Ganglioside GM3 inhibits VEGF/VEGFR-2-mediated angiogenesis: Direct interaction of GM3 with VEGFR-2

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Angiogenesis is associated with growth, invasion, and metastasis of human solid tumors. Aberrant activation of endothelial cells and induction of microvascular permeability by a vascular endothelial growth factor (VEGF) receptor-2 (VEGFR-2) signaling pathway is observed in pathological angiogenesis including tumor, wound healing, arthritis, psoriasis, diabetic retinopathy, and others. Here, we show that GM3 regulated the activity of various downstream signaling pathways and biological events through the inhibition of VEGF-stimulated VEGFR-2 activation in vascular endothelial cells in vitro. Furthermore, GM3 strongly blocked VEGF-induced neovascularization in vivo, in models including the chick chorioallantoic membrane and Matrigel plug assay. Interestingly, GM3 suppressed VEGF-induced VEGFR-2 activation by blocking its dimerization and also blocked the binding of VEGF to VEGFR-2 through a GM3-specific interaction with the extracellular domain of VEGFR-2, but not with VEGF. Primary tumor growth in mice was inhibited by subcutaneous injection of GM3. Immunohistochemical analyses showed GM3 inhibition of angiogenesis and tumor cell proliferation. GM3 also resulted in the suppression of VEGF-stimulated microvessel permeability in mouse skin capillaries. These results suggest that GM3 inhibits VEGFR-2-mediated changes in vascular endothelial cell function and angiogenesis, and might be of value in anti-angiogenic therapy.

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Introduction

Angiogenesis, the formation of new blood capillaries from pre-existing blood vessels, is an important biological process for embryonic and early postnatal development, skeletal growth, and reproductive functions related to physiological conditions. Pathological angiogenesis is associated with solid tumors, hematological malignancies, intraocular neovascular syndromes, rheumatoid arthritis, psoriasis, and others (Risau 1997; Ferrara et al. 2003). Vascular endothelial growth factor (VEGF) is a significant angiogenic-factor in physiological and pathological angiogenesis (Ferrara et al. 2003; Ferrara 2004). Several studies have shown that the expression of the VEGF gene is enhanced in a variety of angiogenic diseases including tumors, diabetic retinopathy, retinal ischemia and inflammatory psoriasis, and others (Folkman 1971; Aiello et al. 1994; Detmar et al. 1994; Ferrara et al. 2003; Ferrara 2004). VEGF affects the migration, permeability, and proliferation of endothelial cells during pathological angiogenesis (McMahon 2000; Ferrara et al. 2003; Ferrara 2004). It is generally known that VEGF binds to two receptor tyrosine kinases including VEGFR-1/Flt-1 and VEGFR-2/Flk-1/KDR exclusively expressed in vascular endothelial cells. However, several studies demonstrated differences in the biological functions of the two receptors in endothelial cells. Among these VEGF receptors, the activation of VEGFR-2 is sufficient to mediate VEGF effects on proliferation, migration, and vascular permeability of endothelial cells for angiogenesis (Waltenberger et al. 1994; Bernatchez et al. 1999; McMahon 2000; Ferrara et al. 2003; Ferrara 2004). Moreover, a number of researches have shown that VEGFR-2 plays an important role in some pathological angiogenesis (McMahon 2000; Ferrara 2004). Thus, many investigators have developed drugs targeting the inhibition of the VEGF-mediated VEGF receptor signal pathway for antiangiogenesis because VEGF and its receptors are closely associated with neovascularization in the progression of pathological diseases including tumor.

Cell surface carbohydrates, which are attached to proteins and lipids, are major components of the outer surface of mammalian cells (Fukuda and Hindsgaul 2000). In mature organisms, the expression of distinct carbohydrates is eventually restricted to specific cell type. However, pattern changes of these cell surface glycans have an effect on various pathological diseases including tumor (Fukuda and Hindsgaul 2000). Gangliosides, sialylated glycosphingolipids, are ubiquitous components of vertebrate cells. Compositional changes of gangliosides, which are localized on the plasma membrane and contribute to the membrane structure and organization, are associated with cellular proliferation, differentiation, adhesion, signal transduction, tumorigenesis, metastasis, and angiogenesis (Varki 1993; Hakomori 2002; Miljan et al. 2002; Miljan and Bremer 2002). It is well known that gangliosides are secreted in the surrounding

microenvironment by tumor cells. Ladisch et al. (1983) have reported that shedding of gangliosides protect tumor cells from host immune destruction. Furthermore, it has been shown that gangliosides shed from tumor enhance tumor progression (Liu et al. 2006). However, a precise molecular mechanism for tumor angiogenesis regulated by gangliosides is poorly understood.

In this study, we have found for the first time that among the gangliosides, GM3 is the only one which directly interacts with VEGFR-2, which inhibits VEGF/VEGFR-2-mediated biological function of vascular endothelial cell and angiogenesis both in vitro and in vivo, the growth of primary tumors in mice inoculated with tumor cells and VEGF-stimulated microvessel permeability in mouse skin capillaries. These results suggest that GM3 is angiogenic inhibitor and might be a therapeutic avenue for antiangiogenesis.

Material and methods

Cell culture and transfection

Human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (MD), cultured in a sterile endothelial growth medium (EGM-2, Cambrex Bio Science), and were maintained at 37°C in a humidified 5% CO_2 incubator. Passages 5–8 of HUVECs were used in this study.

XTT proliferation assay

Cell proliferation was investigated using a commercially available proliferation kit II (XTT, Roche, Mannheim, Germany). Briefly, HUVECs were subcultured into 96-well culture plates at a density of 1×10^3 cells/well in 100 μL of EGM-2 medium. After 24 h of incubation, the medium was discarded and replaced with 100 μL of EBM-2 containing 1% FBS for starvation. The cells were treated in the presence or absence of VEGF (R&D System Inc., MN) with various concentrations of GM3 (Alexis Biochemicals, ALX-302-005, CA). The plates were incubated in a 37°C humidified incubator in a 5% CO_2 atmosphere for 24 or 48 h. At the end of the incubation, 50 μL of XTT test solution prepared by mixing 5 mL of XTT-labeling reagent and 100 μL of electron coupling reagent was added to each well. After 4 h of incubation at 37°C and a 5% CO_2 incubator, the absorbance was measured on an ELISA reader (Molecular Devices, CA) at a test wavelength of 490 nm.

Western blot analysis

After treatments, each HUVEC was homogenized in a buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% NaN_3, 100 μg/mL phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL aprotinin, and 1% Triton X-100. Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, CA). Thirty-microgram samples of total cell lysates were separated by SDS–PAGE and transferred to nitrocellulose membranes using the Hoefer electrotransfer system (Amersham Biosciences, UK). To detect target proteins, the membranes were incubated with the PY20, AKT, p-AKT, ERK, p-ERK, p-IκBα, N-terminus-specific VEGFR-2 (Q20), C-terminus-specific VEGFR-2 (A-3), VE-Cadherin, FAK, paxillin (SantaCruz Biotechnology, USA), and p-VEGFR-2 (Cell Signaling Technology, Danvers, MA, USA) antibodies, respectively. The detection was performed using a secondary horseradish

peroxidase-linked anti-rabbit IgG antibody, anti-goat IgG antibody and anti-mouse IgG antibody, and the ECL chemiluminescence system (Amersham Biosciences).

Immunoprecipitation

The total protein (100 μg) of HUVECs was incubated with the anti-VE-cadherin or anti-FAK antibodies (2 μg) at 4°C overnight with shaking in a 250 μL of immunoprecipitation (IP) buffer (50 mM Tris–HCl, pH 7.4, 0.5% NP-40, 150 mM NaCl, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM dithiothreitol (DTT), 20 μg/mL aprotinin, 20 μg/mL leupeptin, and 1 mM PMSF), followed by incubation with 20 μL of the protein A/G-agarose beads (SantaCruz Biotechnology) for 4 h at 4°C. Protein complexes were washed five times with the IP buffer and released from the beads by boiling in a 2 × SDS sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 2% glycerol, and 0.02% bromophenolblue) for 5 min. The reaction mixture was resolved by a 10% SDS–PAGE gel, and then transferred onto a nitrocellulose membrane by electroblotting and probed with the anti-p-tyrosine, anti-VE-cadherin, and anti-FAK antibodies. The blot was developed using an ECL kit.

Immunofluorescence microscopy

HUVECs were seeded on 12 mm sterilized and gelatin-coated coverslips in 6-well tissue culture plates. After the treatment of HUVECs with or without VEGF in the presence or absence of GM3, they were fixed in 3.7% formalin. The cells were then permeabilized with 0.5% Triton X-100 in PBS and washed three times with PBS. Non-specific sites were then blocked with 1% bovine serum albumin-containing PBS for 30 min at room temperature with gentle rocking. Thereafter, a solution of FAK or paxillin antibodies was flooded over the cells and the cultures were incubated at 4°C for overnight. After washing with PBS, the cells were further incubated with Alexa Flour 594-conjugated goat anti-mouse IgG (Molecular Probes, USA) and Alexa Flour 488-conjugated goat anti-rabbit IgG (Molecular Probes) for 1 h at room temperature, followed by washing with PBS, and then analyzed using a fluorescence microscope. The preabsorbed primary antibody or the secondary antibody alone was used as a negative control.

Immunohistochemistry

Tumor specimens were immediately removed from euthanized mice and fixed with 3.7% formalin in PBS, and then embedded in paraffin. All samples were cut into 4 μm serial sections. The sections were immunostained with anti-CD34 (Dako, USA) and proliferating cell nuclear antigen (PCNA, Dako) antibodies, visualized with a Dako EnVision kit (Dako), and counterstained with hematoxylin.

Tube formation assay

The inhibitory effect of GM3 on the activity of VEGF-induced HUVECs to form a capillary-like network was investigated with Matrigel-coated 24-well culture plates. Matrigel (13.9 mg/mL) was thawed at 4°C for overnight and mixed with an EBM-2 medium at a 1:1 ratio. The 70 μL of EBM-2-diluted Matrigel (6.95 mg/mL) was added to each well of the 24-well culture plates and allowed to polymerize at 37°C for 1 h. The HUVECs, to be tested for tube formation, were detached from the tissue

culture plates, washed, resuspended in the EBM-2 medium containing 1% FBS (1×10^4 cells/well), and subsequently were added to the Matrigel-coated wells with various concentrations of GM3 in the presence or absence of VEGF. The plates were incubated at 37°C for 24 h in 5% CO_2 atmosphere. After incubation, capillary-like tube formation of each well in the culture plates was photographed with a Nikon light microscope.

Migration assay

The migration assay of HUVECs was performed using 24-well chambers containing polycarbonate filter inserts (Corning Incorporated, USA). The upper sides of the filters with 8 µm pore size were coated with 100 µL of 0.2% gelatin. The EBM-2 medium containing 1% FBS with or without VEGF was added to the lower compartment of the chamber. The HUVECs being tested for migration were detached from the tissue culture plates, washed, resuspended in the EBM-2 medium containing 1% FBS (5×10^4 cells/200 µL), and then added to the upper compartment of the chamber in the presence or absence of GM3. The chambers were incubated at 37°C for 24 h in 5% CO_2 atmosphere. After incubation, the filter inserts were removed from the wells of chamber and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, stained, and mounted on microscope slides. The cells that invaded through the gelatin and that were located on the underside of the filter were counted. The values obtained were calculated by averaging the total number of cells from three filters.

Matrigel plug assay

For the Matrigel plug assay, C57BL/6 mice (Koatech, Kyunggi-Do) were subcutaneously injected with 500 µL of Matrigel and heparin (50 Unit/mL) mixture without or with VEGF (100 ng/mL) in the presence or absence of GM3 (30 and 50 µM). After 7 days, the mice were euthanized, and the Matrigel plugs were then removed, fixed with 3.7% formalin in PBS, and embedded in paraffin, and then they were cut into 4 µm serial sections. The sections were stained with hematoxylin and eosin (H&E) solutions for microscopic observation. To quantify the formation of functional blood vessels, the amounts of hemoglobin (Hb) in the Matrigel plugs were measured using Drabkin's reagent (Sigma, USA).

Chorioallantoic membrane assay

The in vivo angiogenic activity was assayed using a chorioallantoic membrane (CAM) assay. GM3 (30, 50 µM/egg) and VEGF (100 ng) were loaded onto a 1/4 piece of thermonox disk (Nunc, USA) and the dried thermonox disk was applied to the CAM of a 7-day-old embryo. After 13 days of incubation, a fat emulsion was injected under the CAM for the better viewing of the blood vessels. A number of newly formed blood vessels were counted and the experiment was repeated twice with 15 eggs in each group.

Assay determining the interaction of GM3 with VEGFR-2 or VEGF

To confirm whether GM3 binds to the VEGFR-2 expressed on the surface of HUVECs or VEGF, the total protein (100 µg) of HUVECs or VEGF (1 µg) was incubated with GM3 (30 µM) in a 300 µL of IP buffer at 4°C overnight. Ten micrograms of monoclonal GM3 antibody (M2590, Biotest Laboratories,

Japan) was added to the GM3 proteins or GM3-VEGF mixture at 4°C overnight with shaking, followed by incubation with 20 µL of the protein L-agarose beads (SantaCruz Biotechnology) for 4 h at 4°C. The next steps were performed like the immunoprecipitation method for SDS–PAGE and electroblotting. The membranes were incubated with the VEGF antibody or VEGFR-2 antibody for ExD detection. The immunodetection was performed using an ECL chemiluminescent kit.

Assay confirming the interaction of GM3 or other gangliosides with ExD of VEGFR-2

To investigate whether GM3 interacts with ExD of VEGFR-2, first of all, mRNAs isolated from HUVECs were used as a template for the construction of a subclone containing the VEGFR-2 ExD region (2238 bp). The primers used were 5′-AAGGTACCATGCAGAGCAAGGTGCTGCT-3′ (sense) and 5′-ACTCTAGATGCACAGCCAAGAACACTGC-3′ (antisense). The PCR product of the VEGFR-2 ExD region amplified from mRNAs of HUVECs was subcloned into the *Kpn* I-*Xba* I multicloning sites of pcDNA^TM3.1/*myc*-His A (Invitrogen, USA). After sequencing, VEGFR-2 ExD, used in this study, is identified through BLAST search of National Center for Biotechnology Information (ref. accession no. NP_002244.1). The DNA construct of VEGFR-2 ExD was transfected into ECV304 cells. The VEGFR-2 ExD-His fusion proteins were purified using a Chelating Excellose Spin Kit (Bioprogen Co., Korea) and Sephadex G50 gel filtration. The isolated VEGFR-2 ExD proteins were identified using SDS–PAGE and Western blot analysis. The purified and identified VEGFR-2 ExD proteins were used to confirm whether the VEGFR-2 ExD protein (1 µg) interacts with GM3 or other gangliosides (30 µM). The VEGFR-2 ExD protein was incubated with each ganglioside in 300 µL of IP buffer at 4°C overnight. Antiganglioside monoclonal antibodies (10 µg, Seikagaku Co., Japan) were incubated with corresponding ganglioside and VEGFR-2 ExD protein mixtures at 4°C overnight with shaking, followed by incubation with 20 µL of the protein L-agarose beads for 4 h at 4°C. The following steps were performed like the immunoprecipitation method for SDS–PAGE and electroblotting. The membranes were incubated with VEGFR-2 antibodies for N- and C-terminus detection. The immunodetection was performed using an ECL chemiluminescence kit. In addition, to further confirm the interaction of GM3 or other gangliosides with ExD of VEGFR-2 using HPTLC, the VEGFR-2 ExD protein was incubated with each of ganglioside in a 300 µL of IP buffer at 4°C overnight. The anti-VGFER-2 ExD antibody (2 µg) was added to each ganglioside and VEGFR-2 ExD protein mixtures and incubated at 4°C overnight with shaking, followed by incubation with 20 µL of the protein G PLUS-agarose beads for 4 h at 4°C. Each of gangliosides interacting with VEGFR-2 ExD was released from the beads with methanol. The gangliosides solved in methanol were applied and developed onto a HPTLC plate with chloroform–methanol–0.2% $CaCl_2$ (55:45:10). After the separation step, the gangliosides were viewed by spraying the plates with a resorcinol-hydrochloric acid reagent.

VEGFR-2 dimerization assay

HUVECs were confluently cultured in 100 mm tissue culture dishes, washed twice with PBS, preincubated with GM3 in the EBM medium containing 1% FBS for 6 h, and exposed to VEGF

(50 ng/mL) for 5 or 10 min. The cells were then washed twice with ice-cold PBS, incubated with 5 mL PBS containing BS[3], (bis(sulfosuccinimidyl) substrate) (1 mg/mL) on ice for 30 min, washed twice with ice-cold PBS, and lysed in a 1 mL lysis buffer. The total VEGFR-2 was immunoprecipitated with an anti-VEGFR-2 antibody. About 500 µg of lysate protein was loaded onto a 6% SDS–PAGE gel. The dimerization of VEGFR-2 was detected by Western blot using an anti-VEGFR-2 antibody.

Animal studies with Lewis lung carcinoma, melanoma, and colon carcinoma

Lewis lung carcinoma (LLC), melanoma (B16F10), and colon carcinoma (CT26) cells were separately suspended in PBS and inoculated in the subcutaneous dorsa of 4- to 6-week-old C57BL/6 and BALB/c mice. Three days after the inoculation of tumor cells, GM3 or PBS were intraperitoneally injected in the mice each day. Tumor volumes were measured with a caliper every 3 days and calculated according to the formula $((l \times w^2)/2)$, where l and w stand for length and width, respectively. All mice were euthanized 18 days after the inoculation of tumor cells and the tumors were excised and weighed. Tumor specimens were immediately removed from euthanized mice and were prepared for histological examination.

Miles assay

BALB/c mice were injected subcutaneously with GM3 or PBS each day. Three days later, they anesthetized with ethyl ether, and 100 µL of 1% Evans blue solution in PBS was injected into the tail vein. After 20 min, 50 µL of VEGF (1 ng/µL) or PBS was injected intradermally into the preshaved back skin. After 20 min, the mice were euthanized. The extravasation of Evans blue in the injected area was photographed.

Results

Suppression of VEGF-induced angiogenesis in vitro and in vivo by GM3

In some pathological angiogenesis, VEGF-mediated VEGFR-2 activation is closely associated with angiogenic activities such as proliferation, migration, survival, and tube formation of endothelial cell via the activation of VEGFR-2 downstream signal pathways (McMahon 2000; Matsumoto and Claesson-Welsh 2001; Ferrara 2004). VEGF-induced cell survival of endothelial cells is associated with PI-3K/AKT activation as a VEGFR-2 downstream activation (Gerber et al. 1998). In addition, VEGF-mediated VEGFR-2 activation results in the activation of PI-3K/AKT-dependent cell migration (Gille et al. 2001). Furthermore, PKC-dependent RAF/MEK/ERK activation in response to VEGF-stimulated VEGFR-2 activation is required for endothelial cell proliferation (Takahashi et al. 1999). The tyrosine phosphorylation of VE-cadherin and catenin stimulated by VEGF-mediated VEGR-2 activation is involved in the loosening of cell–cell contacts in established vessels and results in the modulation of endothelial cell permeability and the sprouting and migration of endothelial cells (Esser et al. 1998). The tyrosine phosphorylation of FAK and paxillin induced by VEGF plays a significant role in cell migration through the activation of VEGFR-2 (Abedi and Zachary 1997). Thus, we checked antiangiogenic activities of GM3 in VEGF-stimulated HUVECs. Al-

though GM3 slightly inhibited VEGF-induced proliferation of HUVECs at 24 h after GM3 treatment in a dose-dependent manner (data not shown), as shown in Figure 1A, GM3 significantly inhibited VEGF-induced proliferation of HUVECs in a dose-dependent manner at 48 h after GM3 treatment. However, GM3 had no effect on proliferation of HUVECs untreated with VEGF even in various different doses. GM3 significantly suppressed the VEGF-induced migration of HUVECs. In VEGF-stimulated signal pathways of HUVECs, GM3 markedly diminished the activation of VEGF-induced AKT and ERKs (Figure 1D). Furthermore, VEGF-stimulated tyrosine phosphorylation of VE-cadherin was clearly inhibited by GM3 in HUVECs (Figure 1E). In addition, GM3 effectively suppressed VEGF-mediated the activation of FAK (Figure 1F). As shown in Figure 1G, anti-FAK stained dots cluster at the cell edges indicated with white arrows and the increase of focal adhesions immunostained with anti-FAK in VEGF-induced HUVECs were prominently inhibited by GM3. Moreover, GM3 obviously blocked the increase of antipaxillin immunostaining in focal adhesions and in filamentous arrays of VEGF-stimulated HUVECs. These results suggest that GM3 effectively inhibits angiogenic activities of VEGF-induced HUVECs. To further confirm whether GM3 inhibits VEGF-stimulated neovascularization in vivo, Matrigel plug and CAM assays were performed. As shown in Figure 1H and I, the Matrigel plugs with VEGF excised from mice revealed a dark red color and the formation of new blood vessels. In addition, hemoglobin content was 6.1-fold higher in the Matrigel treated with VEGF than the Matrigel alone (Figure 1J). In contrast, the Matrigel plugs treated with VEGF and GM3 were light red and yellowish in color (Figure 1H), indicating the reduction of neovascularization in the GM3 treatment (Figure 1I). GM3 also decreased VEGF-induced hemoglobin contents in a dose-dependent manner (Figure 1J). These results indicate that GM3 significantly suppresses the VEGF-induced formation of new blood vessels including abundant red blood cells and hemoglobins in the Matrigel plugs. The inhibitory effect of GM3 on VEGF-stimulated angiogenesis was further investigated using the CAM assay. As shown in Figure 1K and L, GM3 markedly inhibited the formation of new blood capillaries from preexisting blood vessels induced by VEGF. The results demonstrate that GM3 clearly blocks VEGF-induced neovascularization in vivo.

Direct interaction of GM3 with VEGFR-2 expressed in HUVECs

Since GM3 inhibits the activation of downstream signal pathways through VEGF-induced VEGFR-2 activation and angiogenic activity in vitro and in vivo (Figure 1), we presumed that GM3 directly interacts with VEGFR-2 or VEGF. Thus, we confirmed whether GM3 directly binds to VEGFR-2 expressed on the surface of HUVECs or VEGF itself using the immunoprecipitation method which is described in *Material and methods*. The total proteins of HUVECs without the exogenous addition of GM3 as a positive control were immunoprecipitated by VEGFR-2 ExD-specific antibody and protein G PLUS-agarose. As shown in Figure 2A, the positive control shows that the VEGFR-2 protein is detected in the immunoprecipitates obtained by the VEGFR-2 ExD-specific antibody as evidenced by Western blot analysis. Next, the total proteins of HUVECs with the exogenous addition of GM3 were immunoprecipitated by
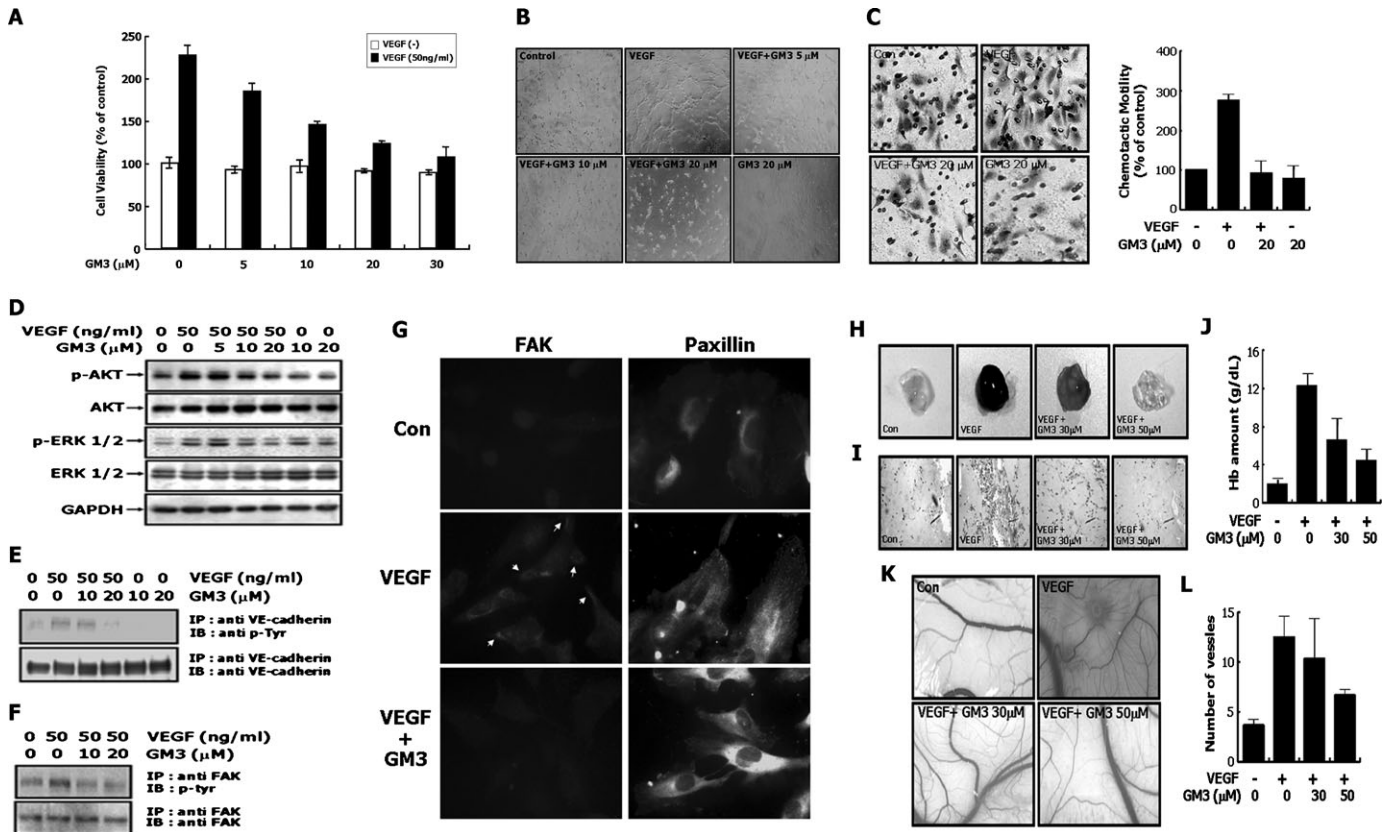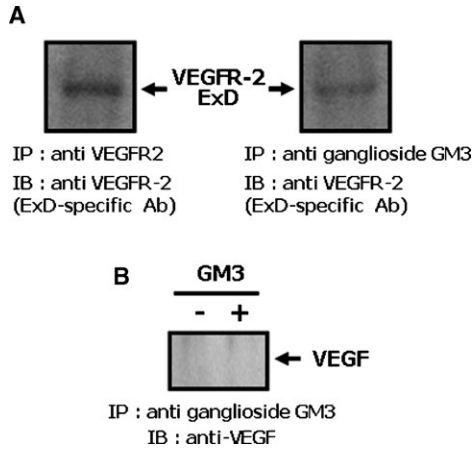
Fig. 1. Inhibitory effects of GM3 on neovascularization stimulated by VEGF in vitro and in vivo. (**A**) The proliferation of HUVECs was determined using an XTT proliferation kit. Data are expressed as the mean ± SD of three independent experiments. (**B**) HUVECs were cultured in the Matrigel-coated plates with various concentrations of GM3 in the presence or absence of VEGF (50 ng/mL) for tube formation assay. Twenty-four hours after incubation, capillary-like tube formation of each well in the culture plates was photographed using a Nikon light microscope. (**C**) Migration assay was performed with or without VEGF (50 ng/mL) in the absence or presence of GM3, as described in *Material and methods*. The total number of cells that migrated in the underside of the filters was counted at 24 h after incubation. Data are expressed as the mean ± SD of three independent experiments. (**D**) Confluent HUVECs were starved in EBM-2 containing 1% FBS for 6 h. The cells were pretreated with various concentrations of GM3 for 1 h and stimulated by the addition of VEGF for 15 min. The cell lysates were analyzed by Western blot analysis with anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-AKT, and anti-AKT antibodies. The cell extracts immunoprecipitated with (**E**) anti-VE-cadherin and (**F**) anti-FAK were separated by SDS–PAGE (IP). Immunoblotting (IB) was performed with anti-phospho-tyrosine, anti-VE-cadherin, and anti-FAK antibodies. (**G**) HUVECs were seeded on gelatin coated-coverslips in 6-well tissue culture plates. The cells were preincubated with GM3 (20 μM) for 1 h and then treated with VEGF (50 ng/mL) for 15 min. The cells were fixed, permeabilized, and then immunostained with FAK and paxillin antibodies. The immunostained cells were analyzed using a fluorescence microscope. For in vivo studies, C57BL/6 mice were subcutaneously injected with a 500 μL of Matrigel and heparin (50 Unit/mL) mixture with or without VEGF (100 ng/mL) in the presence or absence of GM3 (30 and 50 μM). After 7 days, mice were euthanized and the Matrigel plugs were removed. (**H**) The Matrigel plugs with or without VEGF in the presence or absence of GM3 were photographed. (**I**) The Matrigel plugs were excised, fixed with 3.7% formalin in PBS, and embedded in paraffin, and then they were cut into 4 μm serial sections. The sections were stained with hematoxylin and eosin (H&E) solutions. The stained sections were observed using a microscope and photographed. (**J**) To quantify the formation of functional blood vessels, the amount of hemoglobin (Hb) in the Matrigel plugs was measured using Drabkin's reagent. Data are expressed as the mean ± SD. For each group, $n = 5$. (**K**) GM3 (30, 50 μM/egg) with or without VEGF (100 ng) were loaded on the CAMs of a 7-day-old embryo. After a 13-day incubation period, a fat emulsion was injected under the CAM for better visualization of the blood vessels. Thermonox and surrounding CAMs were photographed. (**L**) The number of newly formed blood vessels was counted. Data are expressed as the mean ± SD. For each group, $n = 15$.

GM3-specific antibody (M2590) and protein L-agarose. Thus, the immunoprecipitates containing VEGFR-2 were detected by the VEGFR-2 ExD-specific antibody (Figure 2A). In contrast, as shown in Figure 2B, the immunoprecipitates of VEGF and exogenously added GM3 with an anti-GM3 antibody did not contain VEGF. These results indicate that GM3 did not bind to VEGF but directly interacts with the VEGFR-2 of HUVECs.

GM3-specific suppression of VEGF-stimulated VEGFR-2 activation

We confirmed the direct and specific interaction of VEGFR-2 ExD and GM3 using other gangliosides. The purified VEGFR-2 ExD with or without gangliosides in the IP buffer was incubated

with each ganglioside-specific antibody. As shown in Figure 3A, the immunoblot analysis shows that the band indicating the interaction of VEGFR-2 ExD with GM3, which was immunoprecipitated with two anti-GM3 antibodies (M2590 and GMR6), was detected by the VEGFR-2 ExD-specific antibody, confirming that GM3 directly binds to VEGFR-2 ExD. However, we found that GM1, GD3, GD2, GD1a, or GT1b did not interact with VEGFR-2 ExD (Figure 3A). To further confirm the interaction of GM3 or other gangliosides with ExD of VEGFR-2, the purified VEGFR-2 ExD with or without gangliosides in the IP buffer was immunoprecipitated with the VEGFR-2 ExD-specific antibody and the extracted gangliosides from the immunoprecipitates were analyzed by HPTLC. As shown in Figure 3B, only the GM3 ganglioside was detected by HPTLC

A

IP : anti VEGFR2
IB : anti VEGFR-2
(ExD-specific Ab)

IP : anti ganglioside GM3
IB : anti VEGFR-2
(ExD-specific Ab)

B

IP : anti ganglioside GM3
IB : anti-VEGF

Fig. 2. Direct interaction of GM3 with VEGFR-2 of HUVECs. The total protein of HUVECs or VEGF was incubated with GM3 in a 300 μL of IP buffer. The GM3-total proteins of HUVECs or GM3-VEGF mixture were immunoprecipitated with a monoclonal GM3 antibody (M2590, Biotest Laboratories) and the protein L-agarose beads. The immunoprecitates were analyzed by SDS–PAGE. Western blot analysis was performed with (**A**) the VEGFR-2 antibody for ExD detection and (**B**) VEGF antibody.

in the precipitates prepared from the incubation mixture containing VEGFR-2 and GM3. No bands were detected in the immunoprecipitates prepared from the mixture containing exogenously added ceramide, lactosylceramide, GM2, GM1, GD3, GD2, GD1a, and GT1b. We have concluded that of the tested gangliosides only GM3 directly interacts with ExD of VEGFR-2 but not VEGF. In our previous studies, GM3 had an inhibitory effect on VEGF-induced angiogenic activities in vitro and in vivo. Thus, we assumed that the activation of VEGFR-2 on the surface of VEGF-induced endothelial cells is negatively regulated by GM3. As shown in Figure 3C, GM3 strongly reduced VEGF-stimulated VEGFR-2 phosphorylation of endothelial cells in a dose-dependent manner. This result suggests that GM3 is related to antiangiogenic activities by inhibiting various downstream signalings triggered by VEGF-enhanced VEGFR-2 activation in endothelial cells. Further, we investigated whether GM3 is the only ganglioside which inhibits VEGFR-2 activation stimulated by VEGF. Interestingly, only GM3 significantly inhibited VEGF-induced VEGFR-2 activation (Figure 3D). The preincubation of the anti-GM3 antibody canceled the suppression of VEGF-induced VEGFR-2 activation with GM3 (Figure 3D). These results clearly indicate that GM3 plays a critical role in the VEGF-mediated antiangiogenic activity through the direct interaction of GM3 and VEGFR-2 ExD. The binding of VEGF to VEGFR-2 induces dimerization and phosphorylation of VEGFR-2 (Matsumoto and Claesson-Welsh 2001). Furthermore, our previous data suggest that GM3 suppresses VEGF-stimulated VEGFR-2 phosphorylation through the direct binding of GM3 with VEGFR-2 (Figure 3). Thus, we further examined whether this direct interaction of GM3 with VEGFR-2 ExD results in the blocking of VEGF binding to VEGFR-2 ExD using the immunoprecipitation method which is described in *Material and methods*. One of the two samples was allowed to incubate GM3 and VEGFR-2 ExD together with VEGF in the IP buffer. The reaction of the other sample occurs as follows: VEGFR-2 ExD in the IP buffer was preincubated with GM3 and subsequently incubated with VEGF. After the incubations,

the two samples were immunoprecipitated by the VEGFR-2 ExD-specific antibody and the protein G PLUS-agarose. VEGF and VEGFR-2 ExD without the addition of GM3 were used as a positive control, incubated, and immunoprecipitated similar to the above-mentioned method. As shown in Figure 3E, the positive control shows that VEGF is detected in the immunoprecipitates obtained by the VEGFR-2 ExD-specific antibody on both Western blots with anti-VEGF and VEGFR-2 ExD antibodies. However, the immunoprecipitates, which were obtained by the VEGFR-2 ExD-specific antibody from the sample of GM3, VEGFR-2 and VEGF mixture, or the sample of VEGF and VEGFR-2 preincubated with GM3, contained only small amounts of VEGF or hardly contain VEGF, indicating that the interaction of GM3 with VEGFR-2 ExD interrupts binding of VEGF to VEGFR-2 ExD. We further investigated whether GM3 inhibits VEGF-stimulated VEGFR-2 dimerization in HUVECs because GM3 suppresses VEGF-mediated VEGFR-2 activation as well as the interaction of VEGF with VEGFR-2. As shown in Figure 3F, VEGF enhanced the dimerization of VEGFR-2 in HUVECs. However, GM3 clearly hindered the dimerization of VEGFR-2 induced by VEGF. These results suggest that GM3 suppresses dimerization and phosphorylation of VEGFR-2 by blocking the interaction between VEGF and VEGFR-2.

Antiangiogenic effect of GM3 on tumor growth in mice with tumor cells and VEGF-mediated vascular permeability

To confirm the inhibitory effect of GM3 on the progression of a primary tumor, GM3 (0.1, 0.3, 0.5 and 1 mg/kg) or PBS was intraperitoneally injected in C57BL/6 mice inoculated with LLC cells each day. Tumor volumes were measured with caliper every 3 days. As shown in Figure 4A, GM3 significantly reduced tumor volume in a dose-dependent manner. Furthermore, tumor weight showed a marked reduction in the GM3-treated group compared with PBS-treated group (Figure 4B). As shown in Figure 4C, GM3 obviously resulted in a decrease of the tumor size in a dose-dependent manner. These results suggest that GM3 suppresses the growth of a primary tumor in mice. Because the inhibition of primary tumor growth is associated with antiangiogenic activity and antiproliferation, we further investigated whether GM3 suppresses tumor angiogenesis and proliferation in primary tumor using CD34 and PCNA which are known as microvessel and proliferation markers, respectively. As shown in Figure 4D, immunohistochemical analysis of the residual tumor section showed a significant decrease of CD34 and PCNA staining in the GM3-treated group compared with the PBS-treated control group. These results suggest that GM3 triggers tumor regression via antiangiogenic activity. To further confirm the inhibitory effect of GM3 on the progression of other primary tumor, GM3 (0.5 and 1 mg/kg) or PBS was intraperitoneally injected in mice inoculated with melanoma and colon carcinoma cells each day. Tumor volume and weight were measured after euthanization. As shown in Figure 4E, GM3 significantly reduced tumor volume in a dose-dependent manner. Furthermore, tumor weight showed a marked reduction in the GM3-treated group compared with PBS-treated group. Additionally, GM3 obviously resulted in a decrease of the tumor size in a dose-dependent manner. These results suggest that GM3 suppresses the growth of primary tumors in mice melanoma and colon carcinoma cells as well as LLC cells. VEGF in pathological angiogenesis such as tumors, wound healing, arthritis, psoriasis,
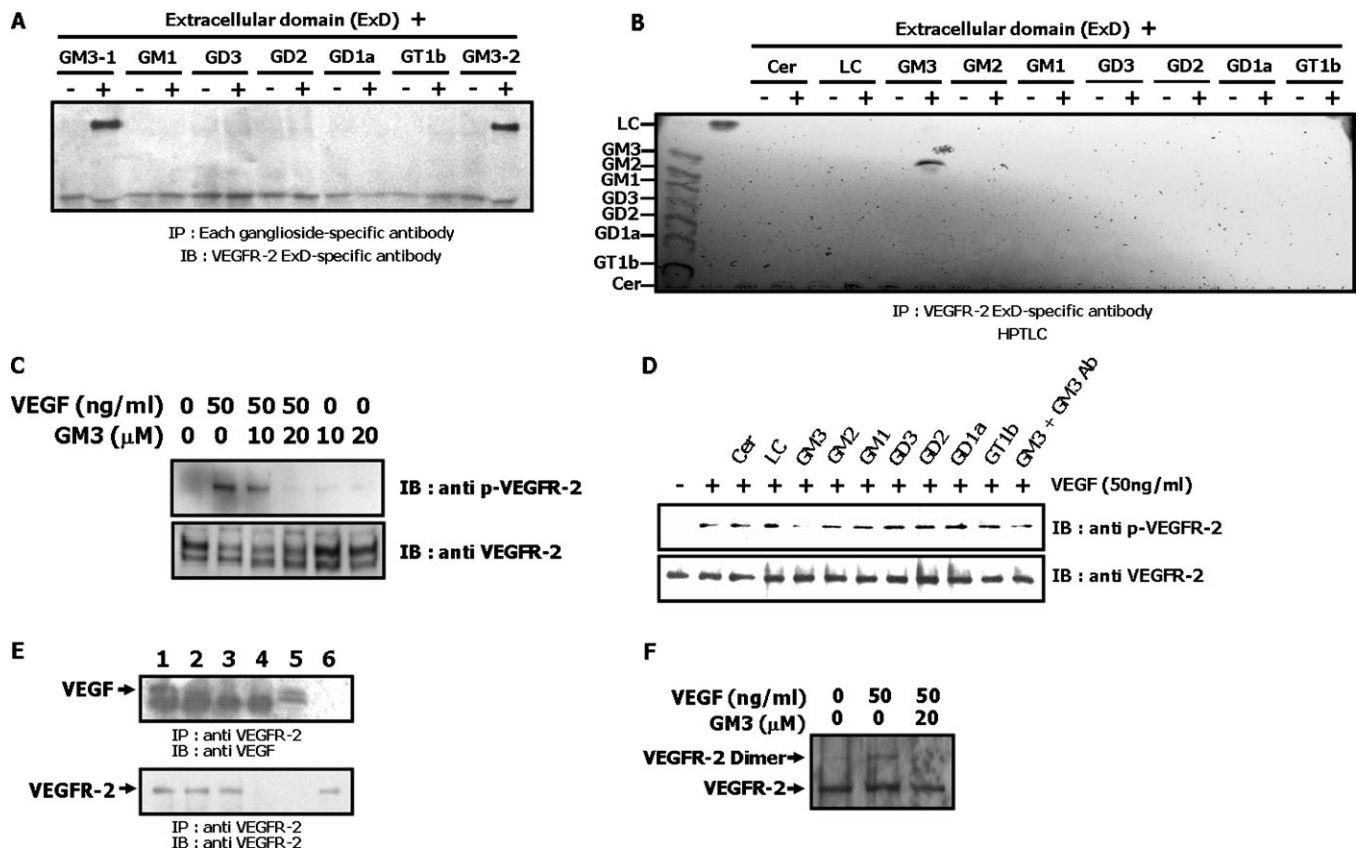
Fig. 3. Direct binding of GM3 to ExD of VEGFR-2. (**A**) The interaction of VEGFR-2 ExD protein with GM3 or other gangliosides. The VEGFR-2 ExD protein was incubated with GM3 and other gangliosides in a 300 μL of IP buffer. The ganglioside–protein mixtures were immunoprecipitated with corresponding ganglioside-specific antibodies and the protein L-agarose beads. The immunoprecitates were analyzed by SDS–PAGE. Western blot analysis was performed with the VEGFR-2 ExD-specific antibody. Ganglioside antibodies used are as follows: GM3-1 (M2590), GM1 (GMB16, Seikagaku, Japan), GD3 (GMR19, Seikagaku), GD2 (GMR7, Seikagaku), GD1a (GMR17, Seikagaku), GT1b (GMR5, Seikagaku), and GM3-2 (GMR6, Seikagaku). (**B**) The interaction of GM3 or other gangliosides with ExD of VEGFR-2 analyzed by HPTLC, VEGFR-2 ExD protein was incubated with each of gangliosides in a 300 μL of IP buffer. Each ganglioside and VEGFR-2 ExD protein mixture was immunoprecipitated with an antibody which react with VGFER-2 ExD and the protein G PLUS-agarose beads. Gangliosides interacting with VEGFR-2 ExD were extracted from the beads with methanol. Ganglioside solved in methanol were applied and developed on a HPTLC plate with chloroform–methanol–0.2% CaCl$_2$ (55:45:10). After separation, gangliosides were visualized by spraying the plate with the resorcinol-hydrochloride reagent. (**C**) Confluent HUVECs were starved in EBM-2 containing 1% FBS for 6 h. The cells were pretreated with various concentrations of GM3 or (**D**) various gangliosides (20 μM) for 1 h and stimulated by the addition of VEGF for 15 min. The cell extracts were analyzed by SDS–PAGE. Immunoblotting analysis was performed with antibodies against VEGFR-2 and phospho-VEGFR-2. (**E**) One of two samples was allowed to incubate GM3 and VEGFR-2 ExD together with the VEGF in an IP buffer. The reaction of the other sample is as follows. VEGFR-2 ExD in the IP buffer was preincubated with GM3, and then incubated with VEGF. After incubation, two samples were immunoprecipitated by the VEGFR-2 ExD-specific antibody/protein G PLUS-agarose. Furthermore, VEGF and VEGFR-2 ExD without the addition of GM3 as a positive control were incubated and immunoprecipitated by the VEGFR-2 ExD-specific antibody and protein G PLUS-agarose. The immunoprecitates were analyzed by SDS–PAGE. Western blot analysis was performed with anti-VEGF and VEGFR-2 antibodies. Lane 1, incubation of VEGF and VEGFR-2 in the IP buffer; lane 2, incubation of GM3, VEGFR-2 and VEGF in the IP buffer; lane 3, after incubation of GM3 and VEGFR-2 in the IP buffer, addition with VEGF; lane 4 incubation without GM3, VEGFR-2 and VEGF in the IP buffer; lane 5, loading of only VEGF for electrophoresis and Western blot; lane 6, loading of only VEGFR-2 for electrophoresis and Western blot. (**F**) Confluent HUVECs preincubated with GM3 in an EBM medium containing 1% FBS for 6 h, and exposed to VEGF (50 ng/mL) for 5 or 10 min. The cells were cross-linked with 5 mL PBS containing BS^3 (1 mg/mL) and immunoprecipitated with an anti-VEGFR-2 antibody. The dimerization of VEGFR-2 was confirmed by Western blot using an anti-VEGFR-2 antibody.

and retinopathies has been shown to induce microvascular permeability which results in extravasation of plasma and plasma proteins, leading to edema and the laying down of a fibrin-rich extracellular matrix (Feng et al. 2000; Pal et al. 2000; Dvorak 2002; Satchi-Fainaro et al. 2005). Thus, to determine whether GM3 and its carbohydrate moiety inhibit VEGF-mediated vascular hyperpermeability as an early step in the pathological angiogenesis, we performed the Miles assay in vivo. As shown in Figure 4F, intradermal injection of VEGF resulted in the induction of vascular permeability in PBS subcutaneously pretreated mice as evidenced by extravasation of Evans blue dye injected intravenously. However, VEGF-mediated vascular permeability was suppressed in GM3 subcutaneously pretreated mice. These

results suggest that GM3 clearly inhibits VEGF-induced vascular permeability for tumor and other pathological angiogenesis.

Discussion

Pathological diseases such as diabetic retinopathy, retinal ischemia and inflammatory psoriasis, and others as well as the growth of many human solid tumors depend on the formation of new capillaries from pre-existing blood vessels. The initiating factors of pathological angiogenesis are often a variety of microenvironmental changes. Among these, lowered oxygen pressure, hypoxia, is one of the potent inducers of an angiogenic
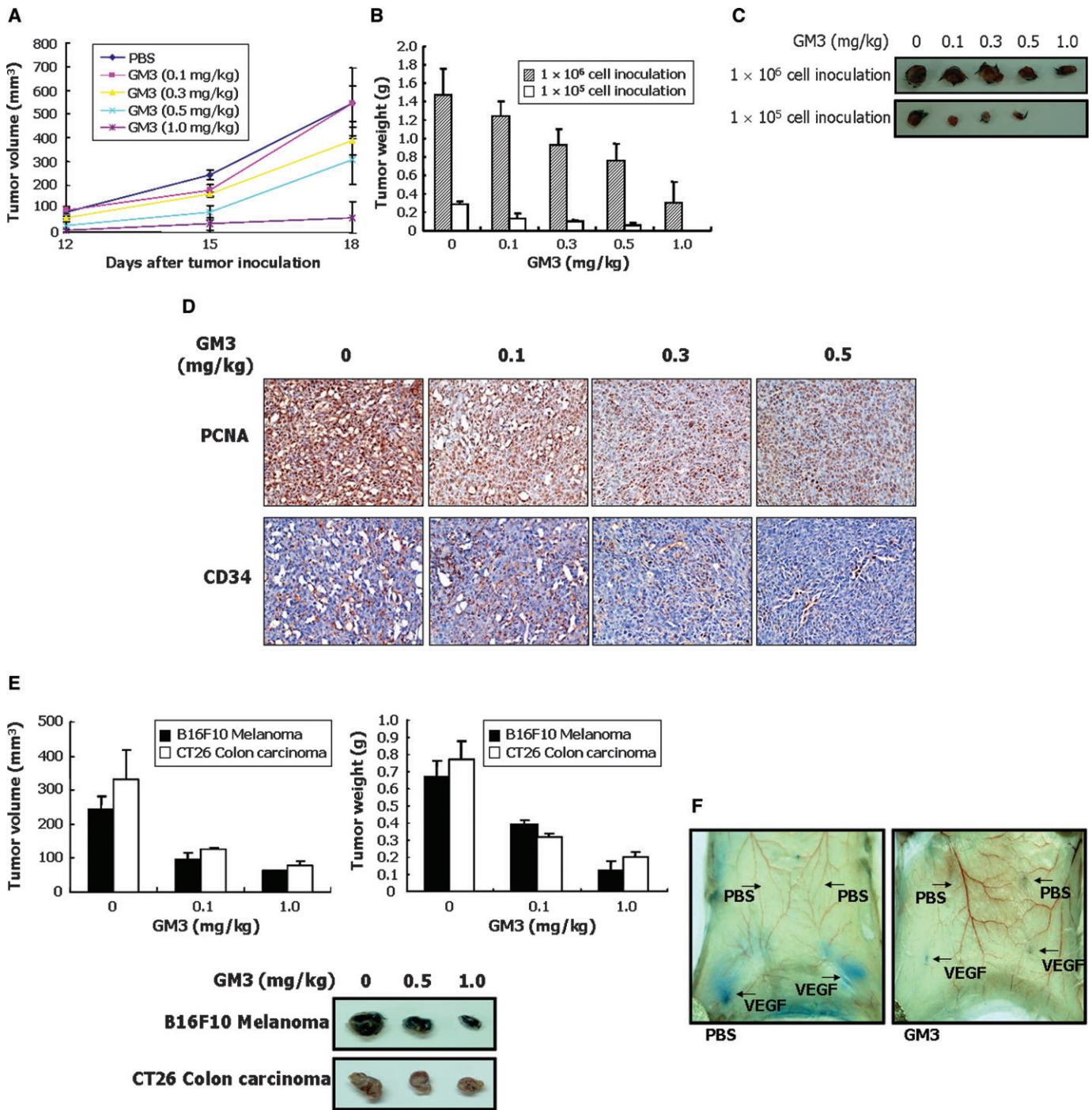
Fig. 4. Inhibitory effects of GM3 on mice model with tumor cells and on VEGF-stimulated vascular permeability in the Miles assay in vivo. Lewis lung carcinoma cells (1×10^6 or 1×10^5/100 µL) suspended in PBS were inoculated on the subcutaneous dorsa of 6-week-old C57BL/6 mice. Three days after the inoculation of tumor cells, GM3 (0.1, 0.3, 0.5 and 1 mg/kg) or PBS was intraperitoneally injected in mice every day. (**A**) Tumor volumes were measured with a caliper every 3 days. (**B**) Tumor weight was measured after euthanization. Data are expressed as the mean ± SD. For each group, $n = 5$. (**C**) Tumor size was photographed after sacrifice. (**D**) Tumor specimens immediately removed from euthanized mice were fixed with 3.7% formalin in PBS and then embedded in paraffin for immunohistochemical analysis. The paraffin sections were immunostained with PCNA and CD34 antibodies, visualized with a Dako EnVision kit (Dako) and counterstained with hematoxylin. (**E**) Melanoma and colon carcinoma cells (5×10^5/100 µL) suspended in PBS were inoculated on the subcutaneous dorsa of 6-week-old C57BL/6 and BALB/c mice, respectively. Three days after the inoculation of tumor cells, GM3 (0.5 and 1 mg/kg) or PBS was intraperitoneally injected in mice every day. Tumor volume and weight were measured after euthanization. Data are expressed as the mean ± S.D. For each group, $n = 5$. Tumor size was photographed after sacrifice. (**F**) BALB/c mice were injected subcutaneously with GM3 or PBS each day. Three days later, the Evans blue solution in PBS was injected into the tail vein. The extravasation of Evans blue after intradermal injection of VEGF or PBS was photographed.

response (Giordano and Johnson 2001). Under hypoxic conditions, various angiogenic factors released from avascular tissues trigger neovascularization. Of these factors, VEGF is the significant endothelial-specific mitogen and is induced by hypoxia-inducible factor-1 (Hif-1) as a transcription factor (Shweiki et al. 1992; Choi et al. 2003). VEGF especially recruits endothelial cells into the hypoxic and avascular area and stimulates the proliferation of endothelial cells for pathological angiogenesis. VEGF expressed in hypoxia state binds to VEGFR-2 exclusively expressed in endothelial cells (Choi et al. 2003). Consequently, the interaction of VEGF with VEGFR-2 results in the dimerization, kinase activation, and autophosphorylation of specific tyrosine residues within the dimeric complex (Neufeld et al. 1999; Matsumoto and Claesson-Welsh 2001). The VEGFR-2 activation of endothelial cells in response to VEGF leads to the activation of intercellular signaling such as intercellular Ca^{2+} mobilization, ERK, PLC-γ, Src family of tyrosine kinases, RAS GTPase-activating protein, eNOS, focal adhesion kinase, PI-3K, AKT, PKC, p38 mitogen-activated protein kinase, Grb, Shc, STAT, tyrosine phosphatase SHP-1, 2, and VE-cadherin. In addition, the VEGF-mediated VEGFR-2 activation is required for the proliferation, migration, survival, tube formation of endothelial cells via the activation of various internal signal pathways to supply new blood vessels at pathogenic sites (Matsumoto and Claesson-Welsh 2001; Ferrara et al. 2003; Ferrara 2004). Taken together, VEGF and its receptor, VEGFR-2, are essential for the activation of endothelial cell in the formation of new blood vessels for the progression of angiogenic diseases.

In previous studies, it has reported that gangliosides modulate the angiogenic response of microvessels stimulated by angiogenic factors (Alessandri et al. 1997). Several reports show that GD3 is overexpressed in many types of tumors and induces angiogenesis by modulating the VEGF expression of tumor cells (Zeng et al. 2000). Furthermore, gangliosides, GM1, GD3, and GT1b have a positive effect on formation of new blood vessels in rabbit corneas (Ziche et al. 1989, 1992). Moreover, VEGFR-2 dimerization and activation by GD1a trigger the migration and proliferation of endothelial cells (Liu et al. 2006). On the other hand, depletion of GM3 in N-acetylgalactosaminyltransferase-transfected mouse brain tumor cells and the reduction of GM3/GD3 ratio in the cornea enhance angiogenesis (Ziche et al. 1992; Manfredi et al. 1999). Furthermore, GM3 treatment inhibits the growth and motility of microvascular endothelium in vitro (Ziche et al. 1992). The tumor microenvironment has an inhibitory effect on tumor progression, indicating suppression of tumor angiogenesis by endogenous angiogenic inhibitors including hormones, proteolytic fragments, tissue-specific inhibitors, and endothelium-derived inhibitors (Sato 2006). Gangliosides are important components of the cell membrane which usually are shed in the surrounding microenvironment by neoplastic cells. Furthermore, gangliosides shed into extracellular microenvironment from tumor cells, which may influence host cells (Ladisch et al. 1983; Manfredi et al. 1999; Liu et al. 2006). In this report, we assumed that exogenous GM3 may be involved in the antiangiogenic microenvironment released from the plasma membrane of several cells around pathological tissues. Thus, we investigated whether GM3, which may be modulated by a local balance between the levels of angiogenic and antiangiogenic factors, plays a negative role in the activation of VEGF/VEGFR-2 mediated endothelial cells in the delay of pathological angiogenesis. We demonstrated

that GM3 inhibits the VEGF-stimulated activation of PI-3K/AKT, ERK, VE-cadherin, and FAK/paxillin (Figure 1D–G) and suppresses proliferation, migration, and the tube formation of endothelial cells induced by VEGF (Figure 1A–C). We confirmed that exogenous GM3 inhibits VEGF-enhanced tyrosine phosphorylation of VEGFR-2 in endothelial cells, suggesting the suppression of the VEGF-induced downstream signal pathway of VEGFR-2 and cellular events (Figures 1 and 3). These results suggest that exogenous GM3 clearly abrogate VEGFR-2 activation induced by VEGF, suggesting the antiangiogenic function of GM3 which may be shed from various cells around tissues of angiogenic diseases.

Our supporting data for the suppression of VEGFR-2 activation in VEGF-induced endothelial cells herein show that GM3 directly interacts with VEGFR-2 expressed on the surface of endothelial cells, but not VEGF (Figures 2 and 3), indicating the suppression of VEGFR-2 dimerization and binding between VEGF and VEGFR-2 (3). It is well known that VEGFR-2 consists of an extracellular, a transmembrane, and a tyrosine kinase domain. The extracellular domain of VEGFR-2 includes the region interacting with VEGF, and the site responsible for the dimerization of the receptor (Neufeld et al. 1999; Matsumoto and Claesson-Welsh 2001). Taking previous data into consideration, we also assumed that gangliosides produced on the membrane of various cell types to regulate angiogenesis may be secreted into the extracellular microenvironment under specific pathological and physiological conditions or for tumor regression and modify VEGF signaling. Thus, to examine whether exogenous gangliosides directly bind to the extracellular domain of VEGFR-2 for the inhibition of VEGF-induced VEGFR-2 activation as an antiangiogenic effect, the gene of the VEGFR-2 ExD region was cloned as well as expressed and the VEGFR-2 ExD protein was purified (data not shown). Interestingly, we found that GM3 suppressed VEGF-induced VEGFR-2 activation as well as the direct interaction with VEGFR-2 ExD (Figure 3). This is the first documentation of the direct binding of GM3 to VEGFR-2 ExD, and this experimental model is feasible for further narrowing down the binding site in VEGFR-2 ExD. The determination of the precise residues on ExD of VEGFR-2 interacting with GM3 using deletion mutants of VEGFR-2 extracellular domain is in progress.

With respect to the interaction between gangliosides and cellular receptors, it has previously reported that GM3 directly binds to the extracellular domain of epidermal growth factor receptor (EGFR) by Miljan et al. (2002) and Hakomori group (Yoon et al. 2006). Hakomori and his colleagues reported that EGFR interacts with GM3 through the carbohydrate-to-carbohydrate interaction. Our present results have also shown that only GM3 directly interacts with ExD of VEGFR-2. It is generally known that the structures and signal pathways of EGFR and VEGFR are different (Levitzki and Mishani 2006). However, we have analyzed the alignment of two protein sequences, VEGFR-2 and EGFR, using the BLAST of National Center for Biotechnology Information (NCBI) to seek a common binding site of VEGFR-2 and EGFR to GM3. As expected, homology of extracellular domains in two proteins was not found, except only intracellular tyrosine kinase regions of VEGFR-2 and EGFR. We thought that binding of GM3 to ExD of VEGFR-2 is different with EGFR. Thus, we have decided to analyze the crystal structure of the GM3 and VEGFR-2 ExD complex and this is in progress.

The present results clearly demonstrate that GM3 plays a significant role in antiangiogenic in vitro activity. The in vivo antiangiogenic activity of GM3 also was evaluated using the chick chorioallantoic membrane and Matrigel plug assays well established as in vivo animal models for neovascularization in response to VEGF. GM3 dramatically inhibited the formation of new blood vessels in the Matrigel plug with VEGF in C57BL/6 mice (Figure 1H–J). GM3 significantly suppressed VEGF-induced neovascularization in the chick chorioallantoic membrane assay (Figure 1K and L). Furthermore, we assumed that the antiangiogenic activity of GM3 is clearly associated with the antitumor progression because angiogenesis is required for tumor progression. Thus, we checked whether GM3 abrogates the growth of primary tumors in C57BL/6 mice inoculated with LLC and melanoma cells, respectively, and BALB/c mice inoculated with colon carcinoma cells. Interestingly, GM3 led to regression of primary LLC, melanoma, and colon carcinoma. An immunohistochemical study indicates the inhibition of angiogenesis and proliferation of tumor cells in mice treated with GM3 (Figure 4). These results demonstrate that GM3 is a negative angiogenic factor in vivo as well as in vitro for tumor regression.

With regard to in vivo angiogenesis, enhanced vascular permeability is one of the earliest steps in the cascade of events leading to tumor angiogenesis. VEGF expressed in many tumors induces microvascular permeability, which leads to tumor angiogenesis. Microvascular hyperpermeability by VEGF is involved in edema and the laying down of the fibrin-rich extracellular matrix through the extravasation of plasma and plasma proteins. Furthermore, vascular permeability induced by VEGF is generally observed in other pathological angiogenesis such as wound healing, arthritis, psoriasis, and retinopathies (Feng et al. 2000; Pal et al. 2000; Dvorak 2002; Satchi-Fainaro et al. 2005). Our results show that GM3 obviously suppresses VEGF-mediated vascular permeability for various pathological angiogenesis as evidenced by the Miles assay, an in vivo model to measure antivascular permeability (Figure 4).

In conclusion, here we have demonstrated for the first time that angiogenic inhibitor GM3 suppresses angiogenesis through the inactivation of VEGF-induced signaling by directly interacting with VEGFR-2.

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Conflict of interest statement

None declared.

Abbreviations

CAM, chorioallantoic membrane; EGFR, epidermal growth factor receptor; EGM-2, endothelial growth medium; HUVECs, human umbilical vein endothelial cells; LLC, Lewis lung carcinoma; PCNA, proliferating cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride; VEGF, vascular endothelial growth factor.

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