[CANCER RESEARCH 60, 5117–5124, September 15, 2000]

Selective Inhibition of Vascular Endothelial Growth Factor (VEGF) Receptor 2 (KDR/Flk-1) Activity by a Monoclonal Anti-VEGF Antibody Blocks Tumor Growth in Mice[1]

Rolf A. Brekken,[2] Jay P. Overholser, Victor A. Stastny, Johannes Waltenberger, John D. Minna, and Philip E. Thorpe[3]

Cell Regulation Program [R. A. B., P. E. T.], Hamon Center for Therapeutic Oncology Research [R. A. B., J. P. O., V. A. S., J. D. M., P. E. T.], and the Department of Pharmacology [R. A. B., J. D. M., P. E. T], University of Texas Southwestern Medical Center, Dallas, Texas 75235-8593, and the Department of Internal Medicine II (Cardiology), Ulm University Medical Center, Ulm D-89081 Germany [J. W.]

ABSTRACT

Vascular endothelial growth factor (VEGF) is a multifunctional angiogenic growth factor that is a primary stimulant of the development and maintenance of a vascular network in embryogenesis and the vascularization of solid tumors. At the present time there are two well-characterized receptors for VEGF that are selectively expressed on endothelium. VEGF receptor 2 [VEGFR2 (KDR/Flk-1)] mediates endothelial cell mitogenesis and permeability increases, whereas the role of VEGF receptor 1 [VEGFR1 (Flt-1)] has not been clearly defined. In the present study, a monoclonal antibody, 2C3, is shown to block the interaction of VEGF with VEGFR2 but not with VEGFR1 through ELISA, receptor binding assays, and receptor activation assays. 2C3 blocks the VEGF-induced vascular permeability increase in guinea pig skin. 2C3 has potent antitumor activity, inhibiting the growth of newly injected and established human tumor xenografts in mice. These findings demonstrate the usefulness of 2C3 in dissecting the pathways that are activated by VEGF in cells that express both VEGFR1 and VEGFR2, as well as highlighting the dominant role of VEGFR2 in mediating VEGF-induced vascular permeability increases and tumor angiogenesis.

INTRODUCTION

Angiogenesis is the development of new vasculature from preexisting blood vessels and/or circulating endothelial stem cells (1, 2). Angiogenesis plays a critical role in many physiological processes, such as embryogenesis, wound healing, and menstruation and in certain pathological events, such as solid tumor growth and metastasis, arthritis, psoriasis, and diabetic retinopathy (3, 4).

Angiogenesis is regulated in normal and malignant tissues by the balance of angiogenic stimuli and angiogenic inhibitors that are produced in the target tissue and at distant sites (5, 6). VEGF[4] (also known as vascular permeability factor) is a primary stimulant of angiogenesis. VEGF is a multifunctional cytokine that is induced by hypoxia and oncogenic mutations and can be produced by a wide variety of tissues (7, 8). VEGF functions as a potent permeability-inducing agent, an endothelial cell chemotactic agent, an endothelial survival factor, and endothelial cell proliferation factor (9, 10). Its activity is required for normal embryonic development because targeted disruption of one or both alleles of VEGF results in embryonic lethality (11, 12). There are at least five splice variants of VEGF, encoding proteins of 121, 145, 165, 189, and 206 amino acids. The smaller versions having 121, 145, or 165 amino acids are secreted from cells (13, 14). Secreted VEGF is an obligate dimer of between M_r 38,000 and M_r 46,000 in which the monomers are linked by two disulfide bonds. The VEGF dimer binds to one of two well-characterized receptors, VEGFR1 (FLT-1) and VEGFR2 (KDR/Flk-1), that are selectively expressed on endothelial cells. A recently identified third cell surface protein, neuropilin-1, binds VEGF165 with high affinity (15–17).

VEGFR1 and VEGFR2 are members of the type III receptor tyrosine kinase family that is characterized by seven extracellular IgG-like repeats, a single spanning transmembrane domain, and an intracellular split tyrosine kinase domain (18). VEGF binds to VEGFR1 and VEGFR2 with high affinities having a K_d (dissociation constant) of 15–100 pM and 400–800 pM, respectively (19). VEGFR2 appears to be the dominant signaling receptor in VEGF-induced mitogenesis and permeability (20–22). Binding of the VEGF dimer to VEGFR2 induces receptor dimerization, causing autotransphosphorylation of specific tyrosine residues on the intracellular side of the receptor that leads to a signal transduction cascade, which includes activation of phospholipase Cγ, an increase in intracellular calcium ions, and an increase in nitric oxide production (23–25). Activation of VEGFR2 by VEGF has also been shown to activate src and the *ras*-MAP kinase cascade (20, 26, 27). The role of VEGFR1 in endothelial cell function is much less clear. Whereas Flt-1 tyrosine kinase-deficient mice are viable and develop normal vessels (28), Flt-1-null mice die *in utero* because of increased hemangioblast commitment that results in an overgrowth of endothelial cells and a disorganized vasculature (29, 30). This latter observation, together with recent findings by Rahimi *et al.* (31), suggest that VEGFR1 may negatively regulate the activity of VEGFR2.

The recognition of VEGF as a primary stimulus of angiogenesis in pathological conditions has led to the generation of many strategies to block VEGF activity. Inhibitory anti-VEGF receptor antibodies, soluble receptor constructs, antisense strategies, RNA aptamers against VEGF, and low molecular weight VEGF receptor tyrosine kinase inhibitors have all been developed to interfere with VEGF signaling (32). Most work has been done with neutralizing monoclonal anti-VEGF antibodies that block VEGF from binding its receptors. Monoclonal antibodies, A4.6.1 (33) and MV833 (34), have both been shown to inhibit human tumor xenograft growth and ascites formation in mice (33, 35–39). These efforts underscore the importance of VEGF in solid tumor growth and its potential as a target for tumor therapy.

We previously described the properties of several monoclonal antibodies directed against human VEGF and the VEGF:VEGFR2 complex (40). One of the antibodies, 2C3, blocked the binding of VEGF to Flk-1, inhibited VEGF-mediated growth of endothelial cells *in vitro*, and localized strongly to connective tissue and blood vessels in tumors after injection into mice bearing various human tumor

Received 2/7/00; accepted 7/18/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH Grants 1RO1 CA74951 and 5RO CA54168, Predoctoral Training Grant T32 GM07062, and University of Texas Specialized Programs of Research Excellence in Lung Cancer P50 CA70907.

[2] Present address: Department of Vascular Biology, The Hope Heart Institute, 528 18th Avenue, Seattle, WA 98122-5798.

[3] To whom requests for reprints should be addressed, at Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9111. Phone: (214) 648-1268; Fax: (214) 648-1613.

[4] The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR1, VEGF receptor 1 (Flt-1); VEGFR2, VEGF receptor 2 (Flk-1/KDR); PAE, porcine aortic endothelial; NSCLC, non-small cell lung carcinoma; GPS, L-glutamine, penicillin, and streptomycin; PVDF, polyvinylidene difluoride; i.d., intradermal.

xenografts. The antibody recognized human but not mouse VEGF. Another antibody, 3E7, bound to both human VEGF complexed with KDR/Flk-1 and to free VEGF and localized selectively to tumor endothelium after injection into mice bearing human tumors. 3E7 recognized an NH_2-terminal sequence on human VEGF and mouse VEGF.

In the present study, we show that 2C3 blocks the binding of human VEGF to VEGFR2 but not to VEGFR1. 2C3 inhibits VEGF-induced phosphorylation of VEGFR2 and inhibits VEGF-induced vascular permeability increases. The antibody has potent antitumor activity, inhibiting the growth of newly injected human tumors in mice and arresting the growth of various established human solid tumors in mice. These results suggest that VEGFR2 has a dominant role in mediating the effects of VEGF on vascular permeability and tumor angiogenesis.

MATERIALS AND METHODS

Cell Lines and Antibodies. PAE cells transfected with either VEGFR1 (PAE/FLT) or VEGFR2 (PAE/KDR; Ref. 20) were grown in F-12 medium containing 5% FCS, L-glutamine, penicillin, and streptomycin (GPS). bEND.3 cells were provided by Dr. Werner Risau (Bad Nauheim, Germany) and were grown in DMEM medium containing 5% FCS and GPS. NCI-H358 NSCLC (received from Dr. Adi Gazdar, University of Texas Southwestern Medical Center, Dallas, TX), A673 human rhabdomyosarcoma, and HT1080 human fibrosarcoma (both from American Type Culture Collection) were grown in DMEM medium containing 10% FCS and GPS. 2C3, a mouse IgG2a anti-VEGF monoclonal antibody, was raised against recombinant human VEGF and recognizes epitope group 4 on VEGF, as defined by Brekken *et al.* (40). 3E7, an IgG1 monoclonal antibody directed against VEGF and VEGF complexed with VEGFR, was raised against the NH_2-terminal sequence of human VEGF and recognizes epitope group 2, as defined by Brekken *et al.* (40). 1A8, a monoclonal anti-Flk-1 antibody, and T014, a rabbit polyclonal anti-Flk-1 antibody, have been described previously (40, 41). A4.6.1, an IgG1 mouse antihuman VEGF monoclonal antibody, was provided by Dr. Jin Kim (Genentech Inc.) and has been described previously (42). Negative control antibodies used were OX7, an IgG1 mouse antirat Thy1.1 monoclonal antibody (43) provided by Dr. A. F. Williams (MRC Cellular Immunology Unit, Oxford, United Kingdom), and C44, an IgG2a mouse anticolchicine monoclonal antibody (Ref. 44; American Type Culture Collection).

ELISA Analysis. The extracellular domain of VEGFR1 (Flt-1/Fc, R&D Systems, Minneapolis, MN) or VEGFR2 (sFlk-1-biotin) was coated directly on wells of a microtiter plate or captured by NeutrAvidin (Pierce, Rockford, IL) coated wells, respectively. VEGF at a concentration of 1 nM (40 ng/ml) was incubated in the wells in the presence or absence of 100-1000 nM (15 μg-150 μg/ml) of control or test antibodies. The wells were then incubated with 1 μg/ml of rabbit anti-VEGF antibody (A-20, Santa Cruz Biotechnology, Santa Cruz, CA). The reactions were developed by the addition of peroxidase-labeled goat antirabbit antibody (Dako, Carpinteria, CA) and visualized by the addition of 3,3′5,5′-tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Inc). Reactions were stopped after 15 min with 1 M H_3PO_4 and read spectrophotometrically at 450 nM. The assay was also done by coating wells of a microtiter plate with either control or test IgG. The wells were then incubated with VEGF:Flt-1/Fc or VEGF:sFlk-1-biotin and developed with either peroxidase-labeled goat antihuman Fc (Kirkegaard & Perry Laboratories, Inc.) or peroxidase-labeled streptavidin, respectively, and visualized as above (data not shown).

Coprecipitation Assay. Forty ng of VEGF were preincubated with the $F(ab')_2$ of either of 2C3 (20 μg) or A4.6.1 (10 and 1 μg) for 30 min in binding buffer (DMEM with 1 mM $CaCl_2$, 0.1 mM $CuSO_4$, and 0.5% tryptone). Two hundred ng of soluble forms of VEGFR1 (Flt-1/Fc) or VEGFR2 (KDR/Fc, R&D Systems, Minneapolis, MN) were added for a total volume of 50 μl and incubated for 2 h. The receptor/Fc constructs were precipitated using protein A-Sepharose beads, and the resulting precipitate was washed four times with binding buffer. The pellet and supernatant of each reaction were boiled for 2 min in sample buffer that contained 10 mM DTT to reduce the $F(ab')_2$ constructs and release the receptor/Fc constructs from the protein A-Sepharose beads. These conditions, however, were not harsh enough to completely reduce all of the VEGF from dimer to monomer. The samples were separated by 12% SDS-PAGE and transferred to PVDF membranes. The membranes were then probed with 12D7 (1.0 μg/ml), a mouse anti-VEGF antibody (40), and developed after incubation with peroxidase-labeled goat antimouse IgG (Kirkegaard & Perry Laboratories, Inc.) by Super Signal chemiluminescence substrate (Pierce, Rockford, IL). The soluble receptor/Fc constructs were also detected through the use of peroxidase-conjugated goat antihuman Fc (Kirkegaard & Perry Laboratories, Inc.; data not shown).

Immunoprecipitation and Western Blot Analysis. PAE/KDR, PAE/FLT, and bEND.3 cells were grown to 80–90% confluency in 100-mm tissue dishes in media containing 5% serum. The cells were then serum starved for 24 h in media containing 0.1% serum. After pretreatment with 100 nM sodium orthovanadate in PBS for 30 min, the cells were incubated with 5 nM (200 ng/ml) VEGF165, 5 nM (100 ng/ml) basic fibroblast growth factor (R&D Systems, Minneapolis, MN), or A673 tumor-conditioned media in the presence or absence of control or test antibodies for an additional 15 min. The cells were then washed with ice-cold PBS containing 10 mM EDTA, 2 mM sodium fluoride, and 2 mM sodium orthovanadate and lysed in lysis buffer [50 mM Tris, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 5 mM EDTA, 1.5 mM MgCl2, 2 mM sodium fluoride, 2 mM sodium orthovanadate, 10% glycerol, and protease inhibitors (Complete Protease Inhibitor Cocktail tablets, Boehringer Mannheim)]. The lysates were clarified by centrifugation, and resulting supernatant was used for immunoprecipitation. VEGFR1 and VEGFR2 were immunoprecipitated with 5 μg of chicken anti-FLT-1 NH_2 terminus (Upstate Biotechnology, Lake Placid, NY) or 5 μg of T014 (affinity purified anti-Flk-1), respectively. The reactions using the chicken anti-FLT-1 antibody were subsequently incubated with a bridging goat antichicken antibody (Kirkegaard & Perry Laboratories, Inc.) for 1 h at 4°C. The immune complex was precipitated with protein A/G-Sepharose, washed four times with 10% lysis buffer in PBS-tween (0.2%) and boiled in SDS sample buffer containing 100 mM β-mercaptoethanol and 8 M urea. The samples were separated by SDS-PAGE and transferred to PVDF membranes, which were blocked for 30–60 min with PP81 (East Coast Biologicals, Berwick, ME) and probed for phosphotyrosine residues with 0.5 μg/ml of 4G10 (Upstate Biotechnology, Lake Placid, NY). The membranes were developed after incubation with peroxidase-labeled rabbit antimouse IgG (Dako, Carpinteria, CA) by Super Signal chemiluminescence substrate (Pierce, Rockford, IL). The membranes were then stripped with ImmunoPure Elution buffer (Pierce, Rockford, IL) for 30 min at 55°C and reprobed for receptor levels with either 0.5 μg/ml chicken anti-FLT-1 or 1.0 μg/ml T014 and developed as above after incubation with the appropriate peroxidase-conjugated secondary antibody.

Miles Permeability Assay. The protocol followed was essentially as described by Murohara *et al.* (45). In brief, 400–450-g male, IAF hairless guinea pigs (Charles River, Wilmington, MA) were anesthetized and then injected i.v. with 0.5 ml of 0.5% Evan's blue dye in sterile PBS through an ear vein. Twenty min later, 20 ng of VEGF in the presence or absence of control or test antibodies were injected i.d. The resultant blue spots in the back of the guinea pig were photographed 30 min after the i.d. injections.

***In Vivo* Tumor Growth Inhibition.** Male *nu/nu* mice weighing ~25 g were injected s.c. with either 1×10^7 NCI-H358 NSCLC cells or 5×10^6 A673 rhabdomyosarcoma cells on day 0. On day 1 and twice per week thereafter, the mice were given i.p. injections of 2C3 at 1, 10, or 100 μg or of control immunoglobulin as indicated. The tumors were measured twice per week for a period of ~6 weeks for the NCI-H358-bearing mice and 4 weeks for the A673-bearing mice. Tumor volume was calculated according to the formula: volume $= L \times W \times H$, where L = length, W = width, and H = height.

***In Vivo* Tumor Therapy.** Male *nu/nu* mice bearing s.c. NCI-H358 tumors or HT1080 fibrosarcoma 200–400 mm^3 in size were injected i.p. with test or control antibodies. The NCI-H358-bearing mice were treated at 100 μg/injection three times per week during the first week and twice per week during the second and third weeks. The mice were then switched to 50 μg per injection every 5 days. The HT1080-bearing mice were treated with 100 μg of the indicated antibody or with saline every other day throughout the experiment. In both experiments, mice were sacrificed when their tumors reached 2500 mm^3 in size or earlier if tumors began to ulcerate.

RESULTS

2C3 Blocks VEGF Binding to VEGFR2 but not to VEGFR1 in ELISA. The anti-VEGF antibody 2C3 blocked VEGF from binding to VEGFR2 (KDR/Flk-1) but not to VEGFR1 (Flt-1) in this ELISA assay. In the presence of a 100-fold and 1000-fold molar excess of 2C3, the amount of VEGF that bound to VEGFR2-coated wells was reduced to 26% and 19%, respectively, of the amount that bound in the absence of 2C3 (Fig. 1). In contrast, in the presence of a 100-fold and 1000-fold molar excess of 2C3, the amount of VEGF that bound to VEGFR1-coated wells was 92% and 105%, respectively, of the amount that bound in the absence of 2C3. Similarly, the amounts of VEGF that bound to VEGFR1 or VEGFR2 were unaffected by the presence of a 100- to 1000-fold excess of the nonblocking monoclonal anti-VEGF antibody 3E7 or of a control IgG of irrelevant specificity.

2C3 Blocks VEGF Binding to VEGFR2 but not to VEGFR1 in Solution. The ability of 2C3 to block the binding of VEGF to VEGFR1/Fc or VEGFR2/Fc in solution was assessed in coprecipitation assays. The results are shown in Fig. 2. VEGF mixed with either VEGFR1/Fc or VEGFR2/Fc was coprecipitated by protein A-Sepharose, showing that VEGF binds to both receptors (Fig. 2, *Lanes 6* and *7*). The addition of 2C3 $F(ab')_2$ blocked the binding of VEGF to VEGFR2/Fc (Fig. 2, *Lane 3*) but not to VEGFR1/Fc (Fig. 2, *Lane 4*). In contrast, 4.6.1 $F(ab')_2$ blocked the binding of VEGF to both VEGFR2/Fc (Fig. 2, *Lanes 9* and *11*) and VEGFR1/Fc (Fig. 2, *Lanes 10* and *12*). The results affirm that 2C3 inhibits the binding of VEGF to VEGFR2 but not to VEGFR1.

2C3 Blocks VEGF-induced Phosphorylation of VEGFR2 and ERK 1/2. Fig. 3 shows that 2C3, along with A4.6.1, blocks VEGF-induced phosphorylation of VEGFR2 in PAE/KDR cells. This is in agreement with previous results that demonstrate that both 2C3 and A4.6.1 block VEGF-mediated growth of endothelial cells (40). 3E7, which sees an NH_2-terminal epitope of VEGF, did not block VEGF-induced phosphorylation of VEGFR2, nor did a control IgG of irrelevant specificity. Also shown in Fig. 3 is a Western blot that demonstrates the amounts of VEGFR2 protein in each lane. Densitometric scanning of the gels confirmed that the ratio of phosphotyrosine: protein was reduced in immunoprecipitates derived from cells that had been treated with 2C3 and A4.6.1 but not with 3E7 or IgG of irrelevant specificity. 2C3 also inhibited VEGF-induced phosphorylation of VEGFR2 in bEND.3 cells (data not shown). We were unsuccessful in demonstrating convincing VEGF-induced phosphorylation of VEGFR1 to examine the effect of 2C3 on VEGFR1 activity. As other investigators have shown, VEGF-induced phosphorylation of VEGFR1 in PAE/FLT cells is difficult to demonstrate, possibly because of the low intrinsic kinase activity of VEGFR1 (15, 20, 46, 47).

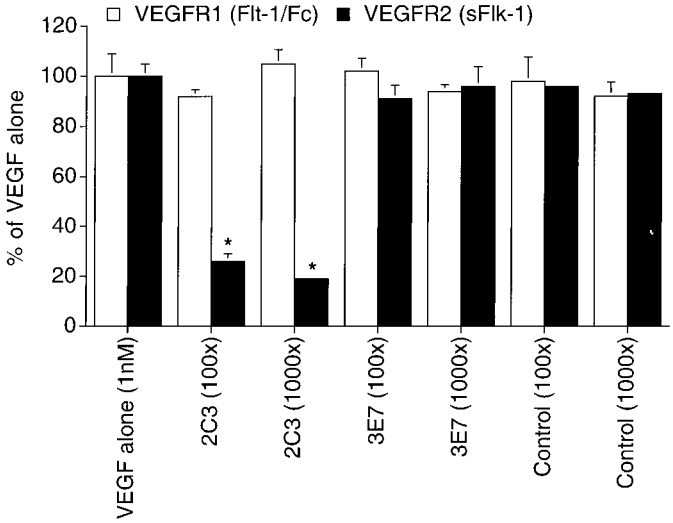

Fig. 1. 2C3 blocks VEGF binding to VEGFR2 but not VEGFR1 in ELISA. Wells were coated with the extracellular domain of VEGFR1 (Flt-1/Fc) or VEGFR2 (sFlk-1) and were then incubated with VEGF alone at 1 nM or VEGF in the presence of the indicated IgG at either 100 nM or 1000 nM. The plate was then incubated with rabbit anti-VEGF (A-20, Santa Cruz Biotechnology, Inc.) at 1 μg/ml and developed using a peroxidase-conjugated goat antirabbit antibody. Assays were performed in triplicate. Mean percent binding in the absence of antibody is shown together with the SD. *, values that are statistically significantly different ($P < 0.002$) from those in the absence of antibody by Student's paired *t* test.

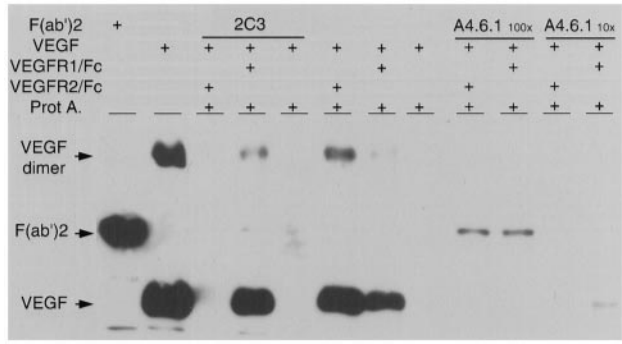

Fig. 2. 2C3 prevents VEGF from associating with VEGFR2 but not VEGFR1 in a coprecipitation assay. Forty ng of VEGF were incubated with 200 ng of extracellular domain of VEGFR1 (Flt-1/Fc) or VEGFR2 (KDR/Fc) in the presence or absence of the indicated $F(ab')_2$. The receptor/Fc constructs were precipitated by incubation with protein A-Sepharose beads. The precipitate was washed and resuspended in reducing sample buffer and separated by 12% SDS-PAGE and transferred to PVDF. The membrane was blocked with PP82 and probed with 12D7 (1 μg/ml) mouse anti-VEGF antibody and developed under standard chemiluminescence conditions. VEGF monomer and dimer along with $F(ab')_2$ are shown.

2C3 Blocks VEGF-induced Vascular Permeability Increases in Guinea Pig Skin. 2C3, which blocks VEGF from activating VEGFR2, inhibited VEGF-induced vascular permeability increases in the Miles permeability assay in guinea pig skin (Fig. 4). This effect was evident with 2C3 at a 10-fold, 100-fold, or 1000-fold molar excess over VEGF. A4.6.1, which blocks VEGF from activating both VEGFR1 and VEGFR2, blocked VEGF-induced permeability at 10-fold molar excess (Fig. 4 and Ref. 42). 3E7, which does not block the VEGF:VEGFR2 interaction, also does not block VEGF-induced vascular permeability increases in the Miles permeability assay. These results suggest that the enhancing effect of VEGF on endothelial permeability is mediated mainly or entirely through VEGFR2 activation. These results accord with those of other investigators who have shown that the tyrosine kinase activity of VEGFR2 is necessary for VEGF-induced permeability (45, 48, 49).

Inhibition of Growth of Newly Implanted Human Tumor Xenografts by 2C3. 2C3 inhibited the *in vivo* growth of both NCI-H358 NSCLC and A673 rhabdomyosarcoma in *nu/nu* mice in a dose-dependent manner (Fig. 5). One hundred μg of 2C3 given i.p. twice per week to mice that had been injected with tumor cells s.c. 1 day earlier inhibited the growth of both human tumor types. The final tumor volume in the 2C3 recipients was ~150 mm^3 in both tumor systems, as compared with ~1000 mm^3 in controls. Treatment with 10 or 1 μg of 2C3 twice per week was less effective at preventing tumor growth. The nonblocking anti-VEGF antibody, 3E7, at a dose of 100 μg twice per week stimulated the growth of A673 tumors (Fig. 5*B*) but not of NCI-H358 tumors (not shown). It is possible that 3E7 increases angiogenesis in A673 tumors by cross-linking VEGF and increasing receptor dimerization.

Treatment of Established Human Tumor Xenografts with 2C3. Mice bearing s.c. NCI-H358 NSCLC tumors that had grown to a size of 300–450 mm^3 were treated with 2C3, A4.6.1, 3E7, or an IgG of

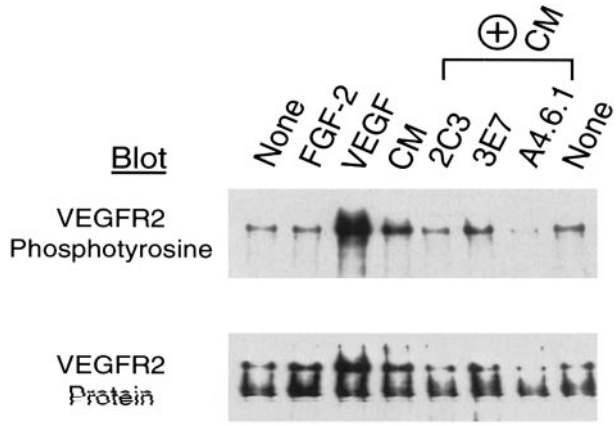

Fig. 3. 2C3 inhibits VEGF-induced phosphorylation of VEGFR2. PAE/KDR cells were stimulated for 15 min with PBS, basic fibroblast growth factor (5 nM, 100 ng/ml), VEGF165 (5 nM, 210 ng/ml), A673-conditioned media (*CM*), or CM + the indicated IgG (100 nM, 15 μg/ml). The cells were than incubated in lysis buffer, and the receptor was immunoprecipitated, separated by SDS-PAGE under reducing conditions, transferred to PVDF membranes, probed with mouse antiphosphotyrosine antibody (4G10), and developed under standard chemiluminescence conditions. The membranes were then stripped and reprobed with the immunoprecipitating IgG (T014) to determine the level of receptor protein in each lane.

irrelevant specificity (Fig. 6*A*). Doses were 50–100 μg given i.p. every 3–5 days. A4.6.1 was used as a positive control because it has been shown by other investigators to block VEGF activity *in vivo*, resulting in an inhibition of tumor growth (33, 35). Treatment with either 2C3 or A4.6.1 led to a slow regression of the tumors over the course of the experiment. The mean tumor volume at the end of the experiment was 30% or 35% of the initial mean tumor volume, respectively. However, these results are complicated by the fact that tumors stopped growing in the control groups of mice between 40 and 60 days after tumor cell injection. Thereafter, the tumors grew progressively. This spontaneous retardation in growth may have contributed to the tumor regressions in the 2C3- and A4.6.1-treated groups. The results up to 40 days, before the spontaneous retardation in growth was evident, show that both 2C3 and A4.6.1 treatment prevent tumor growth.

Fig. 6*B* shows the tumor growth curves of mice bearing a human fibrosarcoma, HT1080, that were every treated every 2 days with 100 μg of 2C3, 3E7, a control IgG of irrelevant specificity, or saline. 2C3 arrested the growth of the tumors for as long as treatment was continued. Tumors in mice treated with 3E7, control IgG, or saline grew progressively to a size that led to sacrifice of the mice less than 4 weeks after tumor cell injection.

No signs of toxicity (weight loss, ruffled fur, behavioral changes) were observed with any of the treatments.

DISCUSSION

The major findings to emerge from this study are: (*a*) 2C3, a monoclonal anti-VEGF antibody, selectively blocks VEGF from binding and activating VEGFR2 but not VEGFR1; (*b*) 2C3 blocks VEGF-induced increases in vascular permeability; and (*c*) 2C3 treatment of mice bearing various types of human tumors can prevent the growth of the tumors.

In vitro binding experiments using ELISA in various configurations and coprecipitation assays with purified receptor proteins demonstrated that 2C3 blocks the binding of VEGF to VEGFR2 but not to VEGFR1. By contrast, 3E7, a nonblocking monoclonal antibody directed against an epitope in the NH_2 terminus of VEGF, did not block VEGF from binding to either VEGFR1 or VEGFR2, and A4.6.1, a blocking anti-VEGF antibody, blocked the binding of VEGF to both VEGF receptors. Crystallographic and mutagenesis studies have shown that the binding epitopes for VEGFR2 and VEGFR1 are concentrated toward the two symmetrical poles of the VEGF dimer (50). The binding determinants on VEGF that interact with the two receptors overlap partially and are distributed over four different segments that span across the dimer surface (51). Antibody 4.6.1 binds to a region of VEGF within the receptor binding region of both receptors (51). Possibly, 2C3 binds to a region that lies close to the VEGFR2 binding site but not to the VEGFR1 binding site.

Using a probe for phosphotyrosine, we demonstrated that 2C3 blocked the VEGF-induced phosphorylation of VEGFR2. However, like other investigators, we were unable to demonstrate consistent VEGF-induced phosphorylation of VEGFR1 (15, 20, 46, 47) and therefore could not reliably judge whether 2C3 inhibits VEGF-induced phosphorylation of VEGFR1. The low activity of VEGF on VEGFR1 phosphorylation has lead others to suggest that VEGFR1 might not be a signaling receptor on endothelial cells (28). However, tyrosine phosphorylation of VEGFR1 by VEGF binding has been reported by Kupprion *et al.* (52) using human microvascular endothelial cells and by Sawano *et al.* (53) using NIH 3T3 cells that overexpress VEGFR1. Additionally, Waltenberger *et al.* (20) have shown that VEGF-induced VEGFR1 activation can be followed using an *in vitro* kinase assay. It is possible that the effect of 2C3 on VEGF-induced phosphorylation of VEGFR1 might be determined using one of these cell types or an *in vitro* kinase assay.

2C3 and 4.6.1 blocked VEGF-induced increases in vascular permeability in the Mile's permeability assay in guinea pigs. The nonblocking anti-VEGF antibody, 3E7, had no effect. These results demonstrate that VEGFR2 is mainly or entirely responsible for mediating VEGF-induced permeability increases. This finding accords with recent findings that a novel form of VEGF-C and two virus-derived VEGF-E variants bind VEGFR2 but not VEGFR1, yet retain the ability to enhance vascular permeability (48, 49, 54). Probably, the various forms of VEGF transmit signals via VEGFR2 that cause NO production, which, in turn, causes the increase in vascular permeability (23–25, 45, 55, 56). However, there is also some evidence to the contrary because Couper *et al.* (57) found a strong correlation between increased vascular permeability induced by VEGF and VEGFR1 expression *in vivo* and Stacker *et al.* (58) found that VEGF could be

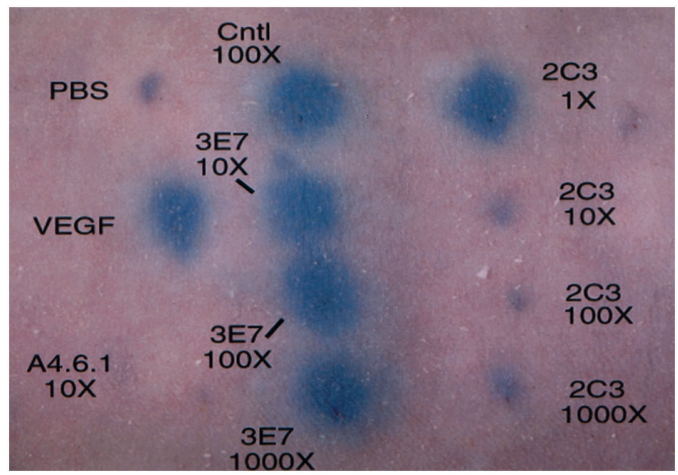

Fig. 4. 2C3 inhibits VEGF-induced permeability. IAF hairless guinea pigs (Hartley strain) 400–450 g in size were anesthetized and injected i.v. with 0.5 ml of 0.5% Evan's blue dye in sterile PBS through an ear vein. Twenty min later, 25 ng of VEGF in the presence or absence of control or test antibodies were injected i.d. The resultant *blue* spots in the back of the guinea pig were photographed 30 min after the i.d. injections. The results shown are representative of three independent experiments.

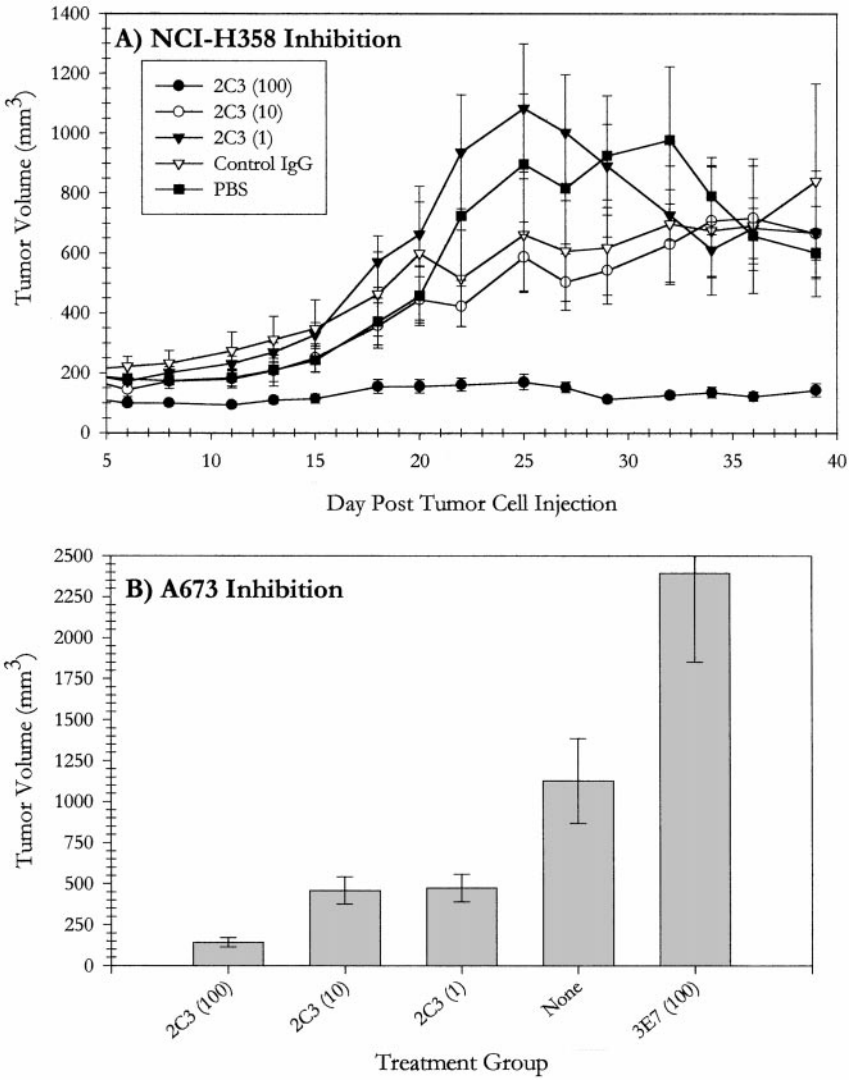

Fig. 5. 2C3 inhibits the *in vivo* growth of human tumor xenografts. NCI-H358 NSCLC (1×10^7; *A*) or A673 rhabdomyosarcoma (5×10^6; *B*) cells were injected s.c. into *nu/nu* mice on day 0. Mice were injected i.p. with the indicated IgG on day 1 and two times a week thereafter. 2C3 was given at a dose of 100, 10, or 1 μg/injection while a control IgG of irrelevant specificity (*A*) and 3E7 (*B*) were also given at 100 μg/injection. Tumors were measured two to three times a week. Mean and SE are shown for the duration of the experiment in *A*, while data for the last day of the experiment (day 26) are shown in *B*.

mutated such that it still activated VEGFR2 but no longer induced an increase in vascular permeability.

Treatment of mice bearing newly transplanted NCI-H358 NSCLC or A673 rhabdomyosarcomas with 2C3 limited the growth of the tumors to small nodules of ~150 mm^3 in size. Similar responses were observed in mice bearing HT29 and LS174T tumors, both human adenocarcinomas of the colon (data not shown). Tumor growth suppression has been demonstrated previously by Mesiano *et al.* (35) and Asano *et al.* (34) for other neutralizing anti-VEGF antibodies, and by Skobe *et al.* (59) for an antimouse VEGFR2 antibody. DC101, a monoclonal anti-Flk-1 antibody, has been shown to prevent the growth of a variety of tumors in mice (60). Additionally, Klement *et al.* (61) have demonstrated that human neuroblastoma tumors grown s.c. in mice can be effectively treated by continuous low dose therapy with vinblastine in combination with DC101. In both studies with DC101, 0.8–1.2 mg of the antibody was given every 3 days, a dose that is 8–12-fold higher than the dose of 2C3 that gave similar antitumor effects in the present studies. Perhaps, in addition to blocking the VEGF:VEGFR2 interaction, 2C3 binds to and cross-links the VEGF:VEGFR1 complex and enhances its reported negative effect on R2-mediated angiogenesis.

Treatment of mice bearing *established* NCI-H358 NSCLC and HT1080 fibrosarcomas with 2C3 caused significant tumor regressions. NCI-H358 tumors treated with 2C3 or A4.6.1 regressed to 30 and 35%, respectively, of their original size after ~10 weeks of treatment. The antitumor effects were attributable to neutralization of tumor-derived (human) VEGF rather than of stromally derived (mouse) VEGF because neither 2C3 nor A4.6.1 bind to mouse VEGF. The fact that regression, rather than tumor stasis, was observed suggests that VEGF is providing more than just a proliferation signal for tumor endothelium. Benjamin *et al.* (62) recently reported that tumors contain a large fraction of immature blood vessels that have yet to establish contact with periendothelial cells and that these blood vessels are dependent upon VEGF for survival. It is possible that neutralization of VEGF causes these immature blood vessels to undergo apoptosis, thereby reducing the existing vascular network in the tumor (62). It is also likely that a dynamic process of vascular remodeling occurs in tumors, involving both vessel formation and vessel regression, and that neutralization of VEGF prevents vessel formation leading to a net shift toward vessel regression. This is supported by Helmlinger *et al.* (63) who have recently shown that VEGF induces elongation, network formation, and branching of nonproliferating endothelial cells under hypoxic conditions. The authors show that inhibition of VEGF activity prevents vessel network formation (63), supporting the view that 2C3 and other anti-VEGF therapies exert their antitumor action by preventing vascular remodeling and endothelial cell survival in addition to preventing endothelial cell proliferation in tumors.

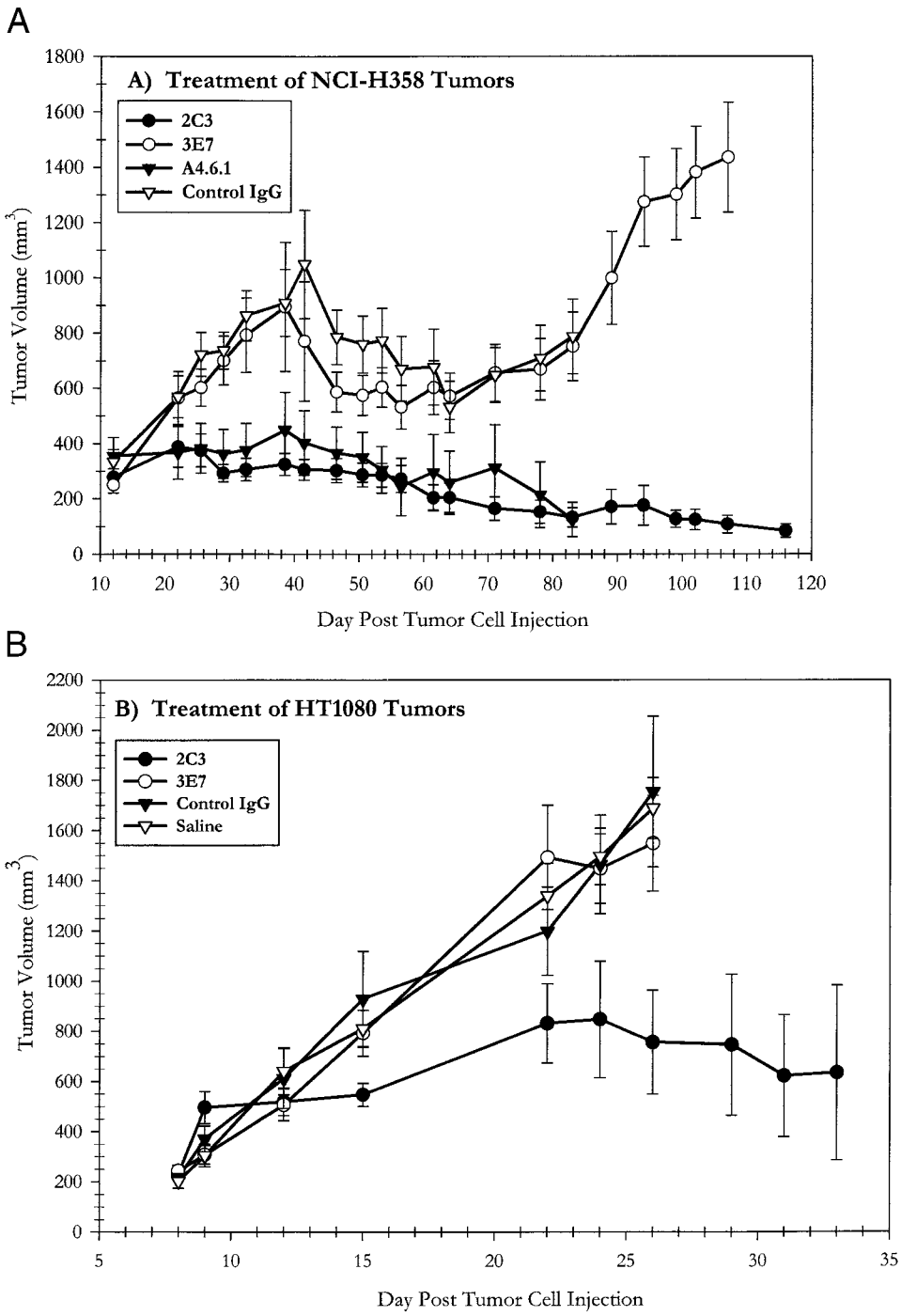

Fig. 6. 2C3 treatment of established human tumor xenografts. *A*, mice bearing s.c. NCI-H358 tumors, ~300–450 mm^3 in size, were treated i.p. with 50 or 100 μg of 2C3 ($n = 14$), monoclonal antibody 4.6.1 ($n = 5$), 3E7 ($n = 12$), or a control IgG ($n = 9$) as indicated in "Materials and Methods." Mean tumor volume along with the SE over a 116-day period is shown. *B*, mice bearing s.c. HT1080 tumors ~200–250 mm^3 in size were treated i.p. with 100 μg of 2C3 ($n = 9$), 3E7 ($n = 11$), control IgG ($n = 11$), or saline ($n = 11$). The mice were treated every other day. The non-2C3-treated mice were sacrificed on day 26 because of >50% of each group having large ulcerated tumors. Mean tumor volume along with the SE is shown.

The finding that 2C3 suppressed tumor growth as completely as did A4.6.1 indicates a dominant role for VEGFR2 in promoting tumor angiogenesis. The multistep process of angiogenesis requires endothelial cell chemotaxis, metalloproteinase production, invasion, proliferation, and differentiation. If VEGFR1 participates at all in these processes, its participation does not appear to limit the overall rate of the angiogenic process. In fact, the opposite may be true: recent evidence indicates that VEGFR1 suppresses VEGFR2 activity (31). VEGFR1 may, however, play an important role in the recruitment of macrophages and monocytes into the tumor because these cells express VEGFR1 and respond chemotactically to VEGF via VEGFR1 signaling (28, 64). 2C3 may therefore have the advantage over A4.6.1 for therapy in which macrophage infiltration is not impaired, enabling these cells to remove tumor cell debris from necrotic tumors and promote tumor shrinkage. Also, it should not interfere with other VEGF-dependent physiological processes that are mediated through VEGFR1, such as the recruitment and differentiation of chondroclasts and other cells involved in cartilage remodeling and bone formation (65).

ACKNOWLEDGMENTS

We thank Dr. E. Helene Sage for review of the manuscript; Dr. Xianming Huang for preparing the $F(ab')_2$ of 2C3 and A4.6.1; Drs. Boning Gao, Claudia Gottstein, and Sophia Ran for helpful discussions; and Linda Watkins for excellent technical assistance.

REFERENCES

1. Springer, M. L., Chen, A. S., Kraft, P. E., Bednarski, M., and Blau, H. M. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. Mol. Cell, *2:* 549–558, 1998.
2. Folkman, J., and Shing, Y. Angiogenesis. J. Biol. Chem., *267:* 10931–10934, 1992.
3. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, *86:* 353–364, 1996.
4. Fidler, I. J., and Ellis, L. M. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell, *79:* 185–188, 1994.
5. Fidler, I. J., Kumar, R., Bielenberg, D. R., and Ellis, L. M. Molecular determinants of angiogenesis in cancer metastasis. Cancer J. Sci. Am., *4* (Suppl. 1)*:* S58–S66, 1998.
6. McNamara, D. A., Harmey, J. H., Walsh, T. N., Redmond, H. P., and Bouchier-Hayes, D. J. Significance of angiogenesis in cancer therapy. Br. J. Surg., *85:* 1044–1055, 1998.
7. Kerbel, R. S., Viloria-Petit, A., Okada, F., and Rak, J. Establishing a link between oncogenes and tumor angiogenesis. Mol. Med., *4:* 286–295, 1998.
8. Mazure, N. M., Chen, E. Y., Yeh, P., Laderoute, K. R., and Giaccia, A. J. Oncogenic transformation and hypoxia synergistically act to modulate vascular endothelial growth factor expression. Cancer Res., *56:* 3436–3440, 1996.
9. Thomas, K. A. Vascular endothelial growth factor, a potent and selective angiogenic agent. J. Biol. Chem., *271:* 603–606, 1996.
10. Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J., *13:* 9–22, 1999.
11. Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature (Lond.), *380:* 435–439, 1996.
12. Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J., and Moore, M. W. Heterozygous embryonic lethality induced by targeted inactivation of the *VEGF* gene. Nature (Lond.), *380:* 439–442, 1996.
13. Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol. Endocrinol., *5:* 1806–1814, 1991.
14. Anthony, F. W., Wheeler, T., Elcock, C. L., Pickett, M., and Thomas, E. J. Short report: identification of a specific pattern of vascular endothelial growth factor mRNA expression in human placenta and cultured placental fibroblasts. Placenta, *15:* 557–561, 1994.
15. de Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science (Washington DC), *255:* 989–991, 1992.
16. Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D., and Bohlen, P. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem. Biophys. Res. Commun., *187:* 1579–1586, 1992.
17. Soker, S., Takashima, S., Miao, H. Q., Neufeld, G., and Klagsbrun, M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform- specific receptor for vascular endothelial growth factor. Cell, *92:* 735–745, 1998.
18. Mustonen, T., and Alitalo, K. Endothelial receptor tyrosine kinases involved in angiogenesis. J. Cell Biol., *129:* 895–898, 1995.
19. Terman, B., Khandke, L., Dougher-Vermazan, M., Maglione, D., Lassam, N. J., Gospodarowicz, D., Persico, M. G., Bohlen, P., and Eisinger, M. VEGF receptor subtypes KDR and FLT1 show different sensitivities to heparin and placenta growth factor. Growth Factors, *11:* 187–195, 1994.
20. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C. H. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J. Biol. Chem., *269:* 26988–26995, 1994.
21. Zachary, I. Vascular endothelial growth factor: how it transmits its signal. Exp. Nephrol., *6:* 480–487, 1998.
22. Korpelainen, E. I., and Alitalo, K. Signaling angiogenesis and lymphangiogenesis. Curr. Opin. Cell Biol., *10:* 159–164, 1998.
23. Hood, J., and Granger, H. J. Protein kinase G mediates vascular endothelial growth factor-induced Raf-1 activation and proliferation in human endothelial cells. J. Biol. Chem., *273:* 23504–23508, 1998.
24. Hood, J. D., Meininger, C. J., Ziche, M., and Granger, H. J. VEGF up-regulates ecNOS message, protein, and NO production in human endothelial cells. Am. J. Physiol., *274:* H1054–H1058, 1998.
25. Kroll, J., and Waltenberger, J. VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). Biochem. Biophys. Res. Commun., *252:* 743–746, 1998.
26. Waltenberger, J., Mayr, U., Pentz, S., and Hombach, V. Functional up-regulation of the vascular endothelial growth factor receptor KDR by hypoxia. Circulation, *94:* 1647–1654, 1996.
27. Kroll, J., and Waltenberger, J. The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. J. Biol. Chem., *272:* 32521–32527, 1997.
28. Hiratsuka, S., Minowa, O., Kuno, J., Noda, T., and Shibuya, M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc. Natl. Acad. Sci. USA, *95:* 9349–9354, 1998.
29. Fong, G. H., Rossant, J., Gertsenstein, M., and Breitman, M. L. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature (Lond.), *376:* 66–70, 1995.
30. Fong, G. H., Zhang, L., Bryce, D. M., and Peng, J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development, *126:* 3015–3025, 1999.
31. Rahimi, N., Dayanir, V., and Lashkari, K. Receptor chimeras indicate that the VEGFR-1 modulates mitogenic activity of VEGFR-2 in endothelial cells. J. Biol. Chem., *278:* 16986–16992, 2000.
32. Siemeister, G., Martiny-Baron, G., and Marme, D. The pivotal role of VEGF in tumor angiogenesis: molecular facts and therapeutic opportunities. Cancer Metastasis Rev., *17:* 241–248, 1998.
33. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., and Phillips, H. S. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. Nature (Lond.), *362:* 841–844, 1993.
34. Asano, M., Yukita, A., Matsumoto, T., Hanatani, M., and Suzuki, H. An anti-human VEGF monoclonal antibody, MV833, that exhibits potent anti-tumor activity *in vivo*. Hybridoma, *17:* 185–190, 1998.
35. Mesiano, S., Ferrara, N., and Jaffe, R. B. Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. Am. J. Pathol., *153:* 1249–1256, 1998.
36. Luo, J. C., Toyoda, M., and Shibuya, M. Differential inhibition of fluid accumulation and tumor growth in two mouse ascites tumors by an antivascular endothelial growth factor/permeability factor neutralizing antibody. Cancer Res., *58:* 2594–2600, 1998.
37. Yuan, F., Chen, Y., Dellian, M., Safabakhsh, N., Ferrara, N., and Jain, R. K. Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. Proc. Natl. Acad. Sci. USA, *93:* 14765–14770, 1996.
38. Borgstrom, P., Hillan, K. J., Sriramarao, P., and Ferrara, N. Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neutralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy. Cancer Res., *56:* 4032–4039, 1996.
39. Borgstrom, P., Bourdon, M. A., Hillan, K. J., Sriramarao, P., and Ferrara, N. Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors *in vivo*. Prostate, *35:* 1–10, 1998.
40. Brekken, R. A., Huang, X., King, S. W., and Thorpe, P. E. Vascular endothelial growth factor as a marker of tumor endothelium. Cancer Res., *58:* 1952–1959, 1998.
41. Huang, X., Gottstein, C., Brekken, R. A., and Thorpe, P. E. Expression of soluble VEGF receptor 2 and characterization of its binding by surface plasmon resonance. Biochem. Biophys. Res. Commun., *252:* 643–648, 1998.
42. Kim, K. J., Li, B., Houck, K., Winer, J., and Ferrara, N. The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. Growth Factors, *7:* 53–64, 1992.
43. Bukovsky, A., Presl, J., Zidovsky, J., and Mancal, P. The localization of Thy-1.1, MRC OX 2 and Ia antigens in the rat ovary and fallopian tube. Immunology, *48:* 587–596, 1983.
44. Rouan, S. K., Otterness, I. G., Cunningham, A. C., Holden, H. E., and Rhodes, C. T. Reversal of colchicine-induced mitotic arrest in Chinese hamster cells with a colchicine-specific monoclonal antibody. Am. J. Pathol., *137:* 779–787, 1990.
45. Murohara, T., Horowitz, J. R., Silver, M., Tsurumi, Y., Chen, D., Sullivan, A., and Isner, J. M. Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. Circulation, *97:* 99–107, 1998.
46. Davis-Smyth, T., Chen, H., Park, J., Presta, L. G., and Ferrara, N. The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade. EMBO J., *15:* 4919–4927, 1996.
47. Landgren, E., Schiller, P., Cao, Y., and Claesson-Welsh, L. Placenta growth factor stimulates MAP kinase and mitogenicity but not phospholipase C-γ and migration of endothelial cells expressing Flt 1. Oncogene, *16:* 359–367, 1998.
48. Joukov, V., Kumar, V., Sorsa, T., Arighi, E., Weich, H., Saksela, O., and Alitalo, K. A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation, and vascular permeability activities. J. Biol. Chem., *273:* 6599–6602, 1998.
49. Ogawa, S., Oku, A., Sawano, A., Yamaguchi, S., Yazaki, Y., and Shibuya, M. A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. J. Biol. Chem., *273:* 31273–31282, 1998.
50. Wiesmann, C., Fuh, G., Christinger, H. W., Eigenbrot, C., Wells, J. A., and de Vos, A. M. Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor. Cell, *91:* 695–704, 1997.
51. Muller, Y. A., Chen, Y., Christinger, H. W., Li, B., Cunningham, B. C., Lowman, H. B., and de Vos, A. M. VEGF and the Fab fragment of a humanized neutralizing antibody: crystal structure of the complex at 2.4 A resolution and mutational analysis of the interface. Structure, *6:* 1153–1167, 1998.
52. Kupprion, C., Motamed, K., and Sage, E. H. SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells. J. Biol. Chem., *273:* 29635–29640, 1998.
53. Sawano, A., Takahashi, T., Yamaguchi, S., Aonuma, M., and Shibuya, M. Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor. Cell Growth Differ., *7:* 213–221, 1996.

54. Meyer, M., Clauss, M., Lepple-Wienhues, A., Waltenberger, J., Augustin, H. G., Ziche, M., Lanz, C., Buttner, M., Rziha, H. J., and Dehio, C. A novel vascular endothelial growth factor encoded by Orf virus, VEGF- E, mediates angiogenesis via signaling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. EMBO J., *18:* 363–374, 1999.
55. Fujii, E., Irie, K., Ohba, K., Ogawa, A., Yoshioka, T., Yamakawa, M., and Muraki, T. Role of nitric oxide, prostaglandins and tyrosine kinase in vascular endothelial growth factor-induced increase in vascular permeability in mouse skin. Naunyn-Schmiedebergs Arch. Pharmacol., *356:* 475–480, 1997.
56. Parenti, A., Morbidelli, L., Cui, X. L., Douglas, J. G., Hood, J. D., Granger, H. J., Ledda, F., and Ziche, M. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. J. Biol. Chem., *273:* 4220–4226, 1998.
57. Couper, L. L., Bryant, S. R., Eldrup-Jorgensen, J., Bredenberg, C. E., and Lindner, V. Vascular endothelial growth factor increases the mitogenic response to fibroblast growth factor-2 in vascular smooth muscle cells *in vivo* via expression of fms-like tyrosine kinase-1. Circ. Res., *81:* 932–939, 1997.
58. Stacker, S. A., Vitali, A., Caesar, C., Domagala, T., Groenen, L. C., Nice, E., Achen, M. G., and Wilks, A. F. A mutant form of vascular endothelial growth factor (VEGF) that lacks VEGF receptor-2 activation retains the ability to induce vascular permeability. J. Biol. Chem., *274:* 34884–34892, 1999.
59. Skobe, M., Rockwell, P., Goldstein, N., Vosseler, S., and Fusenig, N. E. Halting angiogenesis suppresses carcinoma cell invasion. Nat. Med., *3:* 1222–1227, 1997.
60. Prewett, M., Huber, J., Li, Y., Santiago, A., O'Connor, W., King, K., Overholser, J., Hooper, A., Pytowski, B., Witte, L., Bohlen, P., and Hicklin, D. J. Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res., *59:* 5209–5218, 1999.
61. Klement, G., Baruchel, S., Rak, J., Man, S., Clark, K., Hicklin, D. J., Bohlen, P., and Kerbel, R. S. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J. Clin. Invest., *105:* R15–24, 2000.
62. Benjamin, L. E., Golijanin, D., Itin, A., Pode, D., and Keshet, E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J. Clin. Invest., *103:* 159–165, 1999.
63. Helmlinger, G., Endo, M., Ferrara, N., Hlatky, L., and Jain, R. K. Growth factors: formation of endothelial cell networks. Nature (Lond.), *405:* 139–141, 2000.
64. Clauss, M., Weich, H., Breier, G., Knies, U., Rockl, W., Waltenberger, J., and Risau, W. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. J. Biol. Chem., *271:* 17629–17634, 1996.