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VEGF recruits lactosylceramide to induce endothelial cell adhesion molecule expression and angiogenesis *in vitro* and *in vivo*

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Abstract Angiogenesis is largely driven by vascular endothelial growth factor (VEGF). However, the role of lipid second messengers such as lactosylceramide (LacCer) and LacCer synthase in angiogenesis is not well understood. We have determined the distribution of various LacCer synthase mRNA transcripts using sequential analysis of gene expression (SAGE). Endothelial cells from colon cancer tissues had a 4.5-fold increase in a LacCer synthase transcript (β1,4GalT-V) as compared to normal colon tissue endothelial cells. Consequently, our focus turned to understanding the role of this enzyme in regulating VEGF-induced angiogenesis *in vitro* and *in vivo*. Herein, we show that in human endothelial cells, VEGF-induced angiogenesis is mitigated by dimethylsphingosine and suramin; inhibitors of sphingosine kinase 1(SphK-1) and sphingosine1-phosphate receptor 1(S1P (1)), respectively, and this were bypassed by LacCer but not by S1P. VEGF and basic fibroblast growth factor-induced angiogenesis was mitigated by PDMP; an inhibitor of glucosylceramide synthase and LacCer synthase in human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC). Likewise, GalT-V gene ablation using corresponding siRNA also mitigated VEGF-induced angiogenesis. In Matrigel plug angiogenesis assay in nude mice, angiogenesis was markedly inhibited by D-PDMP with concordantly diminished LacCer synthase activity. Mechanistic studies revealed that the use of LY294002, a PI3 kinase inhibitor, mitigated VEGF-induced expression of platelet-endothelial cell adhesion molecule (PECAM-1/CD31); the trans-endothelial migration of a monocyte cell line (U-937) and angiogenesis in HAEC cells. Since this enzyme is a target for VEGF action and LacCer serves as a lipid second messenger in inducing angiogenesis *in vitro* and *in vivo*, novel therapeutic approaches may be developed using our findings to mitigate colon cancer.

Keywords VEGF · Lactosylceramide · Glycosyltransferases · Angiogenesis · Platelet endothelial cell adhesion molecule-1

Abbreviations

LacCer	Lactosylceramide
GlcCer	Glucosyl ceramide
SphK-1	Sphingosine kinase 1
S1P	Sphingosine 1 phosphate
S1P(1)	Sphingosine 1 phosphate receptor
D-PDMP	D-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol
SAGE	Serial analysis of gene expression
PDTC	Pyrrolodinecarbodithiocacid
PECAM-1	Palteled endothelial cell adhesion molecule
L-NAME	N^{ω}-nitro-L-arginine methyl ester

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Introduction

Angiogenesis is a physiological process wherein new blood vessels are formed from existing ones. Angiogenesis is essentially required during embryonic development, formation of vasculature, wound healing and rebuilding of the uterus lining following menstruation in women. Angiogenesis is controlled by on and off switches. For example, vascular endothelial growth factor (VEGF) is the major growth factor that stimulates *in vitro*/*in vivo* angiogenesis. On the other hand, angiogenesis is inhibited by diverse molecules. The first FDA approved angiogenesis inhibitor has been Avastin; a humanized antibody against VEGF (Genentech, California). The process of angiogenesis entails endothelial cell proliferation, adhesion to extra cellular matrix, production of proteases that degrade the extra cellular matrix, elude apoptosis and finally form lumen that transdifferentiate to form mature neo-vessels [1]. Lack of regulation of angiogenesis may result in vascular pathologies, such as in diabetic vasculopathies, tumor growth, arthritis and other inflammatory diseases. The signal transduction pathway operating between the surface receptors and angiogenesis and the final effectors of cell behavior are poorly understood. Therefore, understanding the signaling mechanisms is important, as novel and critical pathways in the endothelium may present new targets for anti- and pro-angiogenic therapies.

Lactosylceramide (LacCer) is a member of the glycosphingolipid family. It consists of a non-polar component ceramide (sphingosine plus a fatty acid) to which is attached glucose and galactose via a Galβ1- 4Glcβ1-1Sph(*N*-fatty acyl) linkages, respectively. LacCer synthesis is catalyzed by an enzyme LacCer synthase, a Golgi localized enzyme that transfers galactose residues from UDP-galactose to glucosyl ceramide (GlcCer). Gene mapping studies and recent nomenclature suggest the presence of at least two LacCer synthases in mammalian tissues. For example, β1,4GalT-V is a constitutionally expressed LacCer synthase [2]. In contrast β1,4 GalT-VI has a tissue-specific expression. Moreover, an alternatively spliced variant of β1,4GalTVI has also been reported recently [3]. The exciting feature of this enzyme is that its activity can be transiently increased by diverse physiologically relevant proteins implicated in health and disease. For example, minimally modified LDL, VEGF and TNF-α all have been shown to induce the activity of this enzyme to generate LacCer and expression of cell adhesion molecules such as intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecular-1 (VCAM-1) and platelet cell adhesion molecule (PECAM-1) and regulate cell proliferation and angiogenesis [4–11]. Some studies have also suggested the role of sphingosine-1-phosphate in PECAM-1 gene expression [12] and angiogenesis [13]. Interestingly, such phenotypic changes observed *in vitro* were mitigated by D-PDMP and inhibitor of GlcCer synthase and LacCer synthase and this was specifically by passed by LacCer. Such studies point to a potential role of LacCer synthase/LacCer in cell proliferation and inflammation.

The purpose of the present study was to determine if VEGF/bFGF-induced angiogenesis *in vitro* and *in vivo* requires LacCer synthase/LacCer and to determine the mechanism by which VEGF/LacCer induce angiogenesis. Also our goal was to assess the expression of LacCer synthase in endothelial cells derived from human colon cancer tissue. Our data suggest that LacCer can mediate VEGF induced PECAM-1 expression and angiogenesis independent of S1P involvement. We found that the expression of β1,4 GalT-V mRNA transcript was markedly and specifically increased in colon cancer-derived endothelial cells as compared to normal colonic endothelial cells. Also we demonstrate that the LacCer synthase/LacCer pathway is relevant in VEGF/bFGF-induced angiogenesis *in vivo*.

Materials and methods

Human recombinant $VEGF_{165}$ and bFGF were purchased from R&D systems (Minneapolis, MN). LacCer from bovine milk and brain, glucosylceramide sphingosine1-phosphate and LacCer synthase inhibitor D-PDMP were obtained from Matreya Inc (Pleasant Gap, PA). Anti-human PECAM-1 mAb was purchased from R&D systems. Secondary antibodies conjugated with horseradish peroxidase (HRP), Super Signal West Pico chemiluminescence™ signal substrate solution and M-PER™ protein extraction kits were obtained from Pierce Biotechnology (Rockfield, IL). LY294002, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and 1-pyrrolodinecarbodithioicacid (PDTC) and suramin were obtained from Calbiochem (San Diego, CA). Dimethyl sphingosine was from Avanti Polar Lipids. Matrigel was purchased from BD-biosciences (Bedford, MA).

Collagenase A, elastase, DNase1 were from Roche (GmbH). BerEP4antibody against epithelial specific antigens, anti CD-45(leukocyte common antigen) beads, anti CD14 beads and anti CD 64 beads were from Invitrogen (Carlsbad, CA). Antibodies against von Willibrand factor (vWF) and mouse anti-human endothelial cell antibody (clone P1HiH12) were from Chemicon (Temecula, CA).

Cell culture Human umbilical vein endothelial cells (HUVEC), aortic endothelial cells (HAEC) and the endothelial cells growth mediums EGM™ were purchased from Lonza (Walkersville, MD) and were cultured in EGM™ growth medium. Cells were grown in either 100 mm dishes or six-well multi-dish chambers coated with 0.2% gelatin. Cells within passages 3 to 5 were used for the study. Prior

to treatment, cells were maintained in serum-free EGM for 12 h and then stimulated with either agonists or antagonists. A human promonocytic cell line U-937 was obtained from ATCC (Manassas, VA) and maintained in RPMI-1640 medium (Lonza, Walkersville, MD) supplemented with 10% FBS.

Western immunoblot analysis Cells treated with agonists/antagonists were washed twice in PBS and lysed with mammalian protein extraction reagent (Pierce Biotechnology) supplemented with protease inhibitor cocktail (Roche, GmbH). Protein content was determined using Bio Rad-Bradford dye binding assay kit (Richmond, CA) using BSA as standard. 25 μg of cellular protein was resolved by 10% SDS-PAGE and then transferred to nitrocellulose membrane. After blocking (5% non-fat dry milk powder in Tris-buffered saline, pH 8.0 containing 0.05% Tween 20) for 1 h at RT, membranes were incubated with appropriate primary antibodies. The membrane-bound primary antibodies were visualized by horseradish peroxidase-conjugated secondary antibody using chemiluminescence kit. To verify equal loading, membranes were stripped and re-probed with β-actin antibody. The X-ray films were then densitometrically scanned using Molecular Dynamics image scanner and analyzed using Image Quant software.

In vitro angiogenesis/tube formation assay HUVECs were grown on 24 well culture plates and then exposed to various agonists/antagonists. After stipulated time points, cells were trypsinized, washed in sterile PBS twice and then reconstituted in EGM™ containing 2% FBS. *In vitro* angiogenesis assay was performed using a commercially available kit from Chemicon Inc (Temecula, CA). In brief, 50 μl of ECMatrix™ were placed on 96-well plates and allowed to polymerize at 37°C for 2 h. Then HUVEC/HAEC (5×10^3) were suspended in 200 μl of EGM containing 2% FBS and pretreated with inhibitors ± VEGF/bFGF/LacCer/S1P for an hour at 37°C, then the cells were added on top of the polymerized ECMatrix™ and incubated at 37°C in 5% CO_2 atmosphere for 8–12 h. Tubes formed were documented using phase contrast microscope (NIKON) at 10× magnification. Images were acquired using CCD camera connected to computer with online image acquiring software AxioVision software (ZEISS). For quantification of tube lengths, images were exported to NIH Image J Software. Results are expressed as mean tube length±SD (in μm) for three photographic fields per experiment/well and each experiment was repeated in triplicate.

Transendothelial migration (TEM) assay TEM assays were performed as previously described [14]. Briefly, HUVECs 3×10^5/ml were placed on 0.2% gelatin coated upper side of Transwell inserts (12 mm diameter, 3.0 μm pore size, Costar. MA) and allowed to reach confluence. Following this, the cells were incubated with 2% FBS plus growth factor free EGM for 6 h. Subsequently, 3×10^6/ml U-937 promonocytic cell line was added to the upper chamber of the insert and allowed to migrate for 10–12 h. At the end of incubation, U-937 cells that migrated to the lower chamber were carefully aspirated and washed in PBS twice (1,500 rpm, 10 min, 4°C) and then counted using a Neubauer hemocytometer.

In vivo assay of angiogenesis in nude mice Female nude mice were injected subcutaneously with 200 μl of Matrigel mixture, containing VEGF (4 μg/ml) and b-FGF (4 μg/ml). Two days later, D-PDMP (10 mg/kg) suspended in 5% Tween-80/0.85% NaCl and was injected intraperitoneally daily for 10 days. Mice, injected with vehicle alone, served as control [15]. Next, Matrigel plugs were removed, fixed in 10% formalin/PBS, embedded in paraffin and sectioned. Sections were stained with trichrome-Masson stain and photographed. Tissues were also flash-frozen and the activity of LacCer synthase was measured.

LacCer synthase activity assay The activty of LacCer synthase was measured using ^{14}C-UDP-Gal as donor and GlcCer as substrate as described previously [16].

Isolation of endothelial cells form human colon cancer, normal colon and SAGE analysis of various isoforms of human LacCer synthases These studies were carried out in the laboratory of Dr. Kenneth Kinzler, Oncology Research Center at our institution as follows. Institutional approval for the use of discarded human tissue material was obtained and all procedures were conducted at 4°C. Strips of mucosa from the ascending colon from normal subjects and half of a tumor were sliced and stored in 50 mL of DMEM. The crypts were removed and the samples were next bathed in 5 mM DTT for 20 min and 10 mM EDTA in PBS for 30 min. The latter procedure was repeated once. Then the samples were transferred to PBS and shaken for 1–2 min. The lamina propria and submucosa are minced into small pieces and digested with 2 mg/mL collagenase A, 250 μg/mL elastase, 25 μg /mlDNAse1 in DMEM by shaking for 2 h at 37°C. Next, the tissue digests were filtered sequentially through 500, 250, 100 and 40 μm nylon filter mesh (Tetko). The cells were washed with PBS/BSA and centrifuged (1,200 rpm, 15 min at 4°C). The clumps were removed by filtration using a 40 μm mesh filter. The pelleted cells were resuspended in PBS/BSA solution and loaded onto a preformed 30% percoll gradient and separated at 800 g for 15 min (4°C). The top layer of cells, which contains the majority of endothelial cells was harvested, washed with PBS/BSA and centrifuged

(1,200 rpm, 15 min). The cell pellets were resuspended in PBS and transferred through a 25 μm nylon filter mesh. The filtrate was centrifuged for 7 min at 600×*g* at 4°C. The remaining enterocytes and tumor cells, which can bind non-specifically to beads in the final magnetic separation were removed using M450 beads, which were pre-bound to the BerEP4antibody against epithelial specific antigens. Likewise, most of the remaining leukocytes were removed using a cocktail of anti CD-45(leukocyte common antigen), anti CD14 and anti CD 64 beads, respectively. Following isolation, batches of endothelial cells were subject to immunostaining using antibodies against von Willebrand factor located in Weibel–Palade bodies and mouse anti-human endothelial cell antibody (clone P1HiH12). Freshly isolated endothelial cells from normal colon and tumor tissue were subject to SAGE analysis using standardized protocols described by the Kinzler–Vogelstein laboratory, previously [17]. The probes used to detect various mRNA transcripts of LacCer synthases, *e.g.* β1,4 GalT-V, β1,4GalT-VIa and β1,4GalTVIb were synthesized at the JHU core facility and are presented in the Table 1.

Statistical analysis All assays were performed in duplicate or triplicate and values were expressed as mean±SD. Student's *t* test or ANOVA was used to evaluate the statistical significance of data. $P<0.05$ were considered significant.

Results

β1,4-GalT-V is the major LacCer synthase in human tumor endothelial cells and is significantly upregulated Up to date three isoforms of LacCer synthase have been described in the literature. These are: β1,4GalT-V, β1,4GalT-VIa and an alternatively spliced variant of β1,4GalT-VIa termed β1,4GalT-VIb. In collaboration with Dr. Kinzler of the Department Oncology at our institution we have compared by serial analysis of gene expression (SAGE) the mRNA levels of three LacCer synthase isoforms in normal human endothelial cells with human colorectal cancer endothelial cells. A tag is a quantification of transcripts. We approximately quantified that there are six and 27 transcripts/cell for normal and tumor endothelial cells, respectively. However, since sometimes more than one transcript can have the same tag, so it is not an absolute proof of expression.

We found no significant difference in the mRNA level for β1,4GalT-VIa and β1,4GalT-VIb in normal human endothelial *vs* human tumor endothelial cells (present in insignificant amounts) in these two cell types. However, the most significant difference was with the mRNA level for β1,4GalT-V. This transcript was increased ~4.5-fold in human tumor endothelial cells as compared to normal human endothelial cells (Table 1). Our previous RT-PCR studies have revealed that in HUVEC β1,4GalT-V is the predominant LacCer synthase. Also Furukawa and co-workers [18] have shown β1,4GalT-V to be the only LacCer synthase isoform whose mRNA levels are increased two to three fold in cancer cells such as NIH3T3 and MTAg. In contrast, β1,4GalT-II transcript was decreased and no change was seen with the other β1,4GalT transcripts including the β1,4GalT-VI. Collectively, our observations point to the suggestion that in human tumor endothelial cells β1,4GalT-V is the predominant LacCer synthase whose transcript is significantly increased.

VEGF-induced tube formation is inhibited by dimethyl-sphingosine and bypassed by LacCer, but not sphingosine-1-phosphate Treatment of human umbilical vein endothelial cells (HUVEC) with VEGF (10 ng/ml) lead to marked tube formation (Fig. 1b), as compared to control (Fig. 1a). VEGF-induced tube formation was abrogated by pretreatment with dimethyl sphingosine (DMS; Fig. 1c), a potent inhibitor of

Table 1 The expression levels of mRNA for different isoforms of LacCer synthase in normal human colonic endothelial cells and human colonic tumor endothelial cells

LacCer synthase isoform	SAGE tag sequence	Tags (number of mRNA transcripts/cell)	
		Normal endothelial cells	Tumor endothelial cells
β1,4GalT-V	TCACAAAAGA	6	27
β1,4GalT-VIa	AGTGTCAGGG	0	0
β1,4GalT-VIb	TACCTCTGGT	0	0

In brief, a polyadenylated RNA is converted to double-stranded cDNA using primer biotin-5′-T 18-3′. The cDNA was cleaved with an anchoring enzyme (a restriction enzyme—*N1a*III) and the 3′ terminal cDNA fragments are bound to streptavidin-coated beads, after ligation of oligonucleotide linker containing recognition sites for a tagging enzyme (a type IIS restriction endonuclease, *e.g. Bsm*FI). The linked cDNA (linker +10–14 bp long cDNA, SAGE tag) is released from the beads by digestion with *Bsm*FI. The released tags are ligated to one another, concatemerized, cloned into the sequencing vector [36]. Sequenced data was analyzed by SAGE program as described in reference [36] to determine the gene expression patterns

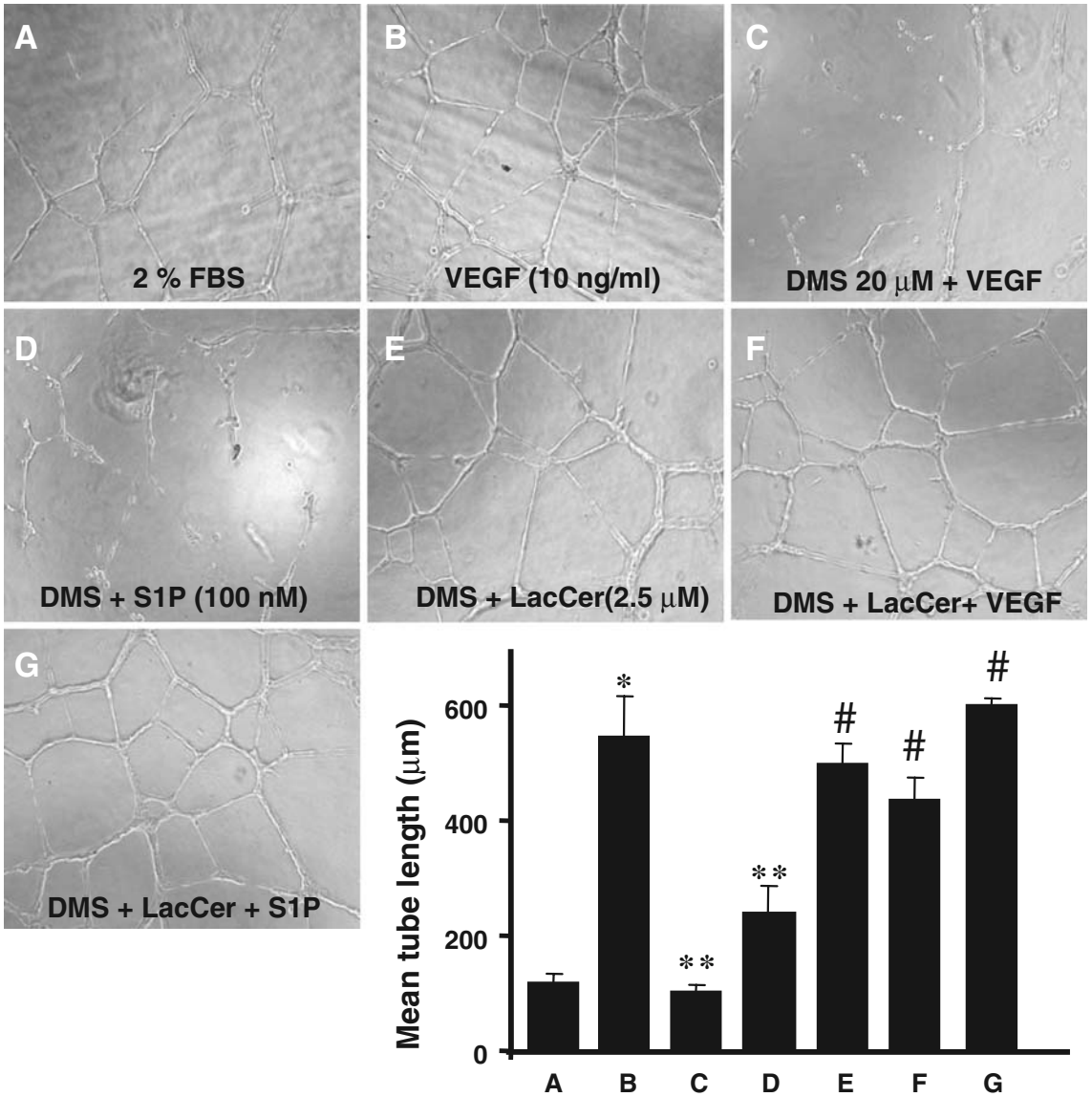
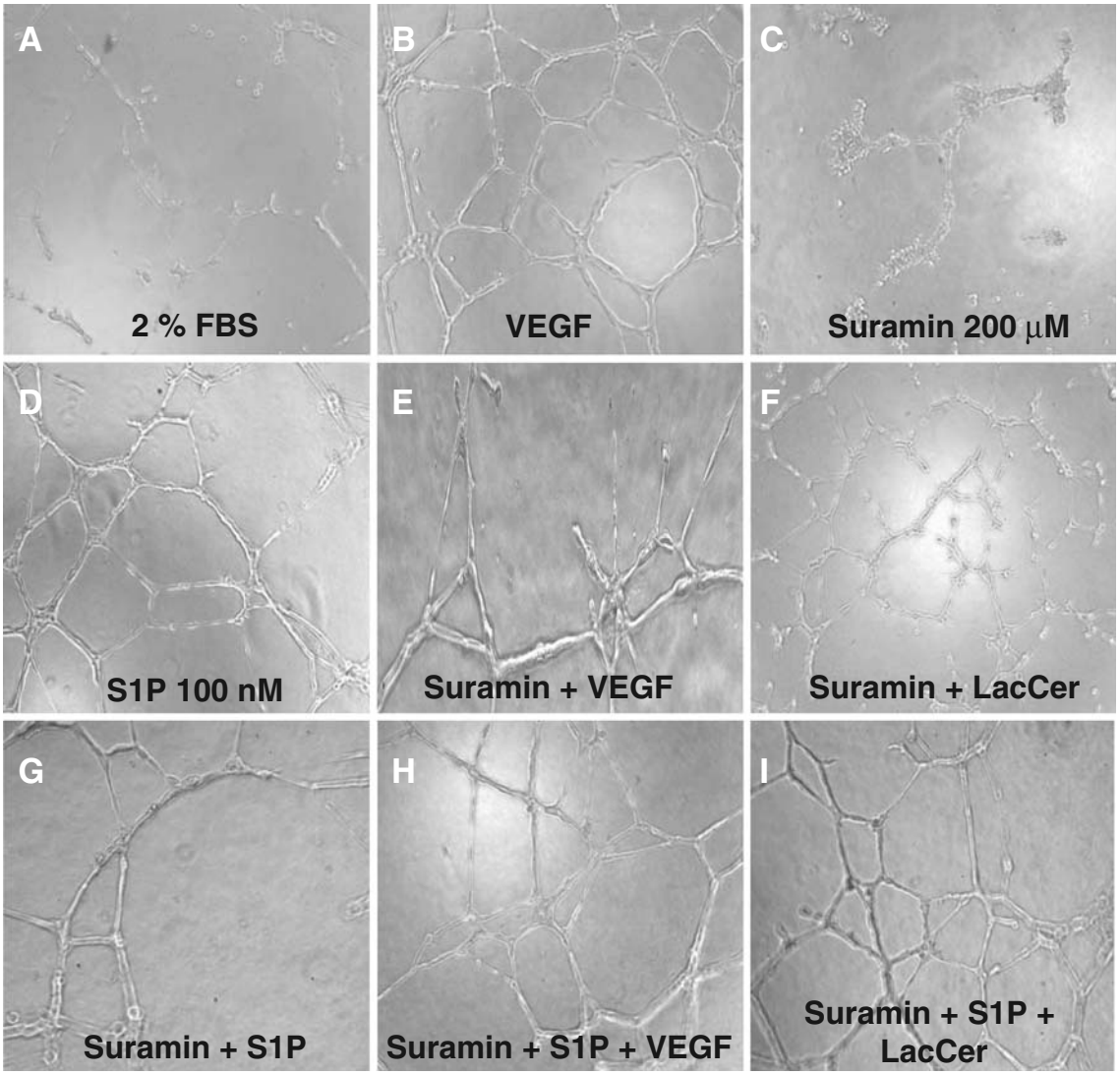


Fig. 1 VEGF induced tube formation was inhibited by DMS and this was bypassed by LacCer but not by S1P. HUVECs were pretreated with the either VEGF alone or pretreated with inhibitor DMS for an hour, followed by incubation with VEGF/LacCer/S1P as indicated and then cells were overlaid on the polymerized Matrigel and tube formation was observed after 8 h of incubation at 5% CO_2 environment. Tubes formed were documented using phase contrast microscope and later quantified using NIH image J program. Each treatment was performed in triplicate and the experiments were repeated in thrice. The *alphabets indicated in the bar diagram* represents the treatments as indicated in the panels. *$P<0.001$ *vs*. 2% FBS; **$P<0.001$ vs. VEGF; $^{\#}P<0.001$ *vs*. DMS or DMS+S1P ($n=9$)

sphingosine kinase (SphK-1). DMS also inhibited S1P-induceded tube formation in HUVECs (Fig. 1d). Further, DMS inhibition of angiogenesis was by-passed by LacCer (Fig. 1e) but not by sphingosine-1-phosphate (S1P; Fig. 1g). These observations suggest that LacCer could induce angiogenesis independent of S1P in endothelial cells.

VEGF-induced tube formation is mitigated by suramin and this was bypassed by LacCer but not S1P Suramin is a specific inhibitor of G-protein coupled receptor (GPCR) activity and has been shown to inhibit VEGF- and S1P-induced angiogenesis *in vitro* [13]. Since S1P mediates its action via S1P1 a GPCR, we used this inhibitor to investigate whether LacCer could bypass the inhibitory effect of suramin on angiogenesis. We found that suramin inhibited VEGF and S1P (Fig. 2e, g) but not LacCer induced angiogenesis (Fig. 2f) in HUVECs. The inhibition of S1P-induced angiogenesis following treatment with suramin was by passed by LacCer (Fig. 2h, i).

VEGF-induced tube formation is mitigated by D-PDMP and this was bypassed by LacCer, but not S1P When HUVECs were treated with either VEGF or LacCer, they



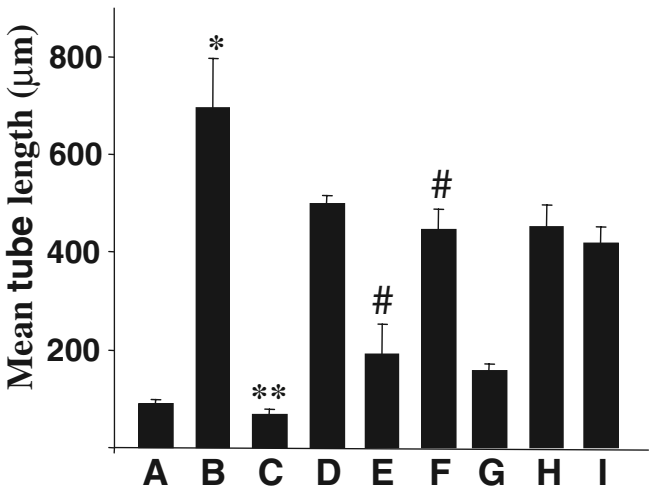


Fig. 2 VEGF induced tube formation was mitigated by suramin and this was bypassed by LacCer. HUVECs were treated with VEGF/S1P alone for 6 h or first pretreated with suramin for an hour followed by treatment with VEGF/S1P/LacCer and then tube formation assays were performed as described in "Materials and methods". *P<0.001 *vs*. 2% FBS; **P<0.001 vs. VEGF; ^{+}P<0.001 *vs*. VEGF; $^{\#}P$<0.001 vs. suramin + VEGF (n=9). The *alphabets indicated in the bar* diagram represents the treatments as indicated in the panels

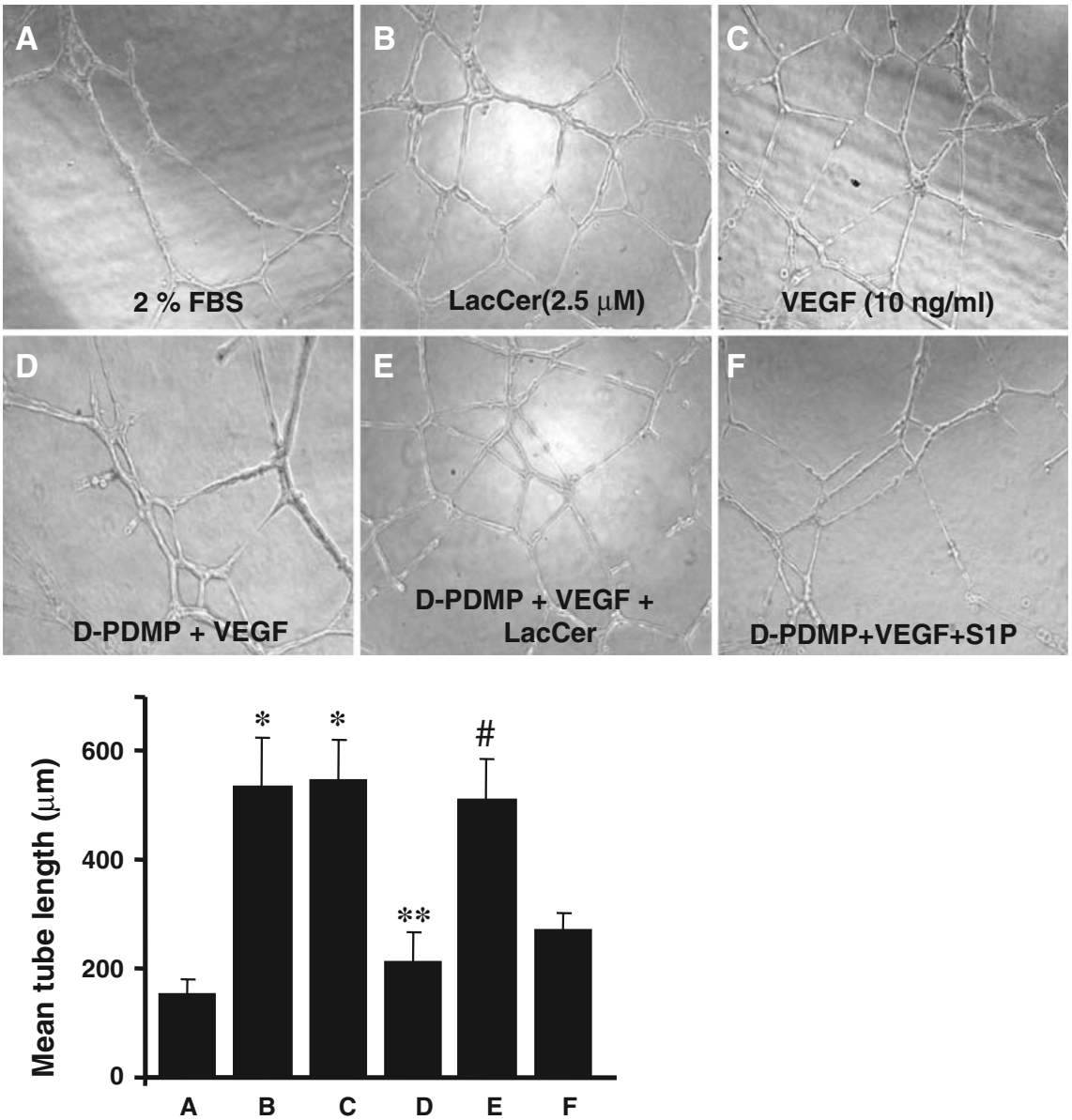


Fig. 3 VEGF induced tube formation was inhibited by D-PDMP and this was bypassed by LacCer but not S1P. HUVECs were treated with LacCer/VEGF alone for 6 h or first pretreated with inhibitor D-PDMP, followed by incubation with VEGF/LacCer/S1P and tube formation assays were performed. *$P<0.001$ *vs*. 2% FBS; **$P<0.001$ vs. VEGF; $^{\#}P<0.001$ vs. D-PDMP+VEGF ($n=9$). The *alphabets indicated in the bar diagram* represents the treatments as indicated in the panels

both induced tube formation (Fig. 3b, c) and this was blunted by pretreatment with D-PDMP a specific inhibitor of glucosylceramide synthase and LacCer synthase. After pretreatment with D-PDMP, when the cells were co-incubated in the presence of VEGF and either LacCer/S1P, we observed that only LacCer, but not S1P bypassed the inhibitory effect of VEGF-induced tube formation in the endothelial cells. Collectively, these results suggest that LacCer could induce angiogenesis independent of S1P receptor.

VEGF and bFGF induce and D-PDMP inhibits tube formation in human arterial endothelial cells and this is reversed by LacCer Next, we tested whether VEGF/bFGF tube formation could be inhibited by D-threo-1-phenyl-2-decanoyl-3-morpholino-1-propanol (D-PDMP). As shown in Fig. 4A the treatment of HAEC with VEGF markedly induced tube formation compared to control and this was inhibited by D-PDMP and could be reversed by pretreatment with LacCer. Similar results were obtained in HAEC with basic fibroblast growth factor (bFGF; Fig. 4b). Thus, both VEGF and bFGF recruit the LacCer synthase and LacCer pathway to induce tube formation in HAEC's as well in HUVEC's [7].

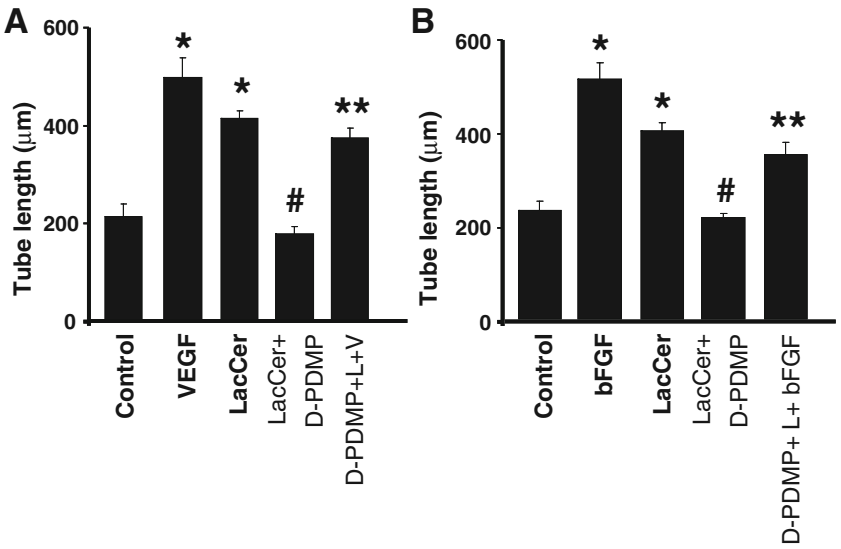


Fig. 4 VEGF , bFGF and LacCer induces tube formation in HAEC and this was inhibited by D-PDMP. **a** HAECs were treated with VEGF (10 ng/ml) or LacCer (2.5 μM) for 6 h, or pretreated with D-PDMP (15 μM) for an hour, followed by treatment with either LacCer/VEGF. VEGF induced marked tube formation, which was inhibited by D-PDMP (15 μM) and this could be reversed by co-incubation of cells with LacCer (2.5 μM and VEGF). **b** bFGF treatment of HAEC induced marked tube formation, which was inhibited by D-PDMP (15 μM) and this could be reversed by co-incubation of cells with LacCer (2.5 μM and bFGF). *P<0.001 *vs*. control; #P=0.01 *vs*. VEGF/bFGF/LacCer; **P<0.05 *vs*. LacCer + D-PDMP (n=6)

D-PDMP is not cytotoxic and does not induce apoptosis in HUVEC We observed that VEGF stimulated proliferation in HUVEC and D-PDMP (10–20 μM) reduced this effect significantly. However, D-PDMP alone did not alter cell proliferation and/or apoptosis in these cells. Apoptosis was estimated by DAPI staining for nuclear fragmentation and immunostaining for the release of cytochrome c from mitochondria. Since the basal medium in our assays contain 2% fetal bovine serum, it may exert a protective effect on these cells. Thus D-PDMP does not impart toxic effects in HUVEC by way of decreasing basal cell proliferation or by inducing apoptosis (data not shown).

D-PDMP inhibits VEGF/bFGF-induced angiogenesis in vivo in mice As shown in Fig. 5, Matrigel plug containing VEGF/bFGF (4 μg/ml) implantation in nude mice, induced marked angiogenesis. I.P. administration of D-PDMP (10 mg/kg) for 10 days, significantly inhibited angiogenesis in mice (n=6). The percentage of area occupied by blood vessels was measured by an imaging system (Image Pro).The corresponding bar graph shows that the PDMP effect on mitigating VEGF/bFGF-induced angiogenesis was statistically significant (P<0.001). This was accompanied by a significant (39%) decrease in the activity of LacCer synthase (0.200±0.015 nmol/mg protein) in D-PDMP treated animals versus agonist treated mice (0.3139±0.015 nmol/h/mg protein).The results strongly suggest that indeed VEGF/bFGF can induce angiogenesis in mice and this can be mitigated by the inhibition of LacCer synthase activity with D-PDMP.

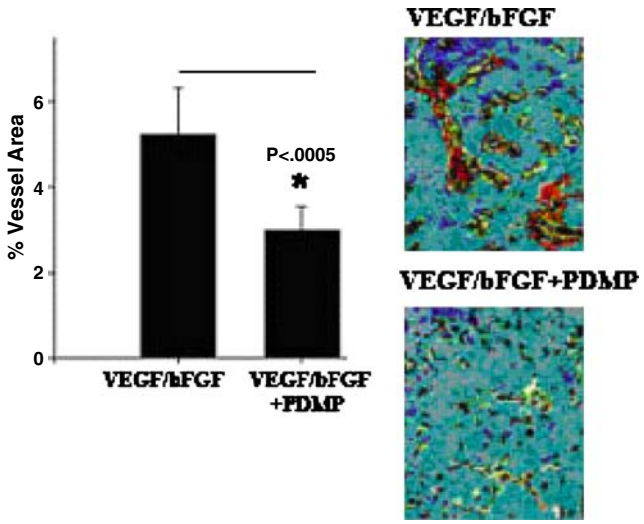


Fig. 5 VEGF/bFGF induce and PDMP mitigate *in vivo* angiogenesis in nude mice. Matrigel plug *in vivo* angiogenesis assays were performed in nude mice as described in "Materials and methods". In brief, Matrigel was mixed with VEGF and bFGF (4 μg/ml each) and injected subcutaneously to induce plugs. In some experiments, 2 h prior to Matrigel implantation, mice were administered D-PDMP (10 mg/kg) I.P. Then treatment with D-PDMP was performed for ten consecutive days. After which mice were sacrificed and the Matrigel plugs were removed and snap frozen for the determination of LacCer synthase activity or fixed in 10% buffered formalin for histological evaluation. Shown are the representative images of trichrome staining of Matrigel plugs indicating the blood vessels stained *red* and collagen *blue*, respectively. Approximately ten fields were taken for each plug, and five plugs from each treated or control group were analyzed by Image Pro software. Results are expressed as percent micro vessel area means±SD. *P<0.001 *versus* untreated control mice (n=6)

Inhibition of VEGF-induced phosphorylation inhibits PECAM-1 expression and transendothelial migration of monocyte-like cells and angiogenesis in HUVEC Previously, we have observed that VEGF/LacCer-induced angiogenesis requires the expression of PECAM-1, an integral protein in human endothelial cells. Therefore, we next investigated the upstream and downstream regulators of VEGF induced PECAM-1 expression. Treatment of HUVEC with VEGF induced marked expression of PECAM-1 (Fig. 6a), angiogenesis/tube formation (Fig. 6b) and in the transendothelial migration (TEM) of U-937 cells (Fig. 6c). However, pretreatment of cells with LY294002, an inhibitor of PI3-kinase, reversed the stimulatory effect of VEGF on TEM as well as PECAM-1 protein expression and angiogenesis. Most importantly, VEGF-induced increase in angiogenesis in HUVEC was also markedly inhibited by the use of LY294002 (Fig. 6c). Further pretreatment of HUVEC with 1-pyrrolodinecarbodithioicacid (PDTC) an inhibitor of NF-κB, also blunted VEGF induced PECAM-1 expression, angiogenesis and monocyte TEM. These results indicate that PI3K and NF-κB are upstream and downstream intermediates that VEGF recruits to induce PECAM-1 expression, tube formation and monocyte TEM.

Discussion

The following suggestions may be drawn from our present study implicating the role of LacCer synthase (β1,4GalT-V) and LacCer in VEGF-induced angiogenesis *in vitro* and *in vivo*. First, β1,4GalT-V mRNA is highly and specifically enriched in human colon cancer endothelial cells as compared to normal human colonic tissue endothelial cells. Second, LacCer independently mediates VEGF-induced angiogenesis in human endothelial cells (despite the presence of inhibitors of S1P(1) and sphingosine kinase). Third, LacCer synthase/LacCer pathway is involved in VEGF/bFGF-induced angiogenesis in human arterial endothelial cells in addition to human umbilical vein endothelial cells. Fourth, most importantly, the VEGF/bFGF-induced angiogenesis *in vivo* in mice was mitigated by the use of D-PDMP. Fifth, VEGF may post-transcriptionally activate PI3 kinase, PECAM-1 expression and contribute to angiogenesis.

The deciphering of the human genome and subsequent molecular studies by several investigators have revealed that there are at least three mRNA transcripts for LacCer synthase. These are: β1,4GalT-V, β1,4GalT-VIa and β1,4GalT-Vb. β1,4GalT-V is the constitutionally expressed LacCer synthase transcript and is present predominantly in most fetal and adult human tissues except in brain, where it is present in small amounts [19]. On the other hand, β1,4GalT-VI (also termed β1,4GalT-VIa transcript) has a

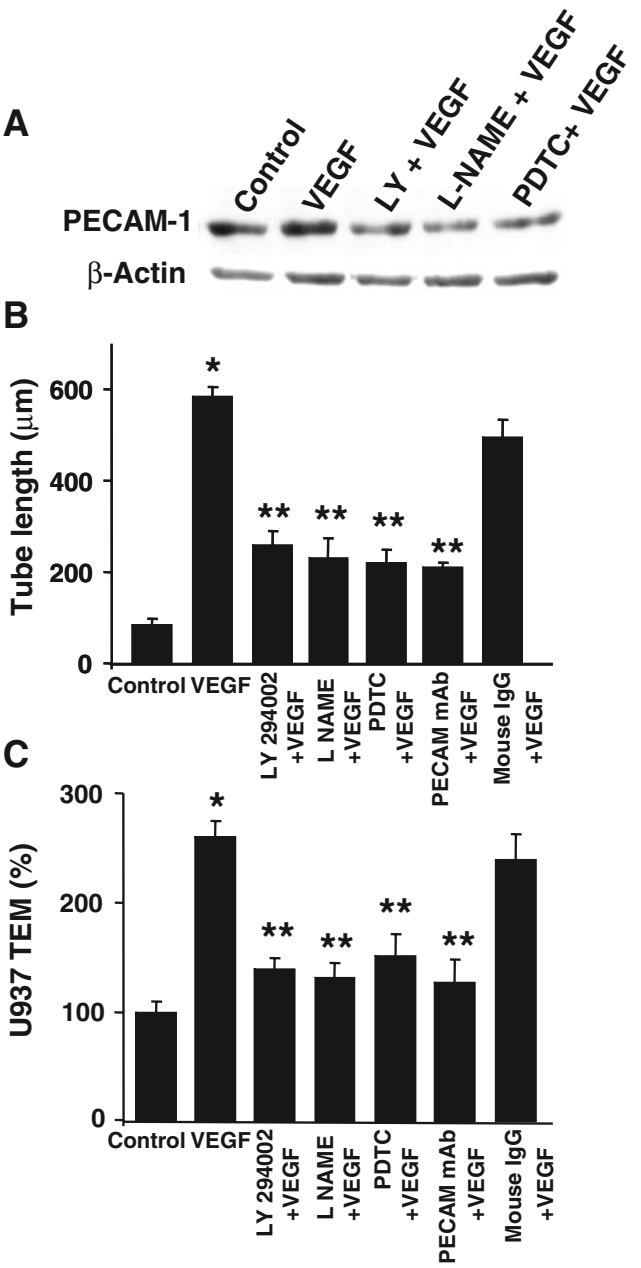


Fig. 6 VEGF induced PECAM-1 expression, tube formation and monocyte TEM was abrogated by PI3 K/eNOS/NF-κB pathway inhibitors. **a** HUVECs were treated with VEGF (10 ng/ml) alone for 4 h or first pretreated with inhibitors LY294002 (75 μM), L-NAME (100 μM) or PDTC (25 μM) for 90 min, followed by incubation with VEGF (25 ng/ml) for 4 h and then cell lysates were prepared. Then PECAM-1 expression was determined by Western immunoblot assay. Shown is the representative blot from three experiments. **b** HUVECs were treated as indicated and then tube formation assays were performed with Matrigel as described previously. The bar diagram shows the quantification data for VEGF induced tube formation and inhibition by PI3K/eNOS/ NF-κB inhibitors respectively. *$P<0.001$ vs. vehicle control; **$P<0.001$ vs. VEGF ($n=9$). **c** HUVECs were pretreated with either LY294002 (75 μM), L-NAME (100 μM), PDTC (25 μM), PECAM-1 monoclonal antibody (4 μg/ml) or mouse IgG (4 μg/ml) for 90 min, followed by incubation with VEGF (25 ng/ml) for 4 h and then U937 (monocyte) TEM assays were performed as described in "Materials and methods". *$P<0.001$ *vs*. vehicle control; **$P<0.001$ *vs*. VEGF ($n=6$)

tissue specific expression mostly in brain tissue. Further, alternatively spliced variant of β1,4GalT-VI containing one more exon, termed β1,4GalT-VIb, has been shown as well [3]. Since some studies have associated increased expression of β1,4GalT-V with cancer [20, 21], we were interested to determine the LacCer synthase mRNA transcripts in endothelial cells derived from human colon cancer and normal human colonic endothelial cells. These cells were freshly isolated and not passaged. We used SAGE in our study although this procedure is more labor intensive. However, SAGE and the newer derivatives practiced by the new generations of sequencing technology have advantage over hybridization-based approach. In addition to allowing the identification of new transcripts SAGE approaches provide absolute expression levels rather than relative expression levels. Since SAGE is not subject to cross-hybridization, it has a wide linear range and can easily be compared from one experiment to another.

We observed a 4.5-fold increase in the β1,4 GalT-V mRNA level in tumor endothelial cells as compared to normal colon endothelial cells. The level of mRNA for the other two LacCer synthase isoforms was not different between normal and tumor endothelial cells. Other investigators have also shown that the β1,4GalT-V transcripts in cancer cells such as NIH3T3 and MTAg is increased two to threefold [18]. In contrast, β1,4GalT-II transcript was decreased and no difference was observed with the other β1,4GalT-transcript [18] We have previously shown that LacCer synthase activity and levels of LacCer and others glycosphingolipids are elevated in human renal cancer cells as compared to normal renal cells [22]. Also we have shown that the activity of LacCer synthase and LacCer levels are elevated in urinary endothelial cells in patients with homozygous familial hypercholesterolemia [23]. Why the mRNA level for β1,4GalT-V is increased in human colon cancer endothelial cells is not discerned from our current study. Studies using neuroblastoma cells and human lung carcinoma cells suggest that the S1P-binding site plays an important role in the transcriptional regulation of β1,4GalT-V. Clearly, further studies on translational and post-translational regulation of β1,4GalT-V in endothelial cells is warranted.

Several studies have elaborated the role of S1P and S1K in angiogenesis [24, 25]. Moreover, over-expression of S1K resulted in an increase in the expression of PECAM-1 and angiogenesis [12]. Our studies are in full agreement with such reports (Figs. 1, 2 and 3) and extend the observation wherein inhibitors of S1K (suramin) and S1P (dimethylsphingosine) inhibited VEGF-induced angiogenesis and this was by passed by LacCer. Moreover, an unexpected observation was that the use of D-PDMP, an inhibitor of glucosylceramide synthase as well as LacCer synthase [26] could also inhibit VEGF-induced angiogenesis and this was bypassed by LacCer, but not by S1P. These observations suggest that LacCer mediated and VEGF-induced angiogenesis is independent of S1P-induced angiogenesis. Previously [7], we have also used PPMP, a relatively more specific inhibitor of GlcCer synthase and observed that its effect on angiogenesis was bypassed by LacCer. We have previously shown that LacCer synthase (GalT-V) gene ablation in HUVEC can markedly mitigate VEGF-induced angiogenesis [7]. We have reproduced these findings in HAEC using GalT-V siRNA electroporation technology (data not shown). Collectively, such observations lend support to the notion that in human endothelial cells LacCer synthase is the target for VEGF-induced angiogenesis.

Since its discovery, basic fibroblast growth factor has been used extensively to study angiogenesis. Although this growth factor is not as effective as a pro-angiogenic molecule, compared to VEGF, it is highly effective in inducing *in vivo* angiogenesis, when used in combination with VEGF [27]. In fact, VEGF alone is not very effective in inducing *in vivo* angiogenesis. Therefore, we have conducted additional studies to determine if bFGF could also use the LacCer synthase-LacCer pathway to induce *in vitro* and *in vivo* angiogenesis. We observed that bFGF alone can induce angiogenesis and this was mitigated by D-PDMP and bypassed by LacCer. A similar observation was made when HAEC were incubated with VEGF. Thus, the LacCer synthase/LacCer pathway is versatile in conducting signaling events via diverse growth factors and a variety of endothelial cells derived from human tissues.

Although *in vitro* angiogenesis/tube formation provides a rapid tool for examining the efficacy of modulators of angiogenesis, there is significant variation in response to growth factors as detailed in [28]. Therefore, the *in vitro* studies should be validated by conducting similar experiments *in vivo*. As compared to other *ex vivo* and *in vivo* angiogenesis assays, the Matrigel assay induces immature tumor-like blood vessels and allows histological examination. When Matrigel containing the mixture of VEGF and bFGF was injected subcutaneously into nude mice, it markedly induced angiogenesis, as expected from our previous observations [29]. However, when D-PDMP was injected intraperitoneally daily for ten days, it significantly mitigated ($p<0.00005$) VEGF/bFGF-induced angiogenesis. This was accompanied by a significant decrease in the activity of LacCer. D-PDMP has been used extensively *in vitro* and *in vivo* [30]. Some studies point to its effect by way of producing ceramide; that, in turn, may lead to apoptosis [31]. However, we observed that in the presence of 2% fetal bovine serum D-PDMP did not induce apoptosis in HUVEC/HAEC. Also D-PDMP did not alter the basal level of cell proliferation either. Additional and extensive *in vivo* studies on toxicity of D-PDMP, in which 60 mg of D-PDMP were given daily for many months, failed to elicit significant toxic effects in mice [32]. Since we gave only 10 mg/kg

D-PDMP daily, we did not observe any change in weight and appetite in these animals. Because this compound is detoxified rapidly via the P-450 cytochrome-c system may account for its non-toxic effect on animals. In sum, our studies suggest that the LacCer synthase/LacCer pathway is effective *in vivo* in mediating VEGF/bFGF-induced angiogenesis. Moreover, D-PDMP is an effective inhibitor to mitigate angiogenesis *in vivo* induced by VEGF/bFGF.

Previously, we have shown that pretreatment with tryphostin can inhibit the activity of LacCer synthase, stimulated by oxidized LDL and PDGF [33]. This was reversed by vanadate; an inhibitor of phosphatases. Moreover, pretreatment of HUVEC's with LY 294002, a PI3 kinase inhibitor, not only mitigated PECAM-1 expression, but also the transendothelial migration of monocyte-like cells and, most importantly, *in vitro* tube formation/angiogenesis. Tryphostin is a broad spectrum tyrosine kinase inhibitor and mitigate the phosphorylation/activation of receptor tyrosine kinase, although the effects of tryphostin on angiogenesis are not known. Geniestin, a plant flavonoid that inhibits the phosphorylation of receptor tyrosine kinase, also inhibits *in vitro* and *in vivo* angiogenesis [34]. We speculate that several of the tyrosine residues in the C-terminal domain in β1,4GalT-V can be phosphorylated by P13 kinase. It was shown that P13 kinase may serve as a docking site for proteins having the SHP motif, such as PECAM-1, that, in turn, can lead to tyrosine phosphorylation [35]. Moreover, using PECAM-$1^{-/-}$ mutant cells and cells overexpressing the PECAM-1 gene it was shown that VEGF/LacCer-induced PECAM-1 expression is required for angiogenesis [7].

In summary, angiogenesis is a multi-dimensional process critical for growth and development of tissues and also in diseases such as cancer and diabetic retinopathies. Although VEGF is an essential growth factor required for angiogenesis, our studies suggest specificity in regard to recruiting LacCer synthase as its target and LacCer as a lipid second messenger to mediate this critical phenotypic change. Our observations may point to LacCer synthase/LacCer as novel targets for developing anti-angiogenic drugs to mitigate human colon cancer, wherein marked increase in the expression of one of the LacCer sytnahse genes (GalT-V) was reported herein.

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