Cancer Therapy: Preclinical

Effects of Anti-VEGF Treatment Duration on Tumor Growth, Tumor Regrowth, and Treatment Efficacy

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Abstract

Purpose: Inhibition of the vascular endothelial growth factor (VEGF) axis is the basis of all currently approved antiangiogenic therapies. In preclinical models, anti-VEGF blocking antibodies have shown broad efficacy that is dependent on both tumor context and treatment duration. We aimed to characterize this activity and to evaluate the effects of discontinuation of treatment on the dynamics of tumor regrowth.

Experimental Design: We evaluated the effects of anti-VEGF treatment on tumor growth and survival in 30 xenograft models and in genetic mouse models of cancer. Histologic analysis was used to evaluate the effects of treatment on tumor vasculature. We used a variety of treatment regimens to allow analysis of the effects of treatment duration and cessation on growth rate, survival, and vascular density.

Results: Preclinical tumor models were characterized for their varied dependence on VEGF, thereby defining models for testing other agents that may complement or augment anti-VEGF therapy. We also found that longer exposure to anti-VEGF monoclonal antibodies delayed tumor growth and extended survival in established tumors from both cell transplants and genetic tumor models and prevented regrowth of a subset of residual tumors following cytoablative therapy. Discontinuation of anti-VEGF in established tumors resulted in regrowth at a rate slower than that in control-treated animals, with no evidence of accelerated tumor growth or rebound. However, more rapid regrowth was observed following discontinuation of certain chemotherapies. Concurrent administration of anti-VEGF seemed to normalize these accelerated growth rates.

Conclusions: In diverse preclinical models, continuous VEGF suppression provides maximal benefit as a single agent, combined with chemotherapy, or as maintenance therapy once chemotherapy has been stopped. Clin Cancer Res; 16(15); 3887–900. ©2010 AACR.

Angiogenesis has long been associated with aggressive tumor growth (1), leading to the proposal that blocking this process may be a viable for the treatment of cancer (2). The discovery of vascular endothelial growth factor (VEGF) as a major regulator of endothelial cell growth and survival (3, 4) paved the way for translating these concepts into clinical practice, and validation of this approach came in 2004 with the approval of the anti-VEGF monoclonal antibody (mAb) bevacizumab for the treatment of metastatic colorectal cancer in combination with standard chemotherapy (5). Bevacizumab has now been approved by the Food and Drug Administration for use in combination with chemotherapy/immunotherapy in colon, breast, lung, and renal cell cancers and as a single agent in glioblastoma. Additional antiangiogenic agents, such as sunitinib, sorafenib, and pazopanib, have also been approved as single agents in specific indications, further substantiating this approach to cancer therapy.

Debate continues over the optimal duration of bevacizumab treatment, prompting additional preclinical and clinical studies to address these questions (6, 7). This is of clinical importance because the current paradigm for cancer treatment duration is limited to experience with cytotoxic chemotherapy. The prolonged use of cytotoxic therapy in common solid tumors has, in general, not provided a compelling survival benefit, but has instead resulted in increased cumulative toxicities (8, 9). These findings have raised the question whether treatment until tumor progression with current cytotoxics and bevacizumab provides clinical benefit for patients.

Data from clinical studies suggest that longer duration of bevacizumab treatment may result in improved patient benefit. Analysis of a large randomized phase III study (NO16966) evaluating bevacizumab in first- and
Inhibition of the vascular endothelial growth factor (VEGF) axis is the basis of most antiangiogenic therapies used in the treatment of human cancer. These studies provide evidence that continuous VEGF blockade provides significant benefit in the setting of established progressing tumors and in models of residual disease, either alone or in combination with chemotherapy. Quantification of tumor growth rates both during and after treatment with anti-VEGF found no evidence of tumor "rebound" secondary to selective VEGF inhibition. These studies provide evidence that continuous, prolonged anti-VEGF treatment delays the growth of established tumors and may prevent regrowth of some residual tumors. Finally, we characterize a large panel of tumor xenografts for their varied dependence on VEGF, thereby defining models for characterization of other antiangiogenic agents that may complement or augment anti-VEGF therapy.

In recent efforts have focused on understanding the biological properties of tumors following discontinuation of first-line therapy, with reports of "tumor rebound" (11–14), or the emergence of a more aggressive and invasive disease following discontinuation of antiangiogenic therapy (15–18). These reports followed the observation of rapid tumor revascularization after discontinuation of a VEGFR tyrosine kinase inhibitor (TKI; ref. 19). Clinical evidence suggestive of rebound following discontinuation of bevacizumab from a limited patient series (12) or from analytic modeling of clinical trial data sets (14), but has not been rigorously addressed.

Based on these clinical results and a number of reports of rebound and adaptive evasion in response to angiogenic blockade, we conducted preclinical studies to better describe the tumor regrowth kinetics after discontinuation of anti-VEGF treatment. These studies are intended to determine if there is rationale for prolonged treatment duration, including treatment beyond progression, and if there is evidence for growth beyond control rates on cessation of an anti-VEGF mAb. A better understanding of these angiogenic mechanisms could have a significant effect on the management of patients.

Materials and Methods

All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the NIH (NIH Publication 85-23, revised 1985). The Institutional Animal Care and Use Committee approved all animal protocols.

Characterization of murine and human cross-reactive anti-VEGF mAbs

Measurement of the relative binding affinity in solution was done by ELISA and surface plasmon resonance as previously described (20).

Pharmacokinetic analysis of anti-VEGF antibodies

A single-dose pharmacokinetic study was done for two anti-VEGF mAbs, B20-4.1 and B20-4.1.1. A single i.v. bolus dose of 5 mg/kg for B20-4.1 and a single i.v. dose of 5, 15, or 25 mg/kg for B20.4.1.1 were administered to mice. Serum samples were collected from three animals in each group at various time points and analyzed in triplicate using an ELISA as previously described (20). Serum concentration-time profiles were used to estimate pharmacokinetic parameters for B20-4.1 and B20-4.1.1 by noncompartmental analysis using WinNonlin Model 201 (WinNonlin-Enterprise, version 5.1.1, Pharsight Corporation).

Tumor model studies

For cell transplant studies, cultured tumor cells were resuspended in PBS and implanted s.c. into the right flank of naïve mice as described in Supplementary Methods. Mice with tumors of a mean volume of 80 to 200 mm³ were grouped into treatment cohorts of 10 mice each. For genetic model studies, we used RIP-TbAg mice, which were a transgenic insulinoma model that phenocopy the RIP1-Tag2 mouse (10) that were treated as noted in Supplementary Methods. Anti-VEGF antibody was administered in PBS at 5 mg/kg twice weekly for 3 to 5 weeks unless otherwise noted. Additionally, chemotherapeutic agents were dosed for selected experiments as noted. Body weights and caliper measurements were taken twice per week during the study.

For analysis of growth rates during and after anti-VEGF treatment, mixed-effect models were used to model tumor growth profiles over time (see Supplementary Methods). All data were fit and plotted using the R

Histology and staining

Tumors were fixed in 10% neutral buffered formalin for 12 to 16 hours before paraffin embedding. Histologic

References

10 Singh et al., in preparation.
11 R: a language and environment for statistical computing, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria; NLME package authored by Douglas Bates and José Pinheiro.
sections (4–5 μm thick) were stained with H&E as described previously (21). Vascular density was assessed in MECA-32 (anti-PLVAP)-stained histologic sections of tumors, essentially as described previously (22). For each tumor, three representative images, centered 500 μm from the tumor margin, were analyzed.

Results

Pharmacokinetic properties of human-murine cross-reactive anti-VEGF antibody

Modeling the therapeutic activity of VEGF blockade in human xenografts requires reagents that can effectively and specifically neutralize both human and mouse VEGF because both tumor and host stromal elements are known to contribute VEGF to drive tumor angiogenesis (20). Furthermore, use of genetically engineered mouse models (GEMM) requires "murinized" antibodies that recognize murine VEGF and have nonimmunogenic backbones to allow for extended dosing in immunocompetent animals (23). Many of the issues related to species compatibility of VEGF inhibitors could be avoided through the use of VEGFR-targeted TKIs. However, such agents do not serve as good surrogates for selective VEGF blockade because their activity within the VEGF axis is much broader than that of a VEGF mAb. These TKIs also have activity against additional receptor tyrosine kinases beyond VEGFR2 (19), making it difficult to attribute any observed biology

Table 1. Growth characteristics and response to anti-VEGF treatment of various tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Control growth rate ([\log_2(\text{mm}^3)/\text{d}])</th>
<th>RGI (%)</th>
<th>Response type</th>
<th>Relative hVEGF expression</th>
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<td>MX-1</td>
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Abbreviations: CRC, colorectal cancer; NSCLC, non–small-cell lung cancer; GBM, glioblastoma.
exclusively to inhibition of VEGF signaling. We therefore chose to use a VEGF-specific function-blocking mAb in our preclinical studies. Bevacizumab is selective for human VEGF, but has barely any detectable binding to murine VEGF (20). Two series of cross-species reactive function-blocking mAbs targeting VEGF (B20, G6) have previously been described (20). These antibodies were identified for their ability to selectively neutralize all isoforms of VEGF-A, with no detectable activity against the related VEGF-family ligands, including placental growth factor, VEGF-B, VEGF-C, and VEGF-D (20). For this study, we selected the B20 series of anti-VEGF antibodies as a surrogate for pre-clinical modeling of bevacizumab activity because they have VEGF affinities similar to bevacizumab, yet effectively block both the human and the murine ligands (20). Two optimized clones were developed for in vivo studies, B20-4.1 and the variant B20-4.1.1, which has been optimized for recombinant production in mammalian cells.

Both B20-4.1 and B20-4.1.1 IgGs have relative affinities to human (EC50 = 0.20 and 0.17 nmol/L, respectively) and murine VEGF (EC50 = 0.39 and 0.17 nmol/L, respectively) that are similar to that of bevacizumab binding to human VEGF (EC50 = 0.40 nmol/L). These affinities are consistent with their potency in inhibiting VEGF-stimulated angiogenesis.

**Fig. 1.** In vivo response of tumor xenograft lines to anti-VEGF treatment. A, RGI of various tumor xenograft models during treatment with anti-VEGF. Models are ordered from the most responsive to the least responsive. B, control growth rate (log2(mm³)/d) for a panel of tumor models. Values are ordered from fastest to slowest growth rates. C and D, representative examples of early- and delayed-onset growth inhibition, which reflect two distinct patterns of response to anti-VEGF treatment. Best-fit curves were generated for control (black) and anti-VEGF (red) treatment groups. In models depicting an early-onset response (C) typified by A375 cell line, the two curves separated before 7 d after initiation of treatment. In models depicting a delayed-onset response (D) typified by DLD-1, the two curves were overlapping for a minimum of 7 d and did not separate until later time points.
endothelial cell growth (data not shown). Single-dose pharmacokinetic data were obtained for each antibody, and dosing regimens were simulated that would result in a minimum trough concentration at steady state ($C_{\text{min \, so}}$) of $\sim 30 \mu$g/mL, similar to that achieved in $>90\%$ of bevacizumab patients. The simulation indicated that this $C_{\text{min \, so}}$ would be predicted for both B20-4.1 and B20-4.1.1 at a dose of 5 mg/kg twice a week or 10 mg/kg weekly (Supplementary Table S1). In addition, serum samples collected from an efficacy study in RIP-TbAg mice with B20-4.1.1 dosed at 5 mg/kg twice a week resulted in a $C_{\text{min \, ss}}$ of $43.9 \pm 12.1 \mu$g/mL. These results are consistent with the predicted $C_{\text{min \, ss}}$ profile and is above both the target trough concentration for bevacizumab in the clinic and the recommended $C_{\text{min \, ss}}$ for antibodies in preclinical mouse models (24). Based on these analyses, the dosing of the cross-reactive anti-VEGF antibodies in preclinical models is predicted to effectively block $>97\%$ systemic VEGF, based on the equilibrium kinetic assumption of $\%$ bound VEGF = $\frac{(\text{anti-VEGF}) \times 100}{[K_D + (\text{anti-VEGF})]}$. This is similar to what is predicted for bevacizumab use in the clinic (25).

**Anti-VEGF has broad efficacy that is influenced by tumor context**

Preclinical tumor models support a role for VEGF in all stages of tumorigenesis (23), including early-stage or dormant tumors, to trigger an "angiogenic switch" and initiate the recruitment of a supportive vasculature (26). In established, late-stage primary and metastatic tumors, VEGF continues to influence the tumor vasculature, as evidenced by decreased microvascular density (MVD) on treatment with anti-VEGF antibodies. However, the resulting effect on tumor growth can vary because additional angiogenic factors can compensate for the loss of VEGF, resulting in tumors with varied sensitivities to angiogenic blockade (27–29). Such models can be used to study escape or evasion from anti-VEGF therapy and can help to identify additional angiogenic targeting approaches to be used alone or in combination with anti-VEGF.

To better define the spectrum of primary dependence that tumor xenograft models have on tumor and host-derived VEGF, we evaluated the in vivo effectiveness of anti-VEGF treatment in 30 tumor xenograft cell lines encompassing a number of different tumor types, including breast, ovarian, colorectal, lung, pancreatic, renal, prostate, and lymphoma. Mice were dosed with 5 mg/kg anti-VEGF (B20-4.1) twice a week for 3 to 5 weeks and tumors were measured twice each week. A number of efficacy measures were evaluated including percent tumor growth inhibition (%TGI), defined as the difference between the median tumor volumes of treated and control groups on the last day when all animals remain on study and expressed as a percentage of the control group; time to end point (TTE), time to reach a predefined end point tumor volume where each tumor is measured twice each week; percent tumor growth delay (%TGD), defined as the percent increase in median TTE in the treatment group compared with the control group; and relative growth inhibition (RGI), percent decrease in the slope of the log$_2$ fitted line for the treated group compared with that of the control group’s growth curve. We found that %TGD is influenced by the growth rate of the tumor line and the duration of treatment, whereas RGI better reflects the on-treatment effect of anti-VEGF in each of the model.

There is a wide range of VEGF dependence among the 30 tumor lines, as evidenced by the RGI values from anti-VEGF-treated mice (Table 1; Fig. 1A). We attribute the range of responses to the biological differences inherent in donor tissues because multiple studies from a given tumor cell line gave similar results (data not shown). RGI ranged from 77% (most VEGF dependent) to 7% (least VEGF dependent; Fig. 1A). Because multiple tumor types were represented by five or more different cell lines (breast, colorectal, and lung), we evaluated the data for any trends of response based on tumor type. No tumor type–specific patterns were observed. Models such as EL4 and LLC had reduced responsiveness to anti-VEGF and have previously been characterized as refractory models, due in part to the influence of BV8 produced by infiltrating CD11b+Gr1+ monocytes (28, 29). Models most responsive to anti-VEGF treatment included MX-1, OVCAR3, and DLD-1 and were derived from different human tumor donor sources. Percent TGD ranged from 10% (least VEGF-dependent) to $>100\%$ (most VEGF dependent; data not shown). Again, no tumor type–specific patterns were observed. Percent TGD is influenced by the growth rate of the tumor line and the duration of treatment, whereas RGI better reflects the effects on tumor growth either during or after treatment. Thus, we have evaluated a series of transplant tumor models and have characterized their sensitivity to anti-VEGF treatment using two different metrics, thereby defining their dependence on VEGF for growth.

To understand the relationship between RGI and %TGD, we also evaluated the correlation between these two metrics. As predicted, we found a correlation (slope of fit = 0.64; $P = 0.048$) indicating that models that have higher %TGD generally show higher RGI (both being defined as more VEGF dependent; data not shown). In models with high RGI, the near cytostatic response to anti-VEGF translated into an extended time on study and resulted in a delay in reaching the study end point. Interestingly, no such correlation was found between %TGI or TTE and either %TGD or RGI (data not shown). Tumor growth rates varied by 10-fold among the 30 lines, with the most rapid growth observed in the B16, HM7, LL, and A375 models (Table 1; Fig. 1B). Although fast-growing tumors are often stated to be highly angiogenic (30, 31), we saw no correlation between RGI and the control growth rate of these tumors (slope of fit = 0.01; $P = 0.97$), indicating that anti-VEGF sensitivity was not a function of tumor growth rate (Supplementary Fig. S1). We also evaluated if VEGF dependence was a function of VEGF expression levels. Evaluation of a cohort of models indicated that neither %TGD (data not shown) nor RGI (Supplementary Fig. S2) correlated with tumor VEGF expression levels, confirming the observations made in patient populations (32, 33).
Anti-VEGF treatment resulted in two primary patterns of growth inhibition (Fig. 1C and D). In some models (typified by A375), we saw an almost immediate separation of the growth rate curves of the anti-VEGF–treated versus control-treated animals (Fig. 1C). We termed this pattern “early-onset growth inhibition.” In the remaining models, the growth rate of treated animals mirrored the control rate for a brief initial period (4–15 days depending on the cell line) before separating to show reduced growth rate (Fig. 1D). We termed this pattern (typified by DLD-1) as “delayed-onset growth inhibition.” Of note, many of the less VEGF-dependent models (low RGI) displayed a delayed pattern, suggesting that although they lacked an immediate response to anti-VEGF, their growth was delayed with continued VEGF blockade. Notably, two other growth patterns were not observed in any of the xenograft models: transient and cytostatic growth inhibition. Transient inhibition would be reflective of an initial growth delay that then
accelerates back to the rate of control-treated tumors while still on anti-VEGF. This pattern might have implicated an adaptive response, development of resistance to VEGF blockade, or selection of a subpopulation of cells within the xenograft that are less dependent on VEGF for growth. A cytostatic response, characterized by a negative growth rate, might have been anticipated in highly VEGF-driven tumors. Although there may seem to be a cytoreductive response to anti-VEGF in newly engrafted tumors, this pattern was not observed in any of the established and actively growing tumor models used in these studies.

Increased efficacy is observed with longer duration of anti-VEGF treatment

Inhibition of VEGF by selective mAbs may essentially be considered irreversible due to the high \( k_{on} \) and low \( k_{off} \) of these reagents [B20-4.1 hVEGF: \( k_{on} = 2.4 \times 10^4 \text{ (mol/L)}^{-1} \text{ s}^{-1} \), \( k_{off} = 0.41 \times 10^{-4} \text{ s}^{-1} \), \( K_D = 1.7 \text{ nmol/L} \); B20-4.1 mVEGF: \( k_{on} = 4.8 \times 10^4 \text{ (mol/L)}^{-1} \text{ s}^{-1} \), \( k_{off} = 1.1 \times 10^{-4} \text{ s}^{-1} \), \( K_D = 2.3 \text{ nmol/L} \); B20-4.1.1 hVEGF: \( k_{on} = 7.1 \times 10^4 \text{ (mol/L)}^{-1} \text{ s}^{-1} \), \( k_{off} = 5.94 \times 10^{-4} \text{ s}^{-1} \), \( K_D = 8.3 \text{ nmol/L} \); B20-4.1.1 mVEGF: \( k_{on} = 5.6 \times 10^4 \text{ (mol/L)}^{-1} \text{ s}^{-1} \), \( k_{off} = 5.66 \times 10^{-4} \text{ s}^{-1} \), \( K_D = 10.1 \text{ nmol/L} \), based on 1:1 monovalent binding fitting]. However, the continuous production of VEGF by both tumor and host stromal cells and the local sequestration of VEGF by heparin binding matrix components (34) would suggest that prolonged and continuous inhibition might be necessary for most effective inhibition of tumor growth. To evaluate this, we conducted a number of preclinical studies in established xenograft tumors, comparing short-term anti-VEGF treatment with treatment for the entire duration of the study (end of study). SW620 colon cancer xenografts were allowed to grow to 600 mm\(^3\), after which they were treated with control antibody, anti-VEGF for 3 weeks, or anti-VEGF until the end of study. Short-term anti-VEGF treatment caused a rapid and constant growth delay consistent with the 45% RGI seen on treatment of smaller 200-mm\(^3\) tumors (Table 1). There was also a 16-day extension of the median TTE compared with control animals. Long-term, continuous exposure to anti-VEGF maintained this level of growth inhibition with a 25-day extension of the median TTE. The Kaplan-Meier plot reveals the survival advantage offered by short- and long-term anti-VEGF treatment, with longer-duration VEGF blockade resulting in optimal outcome (Fig. 2B). Interestingly, the short-term treatment group exhibited tumor regrowth and a reduction in survival starting approximately 1 week after the last dose of anti-VEGF. Additional preclinical studies in moderately anti-VEGF-responsive human breast (MDA-MB-231) and non-small-cell lung (A549) cancer models also showed a delay in tumor growth (data not shown) and improved survival (Supplementary Fig. S3) with both short- and long-term VEGF blockade. This effect was independent of anti-VEGF sensitivity because Bx-PC3 (anti-VEGF sensitive model) and H460 (anti-VEGF refractory model) both showed improved survival (Supplementary Fig. S3) with longer-duration therapy. Together, these data are consistent with the idea that continuous VEGF suppression exerts a

\[ \text{ anti-VEGF Treatment in Preclinical Models } \]

\[ \text{ E, representative examples of MECA-32–stained tumor vasculature (green) in the three groups [control (left), short-term anti-VEGF treatment (anti-VEGF q7d × 3; middle), and longer-term anti-VEGF treatment (anti-VEGF q7d to end of study; right)]. Micrographs are shown from tumors harvested at approximately 5 wk after the start of treatment at low magnification (×100; top row) and at higher magnification (×200; bottom row). Note that tumors on longer-term anti-VEGF treatment have less vasculature than control tumors or short-term anti-VEGF–treated tumors. Tumor nuclei are stained with 4,6-diamidino-2-phenylindole (blue) in the low-magnification images to facilitate visualization of the tumor mass. } \]
cytostatic effect and delays the growth of established tumors, and that treatment withdrawal is followed by regrowth of tumors likely due to the ability of active VEGF to continue to promote angiogenesis.

The vasculature of implanted human xenograft tumors is structurally distinct from that of human tumors (35, 36). We therefore wanted to evaluate the effect of longer-duration anti-VEGF treatment on tumors that arise endogenously in orthotopic organs. The RIP-Tag genetically engineered model of pancreatic islet cell carcinoma has been extensively used for evaluating the role of angiogenic therapy in tumor treatment (37). This model has a stereotypic and autochthonous vasculature that develops through neoplastic progression of the lesions. These GEMMs of cancer are thought to be more representative of human tumor vasculature. Previous studies in this model have shown that the early preneoplastic lesions are highly dependent on VEGF through week 10, but following activation of the angiogenic switch and development of multiple solid tumors, they recruit additional angiogenic factors and become less dependent on VEGF (26). We have used a conditional variant of this model, termed RIP-TbAg, in which the expression of SV40 large T-antigen is driven by a RIP-FLP transgene. This RIP-TbAg model maintains the same temporal progression of multistage islet cell carcinoma as RIP-Tag2 and also exhibits a similar response to early intervention (before week 10) with anti-VEGF that results in a dramatic reduction of tumors. However, late intervention, at 11.7 weeks, with short-term anti-VEGF treatment (2 weeks) showed little effect on overall survival (Fig. 2C, blue versus red lines). However, when these older animals remained on anti-VEGF until end of study, there was a modest but significant improvement in overall survival compared with the control-treated group (15.1 versus 14.3 weeks, P < 0.05; Fig. 2C, green versus red lines). To better understand the lack of benefit following short-term anti-VEGF treatment, we evaluated the vasculature of the treated tumors, both early (1 week) and late (∼3 weeks) after stopping treatment. At the early time point, treatment with anti-VEGF caused a significant decrease in MVD (data not shown). Thus, although there was not a robust effect on tumor growth in response to 2 weeks anti-VEGF treatment, this unresponsiveness was not the result of “resistance” to VEGF blockade. Instead, it seems that the Rip-TbAg tumors do not require excessive vascularization for their growth. Three weeks after stopping anti-VEGF treatment, the MVD returned to the level of control-treated animals (Fig. 2D and E). However, mice on extended anti-VEGF treatment maintained a reduced MVD, suggesting that continuous suppression of tumor vasculature translates into increased efficacy.

GEMMs have recently been generated that recapitulate the development, progression, and pathophysiology of human lung and pancreatic cancers (35, 36). These models are based on tissue-specific activation of key molecular drivers for each disease (KRas and p53 for non–small-cell lung cancer, Rb and p53 for small-cell lung cancer, and KRas and p16/p19 for pancreatic ductal adenocarcinoma) and exhibit similar histopathology to their human counterparts. The endogenously derived vasculature present in these tumors is structurally similar to that observed in human disease and provides a unique opportunity to evaluate the efficacy of therapeutic regimens containing antiangiogenic agents. Response to anti-VEGF in these models correlated with the responses observed in the clinic because VEGF blockade promoted a significant and durable response, both alone and in combination with chemotherapy in the non–small-cell lung cancer model, yet had only a modest and transient response in the pancreatic adenocarcinoma model (data not shown). These GEMMs complement the xenograft models and provide further support that continuous VEGF blockade delays progression and prolongs survival.

Increased efficacy of anti-VEGF in combination with chemotherapy

Bevacizumab is approved for use in combination with a number of chemotherapy regimens in colon, lung, and breast cancers. We therefore tested the ability of anti-VEGF combined with the maximum tolerated dose of several cytotoxic agents to characterize the influence of chemotherapy class, tumor type, and disease setting on response. In the moderately VEGF-dependent MDA-MB-231 breast tumor model, 5 days of treatment with paclitaxel had a strong cytoreductive effect, resulting in a 40% to 100% objective response rate in several studies. A representative study is shown in Fig. 3. However, in all studies, the tumors regrew at approximately 4 weeks after completion of paclitaxel treatment (Fig. 3A and C), indicating that residual cells were able to survive chemotherapy and subsequently reconstitute the tumor. Concurrent treatment with anti-VEGF had both early and late effects on tumor growth. The combination resulted in an initial objective response rate of 90%, with more complete responses than seen with chemotherapy alone (50% versus 10%, respectively). However, if anti-VEGF was stopped after 3 weeks, the objective response rate deteriorated by the end of study (Fig. 3C) and tumors regrew at a rate similar to animals receiving single agent paclitaxel (Fig. 3C). We then asked what effect extended anti-VEGF maintenance therapy would have on recurrence of tumors in this residual disease model. When anti-VEGF was continued for 7 weeks after chemotherapy, 60% of these early responses were sustained, often resulting in no detectable tumors even at 50 days after stopping the anti-VEGF treatment (Fig. 3D). This posttreatment disease-free interval is longer than the dormancy period of 4 weeks observed with single agent paclitaxel and extends beyond the time of systemic VEGF inhibition, suggesting additional synergistic activity with prolonged anti-VEGF treatment. The Kaplan-Meier and TTE plots (Fig. 3B and E) highlight the benefit of extended maintenance anti-VEGF following chemotherapy (Fig. 3B). The early and late effects of anti-VEGF in combination with taxanes were observed in several other tumor xenograft models of various solid tumor origins, including the anti-VEGF–refractory model SKMES and the anti-VEGF–sensitive models Bx-PC3.
(Supplementary Fig. S4) and MV-522 (data not shown), suggesting that this finding is not specific to a single tumor type and is consistent across models with varied anti-VEGF sensitivities. Future studies will follow animals for several months after stopping all therapies and will evaluate for histochimical evidence of residual tumor.

Similar combination studies were extended to additional cytotoxic agents to determine if the anti-VEGF effects were limited to a particular class of agents. We observed combination activity with a number of cytotoxic agents including nucleoside analogues (gemcitabine and 5-fluorouracil), anthracyclines (doxorubicin), platins (carboplatin and cisplatin; Supplementary Fig. S4), topoisomerase I inhibitors (CPT-11), and other taxanes (docetaxel; data not shown). Additionally, this effect did not seem to be model or tumor type dependent because a number of agents including paclitaxel and gemcitabine showed efficacy in multiple tumor models (Supplementary Fig. S4). Generally, these models followed a similar pattern of a profound response to chemotherapy followed by eventual regrowth weeks after the cessation of treatment. In many instances, anti-VEGF treatment was able to significantly delay or prevent the regrowth of the tumors. More than 100 studies were done. We saw additive activity of anti-VEGF and chemotherapy in ∼25% of the studies, and importantly, in the remaining 75%, we saw no evidence of antagonism in any of these combinations (data not shown). These findings suggest that anti-VEGF is able to extend the activity of many cytotoxic agents and that this is likely mechanism specific.

These residual disease models provide preliminary evidence that longer-duration anti-VEGF not only delays regrowth of residual tumors but may also result in sustained and durable inhibition of tumor growth. Conceptually, there may be a limited length of time in which these residual tumors can survive without activating an angiogenic switch and recruiting vasculature. What is currently unclear is whether extended VEGF depletion may ultimately lead to the demise of such tumors, leading to prevention rather than just delay of recurrence.

No evidence of rebound on cessation of anti-VEGF therapy

Our analysis in a diverse collection of tumor lines shows that, during treatment, anti-VEGF causes a reduction in the tumor growth rate. Recent reports have postulated a “rebound phenomena” of increased growth of tumors and their vasculature following discontinuation of an angiogenic blockade (11–19). These studies generally use multispectrum VEGFR-targeted kinase inhibitors, or VEGFR2-targeted antibodies, and surprisingly, none have actually quantified the growth rates of treated and recovering tumors. We sought to quantify tumor growth rates following selective blockade of VEGF with anti-VEGF neutralizing antibodies. We calculated the growth rate of tumors during the recovery period, which we defined as the period following the last dose of anti-VEGF, and compared this rate to that observed in control-treated tumors. A 10-day period for antibody washout was used to ensure that no direct anti-VEGF-mediated effects were included in the calculation. In 25 of 26 cases (96%), we found the recovery rate to be less than the control rates, arguing against the existence of rebound following release of an angiogenic blockade (Fig. 4A). Only a single tumor line (SKMES) showed an increased recovery rate following anti-VEGF treatment. To identify the factors that influenced the tumor regrowth rates, we evaluated a number of parameters, including VEGF expression levels, control tumor growth rate, and growth rate while on anti-VEGF treatment. The only significant correlation identified was between the rates of tumor growth during and after treatment with anti-VEGF (Supplementary Fig. S5). Examples of tumor growth curves for anti-VEGF-sensitive (MX-1, SKOV-3), moderately anti-VEGF-sensitive (MCF-7), and anti-VEGF-refractory (Panc-1) tumor models show that no increased growth rates are observed regardless of anti-VEGF sensitivity (Supplementary Fig. S6).

Because bevacizumab is more commonly used in combination with chemotherapy, we also evaluated the in vivo effects of chemotherapy, both alone and in combination with anti-VEGF on rebound rates. Most tumors grew slower during the regrowth phase after cytoreduction, compared with control rates (black columns, Fig. 4B and C). This blunted growth was seen with multiple chemotherapies in the same tumor model (Fig. 4B) and with the same chemotherapy in different tumor cell lines (Fig. 4C). In a few instances, a significant increase in growth rate was noted following discontinuation of single-agent chemotherapy (gemcitabine in MDA-MB-231 and paclitaxel in Bx-PC3). However, regrowth rates were always lower when anti-VEGF was combined with chemotherapy compared with regrowth rates following single-agent chemotherapy. In the Bx-PC3 model, anti-VEGF cotherapy prevented “rebound” elicited by paclitaxel (Fig. 4C). Taken together, these data suggest that tumor rebound can occur following discontinuation of paclitaxel or gemcitabine, but these uncommon examples of rebound are not associated with discontinuation of anti-VEGF treatment. Furthermore, anti-VEGF treatment has a beneficial effect on regrowth rates both alone and in combination with chemotherapy.

Discussion

Anti-VEGF treatment showed broad activity in a diverse panel of ∼30 tumor models. These results were expected based on the proposed antiangiogenic mechanism and the acknowledged importance of VEGF to tumor angiogenesis (3, 4). However, we have quantified a surprisingly broad range of responses to anti-VEGF among these models, allowing them to be classified based on their dependence on VEGF for growth. Furthermore, we confirmed the sensitivity to VEGF-axis inhibition in a subset of these models by comparing the effects of anti-VEGF and an intermediate dose of the small-molecule inhibitor of VEGF receptors, ABT-869 (ref. 38; Supplementary Fig. S7). VEGF-dependent models could potentially be
used to more thoroughly understand the anti-VEGF mechanism of action (20). By the same token, the VEGF-independent models can be used to study anti-VEGF escape and to identify additional factors (29) that may drive angiogenesis in the context of maximal VEGF blockade. Indeed, such approaches have already provided insights into the tumor microenvironment and have led to the identification of angiogenic and metastatic factors produced by infiltrating immune cells and activated stromal cells (28, 39).

There are multiple end points used in clinical trials to measure response and efficacy. In these preclinical studies, we used %TGD and RGI as distinct metrics of efficacy. We found a correlation between these independent measures, thereby providing increased confidence in each. However, we did not identify any correlation between tissue of tumor origin and efficacy. These findings support the notion that blocking tumor angiogenesis is a broadly applicable approach to cancer therapy but may also imply that the vasculature of cell-transplant tumor models may not faithfully represent the disease from where tumor cells originate. Among the models used in this article, GEMMs may more accurately model human disease because their tumor vasculature develops in concert with malignant
progression and may be more suitable for investigating the disease-specific effects of antiangiogenic agents (35, 36).

Target overexpression or pathway activation may correlate with response to a targeted therapy and has led to the development of diagnostics and the selection of patient subsets more likely to respond. Although human tumors that express high levels of VEGF, including renal, ovarian, and glioblastoma, seem to be more responsive to single-agent antiangiogenic therapy than other solid tumors (40), no predictive biomarker has yet been identified for bevacizumab or other antiangiogenic agents (32). VEGF levels have been found to be prognostic for more aggressive tumors, but they have not conclusively been shown to be predictive of response to bevacizumab or other VEGFR-targeted agents (32). VEGF levels have been found to be prognostic for more aggressive tumors, but they have not conclusively been shown to be predictive of response to bevacizumab or other VEGFR-targeted agents (32). In our diverse collection of tumor models, we did confirm high levels of VEGF expression in renal and glioblastoma tumors (Table 1), but found no correlation between tumor VEGF expression and either the growth rate of untreated tumors or their RGI in response to anti-VEGF (Supplementary Fig. S2).

We also examined the effect of treatment duration on response to VEGF blockade. In established tumors, we found that anti-VEGF treatment resulted in two primary patterns of growth inhibition that we refer to as “delayed onset” or “early onset.” The majority of models had a delayed onset pattern of growth inhibition where VEGF blockade was not immediately reflected by a change in tumor growth rate. Given that a majority of the antivascular effects of anti-VEGF manifest within the first 48 hours of treatment (42), the exact cause for this delay is unclear and may reflect the excess vascular reserve that is generally found in many biological contexts. Not surprisingly, we also observed improved tumor growth control and survival in mice maintained on an extended course of anti-VEGF. In xenografts, we saw no evidence for an increased rate of tumor growth while remaining on anti-VEGF therapy. However, robust yet transient responses to anti-VEGF have been observed in non–small-cell lung cancer GEMMs, suggesting that these genetic models may be useful for identifying factors responsible for escape or evasion from anti-VEGF treatment.

The influence of treatment duration is likely due to the fact that tumors and host stroma continue to produce VEGF, which, on withdrawal of anti-VEGF, is capable of activating angiogenesis to support tumor growth. This is confirmed by the regrowth of tumor vasculature up to, but not exceeding, the density observed in the control tumor within weeks of stopping anti-VEGF treatment. Similar results are also observed on withdrawal of VEGFR TKIs (11, 19). Additionally, we have observed a similar trend when anti-VEGF was used in combination with chemotherapy both in human tumor xenografts (Fig. 3) and in GEMMs.

We also examined the effect of prolonged VEGF blockade in the setting of residual disease. Cytotoxic chemotherapy can effectively reduce established tumors to yield a partial or complete response. However, residual tumor cells or clusters often remain behind and can lie dormant.

Fig. 3 Continued. D, individual tumor growth volumes at the end of study (d90) show that whereas paclitaxel treatment resulted in inhibition of tumor regrowth in 1 animal, the addition of 3 wk of anti-VEGF to paclitaxel resulted in 4 animals with no tumor regrowth and addition of 7 wk of anti-VEGF to paclitaxel resulted in 6 animals with no tumor regrowth. E, TTE for each animal in the study shows that whereas 2 animals did not reach end point during the course of the experiment when treated with paclitaxel, addition of 3 wk of anti-VEGF to paclitaxel increased this number to 6 animals and addition of 7 wk of anti-VEGF to paclitaxel further increased this number to 9 animals.
for weeks to years. Our studies show that these lesions are highly dependent on VEGF and, when appropriately suppressed, exhibit dramatic and durable growth inhibition. Such efficacy is likely due to a failure in their ability to recruit a vasculature and enable these isolated cells to progress and grow to a size that will be detectable by conventional computed tomography scans. Such residual disease models have been used to study the effect of prolonged VEGF blockade following chemotherapy (43–46) and have shown that anti-VEGF therapy primarily functions to delay rather than prevent tumor regrowth. However, we believe that these studies are the first to show that in some models of residual tumor burden, prolonged anti-VEGF therapy can prevent tumor regrowth for prolonged periods. Further studies with longer follow-up and histochemical analysis will be required to determine if this extended treatment would result in durable relapse-free survival.

Recent reports have questioned whether there is a rebound in tumor growth following discontinuation of anti-angiogenic therapy with VEGFR TKIs. Because of the multiple targets inhibited by current VEGF TKIs (19), such an effect cannot be attributed exclusively to inhibition of VEGF signaling. Rebound can be defined as an increase in tumor growth rate following treatment withdrawal that exceeds that of placebo-treated controls. To address the specific effect of VEGF inhibition on tumor regrowth, we measured these rates following cessation of therapy with a VEGF-specific mAb in more than 20 models. We found no evidence of accelerated growth beyond that of control tumors. Although it is possible that accelerated regrowth may occur in another time window, we believe this is unlikely to be related to discontinuation of VEGF suppression because our studies spanned a diverse collection of models representing multiple tumor types, varied primary growth rates, and a broad range of VEGF dependence.
Additionally, these studies do not address the altered metastatic patterns described with TKI inhibition (47, 48) because most of the models do not have observable metastases. In contrast, we observed a few examples of an increased rate of tumor regrowth following cessation of chemotherapy. This is in agreement with previous reports (44) of tumor rebound following treatment with certain cytotoxic agents, and it may be related to reports of enhanced invasive and metastatic properties of tumors treated with broad-spectrum TKIs (47, 48). However, our studies found that, when combined with chemotherapy, anti-VEGF delays, rather than exacerbates, tumor regrowth. These results suggest that the action of anti-VEGF is mechanistically distinct from those of less specific antiangiogenic agents (17, 48). Although tumor and tumor vasculature regrow after anti-VEGF is cleared from circulation, they do so at a rate, and to a degree (density), consistent with that of an untreated tumor of comparable size.

Conclusion
Tumor models with a wide range of dependence on VEGF provide evidence that continuous VEGF blockade is required to maximize benefit as a single agent, combined with chemotherapy, or in the maintenance setting.

We also find no evidence of excessive or aggressive tumor regrowth or vascular “rebound” once VEGF blockade is discontinued. These data provide a rationale for considering the use of bevacizumab with various lines of chemotherapy or for considering longer-maintenance use in early-stage disease.

Disclosure of Potential Conflicts of Interest
All authors except B. Hollister are full-time employees of Genentech, Inc.

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References


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