Globo-H Ceramide Shed from Cancer Cells Triggers Translin-Associated Factor X-Dependent Angiogenesis

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Abstract

Tumor angiogenesis is a critical element of cancer progression, and strategies for its selective blockade are still sought. Here, we examine the angiogenic effects of Globo-H ceramide (GH Cer), the most prevalent glycolipid in a majority of epithelial cancers and one that acts as an immune checkpoint. Here, we report that GH Cer becomes incorporated into endothelial cells through the absorption of microvesicles shed from tumor cells. In endothelial cells, GH Cer addition induces migration, tube formation, and intracellular Ca2+ mobilization in vitro and angiogenesis in vivo. Breast cancer cells expressing high levels of GH Cer displayed relatively greater tumorigenicity and angiogenesis compared with cells expressing low levels of Globo-H. Clinically, GH Cer breast cancer specimens contained higher vessel density than GH Cer breast cancer specimens. Mechanistic investigations linked the angiogenic effects of GH Cer to its endocytosis and binding to TRAX, with consequent release of PLCβ1 from TRAX to trigger Ca2+ mobilization. Together, our findings highlight the importance of GH Cer as a target for cancer therapy by providing new information on its key role in tumor angiogenesis. Cancer Res; 74(23); 6856–66. ©2014 AACR.

Introduction

A growing body of evidence supports crucial roles for cancer-associated glycans and glycosphingolipids (GSL) in tumor progression and sets the stage for therapeutics that target these molecules (1, 2). Among them, GD2 has been shown to be effective targets for cancer immunotherapy (3). The most prevalent cancer-associated GSL is Globo-H ceramide (GH Cer), which is overexpressed in most common cancers, including breast cancer, prostate cancer, lung cancer, and pancreatic cancer (4, 5). The specific expression of GH Cer in tumor stem cells (6) and its function as an immune checkpoint (7) made it an ideal target for immunotherapy and provided the impetus for the ongoing phase II/III clinical trial of a Globo-H vaccine in breast cancer (8, 9). In light of the accumulating evidence that immunosuppression and angiogenesis often go hand in hand in response to specific stimuli in the tumor microenvironment (10), we investigated a possible role for GH Cer in angiogenesis.

Augmented angiogenesis is a key feature of malignant tumors and is dependent on communication between cancer cells and surrounding endothelial cells. Tumor-secreted soluble factors like VEGF-A and Sphingosine-1-phosphate (SIP) bind to their respective receptors on endothelial cells, trigger phospholipase C (PLC) activation and intracellular calcium release, and promote proliferation, migration, and tube formation of endothelial cells. PLC generates second messenger inositol 1,4,5-triphosphate (IP3) by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), thereby increasing intracellular calcium from the endoplasmic reticulum (11). Thus, the PLC serves as an ‘early generator’ of second messengers, driving early stages of angiogenesis. Knockdown of PLCβ3 in human umbilical vein endothelial cells (HUVEC) impairs migration but enhances proliferation (12). PLC activity is regulated by several binding partners, including translin-associated factor X (TRAX), which blocks PLCβ1 activity through binding to its C-terminus (13). In the current study, we demonstrate that GH Cer shed from cancer cells through microvesicles is incorporated by HUVECs, resulting in enhanced angiogenic activity. The molecular mechanism of this event involves the binding of GH Cer to TRAX with consequent release and activation of PLCβ1.

Materials and Methods

Female BALB/c mice (8–12 weeks old) were obtained from National Laboratory Animal Center (Taipei, Taiwan). NOD/SCID mice were obtained from Tzu Chi University (Hualien, Taiwan), and NSG mice were purchased from Jackson Laboratory. All animals were housed under specific pathogen-free conditions.
condition and studies were approved by the Institutional Animal Care and Use Committee of the Academia Sinica (Taipei, Taiwan). Tissue sections of human breast cancer were obtained from Chunghua Christian Hospital (Chunghua, Taiwan) and were fully encoded to protect patient confidentiality. This study was approved by the Institutional Review Board of Human Subjects Research Ethics Committees of Academia Sinica (Taipei, Taiwan) and Chunghua Christian Hospital (Chunghua, Taiwan).

Cell culture
The human umbilical vein endothelial cells (HUVEC) were obtained from Lonza and maintained in EBM-2 medium (Lonza) supplemented with 5% FBS, 3 ng/mL basic fibroblast growth factor, 5 U/mL heparin, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells are routinely tested and authenticated by the ATCC guidelines. MCF-7 cells and MDA-MB-157 cells were obtained from ATCC, which were maintained in complete Eagle’s Minimum Essential Medium (MEM; Invitrogen) supplemented with 10% FBS, 10 μg/mL insulin, and complete DMEM with 10% FBS, respectively. Both breast cancer cell lines have been tested and authenticated by Bioresource Collection and Research Center (Taiwan). All cells were cultured for less than 6 months after resuscitation for experiments.

Microvesicle collection
Microvesicles were collected as previously described protocol with slight modifications (14). Briefly, confluent MCF-7 cells and MDA-MB-157 cells were grown in 10% or 1% FBS. At 24 hours, conditioned medium was removed, passed through 0.22 μm filter, and centrifuged at 20,000 × g for 10 minutes and then at 10,000 × g for 30 minutes twice. The supernatant was centrifuged at 100,000 × g for 1 hour. The resulting pellet microvesicles were resuspended in PBS and then the protein concentration was determined by Micro-BCA kit (Pierce). Microvesicles were aliquoted and stored at 20°C until use. For heat treatment, microvesicles were boiled for 10 minutes before use. Anti-Globo-H mAb VK9 (provided by Dr. G. Ragupathi, Memorial Sloan-Kettering Cancer Center, New York, NY; ref. 15) was incubated with microvesicles at 37°C for 30 minutes.

Tube formation assay
HUVECs were cultured for 4 hours under serum-free conditions. Then, the growth factor–reduced Matrigel (BD Discovery Labware) was placed in 12-well plate and allowed to polymerize for 30 minutes at 37°C. Next, 6 × 10^4 cells per well were seeded on the Matrigel and stimulated for overnight in polymerize for 30 minutes at 37°C on a rocker. The resulting pelleted microvesicles were resuspended in PBS and then the protein concentration was determined by Micro-BCA kit (Pierce). Microvesicles were aliquoted and stored at 20°C until use. For heat treatment, microvesicles were boiled for 10 minutes before use. Anti-Globo-H mAb VK9 (provided by Dr. G. Ragupathi, Memorial Sloan-Kettering Cancer Center, New York, NY; ref. 15) was incubated with microvesicles at 37°C for 30 minutes.

Adapted Boyden chamber assay
All cell migration assays were conducted in a modified Boyden chamber, with 24-well plates in which Transwell inserts (polycarbonate membrane insert with 6.5 mm diameter and 8.0 μm pores; Corning) were placed. To assess endothelial cell migration, 5 × 10^4 HUVECs were seeded into the top compartment and incubated for 24 hours. The nonmigrated cells on the top side of the surface were removed. The migrated cells on the bottom side of the membrane were counted in five standardized fields.

Proliferation assay
HUVECs were plated at 1 × 10^4 cells per well on a gelatin-coated 96-well plate. After starvation in serum-free EBM-2 for 4 hours, the cells were incubated with GH Cer, Cer, or VEGF-A for 24 hours. The cells were treated with 1 μCi [3H]-thymidine per well and further incubated for 6 hours. The incorporation of [3H]-thymidine was determined in a liquid scintillation counter.

Matrigel plug angiogenesis in vivo assay
Balb/c mice were anesthetized by metofane inhalation and given a subcutaneous injection of sterile Matrigel (500 μL/injection) with a 27-gauge needle. Matrigel plus PBS served as the negative control, Matrigel containing VEGF-A (100 ng) served as the positive control, and Matrigel with GH Cer or Cer (20 μmol/L) was the test substance. After 14 days, the mice were sacrificed and the Matrigel plugs were carefully dissected out and analyzed for hemoglobin content.

Determination of hemoglobin content
The Matrigel plugs were weighed and homogenized for 5 to 10 minutes on ice. Supernatants (50 μL) were mixed with 950 μL Drabkin reagent and incubated at room temperature for 30 minutes, and the absorbance was read with an ELISA reader at 540 nm.

Measurement of intracellular free calcium mobilization
Intracellular calcium mobilization in HUVECs was determined with the fluorescent calcium indicator fluo-4/AM as described previously (16). Briefly, 4 × 10^4 HUVECs were seeded in 96-well black plate with clear bottom plate and loaded with fluo-4/AM at 40 μmol/L in serum-free EBM-2 at 37°C for 30 minutes. The cells were then washed twice with calcium-free Locke solution (158.4 mmol/L NaCl, 5.6 mmol/L KCl, 1.2 mmol/L MgCl2, 0.2 mmol/L EGTA, 5 mmol/L HEPES, and 10 mmol/L glucose, pH 7.3) to remove extracellular dye. Probenecid at 2.5 mmol/L was added to both the loading medium and the washing solution to prevent dye leakage. Cells were exposed to 100 ng/mL S1P, 20 ng/mL VEGF-A, GH Cer, or ceramide with or without pretreatment with 30 μmol/L BAPTA-AM, a calcium chelator, or 10 μmol/L U73122, an inhibitor of phospholipases. Fluorescence intensity was taken by Victor3 by alternative wavelength time scanning method.

Fluorescence resonance energy transfer
We applied fluorescence resonance energy transfer (FRET) analysis by flow cytometry to study the colocalization of GH Cer and TRAX (17). Briefly, cells were incubated with various amount of GH Cer for 5 minutes at 37°C then fixed and permeabilized, washed twice in cold PBS, and then labeled with antibodies tagged with donor (PE) or acceptor (Cy5) dyes. GH Cer staining was performed using unlabeled mouse antibody (mAbVK9) and a saturating amount of PE-
labeled anti-mouse IgG (Biolegend). TRAX was detected by anti-TRAX antibodies (Santa Cruz Biotechnology), followed by biotinylated anti-rabbit IgG, and then saturating concentrations of streptavidin-Cy5 (Biolegend). After staining, the cells were washed, resuspended in PBS, and analyzed with a dual-laser FACSaria II flow cytometer (BD Biosciences) with 594 laser power-off.

Endocytosis and immunofluorescence

HUVECs were incubated in the presence or absence of Filipin III for 45 minutes at 37°C, followed by incubation with GHCer or Cer for another 6 hours on ice. The cells were then washed with EB2 and warmed to 37°C immediately to start endocytosis. At the specified time points, cells were fixed with 4% PFA in PBS and permeabilized with 0.1% Triton-X 100 before immunofluorescence staining. GHCer localization was detected by staining with VK-9 conjugated with Alexa594; TRAX was detected by anti-TRAX antibody and anti-rabbit IgG conjugated with Alexa488; TRAX was detected by anti-TRAX antibody and anti-mouse IgG (Santa Cruz Biotechnology), followed by antigen retrieval by autoclave for 121°C.

Flow cytometry and Globo-H subpopulation sorting for in vivo tumorigenicity

FACS analysis and cell sorting were performed with FACSCanto flow cytometer and Aria Cell Sorter (Becton Dickinson), respectively. MCF-7, MDA-MB-157, and BC0244 cells (a patient-derived xenograft of breast cancer; ref. 6) were stained with mAbVK9 followed by goat anti-mouse IgG-conjugated FITC secondary antibody (Biolegend), and the lowest and highest Globo-H-expressing subpopulations were sorted as Globo-Hflow and Globo-Hhigh, respectively. Sorted cells were resuspended in complete MEM with 2 mg/mL Matrigel and injected into mammary glands of female NSG or NOD/SCID mice. The resulting tumors were harvested, sliced to square fragments of 1 mm2 and digested by collagenase (1,000 U/mL), hyaluronidase (300 U/mL), and DNase I (100 μg/mL) at 37°C for 2 hours. The number of mouse endothelial cells in the harvested cells was determined by flow cytometry using anti-mouse CD31 antibody (Biolegend).

Immunohistochemistry

For Globo-H staining, tissue sections were deparaffinized followed by antigen retrieval by autoclave for 121°C, 5 minutes in AR-10 solution (Biogenex). Slides were incubated with mAbVK9 antibody (1:100 dilutions in antibody dilution buffer, Ventana Medical Systems, Inc.) overnight at 4°C followed by polymer-HPF IHC detection system (Biogenex). The slides were counter stained with hematoxylin and mounted. Human CD31 immunohistochemical staining was performed automatically with Ventanas Benchmark XT by using the rabbit anti-human CD31 (DAKO Corporation). Breast cancer tumors were fixed with zinc solution for 24 hours. Slides were incubated with anti-mouse CD31 (1:20, BD at room temperature followed by polymer-HPF IHC detection system). Digital images were captured by Aperio ScanScope XT Slide Scanner (Aperio Technologies) under ×20 magnification. The number of CD31+ cell were evaluated independently by two researchers and confirmed by pathologists.

Statistical analysis

All values are presented as means ± SD. Three independent experiments were performed and representative results were shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001. P value was calculated by using the student t test or one-way ANOVA.

Results

HUVECs incorporate GHCer via microvesicles shed from breast cancer cells

To test whether Globo-H could be transferred from cancer cells to endothelial cells, HUVECs were cultured in the presence/absence of MCF-7. Three days after incubation, Globo-H was detected on the surface of HUVECs (Fig. 1A, top). Next, we investigated whether Globo-H was released from tumor cells into the microvesicles. The MCF-7, a breast cancer cell line with strong Globo-H expression (Supplementary Fig. S5A), was cultured in medium supplemented with 10% or 1% serum. At 24 hours, the supernatants were collected for preparation of microvesicles. The harvested MCF-microvesicles were confirmed by the presence of caveolin-1 (cav-1). Culture of HUVECs with MCF-microvesicles (100 μg) resulted in the expression of GHCer in 37.4% of these cells as detected by anti-Globo-H mAbVK9 (Fig. 1A, left bottom). Furthermore, incubation of HUVECs with synthetic-GHCer at 10 and 20 μmol/L resulted in the expression of Globo-H in 57.4% and 91.5%, respectively, of HUVECs (Fig. 1A, right bottom). As shown in Fig 1B, GHCer was indeed detected in MCF-microvesicles derived from MCF-7 cell culture. Similar findings were observed in another Globo-H-expressing breast cancer cell line, MDA-MB-157 (Supplementary Fig. S1B–S1D).

GHcer induces tube formation and migration of HUVECs

We next examined the biologic effects of MCF-microvesicles on tube formation of HUVECs. To minimize the contribution of proteins, the MCF-microvesicles were heat-treated before adding to HUVECs. Both MCF-microvesicles and heat-treated MCF-microvesicles induced significantly more tube formation than medium-only control by 2.5 ± 0.2 and 2.1 ± 0.2 folds, respectively. Moreover, the tube formation was blocked by preincubation of MCF-microvesicles with anti-Globo-H antibody mAbVK9 (Fig. 1C). Synthetic GHCer had similar biologic activity as MCF-microvesicles, enhancing the tube formation by 1.5 ± 0.2, 1.9 ± 0.3, and 1.9 ± 0.2 folds at concentrations of 10, 20, and 30 μmol/L, respectively. On the other hand, ceramide (Cer) had no effect at 30 μmol/L, whereas VEGF-A increased tube formation by 3.6 ± 0.5 folds (Fig. 1D). These results suggest that GHCer acts as an angiogenic factor.

To further delineate the angiogenic activity of GHCer, we examined the effect of GHCer on migration by Transwell assay. GHCer at 20 μmol/L markedly promoted the migration of HUVECs (168.7 ± 48.5 cells) as compared with PBS (2.0 ± 2.6 cells) and Cer (3.3 ± 3.5 cells; Fig. 1E). In contrast, GHCer had no effect on the proliferation of HUVECs (Fig. 1F).
Globo-H promotes angiogenesis in vivo

Next, we evaluated the angiogenic activity of GHCer in vivo by using a Matrigel plug assay to detect the newly formed blood vessels within the transplanted gel plugs. Both GHCer and VEGF-A induced greater formation of new blood vessels than PBS and Cer controls (Fig. 1G). The amount of hemoglobin was significantly higher in GHCer (5.0 ± 2.0 folds) than Cer (1.5 ± 0.7 folds). These results suggest that GHCer can promote angiogenesis in vivo.

Globo-H expressing breast cancer cells display enhanced tumorigenicity and angiogenesis

To further investigate the contribution of Globo-H to tumoral angiogenesis in vivo, we compared the relative abundance of murine endothelial cells in tumors derived Globo-Hhigh and Globo-Hlow subpopulations of breast cancers. MCF-7 cells were sorted into Globo-Hhigh and Globo-Hlow cells with high purity (83% and 93%, respectively). Although the growth rate of these two subpopulations in vitro was similar (Fig. 2A), the in vivo tumor expansion was significantly greater for Globo-Hhigh subpopulation than Globo-Hlow group (P = 0.0007; Fig. 2B). This was accompanied by 3.0-fold higher density of mouse CD31-expressing endothelial cells in Globo-Hhigh group than Globo-Hlow group, as assessed by immunohistochemical (IHC) staining (Fig. 2C) and 2.3-fold higher by flow cytometry (Fig. 2D). The greater blood vessel density was further confirmed by higher hemoglobin contents in Globo-Hhigh group (Fig. 2E). MDA-MB-157 and BC0244 showed similar trends in tumorigenicity (tumor size 1.2- and 3.3-fold over Globo-Hlow group, respectively) and mouse CD31+ cells (2.4- and 2.1-fold over Globo-Hlow group) (Table 1).

Next, we explored the possible impact of Globo-H on the density of blood vessels in clinical samples. Paraffin tissue sections from 50 breast cancer specimens were examined by IHC staining with mAbVK9 and anti-CD31. As shown in Fig 2F, Globo-H+ tumors (n = 24) harbored significantly greater number of blood vessel than Globo-H- tumors (n = 26; P = 0.04). These results confirmed that Globo-H promotes tumoral angiogenesis and enhanced tumorigenicity.

The interaction between GHCer and TRAX in HUVECs

To explore the molecular mechanism of angiogenesis induced by GHCer, we performed immunoprecipitation
with mAbVK9 to identify the GHCer interaction proteins. An obvious band at the position of 30–41 kDa detected by silver stain in mAbVK9-probed cell lysate was identified as TRAX by mass spectrometry (Fig. 3A). This result was confirmed by Western blot analysis with anti-TRAX antibody in immunoprecipitate with mAbVK9 (Fig. 3B, left). Moreover, no TRAX was observed when GHCer was not added to the cell lysate. On the other hand, GHCer could be detected only in the immunoprecipitate with anti-TRAX antibody (Fig. 3B, right). Next, we performed immunofluorescence staining to determine the localization of GHCer and TRAX by confocal microscopy. Five minutes after adding GHCer to HUVECs, GHCer was detected in the cytoplasm, which colocalized with TRAX. As expected, only TRAX was observed with Cer control (Fig. 3C). The GHCer–TRAX interaction was further confirmed by fluorescence resonance energy transfer (FRET) assay. As shown in Fig. 3D, a dose-dependent increase in FRET was observed with mean fluorescence intensity (MFI) 18.7, 33.1, and 87.6 at GHCer concentrations of 5, 10, and 20 μmol/L, respectively. These findings support the notion that GHCer interacts with TRAX in HUVECs.

**GHCer disrupts the interaction of TRAX and PLCβ1**

Previously, PLCβ1 was shown to bind to TRAX, which blocks its enzymatic conversion of PIP2 to IP3 (13). As IP3 can trigger calcium influx from ER, resulting in angiogenesis (11), we hypothesized that binding of GHCer to TRAX released PLCβ1, leading to its activation and increased angiogenesis. We subsequently showed that the level of PLCβ1 in the immunoprecipitate of TRAX from GHCer-treated lysate decreased to 43% of Cer control (Fig. 3E). On the other hand, PLCγ1 was not detected in the immunoprecipitate of TRAX, which is consistent with the known difference in the C-terminus of PLCβ1 and PLCγ1, where binding of PLCβ1 to TRAX occurs (11, 13).
Table 1. Tumorigenicity and blood vessel density of Globo-H–expressing breast cancer cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Globo-Ha</th>
<th>No. of mice</th>
<th>Frequency (%)</th>
<th>Tumor size (mm³)b</th>
<th>CD31⁺(%)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 Low</td>
<td>20%</td>
<td>18</td>
<td>18/18 (100)</td>
<td>46.5 ± 15.6</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>MCF-7 High</td>
<td>20%</td>
<td>18</td>
<td>18/18 (100)</td>
<td>77.4 ± 11.3d</td>
<td>1.5 ± 1.3f</td>
</tr>
<tr>
<td>MDA-MB-157 Low</td>
<td>20%</td>
<td>12</td>
<td>11/12 (92)</td>
<td>54.8 ± 8.3</td>
<td>0.5 ± 0.6</td>
</tr>
<tr>
<td>MDA-MB-157 High</td>
<td>20%</td>
<td>12</td>
<td>12/12 (100)</td>
<td>66.8 ± 8.9e</td>
<td>1.2 ± 0.9f</td>
</tr>
<tr>
<td>BC0244 Low</td>
<td>10%</td>
<td>18</td>
<td>16/18 (89)</td>
<td>147.3 ± 155.6</td>
<td>1.5 ± 1.4</td>
</tr>
<tr>
<td>BC0244 High</td>
<td>10%</td>
<td>15</td>
<td>15/18 (83)</td>
<td>497.8 ± 220.8d</td>
<td>3.2 ± 1.8f</td>
</tr>
</tbody>
</table>

aBreast cancer cells were sorted into the highest and lowest Globo-H–expressing subpopulations for use in tumorigenesis experiment. The percentage of each subpopulation in total unsorted cells is shown in parenthesis.
bTumor size was measured on day 42 for MCF-7 and MDA-MB-157 and day 60 for BC0244. Tumor volume = 1/2(length x width²).
cMouse CD31 was determined by FACS analysis.
dP < 0.001.
eP < 0.01.
fP < 0.05.
P values were calculated using the Student t test.

![Figure 3](Image)

**Figure 3.** GHCer is associated with TRAX in HUVECs. A, cell lysates of HUVECs were incubated with 20 µmol/L GHCer at 4°C for 8 hours and then immunoprecipitated with mAbVK9 or isotype antibody. The immunoprecipitates were subjected to SDS/PAGE followed by silver staining. The band marked by arrow was identified as TRAX by mass spectrometry. B, cell lysates immunoprecipitated with mAbVK9 (left) or anti-TRAX antibody (right) were subjected to lipid extraction for dot blotting with mAbVK9. C, colocalization of GHCer and TRAX in HUVECs. After treatment with Cer (top) or GHCer (bottom), cells were fixed and stained with mAbVK9 and anti-TRAX antibody. Nucleus was stained with Hoechst 33342. D, the GHCer–TRAX interaction was evaluated by FRET assay. After incubation with GHCer (0–20 µmol/L) for 10 minutes, HUVECs were fixed and stained with anti-TRAX antibody (Cy3), and mAbVK9 (PE). FRET of APC was detected in BD FACSAria II with APC laser off. E, cell lysates preincubated with 60 µg GHCer or Cer were immunoprecipitated with anti-TRAX antibody and subjected to immunoblotting with anti-TRAX, anti-PLCβ1 antibody, and anti-PLCγ1. Each result shown here is one representative from at least three independent experiments.
Endocytosis of GHCer is necessary for forming complex with TRAX

As TRAX is located in the cytoplasm, the next question is how GHCer enters into intracellular compartment. Caveola-dependent endocytosis is a well-known mechanism of glycosphingolipid internalization (18, 19). We examined the effects of a caveolae inhibitor, Filipin III, on the internalization of GHCer. As shown in Fig. 4A, top), GHCer was detected only at the surface membrane before starting endocytosis. GHCer appeared in the cytoplasm as early as 1 minute after warming to 37°C and increased with time. On the other hand, in the presence of Filipin III, GHCer persisted on the surface membrane with very little GHCer detectable in the cytoplasm for up to 10 minutes (Fig. 4A, bottom). Filipin III treatment also inhibited the interaction of endocytosed GHCer with TRAX, as reflected by a reduction of the MFI in FRET analysis from 58.4 to 14.7 (Fig. 4B). In addition, after allowing endocytosis for 10 minutes, the lysate were immunoprecipitated with anti-TRAX antibody and probed for the presence of GHCer and PLCβ1 by Western blot analysis. As shown in Fig 4C, lane 2, TRAX and PLCβ1 formed a complex in HUVECs. The addition of GHCer reduced the level of PLCβ1 in immunoprecipitation of TRAX to 57% of Cer control (lane 3). Furthermore, Filipin III decreased the GHCer content in TRAX immunoprecipitation to 55%, but raised PLCβ1 level back to 82% (Fig. 4C, lane 4). These results clearly demonstrate that after endocytosis, GHCer forms a complex with TRAX, which disrupts the interaction of TRAX and PLCβ1 with ensuing angiogenesis.

Globo-H is predicted to bind TRAX, thereby interfering with the association of PLCβ1

Based on the X-ray crystallographic structures of human TRAX (20) and PLCβ3 (21), and the known association of TRAX with the C-terminal region of PLCβ1, we predicted the potential
binding region of PLCβ1 on TRAX to be predominantly distributed around a concave region formed by α4, α5, and α6 (Fig. 5A). The carbon chain of sphingosine of GHCer binds to the hydrophobic groove formed by α4, α5, and α6, which was exactly the predicted binding region for PLCβ1 as described above (in Fig. 5B). In addition, the hexasaccharide motif of Globo-H interacts with a polar amino-acid region (Fig. 5D), which overlapped with part of the predicted PLCβ1-binding region (Fig. 5C). Thus, Globo-H was predicted to bind TRAX, thereby interfering with the association of PLCβ1 (Fig. 5E).

The detailed description of computer modeling was provided in the Supplementary Data.

**GHCer promotes intracellular calcium mobilization**

To test our hypothesis that GHCer induces calcium influx through disruption of the interaction of TRAX and PLCβ1, we monitored the effect of GHCer on calcium influx in HUVECs. As shown in Fig. 6A, VEGF-A and SIP displayed very different time kinetics in calcium influx. SIP triggered a rapid calcium influx within 5 to 10 seconds after stimulation. VEGF-A induced a slower response that started at approximately 40 seconds. Stimulation with both ligands maintained an elevated cytosolic calcium ion state for up to 4 minutes (Fig. 6A). In contrast, GHCer induced calcium influx starting at approximately 15 seconds, as compared with Cer and PBS controls that did not show any obvious signal (Fig. 6B). The stimulation of PLCβ1 by GHCer evokes the induction of calcium influx in HUVECs, which might be important to the GHCer-induced migration and differentiation of HUVECs. To confirm this, we examined the effects of the specific intracellular Ca2+ chelator BAPTA/AM and PLC inhibitor, U73122, on GHCer-induced calcium influx. Almost all calcium influx induced by GHCer was abolished by preincubation of HUVECs with BAPTA/AM or U73122 (Fig. 6C). The similarity in the time kinetics of SIP- and GHCer-induced calcium influx raised the possibility that GHCer might exert its action by signal transduction via the SIP receptor (e.g., S1P1). Thus, we examined the effect of S1P1 antagonist, W146 on GHCer-induced calcium influx. As shown in Fig. 5D, preincubation of HUVECs with 1 mmol/L W146 failed to dampen calcium influx induced by GHCer. In contrast, blocking the caveolae-dependent endocytosis by Filipin III at 5, 7.5, and 15 μmol/L reduced the GHCer-induced calcium influx to 68.0% ± 31.6%, 21.3% ± 17.9%, and 3.0% ± 2.0%, respectively, of DMSO controls (Fig. 6E). Moreover, BAPTA/AM or U73122 reduced the GHCer-induced tubule formation to 52% ± 11% and 55% ± 16%, respectively, and suppressed GHCer-induced migration to 1.4% ± 1.3% and 9.3% ± 4.8%, respectively, of DMSO controls (Fig. 6F).

Taken together, the results of above studies indicated that GHCer-induced angiogenic activity of HUVECs might be mediated by modulating PLC, leading to intracellular Ca2+ mobilization.

**Discussion**

In this study, we have demonstrated that the uptake of synthetic GHCer or GHCer present in microvesicles by HUVECs stimulates the migration and differentiation of endothelial cells in vitro and promotes angiogenesis in vivo. Although similar angiogenic effects have been reported with certain tumor-associated gangliosides (22–24), the molecular process involved has remained obscure. We have shown that, upon endocytosis, GHCer disrupts the interaction between TRAX and PLCβ1, allowing signal transduction of the latter to induce calcium influx, thus leading to enhanced angiogenesis and tumor growth.

Microvesicles and exosomes shed from cells have been shown to play important roles in many biologic processes (25). However, the biologic impact of transfer of lipid
components, especially glycosphingolipids, within these vesicles is rarely addressed except for a report that GM1-containing exosomes induced extracellular amyloid fibril formation (26). The attribution of the angiogenic effect of MCF-microvesicles to GHCer is supported by several lines of evidence. First, the function of MCF-microvesicles was not affected by heat inactivation, implying the involvement of lipid components. In fact, synthetic GHCer had similar angiogenic activities as MCF-microvesicles. In addition, although sphingomyelin present in microvesicles might contribute to angiogenesis (14), the angiogenic activity of MCF-microvesicles was reduced by mAbVK9. We could not entirely exclude the possibility that globosides could be metabolized into bioactive lipid, such as sphingomyelin or S1P (27, 28), both of which displayed the ability to activate endothelial cells. However, addition of Cer did not affect angiogenesis, suggesting the lack of contribution from hydrolysis products of GHCer.

In this study, we found that GHCer promotes migration and tube formation but not proliferation of HUVECs. These results are reminiscent of S1P-induced lymphangiogenesis of human lymphatic endothelial cells (16), implying that GHCer and S1P might share a common signal transduction pathway. The lack of GHCer and S1P-induced proliferation might be attributed to the upregulation of nuclear PLC\(\gamma\)1 activity, which was reported to correlate with G1 phase accumulation of the cell cycle (29–31). In spite of the similarity of the effects of GHCer and S1P on endothelial cells, W146, an inhibitor of S1P, failed to dampen the GHCer-induced calcium influx, suggesting that angiogenic effects of GHCer is not mediated by S1P signaling. Although VEGF-A–induced calcium signals specifically through activation of PLC\(\gamma\)1, which contributes to tubulogenesis, migration, and DNA synthesis (32), we found that PLC\(\gamma\)1 did not interact with TRAX in the coimmunoprecipitation experiment, consistent with a previous report (13). Moreover, the time kinetics of GHCer-induced calcium influx was not similar to those observed with VEGF-A. These findings suggested that the angiogenic activities of GHCer were not mediated through VEGF-A signaling.

In light of the cytoplasmic localization of TRAX, GHCer incorporated into the surface membrane must be internalized to interact with TRAX. Indeed, we observed caveolin-dependent redistribution of GHCer from the plasma membrane to the cytoplasm. However, blocking the caveolin-dependent
endocytosis by Filipin III did not completely abate the appearance of GH Cer in the cytoplasm, suggesting the possible involvement clathrin- and/or caveolin-independent pathway (19, 33). It also indicates that endocytosis of GH Cer, rather than clustering in lipid raft, is necessary for disrupting the TRAX–PLCβ1 interaction and allowing downstream signal transduction of PLCβ1 (34, 35).

During the synthesis of Globo-H, the terminal sugar fucose is added by fucosyltransferases 1 and 2 (FUT1 and 2; refs. 6, 36). Several lines of evidence suggest that FUT1 and FUT2 play important roles in angiogenesis (37, 38). Our findings of GH Cer-induced angiogenesis provide yet another mechanism by which FUT1 and FUT2 contribute to increased angiogenesis.

Globo-H<sup>high</sup> breast cancer cells from two breast cancer cell lines and a patient-derived xenograft grew faster and contained greater blood vessel density than the Globo-H<sup>low</sup> population in vivo, although there were no differences in their growth in vitro. This is consistent with our observation that Globo-H in the shed microvesicles might influence the behaviors of endothelial cells within the tumor microenvironment, leading to increased blood vessels in tumors derived from Globo-H<sup>high</sup> breast cancer cells and subsequent tumor progression. Aside from the promotion of angiogenesis, GH Cer also functioned as an immune checkpoint by suppressing activation of lymphoid cells via downregulation of Notch1 signaling in T and B cells (7). Blockade of immune checkpoints with antibodies to CTLA-4, PD-1, or PD-L1 to unleash the immune system has generated encouraging results in recent clinical trials (39). Such effects of GH Cer on both angiogenesis and immunosuppression are reminiscent of the dual activities of many soluble factors such as IL-4, VEGF, PDGF, etc., which are crucial for driving tumor progression (10).

Taken together, Globo-H is an ideal target for cancer immunotherapy, due to the angiogenic activities of GH Cer and our previous reports of its immunosuppressive effects (7) as well as the expression of Globo-H on the breast cancer stem cells (6). Our data further strengthen the scientific rationales for the ongoing multinational phase II/III clinical trial of a Globo-H vaccine in breast cancer (8, 9).

Disclosure of Potential Conflicts of Interest
A.L. Yu has ownership interest (including patents) in Globo H-DT vaccine. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.-Y. Cheng; S.-H. Wang
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.-Y. Cheng; J. Yu; J.-C. Wu; J.-J. Lin; Y.-Y. Wu; K.-T. Yeh
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