioside distribution of tumors grown in vivo can differ significantly from that of tumor cells grown in culture because of the presence of host infiltrating cells (e.g., macrophages) in the solid tumor (37).

Fig. 3. HPTLC of gangliosides isolated from EPEN, EPEN-V, and EPEN-GNT tumors grown in vivo. A. ~1.5 μg of ganglioside sialic acid was spotted for each lane. The plate was developed as described in Fig. 1. The bands were visualized by resorcinol spray. STD, standard. B, densitometry of the HPTLC plate was used to calculate the GD1a/GM3 ratio. The values are expressed as the means of three independent tumors; bars, SE.
findings indicate that the expression of complex gangliosides with a concomitant decrease in $G_{M3}$ enhanced both angiogenesis and proliferation of the EPEN-GNT tumor.

**Complex Ganglioside Synthesis Enhanced VEGF Gene Expression in Vivo but not in Vitro.** Because VEGF expression is correlated with tumor angiogenesis, we measured VEGF mRNA levels in the EPEN, EPEN-V, and EPEN-GNT tumors (Fig. 7). Although the tumor cells grown in culture did not differ in VEGF gene expression (Fig. 7B), the EPEN-GNT tumor had significantly greater VEGF mRNA levels than controls when grown in vivo (Fig. 7D). These data suggest that complex ganglioside synthesis stimulated VEGF gene expression in the EPEN-GNT tumor grown in vivo.

**Complex Ganglioside Biosynthesis Enhanced Vascularity in the in Vivo Matrigel Model of Angiogenesis.** The Matrigel angiogenesis model is highly dependent upon host stromal cell infiltration and activation, including monocytes, macrophages, and endothelial precursors. Also, when tumor cells are implanted within Matrigel, their effect on the developing stroma is more selective for soluble factors such as tumor-derived gangliosides. In addition, this model is representative of the early events initiated when tumors are formed, and angiogenic factors and inhibitor balance are critical to the overall angiogenic response. In this case, we determined whether implanting the EPEN-V control vector or EPEN-GNT cell lines would have selective effects upon initial angiogenic events as opposed to alterations in the vascularity of the tumors observed over a period of several weeks. Although both the EPEN-V and EPEN-GNT tumor cells induced angiogenesis, the number and dilation of the vessels was greater in the Matrigel plugs containing EPEN-GNT tumor cells than in plugs containing EPEN-V tumor cells (Fig. 8). Taken together, these findings indicate that EPEN-GNT tumor cells were more angiogenic than the control tumor cells in multiple assays for tumor angiogenesis.

**DISCUSSION**

In this study, we showed that a gene-linked change in ganglioside expression significantly influenced the angiogenic response and growth of an experimental mouse brain tumor. Specifically, the neosynthesis of complex gangliosides $G_{M2}$, $G_{M1}$, and $G_{D1}$ with a concurrent decrease in $G_{M3}$ enhanced the angiogenic phenotype and growth rate of the EPEN brain tumor. This is the first report, to our knowledge, of a ganglioside influence on brain tumor angiogenesis in vivo arising through the genetic manipulation of ganglioside synthesis in the tumor cells. Interestingly, the change in ganglioside biosynthesis did not influence endogenous tumor cell proliferation because the growth rate in vitro was similar in the EPEN-GNT tumor cell line stably transfected with the GalNAc-T gene and in the untransfected or mock-transfected EPEN lines. These findings suggest that gangliosides influence angiogenesis by modulating the response of the host to the tumor.

Support for this hypothesis came from our findings that both the number and quality of blood vessels were greater in the EPEN-GNT
GANGLIOSIDES AND ANGIOGENESIS

Fig. 7. Northern blot analysis of VEGF gene expression in the EPEN, EPEN-V, and EPEN-GNT grown as cell lines in vitro (A) and as solid tumors in vivo (C). Bands were visualized and quantified using a phosphorimager. VEGF mRNA levels in vitro (B; EPEN, n = 3; EPEN-V, n = 6; and EPEN-GNT, n = 6) and in vivo (D; n = 6 for each tumor) were standardized to actin levels and expressed as the means; bars, SE. * VEGF mRNA expression is significantly greater in the EPEN-GNT tumor grown in vivo than in the control EPEN and EPEN-V tumors at the P < 0.01 level (two-tailed t test).

Fig. 8. Tumor cell-induced vascularity in the in vivo Matrigel plug assay. The EPEN-V and EPEN-GNT cells in Matrigel were injected s.c. in nude mice as described in “Materials and Methods.” The Matrigel plugs, with the surrounding skin, were removed on day 7 after implantation. The plugs were photographed at the same magnification (×12.5).

The mechanisms by which gangliosides influence angiogenesis and tumor growth in vivo are not clear. The induction of complex ganglioside biosynthesis in the EPEN-GNT tumor enhanced VEGF gene expression only when the tumor cells were grown in vivo but not when they were grown in vitro. VEGF is an endothelial cell-specific mitogen and is one of the most potent inducers of angiogenesis (13, 16). Although most VEGF synthesized in solid tumors is derived from the neoplastic tumor cells, various host cells (macrophages and fibroblasts) can also synthesize VEGF (12, 48, 49). It is therefore possible that gangliosides significantly influence VEGF gene expression in tumor-associated host cells. Alternatively, gangliosides may influence the number of tumor-infiltrating host cells. Indeed, there is a strong positive correlation between the number of tumor-infiltrating macrophages and the degree of tumor neovascularization (48, 50, 51). Further studies will be needed to determine how tumor gangliosides influence the number and angiogenic properties of tumor-associated host cells.

The shedding of gangliosides from the tumor cell surface into the extracellular milieu is believed to influence tumor-host cell interactions (21, 52, 53). We have shown that the EPEN-V and EPEN-GNT cells shed significant amounts of gangliosides into the culture medium. The EPEN-V cells shed only GM3, whereas the EPEN-GNT cells shed GM1 and the predominant gangliosides (GM1 and GD3). The total amounts shed and the preferential shedding of shorter chain fatty acid species are consistent with previous findings in other murine tumor models and in human brain tumors (21, 54, 55).

Because shed tumor gangliosides can be incorporated into the membranes of neighboring host cells, it is possible that shed gangliosides modulate endothelial cell-specific growth factor receptors, such as the VEGF receptor (56). Previous studies showed that gangliosides modify the function of tyrosine kinase receptors similar to the VEGF receptor (22). It is also possible that shed gangliosides alter the extracellular matrix, which could influence endothelial cell/matrix adhesion, thereby altering endothelial cell proliferation and migration (20). Taken together, these findings suggest that tumor angiogenesis may be significantly influenced by both the content and composition of shed gangliosides.

The most malignant human brain tumors (anaplastic astrocytoma and glioblastoma multiforme) are highly vascular and generally contain GM1 and GD1a as the predominant gangliosides (57–62). Because GD3 stimulates the release of VEGF in glioblastoma cell lines and colocalizes with areas of vascular hot spots, it is suggested that GD3 enhances malignancy by stimulating angiogenesis (28, 30). Although GM3 is also elevated in these tumors, its concentration is generally less than that of GD3 and may, therefore, be unable to counter the proangiogenic effects of GD3. According to our findings and those of Ziche et al. (28), it is the balance of GM3 to complex gangliosides, including GD3, that is most critical for determining angiogenesis. We suggest that increasing the ratio of GM3 to complex gangliosides may be
GANGLIOSIDES AND ANGIOGENESIS

effective in reducing angiogenesis and growth in human glioblastomas.

Our findings suggest that the composition of brain tumor gangliosides can significantly influence the angiogenic response of the host and the growth of the tumor. Because gangliosides are synthesized and shed by all tumors examined to date, it will be important to define the mechanism(s) by which gangliosides modulate tumor angiogenesis.

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