Complexation of VEGF with Bevacizumab Decreases VEGF Clearance in Rats

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INTRODUCTION

Angiogenesis plays a critical role in several physiologic and pathologic processes, particularly in tumor growth, invasion, and metastasis (1). New blood vessels provide tumors with nutrients necessary for growth and also remove metabolic waste from the tumor (2). Several growth factors have been implicated in tumor angiogenesis, and one such factor is vascular endothelial growth factor (VEGF), which is a selective mitogen for endothelial cells. VEGF, a 43- to 46-kD glycoprotein, induces proliferation and migration of vascular endothelial cells and functions as a vascular permeability factor through two receptors: flt-1 and KDR (3). There are also several isoforms of VEGF ($VEGF_{206}$, $VEGF_{189}$, $VEGF_{165}$, $VEGF_{121}$, and $VEGF_{110}$), and in humans, recombinant humanized $VEGF_{165}$ (rhVEGF) displays nonlinear pharmacokinetics, which is attributed to binding of the drug to endothelial cells (4). In numerous preclinical animal models, administration of an antibody-targeting VEGF has been found to be a potent suppressor of tumor growth and is being considered as a potential, novel anticancer therapy (5).

Bevacizumab (Avastin™, rhuMAb VEGF) is a recombinant humanized monoclonal antibody that binds all isoforms of VEGF and inhibits binding of VEGF to its receptors. The antibody was engineered by combining VEGF-binding residues from a murine-neutralizing antibody with the framework of a human immunoglobulin G (IgG_1) (6). Bevacizumab is believed to be cleared through the FcRn system, a MHC class I-related receptor that has been shown to protect circulating IgG_1s from catabolism and thereby contribute to the long terminal half-life of antibodies (7). Bevacizumab binds to primate VEGF and to rabbit VEGF (with lower affinity) but does not bind to rodent VEGF (8). The monoclonal antibody has been shown to inhibit tumor growth in a dose-dependent manner in various animal models (9). Several clinical studies have also been conducted to characterize the pharmacokinetics, safety, and efficacy of bevacizumab in cancer subjects. In two phase II studies in cancer subjects, bevacizumab, in combination with 5-fluorouracil/leucovorin or carboplatin/paclitaxel, has been shown to be safe and has inhibited tumor growth (10,11).

Results from clinical studies have also shown a rise in serum concentrations of endogenous VEGF over baseline after single and multiple intravenous (IV) administration(s) of bevacizumab at doses >1 mg/kg (12). This rise in concentrations was ~3- to 4-fold above baseline and seemed to return to baseline as the antibody cleared systemically. An increase in VEGF synthesis/distribution and/or decrease in VEGF clearance upon complexation with bevacizumab are possible causes for this phenomenon. The latter hypothesis was explored in a pharmacokinetic study conducted in rats where recombinant humanized $VEGF_{165}$ (rhVEGF) was administered intravenously in the presence or absence of bevacizumab. Rats were selected because (i) bevacizumab does not bind rat VEGF, therefore diminishing any competition for binding of bevacizumab to the administered rhVEGF and (ii) serial sampling was possible in the same animal for measurement of drug concentrations. Results presented here confirm that complexation of VEGF with bevacizumab decreases the clearance of circulating VEGF.

MATERIALS AND METHODS

Animals

Male and female Sprague-Dawley rats (Charles River: Hollister, CA; 269–331 g) were used in the study. Animals were housed individually and acclimated for 48 h before the study began. Three males and two females were assigned to three groups according to body weight: group 1, rhVEGF alone; group 2, rhVEGF:Bevacizumab complexes; group 3, Bevacizumab alone. Group means ± SD weights for groups 1, 2, and 3 were 299 ± 24.8, 296 ± 22.5, and 297 ± 19.0 g, respectively. Access to food and water was provided *ad libitum*. All procedures were conducted in adherence with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Materials

Recombinant human $VEGF_{165}$ (rhVEGF) and bevacizumab were manufactured by Genentech, Inc., by using recombinant DNA technology and expressed in genetically engineered *E. coli* and Chinese hamster ovary cell lines, respectively. Dose material was supplied as a clear, sterile liquid at a concentration of 5 and 10 mg/mL for rhVEGF and bevacizumab, respectively.

Dose Preparation

Iodination of rhVEGF

rhVEGF (10 μg) was radiolabeled with [^{125}I] by using the lactoperoxidase method, as previously described (13). rhVEGF was labeled with ^{125}I (New England Nuclear, Boston, MA) by using the addition of 5 μL of a 100 IU/mL solution of bovine milk lactoperoxidase and a 5-μL solution of 0.02% hydrogen peroxide to initiate the reaction. After the

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ABBREVIATIONS: rhVEGF, recombinant human vascular endothelial growth factor; LTR, less than reportable.

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mixture was incubated for 5 min, an additional 5 μL of hydrogen peroxide was added for another 5 min, and the reaction was then quenched with 5 μL of N-acetyl-L-tyrosine 5 min later. Labeled rhVEGF was purified from free iodine with NAP-5 column, which was preequilibrated with 10 mL of column buffer (5 mM sodium succinate, 130 mM sodium chloride, 0.01% tween 20 pH 5). [^{125}I]-VEGF was eluted in a 1-mL volume. Resulting tracer had a specific activity of ~42.06 μCi/μg and had >95% trichloroacetic acid (TCA) precipitability. The radiolabeled material was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and showed bands that migrated at ~22 and 40 kDa under reducing and nonreducing conditions (data not shown).

Dose Solutions for Preformed Complexes (Group 2)

rhVEGF:bevacizumab complexes were preformed before injection (group 2 material). The dosing solution was prepared by gently mixing bevacizumab and rhVEGF at 10:1 molar ratios in a tube (1 mg/kg bevacizumab, 0.723 μg/kg [^{125}I]rhVEGF (30.4 μCi/kg), 25 μg/kg rhVEGF). The solution was stored at 2–5°C before administration. Formation of complexes was confirmed by size exclusion chromatography–high performance liquid chromatography (SEC-HPLC) using a Hewlett Packard 1090 with a TSK G4000SWXL column and HBSS as the mobile phase at a flow rate of 0.5 mL/min. The column eluant was monitored optically at 280 nm and monitored for radioactivity with a Packard Radiomatic 525TR inline gamma detector. Approximate molecular weights were interpolated from gel filtration standards (Bio-RAD, Hercules, CA).

Dose Administration

Rats were administered a single IV bolus dose of either rhVEGF, rhVEGF:bevacizumab complexes, or bevacizumab alone, through a femoral vein cannula. Animals in group 1 received a trace dose of 30.4 μCi/kg [^{125}I]rhVEGF + 25 μg/kg rhVEGF. Animals in group 2 received administration of preformed rhVEGF:bevacizumab complexes, whereas group 3 animals received 1 mg/kg bevacizumab alone. Group mean weights before the study began were used to determine dose.

Blood Sampling/Analytical Methods

Blood samples were collected up to 8 h (group 1) or 14 days (groups 2 and 3) via a jugular catheter or tail vein (dependent on catheter patency) for measurement of serum [^{125}I]rhVEGF and serum bevacizumab concentrations. Serum rhVEGF was analyzed for TCA precipitable [^{125}I]rhVEGF in groups 1 and 2 using a gamma counter (Auto-gamma 5000 series; Packard Corporation). Based on previous studies, TCA precipitable radioactivity represents mostly homogenous and intact [^{125}I]rhVEGF in circulation after IV administration in rats. VEGF, free and bound to bevacizumab, was measured in this method. The mean background of all samples (112 cpm) was subtracted from all radioactivity data. rhVEGF concentrations were calculated by cpm conversion to ng equivalent/mL (ng-eq/mL) based on [^{125}I]rhVEGF specific activity. In addition, serum samples from groups 2 and 3 were analyzed for bevacizumab concentrations by an enzyme-linked immunosorbent assay (ELISA) developed at Genentech, Inc., which uses truncated rhVEGF for capture and a goat antibody to human IgG conjugated to horseradish peroxidase (HRP) for detection [less than reportable (LTR) < 0.036 μg/mL]. This assay detected all bevacizumab that has at least one available VEGF-binding site. At a 10:1 ratio of bevacizumab:VEGF, most VEGF will be complexed as an heterotrimer where each molecule of bevacizumab is expected to have one free site available for binding in the ELISA. Similar complexes were formed for another monoclonal antibody with the same IgG_1 backbone at these molar ratios (14).

Pharmacokinetic Analysis

Serum rhVEGF concentration-time data were analyzed with a two-compartment pharmacokinetic model using an iteratively reweighted least-square scheme with weights inversely proportional to the predicted concentration values ($1/\hat{y}$; Nelder-Mead minimization algorithm) (WinNonlin Pro, version 1.5, Pharsight Corporation, Inc., Mountain View, CA). Concentration levels < 0.116 ng-eq/mL (~3 times the level of background cpm) were excluded from the analyses.

Serum bevacizumab concentration-time data were also analyzed with a two-compartment pharmacokinetic model using weights inversely proportional to the concentration values squared ($1/y^2$; Nelder-Mead minimization algorithm) (WinNonlin Pro, version 1.5). Bevacizumab concentrations at the LOQ (<0.036 μg/mL) were excluded from the analyses. Pharmacokinetic parameters between groups 1 and 2 and groups 2 and 3 were compared by using Student's *t* test ($\alpha < 0.05$).

RESULTS

rhVEGF:Bevacizumab Complexes

By SEC-HPLC, [^{125}I]rhVEGF:bevacizumab complexes were visible with retention times of ~18.6 min. There were no other peaks, suggesting uniformity of the complex. The apparent molecular weight was estimated to be 300–400 kDa, corresponding to the expected heterotrimers of one molecule of VEGF to two molecules of bevacizumab (net molecular weight of ~345 kDa).

Pharmacokinetics of rhVEGF

The mean ± SD clearance of rhVEGF when administered alone was 225 ± 111 mL/h/kg after IV administration, with a volume of distribution at steady state (V_{ss}) of 1580 ± 1050 mL/kg and terminal half-life of 9.37 ± 10.6 h (Table 1). The large SDs were due to one animal, where rhVEGF had a 3-fold lower clearance (92.7 mL/h/kg), 6-fold longer terminal half-life (27.8 h), and 3-fold greater V_{ss} (3410 mL/kg) than the values observed from other animals in its group. Excluding this animal from the determination of means ± SD parameters, rhVEGF clearance was 258 ± 95.6 mL/h/kg, terminal half-life was 4.75 ± 2.96 h, and V_{ss} was 1120 ± 280 mL/kg. In the presence of bevacizumab, the mean clearance of rhVEGF was 65.9 ± 12.5 mL/h/kg, with a volume of distribution at steady state of 1000 ± 105 mL/kg and terminal half-life of 11.5 ± 3.91 h (Table 1). When comparing the pharmacokinetics of rhVEGF alone (including all animals) with rhVEGF bound to bevacizumab (Fig. 1), the clearance of rhVEGF decreased ~3.4-fold in the presence of bevacizumab ($p < 0.013$). The mean V_{ss}, however, did not change when complexed to bevacizumab.

Table I. Means ± SD rhVEGF Pharmacokinetic Parameters

	rhVEGF	rhVEGF:Bevacizumab complexes
Group No.	1	2
No.	5	5
C_{max} (ng-eq/mL)[a]	2.71 ± 0.636	2.37 ± 0.168
CL (mL/h/kg)[b]	225 ± 111	65.9 ± 12.5
$t_{1/2}$ α (min)	13.8 ± 5.13	9.53 ± 4.36
$t_{1/2}$ β (h)	9.37 ± 10.6	11.5 ± 3.91
AUC (h · ng/mL)	4.14 ± 2.44	11.4 ± 2.64
%AUC_{α} (%)	22.1 ± 10.3	3.71 ± 1.04
%AUC_{β} (%)	77.5 ± 9.83	96.4 ± 0.877
V_c (mL/kg)	280 ± 61.0	280 ± 36.4
V_{ss} (mL/kg)	1580 ± 1050	1000 ± 105

[a] Observed maximum concentration.
[b] $p < 0.05$ comparing groups 1 and 2.

Table II. Means ± SD Bevacizumab Pharmacokinetic Parameters

	rhVEGF:Bevacizumab complexes	Bevacizumab alone
Group No.	2	3
No.	5	5
C_{max} (μg/mL)[a]	19.6 ± 3.40	24.7 ± 1.73
CL (mL/day/kg)	7.83 ± 2.82	8.97 ± 2.36
$t_{1/2}$ α (h)	8.67 ± 2.83	7.37 ± 2.59
$t_{1/2}$ β (day)	13.0 ± 7.07	8.68 ± 3.90
AUC (day · μg/mL)	144 ± 61.5	120 ± 40.4
%AUC_{α} (%)	3.87 ± 1.69	5.18 ± 1.78
%AUC_{β} (%)	96.1 ± 1.70	94.8 ± 1.78
V_c (mL/kg)[b]	57.8 ± 6.98	43.8 ± 4.38
V_{ss} (mL/kg)[b]	121 ± 13.5	97.4 ± 13.0

[a] Observed maximum concentration.
[b] $p < 0.05$ comparing groups 2 and 3.

Pharmacokinetics of Bevacizumab

Pharmacokinetics of bevacizumab was compared when administered alone or in the presence of rhVEGF (Table 2, Fig. 2). There was no statistically significant difference in bevacizumab clearance or terminal half-life when bound to rhVEGF. The volumes of distribution (V_c and V_{ss}) of bevacizumab once complexed with VEGF were, however, ~20% larger than the volume of distribution estimated for bevacizumab alone ($p < 0.03$).

DISCUSSION

To investigate the rise in serum VEGF concentrations observed in clinical studies after administration of bevacizumab, rats were administered rhVEGF alone or rhVEGF-:bevacizumab complexes, and the pharmacokinetics of both entities were evaluated and compared. Because in patients, bevacizumab concentrations were in excess over endogenous VEGF concentrations, complexes in this study were formed with a 10-fold molar excess of the antibody compared to rhVEGF. At these molar ratios, most of the complexes formed were expected to be heterotrimers, where one rhVEGF molecule is bound to two bevacizumab, as observed with another monoclonal antibody (14). Because bevacizumab does not bind to rodent VEGF, the impact of endogenous rat VEGF on the pharmacokinetics was considered negligible, and results from this study confirm that complexation of VEGF leads to a significant decrease in VEGF clearance. When bound to bevacizumab, the mean clearance of rhVEGF in rats decreased ~3.4-fold, whereas the volume of distribution remained unchanged.

This effect on antigen clearance due to complexation with an antibody was also observed in pharmacokinetic studies with another monoclonal antibody, omalizumab (Zolair™) (15). Omalizumab binds immunoglobulin E (IgE) and has the same humanized IgG framework as bevacizumab. After IV administration of omalizumab in cynomolgus monkeys, the clearance of IgE decreased resulting in a rise of IgE concentrations in serum (16). Similar increases in IgE concentrations were also found in clinical studies (17). As observed with bevacizumab, the change in antigen clearance was attributed to its complexation with a slower clearing antibody and, therefore, increased serum concentrations of circulating antigen that is predominately inactive pharmacologically.

Unlike rhVEGF, bevacizumab clearance did not appear

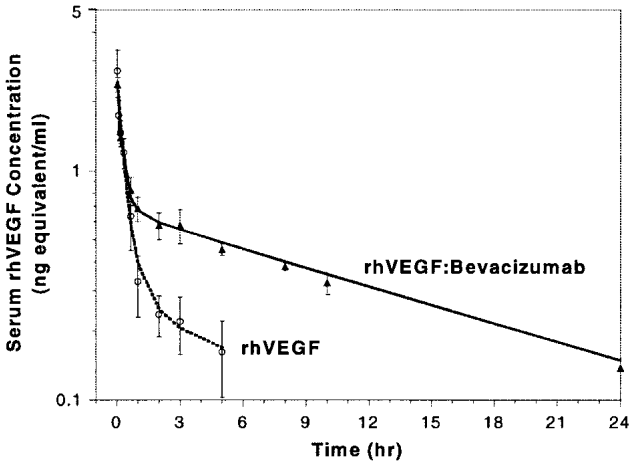

Fig. 1. Mean concentration-time profiles of rhVEGF after IV administration of [^{125}I]rhVEGF + cold rhVEGF or preformed rhVEGF (hot + cold):bevacizumab complexes; compartmental fit superimposed on observed values (ng-eq/mL).

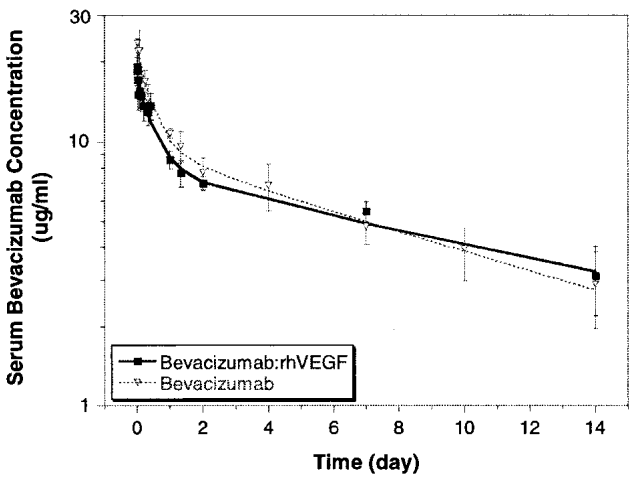

Fig. 2. Group mean concentration-time profiles of serum bevacizumab with compartmental fit superimposed on observed values (LTR excluded).

to be different when comparing the pharmacokinetics of bevacizumab alone or in the presence of rhVEGF. However, the sample size in this study may have been too small to detect differences in clearance. The volumes of distribution, however, were significantly larger with ~20–25% increase when bevacizumab was bound to rhVEGF. This increase in bevacizumab volume of distribution may indicate that bevacizumab, when bound to rhVEGF, distributes more widely than when unbound, because the volume of distribution of free rhVEGF is 5-fold larger than that of bevacizumab alone.

Although there may be other possible contributing factors, such as changes in VEGF synthesis and/or distribution, results from the present study indicate that the rise in VEGF concentrations observed in the clinic after administration of bevacizumab can be explained by the decrease in VEGF clearance due to complexation with bevacizumab.

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