[CANCER RESEARCH 64, 6616–6625, September 15, 2004]

Increased Plasma Vascular Endothelial Growth Factor (VEGF) as a Surrogate Marker for Optimal Therapeutic Dosing of VEGF Receptor-2 Monoclonal Antibodies

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ABSTRACT

A major obstacle compromising the successful application of many of the new targeted anticancer drugs, including angiogenesis inhibitors, is the empiricism associated with determining an effective biological/therapeutic dose because many of these drugs express optimum therapeutic activity below the maximum tolerated dose, if such a dose can be defined. Hence, surrogate markers are needed to help determine optimal dosing. Here we describe such a molecular marker, increased plasma levels of vascular endothelial growth factor (VEGF), in normal or tumor-bearing mice that received injections of an anti-VEGF receptor (VEGFR)-2 monoclonal antibody, such as DC101. Rapid increases of mouse VEGF (*e.g.*, within 24 hours) up to 1 order of magnitude were observed after single injections of DC101 in non–tumor-bearing severe combined immunodeficient or nude mice; similar increases in human plasma VEGF were detected in human tumor-bearing mice. RAFL-1, another anti-VEGFR-2 antibody, also caused a significant increase in plasma VEGF. In contrast, increases in mouse VEGF levels were not seen when small molecule VEGFR-2 inhibitors were tested in normal mice. Most importantly, the increases in plasma VEGF were induced in a dose-dependent manner, with the maximum values peaking when doses previously determined to be optimally therapeutic were used. Plasma VEGF should be considered as a possible surrogate pharmacodynamic marker for determining the optimal biological dose of antibody drugs that block VEGFR-2 (KDR) activity in a clinical setting.

INTRODUCTION

One of the newest anticancer treatment strategies to reach advanced clinical development is antiangiogenic therapy (1). The rationale for this therapeutic approach is based on the requirement of new blood vessel formation for relentless expansion of tumor mass and hence the possibility to control tumor growth for extended periods of time by inhibiting this process, known as tumor angiogenesis (2). There are now a large number of antiangiogenic drugs being evaluated in clinical trials for both safety and antitumor treatment efficacy (1). Among the most advanced of these drugs are those that block the proangiogenic function of vascular endothelial growth factor (VEGF) (3).

The VEGF family consists of VEGF-A, also called VEGF, which is produced in a number of splice variant isoforms, as well as VEGF-B, VEGF-C, and VEGF-D (4). VEGF-A is considered the most important of these with respect to tumor angiogenesis, especially the $VEGF_{121}$ and $VEGF_{165}$ splice variant isoforms (4). Many tumor types express abundant levels of VEGF, and the tumor cell population itself is usually the main source of this growth factor; infiltrating host stromal cells may also make a significant contribution in some cases (4). Both environmental and epigenetic factors, especially hypoxia (5) and genetic changes, including mutations that lead to activation of oncogenes or inactivation of tumor suppressor genes, can lead to VEGF induction or up-regulation in tumor cells (6). $VEGF_{121}$ and $VEGF_{165}$ can be secreted and bind to specific high-affinity receptor tyrosine kinases expressed by activated endothelial cells of newly formed blood vessels (4). These receptors are known as VEGF receptor (VEGFR)-2, also called KDR (in humans) or flk-1 (in mice), and VEGFR-1, or flt-1 (4). VEGFR-2 appears the more functionally important of the two and can mediate signaling events involved in endothelial cell mitogenesis, migration, survival, and vascular permeability (4). In addition, VEGF can mobilize endothelial precursor cells out of the bone marrow and into the peripheral circulation, where they can differentiate and become incorporated into sites of ongoing angiogenesis (4, 7).

Given the importance of the VEGF family and VEGFRs in angiogenesis, including tumor angiogenesis, considerable effort has been made to develop molecularly targeted therapies to block VEGF function. Such drugs include humanized monoclonal antibodies to VEGF (3, 4), chimeric or humanized monoclonal antibodies to VEGFR-2 (8) and VEGFR-1, and a variety of small molecule VEGFR-2 inhibitors, which include synthetic organic receptor kinase antagonists, soluble receptor proteins, and antisense-based drugs (4). These drugs have highly reproducible antiangiogenic and antitumor properties in preclinical models and usually result in either significant tumor growth delays or stabilization of disease when used as monotherapies (4). They rarely cause rapid tumor regression, *i.e.*, they are not overtly cytotoxic agents, and in some cases they may not have dose-limiting toxicities either. In these respects, they are similar to certain other molecularly targeted anticancer drugs such as anti-oncogene–directed signal transduction inhibitors (9). Not surprisingly, therefore, antiangiogenic drugs have the handicap of lacking conventional activities normally used to assess optimal dosing and monitoring antitumor efficacy, such as induction of significant tumor regressions ("objective responses") that are used in phase I or II clinical trials for evaluating the effects of cytotoxic agents. Consequently, evaluation of this new class of drugs in such clinical trials can be difficult (9). This is compounded by the fact that maximal antitumor activity may not coincide with dose-limiting toxicity and the maximum tolerated dose, but rather with lower drug doses, *i.e.*, the optimal (or effective) biological dose (9). Thus, molecular, cellular, or functional imaging pharmacodynamic surrogate markers of antiangiogenic activity are urgently needed to help circumvent these problems and increase the probability of successful implementation of antiangiogenic drugs in the clinic. Some promising possibilities currently include detection of reduction in blood flow and

Received 2/5/04; revised 6/17/04; accepted 7/21/04.

Grant support: Grants from the National Cancer Inst. of Canada), 41223), and the Canadian Institutes of Health Research (R. Kerbel) and a sponsored research contract with ImClone Systems, Inc. G. Bocci was financially supported by a fellowship from the Sunnybrook Trust for Medical Research. U. Emmenegger was financially supported by the Swiss National Science Foundation and the Swiss Cancer League/Oncosuisse (BIL SKL 1237-02-2002). S. Green was financially supported by an OGSST scholarship from the Government of Ontario, Canada. R. Kerbel was financially supported by a Canada Research Chair.

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vascular permeability by dynamic contrast enhanced-magnetic resonance imaging (10) and reductions in the levels of circulating bone marrow-derived circulating endothelial progenitor cells (11).

The purpose of this study is to report a possible pharmacodynamic molecular marker that is easy to measure and that may be exploited as a surrogate marker to help determine optimal therapeutic dosing for a particular antiangiogenic drug that blocks VEGF function, namely, monoclonal antibodies to VEGFR-2. During the course of studies designed to evaluate the possible changes in VEGF expression as a result of administering continuous low-dose (metronomic) chemotherapy as an antiangiogenic treatment strategy (12–14), we unexpectedly discovered significant and rapidly induced dose-dependent increases in the levels of circulating plasma VEGF after administration of a neutralizing antimouse VEGFR-2 antibody, DC101, the magnitude of which correlated with the efficacy of antitumor therapy using various drug doses including the optimal biological/therapeutic dose of the drug. In addition, another antibody directed to VEGFR-2 was also found to cause rapid and marked increase in plasma VEGF levels.

MATERIALS AND METHODS

Materials and Drugs

All chemicals not listed in this section were obtained from Sigma Chemical Co. (St. Louis, MO). Cyclophosphamide (Carter Horner Inc., Mississauga, Canada), paclitaxel (PTX; Bristol-Myers Squibb, Montreal, Canada), and vinblastine sulfate (VBL; Ely Lilly, Toronto, Canada) were purchased from the Sunnybrook hospital pharmacy. DC101 and MF-1 are neutralizing monoclonal antibodies directed against mouse VEGFR-2 and VEGFR-1, respectively (ImClone Systems, Inc, New York, NY). The rat monoclonal antibody RAFL-1 against mouse VEGFR-2 was described recently (15). Normal saline and nonspecific rat IgG (ImClone Systems, Inc.) were used as controls. PTK787 (Novartis, Basel, Switzerland; ref. 16) and SU5416 (SUGEN, South San Francisco, CA; ref. 17), partially selective inhibitors of the VEGFR-2 tyrosine kinase, were dissolved in dimethyl sulfoxide. PTK787 was diluted 1:20 with 1% Tween 80 in sterile 0.9% NaCl solution.

Tumor Cell Lines, Tumors, and Animals

The human prostate cancer cell line PC-3 and the human colorectal adenocarcinoma cell line HT-29 were from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum (FBS; Gibco Invitrogen Corp., Burlington, Canada) and McCoy's 5A medium (Gibco) with 10% FBS, respectively. The human breast cancer cell line MDA-MB-231 was obtained from Dr. Jeff Lemontt (Genzyme Corp., Cambridge, MA) and maintained in 5% FBS and RPMI 1640.

To determine human VEGF protein levels in conditioned medium from the PC-3 and MDA-MB-231 tumor cell lines, cells were plated at a density of 10^5 cells/0.5 mL/well in a 24-well plate and allowed to reach 80% confluence, at which point the medium was replaced by RPMI 1640 or Dulbecco's modified Eagle's medium/1% FBS/10 units/mL heparin. Medium was collected after 24 hours, and cells were counted as described previously (18); the final concentration of human VEGF was normalized for 10^6 cells.

Male and female CB-17 severe combined immunodeficient (SCID) mice (6–8 weeks old) were purchased from Charles River (Charles River, Canada). PC-3 cells (2×10^6 cells/0.2 mL) were injected subcutaneously in the flank of each male mouse, and MDA-MB-231 cells (2×10^6 cells/50 μL) were implanted orthotopically into the mammary fat pad of each female mouse, whereas HT-29 cells (5×10^6) mixed with Matrigel (Collaborative Research Biochemicals, Bedford, MA) were injected subcutaneously into the left flank of female athymic (*nu/nu*) mice (Harlan Sprague Dawley, Indianapolis, IN). The mice were randomized into groups of five, and tumor volumes and animal weights were measured as described previously (19). When the primary tumor volume reached 150 to 200 mm^3, therapy was initiated. Institutional guidelines were strictly followed for maintenance of animals and end point of tumor studies. The mice were sacrificed when the tumor volume reached approximately 1,700 mm^3.

In vivo Mouse and Human VEGF Kinetic Study after Administration of Anti-VEGFR-2 and Anti-VEGFR-1 Antibodies or Anti-VEGFR-2 Small Molecule Drugs

To obtain plasma samples, mice were anesthetized with isofluorane and bled from the retroorbital sinus or cardiac puncture. The blood samples were collected in Microtainer plasma separating tubes (Becton Dickinson, San Jose, CA) and centrifuged at 4°C, and plasma was frozen immediately and stored at −20°C until assayed. All capillary tubing, syringes, and needles used for bleeding were first rinsed with heparin to avoid any clotting, because this can result in release of platelet-derived VEGF. All of the samples were thawed at 4°C only at the time of the enzyme-linked immunosorbent assay (ELISA) analysis.

To determine the kinetics of murine-derived VEGF release after a single intraperitoneal injection of different neutralizing antibodies, escalating doses of DC101 (from 100 to 2,000 μg/mouse; five mice per group), RAFL-1 (from 50 to 100 μg/mouse; five mice per group), and MF-1 (from 0.2 to 2.0 mg/mouse; three mice per group) were administered to the non–tumor-bearing and MDA-MB-231 tumor-bearing SCID mice (6–8 weeks old), and blood was collected from all of the mice after 24 hours.

In a separate experiment, to determine the kinetics of murine-derived VEGF release after DC101, RAFL-1, or MF-1 administration, SCID mice (6–8 weeks old) were treated at days 0, 3, and 6 (*i.e.*, every 3 days) with DC101 (800 μg/mouse intraperitoneally; five mice per group), RAFL-1 (100 μg/mouse intraperitoneally; five mice per group), or MF-1 (1.2 mg/mouse intraperitoneally; three mice per group), whereas the control groups were given saline intraperitoneally. At day 0 (*i.e.*, before treatment) and each day after the injection until day 12 and later at days 15, 18, and 21, five or three mice per time point were anesthetized, bled, and sacrificed.

Furthermore, to investigate a possible correlation between the administration of small molecule VEGFR-2 tyrosine kinase inhibitors and plasma mouse VEGF levels, SCID mice (6–8 weeks old) were also treated with (*a*) 25 mg/kg/mouse SU5416 intraperitoneally (17, 20) or vehicle alone (five mice per group) twice a week, and blood was collected at day 0 (before the treatment) and days 1, 4, 5, 8, and 12 after the beginning of the treatment; or (*b*) 50 mg/kg/mouse PTK787 (ref. 16; orally; by gavage; five mice per group) every day for 12 days, and blood was collected before treatment, 0.5 to 2 hour(s) after treatment, and from day 1 to day 21.

To confirm the previously published (21) DC101 optimal therapeutic dose in an *in vivo* tumor model, HT-29–injected animals were treated with escalating intraperitoneal doses of DC101 alone (200, 400, 800, and 1,200 μg/mouse) every 3 days for 36 days; intraperitoneal rat IgGs were used as a control.

In vivo Mouse VEGF Kinetic Comparative Study between Two Different Strains of Mice after DC101 Administration

Non–tumor-bearing SCID and NIH Swiss athymic nude mice (6–8 weeks old; Taconic Laboratories, Germantown, NY) were treated every 3 days with intraperitoneal 800 μg/mouse DC101 for 28 days and with intraperitoneal rat IgG at the same concentration as a control (five mice per group). All of the mice of both strains were simultaneously bled at weekly intervals up to 28 days, and the plasma was immediately stored and processed as described above.

Combination DC101

Metronomic Chemotherapy Schedules and Blood Samples. The antitumor/antiangiogenic treatments were performed on the basis of previously published experiments by Klement *et al.* (22) and Man *et al.* (19) as follows.

Treatment groups for the MDA-MB-231 orthotopic xenograft experiments (five mice per group) were as follows: (*a*) control group, control mice were given normal saline in their drinking water daily and intraperitoneally every 3 days; (*b*) DC101 monoclonal antibody, the DC101 antibody was administered at 800 μg/mouse intraperitoneally every 3 days; (*c*) low-dose metronomic schedule of continuous oral administration of cyclophosphamide through drinking water (an estimated cyclophosphamide dose of 20 mg/kg/d); (*d*) low-dose metronomic schedule of intraperitoneal administration of vinblastine, 0.3 mg/kg/mouse (1 mg/m^2) VBL sulfate was injected intraperitoneally three times weekly; (*e*) low-dose metronomic schedule of intraperitoneal administration of PTX, 1 mg/kg/mouse PTX was administered intraperitoneally three times weekly; (*f*) combination therapy with DC101 and cyclophosphamide; (*g*) combination therapy with DC101 and VBL; and (*h*) combination therapy with DC101 and PTX.

PC-3 Subcutaneous Xenograft Experiment

The treatments were performed as described in the previous experiment, with the exception of the DC101 intraperitoneal schedule (the antibody was administered twice a week, rather than every 3 days) and the absence of the PTX and PTX+DC101-treated groups.

Before treatment and on days 14 to 51 days after initiation of treatment, all tumor-bearing SCID mice were anesthetized and bled.

Murine and Human VEGF Detection in Culture Media and Plasma Samples by ELISA

Each culture supernatant and plasma sample was assayed twice (two replicates) for human and murine VEGF concentrations by an ELISA that measures the free (soluble) forms of VEGF. The plasma samples were diluted (1:5 of the specific diluent) and tested; the absorbance was determined using the microplate reader Benchmark Plus (Bio-Rad Laboratories, Canada) set to 450 nm with a wavelength correction set to 540 nm.

The human VEGF ELISA kit was purchased from R&D Systems (Quantikine; R&D Systems, Minneapolis, MN). This kit detects $VEGF_{165}$ levels in cell culture supernatants and plasma with a limit of detection of less than 5 and 9 pg/mL, respectively.

The murine VEGF ELISA kit was purchased from R&D Systems (Quantikine M murine). It recognizes both 164- and 120-amino acid isoforms of mouse VEGF; the minimum detectable concentration of mouse VEGF is <3 pg/mL.

Cross-Reactivity Tests

To investigate possible cross-reactions in both the human and mouse ELISA assays, increasing amounts of DC101, MF-1, and RAFL-1 (from 0 ng/mL to 1 mg/mL) were assayed in manufacturer's diluent as well as in mice plasma.

Immunodepletion Experiments

Protein G-Sepharose 4 Fast Flow beads (Pharmacia Biotech, Uppsala, Sweden) were washed twice in PBS before being reconstituted to 50% (v/v) protein G-Sepharose in PBS. To deplete plasma samples of DC101 antibody, 100 μL of protein G slurry were added to 200 μL of murine plasma samples pooled from each treatment group (five mice per group) and incubated at 4°C for 4 hours. After centrifugation (2 minutes at 10,000 rpm), 200 μL of plasma supernatants were removed, and the immunodepletion was repeated by the addition of 100 μL of protein G slurry and overnight incubation at 4°C. Immunodepleted plasma supernatants were collected for subsequent analysis by ELISA for mouse VEGF.

Western Blotting

Protein in pooled plasma samples was quantified by Bradford assay (Bio-Rad, Hercules, CA), and 50 μg of denatured protein from each sample were resolved on a 10% SDS-PAGE gel under reducing conditions. Proteins were transferred onto polyvinylidene membranes (Immobilon-P; Millipore, Bedford, MA), blocked in 10% nonfat milk in TBS-T buffer for 1 hour, and blotted with horseradish peroxidase-conjugated goat antirat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in 5% milk in TBS-T for 1 hour. Membranes were rinsed with TBS-T, and then proteins were visualized by chemiluminescence (Amersham, Piscataway, NJ).

Aggregation- or Non-Aggregation-Associated Platelet VEGF Release

Platelet-rich plasma was prepared as described previously (23). Briefly, blood was collected from SCID mice (6–8 weeks old) by cardiopuncture with a 1-mL syringe that contained 3.4% sodium citrate [8:1 (v/v)]. The blood was centrifuged at 200 $\times$ *g* for 15 minutes. The platelet-rich plasma was removed by aspiration. The remaining blood was further centrifuged at 400 $\times$ *g* for 10 minutes to obtain platelet-poor plasma. The number of platelets in platelet-rich plasma was counted and adjusted to 2.5×10^8/mL with Tyrode buffer. Platelet-poor plasma was also diluted correspondingly, in the same way. Platelet suspension was incubated with control IgG or DC101 (80 and 160 μg/mL) at 37°C for 10 minutes or 2 hours. The supernatants were collected after centrifugation (9,000 $\times$ *g*) and stored at −70°C until the concentrations of VEGF were measured. In another set of experiment, 0.5 unit/mL thrombin was added to the platelet suspension incubated with control IgG and DC101 for 10 minutes and 2 hours. Platelet aggregation was monitored by using a platelet aggregometer, and the supernatant of the resulting aggregate of platelets was collected after centrifugation and stored at −70°C until ELISA for mouse VEGF was performed.

RNA Isolation and Northern Blotting

At day 12 of treatment, both control and DC101-treated mice (injected every 3 days; two mice per group; 6–8 weeks old) were sacrificed. The liver, heart, lungs, kidneys, and skeletal muscles were explanted and immediately collected in tubes containing RNAlater (Ambion, Austin, TX) and stored at 4°C. Total RNA was isolated from the tissues using the Trizol Kit (Gibco Invitrogen Corp.). Northern blotting was carried out as described previously (24). Briefly, 20 μg of total RNA were size fractionated on a 1% agarose-formaldehyde gel and transferred onto Hybond N^+ filters (Amersham). Mouse VEGF, VEGFR-2, and β-actin probes were generated by reverse transcription-polymerase chain reaction, gel purified, [α-^{32}P]dCTP labeled, and hybridized to filters overnight at 42°C. Filters were washed twice (20 minutes each) in 1$\times$ SSC/0.1% SDS at room temperature followed by two washes (20 minutes) in 0.25$\times$ SSC/0.1% SDS at 60°C and autoradiographed for at least 2 weeks.

Mouse VEGF mRNA ELISA

Tissues from DC101 (800 μg/injection/mouse every 3 days, 4 doses) or normal saline-treated SCID mice (6–8 weeks old) were removed and stored in RNAlater (Ambion) at −4°C until processing. Bone marrow cells flushed from both femoral bones were stored at −80°C without further manipulation. Total RNA was obtained using Trizol. The amount of mouse VEGF RNA was determined by the Quantikine mRNA kit (R&D Systems) following the manufacturer's instructions. Two micrograms of RNA were loaded per well. The analysis was performed in duplicates with two to three animals per group.

Statistical Analysis

Results of the ELISA assays are reported as the mean ± SD. Statistical significance of differences was assessed by the one-way analysis of variance, followed by the Student-Newman-Keuls test, when the control group was compared with two or more treated groups or two or more dose levels. The level of significance was set at $P < 0.05$. Student's *t* test was performed to compare the control and MF-1– or RAFL-1–treated sample of the same day (two groups) in the 21-day experiments. All statistical calculations were performed using the GraphPad Prism software package version 4.0 (GraphPad Software Inc., San Diego, CA). In case of pooled samples (depleted and undepleted), no mean and SD could be calculated, and further statistical analysis was not possible. Linear regression analysis between "days of the experiment" and "plasma human VEGF concentration" in the MDA-MB-231 experiment was assessed by GraphPad Prism software package version 4.0.

RESULTS

Dose-Dependent Effects of DC101 on Plasma VEGF Levels. While undertaking experiments to determine whether administration of low-dose chemotherapy might influence levels of plasma VEGF, we unexpectedly discovered that injection of DC101 could cause a rapid and marked rise in levels of circulating VEGF. We therefore sought to determine whether there was a correlation between relative increases in plasma VEGF induced by DC101 with the known optimal biological/therapeutic dose of the antibody, which has been previously shown to be approximately 800 μg/mouse, given every 3 days (21). Specifically, we asked whether there was a dose-dependent increase of plasma VEGF that peaks when mice are given approximately 800 μg to 1.2 mg of DC101, without commensurate increases in VEGF levels at higher antibody dosages. If such was the case, it would suggest the possibility of exploiting maximal increases in plasma VEGF as a surrogate marker to guide optimal dosing of antibody drugs similar to DC101 in a clinical setting.

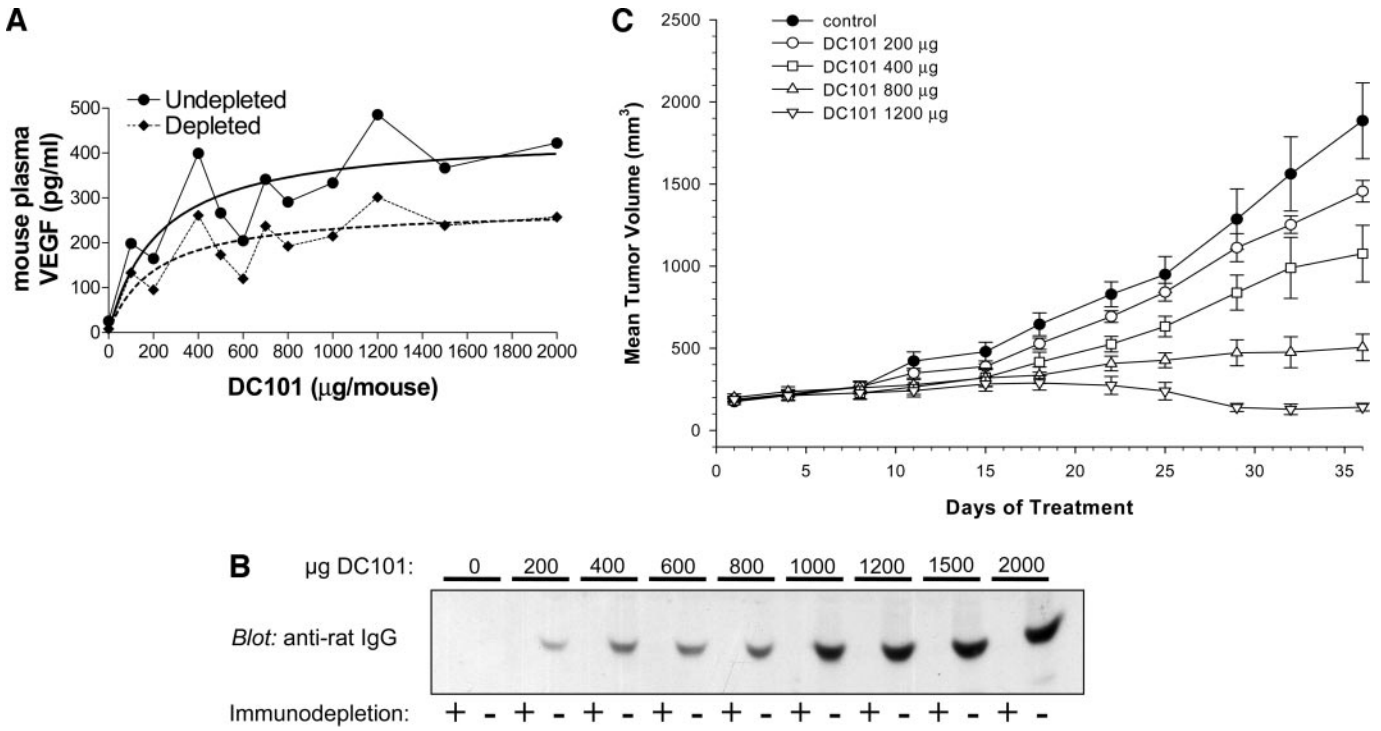

Fig. 1. *A*, plasma mouse VEGF concentrations (24 hours) after escalating single DC101 doses (0–2,000 μg/mouse; five mice per group; age of mice, 6–8 weeks) in non–tumor-bearing SCID mice in undepleted and immunodepleted pooled samples (from five plasma samples). *B*, Western blot analysis of undepleted and depleted plasma samples showing the presence and absence of the DC101 antibody, respectively; membranes were blotted with horseradish peroxidase-conjugated goat antirat IgG antibody. +, immunodepleted plasma samples; −, undepleted samples. *C*, dose-dependent growth inhibition of HT-29 colorectal tumor xenografts after administration of escalating doses of DC101 every 3 days (five mice per group; age of mice, 6–8 weeks). *Symbols and bars*, mean ± SD.

D. Hicklin[5] has found that after an intraperitoneal injection of 800 μg of DC101, the plasma maximal concentration of the drug was 160 μg/mL, which was reached at 4 hours after injection. DC101 plasma disposition revealed an absorption half-life of 1 hour and a distribution half-life of 29 hours. Based on these results, and to ensure that our plasma VEGF detection methods would be accurate, extensive tests were performed to determine whether DC101 demonstrated any cross-reactivity with the detection kits used to measure VEGF levels. At all tested concentrations (from 2 ng to 1 mg), DC101 alone produced no detectable signal in an ELISA kit used to detect human VEGF; in contrast, DC101 started to show a colorimetric response (*i.e.*, cross-reactivity) with the mouse VEGF ELISA kit at a concentration of 200 μg/mL. As such, to avoid any possible overestimation of mouse VEGF concentrations, immunodepletion was performed on plasma samples to remove any DC101 antibody present before assaying VEGF levels.

Neither of the other available antibodies (RAFL-1 and MF-1) showed detectable signals at all of the tested concentrations, confirming the absence of any cross-reactivity when tested on human and mouse VEGF ELISA kits.

Escalating doses of DC101 in non–tumor-bearing mice (Fig. 1*A*) showed a significant increase of mouse VEGF after 24 hours in all of the treated mice when compared with the control groups (which had undetectable levels), reaching a plateau around the experimentally determined optimal therapeutic dose of 800 to 1,200 μg/mouse. Indeed, escalating doses of DC101 in HT-29 tumor-bearing mice (Fig. 1*C*) show a marked dose-dependent antitumor activity, with a maximum effect between 800 and 1,200 μg/mouse. Thus, a dose of 200 or 400 μg only caused slight growth delays, whereas tumor stabilization was induced by 800 μg. A transient regression followed by stabilization was observed using the 1.2 mg dose. A further increase of the administered dose did not result in a corresponding elevation in the mouse VEGF concentrations detected (Fig. 1*A*). The differences in VEGF concentration between undepleted and depleted samples can be ascribed to the aforementioned cross-reactivity of DC101 with the mouse VEGF ELISA test. Fig. 1*B* shows a Western blot detecting the presence of DC101 in undepleted plasma samples and its total absence in plasma samples that had undergone immunodepletion before testing by ELISA.

Furthermore, escalating doses of RAFL-1, a new neutralizing antibody against VEGFR-2 (15), showed a similar significant 24-hour increase of plasma VEGF levels at known biologically active doses (Fig. 2*A*), with a maximum concentration of 66.88 ± 12.67 pg/mL after the injection of 100 μg/mouse.

A similar trend in plasma VEGF levels was found in MDA-MB-231 human breast tumor-bearing mice when levels of human (*i.e.*, tumor-secreted) VEGF levels were evaluated and normalized for the tumor volumes. Before injection into SCID mice, the human tumor cell lines MDA-MB-231 and PC-3 were tested for VEGF production and secretion. The MDA-MB-231 breast cell line secreted substantial amounts of human $VEGF_{165}$ into the conditioned medium (*e.g.*, 7.931 ng/mL/10^6 cells), whereas the prostate cancer cell line PC-3 we used secreted barely detectable quantities (*e.g.*, 0.101 ng/mL/10^6 cells; Fig. 2*B*). As a result, MDA-MB-231 was selected as a preferable human tumor xenograft model for use in this particular set of experiments. The maximum increase of the human VEGF to tumor volume ratio accompanied the administered dose of approximately 700 to 800 μg DC101 antibody/mouse but did not increase with higher doses of the antibody (Fig. 2*C*).

In contrast to the results seen with the anti-VEGFR-2 antibody, no substantial increase of mouse VEGF was found in plasma 24 hours

[5] D. Hicklin *et al.*, unpublished observations.

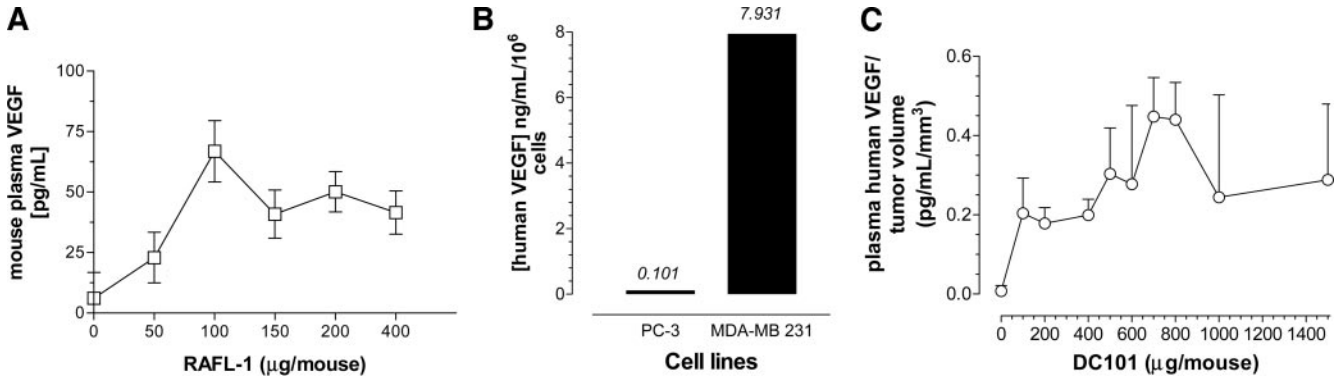

Fig. 2. *A*, plasma mouse VEGF concentrations (24 hours) after single RAFL-1 escalating doses in non–tumor-bearing SCID mice. *Symbols and bars*, mean ± SD; five mice per group; age of mice, 6–8 weeks; $P < 0.05$, treated *versus* untreated control group, one-way analysis of variance, followed by the Student-Newman-Keuls test; two replicates per sample. *B, in vitro* human VEGF secretion by human PC-3 prostate and MDA-MB-231 breast cancer cell lines into conditioned medium. The results were the mean of two different experiments and were normalized for 10^6 cells (two replicates per sample). *C*, plasma human VEGF to tumor volume ratios after escalating single DC101 doses (0–1,500 μg/mouse) in MDA-MB-231 tumor-bearing mice after 24 hours. *Symbols and bars*, mean ± SD; five mice per group; age of mice, 6–8 weeks; $P < 0.05$, treated *versus* untreated control group, one-way analysis of variance, followed by the Student-Newman-Keuls test; two replicates per sample.

after the injection of a monoclonal antibody targeting VEGFR-1, MF-1, at any tested concentration (see below).

Plasma Mouse and Human VEGF Kinetics after Administration of Anti-VEGFR-2 and Anti-VEGFR-1 Antibodies and Anti-VEGFR-2 Small Molecule Antagonists. To further investigate the kinetics of the mouse VEGF increase in plasma samples after DC101 or MF-1 injections, a time course experiment was performed for 21 days, with daily monitoring throughout a three-injection schedule (in the first week) and 2 weeks after discontinuation of treatment. Within 24 hours after the first DC101 intraperitoneal administration in non–tumor-bearing SCID mice, mouse VEGF levels reached a concentration of 89.14 pg/mL in antibody-depleted pooled samples (Fig. 3*A*); after two injections (at day 3 and 6), the VEGF levels increased until they reached the maximum concentration at day 15 (121.14 pg/mL). After day 15, with the discontinuation of the therapy, the plasma VEGF levels slowly decreased to a value of 51.15 pg/mL at day 21 (Fig. 3*A*). The control mice (saline treated) did not show any increase in VEGF levels, ranging around the limit of detection of the ELISA

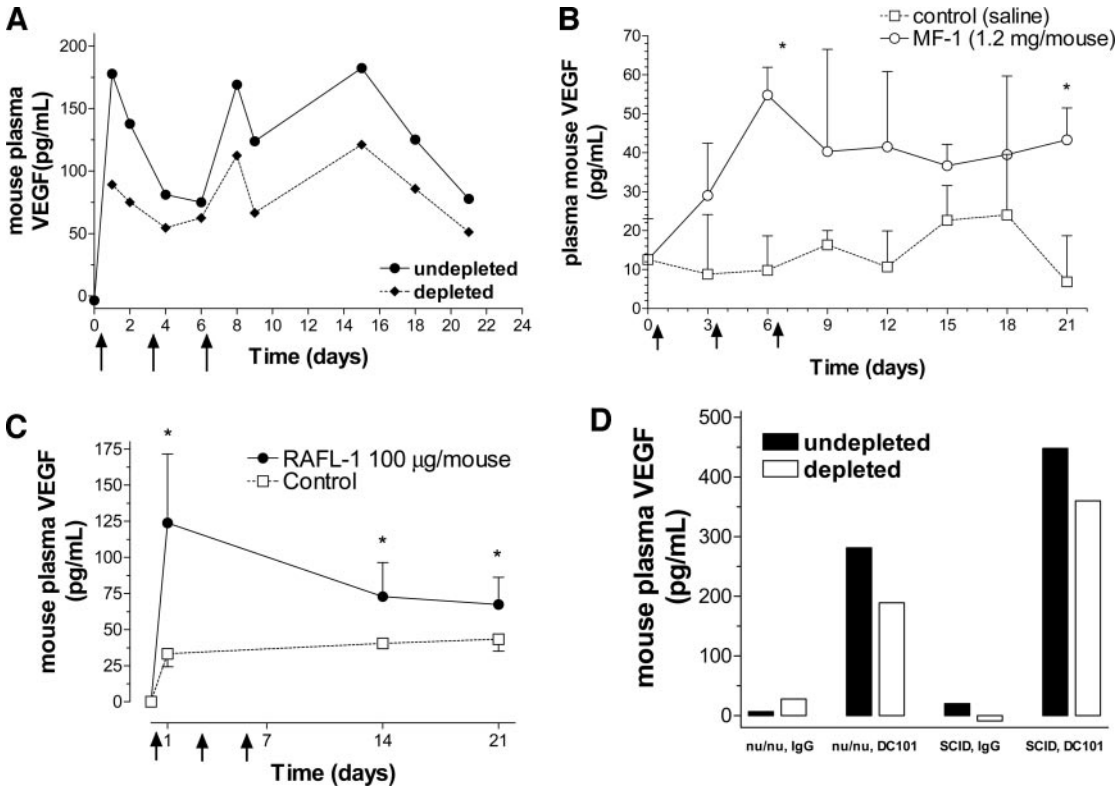

Fig. 3. *A*, plasma mouse VEGF kinetics during and after a three-injection DC101 schedule in non–tumor-bearing SCID mice (five mice per group; age of mice, 6–8 weeks) in immunodepleted and undepleted pooled samples (from five plasma samples); controls samples were at the limit of detection of the ELISA kit. *B*, plasma mouse VEGF concentrations after three-injection MF-1 schedule. *Symbols and bars*, mean ± SD; *arrows*, drug injections; three mice per group; age of mice, 6–8 weeks; ∗, $P < 0.05$, treated *versus* untreated control group of the same day, Student's *t* test analysis; two replicates per sample. *C*, plasma mouse VEGF levels after three-injection RAFL-1 treatment during 21 days of observation. *Symbols and bars*, mean ± SD; *arrows*, drug injections; five mice per group; age of mice, 6–8 weeks; ∗, $P < 0.05$, treated *versus* untreated control group of the same day, Student's *t* test analysis; two replicates per sample. *D*, comparative study between SCID and NIH Swiss nude mice plasma mouse VEGF levels in immunodepleted and undepleted pooled (from five mice) plasma samples after 28 days of simultaneous injections of DC101 (800 μg/mouse) and rat IgG (800 μg/mouse; control) every 3 days (five mice per group; age of mice, 6–8 weeks). *Columns*, mean value of double measurements of pooled samples.

kit. Conversely, after MF-1 intraperitoneal injections, the mouse VEGF levels (not pooled samples) did not significantly increase when compared with the control, with the exception of day 6 (54.8 ± 7.1 *versus* 9.8 ± 8.8 pg/mL, respectively) and day 21 (43.3 ± 8.1 *versus* 6.8 ± 11.8 pg/mL, respectively; Fig. 3*B*). However, this small but statistically significant increase in VEGF concentration is noticeably lower than that observed in DC101-treated mice.

Fig. 3*C* shows that the administration of a different anti-VEGFR-2 antibody, RAFL-1 (15), resulted in a rapid increase, after 24 hours, in VEGF levels, similar to DC101, when compared with control (123.9 ± 47.8 *versus* 33.2 ± 8.9 pg/mL, respectively), and higher VEGF concentrations were maintained even after the discontinuation of the treatment, slowly returning to normal levels.

To determine whether the observed increases in mouse VEGF levels were restricted to only one strain of mice, a simultaneous experiment was performed using SCID and nude mice, employing injection of a rat IgG isotype-matched antibody as a control. In both strains of mice, at day 28 after antibody injections, higher mouse VEGF levels were observed in antibody-depleted plasma samples from mice treated with DC101 *versus* those treated with nonspecific rat IgG (Fig. 3*D*). However, the resultant VEGF increase was higher in SCID mice compared with nude mice.

As a comparison with the DC101 antibody, PTK787 and SU5416, all synthetic (organic) small molecule antagonists of VEGFR-2 (20), were tested to determine whether any analogous increase in circulating mouse VEGF levels would be induced. From 0.5 hour to 21 days after the first PTK787 administration, no differences were detected (data not shown) between treated and untreated animals (ranging around the limit of detection of the ELISA kit). Furthermore, at days 1, 4, 5, 8, and 12 after SU5416 injection, no variations in mouse VEGF plasma concentrations were observed (data not shown). This suggests that therapy-induced increases in free plasma VEGF may be restricted to antibody-mediated therapies that block or displace the VEGF ligand from the external domain of the flk-1 receptor.

Plasma Human VEGF Levels in MDA-MB-231 and PC-3 Human Tumor-Bearing SCID Mice after Combination DC101 Metronomic Chemotherapy Treatments. As discussed at the outset, these experiments were originally undertaken to evaluate whether various frequent low-dose chemotherapy regimens were associated with increases in human plasma VEGF secreted by human tumor xenografts, and, if so, whether the results might be affected by combination treatment with DC101 (12). The rationale for these experiments was to evaluate why low-dose metronomic chemotherapy regimens, which appear to selectively target the endothelial cells of tumor-associated blood vessels (12, 13), may gradually lose treatment efficacy over time (12, 19). Because VEGF is a survival factor for activated endothelial cells (12, 25), chemotherapy-induced increases in VEGF, if they occur, might be expected to compromise the anti-endothelial cell targeting effects of metronomic chemotherapy; if so, concurrent administration of a drug such as DC101 would compromise this potential protective effect and increase the antiangiogenic efficacy of low-dose metronomic chemotherapy regimens (12). Therefore, we decided to test levels of human VEGF in the plasma of human tumor xenograft-bearing SCID mice treated with various chemotherapeutic drugs/regimens. The tumor lines used, as mentioned above, were the MDA-MB-231 breast cancer and PC-3 prostate cancer lines.

Plasma samples were tested for tumor cell-derived human VEGF levels to establish whether changes could be detected as a result of metronomic chemotherapy treatments. Human VEGF levels were under the limit of detection of the ELISA assay in the control group and in the low-dose metronomic chemotherapy-treated groups but surprisingly were already measurable in all of the DC101-treated groups after 14 days, with levels ranging from 182.5 ± 135.8 pg/mL (DC101 alone) to 800.5 ± 140 pg/mL (DC101+VBL; Fig. 4*A*). After 26 to 28 days, the human VEGF levels decreased in all of the DC101-treated groups, compared with the previous measurement, ranging from 100.8 ± 10.1 pg/mL in mice treated with DC101 alone to 392.5 ± 4.2 pg/mL in mice treated with DC101+VBL (Fig. 4*A*). We reasoned that the unexpectedly large increases in human VEGF might be due to a compensatory VEGF response in the human tumor xenografts as a result of hypoxia induced by the DC101 antiangiogenic therapy.

The human VEGF increase in mouse plasma samples occurred after the administration of a DC101 effective dose (800 μg/mouse every 3 days) alone or in combination with metronomic chemotherapy (Fig. 4*A*). Indeed, the groups given DC101 have a better outcome (the animals were still being treated after 77 days), and the measured tumor volumes were smaller than the control ones (Fig. 4*B*). The toxicity profile was favorable and acceptable for a long-term treatment, with no loss or minimal loss of weight throughout the course of the experiment (Fig. 4*C*).

Furthermore, to statistically correlate in this experiment the increased plasma VEGF levels with the efficacy of the antiangiogenic antitumor treatment, a statistical correlation has been calculated (by the GraphPad Prism v. 4.0 software) between the duration of the experiment (expressed in days) per single treatment group and the mean of the latest available VEGF measurement of the same group. Based on the evidence that the success of an antiangiogenic (metronomic) treatment is measured not only by tumor volume but also by the prolonged survival of the treated mice, Fig. 4*D* shows that higher levels of plasma human VEGF significantly correlate ($P = 0.0192$; $r = 0.792$; $n = 8$) with an increase of therapeutic success (a time prolongation in the experiment).

As might be expected from the *in vivo* analysis of VEGF secretion described above, the VEGF-deficient PC-3 subcutaneous tumor-bearing mice showed a much lower average human VEGF level compared with the MDA-MB-231 tumor-bearing mice, yielding undetectable levels of VEGF in all of the bled mice analyzed.

Platelet VEGF Release and Analysis of VEGF/VEGFR-2 mRNA Expression in SCID Mice Tissues after DC101 Treatment. Having established the increase in plasma VEGF in response to treatment with DC101, we next sought to investigate the source of this VEGF increase. Because VEGF is known to be released from the α granules of activated platelets, which can also express VEGF-R (26–29), we tested the release of VEGF from mouse platelets *in vitro* in response to DC101. However, we observed no differences in the amount of VEGF released from either aggregated or nonaggregated platelets when exposed to DC101 or nonspecific rat IgG.[6]

An analysis of VEGF and VEGFR-2 mRNA expression in mouse tissues revealed no significant differences between DC101-treated mice (which received injection every 3 days at a dose of 800 μg/mouse for 2 weeks) and control animals (saline treated) in lung, heart, and kidney tissues (Fig. 5*A*). Lung tissues showed substantial expression of VEGF and VEGFR-2 mRNA compared with other tissues, *e.g.*, heart and kidney (Fig. 5*A*), but these levels were not further elevated after treatment of mice with DC101. In addition to examining VEGF mRNA levels in several tissues by Northern analysis, we also examined RNA levels using a mouse VEGF mRNA ELISA kit on a more extensive panel of mouse tissues before and after DC101 treatments. Results showed elevated expression of mouse VEGF mRNA in treated and untreated lung tissue compared with other tissues. This analysis also revealed a noticeable increase of mRNA VEGF in skin, bone marrow, and spleen tissues after

[6] L. Ma and R. Kerbel, unpublished observations.

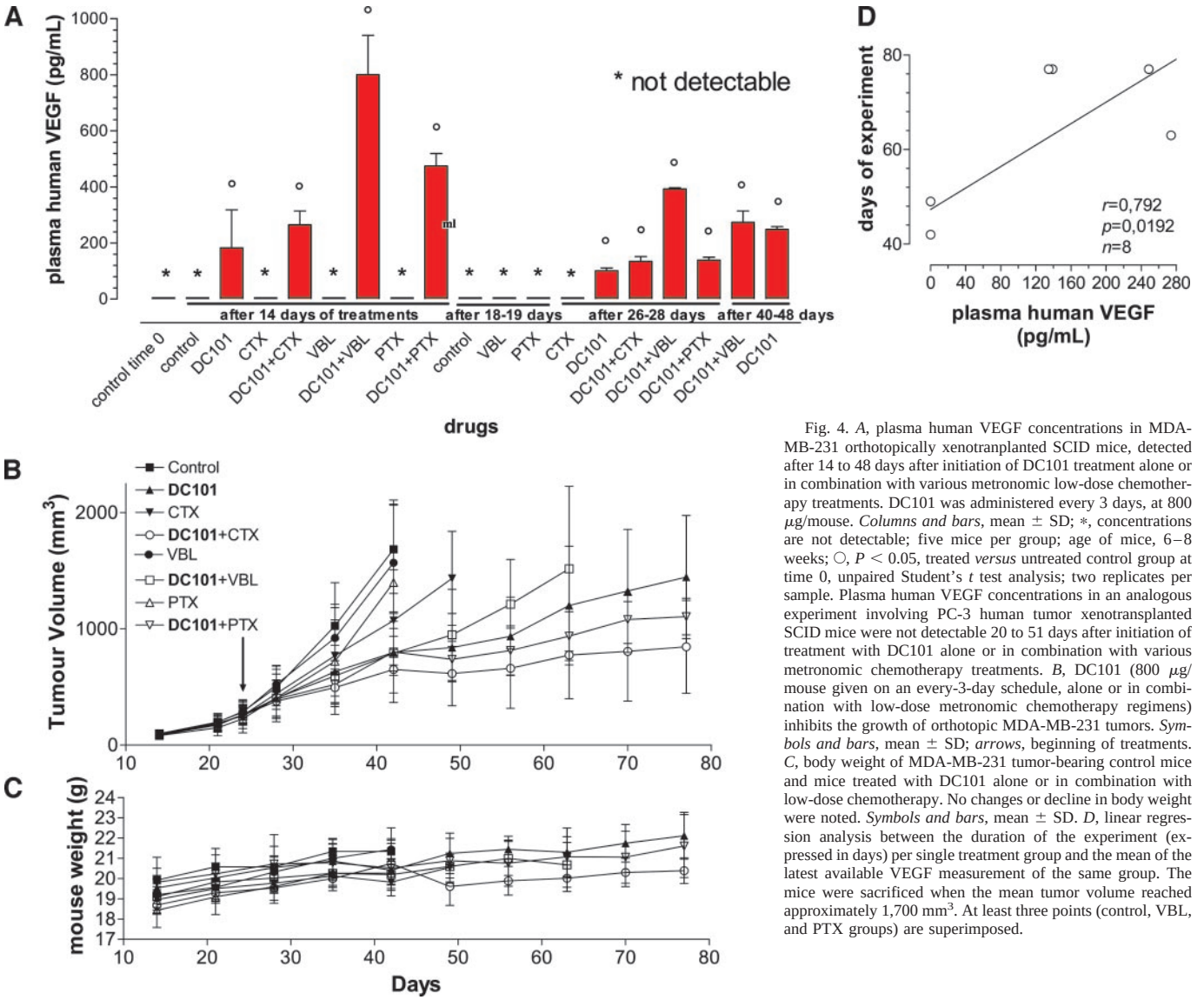

Fig. 4. *A*, plasma human VEGF concentrations in MDA-MB-231 orthotopically xenotranplanted SCID mice, detected after 14 to 48 days after initiation of DC101 treatment alone or in combination with various metronomic low-dose chemotherapy treatments. DC101 was administered every 3 days, at 800 μg/mouse. *Columns and bars*, mean ± SD; ∗, concentrations are not detectable; five mice per group; age of mice, 6–8 weeks; ○, $P < 0.05$, treated *versus* untreated control group at time 0, unpaired Student's *t* test analysis; two replicates per sample. Plasma human VEGF concentrations in an analogous experiment involving PC-3 human tumor xenotransplanted SCID mice were not detectable 20 to 51 days after initiation of treatment with DC101 alone or in combination with various metronomic chemotherapy treatments. *B*, DC101 (800 μg/mouse given on an every-3-day schedule, alone or in combination with low-dose metronomic chemotherapy regimens) inhibits the growth of orthotopic MDA-MB-231 tumors. *Symbols and bars*, mean ± SD; *arrows*, beginning of treatments. *C*, body weight of MDA-MB-231 tumor-bearing control mice and mice treated with DC101 alone or in combination with low-dose chemotherapy. No changes or decline in body weight were noted. *Symbols and bars*, mean ± SD. *D*, linear regression analysis between the duration of the experiment (expressed in days) per single treatment group and the mean of the latest available VEGF measurement of the same group. The mice were sacrificed when the mean tumor volume reached approximately 1,700 mm^3. At least three points (control, VBL, and PTX groups) are superimposed.

DC101 treatment in which the mice received injection twice a week with DC101 (800 μg/mouse) for 2 weeks, and tissues were removed 2 days after the last injection. (Fig. 5*B*).

DISCUSSION

Empirical preclinical studies were required to determine the optimal biological dose of the DC101 anti-VEGFR-2 antibody, which involved treatment of different transplantable human and mouse tumors, as well as assays of antiangiogenic activity *in vivo* (8, 21). For example, a previous dose-response study by Prewett *et al.* (21) showed that the optimum therapeutic dose of DC101 was approximately 800 μg/mouse (administered every 3 days) when treating mouse Lewis lung carcinoma in C57Bl/6 mice. Increasing the dose to 1.2 mg did not enhance the therapeutic efficacy (21). In the present study, a similar type of dose-response analysis showed a dose in the range of 1 mg appeared optimal for treatment of established HT-29 colon cancer xenografts. Undertaking similar studies in humans treated with VEGFR-2 targeting (anti-KDR) antibodies such as 1C11 (30) is clearly difficult, underscoring the critical need for convenient surrogate markers that correlate with effective and, more importantly, optimal, antitumor therapeutic dose activity. Our results therefore represent an example of such a marker because the peak increases in plasma mouse VEGF after injections of DC101 occurred when using doses previously determined to be in the optimal therapeutic range, *i.e.*, 800 μg to 1.2 mg per injection (21), as demonstrated, for example, by the MDA-MB-231 tumor-bearing mice experiment, which also showed an extremely favorable toxicity profile using such drug doses. The apparent correlation between dose escalation and dose response is also evident in the experiment involving HT-29 tumor-bearing mice, in which doses of 800 and 1,200 μg/mouse appeared to give maximum antitumor effect, *i.e.*, tumor stabilization, as opposed to only modest growth delays that were observed using the 200 or 400 μg/mouse doses.

The elevated levels of VEGF rapidly induced by DC101 were detected in non–tumor-bearing and human tumor-bearing mice. In the latter case, both mouse and human plasma VEGF levels were significantly increased within 2 to 24 hours after a single injection of the drug. This suggests that measurement of plasma VEGF levels in cancer patients treated with various doses of a VEGFR-2–specific antibody drug such as 1C11 (30) shortly after a single injection might be used to determine the optimal dose to employ in subsequent phase II and III clinical trials. Alternatively, such measurements could be undertaken in normal healthy volunteers.

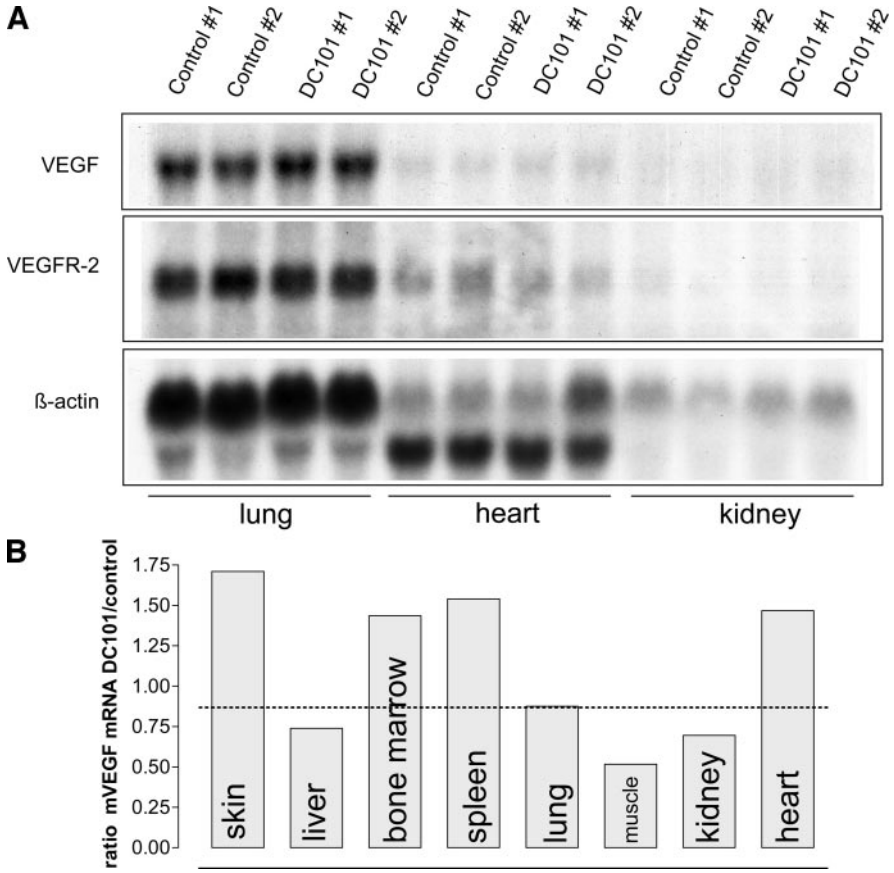

Fig. 5. *A*, Northern blotting analysis of mouse VEGF and VEGFR-2 mRNA in lung, heart, and kidney tissues from SCID mice that were treated with DC101 or saline alone every 3 days. Total RNA was extracted, and approximately 20 μg were loaded per lane. Probes were labeled using [^{32}P]dCTP. β-Actin mRNA is shown as loading control. Grouped blots were generated by stripping and reprobing of the same filter. *B*, mouse VEGF mRNA ELISA analysis of skin, liver, lung, bone marrow, spleen, lung, muscle, kidney, and heart tissues of SCID mice. The results are expressed as a ratio of the amount VEGF mRNA in DC101-treated mice to that in untreated mice. Lung tissues showed a higher expression of VEGF mRNA compared with other organs but no difference between DC101-treated and untreated mice; in contrast, skin, bone marrow, spleen, and heart tissues showed an increase in VEGF mRNA after DC101 treatment of mice.

With respect to testing for VEGF levels in cancer patients (as opposed to normal healthy volunteers), the measurement of VEGF levels could be complicated by the presence of VEGF-secreting tumors. In this regard, human VEGF detected in patients could conceivably be normalized to tumor volume detected using computed tomography and magnetic resonance imaging before, during, and after the injection of a VEGFR-2 antibody to detect changes in VEGF plasma levels.

Clearly, a major question raised by our results is the mechanism(s) responsible for the rapid and marked increases in plasma VEGF, including those observed even when the drug was injected into normal (non–tumor-bearing) mice. The absence of similar marked increases in plasma VEGF detected after administration of various small molecule VEGFR-2 antagonists that we tested suggests an antibody-dependent mechanism, such as dislodgement of VEGF bound to the external domain of VEGFR-2 expressed by cells throughout the body (31); such receptors may act as a "sink" to sequester VEGF. If so, it is interesting to consider whether something similar might occur using antibodies directed toward other receptor tyrosine kinases that bind growth factor ligands. However, it should be noted that we did detect small increases (mostly not significant) in plasma VEGF after injection of MF-1, an anti-VEGFR-1 monoclonal antibody. Other possible mechanisms to account for the rapid increase in circulating plasma VEGF we are currently investigating are based on rapid release of stored VEGF from known sources, *e.g.*, from platelets (26–28), which have been reported to express VEGFR-2 (29), the components of extracellular matrix, where it can be bound by heparin sulfate proteoglycans (32), fibronectin (33), fibrin (34), or thrombospondin-1 (35). In addition, circulating α_2-macroglobulin (or TSP-1) can bind VEGF (35, 36). The rapid increase of human VEGF detected in human tumor-bearing mice after treatment with DC101 would not support the hypothesis that VEGF released from host cells, such as platelets, is involved, nor is a mechanism of acute release of VEGF from circulating TSP-1 or α_2-macroglobulin or from extracellular matrix components induced by an antibody to a receptor for VEGF readily obvious. However, one intriguing new possibility stems from the recent discovery in our laboratory of a circulating, soluble VEGFR-2 (37).

Another possibility to account for the VEGF increase in plasma is some kind of compensatory increase of VEGF in various tissues treated with anti-VEGFR-2 antibodies such as DC101, perhaps secondary to an induced state of local hypoxia. In this regard, we did in fact detect increases in levels of VEGF mRNA in bone marrow, spleen, and skin after DC101 treatment in which mice received injections twice a week for 2 weeks. However, given the rapidity with which increases in plasma VEGF occur, *e.g.*, within 2 hours of a single injection of DC101, it is likely that newly synthesized VEGF would probably make a relatively minor contributory mechanism, compared with release of pre-formed (stored) VEGF. Finally, another possible mechanism for the increased VEGF plasma levels could be the blocking of the VEGF-A clearance by the kidney due to the antibody inhibition of VEGFR-2 that may play a role in the maintenance of glomerular ultrafiltration (38, 39), although the role of VEGF and VEGFR-2 in normal glomerular physiology is poorly understood.

An interesting precedent for highly significant increases in levels of a circulating ligand induced by interference in some manner with its cognate receptor can be found in the studies of Dai *et al.* (40). Normally, 95% of circulating colony-stimulating factor (CSF)-1 is cleared by CSF-1 receptor-mediated endocytosis and its subsequent intracellular destruction by sinusoidally located macrophages. This could explain the elevated levels of circulating CSF-1 observed in CSF-1 receptor genetically deficient (knockout) mice (approximately 20-fold) compared with their wild-type counterparts (40). Perhaps something similar could conceivably occur as well for circulating VEGF when VEGFR-2 receptors are

blocked by a high-affinity antibody, thus resulting, at least in part, in a lack of VEGF clearing by VEGFR-2 in all or most tissues. Whereas the increases in plasma VEGF detected in normal mice clearly distinguished the DC101 and RAFL-1 anti-VEGFR-2 antibodies from the various small molecule VEGFR-2 antagonists that we tested, it is possible that in tumor-bearing hosts, the small molecule drugs may cause increases in plasma VEGF, for example, as a result of hypoxia induced in the tumors secondary to the drug treatment.

With regarding to the differences between the synthetic small molecule inhibitors of VEGFR and antibodies such as DC101 and among different antibodies such as DC101 and RAFL-1, further investigations are needed. However, our findings thus far suggest that the increase of VEGF levels in normal hosts is specifically associated with the use of the neutralizing antibodies. The different doses administered (a higher biologically active dose for DC101 *versus* RAFL-1), the different site of the receptor that is bound, and the differences in the affinity of the antibodies could partially explain the differences in the increase of plasma VEGF obtained experimentally.

Finally, the biological consequences, if any, of the increases in plasma VEGF after administration of drugs such as anti-VEGFR-2 antibodies remain to be determined. The increased circulating VEGF may be bound by other VEGF-binding receptors such as VEGFR-1 or neuropilin-1/2 (4), which conceivably might facilitate salvage mechanisms of VEGF-driven angiogenesis. It will also be of considerable interest to evaluate whether other VEGF targeting strategies such as the VEGF Trap (41) or certain other drugs that target the VEGF/VEGFR-2 axis are also capable of significantly increasing levels of plasma VEGF and, if so, whether they do in a dose-dependent manner that correlates with the optimal biological dose of the drug. Our results also raise the obvious question of whether antibodies to other receptor tyrosine kinases, such as the insulin-like growth factor I receptor (42), the platelet-derived growth factor receptor (43), and so forth may cause rapid and marked increases in the level of the respective ligand in the circulation and, if so, what the biological consequences of this might be. Increases in circulating ligand may be easier to determine in humans treated with such antibodies than in mice because almost all such antibodies target human receptor tyrosine kinases and do not cross-react with the mouse homologue.

ACKNOWLEDGMENTS

The authors thank Cassandra Cheng for her excellent secretarial assistance, and Dr. Lee Ellis and his laboratory for undertaking confirmatory experiments. We are grateful to Dr. Jeanette Wood (Novartis, AG, Basel, Switzerland) for the gift of PTK787 and Douglas Laird (Sugen/Pharmacia, San Francisco, CA) for SU5416, and Dr. Rolf Brekken for discussions.

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Increased Plasma Vascular Endothelial Growth Factor (VEGF) as a Surrogate Marker for Optimal Therapeutic Dosing of VEGF Receptor-2 Monoclonal Antibodies

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Cancer Res 2004;64:6616-6625.