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Protein kinase C regulates FLT1 abundance and stimulates its cleavage in vascular endothelial cells with the release of a soluble PIGF/VEGF antagonist

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Abstract

FLT1 and its soluble form (sFLT1) arise as alternate transcripts from the same gene and sFLT1 can antagonize the effect of vascular endothelial growth factor (VEGF) on its cognate receptors. We investigated the effect of VEGF and protein kinase C (PKC) activation on sFLT1 abundance. We demonstrated that VEGF stimulates sFLT1 and FLT1 mRNA and protein levels in vascular endothelial cells via VEGFR2 and PKC. Using an FLT1 expression vector with N and C-terminal epitope tags, we show that PKC activation increases the cleavage of FLT1 into an N-terminal extracellular fragment and a C-terminal intracellular fragment with the cleavage occurring adjacent to the transmembrane domain. The trafficking and glycosylation inhibitors brefeldin, monensin and tunicamycin substantially reduced cleavage and release of the N-terminal ectodomain of FLT1 and inhibited secretion of the isoforms of sFLT1. The shed FLT1 ectodomain can bind VEGF and PIGF and inhibit VEGF-induced vascular tube formation thus confirming that it is functionally equivalent to the alternately spliced and secreted sFLT1 isoforms.

Keywords

Angiogenesis; proteolytic cleavage; soluble receptor

Introduction

VEGF-A is a potent angiogenic factor that binds to several receptors including VEGFR1 or FLT1 (fms-like tyrosine kinase-1) and VEGFR2 or FLK1 (fetal liver kinase 1) to initiate a number of signaling cascades in endothelial cells that result in increased cell proliferation, migration and vascular permeability. Both FLT1 and FLK1 are transmembrane receptors with an extracellular N-terminal ligand-binding segment characterized by several

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immunoglobulin (Ig) or Ig-like domains, a single membrane-spanning segment and a C-terminal intracellular segment that carries a split tyrosine kinase domain. VEGF-A binds as a homo- or heterodimer to the Ig domains of VEGF receptors which then leads to activation of the receptors. Both FLT1 and FLK1 activate a signaling cascade in endothelial cells that result in the activation of protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3-Kinase) and MAP kinases (1, 2). VEGFR2/FLK1 is the principal receptor that transduces proliferative, migratory and permeability signals in vascular endothelial cells although VEGFR2 has an affinity for VEGF-A which is about 10 fold lower than that of VEGFR1. The actual role of VEGFR1/FLT1 in VEGF signaling is debated. Mice with homozygous inactivation of the *Flt1* gene die *in utero* with disorganized embryonic vasculature (3). However, mice with deletion of the tyrosine kinase (signaling) domain of *Flt1* have no defect in development or in angiogenesis suggesting that FLT-1 may serve to tether VEGF to the cell surface and regulate access of free VEGF to its second receptor, VEGFR2/FLK1 (4, 5).

In addition to the full length VEGF receptor, FLT1, multiple soluble forms of the receptor (sVEGFR1/sFLT1) that can bind VEGF/PlGF with an affinity equal to FLT1 have been identified (6, 7). The mRNAs for sFLT1 and FLT1 have a common transcription start site and a common promoter and upstream regulatory elements. sFLT1 isoforms, i13 and e15a, are encoded by transcripts that arise by intronic polyadenylation or by alternate splicing and result in a secreted protein that lacks the membrane-spanning and C-terminal domains (8, 9). Other transcripts that arise from FLT1 have intronic transcription start sites and encode isoforms that just contain portions of the intracellular tyrosine kinase domains and C-terminal tail (10). sFLT1 effectively functions as a natural VEGF antagonist regulating the actions of VEGF locally where it is produced and also in distant sites. The physiological role of sFLT1 in angiogenesis and maintaining corneal avascularity is now better understood and there is increasing recognition that the hypertension and proteinuria of preeclampsia may be caused by excessive placental production of sFLT1 (11-13). We have previously shown that phorbol myristic acid (PMA) increases the abundance of sFLT1 mRNA and protein (14). We now show that PMA also stimulates the cleavage of FLT1 with the generation of a N-terminal extracellular sFLT1 fragment that binds VEGF and PlGF, and reduce VEGF stimulated vascular tube formation, demonstrating that sFLT1 can be derived post-translationally from the endoproteolytic cleavage of FLT1.

Methods

Materials

Brefeldin A, 3-[1-(Dimethylaminopropyl)indol-3-yl]-4-(indol-3-yl)maleimide hydrochloride, Bisindolylmaleimide I hydrochloride (GF 109203X hydrochloride), heparin, monensin, phorbol 12-myristate 13-acetate (PMA), hexadimethrine bromide and tunicamycin were purchased from Sigma-Aldrich (St. Louis, MO). SKLB1002, GF109293X and cycloheximide were from EMD Millipore (Billerica, MA). Recombinant human VEGF 165, ELISA Kits for human PlGF, VEGF and human sVEGF R1 and anti-sFlt1 antibody (AF321) were obtained from R&D Systems (Minneapolis, MN). Other antibodies, anti-HA antibody (Y-11), anti-Flt1 antibody (sc-316), HRP-conjugated goat anti-mouse IgG and HRP-conjugated donkey anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated goat anti-rabbit IgG was from Cell Signaling Technology (Danvers, MA) and anti-Flag antibody was from Sigma-Aldrich. Peptide N glycosidase F (PNGase F) was purchased from QA-Bio (Palm Desert, CA).

Plasmids

C-terminal Myc-Flag-tagged open reading frame (ORF) clone of full length human FLT1/VEGFR1 was purchased from OriGene (Rockville, MD). An additional HA-tag was inserted 30 AA downstream of the FLT1 start codon in Myc-Flag-tagged FLT1. This FLT1 construct was also subcloned into an adenoviral shuttle vector, pacAd5CMV K-N pA, under control of the CMV promoter. The resulting plasmid was used to generate recombinant adenovirus expressing FLT1 (Ad5CMV FLT1 HAMycFlag) by the Vector Core Facility at the University of Iowa. The empty adenoviral vector Ad5CMV was used as a control. Full length sFLT1-i13 and sFLT1-e15a plasmid constructs with C-terminal V5 tags have been described previously (9). The sFLT1-i13 construct was also used to generate recombinant adenovirus expressing sFLT1 (Ad5CMVsFLT1-i13-V5).

Cell culture

Primary Human Umbilical Vein Endothelial cells (HUVECs) purchased from Lonza (Walkersville, MD) and human microvascular endothelial cells (HMEC-1) were cultured in endothelial growth medium-2 (EGM-2) containing supplements and growth factors including 2% fetal bovine serum (FBS). African green monkey kidney cell line COS7 and human embryonic kidney cell line HEK293 were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 1% penicillin-streptomycin. Chinese Hamster Ovary (CHO) cells were cultured in DMEM/F12 media with 10% FBS and 1% penicillin-streptomycin. Primary cytotrophoblasts (CTBs) were isolated from human placenta as previously described and cultured in Ham's F10/Waymouth (1:1 vol/vol) media containing 10% FBS (9).

Transient transfections and adenoviral transduction

HEK293 and COS7 cells were transiently transfected with various cDNAs using Lipofectamine 2000 from Life Technologies (Grand Island, NY) as per manufacturer's recommendation. Cell cultures were switched to serum-free medium containing PMA or heparin or both for varying time periods or with monensin, brefeldin or tunicamycin for 24hrs. For some experiments, COS7 cells were transduced with recombinant adenovirus expressing FLT1 or control virus, Ad5CMV (30×10^7 plaque forming units/100mm dish) in serum-free media. In other experiments, CHO cells were treated with CAR Receptor Booster (Clontech, CA) for 2 hours and then transduced with recombinant adenovirus expressing sFLT1-i13, FLT1 or control virus, Ad5CMV (12×10^7 plaque forming units/9cm² dish) in serum-free media supplemented with hexadimethrine bromide. Conditioned media was collected 48 hr following transfections or transductions, centrifuged at 350 *g* for 5 min to remove dead cells and then stored frozen at -70°C. For western blot analysis, conditioned media was concentrated using Amicon Ultra centrifugal filters from EMD Millipore (Billerica, MA). Transfected/transduced monolayers were washed with PBS and kept frozen until lysates were prepared.

Western blotting and ELISA

Cells were washed with PBS and lysed in 2× Laemmli buffer (3% sodium dodecyl sulfate, 12% glycerol, 50mM Tris, pH 6.8 and 80mM dithiothreitol) containing protease inhibitor cocktail from Roche Applied Science (Indianapolis, IN). Equal amounts of whole cell lysate and concentrated media were subjected to SDS-PAGE for the separation of proteins. In some cases, conditioned media were incubated with 10 mU of PNGase for 3 hr before SDS-PAGE. Resolved proteins were then transferred on a polyvinylidene fluoride (PVDF) membrane from EMD Millipore (Billerica, MA). In other cases, cells were trypsinized, washed and then membrane, cytosolic and nuclear protein fractions were separated using ProteoExtract® Subcellular Proteome Extraction Kit (EMD Millipore, Billerica, MA) and

subjected to SDS-PAGE. PVDF membranes were incubated sequentially with primary and secondary antibodies. Signals were detected with SuperSignal West Pico/Femto Chemiluminescent Substrate from Fisher Scientific (Pittsburgh, PA), and the image was captured using VisionWorksL S image acquisition and analysis software and the EC³ imaging system from UVP LLC (Upland, CA). Stripping of membranes for repeated blotting was done using 0.2M NaOH.

Quantitation of sFLT1 from conditioned media was performed by ELISA. Quantification of free VEGF was performed by incubating VEGF in the presence of increasing molar concentrations of secreted sFLT1 or cleaved soluble N-terminal fragment of FLT1 using a VEGF ELISA kit (human VEGF Quantikine Immunoassay, R&D Systems).

RNA Extraction, cDNA preparation and real-time PCR

Total RNA from HUVECs was extracted with Absolutely RNA Miniprep kit from Agilent Technologies (Santa Clara, CA) according to the manufacturer's instructions. RNA was quantified using UV-Visual spectrophotometry at 260nm. Equal amounts of RNA were reverse transcribed to generate cDNA with AffinityScript quantitative qPCR cDNA synthesis kit (Agilent Technologies) with the following conditions: 25°C for 5 min for oligo (dT) and random primer annealing, 42°C for 45 min for cDNA synthesis, and 95°C for 5 min for termination.

Real-time qPCR was performed to measure FLT1 and sFLT1-i13 mRNA levels from HUVECs with PCR primers published earlier (9). Briefly, Brilliant II SYBR Green QPCR master mix with Low ROX was used for the detection of amplicons in an Mx3000p Multiplex PCR system (Agilent Technologies). Results were reported as the relative mRNA fold change compared to controls.

Vascular tube formation assay

Tube formation assays were performed in μ -Slide Angiogenesis wells (ibidi LLC, Verona, WI) where 10 μ l of growth factor-reduced Matrigel™ (BD Bioscience, San Jose, CA) had been added to each well and allowed to polymerize. HUVECs grown in complete EGM-2 media were resuspended in serum-free VEGF-free media at 2×10^5 cells/ml and 50 μ l cell suspension added per well. In some experiments cell suspensions were treated with conditioned media either from FLT1 or control adenovirus-transduced COS7 cells before seeding. In other experiments HUVECs were transduced with FLT1 or control adenoviral vectors 48 hr prior to seeding onto μ -slides. The cells were then incubated at 37°C in a 5% CO₂ incubator for ~20 hrs. Bright field images were taken using an inverted microscope with a 4 $\times$ magnification. Quantification of endothelial tubes was done with Image J software (NIH) and data is presented as relative endothelial tube lengths.

Statistical analysis

The data in all graphs are represented as mean $\pm$ standard error of the mean. They were tested for significance with Mann Whitney Rank Sum test or Kruskal Wallis one-way analysis on ranks (ANOVAR), where applicable, using SigmaPlot® 12 (San Jose, CA). P values <0.05 were considered statistically significant in all analysis.

Results

We have previously reported that PMA, an activator of PKC, robustly stimulates sFLT1 mRNA and protein levels in the vascular endothelial cells, HUVEC, HMEC-1 and UtMVEC (14). Since VEGF stimulates PKC in HUVEC cells we hypothesized that VEGF may itself stimulate the level of sFLT1, a VEGF antagonist that may modulate the function of VEGF.

When tested in HUVEC cells, VEGF stimulated the expression of *sFLT1* and *FLT1* mRNA in a time-dependent manner with *sFLT1* rising 3.4 fold and *FLT1* 2 fold at 24 hr (Figure 1A). The effect of VEGF on *sFLT1* mRNA expression is mediated via VEGFR2/FLK1 as SKLB, a VEGFR2 antagonist, inhibits the effect of VEGF (Figure 1B). We then tested the effect of a PKC antagonist GF109203X on *sFLT1* and demonstrate that there is significant reduction in VEGF-mediated *sFLT1* mRNA expression (Figure 1C). We show that VEGF stimulates sFLT1 release into conditioned media with the effect evident at 8 hr with a robust increase at 24 hr (Figure 1D). The increase in sFLT1 release is substantially inhibited by GF109293X indicating that VEGF increases the abundance of sFLT1 via PKC activation.

We examined the effect of PMA on *sFLT1* and *FLT1* in HUVEC and noted that PMA stimulates *sFLT1* mRNA expression with a more modest effect on *FLT1* mRNA expression although the temporal profile was quite different for *sFLT1* compared to *FLT1* (Figure 2A). The effect of PMA was inhibited by GF109203X indicating that this effect was mediated via PKC (Figure 2B). We also confirmed the effect of GF109293X on PMA-stimulated sFLT1 release into HUVEC conditioned media (Figure 2C). As there appeared to be an effect of PMA to also increase *FLT1* mRNA expression, we examined FLT1 levels by Western blotting. We noted that PMA robustly increases FLT1 protein and that a ~65 kDa band appeared specifically in PMA treated lanes (Figure 2D). Since the Flt1 antibody is directed to the C-terminus, the band could correspond to a C-terminal cleaved product of FLT1 as has been described by others (15). The effect of PMA on FLT1 protein abundance is also inhibited by GF109293X (Figure 2E). To look for preliminary evidence for the N-terminal portion of the cleaved product, expected to be in conditioned media, we tested the effect of cycloheximide, a general protein synthesis inhibitor, on PMA treated cells. Our experiment demonstrates that cycloheximide reduces the amount of measurable sFlt1 in conditioned media at 4 hr by 47% compared to vehicle. As cycloheximide blocks continued synthesis of sFlt1 and Flt1 but does not block cleavage of presynthesized Flt1, this data is consistent with the notion that the shed ectodomain contributes significantly to the total sFlt1 produced by HUVEC. To better examine the effect of PMA on FLT1 cleavage we cloned full length FLT1 inserting a C-terminal myc-FLAG/DDK epitope and then added an HA epitope just downstream of the signal peptide at the N-terminus. Constructs were expressed in HEK cells and then treated with PMA or Heparin for 24 hr. A ~200 kDa band is recognized by an anti-HA and anti-FLAG Ab which corresponds to full length FLT1 (Figure 3A). The FLAG antibody also detects a 65 kDa band in cell lysates confirming that full length FLT1 is cleaved to give rise to a C-terminal fragment bearing the FLAG epitope. PMA substantially increases the abundance of full length FLT1 and its cleaved products whereas heparin has no effect on basal levels of FLT1 or its cleaved C-terminal fragment.

We then examined the time course of the PMA effect and noted that the effect is first evident at 2 hr and maximal by 24 hr (Figure 3B). A C-terminal cleaved Flag fragment is evident in cell lysates and an N-terminal cleaved ~130 kDa HA fragment is evident in conditioned media indicating that the N-terminal cleavage occurs after FLT1 arrives at the cell membrane and the site of cleavage is in the extracellular portion of FLT1. Substantial C-terminal cleavage is evident by 6 hr in cell lysates while N-terminal cleavage is evident only by 24 hr although this may reflect the different sensitivities of various antibodies. When conditioned media blot is probed with an sFlt1 antibody (AF-321) we demonstrate a ~140 kDa band and ~ an 85 kDa band from pcDNA3 transfected cells treated with PMA and heparin that correspond to the natural secreted forms of sFLT1. Heparin when added to PMA increases the abundance of the secreted sFLT1 in conditioned media. In addition to the secreted sFLT1, in conditioned media from FLT1 transfected cell lysates we identify a ~130 kDa band that corresponds to the HA-tagged N-terminal cleaved form of FLT1. We find that heparin also increases the HA-tagged cleaved fragment while there is little effect on full length FLT1 in cell lysates consistent with the concept that heparin increases the release of

the cleaved FLT1 fragment from the extracellular surface as has been described for the secreted form of sFLT1 (16, 17).

We then performed subcellular fractionation of HEK cell lysates and demonstrate that the 65 kDa fragment is entirely cytosolic while full length FLT1 appears to be completely membrane-associated (Figure 4A). The samples were re-blotted with an antibody to EGFR, a known membrane-associated protein which confirmed that > 90% of EGFR is found in the membrane prep. Similarly the samples were re-blotted with an antibody to HSP 90, a cytosolic protein, which showed that ~80% of HSP90 is found in the cytosolic fraction. We did not find FLT1 or its cleaved products in nuclear fractions, and we validated this by demonstrating that the greater part of the transcription factor Sp1 is found in this fraction (data not shown). To confirm that FLT1 can be cleaved to release an extracellular fragment into conditioned media we expressed FLT1 in COS-7 cells, and measured sFLT1 in collected media. In mock-transfected cells there is no detectable sFLT1 in conditioned media while in FLT1 transfected cells we see an increase in measured sFLT1, which confirms that the N-terminal cleavage product of FLT1 is recognized as sFLT1 by the assay (Figure 4B).

We cloned tagged FLT1 into a replication-defective adenovirus and then transduced COS-7 cells and demonstrate abundant levels of FLT1 and the presence of a cleaved C-terminal fragment which is absent in COS-7 cells transduced with empty virus (supplementary data and Figure 4C). To determine if FLT1 is naturally cleaved in other cells and tissues, we used a C-terminal FLT1 antibody to immunoblot lysates of HMEC-1, a microvascular endothelial cell line and primary CTB isolated from human placenta. We demonstrate a C-terminal cleaved product in CTB and in HMEC-1. PMA stimulates cleavage of FLT1 in HMEC-1 similar to that seen in HUVEC cells (Figure 4C).

FLT1 and sFLT1 are heavily glycosylated proteins and to begin to determine the site of extracellular cleavage we immunoblotted FLT1 after deglycosylation of cell lysates. HA-tagged FLT1 or empty plasmid was transfected into HEK cells and the lysates treated with PNGase F. Blotting with an HA antibody shows that the ~130 kDa C-terminal piece of FLT1 is reduced to an ~85-95 kDa fragment (Figure 5A, lane 4). This places the cleavage site very close to the transmembrane domain. When blotted with an anti-sFLT1 antibody (AF-321), two bands are seen at 130-140 and 85-95 kDa which are reduced to 95-110 and 70-80 kDa (Figure 5A, lower panel, lanes 1 and 2) which is different in size from the reduced cleaved FLT1 fragment (Figure 5A, lane 4) and likely represents sFLT1-i13 and sFLT1-e15a. We have previously reported, that the natural forms of sFLT1 (sFLT1-i13 and sFLT1-e15a) can be reduced by PNGase F as these are also heavily glycosylated proteins (9).

We examined the effect of inhibitors of glycosylation and trafficking on FLT1 cleavage. FLT1 transfected cells were exposed to tunicamycin, which interferes with the transfer of N-acetylglucosamine to dolichol, the earliest step in protein glycosylation in the ER (18); to monensin, which inhibits transport of glycoproteins from the ER to the Golgi (19); and to brefeldin A, which induces the fusion of Golgi vesicles with the ER and leads to retention of secretory proteins in the ER (20). We find that FLT1 is expressed with no change in its abundance in the presence of monensin or brefeldin A, although there may be a small reduction in size with brefeldin A (Figure 5B). Tunicamycin, on the other hand reduced the size of FLT1 significantly consistent with inhibition of glycosylation. We then looked at the N-terminal cleavage of FLT1 in both lysates and in conditioned media. We find that monensin, brefeldin A and tunicamycin, almost completely abolished N-terminal cleavage of FLT1 with no detectable cleaved product in lysates or media. However, there is a reduced amount of unglycosylated full length FLT1 in tunicamycin treated cells and therefore the

sensitivity of the assay to detect the C-terminal fragment is diminished. Nevertheless, the results suggest that FLT1 glycosylation and trafficking to the cell surface is required for cleavage. We also examined the effect of these agents on secretion of sFLT1. We expressed both tagged sFLT1-i13 and sFLT1-e15a in HEK cells and treated cells with monensin, brefeldin A and tunicamycin. As we see with full length FLT1, tunicamycin reduced the size of sFLT1-i13 and sFLT1-e15a consistent with inhibition of glycosylation (Figure 5C). Monensin, brefeldin A and tunicamycin inhibited secretion of sFLT1.

To determine if the N-terminal cleaved fragment of FLT1 could bind VEGF/PlGF and function as a VEGF/PlGF antagonist we transduced COS-7 cells with the adenoviral construct expressing tagged FLT1 and collected conditioned media from control vector and FLT1 vector-transduced cells. We tested the concentrated media in a PlGF assay and demonstrate that the cleaved FLT1 fragment reduces the amount of free PlGF measurable in an ELISA plate, presumably by binding PlGF and masking its immuno-reactive epitope (supplementary data). Since COS-7 cells secrete VEGF, we also measured free VEGF in control adenovirus- and FLT1 adenovirus-transduced cells. We find that we cannot detect free VEGF in the media from FLT1 transfected cells compared to control (-8.8 ± 8.5 pg/ml in FLT1 transduced cells vs 761.7 ± 136.9 in control-transduced cells) consistent with the presence of a cleaved FLT1 fragment that binds VEGF and masks its immuno-reactive epitope. To directly compare the effect of alternately spliced and secreted sFLT1 with the cleaved FLT1 we collected conditioned media from CHO cells expressing sFLT1 and FLT1 from adenoviral constructs. Equal amounts of sFLT1 and cleaved FLT1 in conditioned media were added in an increasing molar ratio to a fixed concentration of VEGF and then measured free VEGF using a standard ELISA. We demonstrate a dose dependent reduction in free VEGF and could not detect a difference between sFLT1 and cleaved FLT1 in their ability to bind VEGF (Figure 6A). We then tested the effect of N-terminal cleaved FLT1 in a vascular tube formation assay. In one set of experiments we added conditioned media from COS-7 cells transduced with FLT1 or control vector to HUVEC cells and observed tube formation. Media containing the cleaved N-terminal fragment of FLT1 inhibited endothelial tube formation (Figure 6B). In another set of experiments we transduced HUVEC cells with FLT1 or its control vector and compared endothelial tube formation. We demonstrate that FLT1 vector-transduced cells had significantly fewer endothelial tubes and compared to control vector-transduced cells (Figure 6C and D). These results confirm that functionally competent sFLT1 can arise from post-translational cleavage of FLT1.

Discussion

VEGFs are a family of growth factors that act in an autocrine or paracrine fashion on vascular endothelial cells to regulate vascular permeability, proliferation and migration (2). VEGFs achieve these responses by binding as homo- or heterodimers to its cognate tyrosine kinase receptors, VEGFR1 and VEGFR2, on endothelial cells to activate a number of intracellular signaling cascades. VEGF-induced tyrosine phosphorylation of VEGFR2 can activate phospholipase C which in turn activates PKC isoforms and MAP kinases (21). We explored the possibility that VEGF would increase the abundance of its receptors in HUVEC. We show that VEGF increases both FLT1 and sFLT1 mRNA and that this is mediated via VEGFR2 and activation of PKC. However, the inhibition of PKC does not completely abolish the effect of VEGF on sFLT1 and FLT1 mRNA (Figure 1C) suggesting that other pathways, such as the PI3 kinase pathway, may also be involved in VEGF signaling (22). Interestingly, PKC activation increases the abundance of FLT1 and leads to cleavage of FLT1 with the generation of a ~65 kDa C-terminal fragment in HUVEC and in HMEC-1 cells. Previous studies have shown that PlGF stimulates proteolytic cleavage and ectodomain shedding in SKI-DLBCL and HT cells, lymphoma cell lines but not in HUVEC (23). Ectodomain shedding may be regulated by a metalloprotease of the ADAM/TACE

family as it can be blocked by GM60001 although neither the size of the shed ectodomain fragment nor the site of extracellular cleavage was previously known. The C-terminal fragment likely arises from an intramembrane cleavage by -secretase since this can be inhibited by a dominant negative form of presenilin (24). The C-terminal fragment reported in that study is similar in size to that we find in vascular endothelial cells, and CTB. Other studies however have reported that an ~80 kDa FLT1 fragment appears intracellularly in Pigment Epithelium-derived Factor (PEDF) and VEGF-treated bovine retinal microvascular endothelial cells (BRMECs) but not when these cells were treated with VEGF alone (15). In a related study, mutation of a Valine at position 767 of FLT1 was shown to lead to loss of -secretase mediated cleavage and release of an 80 kDa fragment (24). Given the discordance in size of the cleaved fragment in these reports and to examine the regulation of cleavage we tagged full length FLT1 at the N and C-termini and expressed this in HEK and COS-7 cells. We confirm that PMA regulates the cleavage of FLT1 into a ~65 kDa C-terminal fragment and a ~130 kDa N-terminal fragment. We demonstrated that glycosylation is necessary for cleavage and that inhibitors of intracellular trafficking reduce both N-terminal and C-terminal cleavage. We have previously reported that brefeldin A inhibits the secretion of sFLT1-i13 (25). Here we also demonstrate that monensin, brefeldin A and tunicamycin abolish the secretion of sFLT1-i13 and its variant isoform sFLT1-e15a.

The cleavage of FLT1 at a juxta-membranous extracellular site to release an ectodomain and a second cleavage to release a C-terminal cytosolic fragment appears to be an example of regulated intramembrane proteolysis (26). The extracellular cleavage may serve to limit VEGF/PlGF action by eliminating the ligand binding domain of FLT1. However the phenomenon appears to take several hours and is not likely to be an efficient mechanism for rapid turnoff of FLT1-induced signaling. While we clearly detect abundant cleaved FLT1 in conditioned media of FLT1 transfected cells with an increased release in the presence of heparin, the cleavage of FLT1 could occur after VEGF/PlGF binding and internalization of the bound receptor with intracellular VEGF or its receptor fragment mediating a biological function. It is now well established that VEGF can serve intracrine functions although the mechanisms remain to be identified (27, 28). An alternate transcript of FLT1 encodes an intracellular isoform of the receptor that can activate src and promote cell invasion (10). Alternatively, the cleavage of FLT1 may be yet another mode of generation of bioactive sFLT1 that can serve paracrine or endocrine functions and may contribute to the physiological or pathological roles of sFLT1 in inhibiting vascular tube formation. We were unable to detect a functional difference between alternately spliced and secreted sFLT1 and cleaved FLT1 at least in VEGF binding assays.

In conclusion, we demonstrate that VEGF signaling via VEGFR2 activates PKC which in turn stimulates the cleavage of VEGFR1 to release an N-terminal ectodomain and a C-terminal fragment. This cleavage step appears to be dependent on glycosylation and trafficking of VEGFR1. The cleaved ectodomain of VEGFR1 seems functionally equivalent to transcriptionally derived sFLT1 as it can bind PlGF and VEGF and inhibit VEGF-mediated angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Cross MJ, Dixelius J, Matsumoto T, Claesson-Welsh L. VEGF-receptor signal transduction. *Trends Biochem Sci.* 2003; 28(9):488–94. 2003/9. [PubMed: 13678960]
2. Koch S, Tugues S, Li X, Gualandi L, Claesson Welsh L. Signal transduction by vascular endothelial growth factor receptors. *Biochem J.* Jul 15; 2011 2011 437(2):169–83. [PubMed: 21711246]
3. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature.* Jul 6; 1995 376(6535):66–70. PubMed PMID: 7596436. [PubMed: 7596436]
4. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *PNAS.* Aug 4; 1998 95(16):9349–54. 1998. [PubMed: 9689083]
5. Hiratsuka S, Nakao K, Nakamura K, Katsuki M, Maru Y, Shibuya M. Membrane Fixation of Vascular Endothelial Growth Factor Receptor 1 Ligand-Binding Domain Is Important for Vasculogenesis and Angiogenesis in Mice. *Mol Cell Biol.* Jan 1; 2005 25(1):346–54. 2005. [PubMed: 15601855]
6. He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol.* Apr; 1999 13(4):537–45. PubMed PMID: 10194760. [PubMed: 10194760]
7. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci U S A.* Nov 15; 1993 90(22):10705–9. PubMed PMID: 8248162. [PubMed: 8248162]
8. Thomas CP, Andrews JI, Liu KZ. Intronic polyadenylation signal sequences and alternate splicing generate human soluble Flt1 variants and regulate the abundance of soluble Flt1 in the placenta. *FASEB J.* 2007; 21:3885–95. [PubMed: 17615362]
9. Thomas CP, Andrews JI, Raikwar NS, Kelley EA, Herse F, Dechend R, et al. A recently evolved novel trophoblast-enriched sFlt1 variant is upregulated in hypoxia and in preeclampsia. *J Clin Endocrinol Metab.* Mar 31; 2009 94(7):2524–30. 2009. Pubmed Central PMCID: PMC2708964. [PubMed: 19336504]
10. Mezquita B, Mezquita J, Pau M, Mezquita C. A novel intracellular isoform of VEGFR-1 activates Src and promotes cell invasion in MDA-MB-231 breast cancer cells. *J Cell Biochem.* 2010; 110(3):732–42. [PubMed: 20512933]
11. Ambati BK, Nozaki M, Singh N, Takeda A, Jani PD, Suthar T, et al. Corneal avascularity is due to soluble VEGF receptor-1. *Nature.* 2006; 443(7114):993. [PubMed: 17051153]
12. Chappell JC, Taylor SM, Ferrara N, Bautch VL. Local Guidance of Emerging Vessel Sprouts Requires Soluble Flt-1. *Developmental Cell.* 2009; 17(3):377–86. [PubMed: 19758562]
13. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest.* Mar; 2003 111(5):649–58. PubMed PMID: 12618519. [PubMed: 12618519]
14. Thomas CP, Raikwar NS, Kelley EA, Liu KZ. Alternate processing of Flt1 transcripts is directed by conserved cis-elements within an intronic region of FLT1 that reciprocally regulates splicing and polyadenylation. *Nucl Acids Res.* Apr 12.2010 :gkq198. 2010.
15. Cai J, Jiang WG, Grant MB, Boulton M. Pigment Epithelium-derived Factor Inhibits Angiogenesis via Regulated Intracellular Proteolysis of Vascular Endothelial Growth Factor Receptor 1. *J Biol Chem.* Feb 10; 2006 281(6):3604–13. 2006. [PubMed: 16339148]
16. Sela S, Natanson-Yaron S, Zcharia E, Vlodavsky I, Yagel S, Keshet E. Local Retention Versus Systemic Release of Soluble VEGF Receptor-1 Are Mediated by Heparin-Binding and Regulated by Heparanase / Novelty and Significance. *Circ Res.* Apr 29; 2011 2011 108(9):1063–70. [PubMed: 21415391]
17. Searle J, Mockel M, Gwosc S, Datwyler SA, Qadri F, Albert GI, et al. Heparin Strongly Induces Soluble Fms-Like Tyrosine Kinase 1 Release In Vivo and In Vitro—Brief Report. *Arterioscler Thromb Vasc Biol.* Dec 1; 2011 31(12):2972–4. 2011. [PubMed: 21979436]

18. Elbein AD. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu Rev Biochem.* 1987; 56:497–534. PubMed PMID: 3304143. Epub 1987/01/01. eng. [PubMed: 3304143]
19. Tartakoff AM. Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell.* Apr; 1983 32(4):1026–8. PubMed PMID: 6340834. Epub 1983/04/01. eng. [PubMed: 6340834]
20. Fujiwara T, Oda K, Yokota S, Takatsuki A, Ikehara Y. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J Biol Chem.* Dec 5; 1988 263(34):18545–52. PubMed PMID: 3192548. Epub 1988/12/05. eng. [PubMed: 3192548]
21. Kroll J, Waltenberger J. The Vascular Endothelial Growth Factor Receptor KDR Activates Multiple Signal Transduction Pathways in Porcine Aortic Endothelial Cells. *J Biol Chem.* Dec 19; 1997 197 272(51):32521–7. [PubMed: 9405464]
22. Ahmad S, Hewett PW, Al-Ani B, Sissaoui S, Fujisawa T, Cudmore MJ, et al. Autocrine activity of soluble Flt-1 controls endothelial cell function and angiogenesis. *Vascular cell.* 2011; 3(1):15. PubMed PMID: 21752276. Pubmed Central PMCID: 3173355. [PubMed: 21752276]
23. Rahimi N, Golde TE, Meyer RD. Identification of Ligand-Induced Proteolytic Cleavage and Ectodomain Shedding of VEGFR-1/FLT1 in Leukemic Cancer Cells. *Cancer Res.* Mar 15; 2009 69(6):2607–14. 2009. [PubMed: 19276374]
24. Cai J, Chen Z, Ruan Q, Han S, Liu L, Qi X, et al. -secretase and presenilin mediate cleavage and phosphorylation of vascular endothelial growth factor receptor-1. *J Biol Chem.* Oct 20.2011 2011
25. Jung J-J, Tiwari A, Inamdar SM, Thomas CP, Goel A, Choudhury A. Secretion of Soluble Vascular Endothelial Growth Factor Receptor 1 (sVEGFR1/sFlt1) Requires Arf1, Arf6, and Rab11 GTPases. *PLoS ONE.* 2012; 7(9):e44572. [PubMed: 22962618]
26. Lal M, Caplan M. Regulated Intramembrane Proteolysis: Signaling Pathways and Biological Functions. *Physiology.* Feb 1; 2011 26(1):34–44. 2011. [PubMed: 21357901]
27. Li W, Keller G. VEGF nuclear accumulation correlates with phenotypical changes in endothelial cells. *J Cell Sci.* May 1; 2000 2000 113(9):1525–34. [PubMed: 10751144]
28. Liu Y, Berendsen AD, Jia S, Lotinun S, Baron R, Ferrara N, et al. Intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation. *The Journal of Clinical Investigation.* 2012; 122(9):3101–13. [PubMed: 22886301]

Highlights

- We investigated the effect of VEGF and PKC activation on sFLT1 and Flt1 expression.
- We demonstrate that VEGF stimulates sFLT1 and FLT1 in vascular endothelial cells via VEGFR2 and PKC.
- We show that PKC activation stimulates the cleavage of FLT1 into an N-terminal ectodomain and a C-terminal intracellular fragment.
- The shed FLT1 ectodomain can bind PlGF and VEGF and inhibit VEGF-induced vasculogenesis
- The study confirms that the Flt1 ectodomain is functionally equivalent to the alternately transcribed and secreted sFLT1 isoforms.

Figure 1. Effect of VEGF 165 on sFLT1 and FLT1 levels in HUVECs

Panel A: HUVECs were exposed to 100ng/ml VEGF 165 or vehicle and then performed qRT-PCR for sFLT1 and FLT1 mRNA expressed as fold difference compared to 0 hr. VEGF 165 stimulates sFLT1 and FLT1 expression. * $P < 0.05$ by Kruskal Wallis ANOVA compared to corresponding 0 hr; mean $\pm$ SD of 3 samples. **Panel B:** HUVECs treated with 100ng/ml VEGF 165 or vehicle in the presence or absence of 10 μ M SKLB1002 (SKLB) for 4hrs and then qRT-PCR for sFLT1 and FLT1 mRNA expressed as fold difference compared to corresponding vehicle. SKLB inhibits basal and VEGF 165-stimulated sFLT1 expression. ** $P < 0.001$ compared to respective other groups; $P < 0.05$ compared to vehicle* and VEGF +SKLB $\S$; Kruskal Wallis ANOVA, mean $\pm$ SD of 3 samples. **Panel C:** HUVECs treated with 100ng/ml VEGF 165 or vehicle for 24 in the presence or absence of 1 μ M GF109203X (GF109) and then qRT-PCR for sFLT1 and FLT1 mRNA expressed as fold difference compared to corresponding vehicle. GF109 inhibits VEGF 165 stimulated sFLT1 and FLT1 expression. ** $P < 0.001$ and * $p < 0.05$ compared to corresponding vehicle; Kruskal Wallis ANOVA, mean $\pm$ SD of 3 samples. **Panel D:** sFLT1 protein in HUVEC conditioned media. HUVECs treated with vehicle (veh), VEGF, GF109 or the combination and sFLT1 in conditioned media collected at specified intervals and measured by ELISA. * $P < 0.05$ by Kruskal Wallis ANOVA, for that time point compared to other groups; mean $\pm$ SD of 3 samples.

Figure 2. Effect of PMA on sFLT1 and FLT1 levels in HUVECs

Panel A: HUVECs exposed to 30 nM PMA or vehicle and then qRT-PCR for sFLT1 and FLT1 mRNA expressed as fold difference compared to 0 hr. PMA stimulates sFLT1 and FLT1 mRNA expression. **P < 0.05 by Kruskal Wallis ANOVA; mean ± SD of 3 samples. **Panel B:** HUVECs exposed to PMA or vehicle in the presence or absence of GF109 and then qRT-PCR for sFLT1 and FLT1 expressed as fold difference compared to corresponding vehicle. PMA stimulated sFLT1 and FLT1 mRNA expression is inhibited by GF109. mean ± SD of 4 samples. **P < 0.001 by Kruskal Wallis ANOVA compared to Veh and GF109 alone. **Panel C:** sFLT1 protein in HUVEC conditioned media. HUVECs treated with vehicle (veh), PMA, GF109 or the combination and sFLT1 in conditioned media collected at specified intervals and measured by ELISA. Each measurement represents the mean ± SD of 3 samples. *P < 0.05 by Kruskal Wallis ANOVA, for that time point compared to other groups; \$P < 0.05 compared to Veh & GF109; mean ± SD of 3 samples. **Panel D:** HUVECs treated with PMA and cell lysates immunoblotted with an ab directed to the C-terminal portion of FLT1. PMA increases FLT1 protein levels and leads to the appearance of a 65 kDa fragment in cell lysates. **Panel E:** Effect of GF109 on PMA stimulated FLT1 in HUVECs (pooled data from western on FL only). Bar graph above and immunoblot below. **P < 0.001 for by Kruskal Wallis ANOVA compared to other groups, mean + SD of 3 samples. **Panel F:** Effect of cycloheximide on sFLT1 protein in HUVEC conditioned media. HUVECs were pretreated with PMA for 17 hr and then cycloheximide (CHX) or vehicle (veh) was added and conditioned media collected for the indicated time points. Control cells (ctrl) were not exposed to PMA or CHX. Following CHX treatment for 4 hr, the amount of measurable sFLT1 is reduced to 47% of the response seen with vehicle, indicating that a significant fraction of sFLT1 is CHX resistant. *P < 0.05 by Kruskal Wallis ANOVA for that time point compared to other group; Mean + SD for n=4.

Figure 3. FLT1 cleavage assessed by immunoblotting

Panel A: Empty plasmid (pcDNA3) and FLT1 expression plasmid with C-terminal Flag without (FLT1 Flag) or with an N-terminal HA epitope (FLT1 HA Flag) transfected into HEK293 cells and exposed to 30 nM PMA or 10U/ml heparin for 24 hr. Cell lysates blotted with anti-FLAG, anti-HA Ab and anti-Flt1 Ab (AF-321). PMA increases abundance of full length FLT1 (FL FLT1) and ~65 kDa cleaved C-term product while heparin has no effect on cleavage or abundance in cell lysates. Several non-specific bands are seen including a ~200 kDa band that comigrates with FLT1. **Panel B:** Time course of PMA-stimulated FLT1 cleavage assessed in transfected HEK cell lysates and conditioned media. PMA increases abundance of transfected FLT1 and C-terminal cleaved fragment beginning at 2 hr and maximal at 24 hr. FLT1 comigrates with a non-specific band on Flag blots seen in pcDNA3-transfected cells. PMA also increases a ~130 kDa product seen in conditioned media blotted with anti HA ab consistent with increased N-terminal cleavage. Abundance of this product in conditioned media but not in lysates is increased when heparin (hep) is added to PMA. In lowest panel, an anti-sFLT1 Ab (AF321) identifies native sFLT1 as well as the cleaved FLT1 product in conditioned media in the presence of PMA and hep.

Figure 4. FLT1 cleavage assessed by subcellular fractionation, immunoblotting and ELISA

Panel A: HEK cell lysates, following transfection with empty virus of FLT1, separated into cytosolic, membrane-associated and nuclear fractions and blotted with an anti-FLAG antibody. Full length FLT1 (FL FLT1) is membrane associated, while the C-terminal cleaved fragment is cytosolic. Membranes were reprobed with HSP90 a cytosolic protein marker and with EGFR, a membrane protein marker to confirm the efficiency of separation.

Panel B: sFLT1 ELISA in conditioned media from COS-7 cells transfected with FLT1 or pcDNA3 with 10U/ml Heparin and 30nM PMA added for 8-24 hr. At 24 hr there is increased sFLT1 in FLT1 transfected cells. *P < 0.05 by Kruskal Wallis ANOVA for that time point compared to other group; Mean $\pm$ SD in 4 samples.

Panel C: Lysates from adenoviral FLT1 transduced COS7 cells, CTBs and HMEC-1 treated with PMA, immunoblotted with SC-316, an ab directed to the C-terminal portion of FLT1. A C-terminal cleaved product is seen in FLT1 transduced COS7 cells and in HMEC-1 and CTB. PMA increases FLT1 protein abundance in HMEC-1.

Figure 5. Role of glycosylation and effect of trafficking inhibitors on FLT1 cleavage and on sFLT1 secretion

Panel A: FLT1 expressing vector or an empty vector transfected into HEK293 cells and then conditioned media digested with PNGase F and then immunoblotted with anti-HA and anti-sFLT1 (AF321). On deglycosylation with PNGase F, the ~130 kDa cleaved fragment undergoes a shift in molecular mass and appears as ~90 kDa peptide. Also seen with AF321 are native sFLT1 isoforms that also undergo a shift in mass. **Panel B:** FLT1 transfected HEK293 cells treated with vehicle, monensin, brefeldin A or tunicamycin and then subject to immunoblotting. Tunicamycin reduces the size of full length FLT1 (FL FLT1). All agents reduce cleavage of N and C-term FLT1 fragments. **Panel C.** sFLT1-i13 and sFLT1-e15a transfected HEK293 cells treated with vehicle, monensin, brefeldin A or tunicamycin and then lysates and conditioned media subject to immunoblotting with an anti-V5 antibody.

Tunicamycin reduces the size of each sFLT1 isoform and all agents inhibit secretion of these isoforms.

Figure 6. Effect of cleaved FLT1 N-terminal fragment to bind VEGF and to modulate VEGF-stimulated vascular tube formation

Panel A: Secreted sFLT1-i13 and cleaved FLT1 were collected from CHO cells expressing adenoviral sFLT1 and FLT1 respectively and equal amounts were added in increasing molar excess to a fixed concentration of VEGF and free VEGF measured by ELISA. There is a dose dependent reduction in free VEGF with no difference seen between sFlt1-i13 and cleaved Flt1. **Panel B:** Conditioned media from FLT1 and control vector transduced COS7 cells were added to HUVEC cells and the angiogenic response measured. There is a significant reduction in measured endothelial tube length in HUVEC cells exposed to conditioned media from FLT1 transduced cells. Mean $\pm$ SD; n=5; *p<0.05 by Mann Whitney Rank Sum test. **Panel C:** FLT1 and control vector transduced HUVEC cells tested in a tube formation assay. Endothelial tube lengths were measured in both conditions. HUVEC organization into endothelial tubes was significantly inhibited following FLT1 transduction. Mean $\pm$ SD; n=3; *p<0.05 by Kruskal Wallis ANOVA. **Panel D:** Representative image of endothelial tube formation in control vs Flt1 transduced HUVEC.