Abstract
Angiogenesis and vasculogenesis are regulated in large part by several different growth factors and their associated receptor tyrosine kinases (RTKs). Foremost among these is the vascular endothelial growth factor (VEGF) family including VEGF receptor (VEGFR)-2 and -1. VEGFR ligand binding and biological activity are regulated at many levels, one of which is by a soluble, circulating form of VEGFR-1 (sVEGFR-1). This sVEGFR-1 can act as a competitive inhibitor of its ligand, serve as a possible biomarker, and play important roles in cancer and other diseases such as preeclampsia. Recombinant forms of sVEGFR-2 have been shown to have antiangiogenic activity, but a naturally occurring sVEGFR-2 has not been described previously. Here, we report such an entity. Having a molecular weight of ~160 kDa, sVEGFR-2 can be detected in mouse and human plasma with several different monoclonal and polyclonal anti-VEGFR-2 antibodies using both ELISA and immunoprecipitation techniques. In vitro studies have determined that the sVEGFR-2 fragment can be found in the conditioned media of mouse and human endothelial cells, thus suggesting that it may be secreted, similar to sVEGFR-1, or proteolytically cleaved from the cell. Potential biological activity of this protein was inferred from experiments in which mouse sVEGFR-2 could bind to VEGF-coated plates. Similar to sVEGFR-1 and other soluble circulating RTKs, sVEGFR-2 may have regulatory consequences with respect to VEGF-mediated angiogenesis as well as potential to serve as a quantitative biomarker of angiogenesis and antiangiogenic drug activity, particularly for drugs that target VEGF or VEGFR-2. (Mol Cancer Res 2004;2(6):315–26)

Introduction
Angiogenesis, the formation of new blood vessel capillaries from an existing vasculature, and vasculogenesis, the de novo formation of blood vessels from primitive precursor cells, are dependent on several growth factors and their associated tyrosine kinases. A key regulator of both these processes is vascular endothelial growth factor (VEGF), also called vascular permeability factor. Gene targeting methods have provided powerful evidence for the indispensable role of VEGF signaling systems in angiogenesis and vasculogenesis. For example, disruption of only a single VEGF allele results in embryonic lethality due to impaired vessel formation and function (1, 2). Diseases involving angiogenesis such as cancer and age-related vascular degeneration are often driven by VEGF, and as a consequence, a variety of drugs, which target VEGF or VEGF receptors (VEGFRs), are being developed and evaluated for treatment of such diseases (3, 4). One such drug has already been approved for the treatment of colorectal carcinoma [i.e., bevacizumab, the humanized monoclonal anti-VEGF antibody (5)].

The VEGF family consists of VEGF-A (also called VEGF), VEGF-B, VEGF-C, and VEGF-D as well as placental growth factor (6). The most important of these in the context of tumor angiogenesis is VEGF-A, especially the VEGF121 and VEGF165 splice variant isoforms, which act both as an activator and as a survival factor for endothelial cells of newly formed blood vessels through binding to specific high-affinity VEGF tyrosine kinases. These receptors, known as VEGFR-2 or KDR (human)/flt-1 (mouse) and VEGFR-1 or flt-1, have also been shown to be present on bone marrow–derived cells (7), including hematopoietic stem cells (8) and circulating endothelial progenitor cells (9); dendritic cells (10); trophoblast and placenta cells (11); monocytes (12, 13); retinal progenitor cells (14); and certain types of tumor (15). VEGFR-1 and -2 have an extracellular domain containing seven immunoglobulin-like loops, a transmembrane domain, and an intracellular split catalytic tyrosine kinase domain (16). Of the two receptors, VEGFR-1 has very high affinity for VEGF binding (10-fold
higher than VEGFR-2), but VEGFR-2, nevertheless, appears to play a more important functional role in mediating signaling events involved in endothelial cell mitogenesis, migration, survival, and vascular permeability (17).

VEGFR-1 is not only found in a cell membrane–bound form. Through alternative splicing of the VEGFR-1 mRNA, a soluble, truncated form of VEGFR-1 (sVEGFR-1) is secreted by endothelial cells (17–19). Comprised of six of the seven immunoglobulin-like domains present on the extracellular region, this sVEGFR-1 can act as a natural endogenous inhibitor not only by sequestering VEGF (20, 21) but also by binding and inactivating membrane-bound VEGFR-1 and -2 receptors through a mechanism involving a quasi form of receptor homodimerization or heterodimerization (22). As such, the role of sVEGFR-1 has been studied both as a surrogate marker for disease progression and/or as a potential inhibitor of tumor angiogenesis in cancers such as breast (23–25), ovarian (26), colorectal carcinoma (27), renal cell carcinoma (28), fibrosarcoma (29), and astrocytic tumors (30) as well as for its possible contribution to diseases such as preeclampsia (31, 32). Of interest in this regard is that many prior studies using synthetic recombinant sVEGFR-2, encoding for the extracellular domain of the full-length receptor, have shown similar characteristics to that described for natural sVEGFR-1 such as the ability to bind to VEGF (33–35) and VEGF/placental growth factor heterodimers (36, 37) as well as mediating antitumor effects (38–40). However, no natural form of sVEGFR-2 has been reported in biological fluids.

The purpose of the present study was to investigate the possible presence of a sVEGFR-2 in mouse and human plasma. To do so, we used eight different monoclonal and polyclonal antibodies raised against the extracellular domain of mouse and human VEGFR-2 and tested for the fragment by immunoprecipitation and ELISA both in vitro and in vivo. Our results show that sVEGFR-2 can indeed be detected in mouse and human plasma, the functional significance of which is unknown.

**Results**

**Immunoprecipitation of a sVEGFR-2 Fragment From Mouse Plasma Using Anti-VEGFR-2 Antibodies**

A spliced variant of VEGFR-2 mRNA, encoding for all but a portion of the intracellular catalytic domain, was described previously by Wen et al. (41) in rat retinal cells; however, there have been no reports of a naturally occurring truncated VEGFR-2 protein found in biological fluids. Putative sVEGFR-2 was immunoprecipitated from severe combined immunodeficiency (SCID) mouse plasma using protein G–coated Sepharose beads incubated with one of three different rat-derived monoclonal anti-mouse antibodies raised against the extracellular domain of mouse VEGFR-2. The antibodies used—DC101 and RAFL-1/RAFL-2—were raised independently against recombinant sVEGFR-2 and were derived by different techniques. Immunoblotting using a polyclonal anti-mouse anti-VEGFR-2 antibody revealed an ~160-kDa protein for each sVEGFR-2 immunoprecipitation performed (Fig. 1A, lanes 1, 3, and 5). Recombinant sVEGFR-2, designated (M)-sVEGFR-2/Fc, which encodes for amino acid residues 1 to 762 of the extracellular domain of mouse VEGFR-2, was used for comparison (Fig. 1A, lane 8).

**sVEGFR-2 Depletion After Immunoprecipitation Confirmed by Mouse sVEGFR-2 ELISA**

Supernatants from each immunoprecipitation shown in Fig. 1A were tested for levels of sVEGFR-2 depletion using a mouse sVEGFR-2 ELISA. Results show that the postimmunoprecipitation supernatants contained less sVEGFR-2 (Fig. 1B, lanes 1, 3, and 5) corresponding to 0, 7.36, and 3.49 pg/mL, respectively) when compared with the control sample of plasma alone (Fig. 1B, lane 7 corresponding to 40.26 pg/mL), thus confirming that this mouse sVEGFR-2 ELISA is measuring the same sVEGFR-2 fragment immunoprecipitated in Fig. 1A as well as indicating that each of the antibodies used are binding the same protein.

**Plasma-Derived Mouse sVEGFR-2 Binds to VEGF**

The same samples used in the above experiment (postimmunoprecipitation supernatants) were incubated in parallel on 96-well plates coated with 4 μg per well recombinant mouse VEGF to test whether this sVEGFR-2 fragment found in the plasma could bind VEGF (Fig. 1C). Control sample, containing plasma alone, incubated on the VEGF-coated plate (Fig. 1C, lane 7, 22.6 pg/mL) showed sVEGFR-2 levels similar to levels measured by mouse sVEGFR-2 ELISA (Fig. 1B, lane 7, 40.26 pg/mL). Additionally, as in Fig. 1B, each sample was compared with control sample of plasma alone (Fig. 1B, lanes 1 to 6), confirming again that immunoprecipitation supernatants were depleted of sVEGFR-2. Recombinant mouse (M)-sVEGFR-2/Fc was used to generate standard curves for both sVEGFR-2 ELISA and VEGF-coated plates (Fig. 1D). Curves show that recombinant (M)-sVEGFR-2/Fc is less sensitive at binding the VEGF-coated plate compared with the mouse sVEGFR-2 ELISA, potentially explaining why levels of sVEGFR-2 (Fig. 1B, lane 7 and Fig. 1C, lane 7) differed marginally between the two detection methods.

**Plasma-Derived Mouse sVEGFR-2 Is Heavily Glycosylated**

It has been demonstrated previously that recombinant sVEGFR-2, derived from insect cells, has a molecular weight of ~100 to 110 kDa (34, 35). Additionally, because the cDNA-derived amino acid sequence of sVEGFR-2 has 16 potential N-linked glycosylation sites, Huang et al. (34) further showed that this insect-derived recombinant sVEGFR-2, when deglycosylated, is ~80 to 90 kDa. We hypothesized that the reason on the ~160-kDa mouse plasma-derived sVEGFR-2 we report has slower mobility on SDS-PAGE than the previously reported insect-derived recombinant proteins is due to the more extensive glycosylation that occurs in mammalian cells (42). To test this, sVEGFR-2 was immunoprecipitated from SCID mouse plasma using DC101 and R&D antibodies (Fig. 2, lanes 1 and 3, respectively) and treated with peptide N-glycosidase (PNGase) to remove all N-linked glycoproteins. After treatment, sVEGFR-2 was reduced from ~160 to ~90 kDa (Fig. 2, lanes 2 and 4, respectively) by SDS-PAGE analysis, indicating that the mammalian-derived sVEGFR-2 is more heavily glycosylated than the insect-derived sVEGFR-2 and, when deglycosylated, is of comparable size with the previously reported deglycosylated insect-derived recombinant
FIGURE 1. Immunoprecipitation of a naturally occurring sVEGFR-2 from SCID mouse plasma. A. A sVEGFR-2 (~160-kDa) fragment was immunoprecipitated from SCID mouse plasma using one of three rat-derived monoclonal antibodies specific for mouse VEGFR-2. Antibodies used were DC101, RAFL-1, or RAFL-2. Controls included antibody incubated alone with beads, plasma incubated alone with beads, and recombinant extracellular domain of mouse VEGFR-2 fused to a human Fc [designated (M)-sVEGFR-2/Fc]. B. Postimmunoprecipitation supernatants were tested on sVEGFR-2 ELISA and found to be depleted of sVEGFR-2, indicating that the protein measured by this ELISA is the same as that immunoprecipitated in A. C. Using the same samples as in B, parallel experiments were performed using VEGF-coated plates (4 μg per well) and found to yield a similar result, suggesting potential biological activity of this sVEGFR-2 protein. D. Values for B and C were derived from curves using recombinant (M)-sVEGFR-2/Fc as a standard.
sVEGFR-2. To further illustrate this difference in glycosylation, mammalian-derived sVEGFR-2 [designated (M)-sVEGFR-2/Fc] and insect-derived recombinant sVEGFR-2 [designated (I)-sVEGFR-2] were compared after treatment with PNGase. (M)-sVEGFR-2/Fc is an ~180-kDa protein and becomes 120 kDa when deglycosylated (Fig. 2, lanes 5 and 6, respectively), while the (I)-sVEGFR-2 is ~110 to 115 kDa and becomes ~90 to 95 kDa when deglycosylated (Fig. 2, lanes 7 and 8, respectively). We attribute the fact that the (M)-sVEGFR-2/Fc is larger than both the glycosylated and the deglycosylated mouse-derived sVEGFR-2 to be due to the human Fc IgG1 portion to which the recombinant is fused (see Materials and Methods for description).

Conditioned Media From Mouse Endothelial Cells Contains sVEGFR-2

Conditioned media (CM) taken from endothelial cells of human origin have been shown to contain sVEGFR-1 (20, 21). Using VEGFR-2 (+) mouse endothelial cell lines PY4.1, MS1, and bEnd3, we assessed the levels of sVEGFR-2 detectable in CM. Immunoprecipitations were performed using both CM and cell lysates (which would contain the full-length VEGFR-2) with the mouse monoclonal VEGFR-2 antibody DC101 or R&D antibody revealed a full-length 180- to 250-kDa VEGFR-2 RAFL-2. Immunoblotting using a polyclonal anti-VEGFR-2 antibody from SCID mouse plasma using DC101 or R&D antibodies (lanes 1 and 3), migrated on SDS-PAGE gel at ~90 kDa after deglycosylation with PNGase F (lanes 2 and 4). Differences in glycosylation between two recombinant sVEGFR-2 proteins, one mammalian derived [designated (M)-sVEGFR-2/Fc; lane 5] and one insect derived [designated (I)-sVEGFR-2; lane 7], were compared. Mobility of (M)-sVEGFR-2/Fc and (I)-sVEGFR-2 by SDS-PAGE analysis was increased to ~120 and 90 to 95 kDa, respectively (lanes 6 and 8), after deglycosylation with PNGase F, indicating that the mammalian-derived sVEGFR-2 is more heavily glycosylated than the insect-derived protein.

**FIGURE 2.** Deglycosylation of sVEGFR-2 and recombinant sVEGFR-2 proteins increases mobility on SDS-PAGE gel. The ~160-kDa naturally occurring sVEGFR-2 protein, immunoprecipitated from SCID mouse plasma using DC101 or R&D antibodies (lanes 1 and 3), migrated on SDS-PAGE gel at ~90 kDa after deglycosylation with PNGase F (lanes 2 and 4). Differences in glycosylation between two recombinant sVEGFR-2 proteins, one mammalian derived [designated (M)-sVEGFR-2/Fc; lane 5] and one insect derived [designated (I)-sVEGFR-2; lane 7], were compared. Mobility of (M)-sVEGFR-2/Fc and (I)-sVEGFR-2 by SDS-PAGE analysis was increased to ~120 and 90 to 95 kDa, respectively (lanes 6 and 8), after deglycosylation with PNGase F, indicating that the mammalian-derived sVEGFR-2 is more heavily glycosylated than the insect-derived protein.
measured by the sVEGFR-2 ELISA. VEGFR-2 and sVEGFR-2 could not be detected in lysates or CM of VEGFR-2 (−) cell lines such as KS.1, HB60, and CB357 (data not shown). Taken together, these results demonstrate that the sVEGFR-2 protein immunoprecipitated from the CM and plasma and the VEGFR-2 immunoprecipitated from the cell lysate are the same sVEGFR-2 and VEGFR-2 protein measured by the mouse sVEGFR-2 ELISA.

**Human Plasma and Endothelial Cell CM Contain sVEGFR-2**

Using experimental protocols identical to the studies we undertook using mouse samples, sVEGFR-2 was immunoprecipitated from both human plasma and human VEGFR-2 (+) endothelial cell CM using the highly specific phage display–derived monoclonal antibody, 1121, raised against the extracellular domain of human VEGFR-2. The ~160-kDa sVEGFR-2 protein, revealed by immunoblotting using a horseradish peroxidase–conjugated polyclonal anti-VEGFR-2 antibody, was identified from two human plasma samples taken from healthy volunteers (Fig. 4A, lanes 2 and 4) and compared with the full-length 180- to 250-kDa VEGFR-2 protein detected from cell lysates of human endothelial cell lines, human umbilical vascular endothelial cell (HUVEC) and EA.hy926 (Fig. 4A, lanes 6 and 8, respectively). Levels of VEGFR-2 on EA.hy926 cells were markedly lower than on HUVECs. Immunoprecipitation controls using plasma or cell lysate alone (Fig. 4A, lanes 1, 3, 5, and 7) showed no protein, while a control with 1121 antibody alone (Fig. 4A, lane 9) revealed partially unreduced 1121 antibody which cross-reacted with the anti-VEGFR-2–horseradish peroxidase antibody used for immunoblotting. Using a sandwich ELISA for human sVEGFR-2, postimmunoprecipitation supernatants of plasma-alone controls showed sVEGFR-2 in both plasma samples (Fig. 4B, lanes 1 and 3) and VEGFR-2 in both cell lysate controls (Fig. 4B, lanes 5 and 7). In contrast, supernatants after immunoprecipitation with 1121 were shown to be depleted of sVEGFR-2 (Fig. 4B, lanes 2 and 4) and VEGFR-2 (Fig. 4B, lanes 6 and 8), confirming that the full-length VEGFR-2 protein and its soluble truncated form, detected by immunoprecipitation, is the same as that detected by ELISA.

Additionally, as was shown previously for mouse endothelial cell CM, sVEGFR-2 could be immunoprecipitated from HUVEC CM using the 1121 antibody (Fig. 4C, lane 4), whereas control media contained no such protein (Fig. 4C, lane 3). Human sVEGFR-2 ELISA results showed that postimmunoprecipitation CM (Fig. 4D, lane 4) had lower sVEGFR-2 than before immunoprecipitation (Fig. 4D, lane 2). Taken together, these results confirm that the protein detected in human plasma and human endothelial cell CM, measured by both immunoprecipitation and ELISA, is the same full-length VEGFR-2 protein or sVEGFR-2 fragment.

**sVEGFR-2 Levels in Plasma and Serum of Healthy Volunteers**

From eight healthy volunteers (4 male and 4 female), blood was collected and the concentration of sVEGFR-2 was measured in serum, heparin plasma, EDTA plasma, and citrate plasma by sVEGFR-2 ELISA. Table 1 summarizes the normal values of sVEGFR-2 tested in all materials. No statistical differences (P > 0.05) were found either between the sVEGFR-2 levels in the serum and plasma types or between the male and female groups as determined by the Mann-Whitney test (GraphPad Prism Software Package version 4.0). Furthermore, linear regression analysis was performed to compare the sVEGFR-2 levels in the serum and the plasma samples obtained from the same healthy volunteer. A high correlation (P < 0.0001) between sVEGFR-2 levels of serum and the three types of plasma samples of the same volunteer was found, suggesting that sVEGFR-2 levels could potentially be measured accurately using either method (data not shown). However, before the normal levels of sVEGFR-2 in healthy volunteers can be precisely defined, further measurements will need to be performed with a larger population group.

**Discussion**

Our results demonstrate for the first time that a sVEGFR-2 can be detected in plasma. Five different monoclonal antibodies, raised against the extracellular domain of either human or mouse VEGFR-2, were used to precipitate the ~160-kDa fragment from normal human or SCID mouse plasma. The anti-VEGFR-2 antibodies used included the mouse VEGFR-2 (flk-1) specific DC101, R&D, and RAFL-1/R AFL-2 antibodies as well as the human VEGFR-2 (KDR) specific antibody, 1121, all of which were generated independently. Our studies confirmed that the detected soluble fragment was a truncated form of VEGFR-2 by comparison with the full-length 180- to 250-kDa VEGFR-2, precipitated from mouse and human endothelial cell lysates, and the ~180-kDa recombinant mouse sVEGFR-2 [M]-sVEGFR-2/Fc], containing the extracellular domain fused to a human Fc. Further studies also revealed that the ~160-kDa mouse-derived sVEGFR-2 protein is heavily glycosylated as cleavage of N-linked glycoproteins with PNGase resulted in sVEGFR-2 migration at 90 kDa on SDS-PAGE gel. Using two human and mouse polyclonal anti-VEGFR-2 antibodies, our findings show that both the immunoprecipitated natural sVEGFR-2 and the full-length VEGFR-2, as well as mammalian- and insect-derived recombinant sVEGFR-2, could be distinguished by Western blot analysis.

Because endothelial cells of human origin are known to secrete sVEGFR-1 (20, 21), we asked whether a sVEGFR-2 could be detected in the CM of various endothelial cell lines of both mouse and human origin. Our results, using three mouse and two human VEGFR-2–positive endothelial cell lines demonstrate that the ~160-kDa protein, identical in size to the sVEGFR-2 fragment precipitated from plasma, could be detected in the CM using the aforementioned immunoprecipitation technique. This suggests that, like sVEGFR-1, one source for sVEGFR-2 detected in the plasma might be endothelial cell in origin. For VEGFR-1, the secretion of the soluble fragment is a consequence of alternative mRNA splicing. While Wen et al. (41) have demonstrated that alternative splicing of VEGFR-2 mRNA in rat retinal cells can result in a truncated form of VEGFR-2 encoding for a functional form of receptor lacking a portion of the intracellular
domain, there have been no reports of sVEGFR-2 arising as a result of alternative mRNA splicing. Whether the sVEGFR-2 we detect in the plasma and CM of endothelial cells is a result of alternative mRNA splicing, proteolytic cleavage of the membrane-bound receptor, or another mechanism, is still unknown and currently under investigation.

An important aspect of our study was that the sVEGFR-2 detected in the plasma and CM could be confirmed using commercially available sVEGFR-2 sandwich ELISA kits designed to capture and detect the extracellular domain of both human and murine VEGFR-2 using monoclonal and polyclonal antibodies. Using aliquots of supernatants taken from before and after the immunoprecipitation of sVEGFR-2 from both plasma and CM, we found that detectable levels of sVEGFR-2 were either markedly reduced or depleted entirely. This result was an important confirmation that the ~160-kDa sVEGFR-2 fragment, identified from the aforementioned immunoprecipitations, was the same sVEGFR-2 fragment measured by both human and mouse ELISA kits. Because our findings demonstrate that one possible source of sVEGFR-2 might be endothelial cells, this result raises the possibility that detection of natural sVEGFR-2 in the plasma using such ELISA methods

![Diagram](image_url)

**FIGURE 3.** Immunoprecipitation of a naturally occurring sVEGFR-2 from CM of mouse endothelial cells. A. From confluent plates, PY4.1 mouse endothelial cell lysates and CM were tested for VEGFR-2 and sVEGFR-2 levels, respectively. Immunoprecipitations were performed from media and cell lysates using DC101 or RAFL-2. CM contained the same ~160-kDa sVEGFR-2 fragment shown present in plasma (lanes 4 and 6), while cell lysate revealed the full-length 180- to 250-kDa VEGFR-2 (lane 8). Recombinant murine (M)-sVEGFR-2/Fc is shown for size comparison (lane 9). B. For media and cell lysates, aliquots of preimmunoprecipitation and postimmunoprecipitation supernatants were saved for sVEGFR-2 and VEGFR-2 level comparison. Postimmunoprecipitation supernatant showed depletion of sVEGFR-2 and VEGFR-2 as measured by mouse sVEGFR-2 ELISA. Using the identical protocol as above, experiments with MS1 and bEnd3 yielded the same results by immunoprecipitation and Western blotting (C) and depletion of sVEGFR-2 and VEGFR-2 from postimmunoprecipitation supernatants (D). Parallel experiments performed using the same immunoprecipitation supernatants used in D showed that sVEGFR-2 derived from MS1 and bEnd3 could bind to VEGF-coated plates (4 μg per well) (E) in a similar manner to the mouse sVEGFR-2 ELISA. +, either cell lysate or CM; −, either control media or lysis buffer; *, lysates were not tested on VEGF-coated plate.
may have potential use in the evaluation of sVEGFR-2 in preclinical or clinical samples. Because other soluble receptor forms, such as soluble c-kit in germ cell tumors (43), sTie2 and VEGFR-1 in renal cancers (44), and sHER-2/neu in metastatic breast cancers (45), have been studied as potential surrogate markers for disease progression, and the fact that elevated VEGFR-2 expression has been shown in some diseases to be correlated to tumor progression (46), it is plausible that circulating sVEGFR-2 levels might be similarly useful as an indicator for tumor progression. Experiments to determine whether circulating sVEGFR-2 levels might be used as such a surrogate marker for tumor progression and/or response to certain antiangiogenic drugs are currently under way.

FIGURE 3 continued.
Synthetic recombinant forms of both mouse and human sVEGFR-2, encoding for the extracellular region of the receptor, have been used previously for two main purposes—(1) as an immunogen for development of anti-VEGFR-2 antibodies (including those used in this study) and (2) as a potential anti-angiogenic agent both in vitro (34-37, 39) and in vivo (38, 47, 48)—because recombinant sVEGFR-2 retains ligand binding ability and can therefore reduce bioavailability of VEGF. For this reason, we asked whether the natural form of sVEGFR-2 could bind to VEGF in a similar fashion. Using plates coated with recombinant murine VEGF, we tested whether sVEGFR-2 derived from both SCID mouse plasma and mouse endothelial cell CM could be captured in a manner identical to that used in the sVEGFR-2 ELISA studies. Experiments using the murine sVEGFR-2 ELISA and VEGF-coated plates in parallel demonstrated that the sVEGFR-2 detected in both SCID mouse plasma and mouse endothelial cell CM yield similar results. This finding suggests that, like sVEGFR-1, sVEGFR-2 may have biological activity. Whether this natural sVEGFR-2 can play a significant role in VEGF signaling, as has been described for sVEGFR-1, remains to be established.

Finally, an important point to note is that all monoclonal antibodies used for the immunoprecipitation of sVEGFR-2 from the plasma and CM, with the exception of R&D, were developed to inhibit VEGF binding to VEGFR-2 (49-51). This raises the possibility that these antibodies, shown to mediate antiangiogenic effects, could potentially bind sVEGFR-2 in vivo. Because it is unknown whether sVEGFR-2 is involved in VEGF signaling or if the fragment contributes any significant biological role in angiogenesis, an intriguing possibility to consider is that sVEGFR-2 may influence (or be influenced by) the activity of drugs that are designed to block VEGFR-2. In addition, it may have potential as an indicator for drug efficacy and/or optimal biological dosing, at least for agents that target VEGFR-2 and perhaps other angiogenesis inhibitors.

**FIGURE 4.** sVEGFR-2 detected in human plasma and CM from human endothelial cells. A. Immunoprecipitations of sVEGFR-2 from two human plasma samples and VEGFR-2 from HUVEC and EA.hy926 cell lysates were performed using the human anti-VEGFR-2 antibody 1121 raised against the extracellular domain of VEGFR-2. B. Postimmunoprecipitation supernatants showed marked depletion of sVEGFR-2 and VEGFR-2 compared with plasma-alone or cell lysate-alone controls. C. Similar studies revealed sVEGFR-2 in the CM of HUVEC. D. Postimmunoprecipitation supernatants tested by human sVEGFR-2 ELISA proved to be depleted of sVEGFR-2, further confirming that the ~160-kDa sVEGFR-2 and full-length 180 to 250-kDa VEGFR-2 measured by Western blotting and by ELISA in both plasma and CM are the same protein. +, cell lysate, plasma, or CM; −, either control media or lysis buffer.
Materials and Methods

Antibodies

Several different anti-VEGFR-2 antibodies, raised against the extracellular region of mouse and human recombinant VEGFR-2, were used to identify the full-length VEGFR-2 and sVEGFR-2 fragment by immunoprecipitation. For studies using mouse samples, the rat-derived mouse monoclonal anti-VEGFR-2 antibodies used were DC101 (ImClone Systems, New York, NY; ref. 49), RAFL-1 and RAFL-2 (kind gifts from Dr. Philip E. Thorpe, University of Texas, Dallas, TX; ref. 50), and R&D (R&D Systems, Inc., Minneapolis, MN; clone 91292.11). For human samples, the phage display–derived

![Image of Table 1]

<table>
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<th>Plasma EDTA</th>
<th>Plasma Citrate</th>
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NOTE: sVEGFR-2 levels in serum, heparin plasma, EDTA plasma, and citrate plasma of healthy volunteers (total volunteers = 8; 4 female and 4 male). sVEGFR-2 levels were defined by human sVEGFR-2 ELISA.

*age range: 22-31.
monoclonal antibody 1121 (ImClone Systems; ref. 51) was used. Detection of VEGFR-2 and sVEGFR-2 in Western immunoblotting was performed using goat-derived mouse and human specific anti-VEGFR-2 antibodies conjugated to horseradish peroxidase (R&D Systems).

**Cell Lines**

Transformed VEGFR-2 (+) mouse endothelial cell lines used were PY4.1 (derived from s.c. microvessels of SV40T transgenic mice; ref. 52), MS1 (immortalized with a temperature-sensitive SV40 large T antigen; ref. 53), and bEnd3 (originally derived from mouse brain capillaries; ref. 54). All murine cell lines used were generous gifts of Dr. Daniel J. Dumont (Sunnybrook and Women’s College Health Sciences Centre, Toronto, Ontario, Canada). Murine VEGFR-2 (−) cell lines used as negative controls were HB60, KS.1, and CB357 (55). Human umbilical vein endothelial cells used included both a primary cell line (HUVECs; American Tissue Culture Collection, Manassas, VA; CRL-1730) and an immortalized cell line (EA.hy926; ref. 56). HUVECs were maintained as described previously (57). All other cells were routinely cultured in DMEM supplemented with 5% FCS. Cell cultures were incubated at 37°C and 5% CO₂ in a humidified incubator.

**Recombinant Mouse sVEGFR-2**

Two recombinant mouse sVEGFR-2 proteins were used in this study, one derived from mammalian cells, designated (M)-sVEGFR-2/Fc, and the other from insect cells, designated (I)-sVEGFR-2. Both recombinant proteins encode for amino acid residues 1 to 762 of the extracellular domain of mouse VEGFR-2 (58). (M)-sVEGFR-2/Fc was obtained from R&D Systems (catalogue 443-KD), is used as the standard for the human specific anti-VEGFR-2 antibodies conjugated to horseradish peroxidase (R&D Systems).

**Collection and Concentration of Endothelial Cell CM**

All endothelial cell lines grown to ~80% confluency were incubated for 48 hours with control media incubated in parallel. After collection, CM and control media were centrifuged at 1000 rpm for 10 minutes, debris was removed, and supernatant was filtered through 0.44 μm filters (Millipore, Billerica, MA). Both control media and CM were placed in a cold speedvac (Savant, Albertville, MN) and concentrated to maximize the concentration of sVEGFR-2. Samples reduced by 85% of their original volume, for the both control media and CM, were pooled and used to test sVEGFR-2 expression by immunoprecipitation and ELISA studies.

**Immunoprecipitation**

**Step 1.**

**Plasma or Endothelial Cell CM “Clearing.”** Protein G Sepharose 4 Fast Flow beads (Pharmacia Biotech, Upssala, Sweden) were washed three times in lysis buffer [20 mmol/L Tris (pH 7.5), 137 mmol/L NaCl, 100 mmol/L NaF, 10% glycerol, 1% NP40, and 1 mmol/L Na₂VO₄] to remove ethanol storage solution. Pooled SCID plasma samples were then thawed and incubated at 4°C for 2 to 12 hours with the protein G beads (100 μL beads per 1 mL plasma) on a rotator. Plasma was removed from beads by centrifugation, and pre-clearing procedure was repeated twice more. Beads used for these pre-clearing steps were saved and later analyzed by Western blotting to show removal of proteins binding nonspecifically to the beads but not removal of sVEGFR-2 (data not shown). Preclearing procedure was also performed for both the mouse endothelial cell CM and control media (both reduced by 85% from their original volume).

**VEGFR-2 Antibody Preincubation.** Protein G–coated Sepharose beads (100 μL) were incubated for 24 hours with 100 μg of VEGFR-2 antibody in lysis buffer. Antibody-bound beads were then centrifuged and washed once in lysis buffer before adding the pre-cleared plasma.

**Step 2.**

**Immunoprecipitations for sVEGFR-2 From “Cleared” Plasma and CM.** Precleared plasma was quantified by Bradford assay (Bio-Rad, Hercules, CA), and 10 mg of protein were added to antibody-bound beads (see Step 1) and filled to a volume of 1 mL with lysis buffer. After 24 hours of rotating at 4°C, beads were centrifuged and supernatants were saved for later analysis by sVEGFR-2 ELISA. Beads were washed three times in cold lysis buffer and resuspended in loading buffer and a reducing agent (10% 1,4-DTT; Boehringer Mannheim, Laval, Quebec, Canada). Beads were boiled at 100°C to remove protein-antibody complexes, and samples were analyzed by Western blot. The identical protocol was performed for immunoprecipitations from endothelial cell CM (reduced by 85% original volume), with the exception of one modification—pre-cleared endothelial cell CM was added directly to the beads containing antibody, in equal volumes, and no dilution was made. Endothelial cell control media was incubated in parallel in all cases.

**Deglycosylation of sVEGFR-2**

To assess and compare glycosylation of the sVEGFR-2 protein, protein G–coated Sepharose beads containing the
sVEGFR-2-antibody complexes (see above immunoprecipitation protocol) or the recombinant sVEGFR-2 protein were boiled in glycoprotein denaturing buffer and incubated with PNGase F (New England Biolabs, Beverly, MA; catalogue P0704S) for 1 hour at 37°C as per manufacturer’s instructions prior to SDS-PAGE analysis.

Western Blotting

Proteins were resolved using 7.5% SDS-PAGE gels and blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Nepean, Ontario, Canada) by electrophoretic transfer (Hoefer Pharmacia Biotech, San Francisco, CA). Prior to immunoblotting, membranes were blocked in 10% nonfat milk in TBS-T buffer [10 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween 20] and subjected to immunoblot analysis as described previously (59).

Measurement of sVEGFR-2 Protein Level by ELISA

Human and Murine sVEGFR-2 ELISA. Levels of sVEGFR-2 were assessed using a commercially available mouse and human sVEGFR-2 sandwich ELISA assays (R&D Systems, catalogue MVR200 and DVR200) following manufacturer’s instructions. Immunoprecipitation supernatants from control media and CM were added, with no dilution, to the ELISA to maximize low signals. All other samples were diluted as per manufacturer’s instructions. Both ELISA kits are composed of a monoclonal capture antibody and horseradish peroxidase–conjugated polyclonal antibody for detection. Both human and mouse polyclonal antibodies were used for immunoblotting, and the mouse monoclonal (designated “R&D” above) was used for immunoprecipitation studies.

VEGF-Coated Plates. Plates (96 wells) were coated with 4 μg/mL recombinant mouse VEGF165 at 150 μL per well overnight at room temperature. Plates were then washed twice in wash buffer and blocked overnight with 350 μL per well of a bovine serum albumin–based blocking buffer. On the third day, plates were aspirated and dried in a vacuum oven at 25°C to 30°C. VEGF-coated plates were analyzed using the identical protocol as the mouse sVEGFR-2 ELISA using the same reagents (see above).

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References


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