

## INHIBITION OF SOLID TUMOR GROWTH BY GENE TRANSFER OF VEGF RECEPTOR-1 MUTANTS

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**Vascular endothelial growth factor (VEGF) and the high-affinity VEGF receptor Flk-1/KDR (VEGFR-2) are key regulators of tumor angiogenesis. Strategies to block VEGF/VEGFR-2 signaling were successfully used to inhibit experimental tumor growth and indicated that VEGFR-2 is the main signaling VEGF receptor in proliferating tumor endothelium. Here, we investigated the role of the VEGF receptor-1 (VEGFR-1/Flt-1) in the vascularization of 2 different experimental tumors *in vivo*. VEGFR-1 mutants were generated that lack the intracellular tyrosine kinase domain. Retrovirus-mediated gene transfer of the VEGFR-1 mutants led to a strong reduction of tumor growth and angiogenesis in xenografted C6 glioma and in syngeneic BFS-1 fibrosarcoma. Histological analysis of the inhibited fibrosarcoma revealed reduced vascular density, decreased tumor cell proliferation as well as increased tumor cell apoptosis and the formation of necrosis. The retroviral gene transfer of the full length VEGFR-1 also caused a significant reduction of tumor growth in both models. The inhibitory effects of the VEGFR-1 mutants and the full length VEGFR-1 in BFS-1 fibrosarcoma were mediated through host tumor endothelial cells because the BFS-1 fibrosarcoma cells were not infected by the retrovirus. The formation of heterodimers between VEGFR-2 and full length or truncated VEGFR-1 was observed *in vitro* and might contribute to the growth inhibitory effect by modulating distinct signal transduction pathways. The results of our study underline the central role of the VEGF/VEGFR-1 signaling system in tumor angiogenesis and demonstrate that VEGFR-1 can serve as a target for anti-angiogenic gene therapy.**

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The growth of solid tumors beyond a minimal size requires their vascularization in order to supply the tumor with oxygen and nutrients.<sup>1,2</sup> The rapid proliferation of tumor cells leads to the formation of hypoxic areas in the tumor tissue. Hypoxia stimulates tumor angiogenesis by upregulating angiogenic factors such as vascular endothelial growth factor (VEGF).<sup>3–6</sup> VEGF and its high-affinity tyrosine kinase receptor VEGFR-2 (Flk-1/KDR) are key regulators of both physiological and pathological angiogenesis.<sup>7</sup> The VEGF/VEGFR-2 signal transduction system stimulates endothelial cell proliferation, migration, survival and blood vessel permeability.<sup>4</sup> VEGF was first implicated as a potential tumor angiogenesis factor in human glioma<sup>3,8</sup> and subsequently in various other human or experimental tumors.<sup>9</sup> VEGF is secreted from tumor cells as a homodimeric protein, whereas VEGFR-2 is up-regulated in the tumor endothelium. This expression pattern suggested that VEGF/VEGFR signaling stimulates the proliferation and survival of the tumor vasculature in a paracrine manner.<sup>4,7,10</sup> The pivotal role of VEGFR-2 signaling during tumor angiogenesis was demonstrated by gene transfer experiments in which signaling-defective dominant-negative VEGFR-2 mutants strongly inhibited the neovascularization and consequently the growth of experimental glioma and other tumors.<sup>11,12</sup> The observation that the growth of many different tumors *in vivo* is dependent on VEGF/VEGFR-2 signaling stimulated the development of promising anti-angiogenic molecules for tumor therapy, like VEGF

neutralizing antibodies,<sup>13,14</sup> or low-molecular weight inhibitors of VEGFR-2 tyrosine kinase activity.<sup>15,16</sup>

VEGFR-1 (Flt-1) is also upregulated by tumor endothelial cells.<sup>8,17</sup> It binds VEGF with a higher affinity than VEGFR-2 but is phosphorylated only to a minor extent.<sup>18</sup> Beside the transmembrane receptor, a soluble form containing only the first 6 out of 7 extracellular Ig-domains is expressed by alternative splicing.<sup>19</sup> This soluble VEGFR-1 has been shown to be effective in the inhibition of solid tumor growth *in vivo* by sequestration of VEGF.<sup>20–24</sup> The function of VEGFR-1 as signaling receptor during physiological angiogenesis was questioned by gene targeting experiments in which the intracellular tyrosine kinase domain of VEGFR-1 was deleted. Whereas a VEGFR-1 null mutation resulted in defective vascular development and early embryonic death due to increased angioblast commitment,<sup>25,26</sup> mice lacking the intracellular VEGFR-1 domain developed normally.<sup>27</sup> In the latter case, an impairment of monocyte function was observed, which is in line with the involvement of VEGFR-1 signaling in monocyte migration.<sup>28</sup> These results suggested that VEGFR-1 negatively regulates embryonic angiogenesis primarily by binding of ligand rather than by transducing signals in endothelial cells. However, signaling through VEGFR-1 has been observed in several experimental systems *in vitro*, in which activation of VEGFR-1 led to a stimulation of PI3K, PIC $\gamma$  and DNA synthesis.<sup>18,29–34</sup> Moreover, recent reports suggested a stimulatory role for VEGFR-1 in pathological angiogenesis *in vivo*.<sup>35,36</sup> Tumor cells overexpressing placenta growth factor-2 (PlGF-2), a VEGF-related, VEGFR-1 specific ligand, grew faster and exhibited a higher degree of neovascularization when grown in wild-type mice compared to VEGFR-1 tyrosine kinase domain-deficient mice.

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Furthermore, neovascularization of human epidemoid A431 tumors and C6 gliomas in nude mice was blocked by a VEGFR-1 specific antibody.<sup>36</sup> Recent studies suggested an essential function of VEGFR-1 in the recruitment of bone-marrow derived stem cells.<sup>37</sup> It could be shown that bone-marrow-derived endothelial progenitor cells were integrated in newly formed tumor blood vessels.<sup>38,39</sup> In contrast, recent *in vitro* studies using chimeric receptors in human umbilical vein endothelial cells or porcine aortic endothelial cells indicated an inhibitory effect of VEGFR-1 signaling on VEGFR-2 mediated endothelial cell proliferation.<sup>40–42</sup> Furthermore, VEGFR-1 negatively modulated cell division in the vascular lineage both *in vitro* and *in vivo*,<sup>43</sup> suggesting that VEGFR-1 could potentially function as a negative regulator of pathological vascularization. Thus, the function of VEGFR-1 signaling during tumor neovascularization remained unclear.

In our study, we analyzed the role of VEGFR-1 during tumor angiogenesis by a gene therapy approach in xenografted C6 glioma and syngeneic BFS-1 fibrosarcoma, respectively. C6 glioma cells form large, highly vascularized tumors that exhibit an expression pattern of VEGF and the VEGF receptors similar to human glioma.<sup>17,44</sup> Previous studies have shown that the growth of these tumors is dependent on VEGFR-2 signaling.<sup>11</sup> BFS-1 fibrosarcoma are fast growing tumors, which develop a dense vascular network at early tumor stages. These newly formed blood vessels exhibit a strong induction of VEGFR-2 expression.<sup>45</sup> We observed that the retrovirus-mediated gene transfer of a truncated VEGFR-1 mutant, which lacks the intracellular kinase domain, significantly inhibited tumor growth in both experimental models at least as efficiently as dominant-negative VEGFR-2 mutants. Histological analysis of the growth inhibited BFS-1 tumors exhibited a strong decrease in tumor neovascularization and tumor cell proliferation, an increase in tumor cell apoptosis and the formation of massive necrosis. Interestingly, the retroviral gene transfer of the full length VEGFR-1 also led to a significant reduction in tumor growth. The inhibitory effects were mediated by tumor endothelial cells because the BFS-1 fibrosarcoma cells were not infected by the retrovirus. We demonstrate that the truncated mutVEGFR-1 and the full length VEGFR-1 are capable of forming heterodimers with VEGFR-2 *in vitro*, which might contribute to the growth inhibitory effects by modifying distinct signal transduction pathways. The results of our study show that the VEGF/VEGFR-1 signaling system plays an important role in tumor angiogenesis and demonstrate that VEGFR-1 can be used as a target for gene therapy approaches.

## MATERIAL AND METHODS

### Cell culture

Laboratory reagents were purchased from Sigma Chemical Co. (Deisenhofen, Germany) unless otherwise stated. Media and supplements were obtained from Life Technologies, Inc. (Eggenstein, Germany). C6 glioma cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagles medium (DMEM; Invitrogen, Germany) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Germany) and 1% penicillin/streptomycin (Invitrogen, Germany). BFS-1 fibrosarcoma cells<sup>46</sup> were grown in RPMI (Invitrogen, Germany) supplemented with 10% FCS, 1% penicillin/streptomycin, 1% pyruvate and 2% glutamine. Retrovirus producer cells GP+E86 were grown in DMEM<sup>+</sup> containing 10% calf serum, and 1% penicillin/streptomycin. COS-1 cells were grown in DMEM<sup>+</sup> containing 10% FCS (PAA Laboratories, Linz, Austria). NIH3T3 cells were grown in DMEM<sup>+</sup> containing 10% calf serum (Sumit Biotechnology).

### Cloning of VEGFR-1 mutants

cDNA clones encoding truncated mouse VEGFR-1 mutants (mutVEGFR-1) were generated by PCR. A full length mouse VEGFR-1 clone<sup>47</sup> was used as a template for the amplification. The primers used were mft-ATG, 5'-GGA ATT CTT GCT CAC CAT GGT CAG CTG, and mft-Stop, 5'-GGA ATT CTT ATG GGT

CCA TAA TGA TTG ACA G and contained internal EcoRI restriction enzyme cleavage sites to facilitate cloning. DNA was amplified in 20 cycles using Perkin-Elmer Cetus Taq Polymerase as recommended by the manufacturer. The resultant PCR product was digested with EcoRI and cloned into pLXSN vector DNA.<sup>48</sup> Retrovirus vector encoding the full length VEGFR-1 receptor was generated by inserting a 4.8 kb EcoRI-EcoRI cDNA<sup>47</sup> into pLXSN vector DNA. The nucleotide sequence of *mutVEGFR-1* was determined after subcloning into pBluescript.

### Generation of recombinant retrovirus producer cell lines

GP+E86 cells were transfected with retroviral pLXSN vectors using the method of Chen and Okayama,<sup>49</sup> and cells resistant to G418 were selected. Supernatants were used to reinfect fresh cultures of GP+E86 cells that had been treated for 16 hr with 100 ng/ml tunicamycin (Sigma Chemical Co.). Cell clones resistant to G418 were selected and assayed by RT-PCR analysis for expression of mRNA encoding VEGFR-1. Positive clones were further analyzed for specific binding of <sup>125</sup>I-VEGF as described.<sup>47</sup> Virus titer was determined by infecting NIH3T3 cells as described below and counting of the selected G418-resistant colonies. Virus titers were approx. 1–2×10<sup>6</sup> pfu/ml for GP+E86/mutVEGFR-2 and 0.5–1×10<sup>6</sup> pfu/ml for GP+E86/mutVEGFR-1 or GP+E86/full length VEGFR-1.

### Transfection of COS-1 cells

The COS-1 cells were grown to 50% confluency and calcium-phosphate transfection was performed with cytomegalovirus-based expression vectors (pRC/CMV; Invitrogen BV, Leek, The Netherlands) that contained mouse cDNAs encoding full length VEGFR-2, mutVEGFR-2 and mutVEGFR-1, respectively. Twenty-four hours after transfection, cells were harvested in 2× sample buffer and tested for expression of the respective VEGF-receptor proteins by immunoblot analysis. To assay for heterodimer formation of VEGF receptors, cotransfections of COS-1 cells were performed with expression vectors encoding full length VEGFR-2<sup>50</sup> and VEGFR-1,<sup>47</sup> full length VEGFR-2 and mutVEGFR-1, full length VEGFR-2 and mutVEGFR-2, respectively.

### Infection of NIH-3T3 fibroblasts

The NIH3T3 fibroblast were infected with retrovirus encoding full length VEGFR-1 and protein expression was controlled by immunoblot analysis. For infection, the cells were grown to 50% confluency and infected twice for 2 hr at 37°C with virus-containing conditioned medium of GP+E86 retrovirus producer cell lines<sup>51</sup> supplemented with 8 µg/ml polybrene.

### Immunoprecipitation and Immunoblotting

Two days after transfection (COS-1 cells) or infection (NIH-3T3 fibroblasts), the cells were washed in PBS and starved for 16–18 hours with DMEM<sup>+</sup> medium supplemented with 1% FCS or donor calf serum, respectively. The cells were then stimulated for 6 min at 37°C with recombinant VEGF (50 ng/ml) and washed twice in PBS. Cells were solubilized on ice for 30 min with lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1% Triton X-100) containing a mixture of phosphatase and proteinase inhibitors (1 mM vanadate, 10 µg/ml aprotinin and 1 mM PMSF) with occasional gentle agitation. The cells were scraped from the culture dishes and the lysates were centrifuged at 16,000g for 10 min at 4°C. Supernatants were either subjected to immunoprecipitation or aliquots were removed and boiled for 5 min after addition of 2× sample buffer. Immunoprecipitations were performed by adding the VEGFR-2 monoclonal antibody and the lysates to Protein G-Sepharose beads (Pharmacia, Freiburg, Germany). After incubation of 3 hr at 4°C under continuous mixing, the Sepharose-bound immunocomplexes were washed 4 times with lysis buffer (containing phosphatase and proteinase inhibitors) and then boiled in reducing sample buffer. As a control, immunoprecipitation with antibody-coupled Sepharose beads but without addition of cell lysate was also performed. The immunoprecipitates were then separated by SDS-PAGE in 7.5% gels, transferred onto 0.2 µm

pore size nitrocellulose membranes (Schleicher & Schuell, Inc., Dassel, Germany), blocked with 3% BSA in PBST (0.1% Tween-20 in PBS) and incubated with the appropriate primary antibodies against VEGFR-2<sup>52</sup> or VEGFR-1,<sup>53</sup> kindly provided by H. Weich, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany, respectively (1 hr at room temperature). Immunoreactive bands were detected by using peroxidase-conjugated secondary antibodies and the ECL Western blot detection system (Amersham Buchler GmbH).

#### Tumor experiments

After reaching confluency, tumor cells and GP+E86 cells were washed 2 times with PBS and trypsinized. The cell suspensions were collected by centrifugation (1,200g for 5 min at room temperature). The cell pellets were resuspended in PBS, and  $1.5 \times 10^6$  cells/cell line in 50  $\mu$ l were injected s.c. into adult C57/Bl6 mice. Tumors were harvested 8 and 16 days post injection for determination of tumor weight and for further histological investigation. C6 ( $10^6$ ), GP+E86 cells (as indicated in the figure legends) and NIH3T3-VEGFR-1 cells were trypsinized and resuspended in 50  $\mu$ l PBS. A mixture containing C6 cells and virus producing cells (ratio 1:1) were implanted s.c. into the hind flank of athymic nude mice (obtained from Charles River, Germany) (6 mice/group). The mice were sacrificed 21 days post-injection, the tumors were resected, weighted and snap frozen in Tissue Tek (Sakura) for histological examination.

#### In situ hybridization

The techniques used for *in situ* hybridization were as described.<sup>47,54</sup> In short, single stranded <sup>35</sup>S-labeled antisense or sense RNA probes were generated by *in vitro* transcription using SP6, T3 or T7 RNA polymerases as described by the manufacturer (Stratagene, La Jolla, CA). The mouse *VEGFR-1* probe encoding most of the extracellular domain was generated from a 2,160 bp EcoRI-XbaI fragment of mouse *VEGFR-1* cDNA.<sup>47</sup> The mouse *VEGFR-2* probe<sup>50</sup> encoded the extracellular domain and the transmembrane domain. The VEGF probe was derived from a cDNA encoding the VEGF<sub>120</sub> isoform<sup>54</sup> and detects all known VEGF isoforms. Sense RNA probes were used as a control and did not show specific hybridization. After hybridization, slides were coated with photographic emulsion (Kodak NTB-2) and exposed for 2 weeks. Slides were developed and stained with 0.02% toluidine blue.

#### Immunohistochemistry

After sacrificing the mice, tumors were harvested, embedded in Tissue Tek (Sakura, Giessen, Germany), and stored at  $-80^\circ\text{C}$ . For immunohistochemical analysis, 10  $\mu$ m frozen sections were prepared, air dried and stained. The slides were first fixed for 10 min in Aceton at  $-20^\circ\text{C}$ , washed once in PBS and incubated with goat serum for 15 min at RT to block unspecific binding. Then the section were incubated with the first antibody [monoclonal rat-anti-PECAM-1 antibody,<sup>55</sup> monoclonal rat-anti-VEGFR-2 antibody,<sup>52</sup> and anti-Ki-67 antibody (Dianova, Hamburg, Germany), respectively] and rinsed 3 times in PBS. An incubation with a biotinylated rabbit-anti-rat secondary antibody (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA) followed that was subsequently detected with an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit). The color was developed with an AEC kit (Sigma Chemical Co., Deisenhofen, Germany).

#### Modified TUNEL-assay

For detection of apoptotic cells on frozen sections, the Tumor-Tacs kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions.

#### Reverse transcription-polymerase chain reaction (RT-PCR).

RT-PCR for PIGF was carried out using primers PIGF-FW1 (5'-GTC GCT GTA GTG GCT GCT GTG GTG-3') and PIGF-REV1 (5'-CCG TGG CTG GCT TCT TTC TTT CTC-3') which amplified a 483 bp fragment (35 cycles). Control RT-PCR for

GAPDH was performed using primers GAPDH forw (5'-CAG TAT GAC TCC ACT CAC GGC-3') and GAPDH REV 5'-GAG GGG CCA TCC ACA GTC TTC-3' (30 cycles).

## RESULTS

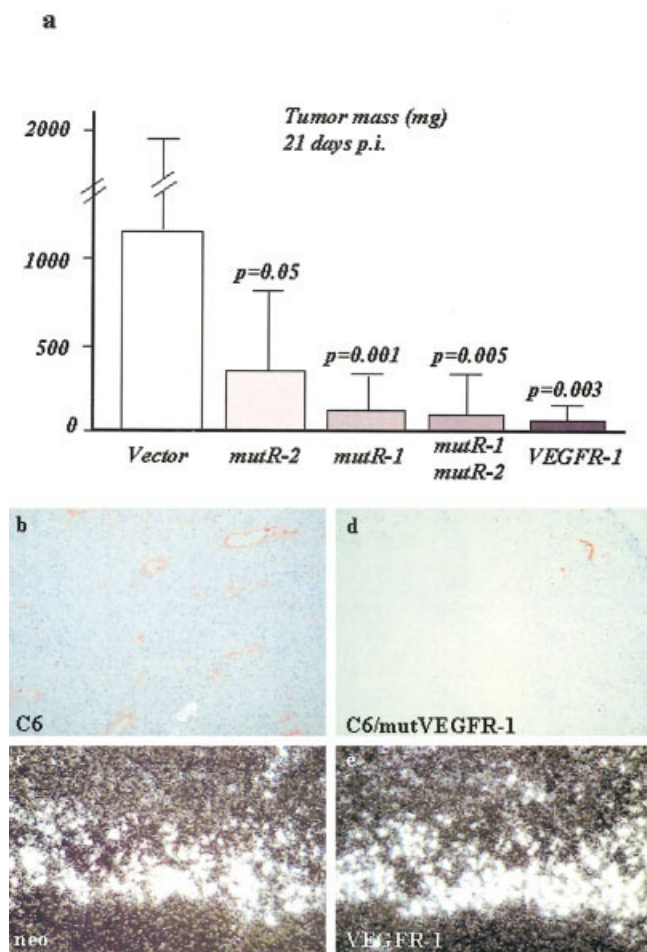
### Generation of VEGFR-1 mutants

Retroviral vectors were generated encoding either the full length mouse VEGFR-1<sup>47</sup> or a truncated VEGFR-1 mutant (mutVEGFR-1) that lacked the intracellular tyrosine kinase domain. In the cDNA for the mutVEGFR-1, a stop codon was introduced C-terminal to the putative transmembrane domain leading to termination of translation. This corresponds to the localization of the stop codon in the dominant-negative VEGFR-2 mutant (mutVEGFR-2).<sup>11,50</sup> Ecotropic retrovirus producer cell lines (GP+E86) were generated that expressed the respective receptors. Retrovirus producer cell clones were assayed for VEGFR-1 mRNA expression by RT-PCR analysis (data not shown), and positive clones were further tested for their ability to specifically bind <sup>125</sup>I-VEGF, as previously described for transfected COS cells.<sup>47</sup> GP+E86 cells did not bind VEGF specifically, whereas high affinity binding of VEGF was observed only for positive clones (data not shown). A clone exhibiting strong specific VEGF binding was selected for *in vivo* experiments.

### Growth inhibition of C6 glioma by truncated VEGFR-1 mutants

C6 glioma cells form large, highly vascularized tumors within 2–3 weeks after subcutaneous injection into nude mice. To investigate whether the retrovirus-mediated gene transfer of the mutVEGFR-1 had an effect on the growth of C6 tumors, C6 cells were coinjected into nude mice with GP+E86 cells that produced recombinant retrovirus encoding mutVEGFR-1, mutVEGFR-2 or full length VEGFR-1. As a negative control, C6 cells were coinjected with GP+E86 cells that contained the empty retroviral vector. In all coinjection experiments, C6 cells and retrovirus producing cells were injected at a ratio of 1:1 ( $10^6$  cells/cell line). Tumor outgrowth was monitored, and 21 days after inoculation, tumors were excised and tumor weight was determined. Large tumors grew when C6 glioma cells were coinjected with GP+E86 cells, containing the empty vector (Fig. 1a), indicating that the retroviral infection itself had no influence on tumor growth. As previously demonstrated by Millauer *et al.*,<sup>11</sup> dominant-negative mutVEGFR-2 strongly inhibited tumor growth. An even stronger reduction in tumor growth was observed by mutVEGFR-1. Immunohistological analysis for the endothelial cell marker, PECAM-1, revealed a decreased blood vessel density in inhibited tumor specimens (Fig. 1d) compared to the dense vascular network of the control tumors (Fig. 1b), indicating that tumor angiogenesis was inhibited. *In situ* hybridization with a neomycin resistance gene-specific probe was performed to detect areas of retroviral infection (Fig. 1c), confirming retroviral infection of tumor tissue. Coinjection of GP+E86/mutVEGFR-1 and GP+E86/mutVEGFR-2 did not result in a stronger inhibition of tumor growth compared to the separate injection of the receptor mutants (Fig. 1a).

Surprisingly, the gene transfer of the full length VEGFR-1 significantly inhibited tumor growth (Fig. 1a) to a similar extent as mutVEGFR-1. This finding was unexpected because the retroviral gene transfer of the full length VEGFR-2 did not influence tumor growth.<sup>11</sup> Therefore, we investigated the possibility that the GP+E86 cells expressing VEGFR-1 might serve as a sink for VEGF produced by tumor cells and thereby remove bioactive VEGF. NIH3T3 cells, which are the parental cells of GPE cells, were used to generate cell lines constitutively expressing VEGFR-1. Binding studies using <sup>125</sup>I-VEGF were performed as described<sup>47</sup> and demonstrated that the infected cells expressed high affinity VEGF receptors (data not shown). Coinjection of NIH3T3 cells, stably expressing VEGFR-1, and C6 glioma cells did not result in an inhibition of tumor growth (data not shown). It is therefore unlikely that the sequestration of VEGF by binding to VEGFR-1 expressing GPE cells caused the inhibition of C6 gli-

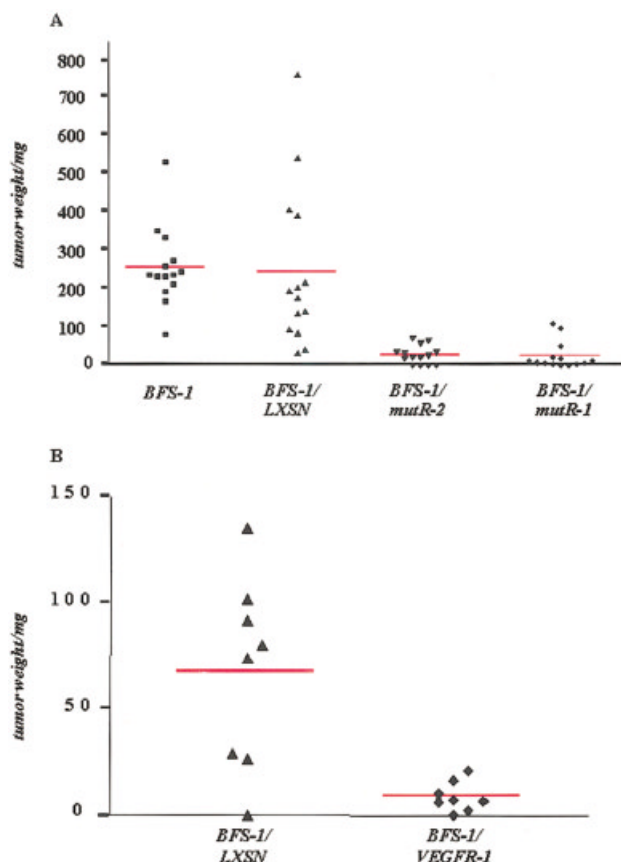


**FIGURE 1** – Growth inhibition of subcutaneous C6 glioma by retroviral gene transfer of VEGF receptor mutants. The tumors were harvested 21 days after subcutaneous injection of tumor cells or coinjection of tumor cells and retrovirus producer cells, respectively, and tumor weight was determined. (a) Significant inhibition of tumor growth by the retroviral gene transfer of VEGF receptor mutants or of full length VEGFR-1. (b,d) Immunohistological staining for PECAM-1 exhibited a strong reduction of blood vessel density in the mutVEGFR-1 treated tumors (d) in comparison to C6 cells injected alone (b). (c,e) *In situ* hybridization of a mutVEGFR-1 treated tumor with a probe specific for the neomycin resistance gene (c) confirmed the retroviral infection of the tumor tissue. *In situ* hybridization with a VEGFR-1 specific probe served as a positive control for the technique (e).

oma growth. In order to monitor the growth behavior of the GP+E86 cells, they were also injected alone. No growth was observed (data not shown), demonstrating that the detected increase in tumor mass was caused by the growth of C6 glioma cells and that the GP+E86 cells were not tumorigenic.

#### Growth inhibition of subcutaneous BFS-1 fibrosarcoma by truncated VEGFR-1 mutants

To investigate the growth inhibitory effect of mutVEGFR-1 in an immunocompetent, syngeneic tumor model, we used mouse BFS-1 fibrosarcomas. These tumors grow rapidly, and their vascularization begins at a very early stage of tumor development.<sup>45</sup> BFS-1 cells and GP+E86 cells that produced recombinant retrovirus encoding mutVEGFR-1, mutVEGFR-2 or wild-type VEGFR-1 were coinjected subcutaneously into C57/B16 mice. Coinjection of BFS-1 cells and GP+E86 cells that contained the empty retroviral vector was performed as a negative control. To

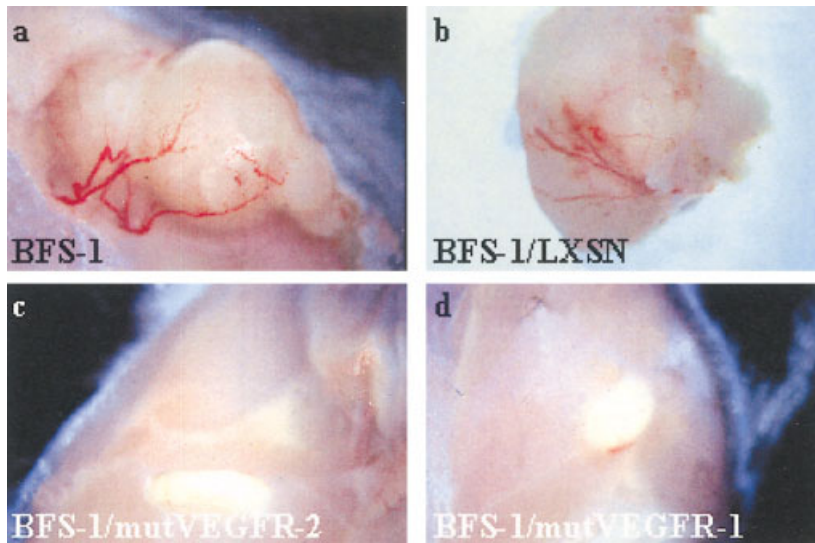


**FIGURE 2** – Growth inhibition of subcutaneous BFS-1 fibrosarcoma by retroviral gene transfer of VEGF receptor mutants. The tumors were harvested 16 days after subcutaneous injection of tumor cells or coinjection of tumor cells and retrovirus producer cells, respectively, and tumor weight was determined. (a) Significant inhibition of tumor growth was observed by the co-injection of GP+E86/mutVEGFR-1 cells. (b) Tumor growth was strongly inhibited by retroviral gene transfer of the full length VEGFR-1

exclude the possibility that tumor growth is affected by the retroviral infection itself, BFS-1 fibrosarcoma cells were also injected alone. The tumors were harvested 8 and 16 days after inoculation and the tumor weight was determined. 16 days post injection, fast growing tumors developed when BFS-1 fibrosarcoma cells were injected alone or in combination with GP+E86 cells containing the empty retroviral vector (Fig. 2a). In contrast, a significant inhibition of tumor growth was observed by the coinjection of GP+E86/mutVEGFR-1 cells (Fig. 2a). This effect was comparable to the growth inhibition by mutVEGFR-2. Eight days post injection, similar results were observed (data not shown). Also in this tumor model, the retroviral gene transfer of the full length VEGFR-1 strongly inhibited the growth of BFS-1 fibrosarcoma, as observed 16 days p.i. (Fig. 2b). The growth inhibition was even slightly more pronounced than the effect of the VEGFR-1 or VEGFR-2 mutants.

#### Tumor neovascularization, but not VEGF/VEGFR expression was inhibited by truncated VEGFR-1 mutants

By macroscopic observation, the control tumors (BFS-1 cells alone, or coinjection of BFS-1 cells and GP+E86 cells containing the empty vector, respectively) exhibited large blood vessels on the tumor surface (Fig. 3a,b). In contrast, fibrosarcomas inhibited by the truncated VEGFR mutants exhibited a pale, mostly avascular surface (Fig. 3c,d). To further analyze the effect of the VEGFR mutants on tumor neovascularization, immunohistological stain-



**FIGURE 3** – Macroscopic analysis of growth inhibited BFS-1 fibrosarcoma 16 days post injection. (a,b) control tumors exhibited large blood vessels on the tumor surface. (c,d) The growth inhibited tumors exhibited pale and nearly avascular surface.

ings with specific antibodies against the endothelial markers PECAM-1 and VEGFR-2 as well as *in situ* hybridization for the expression of *VEGFR-1*, *VEGFR-2* and *VEGF* were performed. The control tumors were well vascularized as shown by PECAM-1 staining (Fig. 4a), and most of the tumor vessels, if not all, expressed VEGFR-2 (Fig. 4b). The expression of *VEGFR-1* on tumor endothelium and of *VEGF* in tumor cells was verified by *in situ* hybridization (Fig. 4c,d). In contrast, the growth inhibition by retroviral gene transfer of the kinase domain deficient VEGF receptor mutants resulted in a decreased tumor vascularization (Fig. 4e,i). The majority of the remaining blood vessels expressed VEGFR-2, as detected by immunohistological staining (Fig. 4f,j). *In situ* hybridization with a *VEGFR-1* specific probe revealed a strong expression on endothelial cells (Fig. 4g,k). The antisense probe used detected both the *mutVEGFR-1* and the full length *VEGFR-1*. Therefore, the observed *VEGFR-1* signal in *mutVEGFR-1*-treated tumors reflected not only the expression of the endogenous full length VEGFR-1 but also of the exogenous *mutVEGFR-1*. By *in situ* hybridization, the characteristically strong *VEGF* expression in tumor cells could be observed (Fig. 4h,l).

*Inhibition of tumor growth by truncated VEGFR-1 mutants was characterized by decreased tumor cell proliferation and increased apoptosis*

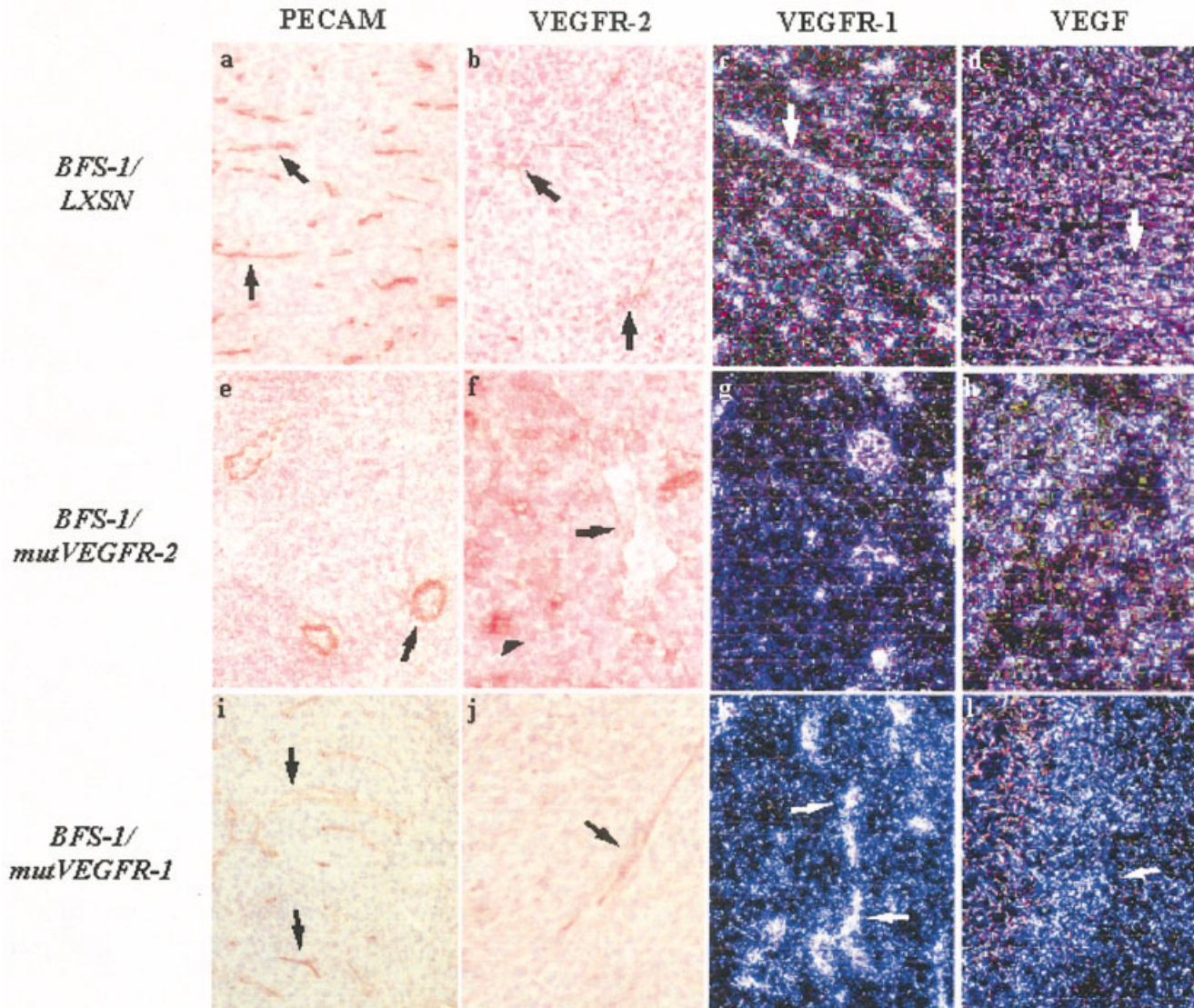
In order to analyze the proliferative and apoptotic activities in the tumor tissue, we performed immunohistological staining for the nuclear cell proliferation-associated antigen Ki-67 as a marker for proliferation and a modified Tunel-assay to monitor apoptosis. In the control tumors, strong tumor cell proliferation was observed (Fig. 5a), whereas only a few apoptotic tumor cells could be detected (Fig. 5d). These results are in line with the rapid growth behavior of the BFS-1 fibrosarcoma. The tumors inhibited by GP+E86/*mutVEGFR-2* exhibited a strongly decreased tumor cell proliferation (Fig. 5b), whereas the apoptosis rate was similar to control tumors (Fig. 5e). In contrast, fibrosarcoma inhibited by *mutVEGFR-1* revealed a strong increase in tumor cell apoptosis (Fig. 5f), while the tumor cell proliferation was strongly reduced (Fig. 5c). Apoptotic tumor cells could be detected mainly around massive necrotic areas. These results suggest that the inhibition of tumor growth by *mutVEGFR-1* started with the inhibition of tumor neovascularization, which was then followed by the reduction of tumor cell proliferation due to the starving conditions and resulted in an increase of tumor cell apoptosis and tumor necrosis.

*The anti-angiogenic effect of the dominant-negative VEGFR mutants was mediated through host tumor endothelial cells and not through transduction of tumor cells*

As shown by immunohistochemistry and *in situ* hybridization, no expression of VEGFR-1 or VEGFR-2 in BFS-1 fibrosarcoma cells was detected in tumors grown after coinjection with the corresponding GP+E86/*mutVEGFR*, suggesting that the tumor cells were not infected by the retroviruses. To verify that the BFS-1 cells are not infected by retrovirus, they were incubated *in vitro* with retrovirus-containing conditioned medium of GP+E86/VEGFR-1 cells. Two days following infection, the cells were harvested, and Western blot analysis of lysates was performed with a monoclonal anti-VEGFR-1 antibody. BFS-1 cells infected with virus-containing conditioned medium of GP+E86/empty vector cells were used as a negative control. NIH-3T3 cells were also infected with GP+E86/VEGFR-1 cells and Western blot analysis was performed. No VEGFR-1 expression could be detected in infected BFS-1 cells, whereas the NIH-3T3 cells exhibited a strong VEGFR-1 expression following retroviral infection with GP+E86/VEGFR-1 (Fig. 6). No VEGFR-1 expression was observed in NIH-3T3 cells infected with the empty retroviral vector. These results indicated that the described inhibitory effects of *mutVEGFR-1* or the full length VEGFR-1 were not caused by sequestration of VEGF by infected tumor cells but by an endothelium-specific mechanism, most likely the modulation of VEGF signaling in tumor endothelial cells.

*Heterodimer formation of VEGFR-1 and VEGFR-2*

Heterodimer formation of related receptor molecules has been demonstrated in various systems, for example in the case of the PDGF receptors - $\alpha$  and - $\beta$ , which are closely related to the VEGF receptors. In order to test the hypothesis that the full length VEGFR-1 might interact with full length VEGFR-2 molecules, we transiently cotransfected COS-1 cells with expression vectors containing the cDNAs for VEGFR-1 or VEGFR-2, respectively. A second cotransfection was performed with expression vectors encoding VEGFR-2 and *mutVEGFR-1*. As a control, COS-1 cells were transfected with the empty expression vector. Two days following transfection the cells were stimulated with VEGF (50 ng/ml), harvested, and immunoprecipitation was performed using anti-VEGFR-2 monoclonal antibody. Immunoblot analysis was performed for VEGFR-1. To check for VEGF receptor expression, cell lysates were directly assayed for VEGFR-1, and additionally, after immunoprecipitation, for VEGFR-2 protein. In immunoprecipitates of COS-1/VEGFR-1/VEGFR-2 cells with monoclonal anti-VEGFR-2 antibody, a 180 kDa molecule was detected, cor-



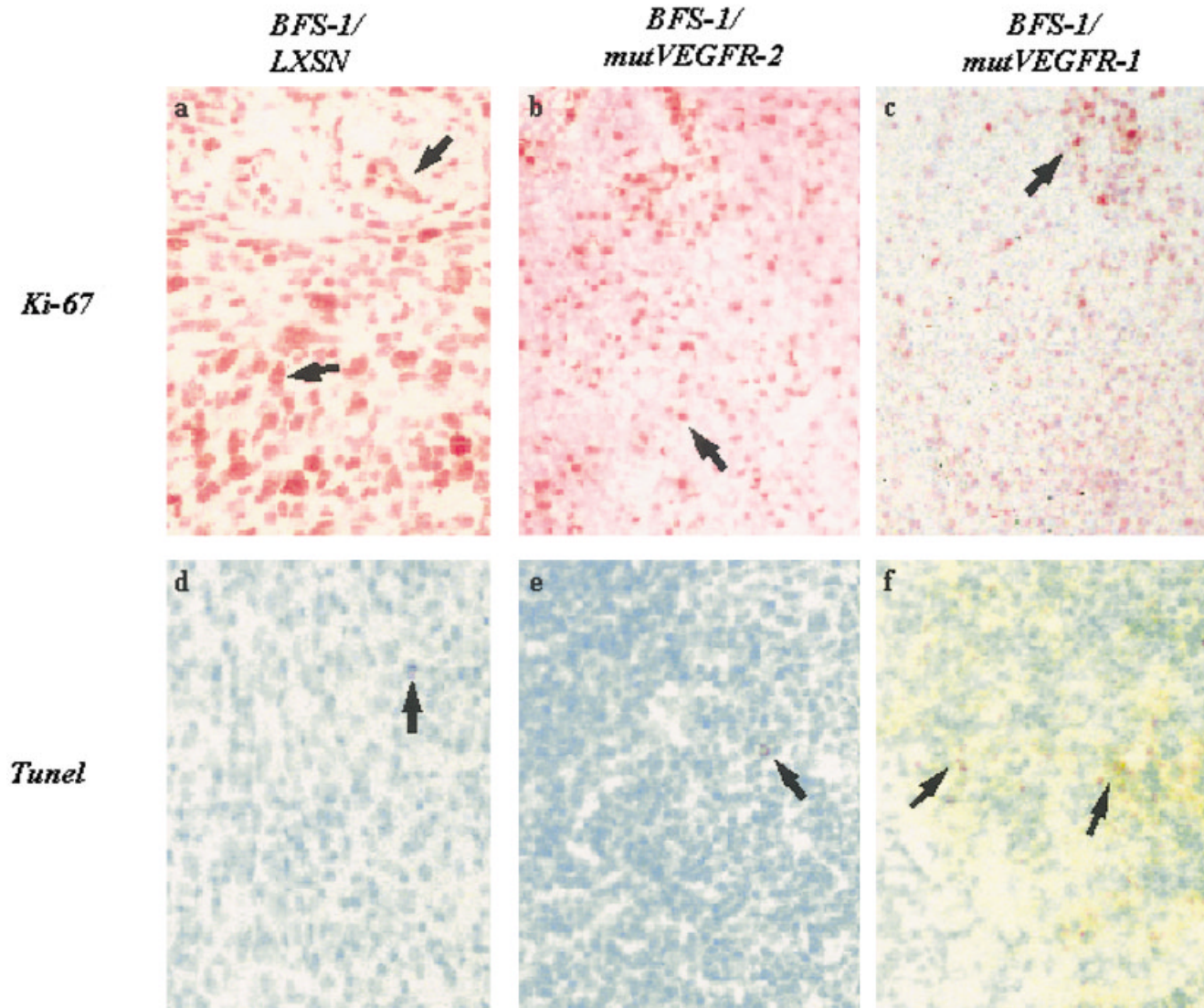
**FIGURE 4** – Histological analysis of growth inhibited BFS-1 fibrosarcoma 16 days post injection. The control tumors exhibited a dense vascular network (a) and most of the blood vessels expressed VEGFR-2 (b). *VEGFR-1* expression on tumor endothelium (c) and *VEGF* in tumor cells (d) was detected by *in situ* hybridization with radioactive-labeled probes. In contrast, growth inhibited tumors exhibited a reduction in blood vessel density (e,i), but most of the remaining vessels expressed VEGFR-2 (f,j). Also these tumors showed *VEGFR-1* expression in tumor endothelium (g,k) and *VEGF* in tumor cells (h,l). Arrows in a–c, e, f, i–k: tumor blood vessels, arrowhead in f: VEGFR-2 expressing tumor cells, arrows in d, l: *VEGF* expressing tumor cells. Magnification: a–e, g–i, k, l:  $\times 200$ , f, j:  $\times 400$ .

responding to the molecular weight of VEGFR-1 (Fig. 7). In addition, a 100 kDa protein was observed in immunoprecipitates of COS-1/VEGFR-2/mutVEGFR-1 cells, corresponding to the molecular weight of the truncated receptor. These results show that the full length VEGFR-1 as well as the mutVEGFR-1 are able to form heterodimers with the full length VEGFR-2. These heterodimers might contribute to the growth inhibitory effect of the VEGFR-1 mutant as well as of the wild-type VEGFR-1, for example, by altering distinct signal transduction pathways in tumor endothelial cells. A potential role of PIGF in activating VEGFR-1 in the experimental tumors used in our study is indicated by the observation that C6 cells and, to much lesser extent, BFS-1 fibrosarcoma cells expressed *PIGF* mRNA (Fig. 7b).

#### DISCUSSION

Based on the hypothesis that the growth of many different tumors *in vivo* is dependent on their neovascularization, tumor

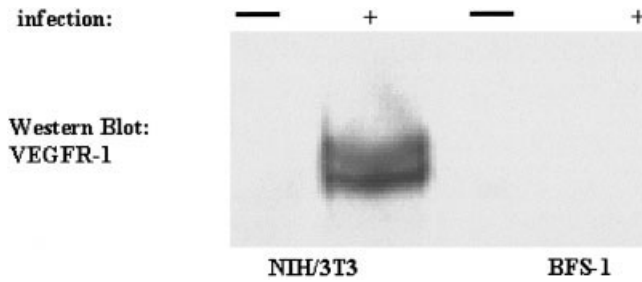
therapy with anti-angiogenic substances is considered as a promising strategy to complement conventional therapeutical strategies. The specificity of anti-angiogenic therapeutical approaches is essential and was aimed at by the targeting of key regulators of tumor angiogenesis, such as the VEGF/VEGFR signal transduction system. The efficacy of anti-VEGF treatment in animal models was demonstrated by the inhibitory effect of neutralizing anti-VEGF antibodies on tumor neovascularization and tumor growth in various animal tumor models.<sup>13,56–58</sup> The central role of VEGFR-2 signaling was demonstrated by the retrovirus-mediated gene transfer of signaling defective dominant-negative VEGFR-2 mutants, which led to a strong inhibition of tumor angiogenesis and tumor growth.<sup>11,12,59</sup> However, the function of VEGFR-1 during tumor neovascularization is still under discussion. Although VEGFR-1 exhibits a higher affinity than VEGFR-2 to its ligand VEGF, it is phosphorylated only very weakly. The relevance of VEGFR-1 signaling in endothelial cells *in vivo* was questioned by



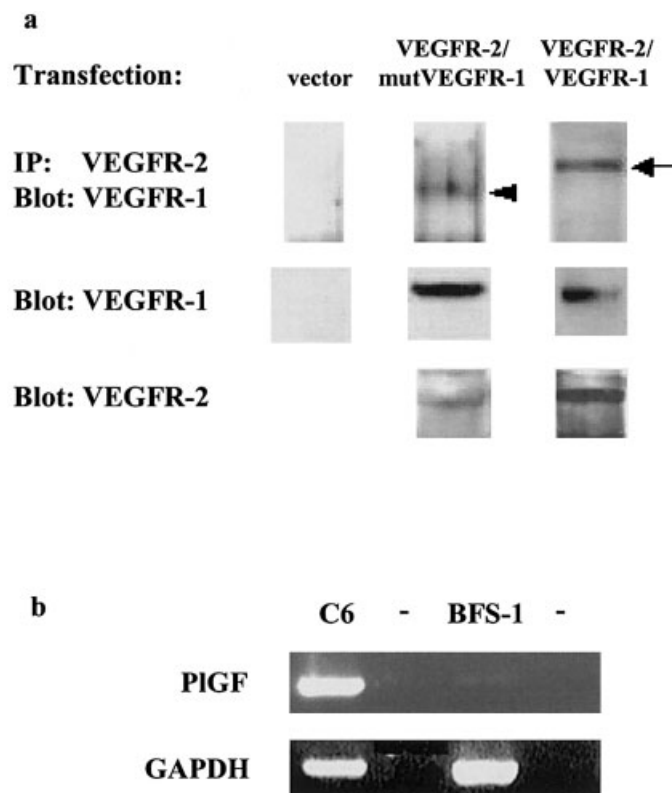
**FIGURE 5** – Analysis of proliferation and apoptosis in growth inhibited BFS-1 fibrosarcoma 16 days post injection. (a–c) Detection of proliferating tumor cells by immunohistological staining with anti-Ki-67 antibody. The control tumors exhibited a strong tumor cell proliferation (a), whereas the growth inhibited tumors exhibited a strong decrease in proliferating tumor cells (b,c). (d–f) TUNEL assay for detection of apoptotic cells. Control tumors and mutVEGFR-2 treated tumors exhibited only a few apoptotic cells (d,e), but mutVEGFR-1 treated tumors exhibited a strong increase in tumor cell apoptosis (f). (a–c)  $\times 200$ ; d–f:  $\times 400$ . Arrows in a–c: proliferating tumor cells; arrows in d,f: apoptotic tumor cells.

gene targeting experiments. Whereas homozygous *VEGFR-1* knock-out mice died *in utero* as a result of severe vascular defects,<sup>25</sup> mice lacking the VEGFR-1 intracellular kinase domain developed normally.<sup>27</sup> These studies suggested that VEGFR-1 has a negative-regulatory function during embryonic blood vessel formation by VEGF-trapping and that VEGFR-1 signaling is dispensable for embryonic angiogenesis. However, recent *in vivo* studies suggested a positive regulatory role for VEGFR-1 signaling in tumor angiogenesis.<sup>35,36</sup> In these reports, tumor cells overexpressing PIGF-2, a VEGFR-1 specific ligand, grew faster and formed better vascularized tumors when injected into wild-type mice compared to VEGFR-1 tyrosine kinase deficient-mice. Furthermore, tumor neovascularization was inhibited by the administration of a VEGFR-1 specific antibody.<sup>35</sup> These results implied a proangiogenic role of VEGFR-1 signaling in pathological angiogenesis. This hypothesis is further supported by a recent study by Autiero *et al.*<sup>60</sup> who reported that activation of VEGFR-1 by PIGF resulted in intermolecular transphosphorylation of VEGFR-2,

thereby amplifying VEGF-driven angiogenesis. In contrast, several *in vitro* studies showed an inhibition of VEGFR-2 mediated endothelial cell proliferation by the stimulation of VEGFR-1 signal transduction.<sup>40–42</sup> A more recent study<sup>43</sup> supported the model of VEGFR-1 as a negative modulator of vascularization both, *in vitro* and *in vivo*. In this report, aberrant endothelial cell division in the absence of VEGFR-1 was observed during ES cell differentiation as well as during embryogenesis, raising the possibility that VEGFR-1 signaling might potentially inhibit also pathological vascularization. To gain more insight into the function of VEGFR-1, we analyzed the effects of VEGFR-1 kinase domain-deficient mutants (mutVEGFR-1) or the wild-type receptor on the growth of xenografted C6 glioma or syngeneic BFS-1 fibrosarcoma, respectively, by a gene therapy approach. Compared to the control injections, a strong inhibition of C6 glioma growth was observed by the truncated VEGFR-2 mutant, as previously shown by Millauer *et al.*<sup>11</sup> The retroviral gene transfer of the VEGFR-1 mutant led to an even stronger growth inhibitory effect, which was



**FIGURE 6** – Retroviral infection of BFS-1 fibrosarcoma cells *in vitro*. NIH-3T3 cells and BFS-1 cells were incubated with retrovirus-containing conditioned medium of GP+E86/VEGFR-1 cells, harvested and Western blot analysis was performed with an VEGFR-1 specific antibody. No VEGFR-1 expression was detected in lysates of infected BFS-1 cells, whereas the NIH-3T3 cells exhibited a strong VEGFR-1 expression.



**FIGURE 7** – Formation of VEGFR-1/VEGFR-2 heterodimers and PIGF expression by tumor cells. (a) COS-1 cells were transiently cotransfected with expression vectors containing the cDNAs for VEGFR-1 or VEGFR-2. A second cotransfection was performed with expression vectors containing the cDNAs for VEGFR-2 or a truncated VEGFR-1 mutant. After immunoprecipitation with a VEGFR-2 specific antibody, a specific band for VEGFR-1 or the VEGFR-1 mutant was detected by Western blot analysis with a VEGFR-1 specific antibody in lysates of the corresponding transfection assay. (b) RT-PCR analysis demonstrates that C6 cells and, to much lesser extent, BFS-1 fibrosarcoma cells expressed *PIGF* mRNA. *GAPDH* mRNA was amplified as a control. Reactions without RT (–) were analyzed as negative control.

accompanied by a significant reduction of blood vessel density, as shown by immunohistology. These results suggested that the over-expression of the kinase deficient VEGFR-1 mutant first led to an inhibition of tumor angiogenesis and consequently to a malnutri-

tion of the tumor tissue, which then in turn caused the growth inhibition. Remarkably, the retroviral gene transfer of the full length VEGFR-1 also resulted in a significant inhibition of tumor growth. Because of the high affinity of the receptor for its ligand VEGF, it seemed possible that the injected GP+E86 cells, which expressed the truncated or the full length VEGFR-1, served as a sink for VEGF, thereby decreasing the amount of bioactive VEGF binding to tumor endothelial cells. This possibility was excluded by coinjections of C6 glioma cells with NIH3T3 cells stably expressing VEGFR-1. No influence on tumor growth was observed. These results suggested an inhibitory effect of mutVEGFR-1 or the full length receptor on the VEGF/VEGFR signal transduction system. However, a sink effect by those tumor cells, which are infected by the retrovirus after coinjection, could not be excluded in this model.

The observed inhibitory effects by retrovirus-mediated gene transfer of mutVEGFR-1 or the wild-type VEGFR-1, respectively, were verified in an immunocompetent BFS-1 fibrosarcoma model *in vivo*. The retrovirus-mediated gene transfer of mutVEGFR-1 led to a similar tumor growth inhibition and a strong reduction in blood vessel density like in C6 glioma, suggesting that this inhibitory effect is not restricted to a single tumor type but is a more general mechanism in angiogenesis-dependent tumor growth. The observed reduction in blood vessel density might lead to a reduced supply with oxygen, nutrients and growth factors essential for tumor cell proliferation. In order to investigate the consequences of the reduced tumor angiogenesis in more detail, tumor cell proliferation and apoptosis were monitored. The control tumors exhibited a high proliferation rate and only single apoptotic tumor cells, reflecting the rapid growth of BFS-1 fibrosarcoma. In contrast, tumor cell proliferation was strongly reduced by the overexpression of VEGFR-1 or VEGFR-2 mutants. Necrotic areas were observed in these tumors, with mutVEGFR-1 overexpressing tumors exhibiting a consistently higher degree of apoptosis. Differences in the degree of apoptosis between VEGFR-1 or VEGFR-2 overexpressing fibrosarcomas might be due to different stages of tumor growth because identical tumor size does not necessarily reflect the same progression level. Apoptosis in inhibited tumors was detectable as early as 8 days post injection, suggesting that the anti-angiogenic effect started at a very early time point of tumor progression.

Our study confirms the central role of the VEGF/VEGFR-2 signal transduction system for tumor angiogenesis and tumor growth. As shown previously, also i.p. injections of an anti-VEGFR-2 antibody led to an inhibition of tumor growth, tumor neovascularization, a decrease of proliferating tumor cells and the formation of necrosis.<sup>61</sup> Interestingly, our investigations showed a similar outcome for the tumor gene therapy using the truncated VEGFR-1 mutant. BFS-1 cells exhibited no detectable expression of the exogenous VEGF receptors. *In vitro* studies confirmed that BFS-1 fibrosarcoma cells could not be transduced by the retrovirus used, indicating that the observed effects were caused by an endothelial cell specific mechanism. The high level of VEGF expressed by the tumor cells was obviously not affected by the overexpression of the VEGF receptor mutants or the full length VEGFR-1 in the tumor endothelium, respectively.

Receptor tyrosine kinases form dimers after ligand binding, resulting in an intracellular cross-phosphorylation that induces further signaling. By the over-expression of receptor mutants that lack the intracellular kinase domain, heterodimers of wild-type and kinase-deficient receptors do not cross-phosphorylate, and thus downstream signal transduction cannot occur. Therefore, if the formation of heterodimers between the wild-type VEGFR-2 and the truncated VEGFR-1 mutants that was observed in our study *in vitro* also occurs *in vivo*, VEGFR-1 mutants might inhibit or modulate VEGFR-2 mediated signal transduction. However, a VEGFR-1 specific inhibitory mechanism independent of VEGFR-1/VEGFR-2 heterodimerization should also be considered. *In vitro* studies by Zeng *et al.*<sup>41</sup> demonstrated a decrease in VEGFR-2



mediated proliferation of HUVECs as a result of VEGFR-1 signaling. Mutational analysis revealed an essential role of the intracellular tyrosine 794 residue for this antiproliferative effect. The truncated VEGFR-1 mutant used in our study still contained this tyrosine residue in the remaining short intracellular portion and may therefore exert negative signaling. Remarkably, the overexpression of the full length VEGFR-1 led to a strong inhibition of tumor growth, supporting the concept of a negative signaling function of VEGFR-1. These results are in line with the recently published study by Kearney *et al.*,<sup>43</sup> in which an increased endothelial mitotic index was shown for ES cells and embryos lacking VEGFR-1, as compared to the corresponding wild types. This demonstrated a modulating effect of VEGFR-1 on endothelial proliferation both *in vitro* and *in vivo*. It seems likely that the alternative VEGFR-1 ligand, PlGF, which is expressed strongly by C6 glioma cells and to a lesser extent also by BFS-1 fibrosarcoma

cells, contributes to the modulating effect of VEGFR-1, possibly through the formation of VEGF-PlGF heterodimers which antagonize angiogenic VEGF signaling.<sup>62</sup> Taken together, the results of our study indicate a key role for VEGFR-1 in modulating VEGF signaling during tumor angiogenesis and show that VEGFR-1 is a promising novel target for anti-angiogenic gene therapy.

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