Vascular Endothelial Growth Factor–Mediated Decrease in Plasma Soluble Vascular Endothelial Growth Factor Receptor-2 Levels as a Surrogate Biomarker for Tumor Growth

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Abstract

Vascular endothelial growth factor (VEGF) is a potent proangiogenic protein that activates VEGF receptor (VEGFR) tyrosine kinases expressed by vascular endothelial cells. We previously showed that one of these receptors, VEGFR-2, has a truncated soluble form (sVEGFR-2) that can be detected in mouse and human plasma. Because activation of VEGFR-2 plays an important role in tumor angiogenesis, clinical interest in monitoring plasma sVEGFR-2 levels in cancer patients has focused on its potential exploitation as a surrogate biomarker for disease progression as well as assessing efficacy/activity of antiangiogenic drugs, particularly those that target VEGF or VEGFR-2. However, no preclinical studies have been done to study sVEGFR-2 during tumor growth or the mechanisms involved in its modulation. Using spontaneously growing tumors and both localized and metastatic human tumor xenografts, we evaluated the relationship between sVEGFR-2 and tumor burden as well as underlying factors governing protein level modulation in vivo. Our results show an inverse relationship between the levels of sVEGFR-2 and tumor size. Furthermore, using various methods of VEGF overexpression in vivo, including cell transfection and adenoviral delivery, we found plasma sVEGFR-2 decreases to be mediated largely by tumor-derived VEGF. Finally, in vitro studies indicate VEGF-mediated sVEGFR-2 modulation is the result of ligand-induced down-regulation of the VEGF-2 from the cell surface. Taken together, these findings may be pertinent to further clinical exploitation of plasma sVEGFR-2 levels as a surrogate biomarker of VEGF-dependent tumor growth as well as an activity indicator of antiangiogenic drugs that target the VEGFR system. [Cancer Res 2008;68(2):521–9]

Introduction

The development of new sprouting blood vessels from a preexisting mature vasculature, termed angiogenesis, is required for tumor growth beyond microscopic sizes, and strategies to inhibit this process now constitute a validated anticancer treatment modality for various tumor types (1). Regulation of angiogenesis is governed, in large part, by an array of growth factors, including those favoring or opposing new blood vessel capillary formation, many of which bind their cognate receptor tyrosine kinases (RTK) expressed on vascular endothelial cells and their bone marrow-derived progenitors (2). Because of this vital role, monitoring changes in circulating levels of angiogenic factors offers the potential for exploitation as surrogate biomarkers for the presence of cancer occurrence, tumor progression, as well as efficacy/activity of certain antiangiogenic treatment strategies such as predicting potential benefit and/or eventual relapse (3). Studies designed to discover and validate such markers have included numerous proteins such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor, and thrombospondin-1, to name only a few (reviewed in ref. 4). Additionally, circulating soluble receptors have also been studied; these are generated either via proteolytic cleavage of the ectodomain from the cell surface or via alternative mRNA splicing, which gives rise to a secreted polypeptide lacking a transmembrane region (5). For example, soluble c-kit (sKIT) in germ cell tumors (6), soluble TIE-2 (sTIE-2) in renal cancers (7), soluble HER-2/neu (sHER-2/neu) in metastatic breast cancers (8), soluble interleukin-2 receptor in lymphomas (9), soluble epidermal growth factor receptor (sEGFR) in cervical neoplasia (10), and many others have been evaluated as potential biomarkers for tumor progression and/or predictors for survival (5).

Perhaps the most studied system in this regard involves the VEGF family of proteins and its associated RTKs [VEGF receptors (VEGFR)]. The VEGF family consists of VEGF-A (also called VEGF), VEGF-B, VEGF-C, and VEGF-D, as well as placental growth factor (11). VEGF-A, especially the VEGF121 and VEGF165 isoforms, plays an integral role in tumor angiogenesis acting both as an activator and survival factor for endothelial cells. Hence, monitoring plasma VEGF levels has been studied as a possible surrogate biomarker of angiogenesis in the context of numerous malignancies (4). Similarly, expression of VEGFRs, including VEGFR-2 or KDR (human)/flk-1 (mouse) and VEGFR-1 or flt-1—both of which are expressed on a number of cell types in addition to endothelial cells (11), including certain types of tumors (12, 13)—have been correlated to various disease stages (14). Interestingly, soluble forms of VEGFR-1 (sVEGFR-1) and VEGFR-2 (sVEGFR-2) have also been studied as potential biomarkers. Whereas both full-length receptors have an extracellular domain containing seven immunoglobulin-like loops, a transmembrane domain, and an intracellular split catalytic tyrosine kinase domain (15), the sVEGFR-1 is the product of alternative mRNA splicing and is composed of only six of the seven extracellular immunoglobulin-like domains. The sVEGFR-1 has been studied both as a potential surrogate marker for disease progression and/or as a potential inhibitor of tumor angiogenesis in cancers such as breast (16), renal cell carcinoma (7), fibrosarcoma (17), and astrocytic tumors (18).
In contrast to sVEGFR-1, little is known about sVEGFR-2. We first reported the existence of this 160-kDa truncated protein, which could be detected in both mouse and human plasma (19). Recent clinical studies have involved investigations into its potential as a surrogate biomarker for tumor progression or survival in melanoma (20), myelodysplastic syndromes (21), as well as in various leukemias (22–24). However, no clear pattern has yet emerged to indicate the utility of monitoring circulating sVEGFR-2 in the clinical setting, how it relates to tumor growth, and the underlying mechanisms governing observed changes. The possible exception to this is that decreased plasma sVEGFR-2 levels is a consistent feature of therapy with small-molecule antiangiogenic multitargeted RTK-inhibiting drugs such as SU11248/sunitinib (25) and many others (26).

We therefore decided to undertake a series of preclinical studies to investigate the modulation of sVEGFR-2 in vivo and evaluate the relationship between circulating sVEGFR-2 and tumor growth in various mouse models, as well as with VEGF itself. Using the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) and transplanted tumor models, we found an inverse relationship between levels of sVEGFR-2 and increasing tumor size. Furthermore, by using various methods of VEGF overexpression in vivo, including VEGF overexpressing tumor cells and adenoviral delivery of VEGF, we found that sVEGFR-2 decreases were mediated by tumor-derived VEGF, likely caused by ligand-induced down-regulation of VEGFR-2 from the cell surface, a finding that was confirmed in a series of in vitro experiments. Taken together, our results represent the first preclinical studies exploring the relationship between sVEGFR-2 and tumor progression and could provide insight into the further utility of monitoring sVEGFR-2 levels as a surrogate biomarker of VEGF-mediated tumor growth.

Materials and Methods

Antibodies

Commercially available antibodies for Western blotting and immunofluorescence were used as follows: human-specific polyclonal anti–VEGFR-2 antibody raised against the intracellular COOH-terminal region (Santa Cruz Biotechnology), human-specific goat polyclonal anti–VEGFR-2 antibody raised against the NH2-terminal region (R&D Systems, Inc.), mouse-specific monoclonal anti–VEGFR-2 raised against the NH2-terminal region (R&D Systems), and anti–β-actin (clone AC-15, Sigma-Aldrich). Secondary antibodies used were antirabbit IgG-HRP and antimouse IgG-HRP (Promega).

ELISA Analysis

Levels of murine sVEGFR-2 and human VEGF were assessed with commercially available mouse sandwich ELISA assays (R&D Systems, Inc.) following the manufacturer’s instructions with the exception of the following protocol modifications: for mouse sVEGFR-2, a 2:20-fold dilution of plasma was made instead of the recommended 1:15-fold; for human VEGF tested in human umbilical vascular endothelial cell (HUVEC), conditioned medium was added, with no dilution, to the ELISA to maximize low signals.

Cell Lines

The human colorectal adenocarcinoma HT29, human metastatic melanoma WM239, human prostate cancer PC-3, and mouse mammary EMT-6 cell lines were obtained from the American Type Culture Collection and maintained in DMEM, RPMI 1640, or Waymouth’s medium with 5% heat-inactivated fetal bovine serum (Life Technologies-Invitrogen Corp.). All cells were incubated at 37°C and 5% CO2 in a humidified incubator.

231/LM2-4 cells, a metastatic variant of the human MDA-MB-231 breast cancer cell line derived after two rounds of in vivo lung metastasis selection in mice, were maintained as previously described (27). HUVEC growth and maintenance as well as information on cell transfections used for this study are detailed in Supplementary Materials and Methods.

Plasma and Ascites Collection

Blood samples were obtained from the retro-orbital sinus or by cardiac puncture of mice under anesthesia with isoflurane. Ascites fluid was obtained directly from the peritoneal cavity using a syringe. Plasma and ascites were collected in Microtainer (Becton Dickinson) plasma separating tubes, centrifuged at 4°C, aliquoted, and stored at –70°C until assayed. All capillary tubing, syringes, and needles used for bleeding were first rinsed with heparin to avoid any clotting.

Adenoviral Delivery of VEGF

Replication-defective adenovirus serotype 5 expressing the human VEGF165 cDNA, including the signal sequence for secretion and under the direction of the cytomegalovirus promoter (Ad-VEGF165), was obtained from the Vector Core facility, Molecular Medicine Institute, University of Pittsburgh. Construction and use of the Ad-VEGF165 both in vitro and in vivo have previously been reported (28, 29). Adult BALB/c mice were injected with 1 × 108 plaque-forming units (pfu) of Ad-VEGF165, i.p. following experimental guidelines described by Thurston et al. (30) with expected peak systemic Ad-VEGF165 levels after 1 to 3 days and morbidity after 6 days due to vascular leakage. Ad-VEGF165–injected mice were sacrificed after 5 days and blood drawn from the retro-orbital sinus (to obtain blood in systemic circulation) and from ascites fluid.

Mouse Tumor Models

For all studies, institutional guidelines were strictly followed for maintenance of animals and determination of experimental end points. TRAMP, TRAMP mice, bred on a C57BL/6 background as previously described (31) or outbred onto a CD1 background, were heterozygous for the probasin-Tag transgene and weaned at 3 weeks. Genotyping for the probasin-Tag transgene was done by PCR using DNA isolated from tail biopsy. Blood was drawn via cardiac puncture and tumor excised and weighed at time of sacrifice. Tumor weights included seminal vesicles, bladder, urethra, and the prostate. If macroscopic metastatic disease was noted, the mouse was excluded from further study. Nontransgenic C57BL/6 and CD1 littersmates were used as controls.

S.C. syngeneic and human xenograft tumors. Tumor cells (2 × 106) were injected s.c. into the flank of 6- to 8-week-old female CB-17 severe combined immunodeficient (SCID) mice for human xenograft studies (using the human WM239, PC3VEGFR-high, PC3VEGFR-low, HT29 cells) or BALB/c mice for syngeneic studies (using mouse EMT-6 cells; Charles River). Blood was then drawn, mice were sacrificed, and tumors were excised and weighed.

Experimental human breast cancer xenograft metastasis. 231/LM2-4LM2-C2 cells (1 × 106) were injected into the tail vein of 6- to 8-week-old female CB-17 SCID mice. Tumor burden was monitored weekly (see imaging details in the following section) and mice were sacrificed at various stages of disease progression between 25 and 45 days after injection.

Bioluminescent Imaging

Bioluminescent imaging was done with a highly sensitive, cooled charge-coupled device camera mounted in a light-tight specimen box (IVIS, Xenogen) as previously described (32). Detailed descriptions of experimental protocol are listed in Supplementary Materials and Methods.

Cell Lysis and Western Blotting

For whole-cell lysis preparation, cells were washed in cold PBS and resuspended in cold lysis buffer [20 mmol/L Tris (pH 7.5), 137 mmol/L
NaCl, 100 mmol/L NaF, 10% glycerol, 1% NP-40, 1 mmol/L Na₂VO₄, and supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin) as previously described (33). Further details of cell lysis and Western blotting can be found in Supplementary Materials and Methods.

Cell-Surface Biotinylation and Immunoprecipitation
Following stimulation of HUVECs with recombinant human VEGF₆₅ [R&D Systems; Minneapolis, MN] at various time points, biotinylation and immunoprecipitation processing of cells were done as previously described (34). Further experimental details are listed in Supplementary Materials and Methods.

Statistical Analysis
Results were subjected to statistical analysis using the GraphPad Prism software package v.4.0 (GraphPad Software, Inc.). Additional methods used in this study are described in Supplementary Materials and Methods.

Results
Plasma sVEGFR-2 levels decrease in transgenic and xenograft tumor models. Recent clinical studies have incorporated evaluations of plasma levels of sVEGFR-2 during tumor progression.
in a variety of cancers, including breast and colorectal carcinomas (35), melanoma (20), and a number of blood cell–related malignancies (21–24); however, the utility of this protein as a potential biomarker remains unclear. To further investigate the relationship between sVEGFR-2 levels and tumor burden, we examined plasma obtained from various preclinical mouse tumor models that included spontaneous and implanted tumors, as well as localized and metastatic disease. Four mouse models were used. The first included the transgenic TRAMP mouse prostate tumor model, which uses the expression of the SV40 early genes (Tag) under the prostate-specific control of the minimal rat probasin promoter, leading to spontaneous prostate tumor development after 16 weeks (31). We found that increased TRAMP tumor burden, as measured by excised tumor weight after mouse sacrifice, correlated negatively with the levels of plasma sVEGFR-2 ($R^2 = 0.4661$ and $P = 0.0018$; Fig. 1A). A second mouse tumor model, which used syngeneic mouse EMT-6 tumors grown s.c. in BALB/c mice, yielded similar findings, with increased tumor weight negatively correlated to levels of plasma sVEGFR-2 ($R^2 = 0.6861$ and $P \leq 0.0001$; Fig. 1B). The third model tested plasma from mice that had been injected s.c. with the human tumor cells HT29, a colorectal carcinoma cell line, and WM239, a melanoma cell line. In these experiments, a negative relationship between sVEGFR-2 levels and excised tumor weight was also observed; however, the correlation was not found to be significant in either case (Fig. 1C). Finally, because the aforementioned tumor models involved testing localized disease, we also tested a metastatic human tumor xenograft model (32). The highly metastatic human breast cancer cell 231/LM2-4LMC-1 expressing luciferase was injected into the tail vein of SCID mice. Tumor distribution and growth was monitored with weekly imaging (see Materials and Methods for details). 231/LM2-4LMC-1 tumor growth was evident at multiple sites including lung, abdomen, femoral hind limb bone or muscle, and spine (Fig. 1D, top). *Ex vivo* imaging showed tumor nodules in the lung, femur, lymph nodes, and, less frequently, in organs such as the brain, kidney, and adrenals (data not shown). Imaging and quantification of bioluminescence at various disease stages were compared with circulating mouse sVEGFR-2 levels. A negative relationship was found between metastatic tumor burden and the sVEGFR-2 levels ($R^2 = 0.4739$ and $P = 0.0047$). Taken together, our results show that increased tumor burden can lead to a decrease in the overall levels of circulating sVEGFR-2 and that the strength of this correlation can vary among tumor types and tumor models, suggesting an underlying characteristic of the tumor responsible for modulation of plasma sVEGFR-2 levels.

**Inverse relationship between VEGF and sVEGFR-2 levels in tumor-bearing mice.** Numerous clinical studies in multiple tumor types have described a positive correlation between VEGF and the stage of disease (4). It has also recently been shown that levels of
VEGF and sVEGFR-2 can be inversely correlated, with increasing VEGF correlating to a decrease in sVEGFR-2. Such opposing VEGF/sVEGFR-2 trends were reported in various diseases, including acute lymphoblastic leukemia patients (23), certain obstetric disorders such as placenta accreta (36), in patients with dengue virus infection (37), and as well as in patients enrolled in numerous clinical trials who were treated with VEGF RTK inhibitors where elevated VEGF and decreased levels of sVEGFR-2 after treatment have been observed. These changes are currently under investigation as potential surrogate biomarkers for drug activity (26, 38). We therefore decided to examine the relationship between plasma levels of VEGF and sVEGFR-2 in the mouse TRAMP and EMT-6 tumor models described in Fig. 1. Levels of sVEGFR-2 showed a negative correlation to levels of VEGF in TRAMP mice ($R^2 = 0.4511$ and $P = 0.0023$), whereas wild-type non–tumor-bearing control mice showed no significant relationship ($R^2 = 0.1379$ and $P = 0.29$; Fig. 2A). Similar results were found in BALB/c mice bearing s.c. EMT-6 tumors described in Fig. 1B. Levels of sVEGFR-2 were negatively correlated to levels of plasma VEGF ($R^2 = 0.2377$ and $P = 0.0292$) but not significantly correlated in the plasma of non–tumor-bearing control BALB/c mice ($R^2 = 0.1232$ and $P = 0.3201$; Fig. 2B). We next tested whether the sVEGFR-2 detected in the plasma may be derived from TRAMP or EMT-6 tumor cells because it has previously been shown that some tumors can express VEGF-2 (12, 13) and could, theoretically, contribute to overall sVEGFR-2 levels. Therefore, we examined whether tumor cells can produce the sVEGFR-2 protein. By screening various human cell lines, we found two lines that expressed VEGF-2, the human melanoma WM239 and human prostate PC3, and a number of lines that were negative, such as the human colorectal carcinoma HT29 (Supplementary Fig. S1A). After injection of these cells s.c. into the flanks of SCID mice, and after tumor size reached institutionally allowed limits, plasma was screened for human (tumor-derived) sVEGFR-2 and expression was found in the WM239 and PC3 models, but not in the HT29 model. However, comparative values show that human (tumor-derived) sVEGFR-2 contributed only minimally to the overall circulating mouse-derived (host) sVEGFR-2 levels (Supplementary Fig. S1B and C). Regardless of this minimal effect, to ensure that the sVEGFR-2 detected in the TRAMP and EMT-6 mouse tumor models described in Figs. 1 and 2 was not derived from VEGFR-2–expressing tumors, and therefore contributing to overall sVEGFR-2 levels, we carried out immunofluorescent staining on tumor sections to show that VEGFR-2 expression can be detected on endothelial cells in blood vessels in TRAMP and EMT-6 tumors (Fig. 2C and D, respectively) but not on the tumor cells, indicating that detected sVEGFR-2 levels in Figs. 1 and 2 were not tumor derived.

**Tumor overexpression of VEGF leads to decreased levels of sVEGFR-2 in vivo.** To determine whether tumor-derived circulating VEGF levels in the plasma can contribute to the modulation of sVEGFR-2 in vivo, we used a xenograft mouse model with human tumor cell lines implanted s.c. into SCID mice. This had the advantage of allowing for the distinction between human (tumor-derived) plasma VEGF and mouse (host-derived) sVEGFR-2 using ELISAs specific for the human and mouse proteins. Cells from human prostate line PC3, previously shown to have a low level of VEGF expression compared with other tumor cell types (39), were transfected to overexpress human VEGF165, and clones expressing various levels of human VEGF165 were isolated. Two distinct cell lines were derived, one with elevated VEGF expression, named “PC3VEG-high”, and one with low levels of VEGF, named “PC3VEG-low”. Parental and transfected variants were incubated for 24 h in vitro in serum-free medium and conditioned medium and then tested for VEGF; PC3VEG-high cells expressed >1,500-fold

![Figure 3.](image-url)
Adenoviral mediated delivery of human VEGF decreases sVEGFR-2 levels in vivo. To determine whether VEGF alone can lead to the decreased levels of circulating sVEGFR-2, we examined the effects of systemic VEGF overexpression in tumor-free mice. Because it has previously been shown that i.v. injection of recombinant human VEGF165 leads to rapid kidney clearance of the protein (40), we used adenoviral-mediated delivery of the VEGF165 gene (Ad-VEGF165) to establish sustained overexpression in vivo. BALB/c mice were injected with $1 \times 10^8$ pfu Ad-VEGF165 i.p. and monitored for 5 days. Mice overexpressing Ad-VEGF165 have been shown to develop widespread tissue edema in most organs, tissue swelling, and separation of cellular elements by interstitial fluid resulting from an increase in vascular permeability (30). For this reason, the experiment was terminated after 5 days and blood drawn from the retro-orbital sinus (to measure levels of systemic blood proteins) and from ascites fluid accumulated in the peritoneum. Levels of mouse plasma sVEGFR-2 showed a 2-fold decrease in the systemic blood ($4,412 \pm 1,983.5$ pg/mL) and a 3.5-fold decrease in the ascites fluid ($2,690 \pm 437.8$ pg/mL) in Ad-VEGF165-injected mice when compared with plasma derived from noninfected BALB/c control mice (9,443 $\pm$ 364.8 pg/mL; Fig. 4A). Measurement of human Ad-VEGF165 in the mouse plasma using human specific ELISA showed that elevated VEGF levels corresponded with a decrease in systemic sVEGFR-2 levels, yielding a negative relationship (Fig. 4B). Taken together, these results indicate that VEGF overexpression alone can lead to decreased sVEGFR-2 levels independent of the tumor.

sVEGFR-2 levels modulated by VEGF-mediated receptor down-regulation in vitro. VEGF has been shown to initiate ligand-dependent VEGF-2 endocytosis and lead to the down-regulation of receptor expression from the endothelial cell surface (41). We therefore tested whether VEGF-mediated VEGFR-2 down-regulation could lead to decreased sVEGFR-2, possibly explaining modulations observed in vivo. We stimulated HUVECs with recombinant VEGF165 as well as with other mitogenic growth factors, including EGF and bFGF, previously shown to increase secretion of granulocyte-macrophage colony stimulating factor (GM-CSF), previously shown to increase secretion of sVEGFR-1 in monocytes (43), and phorbol 12-myristate 13-acetate (PMA) because activation of protein kinase C plays a role in VEGF-2 down-regulation (44) and has been shown to be involved in ectodomain shedding of other RTKs including TIE-2 (45). Conditioned media taken from HUVECs after 24 h of incubation showed that only VEGF stimulation led to the decrease in levels of sVEGFR-2 when tested by ELISA (Fig. 5A). Furthermore, we found that HUVECs stimulated with increasing concentrations of VEGF led to a correlative decrease in sVEGFR-2 in the conditioned media (Fig. 5B, top), which coincided with a decrease in full-length VEGFR-2 in whole-cell lysates analyzed by SDS-PAGE and Western blot (Fig. 5B, bottom). VEGFR-2 was detected with antibodies specific to the intracellular COOH-terminal and extracellular NH2-terminal domains to test whether possible cleavage products (i.e., either extracellular or intracellular components) would have differential expression and could therefore provide evidence of ectodomain shedding. No difference was observed between these two antibodies, suggesting that VEGFR-2 down-regulation was the underlying source of the decreased sVEGFR-2 in the conditioned media. Finally, to further

higher amounts of VEGF than parental controls, whereas PC3VEGF-low variants expressed levels of VEGF below the detectable range of the ELISA kit (Fig. 3A). For in vivo experiments, PC3VEGF-low and PC3VEGF-high cells were injected s.c. into SCID mice to test whether differential expression of tumor-derived VEGF165 in vivo could modulate sVEGFR-2. Mice bearing PC3VEGF-low tumors yielded no correlation between tumor size and the weight of the tumor ($R^2 = 0.5703$, $P \leq 0.0001$). In contrast, in SCID mice implanted with s.c. PC3VEGF-high cells, a strong negative correlation between levels of mouse sVEGFR-2 and excised tumor weight was detected ($R^2 = 0.7496$, $P \leq 0.0001$; Fig. 3C). Taken together, these results indicate that increased expression of human (tumor-derived) VEGF165 is associated with a decrease in the overall circulating levels of mouse (host-derived) sVEGFR-2 and likely is causative of this change in sVEGFR-2 levels.

Adenoviral-mediated overexpression of human VEGF decreases plasma sVEGFR-2 levels in vivo. Replication-defective adenovirus expressing the human VEGF165 (Ad-VEGF165) was injected ($1 \times 10^8$ pfu) i.p. in BALB/c mice and blood collected after 5 d retro-orbitally (circulating levels in plasma) and from the peritoneal cavity (localized ascites accumulation). A, sVEGFR-2 levels were decreased in plasma and ascites plasma of BALB/c mice injected with Ad-VEGF165 compared with control mice. B, human Ad-VEGF165 elevation in mice corresponded to a decrease in circulating sVEGFR-2. Control, $n = 12$; Ad-VEGF165 mice, $n = 8$.

Figure 4. Adenoviral-mediated overexpression of human VEGF decreases plasma sVEGFR-2 levels in vivo. Replication-defective adenovirus expressing the human VEGF165 (Ad-VEGF165) was injected ($1 \times 10^8$ pfu) i.p. in BALB/c mice and blood collected after 5 d retro-orbitally (circulating levels in plasma) and from the peritoneal cavity (localized ascites accumulation). A, sVEGFR-2 levels were decreased in plasma and ascites plasma of BALB/c mice injected with Ad-VEGF165 compared with control mice. B, human Ad-VEGF165 elevation in mice corresponded to a decrease in circulating sVEGFR-2. Control, $n = 12$; Ad-VEGF165 mice, $n = 8$. 

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confirm loss of VEGFR-2 from the cell surface, immunoprecipitation studies were carried out in which cells were tagged with biotin, lysed, and streptavidin agarose beads used to isolate only proteins biotinylated on the cell surface. Loss of VEGFR-2 from the cell surface was seen after 30 min and continued after 24 h (Fig. 5C). Additionally, no difference in expression could be seen between the two VEGFR-2 antibodies, indicating no differential presence of extracellular or intracellular VEGFR-2 domains. VEGFR-2 expression returned after 24 h of VEGF removal from the media, demonstrating the reversibility of this effect (Fig. 5C).

Discussion

Recent clinical studies have evaluated plasma levels of sVEGFR-2 as a potential surrogate biomarker of disease progression in a variety of malignancies based, in part, on the hypothesis that circulating sVEGFR-2 levels may provide insight into VEGFR-2 expression and activation, a critical process in tumor angiogenesis (23). However, there have been no preclinical studies examining the relationship between sVEGFR-2 and tumor progression or the underlying factors governing in vivo modulation. Using four mouse models, including spontaneous and implanted xenograft tumors, as well as an analysis of the effects of both local and metastatic disease, we show that increasing tumor burden leads to decreased sVEGFR-2 levels, which are mediated, at least in part, by tumor-derived VEGF. This was shown using various VEGF overexpression...
models in vivo, including implantation of tumor cells transfigured with VEGF or adenoviral-mediated VEGF delivery, both of which showed that an increase in circulating VEGF led to a relative decrease in sVEGFR-2 levels. Experiments conducted in vitro show that these results may be explained by VEGF- mediated receptor down-regulation from the cell surface, which in turn leads to reduced sVEGFR-2 levels in conditioned media from endothelial cells, thus implying that expression levels of VEGF-2 and its soluble form are linked and may explain modulations of sVEGFR-2 in vivo. Taken together, our results suggest that clinical evaluations of sVEGFR-2 may be indicative of overall circulating VEGF levels and serve as a possible surrogate biomarker for VEGF-dependent tumor growth.

It has previously been shown that circulating levels of soluble receptors such as sKIT (6), sTIE-2 (7), sHER-2/neu (8), sIL-2 (9), sEGFR (10), and many others have utility as potential biomarkers for cancer progression. The majority of these studies show positive correlations between disease and soluble receptor level. However, recent clinical studies investigating sVEGFR-2 and cancer progression have been less clear and shown varied correlations with disease (20, 24), such that the potential use of sVEGFR-2 as a biomarker for tumor growth remains unknown. By using preclinical mouse cancer models to monitor sVEGFR-2 during tumor growth, our studies allowed for the distinction between tumor and host-derived proteins and showed that systemic elevations in VEGF lead to decreased sVEGFR-2 independent of tumor. Because of the varied levels of VEGF known to be expressed between tumor types as well as between individual patients (4), our results may explain varied clinical findings of sVEGFR-2 levels during cancer progression. Further testing of sVEGFR-2 in conjunction with VEGF in malignancies could provide more clues about the value of this protein as a surrogate biomarker for VEGF-dependent tumor growth and possibly even for changes in systemic VEGF levels alone. It is possible that the testing of sVEGFR-2 in this regard may have one advantage over the measurement of VEGF levels because sVEGFR-2 levels do not vary significantly between blood collection methods (i.e., in serum or plasma; ref. 19), unlike VEGF (46).

An intriguing aspect of our preclinical findings with VEGF and sVEGFR-2 is that they may help to explain similar observations of an inverse relationship detected in clinical studies. For example, circulating VEGF and sVEGFR-2 were found to be inversely correlated in acute lymphoblastic leukemia patients (23), placenta acreta (36), and in patients with dengue virus infection (37). Our results may also offer insight into the similar inverse trend seen after treatment with certain antiangiogenic drugs that target VEGF or VEGFRs (i.e., small-molecule RTK inhibitors). Increased levels of plasma VEGF and reduced levels of sVEGFR-2 have now been noted with four different VEGF RTK inhibitors, including SU11248/sunitinib, AG-013736/axitinib, PTK787/vatalanib, and AMG706/motesinib, and at least eight more reported in recent meetings, including BAY-43-9006/sorafenib and BAY-57-9352/telatinib, such that these changes can now be considered a “class” effect of small-molecule antiangiogenic VEGF RTK inhibitors (26, 38). Moreover, these changes have been investigated as surrogate biomarkers for such drugs, potentially providing information on pharmacodynamic drug activity/exposure and patient benefit. Interestingly, we recently showed that this increase in VEGF and corresponding decrease in sVEGFR-2 after drug treatment could be recapitulated in tumor-free mice and involve multiple organs, indicating that these molecular changes are a systemic response to drug treatment and that the changes occur maximally in the optimal anti-tumor dose range (26). Our results reported herein showing that sVEGFR-2 level reduction is induced by VEGF-mediated receptor down-regulation could offer a potential underlying explanation for these findings and suggest that sVEGFR-2 may serve as an indicator of VEGF-induced receptor down-regulation.

It will be interesting to note whether, in addition to the potential exploitation of these changes as surrogate biomarkers, such elevations in VEGF and declines in sVEGFR-2 may have any biological consequence for tumor angiogenesis either during or after cessation of treatment using drugs such as antiangiogenic RTK inhibitors. To determine this, additional studies would be needed to elucidate a potential function, if any, for the sVEGFR-2 fragment and to determine if it plays a role in VEGF activity. Although we have previously shown that sVEGFR-2 can bind to VEGF (19), it remains unknown whether sVEGFR-2, like other soluble receptors such as sVEGFR-1 (47), can bind and sequester VEGF in vivo and therefore influence binding and activation of VEGFRs. If so, then it is conceivable that sVEGFR-2 levels affect certain anti-VEGF and anti−/−VEGF-2 strategies in the clinic that aim to disrupt VEGF binding to VEGFR-2. This could be of considerable importance if such drug-induced sVEGFR-2 changes vary between different VEGF and VEGFR-2 inhibitors, such as between those that are RTK inhibitor or antibody based.

Finally, although some investigators have reported varied consequences of VEGF on VEGFR-2 expression, the general consensus is that, like other RTKs (41), VEGF165 binding and activation of VEGFR-2 leads to internalization and down-regulation of the receptor from the cell surface (41, 44). Our results confirm that VEGF binding and activation of the VEGFR-2 leads to down-regulation, which, in turn, we found to lead to a correlative decreases in sVEGFR-2 production. However, it remains unknown whether the sVEGFR-2 is a product of ectodomain shedding from cell-surface VEGFR-2 or a product of alternative mRNA splice variation. Whereas it has been shown that some alternative splice variation of VEGFR-2 mRNA can occur in rat retinal cells, leading to a slightly truncated but still functional receptor (48), a splice variation for VEGFR-2 encoding for the VEGFR-2 ectodomain has not been detected. Therefore, determination of whether sVEGFR-2 is a product of ectodomain cleavage from the cell surface requires more detailed analysis.

Taken together, our results represent the first preclinical investigation of the relationship between sVEGFR-2 and tumor burden. Additionally, our findings that VEGF levels are an important mediator of sVEGFR-2 levels independent of tumor burden may help explain recent clinical results and allow for further understanding and utilization of sVEGFR-2 as a potential biomarker for monitoring cancer progression.

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