

Levels of Soluble Vascular Endothelial Growth Factor (VEGF) Receptor 1 in Astrocytic Tumors and Its Relation to Malignancy, Vascularity, and VEGF-A¹

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ABSTRACT

Purpose: Vascular endothelial growth factor (VEGF)-A is a key mediator of angiogenesis in malignant gliomas. Soluble VEGF receptor 1 (sVEGFR-1) can complex VEGF-A and reduce its bioavailability. In several animal models sVEGFR-1 inhibited angiogenesis and tumor growth. We analyzed the levels of endogenous sVEGFR-1 in gliomas of different malignancy grades in relation to tumor vascularity and VEGF-A.

Experimental Design: The concentration of sVEGFR-1 was determined by ELISA in 104 gliomas and normal brain. Levels of sVEGFR-1 were compared with malignancy grade, microvessel density, and VEGF-A concentration. Effects of sVEGFR-1 on glioma extract-induced endothelial cell chemotaxis were analyzed *in vitro*.

Results: The concentration of sVEGFR-1 correlated with the malignancy grade and was 12-fold higher in glioblastomas than in diffuse astrocytomas ($P < 0.001$), with intermediate levels for anaplastic astrocytomas. VEGF-A levels were 30-fold higher ($P < 0.001$) in glioblastomas than in diffuse astrocytomas. The sVEGFR-1:VEGF-A ratio was 0.27 in glioblastomas and 0.70 in diffuse astrocytomas. Both sVEGFR-1 and VEGF-A correlated with microvessel density ($P < 0.001$) and with each other ($P < 0.001$); sVEGFR-1 and VEGF-A also correlated with each other when only glioblastomas were analyzed ($P = 0.001$). *In vitro*, recombinant sVEGFR-1 inhibited endothelial cell chemotaxis induced by tumor extracts.

Conclusions: Although absolute levels of sVEGFR-1 are increased in the more malignant gliomas, the sVEGFR-1:VEGF-A ratio is decreased 2.6-fold in glioblastomas com-

pared with diffuse astrocytomas, suggesting that the ensuing increased bioavailability of VEGF-A favors angiogenesis. The inhibition of tumor extract-induced endothelial chemotaxis by sVEGFR-1 suggests that sVEGFR-1 could be useful as an angiogenesis inhibitor in the specific context of human gliomas.

INTRODUCTION

Glioblastomas are among the most densely vascularized human tumors (1). VEGF-A³ is considered to be of crucial importance to angiogenesis in these tumors, and VEGF-A mRNA is strongly expressed by the tumor cells in glioblastomas, preferably around necrotic areas (2). Up-regulation of VEGF-A in malignant gliomas compared with low-grade ones was confirmed at the protein level, and concentrations of VEGF-A were found to correlate with the malignancy grade and MVD in gliomas (3). Correspondingly, increased expression of the endothelial VEGF-A receptors, VEGFR-1 (*Flt-1*) and VEGFR-2 (*Flk-1/KDR*), was demonstrated in glioblastomas compared with normal brain (2).

The effects of VEGF-A on endothelial cell proliferation and vascular permeability are mostly mediated by VEGFR-2 (4). We and others have recently shown (5, 6) that systemic treatment with monoclonal antibodies against VEGFR-2 or VEGF-A could inhibit glioblastoma growth and vascularization in orthotopic animal models. In contrast, the function of VEGFR-1 is less clear; it not only occurs as a transmembrane form but also occurs as a secreted soluble form (sVEGFR-1), which is generated by alternative splicing. sVEGFR-1 was first cloned from endothelial cells (7) and later purified from the endothelial culture medium (8). It consists of the extracellular immunoglobulin-like domains 1–6 of VEGFR-1 with a unique COOH-terminal extension of 31 amino acids derived from an intronic sequence of the VEGFR-1 gene.

The function of sVEGFR-1 is supposed to be mainly inhibitory, complexing VEGF and thus acting as a regulator of VEGF-A bioavailability (9, 10). VEGFR-1 has a more than 10-fold higher affinity to VEGF-A, even in soluble form, but has about a 10-fold lower tyrosine kinase activity than VEGFR-2 (4). In addition to sequestering the ligand, sVEGFR-1 can form heterodimers with transmembrane VEGFR-2, prevent its autophosphorylation, and thus abolish signaling in a dominant-negative fashion (8).

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³ The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; sVEGFR-1, soluble VEGFR-1; vWF, von Willebrand factor; EGFR, epidermal growth factor receptor; MVD, microvessel density; HUVEC, human umbilical vein endothelial cell; S/V, sVEGFR-1:VEGF-A.

In knockout mice, the *VEGFR-1*-null mutation was found to result in early embryonic death, and the animals displayed disorganized blood vessels with overgrowth of endothelial cells (11), suggesting that the absence of VEGFR-1 leads to overstimulation of endothelial cells by VEGF-A. In contrast, knockout mice that lacked only the kinase domain of VEGFR-1 developed normal vessels and survived (12), which provides evidence for an inhibitory function of its extracellular ligand-binding domain. Recent findings suggest that from embryonic day 9.5 onward, most embryonic VEGFR-1 is present as the soluble form and exerts mostly inhibitory function (13). In contrast, during pathological angiogenesis in the adult, increased membrane location of VEGFR-1 occurs concomitant with up-regulation of placenta growth factor, which also acts as a ligand for VEGFR-1 and can amplify angiogenic effects induced by VEGF-A (13).

Most available data on sVEGFR-1 in humans were obtained in the gynecological context, where sVEGFR-1 in sera and amniotic fluids was identified as a pregnancy-associated factor (14). Little is known about natural sVEGFR-1 in human cancer, and nothing is known about sVEGFR-1 in human gliomas. We therefore analyzed tissue extracts of 104 specimens, including 99 human gliomas and 5 samples of normal human brain, for their content of sVEGFR-1 protein. In addition, we addressed the functional relevance of sVEGFR-1 in the complex context of tumor extracts *in vitro*.

MATERIALS AND METHODS

Extraction of Tumor Tissue Samples. All glioma specimens were obtained from patients treated at the Department of Neurosurgery, University Hospital Hamburg-Eppendorf. Tumor tissue that was macroscopically free of adjacent nontumorous tissue was stored at -80°C , and protein extracts were prepared from samples of approximately 1 cm^3 . Specimens were homogenized in extraction buffer [3 ml/g tissue; 20 mM Tris (pH 7.5), 0.5 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 10 $\mu\text{g/ml}$ leupeptin], sonicated, and clarified by microfuging. The precipitates were re-extracted in the same buffer (3 ml/g tissue) with high salt (1 M NaCl). High salt buffer was used to extract soluble factors bound to heparan sulfate proteoglycans in the matrix and on cells. The two clarified supernatants were pooled and dialyzed against Tris-buffered saline [20 mM Tris (pH 7.5) and 150 mM NaCl]. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). All tumors were classified according to the current neuropathological WHO grading system (15). As controls, we used specimens of normal brain that were available from autopsies performed in the Institute of Legal Medicine, University Hospital Hamburg-Eppendorf.

Determination of sVEGFR-1 and VEGF-A Protein Levels. A specific double-antibody ELISA (Bender Med Systems, Vienna, Austria) was used to determine the concentration of sVEGFR-1 in the tumor tissue extracts. The standard curve was linear from 0.15–10 ng/ml sVEGFR-1. A highly sensitive commercial ELISA (R&D Systems, Minneapolis, MN) was used to measure concentrations of VEGF-A in the same tumor extracts. The standard curve was linear from 0.015 to 2 ng/ml VEGF-A.

All values were assessed at least in quadruplicate, using a minimum of four different dilutions.

Immunohistochemistry for vWF, p53, and EGFR.

Four- μm -thick sections were cut from formalin-fixed paraffin-embedded tissue blocks and stained according to standard protocols using the immunoperoxidase technique. Antigen retrieval was achieved by either protease digestion (vWF) or microwave treatment (p53). The primary antibodies used were polyclonal antiserum against vWF (DAKO, Copenhagen, Denmark), anti-p53 (clone DO-7; DAKO), and anti-EGFR (clone H11; DAKO). Intratumoral MVD was determined on vWF-stained sections as described previously (3); briefly, for each tumor section, the area with the highest capillary density was selected, and within this area, the number of microvessels was determined in five high-power fields (0.19 mm^2 each).

Isolation and Culture of HUVECs. HUVEC isolation from freshly obtained human umbilical cords was performed as described previously (16). Cells were grown on 1% collagen I-coated plasticware in M199 medium supplemented with 20% FCS, 90 $\mu\text{g/ml}$ endothelial cell growth supplement, 40 units/ml heparin, 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 0.25 $\mu\text{g/ml}$ fungizone (all from Gibco, Paisley, United Kingdom). HUVECs were used in passages 7 and 8.

Chemotaxis Assay. Chemotaxis of HUVECs in response to VEGF-A (isoform 165; Peprotech, Rocky Hill, NY) or tumor extracts in the absence or presence of sVEGFR-1 (sVEGFR-1_{D1-6}, monomeric form; Biomol, Hamburg, Germany) was analyzed using a modified Boyden chamber assay as described previously (17). VEGF-A or extracts that were normalized to a protein concentration of 500 $\mu\text{g/ml}$ were added to the lower wells of a 96-well modified Boyden chamber (Neuroprobe, Cabin John, MD) using serum-free M199 medium with 0.1% BSA as assay medium. Wells were covered with an 8- μm pore size Nucleopore filter that had been coated with Vitrogen 100 (Cohesion Technologies, Inc., Palo Alto, CA). Endothelial cells were suspended at 1.5×10^4 cells in 50 μl of assay medium and seeded into the upper wells. After incubation for 5 h at 37°C , nonmigrated cells were scraped off the upper side of the filter, and filters were stained with Diff Quick (Dade, Unterschleissheim, Germany). Nuclei of migrated cells were counted in 10 high power fields using a $\times 40$ objective with a calibrated ocular grid. Values were assessed in triplicate.

Methods of Data Analysis. Differences between sVEGFR-1 or VEGF-A concentrations in gliomas of different malignancy grades and in tumors with either EGFR or p53 immunoreactivity were determined using the Mann-Whitney rank-sum test. Correlations between sVEGFR-1, VEGF-A, malignancy grade, MVD, patient age, and survival were analyzed using the Spearman rank order correlation. Effects of sVEGFR-1 on VEGF-A-induced or tumor extract-induced endothelial cell chemotaxis were analyzed using the unpaired *t* test.

RESULTS

Concentration of sVEGFR-1 in Human Gliomas. The concentrations of sVEGFR-1 and VEGF-A were determined by ELISA in tissue extracts prepared from 99 gliomas of different malignancy grades and 5 specimens of normal white matter.

Table 1 sVEGFR-1, VEGF-A, MVD, and patient age in gliomas and normal brain^a

Tissue type (N) ^b	sVEGFR-1 (ng/mg)	VEGF-A (ng/mg)	MVD (0.95 mm ²)	Patient age (yrs)
Glioblastoma (46)	3.26 ± 4.00 ^c	11.90 ± 20.74	162.5 ± 64.6	53.4 ± 13.2
Anaplastic astrocytoma (14)	0.89 ± 1.66	0.67 ± 2.04	102.2 ± 61.0	49.1 ± 14.1
Anaplastic oligoastrocytoma (6)	0.67 ± 1.26	0.00 ± 0.00	91.0 ± 22.4	45.3 ± 6.1
Diffuse astrocytoma (29)	0.28 ± 0.97	0.40 ± 1.79	72.8 ± 52.8	35.6 ± 12.0
Oligoastrocytoma (4)	0.00 ± 0.00	0.00 ± 0.00	89.5 ± 8.5	49.5 ± 17.8
White matter (5)	0.00 ± 0.00	0.06 ± 0.10	71.3 ± 9.1	71.1 ± 8.4

^a Concentrations of sVEGFR-1 and VEGF-A were determined by ELISA and normalized to protein content; to determine the MVD, microvessel counts within the area of highest capillary density were performed in 5 high-power fields (0.19 mm² each).

^b N, number of samples analyzed.

^c Values are means ± SD.

sVEGFR-1 was detectable in 40 of 46 glioblastomas (87%; WHO grade IV), 6 of 14 anaplastic astrocytomas (43%; WHO grade III), 3 of 6 anaplastic oligoastrocytomas (50%; WHO grade III), 9 of 29 diffuse astrocytomas (31%; WHO grade II), and none of the oligoastrocytomas (WHO grade II) that were analyzed.

Intratumoral concentrations of sVEGFR-1 correlated with the tumor malignancy grade ($r = 0.625$; $P < 0.001$) and with patient age ($r = 0.350$; $P < 0.001$). Comparisons between tumors of astrocytic differentiation revealed 12-fold higher concentrations of sVEGFR-1 in glioblastomas than in diffuse astrocytomas ($P < 0.001$), with intermediate levels for the anaplastic astrocytomas (Table 1). Concentrations of sVEGFR-1 ranged from 0 to 16.30 ng/mg protein in glioblastomas, from 0 to 5.82 ng/mg protein in anaplastic astrocytomas, and from 0 to 4.88 ng/mg protein in diffuse astrocytomas. Within the subgroup of glioblastomas, the sVEGFR-1 concentration correlated with the length of patient survival ($r = 0.446$; $P < 0.05$; mean survival, 72 weeks; $n = 26$ newly diagnosed glioblastoma patients for whom survival data could be analyzed). The sVEGFR-1 concentration in recurrent glioblastomas did not differ statistically from that in newly diagnosed ones.

To investigate whether sVEGFR-1 levels were associated with tumor vascularity, microvessel counts were performed on paraffin sections of routinely embedded tumor material (Table 1). Levels of sVEGFR-1 correlated with the MVD ($r = 0.560$; $P < 0.001$). In addition, paraffin sections from 88 gliomas were stained for p53 and EGFR. Within the subgroup of glioblastomas, tumors that were immunoreactive for EGFR tended to contain higher levels of sVEGFR-1 than EGFR-negative ones (4.571 versus 2.242 ng/mg, respectively; $P < 0.05$).

Concentration of VEGF-A in Human Gliomas and Relation to sVEGFR-1. VEGF-A was detectable in 42 of 46 glioblastomas (91%), 6 of 14 anaplastic astrocytomas (43%), 10 of 29 diffuse astrocytomas (34%), and none of the anaplastic or nonanaplastic oligoastrocytomas that were investigated. VEGF-A concentrations correlated with the tumor malignancy grade ($r = 0.665$; $P < 0.001$), patient age ($r = 0.341$; $P < 0.001$), and MVD ($r = 0.431$; $P < 0.001$). Among the astrocytic tumors, glioblastomas contained 30-fold higher concentrations of VEGF-A than diffuse astrocytomas ($P < 0.001$), with intermediate levels found in anaplastic astrocytomas (Table 1). VEGF-A concentrations ranged from 0 to 103.46 ng/mg protein in glioblastomas, from 0 to 7.59 ng/mg protein in anaplastic

astrocytomas, and from 0 to 9.66 ng/mg protein in diffuse astrocytomas.

A significant correlation was found between the levels of sVEGFR-1 and VEGF-A ($r = 0.712$; $P < 0.001$; Fig. 1A), and interestingly, this association was still significant when only the subgroup of glioblastomas ($n = 46$) was evaluated ($r = 0.466$; $P = 0.001$; Fig. 1B). The ratio between sVEGFR-1 and VEGF-A (S/V ratio) was 2.6-fold higher in diffuse astrocytomas (S/V ratio, 0.70) than in glioblastomas (S/V ratio, 0.27). We found no association between the S/V ratio and MVD when all tumor grades were analyzed together or when only the subgroup of glioblastomas was analyzed. Nor did the S/V ratio correlate with the survival of glioblastoma patients. Within the subgroup of glioblastomas, the VEGF-A concentration was slightly (but nonsignificantly) lower in EGFR-positive than in EGFR-negative tumors, so that given the above-mentioned increased sVEGFR-1 concentration in EGFR immunoreactive glioblastomas, consequently the S/V ratio was approximately 3-fold higher in EGFR-positive tumors (S/V ratio, 0.47) than in EGFR-negative tumors (S/V ratio, 0.15).

Effects of sVEGFR-1 on Endothelial Cell Chemotaxis Induced by Tumor Extracts. The response of microvascular endothelial cells to chemotactic stimuli is considered a good *in vitro* correlate of angiogenic activity *in vivo* (18, 19). We used a modified Boyden chamber assay to evaluate the effects of tumor extracts and VEGF-A in combination with sVEGFR-1 on the directional motility of endothelial cells. To test the efficacy of recombinant sVEGFR-1, we first added different doses of sVEGFR-1 (0.03–3 μg/ml) to increasing concentrations of VEGF-A. Maximum stimulation of HUVEC migration was observed at 10 ng/ml VEGF-A (Fig. 2). At 0.03 μg/ml, sVEGFR-1 had no inhibitory effect, whereas at 0.3 μg/ml, almost complete inhibition of VEGF-A-induced HUVEC chemotaxis was observed, and inhibition was complete at 3 μg/ml (Fig. 2).

Tumor extracts to be tested in the modified Boyden Chamber assay were selected for the presence of high VEGF-A levels but relatively low sVEGFR-1 levels (Table 2). All extracts were derived from glioblastomas. Without exception, the extracts induced a significant stimulation of directional HUVEC motility ($P < 0.001$ for all extracts; Fig. 3), with a range from 2.4-fold stimulation (extract 12) to 4.1-fold stimulation (extract 67). In all cases, the extract-induced endothelial chemotaxis could be inhibited by sVEGFR-1 in a dose-dependent fashion. To achieve

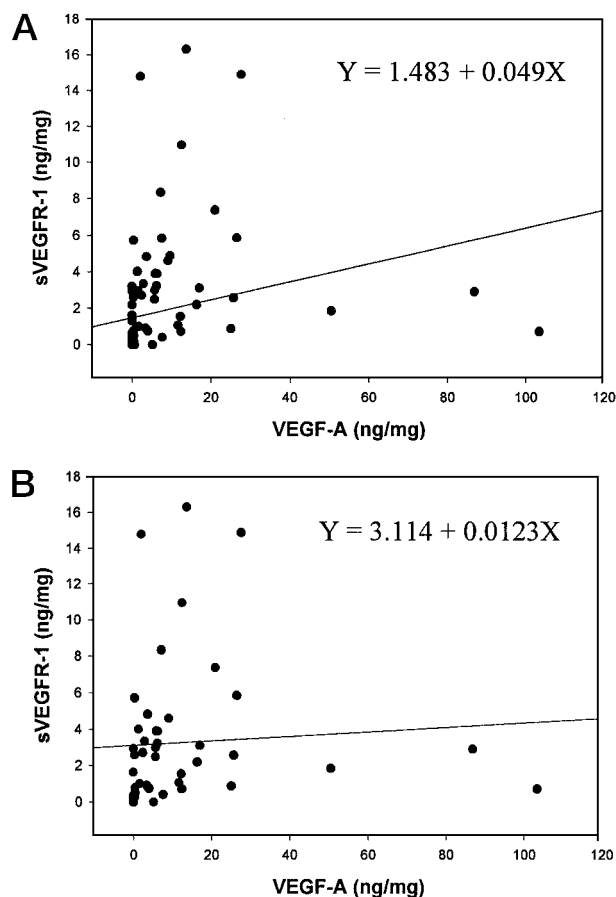


Fig. 1 Correlations between sVEGFR-1 and VEGF-A concentrations in (A) gliomas of WHO grades II to IV ($n = 99$) and (B) glioblastomas only ($n = 46$). (A) There was a significant association between sVEGFR-1 levels and VEGF-A levels ($r = 0.712$; $P < 0.001$), and this association was also significant when (B) only the subgroup of glioblastomas was analyzed ($r = 0.466$; $P = 0.001$).

significant inhibition, molar concentrations of sVEGFR-1 were required that exceeded those of VEGF-A between approximately 2-fold (extract 52) and approximately 200-fold (extract 28; Fig. 3; Table 2). Inhibition by sVEGFR-1 was significant at 3 $\mu\text{g}/\text{ml}$ for all extracts and at 0.3 $\mu\text{g}/\text{ml}$ for approximately half of the extracts (Fig. 3). These results are congruous with the observations for sVEGFR-1-mediated inhibition of VEGF-A effects (Fig. 2), where at VEGF-A concentrations comparable with those present in the tumor extracts (between 1 and 100 ng/ml), sVEGFR-1 at 0.03 $\mu\text{g}/\text{ml}$ was also ineffective but showed strong inhibition of VEGF-A-induced HUVEC migration at 0.3 $\mu\text{g}/\text{ml}$ and even more so at 3 $\mu\text{g}/\text{ml}$.

DISCUSSION

We detected strongly increased concentrations of sVEGFR-1 protein in glioblastomas compared with low-grade gliomas and normal brain. Both *VEGFR-1* expression and *sVEGFR-1* expression are known to be up-regulated by hypoxia in endothelial cells and other cell types, and the *VEGFR-1* gene promoter contains hypoxia response elements (20, 21). There-

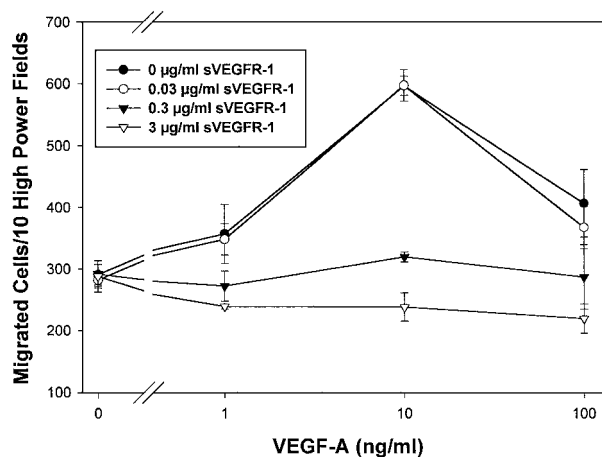


Fig. 2 Inhibition of VEGF-A-induced HUVEC chemotaxis by sVEGFR-1. VEGF-A (0–100 ng/ml) was added to the lower wells of a modified Boyden chamber in the absence or presence of sVEGFR-1 (0–3 $\mu\text{g}/\text{ml}$). HUVECs were seeded into the upper wells. Values are the means \pm SD of triplicate determinations.

Table 2 Glioblastoma extracts selected for chemotaxis experiments as shown in Fig. 3^a

Extract no.	VEGF-A (ng/ml)	sVEGFR-1 (ng/ml)	Inhibitory molar ratio
7	3.82	0.20	32
12	2.61	0.00	47
16	5.83	0.53	21
26	25.23	0.92	5
28	6.15	0.77	200
52	6.18	0.36	2
67	12.59	0.44	98

^a Extracts were assayed at concentrations of 500 $\mu\text{g}/\text{ml}$ protein. Concentrations of VEGF-A and sVEGFR-1 after dilution to 500 $\mu\text{g}/\text{ml}$ protein are listed. The approximate molar ratio between exogenous sVEGFR-1 and endogenous VEGF-A at which significant inhibition of extract-induced HUVEC migration was observed (see Fig. 3) is shown.

fore, in the hypoxic environment endogenous to malignant gliomas especially in the vicinity of necroses, increased levels of sVEGFR-1 could have been expected. In addition, endothelial *sVEGFR-1* is also known to be up-regulated by its ligand, VEGF-A (22), and the high levels of VEGF-A found in malignant gliomas compared with low-grade ones are likely to further contribute to up-regulation of sVEGFR-1.

Functionally, the increase of sVEGFR-1 levels in malignant gliomas, which, in contrast to the less malignant forms, are usually highly vascularized, might seem unexpected. In recent years, a model has been proposed in which the angiogenic switch from a normally quiescent vasculature to neovascularization is controlled by a balance between angiogenesis inducers and inhibitors (23). VEGF-A has been suggested to be a protagonist, controlling this switch at the transition from low-grade glioma to highly vascularized glioma, which is accompanied by the onset of angiogenesis (24). Up-regulation of its main known natural antagonist, sVEGFR-1, in the more malignant gliomas would thus seem contradictory because it would shift the bal-

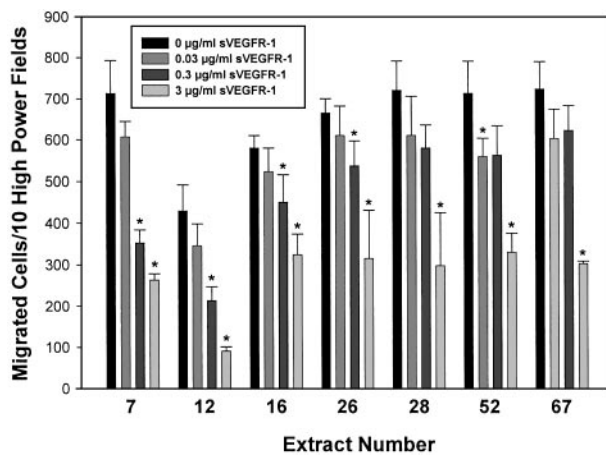


Fig. 3 Inhibition of extract-induced HUVEC chemotaxis by sVEGFR-1. Extracts were assayed at a fixed protein concentration of 500 µg/ml. Final assay concentrations of sVEGFR-1 and VEGF-A are listed in Table 2. Values are the means \pm SD of triplicate determinations. Baseline migration in the absence of extract or sVEGFR-1 was 176 ± 31 cells/10 high-power fields.

ance toward angiogenesis inhibition. However, when comparing the levels of sVEGFR-1 and VEGF-A, the S/V ratio was 2.6-fold lower in glioblastomas than in diffuse astrocytomas because the VEGF-A concentration was increased 30-fold, whereas sVEGFR-1 was increased only 12-fold. Therefore, the net balance between these two molecules would still be tilted toward angiogenesis. Of course, such a reductionistic model does not truly reflect the complex biochemical homeostasis of gliomas *in vivo*, where other factors with angiogenic activity, such as scatter factor/hepatocyte growth factor or fibroblast growth factor 2 (3), are likely to also contribute to tumor neovascularization.

The only other study that has been published thus far on sVEGFR-1 levels in tumor tissue extracts was performed on breast cancer samples (25). In contrast to our observations on gliomas, sVEGFR-1 was not found to correlate with any clinicopathological findings or with prognosis, whereas VEGF-A did. However, sVEGFR-1 levels in breast cancer correlated with VEGF-A levels, a finding similar to our observation on gliomas, where the same association was found not only when specimens of all different WHO grades were included in the analysis, but also when only the subgroup of glioblastomas was analyzed. In the study on breast cancer, the S/V ratio had turned out to be prognostically important; tumors in which concentrations of sVEGFR-1 exceeded VEGF-A concentrations by >10-fold exhibited a markedly more favorable prognosis. In the present study, we were able to compare the survival time of 26 newly diagnosed glioblastoma patients. Interestingly, we found that the sVEGFR-1 concentration correlated with the length of patient survival, although the association between the S/V ratio and survival did not reach significance. This tendency for a protective effect of the sVEGFR-1 concentration is in line with the positive association between the S/V ratio and survival in breast cancer. Survival data for patients with grade II or grade III gliomas could not be analyzed in our study because the vast

majority of these patients are still alive. However, patients with diffuse astrocytoma are known to have an average life expectancy of several years after diagnosis, whereas for glioblastoma, it is usually less than 1 year; therefore, our finding of a lower S/V ratio in glioblastomas than in the diffuse astrocytomas is also in line with the prognostic significance of the S/V ratio detected in breast cancer. Absolute levels of both VEGF-A and sVEGFR-1 were higher in our study on gliomas than in the study by Toi *et al.* (25) on breast cancer specimens. This difference may be due to the different types of tumors investigated and may also be partly explained by the different extraction protocols used, in our case employing high salt to release soluble factors also from the extracellular matrix.

In another recent study, plasma levels of sVEGFR-1 were measured in patients with renal cancer before and 1 month after antiangiogenic therapy with razoxane (26). Higher pretreatment levels of sVEGFR-1 were associated with a lesser chance of stable disease and poorer survival. Up-regulation of VEGF-A (which was not measured in that study) was speculated to account in part for the increased sVEGFR-1 levels in the prognostically worse cases with renal cancer. In addition, it was speculated that sVEGFR-1 may not only be expressed by the endothelial tumor compartment, but also by macrophages, which have been associated with a worse prognosis in renal cancer, and also by the tumor stroma and/or the epithelial component. Several studies indicate that sVEGFR-1 is not only secreted by endothelial cells but also by tumor cells, including malignant hematopoietic cells (27) and melanoma cells (21), at least *in vitro*. Likewise, also in gliomas, sVEGFR-1 could potentially be derived not only from vessels alone but also from the tumor cells. Interestingly, within the group of glioblastomas, we found an association between EGFR immunoreactivity and increased sVEGFR-1 concentration, and the S/V ratio was approximately 3-fold higher in EGFR-positive tumors than in EGFR-negative tumors. Although these differences were not reflected in different MVDs, this finding suggests that different molecular tumor subtypes may be associated with different pathways of angiogenesis regulation. To clarify these mechanisms, it would be important to determine the cellular origin of sVEGFR-1 in gliomas *in vivo* and to study the regulation of sVEGFR-1 in response to EGFR signaling *in vitro*.

sVEGFR-1 is considered to act mostly as a sink for VEGF-A and thus inhibit angiogenesis. Up-regulation of sVEGFR-1 may therefore be interpreted as a negative feedback mechanism. Studies on knockout mice, however, suggest that sVEGFR-1 does not merely inhibit angiogenesis but that the counterbalancing of VEGF-A is also important to fine-tune the angiogenesis cascade, which otherwise results in the formation of disorganized vessels with endothelial overgrowth (11). In addition, complexing by sVEGFR-1 may stabilize VEGF, protect it from proteases, and sequester it in the microenvironment of endothelial cells via binding of sVEGFR-1 to heparan sulfate proteoglycans, from where it can be released when needed. The endothelial overgrowth in *VEGFR-1* knockout mice resembles the microvascular proliferations characteristic of glioblastomas, which were termed "endothelial proliferations" before the latest revision of the WHO classification (15). It is tempting to speculate that these microvascular proliferations in glioblastomas might arise when sVEGFR-1 is present in insufficient local

concentrations, so that overstimulation of endothelial cells by VEGF-A occurs. Detailed histomorphological *sVEGFR-1* expression analyses are required to address this question.

In several animal models, adenovirus-mediated overexpression of sVEGFR-1 in tumor cells was shown to inhibit growth of melanoma, lung cancer, fibrosarcoma, or glioblastoma *in vivo* and to prolong survival (28–30). These effects were ascribed to an inhibition of tumor angiogenesis. We performed *in vitro* studies to analyze the biological effect of recombinant human sVEGFR-1 on endothelial migration in the complex biological context of human glioblastoma extracts. A molar concentration of sVEGFR-1 that exceeded that of VEGF-A by approximately 2–200-fold was required to obtain significant effects on endothelial chemotaxis. With the exception of one extract, the addition of even 30 ng/ml to the extracts was not enough to exert a significant inhibitory effect with extracts containing between 2.6 and 25.2 ng/ml VEGF-A. A comparable result was obtained when human recombinant VEGF-A was used in concentrations ranging from 1 to 100 ng/ml. These results are similar to those obtained by Roeckl *et al.* (31), who observed strong inhibition of VEGF-A-induced endothelial DNA synthesis at sVEGFR-1 concentrations above 100 ng/ml, but only weak effects at lower concentrations. In addition to VEGF-A, the VEGFR-1 ligands VEGF-B and placenta growth factor are also known to be expressed in gliomas and may have contributed to the chemotactic effects of the extracts. This could explain why very different sVEGFR-1 concentrations were necessary to achieve significant inhibition of endothelial cell migration induced by different extracts.

To conclude, our findings suggest that in the complex environment endogenous to human gliomas *in vivo*, sVEGFR-1 can inhibit the biological activity of VEGF-A present in the extracts. Animal studies in which sVEGFR-1 was overexpressed suggest that inhibition of tumor angiogenesis via sVEGFR-1 could have therapeutic potential for a variety of human cancer types. The decrease of the S/V ratio that we detected in malignant gliomas compared with low-grade ones further suggests that sVEGFR-1 does counteract the effects of VEGF-A in glioma angiogenesis *in vivo*. However, the levels of sVEGFR-1 present in the extracts are obviously insufficient to prevent neovascularization in these tumors. Therefore, considerably higher levels appear to be required to effectively inhibit the angiogenic effects of VEGF-A on endothelial cells *in vivo*.

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