Novel Role of Lactosylceramide in Vascular Endothelial Growth Factor–Mediated Angiogenesis in Human Endothelial Cells

Mohanraj Rajesh, Antonina Kolmakova, Subroto Chatterjee

Abstract—Vascular endothelial growth factor (VEGF) has been implicated in angiogenesis associated with coronary heart disease, vascular complications in diabetes, inflammatory vascular diseases, and tumor metastasis. The mechanism of VEGF-driven angiogenesis involving glycosphingolipids such as lactosylceramide (LacCer), however, is not known. To demonstrate the involvement of LacCer in VEGF-induced angiogenesis, we used small interfering RNA (siRNA)-mediated silencing of LacCer synthase expression (GalT-V) in human umbilical vein endothelial cells. This gene silencing markedly inhibited VEGF-induced platelet endothelial cell adhesion molecule-1 (PECAM-1) expression and angiogenesis. Second, we used D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), an inhibitor of LacCer synthase and glucosylceramide synthase, that significantly mitigated VEGF-induced PECAM-1 expression and angiogenesis. Interestingly, these phenotypic changes were reversed by LacCer but not by structurally related compounds such as glucosylceramide, digalactosylceramide, and ceramide. In a human mesothelioma cell line (REN) that lacks the endogenous expression of PECAM-1, VEGF/LacCer failed to stimulate PECAM-1 expression and tube formation/angiogenesis. In REN cells expressing human PECAM-1 gene/protein, however, both VEGF and LacCer-induced PECAM-1 protein expression and tube formation/angiogenesis. In fact, VEGF-induced but not LacCer-induced angiogenesis was mitigated by SU-1498, a VEGF receptor tyrosine kinase inhibitor. Also, VEGF/LacCer-induced PECAM-1 expression and angiogenesis was mitigated by protein kinase C and phospholipase A2 inhibitors. These results indicate that LacCer generated in VEGF-treated endothelial cells may serve as an important signaling molecule for PECAM-1 expression and in angiogenesis. This finding and the reagents developed in our report may be useful as anti-angiogenic drugs for further studies in vitro and in vivo. (Circ Res. 2005;97:796-804.)

Key Words: vascular endothelial growth factor ▪ lactosylceramide ▪ platelet endothelial cell adhesion molecule-1 ▪ angiogenesis

Vascular endothelial growth factor (VEGF) has been implicated in the process of vasculogenesis and angiogenesis.1-2 Aberrant expression of VEGF has been reported in several vascular pathologies such as inflammation, complications of diabetes mellitus, cardiovascular diseases, and tumor metastasis.3 VEGF binds to its receptors KDR/Flik-1 to mediate its effect on angiogenesis in physiological conditions and in human atherosclerosis.4-6

Platelet endothelial cell adhesion molecule-1 (PECAM-1)/CD31 is a constitutively expressed integral protein in endothelial cells.7 In addition, PECAM-1 is expressed in platelets, monocytes, neutrophils, and a certain subset of T cells.8 Recent studies implicate PECAM-1 in angiogenesis and in vitro endothelial cell migration.9,10 For example, human mesothelioma cell line (REN) that is deficient in PECAM-110 and cells from PECAM-1 deficient mice11 did not form tubes in vitro (angiogenesis). In contrast, REN cells overexpressing PECAM-1 did form tubes in vitro. Furthermore, the use of monoclonal PECAM-1 antibody inhibited tumor angiogenesis in mice.12 More recently, the pivotal role of PECAM-1 in angiogenesis was unraveled, wherein transfection of human full-length PECAM-1 cDNA carrying mutation in immunotyrosine-based inhibitory motifs (ITIM) in REN cells inhibited migration of these cells in response to VEGF and failed to form tubes in the in vitro angiogenesis assays.13

Lactosylceramide (LacCer) is a member of the glycosphingolipid (GSL) family. LacCer is ubiquitously present in mammalian tissues and plays a pivotal role as a precursor for the synthesis of complex GSLs.14 Moreover, LacCer has been implicated in critical phenotypic changes such as prolifera-
tion and adhesion in mammalian cells.\textsuperscript{15–21} Recently, in a promonocytic cell line (U-937), we have shown that LacCer stimulates the transcriptional expression and protein expression of PECAM-1 by recruiting protein kinase C (PKC) \(\alpha\) and \(\epsilon\) and phospholipase A\(_2\) (PLA\(_2\)).\textsuperscript{22} An increased level of LacCer has been reported in plasma of patients with familial hypercholesterolemia\textsuperscript{23} and in calcified and un-calcified human atherosclerotic plaques.\textsuperscript{24} Similarly, an increased plasma level of soluble PECAM-1 has been reported in patients with cardiovascular disease\textsuperscript{25,26} and in animal models of atherosclerosis such as the apolipoprotein E knockout mice.\textsuperscript{27}

Because PECAM-1 expression may be a prerequisite for VEGF-induced vasculogenesis and also angiogenesis, and because LacCer can upregulate PECAM-1 expression in U937 cells, we rationalized that LacCer may well play a second messenger role in VEGF-induced PECAM-1 expression and angiogenesis in human endothelial cells. In the present article, we describe how LacCer is critical in mediating VEGF-induced PECAM-1 expression and angiogenesis in human umbilical vein endothelial cells (HUVECs).

Materials and Methods

Expanded Materials and Methods can be found in online data supplement available at http://circres.ahajournals.org.

Cell Culture

HUVECs and endothelial cell growth medium were purchased from Cambrex (Walkersville, Md) and were cultured with 10% fetal bovine serum (FBS). Human mesothelioma cell line (REN-wild type; WT) that lacks endogenous PECAM-1 expression and REN (mt-rhPECAM-1) expressing human PECAM-1 were kindly provided by Dr Steven Albelda, University of Pennsylvania Medical Center, Philadelphia. REN-WT was grown in RPMI 1640 supplemented with 10% FBS. REN (mt-rhPECAM-1) was cultured in the same medium with G418 (0.5g/L Gibco).

Determination of LacCer Synthesis

Determination of LacCer by high performance thin-layer chromatography was performed as described previously.\textsuperscript{19}

Determination of LacCer Synthase Activity

The activity of LacCer syntheses in HUVECs was determined as described earlier.\textsuperscript{19} (In this article, we have used the term GalT-V to specifically designate the HUVEC enzyme. Where we are not sure whether the enzyme is GalT-V or GalT-VI, we have referred to it as LacCer synthase.)

LacCer Synthase (GalT-V) siRNA Synthesis and Transfection

The siRNA sequence for human GalT-V cDNA (Gene Bank Accession No. AF038663) according to the (N19) TT rule was 5'-CGG AGU GAG UGG CUU AAC A dTdT-3' (sense), 5'-UGU UAA GCC ACU CAC UCC G dTdT-3' (antisense) respectively. Scrambled (negative control) siRNA used were 5'- AUG GUG AUU AGA CUG UAC C dTdT-3' (sense), 5'- AAG CUU AGC AUC AUC AGU A dTdT-3' (antisense), respectively. HUVECs were transfected with siRNA duplexes using Oligofectamine reagent (Invitrogen) following protocol supplied by the manufacturer.

Real-Time Reverse Transcriptase Polymerase Chain Reaction

Further information on real-time reverse transcriptase polymerase chain reaction (RT-PCR) is available in the online data supplement.

Western Immunoblot Analysis

Analysis information is available in the online data supplement.

In Vitro Angiogenesis and Tube Formation Assay

In vitro angiogenesis assays were performed using a commercially available kit from Chemicon Inc.

Statistical Analysis

All assays were performed in duplicate or triplicate. Values were expressed as mean±SD. Student’s t test was used to evaluate the statistical significance of data. \(P<0.05\) was considered significant.

Results

VEGF Induces PECAM-1 mRNA and Protein Expression

There was a dose-dependent increase in the expression of PECAM-1 mRNA. Maximal mRNA expression of PECAM-1 was observed when HUVECs were treated with VEGF (30 ng/mL for 4 hours) as determined by real-time RT-PCR (Figure 1A). Similarly, when HUVECs were treated with 25 ng/mL VEGF for different time points, maximal PECAM-1 mRNA expression was observed at 4 to 5 hours and decreased thereafter, as demonstrated by real-time RT-PCR (Figure 1C). Further, PECAM-1 protein expression was maximal after incubation with VEGF (30 ng/mL) for 4 hours (Figure 1B). When HUVECs were incubated with VEGF (25 ng/mL) for various time intervals, PECAM-1 protein expression was maximal at 4 hours (Figure 1D).

Figure 1. Effect of concentration- and time-dependent action of VEGF on PECAM-1 mRNA transcription and protein expression in HUVECs. A, Cells were treated with different concentrations of VEGF (0 to 30 ng/mL) for 4 hours. Equal quantity of total RNA was used for real-time RT-PCR. B, Western blot analysis of PECAM-1 in HUVECs treated with various concentrations of VEGF for 4 hours. Bottom panel shows the densitometric quantification of protein expression. C, Quantitative real-time RT-PCR analyses were performed to determine the change in gene expression of PECAM-1 in HUVECs treated with VEGF (25 ng/mL) for different time points. D, Western blot analysis of PECAM-1 expression to determine the time course of VEGF (25 ng/mL) on PECAM-1 protein expression. Bottom panel shows the densitometric quantification of protein expression. Figures shown are representative of experiments repeated in triplicate yielding similar results and the values presented in the bar graphs are mean±SD.
VEGF Stimulates LacCer Synthesis and This Is Abrogated by D-PDMP

As shown in Figure 2A, treatment of HUVECs with VEGF (25 ng/mL) significantly stimulated the de novo biosynthesis of LacCer (Figure 2A, panel A, open squares) in a time-dependent fashion, which occurred early at 10 minutes of incubation and continued to be higher as compared with control. In sharp contrast, HUVECs pretreated with 20 μmol/L D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) (an inhibitor of glucosylceramide synthase that blocks the synthesis of glucosylceramide [GlcCer] from ceramide) and LacCer synthase mitigated VEGF-induced LacCer biosynthesis (Figure 2A, panel A, solid squares). VEGF also stimulated the biosynthesis of GlcCer (Figure 2A, panel B, open squares) and D-PDMP pretreatment inhibited VEGF-induced GlcCer synthesis as early as at 10 minutes of incubation (Figure 2A, panel B, solid squares).

VEGF-Induced PECAM-1 Expression Is Abrogated by D-PDMP and Reversed by LacCer

Preincubation of HUVECs with D-PDMP (10 to 30 μmol/L) exerted a concentration-dependent inhibition of VEGF-induced PECAM-1 expression (Figure 2B). Preincubation of HUVECs with D-PDMP (20 μmol/L) for 90 minutes followed by incubation with VEGF (25 ng/mL) also abrogated PECAM-1 mRNA and protein expression, and this was bypassed by LacCer (Figure 2C).

LacCer Specifically Reversed the Inhibitory Effect of D-PDMP on PECAM-1 Expression and Tube Formation/Angiogenesis

When HUVECs were incubated with GlcCer, DGDG or C2 ceramide (2.5 μmol/L each) for 4 hours did not induce PECAM-1 expression (Figure 3A). Moreover, treatment of HUVECs with D-PDMP followed by incubation with GlcCer, DGDG, or C2 ceramide failed to bypass the inhibitory effect of D-PDMP on VEGF-induced PECAM-1 expression (Figure 3B) and angiogenesis (Figure 3C, panels e and f, and Figure 3D). In contrast, LacCer significantly induced PECAM-1 expression and angiogenesis independent of the presence/absence of D-PDMP and VEGF (Figure 3B, 3C panel b, and Figure 3D). These observations suggest that VEGF-induced PECAM-1 expression and angiogenesis are closely associated and regulated by LacCer.

PPMP Inhibits VEGF-Induced PECAM-1 Expression and Tube Formation/Angiogenesis and Is Bypassed by LacCer

We found that pretreatment of HUVECs with 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP; 20 μmol/L), a specific inhibitor for glucosylceramide synthase, resulted in mitigation of VEGF-induced PECAM-1 expression and angiogenesis (Figure 4A and 4B). LacCer reversed the inhibitory effect of PPMP on VEGF-induced PECAM-1 expression and angiogenesis (Figure 4A and 4B). GlcCer also reversed the inhibitory effect of PPMP with regard to PECAM-1 expression and angiogenesis (Figure 4A and 4B) but to a lesser extent than LacCer. Thus, these findings suggest that VEGF targeting of LacCer synthase is
critical in PECAM-1 expression and angiogenesis in HUVECs.

LacCer Synthase (GaIT-V) Gene Ablation Mitigates PECAM-1 Expression and Angiogenesis

To investigate whether LacCer is specifically required to mediate VEGF-induced PECAM-1 expression and angiogenesis, we silenced GaIT-V gene expression using siRNA duplex directed for human GaIT-V. Western immunoblot assay using cell lysates prepared from 2 separate preparations of HUVECs and a mutant CHO cell line Pro5Lec20 expressing GaIT-V but missing GaIT-1 and GaIT-VI genes (gift from Dr Pamela Stanley, Albert Einstein University, New York, NY) revealed that the rabbit polyclonal GaIT-V antibody (IgG) specifically reacted with GaIT-V with an apparent molecular weight of 55kDa (Figure 5A). Moreover, transfection of GaIT-V–specific siRNA duplex (100 nmol/L) markedly decreased (~70% GaIT-V) protein expression in HUVECs (Figure 5B). In addition, the activity of GaIT-V enzyme in these cells also decreased ~62% when compared with scrambled siRNA treated cells (Figure 5C). Further, we were interested in understanding the effects of VEGF in GaIT-V–silenced HUVECs on PECAM-1 expression and angiogenesis. We observed no change in PECAM-1 expression (Figure 6A) and blunted angiogenesis (Figure 6B panel D and Figure 6C) in LacCer synthase (GaIT-V) silenced cells treated with VEGF when compared with scrambled siRNA transfected cells (Figure 6B panel B). These results provide convincing evidence that LacCer is critical in mediating VEGF-induced PECAM-1 expression and angiogenesis in HUVECs.

PECAM-1 Is Required for VEGF/LacCer-Induced Tube Formation/Angiogenesis

To investigate whether PECAM-1 is absolutely required for VEGF/LacCer-induced angiogenesis, we pretreated HUVECs with PECAM-1 monoclonal antibody, followed by incubation with VEGF or LacCer. In PECAM-1 monoclonal antibody but not mouse IgG pretreated cells, VEGF/LacCer after treatment did not significantly induce angiogenesis (Figure 7A, panels e and f). Further, to understand whether PECAM-1 is pivotal for VEGF/LacCer angiogenesis, we performed experiments in REN (WT) cells, which phenotypically resemble endothelial cells but lack endogenous PECAM-1 expression. On the other hand, 2.5 kb human PECAM-1 cDNA was cloned in to mammalian expression vectors.
vector pCDNA3 (Invitrogen) under cytomegalovirus promoter for constitutive expression of PECAM-1 and was transfected in REN WT and established REN (mt-rhPECAM-1) as previously described. In REN (WT) cells, VEGF/LacCer did not form tube-like structures in the in vitro angiogenesis assays (Figure 8B and 8C) when compared with 2% FBS treated cells (Figure 8A). On the other hand, in REN cells transfected with human PECAM-1 gene, VEGF/LacCer induced tube formation in the in vitro angiogenesis assays (Figure 8E and 8F) when compared with control (Figure 8D). Therefore, the results from these experiments reveal that PECAM-1 expression is necessary for VEGF/LacCer-induced angiogenesis. In addition, we observed that pretreatment of HUVECs with VEGF receptor (KDR/Flk-1) antagonist SU 1498 followed by incubation with VEGF but not LacCer failed to induce angiogenesis (Figure 7A, panels c, d, and h). These observations suggest that LacCer is downstream of the KDR/Flk-1 in VEGF-induced signaling pathway, leading to PECAM-1 expression and angiogenesis in HUVECs.

**PKC and PLA₂ Inhibitors Mitigate VEGF/LacCer-Induced PECAM-1 and Angiogenesis**

PKC inhibitors CC (5.0 μmol/L), GÖ 6850, and 6976 (50 nmol/L) and PLA₂ inhibitors 10 (10 μmol/L) and MAFP (3.0 μmol/L) abrogated VEGF/LacCer-induced PECAM-1 expression (supplemental Figure IA) and tube formation (supplemental Figure IB) when compared with cells that were treated with vehicle alone. Thus, LacCer induces PECAM-1 expression by recruiting PKC and PLA₂ and these are downstream signaling events, which LacCer can recruit to induce PECAM-1 expression and angiogenesis.

**L-PDMP Stimulates PECAM-1 Expression and Tube Formation/Angiogenesis**

Because L-PDMP is a potent activator of LacCer synthase, we examined whether this compound may alter PECAM-1 expression and angiogenesis. L-PDMP significantly induced PECAM-1 expression (supplemental Figure IIA) and angiogenesis (supplemental Figure IIB). These
observations stress the importance of PDMP stereoisomers in the upregulation and downregulation of LacCer synthase, PECAM-1 expression, and angiogenesis.

**Discussion**

Angiogenesis involves a series of steps wherein endothelial cells degrade their basement membrane locally. Next, the endothelial cells migrate into the connective tissue stroma, proliferate, and finally differentiate into capillary loops. VEGF is a mediator of angiogenesis and is of considerable interest, as it is known to augment collateral blood flow in experimental animals and in patients with limb and myocardial ischemia.29

Although most studies have focused on the role of VEGF in angiogenesis, little is known in regard to mechanisms underlying this critical phenotypic change. Lactosylceramide is a member of the neutral glycosphingolipid family and plays a pivotal role by virtue of serving as a precursor for the biosynthesis of gangliosides such as monosialoganglioside GM3 and disialoganglioside GD3, as well as globotriosylceramide and LacCer sulfate. Although these glycosphingolipids have been shown to impart diverse biological functions, LacCer by its own right has been implicated in cell proliferation, cell adhesion, and cell migration, events that are collectively required for angiogenesis. Most importantly, LacCer was found to induce PECAM-1 gene/protein expression,22 a pre-requisite to initiate angiogenesis.10,11

Consequently, the focus of the current study was to determine whether LacCer is implicated in VEGF-induced angiogenesis. The initial step in VEGF-induced angiogenesis in endothelial cells requires its binding to a receptor having intrinsic tyrosine kinase domain designated as KDR/Flk-1.3 SU1498, a small lipophilic molecule, has been shown to specifically inhibit the tyrosine kinase activity of VEGF and abrogate angiogenesis/tube formation in endothelial cells.30 In our present study, SU1498 abrogated VEGF- but not LacCer-induced angiogenesis. This observation suggests that LacCer synthase/LacCer are downstream components of the VEGF-induced angiogenesis-signaling pathway.

To determine the mechanism by which VEGF may recruit LacCer in inducing angiogenesis, we have used both pharmacological and molecular approaches to manipulate enzymes responsible for LacCer biosynthesis. Because VEGF induced LacCer synthase activity, we first used D-PDMP, initially shown to be an inhibitor of GlcCer synthase31 but later proven to be an inhibitor of purified LacCer synthase.28,32 Our studies provided evidence that VEGF-induced LacCer/GlcCer synthesis, PECAM-1 gene/protein expression, and angiogenesis was inhibited by D-PDMP in a dose-dependent fashion. Moreover, this inhibitory effect was by passed by LacCer but not GlcCer, suggesting that VEGF targets the LacCer synthase to induce angiogenesis. Recently, Pannu et al33 demonstrated that interferon-γ/H9253 or lipopolysaccharide treatment in neuronal cells also recruited LacCer to induce inducible nitric oxide synthase and accelerated spinal cord injury in mice and that tumor necrosis factor-α/H9251 induced proliferation of astrocytes and astrogliosis in spinal cord injury in rats. These events were abrogated by D-PDMP and antisense-mediated silencing of LacCer synthase.34

Previously, D-PDMP has also been shown to mitigate neurite outgrowth and ameliorate osteoclast formation35,36 and aortic smooth muscle cell proliferation.15 Although D-PDMP can also induce apoptosis by raising the cellular level of ceramide, in studies above35,36 and in the present study, D-PDMP (20 μmol/L) up to 4 to 6 hours did not induce apoptosis in HUVEC (data not shown). Collectively, D-PDMP has been widely used to elaborate the role of LacCer synthase/LacCer in multiple phenotypic changes in vivo and in vitro. In contrast, a stereoisomer L-PDMP that stimulates the activity of LacCer synthase28 stimulated...
PECAM-1 expression and angiogenesis in our present study. Thus, stereoisomers of PDMP, by virtue of targeting LacCer synthase, altered phenotypic changes such as cell proliferation in previous studies\(^1\)\(^5\)\(^–\)\(^2\)\(^2\) and angiogenesis/tube formation in the present study.

To further determine that the target for VEGF action was LacCer synthase and not GlcCer synthase, we used PPMP, a specific inhibitor of GlcCer synthase. Again, PPMP, like D-PDMP, also mitigated VEGF-induced LacCer synthase and angiogenesis, and this was bypassed by LacCer. A more direct approach to ascertain the role of LacCer synthase/LacCer in VEGF-induced PECAM-1 expression and angiogenesis in the present study was to use siRNA-mediated gene ablation. Herein, we silenced the LacCer synthase/GalT-V expression in HUVECs and then compared its effect on VEGF-induced PECAM-1 protein expression and angiogenesis. These studies showed that LacCer synthase (GaIT-V) siRNA silencing in HUVECs contributed to a \(\approx70\%\) decrease in the GaIT-V gene/protein ablation and significantly mitigated VEGF-induced PECAM-1 gene expression and angiogenesis. On the basis of Northern blot assays, however, GaIT-V constitutes \(\approx90\%\) of the total LacCer synthase in HUVECs (data not shown), whereas GaIT-VI represents the rest of LacCer synthase. These observations and the \(\approx70\%\) ablation of the GaIT-V gene could explain why complete inhibition of VEGF-induced angiogenesis and inhibition of

LacCer synthase activity (Figure 6B panel D and Figure 6D) in HUVECs was not achieved in our study.

Previous studies have shown a vital role for PECAM-1 in angiogenesis.\(^1\)\(^0\)\(^,\)\(^1\)\(^1\) In our present study, REN cells, which are devoid of PECAM-1, were unresponsive to VEGF/LacCer-induced angiogenesis. In contrast, VEGF/LacCer treatment in REN cells expressing full-length cDNA for PECAM-1 responded strongly with regard to PECAM-1 expression and formation of tube-like structures in the in vitro assay of angiogenesis (Figure 8). Thus, both pharmacological and/or genetic manipulations of LacCer synthase adversely affected PECAM-1 gene/protein expression and angiogenesis. Therefore, our studies accede to the tenet that indeed PECAM-1 gene/protein expression and angiogenesis are closely associated.

Several studies have shown that VEGF recruits PKC/PLA to induce angiogenesis in HUVECs.\(^3\)\(^7\),\(^3\)\(^8\) Using specific inhibitors of PKC/PLA, in the present study we found that VEGF induced de novo synthesis of LacCer, which in turn recruits PKC/PLA to induce angiogenesis/tube formation in HUVECs. In addition, we have also documented that in a promonocytic cell line (U-937), LacCer specifically stimulated the migration of cytosolic PKC \(a/c\) to the cell membrane considered to be due to the activation of these proteins.\(^2\)\(^2\)

---

**Figure 7.** PECAM-1 is necessary for LacCer/VEGF-induced tube formation/angiogenesis. A, HUVECs were pretreated with either SU 1498 for 1 hour or anti-human PECAM-1 monoclonal antibody and new IgG for 1 hour and then exposed to VEGF (25 ng/mL)/LacCer (2.5 \(\mu\)mol/L) for 4 hours. Tube formation assays were then performed. B, Quantitative analysis of tube formation. n=3. *P<0.001 vs vehicle control that received either PBS or DMSO; **P<0.001 vs VEGF.
To date, several in vitro angiogenesis assays have been developed. Often, these assays involve the study of one particular step in the angiogenic cascade, such as proliferation, migration, or differentiation of endothelial cells. Tube formation assay using extracellular matrix derived from Engelbreth-Holm-Swarm sarcoma has been extensively used for studying the angiogenic/anti-angiogenic potential of a molecule over a decade. In vitro angiogenesis, such as the tube formation assay that we have used in our study, offers the opportunity to investigate angiogenic mechanisms with a greater speed and simplicity than with in vivo assays. It is important to note, however, that tube formation in vitro does not mimic the whole process of in vivo angiogenesis. Nonetheless, tube formation assays were widely used because of the ability to study 2 key steps in the angiogenesis process, the migration and differentiation of endothelial cells. Because there could also be variation in the tube formation assays, therefore one has to repeat the assays several times to arrive at the convincing conclusions. Because of these limitations in the in vitro angiogenesis/tube formation assays, one has to verify the in vitro angiogenesis data with in vivo angiogenesis assays. Presently, our laboratory is engaged in selecting and optimizing the assays to investigate the in vivo angiogenic potential of LacCer. Recently, the requirement for sphingosine-1-phosphate receptor-1 in tumor angiogenesis was demonstrated using in vivo RNA interference. The present study suggests that because angiogenesis is a critical multifaceted event, cells may recruit various sphingolipids to meet the demands of organ repair, growth, and development. Our present study indicates that the use of antibodies against PECAM-1, LacCer synthase inhibitors, and/or LacCer synthase siRNA can mitigate VEGF-induced angiogenesis and well may serve as invaluable pharmacological reagents in anti-angiogenic therapy.
Acknowledgments

This work was supported by grants from the Johns Hopkins Singapore Pte. Ltd and the National Medical Research Council of Singapore (NMRC#10618.1200) and by a CAM-NIH grant. We thank Dr Steven Alhelda (University of Pennsylvania Medical Center, Philadelphia) for the gift of REN cells.

References

Novel Role of Lactosylceramide in Vascular Endothelial Growth Factor–Mediated Angiogenesis in Human Endothelial Cells
Mohanraj Rajesh, Antonina Kolmakova and Subroto Chatterjee

Circ Res. 2005;97:796-804; originally published online September 8, 2005;
doi: 10.1161/01.RES.0000185327.45463.A8

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/97/8/796

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2005/09/08/01.RES.0000185327.45463.A8.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
ONLINE DATA SUPPLEMENT

Novel Role of Lactosylceramide in Vascular Endothelial Growth Factor Mediated Angiogenesis in Human Endothelial Cells

Expanded Materials and Methods

Reagents - Human recombinant VEGF_{165} was purchased from R&D systems (Minneapolis, MN), VEGF receptor (FLK-1/KDR) kinase inhibitor – SU1498, chelerythrine chloride, 2-{1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (Gö6850), 12-(2-cyanoethyl)-6,7,12,13tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a) pyrrolo (3,4-c-) carbazole (Gö6976), bromophenacyl bromide (BPB) and methyl arachidonyl fluorophosphonate (MAFP) was obtained from Calbiochem (San Diego, CA). LacCer from bovine milk and brain was purchased from either Sigma or Matreya Inc (Pleasant Gap, PA). Glucosylceramide (GlcCer) digalactosyldiglyceride (DG.DG) and other gangliosides were purchased from Matreya Inc (Pleasant gap, PA). LacCer synthase inhibitor D-threo-1-phenyl-2-decanolylamino-3-morpholino-1-propanol (D-PDMP) activator L-PDMP and GlcCer synthase inhibitor PPMP was obtained from Matreya Inc (Pleasant Gap, PA).

Anti - human PECAM-1 monoclonal antibody (mAb) was purchased from R&D systems. Anti - human NF-κB p65 (goat polyclonal) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated with horseradish peroxidase (HRP), Super Signal West Pico chemiluminescent™ signal substrate solution and M-PER™ protein extraction kits were obtained from Pierce Biotechnology (Rockfield, IL). TRIzol reagent and oligofectamine was purchased from Invitrogen (Carlsbad, CA). Primers used for amplification of PECAM-1 and β Actin genes were
synthesized from 1st Base Pvt. (Singapore). Reagents for RT-PCR were obtained from Promega (Madison, WI). [14C] palmitate (52 mCi/mmol) and [14C] galactose was purchased from New England Nuclear (Boston, MA). All other reagents were obtained from Sigma unless specified.

**Cell Culture** - Human umbilical vein endothelial cells (HUVEC) and endothelial cells growth medium EGM™ were purchased from Cambrex, (Walkersville, MD) and were cultured in EGM™ medium supplemented with 10 % fetal bovine serum (FBS). Cells were grown in either 100 mm dishes or 6 well multi-dish chambers coated with 0.2 % gelatin. Cells within passages 3 to 7 were used for the study. Prior to treatment, cells were maintained in serum-free EGM for 6 hrs and then stimulated with either agonists or antagonists. Human mesoendothelioma cell line [(REN- wild type (WT))] which lacks endogenous PECAM-1 expression and REN (mt-rhPECAM-1) expressing human PECAM-1 was kindly provided by Dr. Steven Albelda, University of Pennsylvania Medical Center, USA. REN-WT was grown in RPMI 1640 supplemented with 10 % FBS, penicillin/streptomycin and 2 mM L-Glutamine. 2.5 kb human PECAM-1 cDNA was cloned in to mammalian expression vector pcDNA3 (Invitrogen) under CMV promoter for constitutive expression of PECAM was transfected in REN (mt-rhPECAM-1) and cultured in the RPMI 1640 medium with G418 (0.5g/L Gibco) as descried earlier. 1,2 β4GalT-V mutant Chinese hamster ovary (CHO) cell line was a gift from Dr. Pamela Stanley, Albert Einstein University, New York and was grown in Minimal Essential Medium (MEM) alpha from Invitrogen (Carlsbad, CA) supplemented with 10 % FBS and antibiotics.
Vehicle for Glycosphingolipids – LacCer, GlcCer and DGDG and other gangliosides were prepared in DMSO. SU1498, GÖ 6976, GÖ 6850, CC, BPB, MAFP stock solutions were also prepared in DMSO. The final DMSO concentration exposed to the cells was 0.01%. VEGF was solubilized in endotoxin free PBS containing 0.1 % BSA.

Determination of LacCer synthesis - Cells were metabolically labeled with $[^{14}\text{C}]$ palmitate (1$\mu$Ci/ml) for 24hrs at 37°C. Next, the cells were washed and incubated for 60 min, with and without D-PDMP (20 $\mu$M). Next, VEGF (25 ng/ml) was added and incubation was continued at 37°C. At the indicated time intervals, cells were washed three times with PBS and lipids were extracted with hexane-isopropanol (3:2 V/V) for 5 min at room temperature. The procedure was repeated and lipid extracts were pooled and dried in nitrogen. Glycosphingolipids were fractionated from the total lipid extracts by the use of silicic acid column chromatography. The glycosphingolipids were separated by high-performance thin layer chromatography (Whatman). The plates were calibrated with standard glycosphingolipids. Following development in chloroform-methanol-water (100:42:6 V/V), the gel area corresponding to LacCer was scrapped and radioactivity measured using a Packard scintillation counter. The solvent extracted cell culture dish was solubilized over night with 1M NaOH and then suitable aliquots withdrawn for the measurement of protein using the BCA protein assay kit (Pierce, Rockford, IL).

Determination of LacCer synthase activity - The activity of LacCer synthase in cells incubated with VEGF was measured employing UDP-$[^{14}\text{C}]$ galactose as a nucleotide sugar donor and glucosylceramide as an acceptor. Briefly, the assay mixture
contained 100 μg of enzyme preparation, 20 μM of cacodylate buffer (pH 6.8), 1.0 mM Mn$^{2+}$/Mg$^{2+}$, 0.2 mg/ml Triton X-100, 30 nmol of GlcCer, and 0.1 mmol of UDP-galactose. Assays without exogenous GlcCer served as blanks and were subtracted from all corresponding data points. The assay was terminated by the addition of 25 μmol of EDTA plus 2.5 μmol of KCl. Chloroform/methanol (2:1, v/v) and 5 μg of human kidney GSL were added, and the products were isolated and separated by Whatman SG-81 paper chromatography by developing in chloroform/methanol/water (60:17:2, v/v/v). Chromatogram areas corresponding to migration with standard LacCer were cut, and radioactivity was measured in a Beckman LS-3800 scintillation spectrometer using the background subtract setting and automatic quench setting.

**LacCer Synthase (GalT-V siRNA) Synthesis and Transfection** – The siRNA sequence for human GalT-V cDNA [Gene Bank Accession No. AF038663] according to the (N19) TT rule was 5’-CGG AGU GAG UGG CUU AAC A dTdT-3’ (sense), 5’-UGU UAA GCC ACU CAC UCC G dTdT- 3’ (antisense) respectively. Scrambled (negative control) siRNA used were 5’- AUG GUG AUU AGA CUG UAC C dTdT – 3’ (sense), 5’- AAG CGU ACU AGG AUC AGU A dTdT-3’(antisense), respectively. Specificity of the 21-nt sequences was confirmed by BLAST search against the human genome. The pre-annealed RNA duplexes (HPLC purified) were purchased from Proligo (Singapore). In preliminary experiments we optimized the conditions for efficient transfection of HUVECs using siRNA duplexes. We observed that transfection efficiency was optimal when the cells were at 60 –70 % confluent and maintained in EGM with 10 % FBS. HUVECs cultured in 35 mm dishes, were transfected with siRNA duplexes using
Oligofectamine, following protocol supplied by the manufacturer. Silencing of GalT-V was evaluated after 48hrs of transfection by performing Western immunoblot assay using polyclonal rabbit anti human GalT-V antibody raised against synthetic peptide corresponding to 61-84 amino acid sequence of human GalT-V. We used lysates of CHO-K1 (MT) cells over expressing human GalT-V (generous gift from Prof. Pamela Stanley, Albert Einstein University, NY) as a positive control.

**Real – Time Reverse Transcriptase PCR** – Total RNA was extracted from cells using Trizol reagent following manufacturer’s protocol. Real-time RT-PCR was performed with Bio-Rad iCycler system. Single-stranded cDNA was prepared from total RNA as described earlier. The primer pairs were designed (Primer Quest – Integrated DNA technologies) and synthesized from 1st BASE (Singapore). The primer sequence for PECAM-1 is as follows: (forward) 5′ TGACCCTTCTGCTCTGTT 3′ and (reverse) 5′TGAGAGGTGGTGCTGACATC3′ respectively. For β - actin primers were (forward) 5′ AGGTCATCACTATTGGCAACGA 3′ and (reverse) 5′ CACTTCATGATGGAATTGAATGTAGTT 3′ respectively. Each 25-µl PCR reaction contained 12.5 µl SYBR Green mix (2X, Applied Biosystems), 0.2 µl cDNA, 1µl primer pair mix (10 pmol/ul each primer) and 11.3 µl nuclease free H2O. Thermal cycling conditions were as follows: initial denaturation at 94°C for 10 min, followed by 40 cycles of amplification at 94°C/30s, 60°C/40s and 72°C/1min, respectively. Final extension was carried out for 10 min at 72°C. A no RT control RNA sample was used with each real-time RT-PCR experiment containing human actin primers to verify no genomic DNA contamination. Amplification products using SYBR Green detection were routinely checked using dissociation curve and by gel electrophoresis on a 1% agarose gel then
visualized under UV light following staining with 0.5% ethidium bromide to confirm the size of the DNA fragment and that only one product was formed. Samples were compared using the relative (comparative) $C_t$ method. The $C_t$ value, which is inversely proportional to the initial template copy number, is the calculated cycle number where the fluorescence signal emitted is significantly above background levels. The fold induction/repression in gene expression by real-time RT-PCR was calculated after adjusting for actin using the formula $2^{-\Delta \Delta C_t}$, where $\Delta C_t = \text{target gene } C_t - \text{actin } C_t$, and $\Delta \Delta C_t = \Delta C_t \text{ control } - \Delta C_t \text{ treatment}$. The calculations were performed using the $2^{-\Delta \Delta C_t}$ formula in Microsoft Excel data spreadsheet as described. Amplifications were performed in triplicates for each sample and experiments were repeated in duplicates.

**Western Immunoblot Analysis** - Cells treated with agonists/antagonists were washed twice in PBS and lysed by mammalian protein extraction reagent (Pierce Biotechnology) supplemented with protease inhibitor cocktail (Roche, Grenzaherstrasse, Switzerland). 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 1hr at RT (room temperature), 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 $\beta$-actin antibody (Chemicon...
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 96 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 hr. Then HUVEC (3 X 10⁴) treated with agonists/antagonists were suspended in 200 µl of EGM containing 2 % FBS was added on to the polymerized ECMatrix™ and incubated at 37°C in 5% CO₂ atmosphere for 8-12 hrs. Tubes formed were documented using phase contrast microscope (ZEISS) at 10X magnification. Images were acquired using CCD camera connected to a computer with online image acquiring software AxioVision software (ZEISS). For quantification of tube lengths, images were exported to NIH Image J Software ([http://rsb.info.nih.gov/ij/download.html](http://rsb.info.nih.gov/ij/download.html)) and quantified as described previously.⁴,⁵ Results are shown as the mean tube length ± SD (in µm) for three photographic fields per experiment/well for at least three experiments per condition.

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


SUPPLEMENTAL FIGURES

Figure Legends

**Online Figure 1.** PKC and PLA₂ inhibitors blocks LacCer mediated angiogenesis in HUVECs. (A) Cells were pretreated with either GÖ6976 (50 nM), GÖ6850 (50 nM), CC (5 μM), BPB (10 μM) or MAFP (3 μM) for an hr, followed by incubation with 2.5 μM LacCer for 4hrs. Subsequently cells were lysed and Western blot analysis was performed to measure PECAM-1 expression. (B) *In vitro* angiogenesis assays were performed as in HUVECs treated with inhibitors followed by incubation with LacCer as mentioned in above. (n = 3; * P< 0.001 vs. LacCer treated cells).

**Online Figure 2.** Effect of L-PDMP on PECAM-1 and in vitro tube formation/angiogenesis in HUVECs. (A) Western immunoblot analysis of PECAM-1 in cells that were treated with different concentrations of L-PDMP is shown. The bar graph indicates quantitative measurement of PECAM-1 protein levels performed in duplicate. (B) Tube formation assay in cells treated with different concentrations of L-PDMP for 4hrs is presented. Data presented are representative of experiments performed in duplicate, yielding similar results.
Figure 1
Figure 2