Vascular Endothelial Growth Factor Trap Blocks Tumor Growth, Metastasis Formation, and Vascular Leakage in an Orthotopic Murine Renal Cell Cancer Model

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Abstract

Purpose: Angiogenesis inhibitors have shown clinical benefit in patients with advanced renal cell cancer, but further therapeutic improvement is needed. Vascular endothelial growth factor (VEGF) Trap is a newly developed VEGF-blocking agent with stronger affinity and broader activity than the anti-VEGF antibody bevacizumab. In this study, we tested the activity of VEGF Trap in an orthotopic murine model of renal cancer with spontaneous lung metastases.

Experimental Design: Murine syngeneic renal cell carcinoma cells (RENCA) transfected with a luciferase-expressing vector were injected into the renal capsule of BALB/c mice. I.p. treatment with VEGF Trap or control protein (10 or 25 mg/kg twice weekly) was started shortly after tumor injection to prevent tumor development (prevention model) or after established tumors were formed to inhibit tumor growth and metastasis formation (intervention model).

Results: In the prevention model, VEGF Trap inhibited tumor growth by 87 ± 14% compared with control ($P = 0.007$) and significantly prolonged survival. In the intervention model, VEGF Trap inhibited tumor growth by 74 ± 9% ($P < 0.001$) and the formation of lung metastases was inhibited by 98% ($P < 0.004$). Microvascular density was reduced by 66% due to VEGF Trap treatment ($P < 0.001$). In addition, VEGF Trap prevented fibrinogen leakage into the tumor microenvironment representative for reduced vascular leaking as shown by immunohistochemical staining.

Conclusions: VEGF Trap is a potent inhibitor of RENCA tumor growth and metastasis formation and blocks the biological function of VEGF *in vivo*. These results support further clinical development of VEGF Trap for renal cell cancer and other cancer types.

Renal cell carcinoma (RCC) is the fourth most common genitourinary cancer with ~38,000 new cases diagnosed and 12,000 deaths annually (1). At the time of diagnosis, ~30% of patients have distant metastases and 25% have locally advanced disease. Treatment options for patients with advanced disease remain limited, and the 5-year survival rate for patients with stage IV disease is <5%. Angiogenesis is a marked feature in clear cell RCC, the most common type of RCC, which accounts for approximately 70% to 80% of cases. A key genetic and epigenetic marker in clear cell RCC is the functional inactivation of the *von Hippel-Lindau* tumor suppressor gene (2). One consequence is the overproduction of the vascular endothelial growth factor (VEGF) through overexpression of hypoxia-inducible factor-1α (3).

Angiogenesis is the process of new vessel formation, which is essential for tumors to grow and metastasize (4). VEGF and its receptors, VEGFR-1 (flt-1) and VEGFR-2 (flk-1/KDR), are key players in stimulating angiogenesis, and inhibition of VEGF or its receptors has been shown to suppress angiogenesis and tumor growth (5). VEGF is released by tumor cells but also by host cells, such as macrophages and platelets (6, 7). VEGF has multiple biological activities by inducing proliferation and migration and by preventing apoptosis of endothelial cells and other cells, including bone marrow–derived cells (5). Initially, VEGF was discovered as the vascular permeability factor responsible for leakage of plasma proteins out of blood vessels into the tumor extracellular matrix and for ascites formation (8–10). VEGF has also been shown to induce tissue factor activity and subsequent activation of the coagulation cascade (11, 12).

Several angiogenesis inhibitors targeting VEGF but also other angiogenic pathways have been developed in the past decades (13, 14). Both humanized antiangiogenic antibodies as well as small-molecule receptor tyrosine kinase inhibitors have shown clinical efficacy in patients with advanced cancer. The

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antibodies primarily showed activity in combination with chemotherapy, whereas the small-molecule tyrosine kinase inhibitors have shown activity as single-agent therapy in patients with advanced RCC. Two receptor tyrosine kinase inhibitors, sorafenib and sunitinib, have been recently approved by the Food and Drug Administration for treatment of patients with advanced renal cell cancer (15, 16).

Bevacizumab (Avastin) is a humanized monoclonal antibody that neutralizes VEGF. A large phase III randomized trial in patients with advanced colorectal cancer showed that treatment with bevacizumab plus standard chemotherapy increased the overall survival by ~5 months compared with those treated with chemotherapy alone (17). Based on these results, bevacizumab has been approved by Food and Drug Administration. In a randomized phase II study, treatment with single-agent bevacizumab of patients with metastatic renal cell cancer resulted in a greater time to tumor progression compared with those receiving placebo, although there was no difference in overall survival between the two groups (18).

VEGF Trap is a soluble decoy receptor comprising parts of VEGFR-1 and VEGFR-2 based on a human IgG1 backbone (19). After modifications of the parent compound that had several interactions with the extracellular matrix, VEGF $Trap_{R1R2}$ (VEGF Trap) has been developed. It avidly binds VEGF and leads to potent suppression of VEGF signaling and angiogenesis at low concentrations (picomolar range). This agent is currently under clinical investigation. Enhanced clinical activity of VEGF Trap compared with the monoclonal antibody bevacizumab against VEGF is expected because VEGF Trap recognizes the whole VEGF family that binds to VEGFR-1 and VEGFR-2, including placental growth factor, and it possesses higher affinity for VEGF than bevacizumab *in vitro*. In preclinical models, VEGF Trap has been shown to potently inhibit tumor growth, metastasis formation, and ascites formation in several murine tumor models (20–23). In early-phase clinical trials, VEGF Trap has been studied as single agent and in combination with chemotherapy, and phase II clinical trials are being conducted (24–26).

In the present study, we tested the activity of VEGF Trap on tumor growth and spontaneous metastasis formation in an orthotopic renal cell cancer model (RENCA). We found that VEGF Trap is a potent inhibitor of primary tumor growth and metastasis formation and significantly prolongs survival of tumor-bearing mice. In addition, we investigated whether blockade of VEGF *in vivo* reduced vascular permeability by assessing extravasated fibrinogen.

Materials and Methods

Cell line and reagents

RENCA cells were obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 (Life Technologies) containing 10% tryptose phosphate broth (Sigma), 1% L-glutamine (Life Technologies), 1% nonessential amino acids (Life Technologies), 1% sodium pyruvate (Sigma), 1% penicillin/streptomycin (Life Technologies), and 10% fetal bovine serum and kept in an incubator at 37°C in an atmosphere containing 5% CO_2. VEGF Trap and its control IgG backbone (hFc) were kindly provided by Regeneron in a blinded fashion.

RENCA proliferation *in vitro* (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt assay)

RENCA tumor cell proliferation was assessed in a 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt assay according to standard procedures as described previously. RENCA cells were plated on day -1 at 1,000 per well in a 96-well plate in 10% RPMI 1640. At day 0, cells were washed extensively and starved without serum. On day 1, cells were incubated in RPMI 1640 (5% serum) with increasing concentrations of VEGF Trap and control (0-1 μg/mL). 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt assay was done on day 1 (as $T = 0$) reading and on day 4 ($T = 72$ h) according to the standard manufacturer's guidelines.

RENCA tumor model *in vivo*

In vivo experiments were conducted according to our animal protocol approved by the Institutional Care and Use Committee at the Johns Hopkins Medical Institutions and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Luciferase-transfected RENCA cells (5×10^5) mixed with Matrigel were orthotopically injected into the subcapsular space of the left kidney of BALB/c female mice (The Jackson Laboratory). On a weekly basis, tumor imaging was done by i.p. administration of luciferin and by using bioluminescence technology (Xenogen system). Before starting the treatment, mice were grouped according to tumor burden as determined by luciferase expression. As an indicator of treatment-related toxicity, mouse weights were measured once weekly. Lung metastases and primary tumor weights were determined at the end of the study when mice were sacrificed. All experiments were repeated at least twice. Representative experiments are shown.

Prevention experiments. Treatment with 10 mg/kg VEGF Trap or its control by i.p. injections twice weekly was started on day 3 or 4 after implantation of the tumor and mice were treated for 30 days. In these experiments, survival of mice was determined as well.

Intervention experiments. Treatment was started when tumor burden had been clearly increased according to luciferase expression measurements. Treatment with 10 or 25 mg/kg VEGF Trap or its vehicle control started on day 10 or 14 after tumor implantation, respectively, and mice were treated for 22 days. In both models, VEGF Trap and control IgG were given in a blinded fashion and the code was broken by Regeneron after completion of the experiments.

Immunohistochemistry

Immunohistochemical studies were done on 4-μm-thick sections derived from zinc-fixed, paraffin wax–embedded tumor tissue blocks. These tumors were harvested at the end of the experiments (after 22 days). Sections were subsequently dewaxed, rehydrated, and had endogenous peroxidase activity quenched before specific immunohistochemical staining. After specific staining or H&E staining, sections were dehydrated in alcohol and xylene and subsequently mounted.

Microvessel density

The tumor vasculature was stained with an antibody against CD31, and microvessel density (MVD) was determined by counting CD31-stained vessels of tumor slides by examining "hotspots" according to standard procedures as described previously (27). In brief, sections were blocked in PBS + 5% rabbit serum (Vector Laboratories, Inc.) and incubated overnight with monoclonal rat anti-mouse CD31 (PECAM-1) IgG (BD PharMingen). Subsequently, sections were incubated with Dako LSAB 2 peroxidase-conjugated streptavidin (biotinylated rabbit anti-rat IgG, mouse absorbed; Vector Laboratories), developed with 3,3′-diaminobenzidine, and counterstained with methyl green (Dako). Vessel density per 200 field was quantified from 6 to 8 fields per tumor section from the treatment and control groups and expressed as percentage per area (200× field). Percentage per area was assessed using ProImage software.

Fibrinogen staining

Sections were blocked in PBS + 5% horse serum (Vector Laboratories). Polyclonal goat anti-mouse fibrinogen IgG (Nordic Immunology) was incubated overnight at 4°C. Sections were developed with the Vectastain Elite avidin-biotin complex system (biotinylated goat

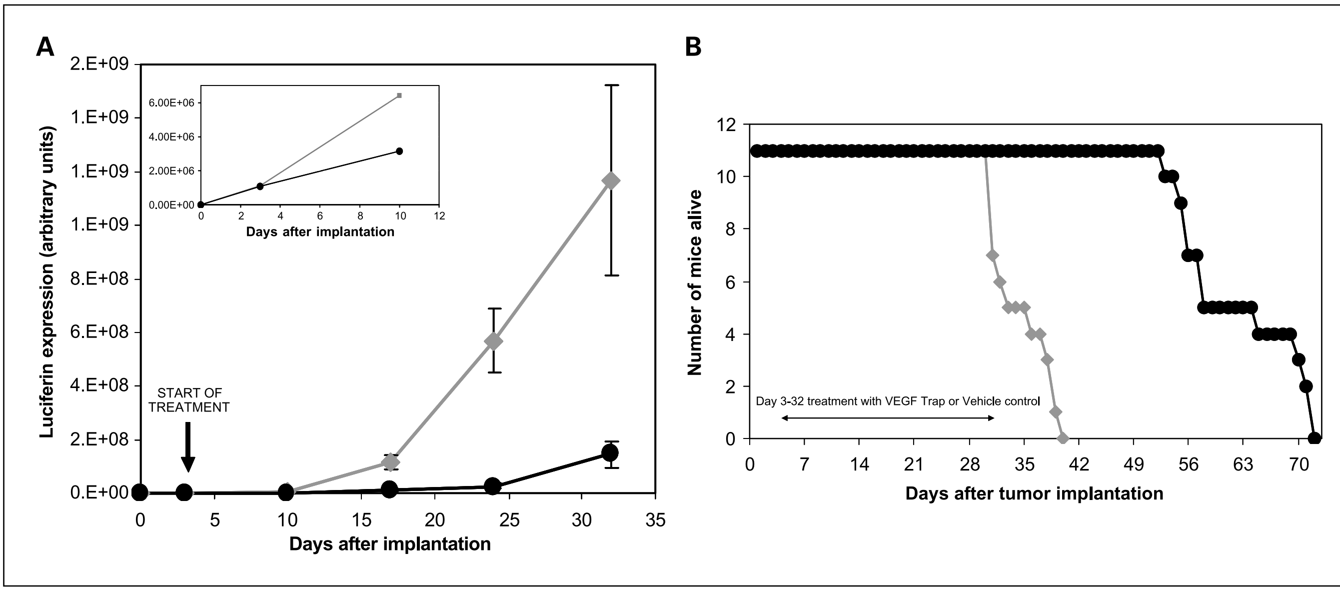

Fig. 1. Delay of tumor development and prolonged survival by treatment with VEGF Trap. *A,* after orthotopic implantation of RENCA cells in the left kidney, VEGF Trap (*black dots*) and control treatment (*gray squares*) were given in a blinded fashion starting day 4. Tumor growth was inhibited by 87 ± 14% (n = 11; P = 0.007) as measured by luciferase expression 32 d after tumor implantation. In the inlay, it is shown that already after 3 d tumors can be identified in these mice. *B,* survival in these animals was monitored. Survival of VEGF Trap – treated mice (*black dots*) was 62 ± 8 d versus 35 ± 4 d in controls (*gray squares*; n = 11 in each group; $P < 0.0001$) after 29 d of treatment.

anti-rabbit antibody; Vector Laboratories) and subsequently by 3,3′-diaminobenzidine (Dako). Counterstaining was done with modified Mayer's hematoxylin (Richard-Allan Scientific).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay and Ki67 staining

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling and Ki67 staining were done as described previously (28). Staining intensity and localization for fibrinogen, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling, and Ki67 were scored by two investigators independently.

Pericyte coverage

Fresh tumor tissue was embedded in OCT medium and quick frozen on liquid nitrogen. Frozen sections of 8 μm were obtained and postfixed with acetone. Sections were blocked in PBS/5% fetal bovine serum for 1 h and then incubated with a 1:200 dilution of anti-CD31 (clone MEC 13.3, BD Biosciences) and a 1:2,000 dilution of anti–smooth muscle actin (clone 1A4, Sigma) overnight at 4°C. The sections were then washed thrice and incubated with a secondary goat anti-rat antibody conjugated with Alexa Fluor 488 and a goat anti-mouse antibody conjugated with Alexa Fluor 555 (both 1:400 dilution) for 4 h at room temperature. Slides were washed, mounting medium (VectorShield) was added, and the specimens were covered with a coverslip. Digital images were acquired at 400× on a Nikon Eclipse E800 fluorescence microscope equipped with a 5 MHz interline CCD camera. The MetaMorph software package (Universal Imaging) was used for acquisition and processing. Representative pictures are shown.

Statistical analysis

Results were analyzed with the Excel software using Student's t test, with $P < 0.05$ considered as statistical significant.

Results

VEGF Trap treatment inhibits RENCA tumor growth and prevents lung metastases. To determine the effect VEGF Trap on tumor development *in vivo*, treatment of mice was started after 3 to 4 days following tumor implantation (prevention model). Tumor growth was inhibited by 87 ± 14% (n = 11; P = 0.007) as measured by luciferase expression (Fig. 1A). Survival in these animals was also monitored. VEGF Trap treatment significantly prolonged survival of these animals after 29 days of treatment (twice weekly) by 27 days compared with controls. Survival of VEGF Trap was 62 ± 8 days versus 35 ± 4 days in controls (n = 11 in each group; $P < 0.0001$; Fig. 1B). No significant changes in weight of the treated animals were observed during treatment. After 4 weeks, treatment was terminated because it is known that mice develop antibodies against VEGF Trap in the following weeks.[3] In the "intervention model," treatment with VEGF Trap at doses of 10 or 25 mg/kg twice weekly i.p. inhibited primary tumor growth by 74 ± 9% (n = 7; $P < 0.001$) and 78 ± 4% (n = 6; P = 0.013), respectively, as measured by luciferase expression. This inhibitory effect was confirmed by tumor weight assessment (Fig. 2A-C). Formation of spontaneous lung metastases was almost completely prevented by VEGF Trap (98% inhibition; 1.2 ± 0.7 lung metastasis nodules per mouse in the lungs versus 44.6 ± 6.5 nodules in control mice; n = 5 mice/group; P = 0.004; Fig. 2D and E). No significant weight changes of the treated animals were observed in these mice as well.

To determine whether the significant inhibitory activity of VEGF Trap on RENCA tumor growth *in vivo* was due in part to a direct effect on tumor cells, we tested this agent *in vitro*. In a 72-h proliferation assay *in vitro* with VEGF Trap versus control, no inhibition of proliferation of RENCA tumor cells was detected at concentrations of VEGF Trap ranging from 0.05 to 1 μg/mL (data not shown).

VEGF Trap–induced RENCA tumor growth inhibition is associated with decreased MVD. To determine whether VEGF

[3] J. Rudge, Regeneron, personal communication.

blockade has an effect on RENCA tumor vasculature, MVD was analyzed. New blood vessel formation was significantly reduced in RENCA tumor by VEGF Trap in the intervention model (~66% inhibition). The percentage area occupied by vasculature in VEGF Trap–treated tumors was 0.58 ± 0.28% versus 1.72 ± 0.27% in controls (n = 10; P < 0.0001; Fig. 3).

VEGF Trap–induced RENCA tumor growth inhibition is associated with decreased vascular leakage and increased pericyte coverage. Fibrinogen staining was done as an indicator for vascular permeability. This plasma protein is produced by the liver and only expressed in tumor extracellular matrix as a consequence of extravasation (10). Fibrinogen staining revealed a diffuse staining pattern in the RENCA tumor extracellular matrix, whereas tumor sections of VEGF Trap–treated mice had a strongly local staining pattern in direct vicinity of the tumor vasculature (Fig. 4A). At the same time, a striking increase in pericyte coverage was observed, indicating a more mature vessel phenotype after treatment with VEGF Trap (Fig. 5). H&E staining of the RENCA tumors of the treatment and control groups revealed also three significant differences caused by the treatment with VEGF Trap. First, H&E staining showed higher cellularity of the VEGF Trap–treated tumors compared with controls. This pattern is in accordance with a lower fibrinogen expression indicative for reduced leakage of plasma proteins and edema in the treated tumors compared with controls. Second, H&E staining revealed that vessels in the control tumors were more dilated compared with the treatment specimens. Third, most of the blood vessels in the treated tumors were packed with erythrocytes and other blood cells compared with the mostly empty vessels in the control tumors. This difference might be indicative for a reduced tumor blood flow in the treated tumors (Fig. 4B).

The percentage of apoptotic tumor cells and proliferating tumor cells (as assessed by Ki67 staining) in both treated versus untreated tumors revealed no significant difference (data not shown). These stainings were done on tumor tissues at the end of the treatment period when treated tumors were growing as well as control tumors (as shown in Fig. 2).

Discussion

In this study, we determined that VEGF Trap is a very potent agent against an aggressive orthotopically implanted kidney tumor in mice (RENCA). VEGF Trap significantly inhibits primary tumor growth and metastasis formation and prolongs survival in this murine model. Compared with other treatment strategies used in this tumor model, VEGF Trap can be considered significantly active (29, 30). For example, two other agents that inhibit the VEGF signaling pathway, ZD6474 and PTK787, reduced tumor growth by maximal 76% and 24% in

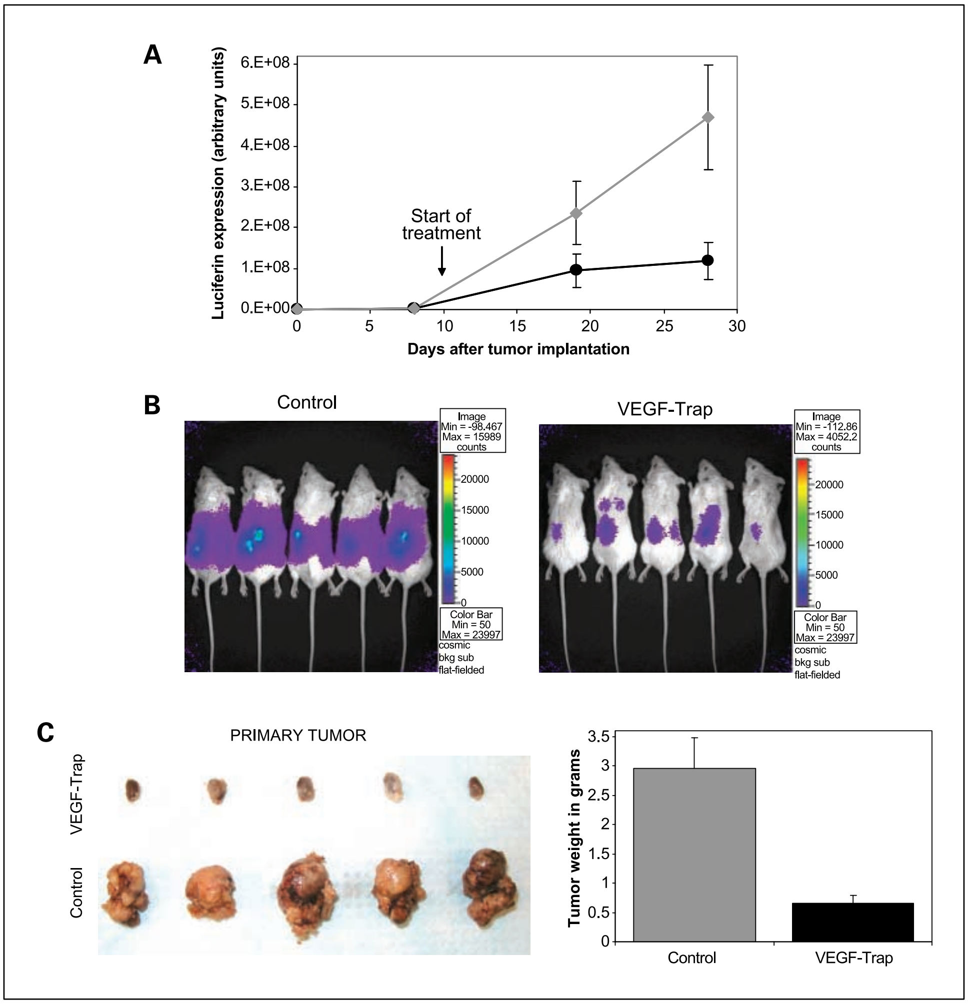

Fig. 2. Tumor growth inhibition and prevention of spontaneous metastasis formation. *A,* treatment with VEGF Trap at 25 mg/kg twice weekly i.p. (*black dots*) inhibited primary tumor growth by 78 ± 4% compared with controls (*gray squares*; n = 6; P = 0.013) as measured by luciferase expression. *B,* representative picture showing the difference in luciferin expression between control (*left*) and VEGF Trap–treated (*right*) mice (five mice of each group of a representative experiment). *C,* the difference in luciferin expression was confirmed (in each experiment) by tumor weight. Control tumor weight at the end of the experiment was 2.96 ± 0.5 g versus 0.66 ± 0.13 g of the VEGF Trap–treated tumors (n = 6; P = 0.016; a calculated 78% inhibition in tumor weight as shown for luciferase expression, *A*).

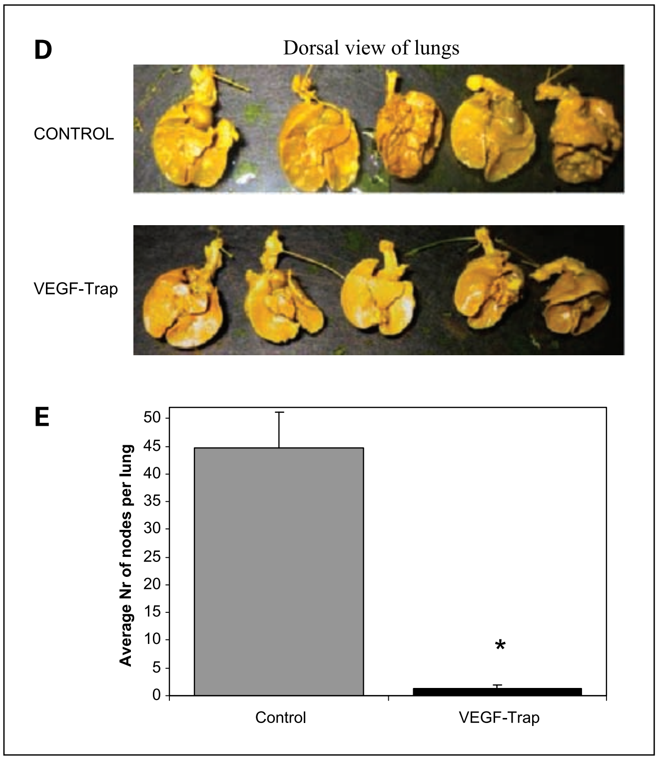

Fig. 2 *Continued*. *D,* representative picture of lungs from VEGF Trap – treated versus control mice shows a significant difference in the number of spontaneous pulmonary metastasis. *E,* the number of macroscopic tumor nodules in VEGF Trap – treated mice was 1.2 ± 0.7 lung nodules per mouse (*black column*) versus 44.6 ± 6.5 nodules in the controls (*gray column*; $n = 5$ mice/group). *, $P = 0.004$.

the prevention model, respectively, whereas VEGF Trap inhibited tumor growth by 98% and 76%, respectively, under similar conditions in this study (29, 30).

Surgery, radiation, chemotherapy, and immune therapy are widely accepted as established anticancer strategies. Recently, inhibition of angiogenesis has been accepted as a new treatment strategy in the fight against cancer (14). Although the antitumor activity of angiogenesis inhibitors as single agents is only modest for most cancer types, in patients with renal cell cancer these agents have shown clinical benefits (16). Small-molecule inhibitors of receptor tyrosine kinases, including the VEGFRs, have shown significant progression-free survival benefits. Because VEGF is known to play a major role in renal cell cancer, it is expected that VEGF Trap will be a potent agent in patients with renal cell cancer as well. It has a higher and broader affinity for the different splice variants and isoforms of VEGF compared with bevacizumab, which was shown to significantly prolong disease-free survival in patients with advanced renal cell cancer in a small phase II study (31).

Despite the clinical benefit, patients with advanced renal cell cancer treated with receptor tyrosine kinase inhibitors develop drug resistance. The mechanism of the observed resistance remains unknown, but several hypotheses have been proposed. For example, (*a*) the sensitivity of the receptor for direct inhibition may decrease due to mutations, (*b*) tumor cells may start to selectively produce other growth factors that are not inhibited by these agents, and (*c*) the growth factors of the targeted pathways are being overproduced (32). Indeed, elevated plasma levels of VEGF have been detected in patients treated with the tyrosine kinase inhibitor sunitinib, although these levels have not been directly linked to drug resistance (15). One of the future strategies to optimize the treatment of patients with renal cell cancer may be combination of tyrosine kinase inhibitors with VEGF-blocking agents to neutralize the biological activity of these increased plasma VEGF levels. Therefore, the efficacy of VEGF inhibition by bevacizumab or VEGF Trap before, during, or after treatment with tyrosine kinase inhibitors should be evaluated.

Blockade of the VEGF signaling pathway predominantly inhibits endothelial cell proliferation, migration, and survival. Initially, Senger et al. (8) discovered VEGF as a permeability factor with a potency of 400-fold greater than histamine and found that tumor-derived VEGF induces vascular leakage, causing extravasation of fibrinogen. The mechanism of VEGF-induced vascular leakage has not been fully elucidated. VEGF has been shown to cause endothelial cell fenestrations, perturbed endothelial cell-cell interactions, and breakdown of the basal membrane, which all may increase vascular leakage (10). Furthermore, altered capillary-venule hierarchy, vascular tortuosity, and variability in vessel diameter have been described as a consequence of VEGF stimulation (33). Inhibition of the VEGF signaling cascade can partially normalize these VEGF-induced features of tumor vessels and can reduce vessel diameter to such an extent that eventually blood flow may stop (34). Inhibition of VEGF by VEGF Trap has been shown to block endothelial cell fenestrations within 24 h (33). In our studies, immunohistochemical and fluorescence staining revealed that VEGF Trap not only reduced MVD but also led

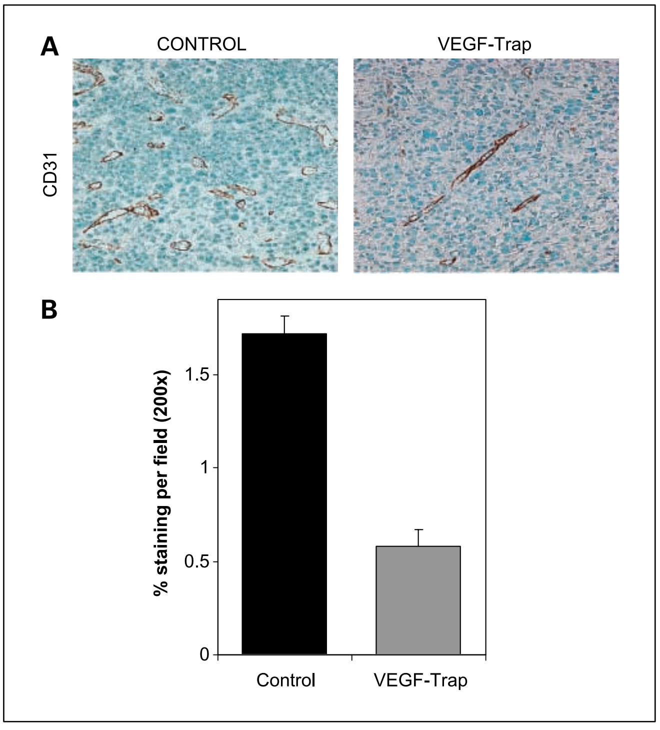

Fig. 3. Inhibition of tumor angiogenesis VEGF Trap as measured by MVD. *A,* representative area of the vascular density of the tumor from control (*left*) versus VEGF Trap – treated (*right*) mice. Brown, endothelial cells. *B,* quantification of the brown staining revealed that the percentage area occupied by vasculature in VEGF Trap – treated tumors was 0.58 ± 0.28% (*black column*) versus 1.72 ± 0.27% in controls (*gray column*; $n = 10$). *, $P < 0.0001$.

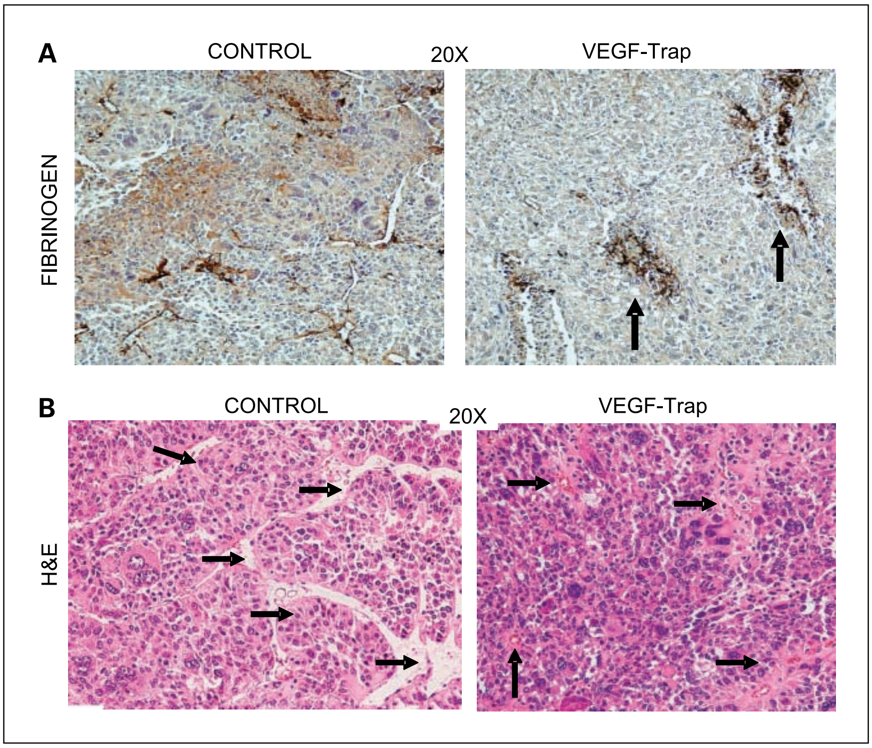

Fig. 4. Immunohistochemistry associated with VEGF function *in vivo. A,* fibrinogen staining was done as an indicator of vascular permeability in brown and counterstained with hematoxylin. Left, this staining showed high fibrinogen expression in the tumor microenvironment of control tumors. In VEGF Trap – treated tumors, fibrinogen expression in the tumor microenvironment was low (*right*), although fibrinogen was expressed in or in direct vicinity of the tumor vasculature (*black arrow*). *B,* H&E staining of the RENCA tumors of the treatment and control groups indicated that treatment with VEGF Trap (*right*) reduced vessel diameter compared with controls (*left*). In addition, the vasculature in the treated tumors was packed with erythrocytes and other blood cells (indicative for stasis) compared with mostly empty vessels in the control tumors (*black arrows*).

to a dramatic increase in pericyte coverage, a marker for vessel maturation, and prevented the extravasation of fibrinogen. These results indicate that VEGF Trap inhibits extravascular leakage into the microenvironment of solid tumors similar to blockade of ascites formation as shown in the ovarian cancer tumor models (22, 23). It is well known that fibrinogen is one of the proteins that can function as an ideal matrix for endothelial cell migration and is important for angiogenesis (35). Several studies have shown that fibrinogen is a component of the tumor microenvironment (36). In addition to fibrin(ogen), other plasma proteins are likely to serve as ideal matrix proteins for endothelial cells. Inhibition of fibrinogen extravasation in this study is an example for inhibited extravasation of other plasma-derived proteins that may serve as an ideal matrix for endothelial cells, such as von Willebrand factor and vitronectin.

VEGF also plays a role in the coagulation cascade by inducing tissue factor expression on endothelial cells (12). Tissue factor expression on endothelial cells is considered to be the main initiator in the activation of the coagulation cascade leading to the conversion of prothrombin into thrombin, which subsequently causes platelet activation and fibrinogen conversion into fibrin (thrombi formation). Tissue factor is also expressed

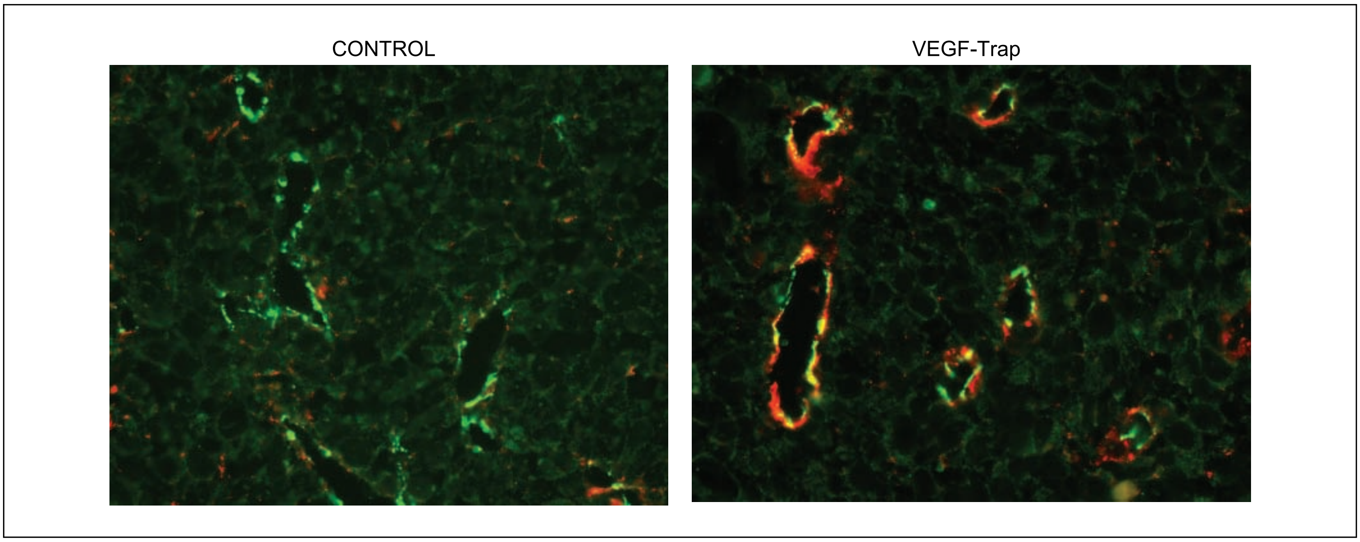

Fig. 5. Pericyte coverage as a marker of vessel maturation. Left, pericyte coverage determined as smooth muscle actin (*red*)/CD31 (*green*) double-positive blood vessel was rarely seen in untreated tumors; right, an abundance of double-positive blood vessels in VEGF Trap – treated tumors suggests an increase in blood vessel maturation.

by a variety of tumor cells as shown in several preclinical models as well as in patient tumor samples (37–39). In this study, no clear differences between intravascular thrombi of treated tumors compared with untreated controls were observed in the H&E-stained slides.

Vascular tone and diameter may be affected by VEGF Trap as well. The diameter of the tumor vessels that were treated with VEGF Trap was reduced compared with control, and most of these vessels were packed with blood cells (especially erythrocytes; Fig. 4B). It is unclear whether this observation means that the blood perfusion per vessel is decreased following treatment with VEGF Trap as previously shown by Yuan et al. (34). Another possibility is that the blood perfusion is improved, as the pruned vasculature shows a mature, stabilized phenotype by decreased permeability and enhanced pericyte coverage. In this case, the presence of RBCs may be a marker of functional blood vessels. In our model, treatment with VEGF Trap led to a halt in tumor growth rather than regression. It is therefore conceivable that the withdrawal of VEGF may lead to a pruning of immature blood vessels required for progressive tumor growth, and it induces compensatory changes in the existing vasculature toward more mature blood vessels. This vasculature phenotype ensures maintenance of the established tumor mass and may be resistant to anti-VEGF treatment. Targeting the compensatory mechanisms, such as the observed increase in pericyte coverage, may increase the efficacy of anti-VEGF agents (40). A better understanding of the vascular variable changes induced by anti-VEGF treatment will be critical to exploit the therapeutic potential of VEGF inhibitors in combination with other agents.

An increase in the apoptotic rate of tumor and endothelial cells was expected based on previous studies (23). However, in our study, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining did not show an induction of apoptosis in RENCA tumor cells by VEGF Trap treatment (data not shown). We speculate that this finding is due to the fact that these rates were determined at termination of the experiments when growth rate of the treated tumors may have become similar to the untreated controls (Fig. 1).

In conclusion, the results of this study indicate that VEGF Trap is a very potent inhibitor of tumor growth and metastasis formation in a RCC model without causing major drug-related toxicity. The immunohistochemical stainings of MVD and fibrinogen confirmed that this agent blocks VEGF activity *in vivo*. It will be of particular interest to study the efficacy of this agent in the clinical setting of advanced renal cell cancer. A National Cancer Institute-Cancer Therapy Evaluation Program–sponsored phase II study will be conducted by Eastern Cooperative Oncology Group to determine the effect of two different doses of VEGF Trap in patients with metastatic RCC who have been previously treated with a receptor tyrosine kinase inhibitor.

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