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Identification of Ligand-Induced Proteolytic Cleavage and Ectodomain Shedding of VEGFR-1/FLT1 in Leukemic Cancer Cells

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Abstract

Vascular endothelial growth factor receptor-1/fms-related tyrosine kinase 1 (VEGFR-1/FLT1) is expressed as a membrane-bound receptor tyrosine kinase and as an alternatively spliced soluble protein (sVEGFR-1) containing the 1-6 IgG-like domain of its ectodomain. sVEGFR-1 is known as a naturally occurring inhibitor of angiogenesis and as a surrogate marker for cancer progression; it is also linked to pregnancy-induced hypertension called preeclampsia and to avascularity of normal cornea. It remains an open question whether alternative mRNA splicing is the only mechanism by which sVEGFR-1 is generated. In this study, we show that in leukemic cancer cells, PlGF and VEGF-A both induce tyrosine phosphorylation of VEGFR-1 and render it susceptible to ectodomain shedding, resulting in the generation of sVEGFR-1 and an intracellular cytoplasmic fragment. Activation of protein kinase C and tumor necrosis factor- α -converting enzyme family metalloproteases are critically required for the occurrence of sVEGFR-1. Following the removal of the ectodomain, the remnant of VEGFR-1 remains attached to the membrane, and the activity of γ -secretase/presenilin is required for its release from the cell membrane. We propose that sVEGFR-1 produced via ectodomain shedding plays a prominent role in the VEGF receptor system by antagonizing VEGF receptor signaling by acting as a dominant-negative form and/or forming a nonsignaling dimerizing complex with VEGF receptors.

Introduction

Vascular endothelial growth factor receptor-1 [VEGFR-1, also called fms-related tyrosine kinase 1 (FLT-1)] is described to play a negative role in angiogenesis mainly by acting as a decoy receptor in endothelial cells. The negative role of VEGFR-1 in angiogenesis was initially suggested based on the observation that loss of *VEGFR-1* in mice causes an increase in the number of endothelial progenitors, resulting in vascular disorganization (1,2), and further by a gene-targeting study in which the entire cytoplasmic region of VEGFR-1, including its kinase domain, was deleted. The knockin truncated *VEGFR-1* mice developed normally with no apparent defect in vasculogenesis (3). Additional observations further showed that VEGFR-1 lacks a significant signaling capacity in endothelial cells as it is poorly tyrosine phosphorylated (4–7) and lacks the capacity to induce gene expression (8) or to stimulate cellular responses such as proliferation and migration in cells with endothelial origin (6,7,9,10).

Interestingly, the expression and activation of VEGFR-1 in nonendothelial cells, in particular cancer cells, are linked to cell proliferation (11,12), and a recent study indicates that preventing

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VEGFR-1 signaling by a VEGFR-1-specific ligand, PlGF-blocking antibody, inhibits tumor growth in mice (13), suggesting a functional role for VEGFR-1 signaling in tumor growth. The potential role of VEGFR-1 in cancer was recently highlighted by the observation that up to 76% of patients with leukemic cancer were identified as positive for VEGFR-1 (14,15). Moreover, the expression and activation of VEGFR-1 in leukemic cancer cells are reported to promote cell proliferation and tumor growth (16).

VEGFR-1 is expressed as a membrane-bound receptor tyrosine kinase and as a soluble form (sVEGFR-1) due to an alternative splicing of VEGFR-1 mRNA, which encodes its extracellular domain containing 1-6 immunoglobulin-like domains of VEGFR-1 (17). sVEGFR-1 is considered to play a key role in a number of physiologic and pathologic conditions; it acts as a surrogate marker for acute myelogenous leukemia (AML) cancer progression (18) and has been identified as an endogenous inhibitor of angiogenesis in certain cancers (19,20). The presence of sVEGFR-1 is also required for avascularity of normal cornea (21) and its expression is linked to pregnancy-induced hypertension characterized as preeclampsia (22).

Despite the clear physiologic importance of sVEGFR-1, little is known about the source of circulating sVEGFR-1 and the molecular mechanism involved in its generation. The soluble ectodomain of cell surface receptors is produced either via alternative mRNA splicing as it has been shown for VEGFR-1 (17) or via proteolytic cleavage of the ectodomain from the cell surface following ligand-induced down-regulation as it has been shown for various receptor tyrosine kinases (RTK), including TIE2, c-kit, HER2, and c-Met (reviewed in ref. 23). In this study, we show that VEGFR-1 is highly active in leukemic cancer cells, undergoes tyrosine phosphorylation, and activates a number of key signaling pathways. Tyrosine phosphorylation predisposes VEGFR-1 for ectodomain shedding and proteolytic cleavage via a protein kinase C (PKC)-dependent pathway. Ligand-induced down-regulation and ectodomain shedding contributes to formation of sVEGFR-1, which may antagonize VEGF receptor signaling by acting as a dominant-negative form and/or forming a nonsignaling dimerizing complex with VEGF receptors.

Materials and Methods

Reagents and antibodies

Recombinant VEGF, recombinant PlGF, and ELISA kit, including anti-VEGFR-1 antibody that specifically recognizes the ectodomain of VEGFR-1 for detection of soluble VEGFR-1, were purchased from R&D. Mouse anti-phosphotyrosine antibody (PY-20) was purchased from Transduction Laboratories. Mouse anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology, Inc. The following antibodies were purchased from Santa Cruz Biotechnology, Inc.: rabbit and goat polyclonal anti-VEGFR-1 antibodies, which specifically recognize the cytoplasmic domain of VEGFR-1; goat anti-rabbit antibody; anti-PLC γ 1 antibody; goat anti-mouse antibody; and donkey anti-goat antibody antibodies. Anti-phospho-PLC γ 1 (pY783) was purchased from Biosource International. GF-109203X was purchased from Calbiochem.

Cell lines and constructs

Construction and expression of wild-type VEGFR-1 antibodies and mutant VEGFR-1, D1050/VEGFR-1, were described elsewhere (9). The following lymphoma cell lines, including HT, SKI-DLBCL (a diffuse large B cell), and Hs602 (a diffuse large cell lymphoma), were grown in RPMI medium as described (24) and were kindly provided by Dr. Malcolm A.S. Moore (Memorial Sloan-Kettering Cancer Center, New York, NY).

Cell proliferation assay

Cell proliferation was measured by the uptake of [³H]thymidine as described before (9). Cells were plated at 5×10^4 /mL in RPMI containing 10% fetal bovine serum (FBS) in 24-well plates and incubated at 37°C for 12 h. Cells were then washed twice in PBS and growth-arrested in RPMI for 12 h at 37°C. Depending on the experimental conditions as indicated in the figure legends, VEGF was added and incubated for 18 to 20 h at 37°C. Cells were pulsed for 4 h with [³H]thymidine (0.2 µCi/mL) and harvested and measured by a scintillation counter. Quadruplicate samples were performed for each group. Three independent experiments were performed.

Immunoprecipitation, cell fractionation, and Western blotting

Equal numbers of cells from the indicated cell lines were grown in 10-cm culture dishes until 80% to 90% confluent. After overnight serum starvation, cells were left either resting or stimulated with 100 ng/mL of PlGF or VEGF at 37°C as indicated in the figure legends. Cells were prepared and lysed as described (7,9). Normalized whole-cell lysates were either subjected to Western blot analysis or to immunoprecipitation by incubating with appropriate antibodies as indicated in the figure legends. Immunocomplexes were captured by incubating with either recombinant Protein-A Sepharose or Protein-G Agarose beads and immunoprecipitated proteins were subjected to immunoblotting analysis using the desired antibody. Proteins were visualized by using an ECL kit. In some instances, cells were lysed in a cold hypotonic buffer containing 20 mmol/L Tris-Cl (pH 7.4), 1 mmol/L MgCl₂, 2 mmol/L EGTA, 0.1 mmol/L sodium orthovanadate, 10 µg of aprotinin/mL, and 10 µg of leupeptin/mL and further lysed by Dounce homogenization after swelling in 500 µL hypotonic buffer. Lysates were centrifuged for 45 min at 10,000 rpm at 4°C to separate the particulate and soluble fractions. The particulate fraction was further lysed and subjected to immunoprecipitation and SDS-PAGE analysis.

Metabolic labeling

The ligand-dependent proteolytic fragmentation of VEGFR-1 in SKI-DLBCL and human umbilical vascular endothelial cells (HUVEC) was evaluated by pulse-chase analysis using a mixture of ³⁵S-labeled L-methionine/L-cysteine as described (25). In brief, equal numbers of cells were plated in 10-cm tissue culture dishes containing RPMI supplemented with 10% FBS. Cells were then starved for 8 h in RPMI medium plus 1% FBS. The medium was removed, and cells were rinsed twice with PBS before an additional 2 h of starvation at 37°C in L-methionine/L-cysteine-free DMEM (Invitrogen) supplemented with L-glutamine (Invitrogen). Cells were pulse labeled by supplementing L-methionine/L-cysteine-free DMEM with 75 µCi/mL of [³⁵S]methionine/cystine (Perkin-Elmer Life Sciences) for 3 h at 37°C and then chased with complete RPMI supplemented with 100-fold excess of unlabeled L-methionine and L-cysteine. Cells were either left unstimulated or were stimulated with rhVEGF-A or PlGF (100 ng/mL) at 37°C for the indicated times during the chase period. Finally, cells were washed and lysed with cold lysis buffer. Cell lysates representing equal numbers of cells were immunoprecipitated using an anti-VEGFR-1 antibody, which recognizes the cytoplasmic domain of VEGFR-1. Immunocomplexed proteins were resolved on 12% SDS-PAGE, and gels were prepared for autoradiography as described (25).

Results

VEGFR-1 is expressed in human leukemic cancer cells, undergoes tyrosine phosphorylation, and activates multiple signaling pathways

Despite its expression in endothelial cells, VEGFR-1 is poorly tyrosine phosphorylated in response to ligand stimulation and lacks proliferative signaling capability in endothelial cells

(4,5,18,26). In contrast, VEGFR-1 in certain cancer cells, including leukemic cancer cells such as SKI-DLBCL (a diffuse large B-cell lymphoma) cells, promotes cell proliferation (24). Our initial observation indicates that in SKI-DLBCL and HT (diffuse large B-cell lymphoma) cells, VEGFR-1 is expressed significantly at lower levels compared with HUVEC (primary human umbilical vein endothelial) cells (Fig. 1A). VEGFR-1, however, is highly tyrosine phosphorylated in response to PlGF (Fig. 1A) and VEGF stimulation (Fig. 1B). Because VEGFR-1 undergoes tyrosine phosphorylation in response to PlGF, we also analyzed the PlGF-dependent activation of key signaling pathways in SKI-DLBCL and HT cells. The results showed that PlGF treatment of SKI-DLBCL and HT cells stimulates the activation of AKT, PLC γ 1, and mitogen-activated protein kinase (MAPK; Fig. 1C). The data indicate that the VEGFR-1 signaling machinery system is fully operational in leukemic cells and may play a pivotal role in proliferation and survival of leukemic cancer cells.

PlGF stimulates ectodomain shedding and proteolytic cleavage of VEGFR-1

The presence of soluble VEGFR-1 (sVEGFR-1, herein referred to as ectodomain of VEGFR-1) is elevated in human lymphoma cell lines and in patients with AML and myelodysplastic syndromes (11,18,24). The elevated level of sVEGFR-1 is also suggested to be an independent prognostic factor in these patients (24,27). sVEGFR-1 is thought to be produced by alternative splicing of VEGFR-1 mRNA (17) and whether it may also originate as a consequence of proteolytic cleavage of VEGFR-1 has not been studied yet. Because SKI-DLBCL cells express and secrete sVEGFR-1 (24), we wished to test whether there is a link between the ligand-induced tyrosine phosphorylation and down-regulation of VEGFR-1 and generation of soluble ectodomain of VEGFR-1. To this end, SKI-DLBCL cells were stimulated with PlGF and conditioned medium was collected. Generation of the ectodomain of VEGFR-1 was analyzed by ELISA using an anti-VEGFR-1 antibody that selectively recognizes the ectodomain of VEGFR-1. To prevent new protein synthesis, cells were also preincubated with cycloheximide. Our analysis showed that treatment of SKI-DLBCL cells with PlGF increases ectodomain shedding of VEGFR-1 in conditioned medium in a time-dependent manner (Fig. 2A). Stimulation of these cells with VEGF-A also resulted in the increased level of sVEGFR-1 (data not shown). Further studies showed that incubation of SKI-DLBCL cells with a general tyrosine kinase inhibitor, genistein, inhibited the formation of sVEGFR-1 in conditioned medium, suggesting that kinase activation is required for occurrence of sVEGFR-1 (Fig. 2A). Taken together, the data suggest that PlGF- and VEGF-dependent accumulation of sVEGFR-1 in the conditioned medium of SKI-DLBCL cells is most likely a result of ligand-mediated down-regulation of VEGFR-1 and potential proteolytic cleavage that subsequently generates sVEGFR-1. In addition, quantitative reverse transcription-PCR analysis consistent with the previous studies (17) showed that both HUVEC and SKI-DLBCL cells express both sVEGFR-1 and VEGFR-1 transcripts, and treatment of these cells with PlGF or VEGF-A up to 60 minutes do not alter their transcription (data not shown).

To show the biological relevance of ligand-mediated generation of sVEGFR-1, we collected conditioned medium of SKI-DLBCL cells treated with PlGF; the concentrated conditioned medium then was used to test whether it is able to antagonize the VEGF-induced proliferation of SKI-DLBCL cells. Treatment of SKI-DLBCL cells with VEGF stimulated cell proliferation in a concentration-dependent manner, where cotreatment of SKI-DLBCL cells with VEGF and conditioned medium resulted in the inhibition of VEGF-mediated cell proliferation (Fig. 2B). Conditioned medium without VEGF treatment of SKI-DLBCL cells only had a minor inhibitory effect on the growth of SKI-DLBCL cells. In addition, boiling the conditioned medium or preincubating the conditioned medium with VEGF inhibited the inhibitory property of the conditioned medium (data not shown). The result suggests that sVEGFR-1 produced as a result of ectodomain shedding antagonizes VEGF function by possibly acting as a “VEGF trap.”

To test whether ectodomain shedding of VEGFR-1 is the result of ligand-induced down-regulation and proteolytic cleavage of VEGFR-1 in SKI-DLBCL cells, we comparatively analyzed down-regulation of VEGFR-1 in response to PIGF in SKI-DLBCL and HUVEC cells. The data show that VEGFR-1 undergoes down-regulation in response to PIGF stimulation in a time-dependent manner in SKI-DLBCL cells but VEGFR-1 is refractory to down-regulation in response to the same stimulation in HUVEC cells (Fig. 2C). Interestingly, the VEGF-mediated disappearance of full-length cell surface VEGFR-1 directly correlates with appearance of an ~60 kDa protein (Fig. 2C). Indeed, after 60 minutes of stimulation with PIGF, the majority of VEGFR-1 protein was in the form of 60-kDa VEGFR-1 fragment (Fig. 2C). The 60-kDa VEGFR-1 fragment was also observed in the HUVEC cells but its level was almost unaffected with PIGF stimulation of HUVEC cells (Fig. 2D). It should be noted that because the anti-VEGFR-1 antibody used for immunoprecipitation specifically recognizes the intracellular domain of VEGFR-1, the appearance of the 60-kDa VEGFR-1 fragment in the autoradiograph of the radiolabeled VEGFR-1 immunoprecipitates highly likely corresponds to the intracellular domain of VEGFR-1. Figure 2D shows the quantification of down-regulation of VEGFR-1 in response to PIGF as shown in Fig. 2C. Furthermore, immunoprecipitation with an anti-VEGFR-1 antibody that selectively recognizes the extracellular domain of VEGFR-1 did not detect the 60-kDa protein band (data not shown). Taken together, the data show that in SKI-DLBCL lymphoma cells, VEGFR-1 undergoes ligand-induced tyrosine phosphorylation and proteolytic fragmentation, resulting in the formation of soluble ectodomain and a 60-kDa cytoplasmic fragment.

To test the hypothesis that tyrosine phosphorylation of VEGFR-1 in SKI-DLBCL cells is responsible for its ectodomain shedding, we initially evaluated the effect of the tyrosine kinase inhibitor genistein on the formation of the sVEGFR-1 and 60-kDa cytoplasmic fragment of VEGFR-1. The result showed that genistein treatment of cells almost completely blocked the formation of the sVEGFR-1 and 60-kDa cytoplasmic fragment of VEGFR-1 in response to VEGF stimulation of SKI-DLBCL cells (Fig. 3A and B). To directly address the role of tyrosine phosphorylation of VEGFR-1 in the formation of the 60-kDa fragment, we analyzed the ability of a mutant VEGFR-1 (D1050-VEGFR-1) that previously has been shown to render VEGFR-1 to undergo robust tyrosine phosphorylation (9). Aspartic acid (D1050) is located in the activation loop and is highly conserved among RTKs; however, in VEGFR-1, it is substituted to asparagines (N; ref. 9). The result shows that D1050-VEGFR-1, unlike the wild-type VEGFR-1, undergoes enhanced proteolytic cleavage and forms the 60-kDa fragment (Fig. 3C). Further analysis also showed that D1050-VEGFR-1 undergoes ectodomain shedding (Fig. 3D) in response to ligand stimulation in PAE cells. Altogether, the data suggest that ligand-directed tyrosine phosphorylation of VEGFR-1 is a paramount requirement for its ectodomain shedding and formation of 60-kDa cytoplasmic fragment.

Ectodomain shedding of VEGFR-1 is regulated by a PKC-dependent pathway

Activation of PKC and a disintegrin and metalloproteinase/tumor necrosis factor- α -converting enzyme (ADAM/TACE) family metalloproteases is suggested to play a key role in the ectodomain shedding of several cell surface receptors (28,29). To analyze the involvement of PKC pathway in the ectodomain shedding of VEGFR-1, we initially treated several lymphoma cell lines with a PKC inhibitor, GFX, and measured the formation of sVEGFR-1. As shown, lymphoma cell lines, including HT, SKI-DLBCL, and Hs602, all produce the soluble ectodomain of VEGFR-1 in various amounts compared with control HUVEC cells, and treatment of these cells with GFX significantly inhibited the production of sVEGFR-1 in these lymphoma cell lines (Fig. 4A). HUVEC cells produces a low level of soluble ectodomain of VEGFR-1, and GFX treatment had no significant effect on its production (Fig. 4A), suggesting that perhaps this sVEGFR-1 protein is related to alternatively spliced variant of VEGFR-1 as previously reported (17).

Because inhibition of PKC by GFX blocks the production of sVEGFR-1 in lymphoma cells, in a complementary approach, we further tested the effect of up-regulation of the PKC pathway in the generation of ectodomain shedding of VEGFR-1 with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a known PKC activator. As shown, treatment of SKI-DLBCL cells and HUVEC cells with TPA induced rapid ectodomain shedding of VEGFR-1 in a time-dependent manner and maximum concentration of sVEGFR-1 was detected after 90 minutes of stimulation with TPA (Fig. 4B). Stimulation of cells with TPA beyond 90 minutes did not result in an increased concentration of sVEGFR-1 (data not shown). Altogether, the data show that VEGFR-1 undergoes ectodomain shedding and that PKC activation is a chief pathway for this process to occur. To further address the mechanism by which PKC induces ectodomain shedding of VEGFR-1, we analyzed the effect of GM6001, a broad inhibitor of ADAM family proteases, including TACE. Treatment of SKI-DLBCL cells with GM6001 inhibited both PLGF- and TPA-induced ectodomain shedding of VEGFR-1 (Fig. 4C), indicating that PKC-induced ectodomain shedding of VEGFR-1 requires activity of ADAM family metalloproteases. Unlike GM6001, treatment of cells with lactacystin, a potent and selective proteasome inhibitor, did not block the formation of sVEGFR-1 (data not shown), further arguing that TACE family metalloproteases are selectively involved in the ectodomain shedding of VEGFR-1.

γ -Secretase activity is required for the removal of the 60-kDa cytoplasmic domain from the cell membrane

To determine if, following the VEGF-stimulated ectodomain shedding of VEGFR-1, the 60-kDa cytoplasmic fragment remains associated with the membrane or is directly deposited to the cytoplasm, we initially lysed VEGF-stimulated SKI-DLCBL cells with a hypotonic lysis buffer followed by a high-speed centrifugation to isolate particulate fraction. The particulate fractions were further analyzed for the presence of VEGFR-1 protein. As shown in Fig. 5A, the presence full-length VEGFR-1 was detected in the particulate fraction and its levels gradually decreased in response to ligand stimulation. On the other hand, the presence of the 60-kDa fragment was absent in unstimulated group, is readily detectable after 10 minutes of stimulation, and highest after 60 minutes of stimulation (Fig. 5A). Based on this observation, it seems that the 60-kDa fragment of VEGFR-1 remains attached to the membrane and it may require further proteolytic processing before it is released into the cytoplasm.

A recent study indicates that γ -secretase activity is up-regulated in response to pigmented epithelial-derived growth factor (PEDF) and that induction of PEDF plays a role in the intramembrane cleavage of VEGFR-1 (30). If VEGF-mediated formation of the cytoplasmic 60-kDa VEGFR-1 protein occurs through γ -secretase, then a membrane-associated 60-kDa remnant consisting of the transmembrane and cytoplasmic domains should be generated in conjunction with a pronounced decline in mature VEGFR-1 following PIGF stimulation. Therefore, inhibition of γ -secretase activity would result in accumulation of 60-kDa VEGFR-1 cytoplasmic domain proteolytic remnant following PIGF stimulation. To test this possibility, we pretreated SKI-DLBCL cells with a γ -secretase inhibitor, L-685,458, followed by PIGF stimulation. The result showed that PIGF-dependent down-regulation of VEGFR-1 is not affected by treatment of cells with L-685,458; however, it resulted in the accumulation of the 60-kDa fragment (Fig. 5B). This suggests that following the removal of the ectodomain of VEGFR-1, the 60-kDa fragment of VEGFR-1 remains associated with the cell membrane and it further undergoes proteolytic processing involving γ -secretase.

γ -Secretase is composed of multiple proteins, including presenilin 1 (PS1) and PS2, which are thought to act as catalytic subunits of γ -secretase (31). To further link γ -secretase activity to proteolytic cleavage processing of VEGFR-1, we expressed either wild-type PS1 or a dominant-negative form of PS1 (PS1-TM2) in HEK-293 cells expressing VEGFR-1 and analyzed the effect of their expression in the formation of the 60-kDa VEGFR-1 fragment (Fig.

5C). The result showed that the expression of wild-type PS1 enhances the formation of the 60-kDa fragment in response to VEGF stimulation, in which the expression of the dominant-negative PS1 significantly inhibited the formation of the 60-kDa fragment (Fig. 5D). Taken together, the data show that VEGFR-1 undergoes two distinct proteolytic cleavages. Ectodomain cleavage, via ADAM family metalloproteases, which results in the ectodomain shedding of VEGFR-1, and the transmembrane cleavage, via γ -secretase/presenilin, which results in the release of intracellular VEGFR-1 from the cell membrane.

Discussion

VEGFR-1 is expressed in endothelial and nonendothelial cell lineages as a full-length cell surface protein and as an alternatively spliced soluble form (17,26). Our study for the first time shows that sVEGFR-1 is also produced as a consequence of ectodomain shedding, suggesting that elevated sVEGFR-1 levels observed in certain cancers such as leukemic cancers (18,27, 32) may originate from full-length VEGFR-1 due to paracrine/autocrine activation of VEGFR-1, aberrant hyperactive signaling events, and metalloproteinase activity in the tumor tissue microenvironment. The data presented in this article also underscores a pivotal role for PKC-mediated ADAM/TACE-dependent proteolytic cleavage of VEGFR-1, resulting in the generation of sVEGFR-1. Moreover, both VEGFR-1 ligands (VEGF-A and PlGF) and TPA-mediated formation of sVEGFR-1 was inhibited by the metalloprotease inhibitor MG6001, suggesting that PKC acts as an upstream signaling pathway to regulate ADAM/TACE protease activity, which results in the cleavage of ectodomain of VEGFR-1. Whether PKC is directly responsible for ectodomain shedding of VEGFR-1 or other PKC-activated serine/threonine kinases, such as MAPKs, which are also involved in protein shedding (reviewed in ref. 23), needs further investigation.

The main *in vivo* function of sVEGFR-1 is its antiangiogenic function; it complexes with VEGF-A with a high affinity and sequester this angiogenic factor. In addition to its ability to sequester the VEGF ligand, sVEGFR-1 can also form a nonsignaling complex with membrane-bound VEGFR-1 and VEGFR-2, by acting as a dominant-negative receptor (17). Our results suggest that sVEGFR-1 produced as a result of ectodomain shedding of VEGFR-1 is a functional protein and it inhibits VEGF-mediated proliferation of SKI-DLBCL cells likely by a mechanism that involves sequestering of VEGF and/or forming homodimeric and heterodimeric complexes with VEGFR-1 and VEGFR-2.

Another interesting aspect of our observation is that once the ectodomain of VEGFR-1 is cleaved by TACE family metalloproteases, the remnant of VEGFR-1 remains attached to the membrane until it is further proteolytically processed by the γ -secretase/presenilin pathway. In this regard, VEGFR-1 down-regulation of VEGFR-1 seems to be regulated by the process that was recently described as regulated intermembrane proteolysis (RIP), which involves proteolytic cleavage of membrane proteins resulting in the shedding of ectodomain, followed by intermembrane proteolytic cleavage resulting in the detachment of cytoplasmic fragment from cell membrane (23,33). Interestingly, the down-regulation of a highly related VEGF receptor, VEGFR-2/FLK-1, is not subjected to RIP-mediated proteolysis (25), suggesting that RIP-mediated processing of VEGFR-1 is selective and it may reflect its distinct biochemical and biological function.

What is the importance of proteolytic cleavage of VEGFR-1? In a nutshell, proteolytic cleavage of VEGFR-1 terminates the ability of cells to interact with VEGF ligands and thus inhibit VEGF-dependent responses in cancer cells. sVEGFR-1 can also associate with VEGF ligands by acting as a VEGF trap, which could further antagonize VEGF function (3). sVEGFR-1 bound to VEGF ligands could also generate a nonsignaling homodimeric and heterodimeric VEGF receptors (17). Finally, sVEGFR-1 may act as a ligand for other cell surface receptors

(34). It has been shown that RIP-mediated proteolytic cleavage of RTKs such as ErbB4 and CSF-1R results in the translocation of their cytoplasmic domain into the nucleus (reviewed in ref. 35). Interestingly, VEGFR-1 has also been reported to transport into the nucleus (30), suggesting that proteolytically cleaved VEGFR-1 may mediate an additional role in nucleus.

sVEGFR-1 is up-regulated in various cancers, including AML, and is suggested to be an independent prognostic factor in these patients (12,18,27,36). Its presence is proposed to be responsible for corneal avascularity (21) and preeclampsia (22). In sum, our observation presented in this study is pertinent to further examination of the source of sVEGFR-1 and the biological implication of RIP-mediated proteolysis of VEGFR-1 in tumor growth as well as in angiogenesis.

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Figure 1.

VEGFR-1 is expressed in leukemic cancer cells, undergoes tyrosine phosphorylation, and activates multiple signaling pathways. Serum-starved SKI-DLBCL, HT, and HUVEC cells were either unstimulated (–) or stimulated (+) with PLGF for 10 min; cells were then lysed and immunoprecipitated with an anti-VEGFR-1 antibody. Immunoprecipitated proteins were resolved in 7% SDS-PAGE gel and subsequently immunoblotted with an anti-phosphotyrosine antibody. The same membranes were reblotted for VEGFR-1 using an anti-VEGFR-1 antibody (A). SKI-DLBCL and HT cells were stimulated with VEGF-A for 10 min, lysed, and immunoprecipitated with an anti-VEGFR-1 antibody and blotted with anti-phosphotyrosine antibody (B). Total cell lysates derived from SKI-DLBCL and HT cells were subjected to Western blot analysis using phospho-Akt, phospho-PLCγ1, and phospho-Erk1/Erk2 (MAPK). Protein levels of Akt, PLCγ1, and MAPK are also shown (C).

Figure 2.

VEGFR-1 undergoes ectodomain shedding and proteolytic cleavage in leukemia cancer cells. SKI-DLBCL cells were stimulated with PIGF (100 ng/mL) for the indicated times in the presence of cycloheximide (CHX; to inhibit protein synthesis); conditioned medium was collected from each group separately and was analyzed for the presence of sVEGFR-1 by an ELISA assay using an anti-VEGFR-1 antibody that specifically recognizes the ectodomain of VEGFR-1 (A). Serum-starved SKI-DLBCL cells were stimulated with different concentrations of VEGF as indicated alone or with the concentrated conditioned medium (CM). Proliferation of cells was measured as described in Materials and Methods. Points, mean number of cells of quadruplicates; bars, SD. B, HUVEC and SKI-DLBCL cells were serum starved in Met/Cys-free medium and then pulse labeled with [³⁵S]Met/Cys (75 μCi/mL) for 3 h. Radiolabeled VEGFR-1 was then chased to the cell surface in the absence (0) or presence of PIGF for the indicated times in the presence of cycloheximide. Cells were lysed, immunoprecipitated with an anti-VEGFR-1 antibody that specifically recognizes the cytoplasmic domain of VEGFR-1, and resolved in SDS-PAGE gel followed by autoradiography. Arrows on the right indicate mature VEGFR-1 and the cytoplasmic fragment of VEGFR-1, respectively. A high molecular weight and nonspecific fragment was also detected on the top of autoradiograph (n.s.; C). The relative amount of VEGFR-1 was measured by densitometry of autoradiographs using the NIH image J software program (D).

Figure 3.

The kinase activity of VEGFR-1 is required for proteolytic cleavage of VEGFR-1. Serum-starved SKI-DLBCL cells were stimulated with PIGF in the absence or presence of genistein. All the groups were also preincubated with cycloheximide. Conditioned medium was collected from each group separately and was analyzed for the presence of the ectodomain of VEGFR-1 (A). Serum-starved SKI-DLBCL cells were subjected to metabolic labeling with [³⁵S]Met/Cys (75 μ Ci/mL) in the presence or absence of the tyrosine kinase inhibitor genistein. Cells were lysed, immunoprecipitated with an anti-VEGFR-1 antibody that recognizes the cytoplasmic domain of VEGFR-1, and resolved in SDS-PAGE gel followed by autoradiography. Arrows on the right indicate the full-length VEGFR-1 protein and the proteolytic cytoplasmic fragment, respectively (B). PAE cells expressing VEGFR-1 and mutant VEGFR-1 (designated D1050-VEGFR-1) were stimulated with the ligand for the indicated times; cells were lysed and immunoprecipitated with an anti-VEGFR-1 antibody and immunoblotted with an anti-VEGFR-1 antibody (C). Also, PAE cells expressing wild-type VEGFR-1, D1050-VEGFR-1, and empty vector (*pLNCX*) were stimulated with VEGF (100 ng/mL) in the presence of

cycloheximide. Conditioned medium was collected from each group separately and was analyzed for the presence of the ectodomain of VEGFR-1 (*D*).

Figure 4.

The ectodomain shedding of VEGFR-1 is regulated by a PKC-dependent pathway. Overnight serum-starved HUVEC cells and lymphoma cell lines, including HT, SKI-DLBCL, and Hs602, were treated with a PKC inhibitor, GFX (5 μ mol/L), and cells were incubated for 12 h in the presence of cycloheximide. Condition medium was collected and analyzed for the presence of sVEGFR-1 in an ELISA assay using an anti-VEGFR-1 antibody that specifically recognizes the ectodomain of VEGFR-1. Columns, mean (ng/mL) of triplicates; bars, SD (A). SKI-DLBCL cells were either stimulated with PLGF or TPA (100 nmol/L) as indicated and conditioned medium was collected as in A and analyzed for the presence of sVEGFR-1. Points, mean (ng/mL) of triplicates; bars, SD (B). SKI-DLBCL cells were stimulated with

PIGF or TPA in the presence or absence of MG6001, and conditioned medium was collected and analyzed for the presence of sVEGFR-1. *Columns*, mean (ng/mL) of triplicates as in *B*; *bars*, SD (*C*).

Figure 5.

γ -Secretase activity is required for the proteolytic processing of VEGFR-1. SKI-DLBCL cells were subjected to metabolic labeling with [35 S]Met/Cys as in Fig. 2C and were stimulated with PLGF for the indicated times. Cells were lysed in a hypotonic buffer and cell lysates were separated into particulate and soluble fractions. The particulate fraction was further analyzed for the presence of VEGFR-1 as described in Materials and Methods. The immunoprecipitated VEGFR-1 was resolved in SDS-PAGE gel followed by autoradiography (A). SKI-DLBCL cells were subjected to metabolic labeling with [35 S]Met/Cys and stimulated with PLGF for various times as indicated in the absence or presence of L685458. Cells were lysed with a hypotonic buffer as in A. VEGFR-1 was immunoprecipitated with an anti-VEGFR-1 antibody and was resolved in SDS-PAGE gel followed by autoradiography (B). HEK-293 cells transiently coexpressing VEGFR-1 with pCDNA vector (V), presenilin 1 (PS1), or dominant-negative presenilin 1 (PS1-DN) were lysed and blotted for the expression of presenilin 1 using an anti-PS1 antibody (C). Metabolically labeled HEK-293 cells coexpressing VEGFR-1 with presenilin 1 or dominant-negative presenilin 1 were stimulated with PLGF for 30 min, and cells were lysed and the particulate fraction was used to immunoprecipitate VEGFR-1. The immunoprecipitated VEGFR-1 was then resolved in SDS-PAGE gel followed by autoradiography (D).