



# VEGF Trap complex formation measures production rates of VEGF, providing a biomarker for predicting efficacious angiogenic blockade

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VEGF is the best characterized mediator of tumor angiogenesis. Anti-VEGF agents have recently demonstrated impressive efficacy in human cancer trials, but the optimal dosing of such agents must still be determined empirically, because biomarkers to guide dosing have yet to be established. The widely accepted (but unverified) assumption that VEGF production is quite low in normal adults led to the notion that increased systemic VEGF levels might quantitatively reflect tumor mass and angiogenic activity. We describe an approach to determine host and tumor production of VEGF, using a high-affinity and long-lived VEGF antagonist now in clinical trials, the VEGF Trap. Unlike antibody complexes that are usually rapidly cleared, the VEGF Trap forms inert complexes with tissue- and tumor-derived VEGF that remain stably in the systemic circulation, where they are readily assayable, providing unprecedented capability to accurately measure VEGF production. We report that VEGF production is surprisingly high in non-tumor-bearing rodents and humans, challenging the notion that systemic VEGF levels can serve as a sensitive surrogate for tumor load; tumor VEGF contribution becomes significant only with very large tumor loads. These findings have the important corollary that anti-VEGF therapies must be sufficiently dosed to avoid diversion by host-derived VEGF. We further show that our assay can indicate when VEGF is optimally blocked; such biomarkers to guide dosing do not exist for other anti-VEGF agents. Based on this assay, VEGF Trap doses currently being assessed in clinical trials are in the efficacious range.

aflibercept | angiogenesis | tumor | endothelial cell

VEGF is critical in many settings of physiological and pathological angiogenesis (1). In particular, high VEGF expression is characteristic of many types of cancers (1), suggesting that it might be an attractive target for therapeutic intervention aimed at preventing tumors from recruiting the blood supply that they need to survive (2). The first attempts at validating this particular approach were taken by Ferrara and colleagues (3), who demonstrated that a murine anti-human VEGF antibody suppressed the growth of human tumor cell lines implanted in nude mice. This led to the generation of a humanized monoclonal antibody, bevacizumab (Avastin; Genentech, South San Francisco, CA), which yielded impressive results in a controlled clinical trial in patients with metastatic renal cell cancer (4, 5). At doses of 3 and 10 mg/kg, bevacizumab treatment resulted in a significant prolongation in time to tumor progression compared with placebo, although the increased efficacy of the higher dose in this study suggested that the maximally efficacious dose may not yet have been attained (4, 5). Bevacizumab was subsequently granted FDA approval based on the demonstration that it significantly improved the progression-free and overall survival in patients with metastatic colorectal cancer when given in combination with irinotecan 5-FU/LV chemotherapy (6). Sev-

eral other drugs designed to block VEGF signaling have since been developed and recently approved [BAY 43-9006 (sorafenib) and SU11248 (sunitinib)] or are proceeding through clinical trials [PTK787 (vatalanib), ZD6474 (zactima), ZD6126, SU5416 (semaxanib), and AG-013736] (7-9).

As new anti-VEGF agents proceed through the clinic, it would be very useful to have biomarkers that could either identify patients whose tumors depend most on VEGF or that could guide dosing by indicating when optimal VEGF blockade has been achieved. Unfortunately, accepted biomarkers do not currently exist for VEGF blockade and are few and far between for other targeted agents, such as epidermal growth factor receptor for colon cancer, Kit for gastrointestinal stromal tumor, and HER2/NEU for breast cancer (10). VEGF itself has been suggested as a candidate biomarker for guiding the application of anti-VEGF therapies. It is widely assumed that VEGF production is quite low in healthy adults in the absence of active angiogenesis. Were that the case, blood levels of VEGF in cancer patients might provide a useful index of tumor VEGF production (11, 12). However, because VEGF is rapidly cleared from the systemic circulation (having a half-life of only minutes), the sensitivity of assays measuring VEGF in the peripheral blood leads to a wide variability for blood levels of VEGF in published reports. Furthermore, VEGF is present at substantial levels within platelets and released upon their lysis such that preparation of peripheral blood samples that avoid contamination from platelet-derived VEGF becomes difficult. These limitations are reflected in the disparate values reported for circulating VEGF levels in cancer patients, which range from 0.04 to 1 ng/ml, calling into question the utility of plasma VEGF levels as a useful biomarker for guiding anti-angiogenic therapy (11, 13-19).

VEGF Trap is a fully human soluble decoy receptor protein that consists of a fusion of the second Ig domain of human VEGF receptor (VEGFR) 1 and the third Ig domain of human VEGFR2 with the constant region (Fc) of human Ig IgG1 (20).

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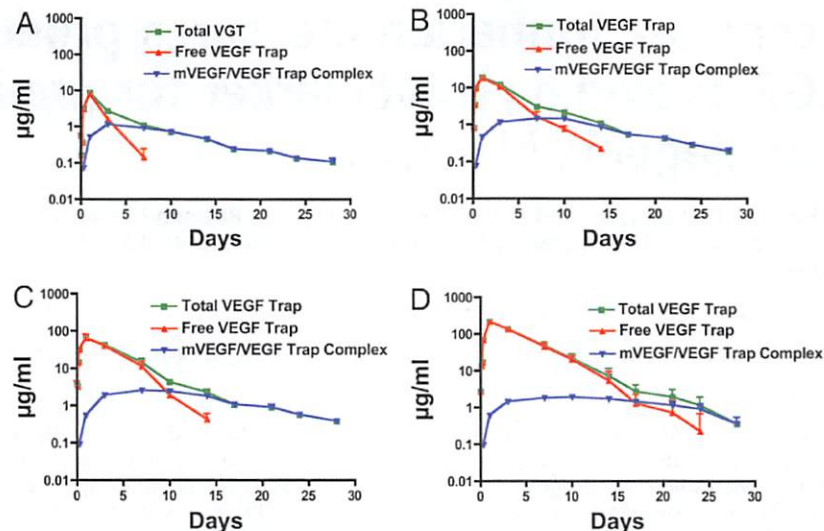
Abbreviations: AMD, age-related macular degeneration; MALLS, multiangled laser light scattering; SEC, size exclusion chromatography; VEGFR, VEGF receptor.

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**Fig. 1.** s.c. injection of VEGF Trap into SCID mice at different doses reveals different levels of circulating free VEGF Trap but similar levels of circulating mouse VEGF–VEGF Trap complex. At all doses ranging from 1 mg/kg (A) to 25 mg/kg (D), a steady-state level of VEGF–VEGF Trap complex is achieved, which plateaus at  $\approx 1$   $\mu\text{g/ml}$ . Dose-dependent levels of free VEGF Trap are observed as follows: 1 mg/kg to 10  $\mu\text{g/ml}$   $C_{\text{max}}$  falling below complex levels at 4 days (A); 2.5 mg/kg to 20  $\mu\text{g/ml}$   $C_{\text{max}}$  falling below complex levels at 7 days (B); 10 mg/kg to 80  $\mu\text{g/ml}$   $C_{\text{max}}$  falling below complex levels at 9 days (C); and 25 mg/kg to 200  $\mu\text{g/ml}$   $C_{\text{max}}$  falling below complex levels at 17 days (D). The half-life of VEGF Trap is  $\approx 2$  days at doses  $>2.5$  mg/kg. ( $n = 6$  for each dose.)

The VEGF Trap was engineered to have optimized pharmacokinetic properties and a very high affinity for all isoforms of VEGF-A ( $<1$  pM), as well as placental growth factor, a closely related angiogenic factor (20). VEGF Trap has shown robust antitumor effects in numerous mouse models of cancer and is now in clinical trials (21, †, §, ¶, ||). Here, we show that—unlike VEGF antibodies that tend to form multimeric immune complexes that are rapidly cleared from the circulation and can form immune complex deposits in tissues—the VEGF Trap forms a stable and inert 1:1 complex with VEGF. This VEGF–VEGF Trap complex has a long plasma half-life and can readily be measured in the systemic circulation, thus affording a reliable way to measure the rates of VEGF production in both tumor-bearing and non-tumor-bearing adult animals and humans. This unique ability to capture and thus precisely measure total VEGF levels, regardless of whether the VEGF comes from tumor or normal host tissues, allows for the unprecedented opportunity to accurately determine tumor and host VEGF production rates. Surprisingly, we find that total body VEGF production rates are quite high in normal adult rodents and humans, with the fractional contribution made by tumors being comparatively small. This finding has the important implication that therapies directed toward neutralizing VEGF produced by tumors must be provided in sufficient amounts so as to avoid being largely consumed by the significant levels of VEGF produced by the rest of the body. Toward this end, measurement of VEGF Trap complex allows the identification of VEGF Trap doses required to completely capture and block tumor-derived VEGF, providing a useful guide for optimizing angiogenic blockade; such assays do not exist for other anti-VEGF agents. Based on this

assay, we report that VEGF Trap doses currently being assessed in clinical trials appear to be in the efficacious range.

## Results

**VEGF Trap Forms an Inert Complex with VEGF That Remains Stably in the Circulation.** Initial studies to determine the clearance rate of VEGF Trap revealed that it could form stable detectable complexes with endogenous VEGF in normal adult mice. After single injections of increasing amounts of VEGF Trap, we measured total VEGF Trap, uncomplexed/unbound or “free” VEGF Trap, and VEGF Trap–mouse VEGF “complex” at various times after injection (Fig. 1 A–D represent increasing amounts of injected VEGF Trap). Because no exogenous VEGF was provided, complexes represent the association of VEGF Trap with endogenous murine VEGF. As expected, total VEGF Trap levels increased proportional to dose (determined by combining free VEGF Trap levels with complex levels) (Fig. 1, see green curves). Somewhat unexpectedly, substantial levels of VEGF Trap complexed with mouse VEGF accumulated rapidly (Fig. 1, see blue curves). At all doses of VEGF Trap tested, maximal levels of complex ( $\approx 1$ – $2$   $\mu\text{g/ml}$ ) were attained within 24–48 h of injection and sustained at this level for at least several days. Consistent with conversion of free VEGF Trap into complexed VEGF Trap, most of the injected VEGF Trap is initially found in the free, unbound form, but after reaching peak levels ( $\approx 24$  h after injection) free VEGF Trap in the circulation declines progressively (Fig. 1, note that red curves, corresponding to free VEGF Trap, initially overlap at early time points with green curves, representing total VEGF Trap, but then drop, as is most obvious at the lowest dose). Levels of free VEGF Trap decline because of a “consumption” (binding VEGF, thus being converted to complex) and clearance, which occurs at an identical rate for free and bound Trap. Thus, as long as free VEGF Trap remains in excess of bound, maximal steady-state levels of complex are maintained in the circulation. VEGF Trap is also able to bind placental growth factor with high affinity and is capable of forming stable circulating placental growth factor–VEGF Trap complexes *in vivo* with the same profile as VEGF–

<sup>†</sup>Rixe, O., Verslype, C., Meric, J. B., Tejpar, S., Bloch, J., Crabbe, M., Khayat, D., Furfine, E. S., Assadourian, S., Van Cutsem, E. (2006) *J. Clin. Oncol.* 24:13161 (abstr.).

<sup>‡</sup>Mulay, M., Limentani, S. A., Carroll, M., Furfine, E. S., Cohen, D. P., Rosen, L. S. (2006) *J. Clin. Oncol.* 24:13061 (abstr.).

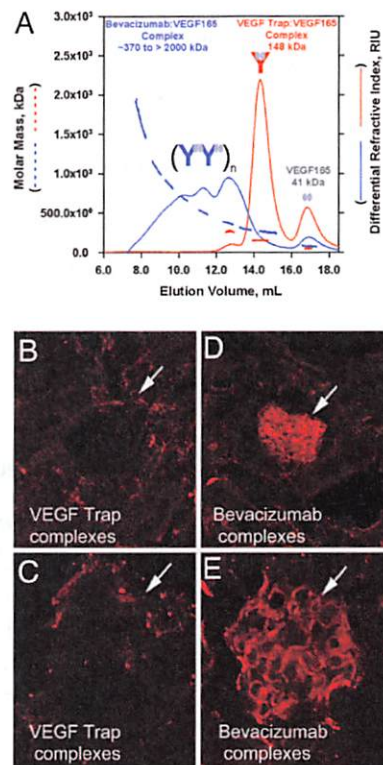
<sup>¶</sup>Tew, W. P., Colombo, N., Ray-Coquard, I., Oza, A., del Campo, J., Scambia, G., Spriggs, D. (2007) *J. Clin. Oncol.* 25:5508 (abstr.).

<sup>||</sup>Massarelli, E., Miller, V. A., Leigh, N., Rosen, P., Albain, K., Hart, L., Melnyk, O., Sterns, L., Akerman, J., Herbst, R. S. (2007) *J. Clin. Oncol.* 25:7627 (abstr.).

VEGF Trap complex, albeit at  $\approx 10$ -fold lower levels (data not shown).

In separate experiments, the bioavailability of VEGF Trap and the efficiency of VEGF capture were determined by injecting s.c. [supporting information (SI) Fig. 7A] or i.v. (SI Fig. 7B) preformed complexes of the Trap and its VEGF target, or both agents separately. The results show that the bioavailability of s.c. (SQ) injected complex was essentially identical to that of i.v. injected complex, indicating that negligible complex was depositing within tissues. Moreover, whether the VEGF Trap was injected as a preformed complex with VEGF (single bolus) or the Trap and its target were injected separately, similar levels of complex were rapidly noted in the circulation, indicating that the Trap efficiently captures its target and brings it into the systemic circulation. In addition, VEGF Trap is also capable of sequestering VEGF already bound in target tissues as shown by injecting VEGF before VEGF Trap (SI Fig. 7). Thus, VEGF Trap efficiently captures and forms inert complexes with VEGF that enter and remain stably in the circulation, readily accessible for measurement.

**Although VEGF Trap Forms a 1:1 Complex with VEGF, VEGF Antibodies Form Heterogeneous, Multimeric Immune Complexes with VEGF.** The above findings suggested that VEGF Trap might behave very differently than VEGF antibodies, because antibodies commonly form multimeric immune complexes that rapidly deposit in tissues and thus are rapidly cleared from the circulation. Because immune complexes rapidly disappear, the amount of captured ligand cannot be determined from levels of bound or unbound antibodies remaining in the circulation. To demonstrate directly that the VEGF Trap behaves in a fundamentally different way than antibodies, we compared VEGF Trap complex formation and clearance with that of a well characterized VEGF antibody, bevacizumab (Avastin). As predicted, size exclusion chromatography (SEC) of a preformed VEGF Trap-VEGF<sub>165</sub> complex revealed a single major homogenous peak, with an approximate molecular mass (as judged by comparison to molecular mass standards, data not shown) of  $\approx 150$  kDa corresponding to that expected of a 1:1 complex between VEGF Trap ( $\approx 110$  kDa) and VEGF<sub>165</sub> ( $\approx 40$  kDa) (Fig. 2A, solid red line); a minor peak of free excess VEGF<sub>165</sub> was also seen, as was a small shoulder of higher molecular mass. The molecular masses of the peaks were confirmed by using coupled multiangled laser light scattering (MALLS) (dashed red lines in Fig. 2A). In contrast, SEC of preformed bevacizumab-VEGF<sub>165</sub> complexes revealed a heterogeneous mixture corresponding to very high molecular masses (Fig. 2A, solid blue line) in addition to the small peak of free excess VEGF<sub>165</sub>. The purity of free VEGF Trap, bevacizumab, and VEGF was  $>97\%$ , as determined by SEC (data not shown). Coupled MALLS analysis revealed molecular masses of the heterogeneous mixture ranging from 370 kDa (corresponding to a multimer consisting of two bevacizumab molecules, each with a molecular mass of  $\approx 145$  kDa, and two VEGF<sub>165</sub> molecules, each with a molecular mass of  $\approx 40$  kDa) to  $>2,000$  kDa (corresponding to much larger multimers) (Fig. 2A, dashed blue line). Consistent with the apparent tendency of bevacizumab to form multimeric immune complexes with VEGF, preformed bevacizumab-VEGF<sub>165</sub> complexes rapidly disappeared from the circulation when injection intravenously, as would be expected for multimeric immune complexes (SI Fig. 8; note that the levels of Bevacizumab when complexed with VEGF rapidly drop compared with the levels of free Bevacizumab that remain much higher), and in contrast to what was described above with VEGF Trap complexes that remain stably in the circulation. Because immune complexes can often be cleared by depositing in the renal glomeruli, we further explored apparent differences in the clearance of bevacizumab-VEGF and VEGF Trap-VEGF complexes by performing im-



**Fig. 2.** The molar masses of VEGF Trap-VEGF and bevacizumab-VEGF complexes were determined by MALLS coupled to SEC. (A) Using a 1:2 molar ratio of VEGF Trap to VEGF<sub>165</sub>, discrete peaks were observed at  $\approx 17$  ml for VEGF (41 kDa) and  $\approx 14.5$  ml for VEGF Trap-VEGF complex (148 kDa) with SEC (red line) and MALLS (dashed red line). In contrast, a 1:2 molar ratio of bevacizumab to VEGF<sub>165</sub> revealed a heterogeneous multimeric complex that ranged in molar mass from  $\approx 370$  kDa to  $>2,000$  kDa (SEC, solid blue line; MALLS, dashed blue line). (B–E) One milligram of a preformed complex of VEGF Trap and VEGF<sub>165</sub> (B and C) or bevacizumab and VEGF<sub>165</sub> (D and E) were injected into the left ventricle of 2- to 3-month-old C57bl6 mice. After 10 min, mice were killed, and their kidneys were processed for immunocytochemistry, using an anti human Fc reporter antibody to the human Fc moiety present on both VEGF Trap and bevacizumab. Significant staining was observed in the glomeruli of bevacizumab/VEGF treated mice but not in the glomeruli of VEGF Trap/VEGF treated mice (white arrows).

munostaining in the kidney. After i.v. administration, renal glomeruli stained strongly for bevacizumab-VEGF complexes (Fig. 2D and E) but not for VEGF Trap-VEGF complexes (Fig. 2B and C). Current evidence indicates that, as a class, pharmacological agents that block VEGF signaling may produce mechanism-based effects on kidney function. Deposition of immune complexes as noted for bevacizumab/VEGF in the renal glomeruli could further accentuate renal toxicity in a nonspecific and non-class-dependent manner.

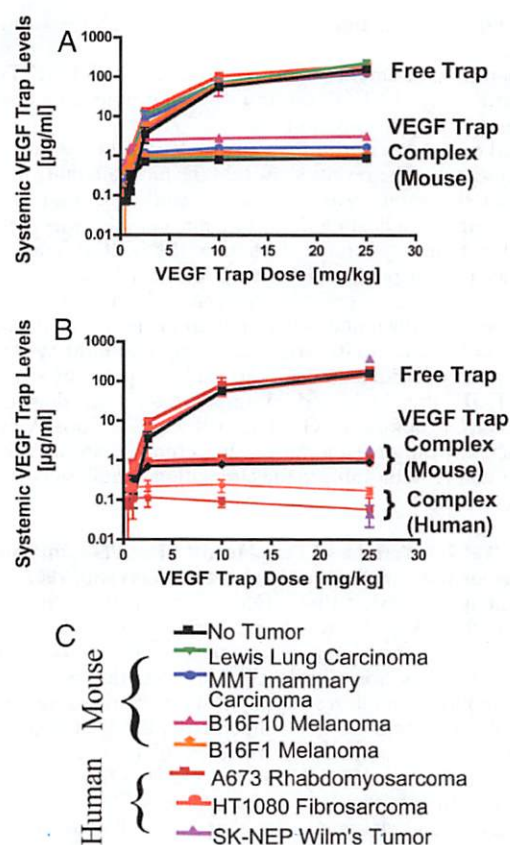
**VEGF Trap Complex Formation Reveals Unexpectedly High Production of Endogenous VEGF in Normal Adult Mice.** As shown above, VEGF antibodies form immune complexes that rapidly deposit in tissues and thus do not allow for easy ascertainment of the amount of complex formed. In contrast, VEGF Trap forms inert complexes with VEGF that remain stably in the circulation and are thus readily accessible for measurement. In fact, the above findings demonstrate that, if VEGF Trap is present at sufficient levels so as to be in excess of Trap bound in complexes, the steady-state levels of VEGF Trap complex in the circulation reflect the total amount of VEGF produced. Daily production

rates of VEGF can be calculated by assuming that steady-state levels of VEGF Trap–VEGF complex reflect a balance between production of VEGF leading to formation of complex, and clearance of the resulting complex. Based on experimentally determined values for the steady-state levels of complex and its clearance (see *Materials and Methods*), we estimate that mice produce  $\approx 0.065 \mu\text{g}$  of VEGF per day per ml of the volume of distribution, or  $\approx 0.006 \mu\text{g}$  per gram of tissue per day. Because VEGF is active at picomolar levels, this at first seems to be a surprisingly high level of production for a normal adult animal (see below for comparison to tumor production rates). However, it should be noted that in the absence of VEGF Trap, any VEGF that enters the systemic circulation is rapidly cleared. For this reason, among others noted above, it has not proven possible to consistently and reliably measure systemic VEGF levels, preventing accurate estimation of VEGF production rates in normal adult animals.

**Tumor-Derived VEGF Represents a Minority of Total Body VEGF Under Conditions of Minimal Tumor Burden.** Next, we compared the total body production rate of VEGF, as determined above, with tumor production rates of VEGF. Toward this end, we implanted mice with tumors, allowed these tumors to grow to 0.5–3% of total body weight (average mouse weight,  $\approx 25 \text{ g}$ ) and measured levels of VEGF Trap complex in these mice to compare them to complex levels found in healthy, non-tumor-bearing mice. Surprisingly, in mice bearing four different types of rodent tumors, the total levels of complex were not markedly different from those seen in non-tumor-bearing mice ( $1\text{--}2 \mu\text{g/ml}$ ; see Fig. 3A and compare with Fig. 1). This finding implies that tumor-derived VEGF represented only a small proportion of total body VEGF or circulating bioavailable VEGF in these mice.

To further validate this unanticipated finding, we analyzed VEGF Trap complex levels in mice bearing human tumors, where it is possible to distinguish complexes formed with endogenous mouse VEGF with those formed with human VEGF derived from the implanted tumors by analyzing human VEGF–VEGF Trap complex levels in mouse serum. The levels of mouse-derived complexes (Fig. 3B) in these animals were equivalent to those of non-tumor-bearing mice (Fig. 2, above) and mice bearing rodent-derived tumors (Fig. 3A). In contrast, the levels of VEGF Trap complexed with tumor-derived human VEGF were an order of magnitude lower ( $0.08\text{--}0.2 \mu\text{g/ml}$ ) (Fig. 3B). This result was seen in mice bearing tumors of three different human cell lines (SK-NEP, A673, and HT1080). Together, these studies demonstrate that normal total body production of VEGF eclipses the production from tumors that may weigh as much as 3% of body weight (mouse weight ranges from 23 to 29 g). Thus, it is unlikely that total levels of free VEGF in the systemic circulation would provide a sensitive index of tumor burden, even if accurate measurement of unbound VEGF in blood samples were readily achievable. Moreover, the above findings suggest that therapeutic compounds designed to bind and inactivate tumor-derived VEGF would have to be provided at sufficient levels to avoid being diverted by significant levels of VEGF normally produced by the rest of the body.

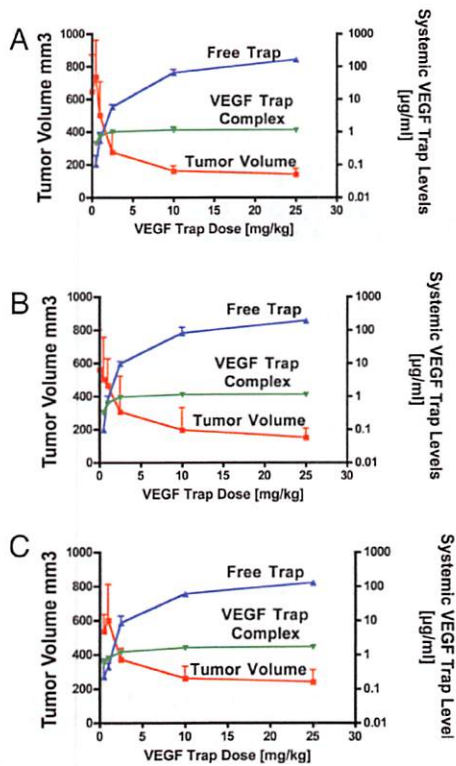
**VEGF Trap Complex Levels Provide Guidance on When Efficacious VEGF Blockade Is Achieved.** Based on the results above, it is evident that drugs that bind and neutralize VEGF must engage significant levels of VEGF derived from normal tissues, in addition to that originating from tumors. Therefore, we reasoned that measurements of VEGF Trap complex might provide a useful guide to when the dose of VEGF Trap sufficient to substantially neutralize both host and tumor-derived VEGF had been achieved. Indeed, for three different tumors [B16F1 mouse melanoma (Fig. 4A); A673 human rhabdomyosarcoma (Fig. 4B); and MMT mouse mammary carcinoma (Fig. 4C)], increasing the VEGF



**Fig. 3.** In mice bearing tumors  $<3\%$  body weight, the tumor pool of VEGF production is modest compared with endogenous mouse tissue VEGF production. (A and B) Mouse (A) or human (B) tumors were allowed to grow to  $\approx 100 \text{ mm}^3$ , and then VEGF Trap was administered twice per week for 1–2 weeks at 0.5, 1, 2.5, 10, and 25 mg/kg. At the termination of the experiment, free VEGF Trap, mouse, and human complex levels were measured in serum. In all cases, regardless of terminal tumor volume, levels of circulating mouse complex were  $\approx 1 \mu\text{g/ml}$ , whereas human complex levels in the mice bearing human tumors were  $\approx 0.1 \mu\text{g/ml}$ . Free Trap levels increased incrementally, with the dose levels rising above complex levels at the 2.5 mg/kg dose and reaching  $\approx 100 \mu\text{g/ml}$  at the 25 mg/kg dose. ( $n = 6$  for each dose). (C) Legend of mouse and human tumor types used.

Trap dose resulted in progressive, marked improvements in anti-tumor efficacy until a dose at which free VEGF Trap substantially exceeded maximal steady-state levels of complex was reached (Fig. 4). For all three tumor types, this was achieved at a dose of 2.5 mg/kg VEGF Trap given twice weekly: at this dose, free VEGF Trap (blue curve) is severalfold the level of complex (green curve), and past this point further dose escalation yields only modest incremental increases in complex levels (green curve) and in anti-tumor efficacy (red curve). In other tumor types, such as U87 glioblastoma, higher levels of VEGF Trap are required to achieve maximal efficacy (22).

**Human VEGF/VEGF Trap Complex Levels Are Directly Related to Tumor Size.** The finding that conventionally sized s.c. tumors in mice produced  $<10\%$  the amount of total body VEGF prompted us to determine whether there is a consistent relationship between tumor size and VEGF production levels. Human tumors (A673 rhabdomyosarcoma) were implanted into mice and allowed to grow to various sizes before injecting VEGF Trap. In this case, we could define a clear linear relationship between tumor size (Fig. 5A) and complex levels (Fig. 5B, note that the assay reflects



**Fig. 4.** VEGF Trap Complex provides guidance on when optimal VEGF blockade is achieved for antitumor purposes. In mice bearing B16F1 mouse melanoma tumors (A), A673 human rhabdomyosarcoma (B), and MMT mouse mammary carcinoma tumors (C) grown to  $\approx 100 \text{ mm}^3$  before treatment, increasing the dose of VEGF Trap from 0.5 mg/kg twice per week to 25 mg/kg twice per week results in a steady-state of mouse complex at  $\approx 1 \mu\text{g/ml}$  at 1–2.5 mg/kg and free circulating VEGF Trap levels of  $\approx 10 \mu\text{g/ml}$  at the 2.5 mg/kg dose, rising to  $\approx 100 \mu\text{g/ml}$  at the 25 mg/kg dose. Tumors remain quite large at the 0.5 and 1 mg/kg doses but begin to show a significant lack of growth at the 2.5 mg/kg dose, where free Trap levels rise above steady-state complex levels ( $n = 6$  for each dose). Tumors were treated with VEGF Trap from 6–13 (B16F1), 4–13 (MMT), and 12–18 (A673) days after implantation.

levels of complexes containing only human VEGF to specifically detect only tumor-derived complex). The amount of complex per unit weight of tumor was similar across different-sized tumors (Fig. 5C), indicating that tumors maintained their rates of VEGF production as they grew. Linear regression analysis confirmed that there was a very strong correlation between A673 tumor volume and circulating human VEGF complex (Fig. 5D).

At these larger tumor sizes, the amount of complex (ranging from  $\approx 0.8$  to  $5 \mu\text{g/ml}$ ) contributed by the tumor matched or even exceeded that contributed by the rest of the body, confirming that tumors do indeed make substantially more VEGF per cell than does the average cell in the normal adult host. For example, in the largest tumors (weighing  $\approx 10\%$  of the total mass of the mouse, Fig. 5A), the tumor-derived human VEGF–VEGF Trap complex levels ( $\approx 5 \mu\text{g/ml}$ , Fig. 5B) were  $\approx 3$ -fold above the levels of murine VEGF–VEGF Trap complex, indicating that the tumors made  $\approx 30$  times the amount of VEGF per unit of weight compared with normal, adult tissues.

**VEGF Trap Complex Formation in Human Subjects With and Without Cancer.** Very large tumors that substantially contribute to VEGF Trap complex formation in mice are generally not seen in the human patient. This in turn suggests that it is unlikely that most tumors in human patients become large enough to make a

readily detectable contribution to total body VEGF production. To determine whether or not this was indeed the case, we studied VEGF Trap complex formation in non-cancer patients [patients suffering from age-related macular degeneration (AMD)] and then compared these results with complex formation in cancer patients. In the AMD patients, the lowest dose of VEGF Trap tested (0.3 mg/kg, i.v.) was insufficient to neutralize all VEGF, as evidenced by the levels of free Trap quickly falling below those of bound VEGF Trap, and bound VEGF Trap did not approach the maximal steady-state levels seen with higher doses (Fig. 6A and B). However, doses of 1.0 and 3.0 mg/kg (i.v.) maintained substantial free Trap levels throughout the dosing period (Fig. 6A), and maximal complex levels were attained, as evidenced by equivalent levels of complex being generated at the two higher doses ( $\approx 1$ – $2 \mu\text{g/ml}$ , see Fig. 6B). In cancer patients with advanced solid tumors or non-Hodgkin’s lymphoma, remarkably similar results were obtained. That is, similar doses of VEGF Trap were required to saturate VEGF binding and complex formation (Fig. 6C–E). In addition, the maximal steady-state levels of VEGF–VEGF Trap complex were similar to those seen in non-cancer patients (Fig. 6B, D, and E). These findings indicate that, consistent with our findings in mice, endogenous VEGF production in adult human subjects is quite high, whether or not the individuals harbor tumors (Fig. 6E).

Using the same approach as was used for the mouse (see *Materials and Methods*), human production rates of VEGF in humans were found to be  $\approx 0.0025 \mu\text{g}$  per gram of tissue per day, which is remarkably similar to that calculated for mice (see above). If our findings in animal models continue to be predictive, these VEGF Trap levels achieved in ongoing clinical studies should be in the efficacious range.

## Discussion

At present, there are a number of anti-angiogenic agents targeting the VEGF pathway that are proceeding through clinical trials or already approved for the treatment of cancer (9). One major challenge is the lack of objective measures to guide dosing to determine when sufficient blockade has been achieved or to inform pharmacological response to these drugs. VEGF itself has been suggested as a potential biomarker for the above purposes, based on the assumption that VEGF in the peripheral circulation was primarily derived from the tumor and therefore accurately reflected tumor burden (19). However, to date it has proven difficult to accurately measure systemic levels of VEGF, correlate these levels with tumor burden, or use them as a guide to dosing (11, 12). Here, we describe the use of the VEGF Trap, a potent VEGF antagonist that forms a stable, inert complex with VEGF, as an index that allows for the accurate assessment of VEGF production rates. In addition, this unique property of the VEGF Trap allows accurate assessment of the amounts of VEGF made by a resident tumor compared with the rest of the body. Furthermore, in animals, this approach has been shown to provide a useful guide to selecting dosing regimens that substantially block available VEGF. This has not been possible with anti-VEGF antibodies, as VEGF–antibody complexes are rapidly cleared.

We find unexpectedly high levels of VEGF production in the normal adult setting, where it has long been assumed that, in the absence of ongoing angiogenesis, VEGF production rates would be quite low (11, 12). However, the unexpectedly high rates of VEGF production in non-tumor-bearing adult mice and humans is consistent with the recent realization that VEGF likely plays an ongoing role in the “quiescent” vasculature of normal adults (23). For example, treating normal adult mice and monkeys with VEGF antagonists can increase hematocrit (a measure of the proportion of the blood volume occupied by red blood cells) (24). Similarly, VEGF antagonists can also increase blood pressure (25), indicating that VEGF is involved in regulating

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