Differential Therapeutic Effects of Anti–VEGF-A Antibody in Different Tumor Models: Implications for Choosing Appropriate Tumor Models for Drug Testing

Dror Alishekevitz¹, Rotem Bril¹, David Loven², Valeria Miller¹, Tali Voloshin¹, Svetlana Gingis-Velistski¹, Ella Fremder¹, Stefan J. Scherer³, and Yuval Shaked¹

Abstract

We previously reported that the host response to certain chemotherapies can induce primary tumor regrowth, angiogenesis, and even metastases in mice, but the possible impact of anti–VEGF-A therapy in this context has not been fully explored. We, therefore, used combinations of anti–VEGF-A with chemotherapy on various tumor models in mice, including primary tumors, experimental lung metastases, and spontaneous lung metastases of 4T1-breast and CT26-colon murine cancer cell lines. Our results show that a combined treatment with anti–VEGF-A and folinic acid/5-fluorouracil/oxaliplatin (FOLFOX) but not with anti–VEGF-A and gemcitabine/cisplatinum (Gem/CDDP) enhances the treatment outcome partly due to reduced angiogenesis, in both primary tumors and experimental lung metastases models. However, neither treatment group exhibited an improved treatment outcome in the spontaneous lung metastases model, nor were changes in endothelial cell numbers found at metastatic sites. As chemotherapy has recently been shown to induce tumor cell invasion, we tested the invasion properties of tumor cells when exposed to plasma from FOLFOX-treated mice or patients with cancer. While plasma from FOLFOX-treated mice or patients induced invasion properties of tumor cells, the combination of anti–VEGF-A and FOLFOX abrogated these effects, despite the reduced plasma VEGF-A levels detected in FOLFOX-treated mice. These results suggest that the therapeutic impact of antiangiogenic drugs varies in different tumor models, and that anti–VEGF-A therapy can block the invasion properties of tumor cells in response to chemotherapy. These results may implicate an additional therapeutic role for anti–VEGF-A when combined with chemotherapy. Mol Cancer Ther; 13(1); 202–13. ©2013 AACR.

Introduction

Tumor angiogenesis entails endothelial cell division from preexisting vessel capillaries and the mobilization of circulating bone marrow–derived endothelial progenitor cells (CEP), which home to active angiogenic sites and incorporate into tumor blood vessel walls (1). A number of antiangiogenic-based drugs have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of several malignancies. Bevacizumab, for example, is a humanized monoclonal antibody aimed at neutralizing VEGF-A. Coadministrating bevacizumab and chemotherapy increases overall survival and/or progression-free survival of patients with advanced metastatic diseases, including colorectal, breast, non–small cell lung, and ovarian cancers (2–5). However, only limited clinical evidence exists about the therapeutic impact of bevacizumab at the adjuvant and neoadjuvant treatment settings. Recently, it has been shown that coadministration of bevacizumab and neoadjuvant chemotherapy significantly increased the pathologic complete response in patients with triple-negative breast cancer (6, 7). In addition, emerging findings from a phase III clinical study in which bevacizumab was coadministered with folinic acid/5-fluorouracil/oxaliplatin (FOLFOX) to patients with colorectal cancer in an adjuvant setting, revealed that this treatment combination failed to meet the predetermined disease-free survival endpoint at 3 years (8, 9). These results raise questions about the therapeutic impact of bevacizumab and the mechanism of action of blocking VEGF-A on tumor growth, when coadministered with conventional chemotherapy.

We have recently studied the impact of antiangiogenic drugs in combination with chemotherapy on tumor growth and angiogenesis. Our goal was to reveal mechanisms that may explain how antiangiogenic drugs act as chemosensitizing agents (10). We showed that certain chemotherapy drugs, including paclitaxel and 5-flourouracil (5-FU), induce rapid mobilization of CEPs from the
bone marrow (11) and subsequently home to treated tumor sites and promote angiogenesis. The administration of antiangiogenic drugs 24 hours before chemotherapy resulted in the inhibition of therapy-induced CEP mobilization and subsequently enhanced treatment efficacy (11). Other chemotherapy drugs, such as gemcitabine and cisplatinum, which do not affect CEP levels, did not show any added therapeutic benefit when combined with an antiangiogenic drug (11). Importantly, some of these studies were conducted in non–tumor-bearing mice, suggesting that antiangiogenic drugs may act as chemosensitizing agents by inhibiting the host systemic-rebound angiogenesis effects mediated by CEPs, leading to improved treatment outcomes when tumors are intact (12).

Counterintuitively, recent preclinical studies have suggested that while antiangiogenic drugs should, in theory, act solely to inhibit the metastatic spread by decreasing angiogenesis in tumors, they may actually promote tumor invasion to normal adjacent tissue or even accelerate metastasis (13). Inhibition of VEGF pathways by small-molecule antiangiogenic drugs led to increased tumor cell invasiveness and metastatic growth in several preclinical tumor models (14, 15). On the other hand, an anti–VEGF-A neutralizing antibody was recently shown not to promote metastasis in genetically engineered mouse tumor models (16, 17). It is suggested that various angiogenic factors are induced by the host in response to antiangiogenic therapy (18). Furthermore, myeloid-derived suppressor cells (MDSC) were also found to home to tumors treated with anti–VEGF-A therapy and contribute to tumor refractoriness and subsequent regrowth (19). Overall, these findings raise the possibility that certain cellular and molecular mechanisms may compensate for the lack of VEGF-A or its receptors by upregulating multiple "bypass" pathways in the tumor or in the host that, in turn, may promote tumor regrowth and metastasis (20–22).

Similar to antiangiogenic drugs, the potential of chemotherapy to accelerate tumor cell invasion and metastasis has also been investigated recently. We and others have previously reported that chemotherapy induces molecular host factors that can contribute to tumor cell growth and tumor colonization in the lungs (11, 23–26). For example, matrix metalloproteinase 9 (MMP9) secreted from bone marrow–derived cells (BMDC) that colonize paclitaxel-treated tumors may explain why mice primed with paclitaxel chemotherapy succumb to metastasis earlier than mice treated with vehicle control (25). Overall, these studies suggest that the host in response to the chemotherapy generates prometastatic and protumorigenic effects that promote tumor regrowth and metastasis. Therefore, drug combinations that inhibit the host’s protumorigenic effects in response to chemotherapy are required.

The aim of the current study is to investigate the effect of combined treatments with anti–VEGF-A and chemotherapy on breast and colon carcinomas in various tumor models extensively used in preclinical studies, which include primary (ectopic), spontaneous lung metastasis, and experimental lung metastasis. Using these models, we explored the therapeutic benefits of an anti–VEGF-A antibody (B20) and its effects on the invasion of tumor cells. The focus of this study is to elucidate the host effects found following the combination of anti–VEGF-A and chemotherapy and its therapeutic impact on various tumor models. The results show an additional role for anti–VEGF-A other than its antiangiogenic effect.

Materials and Methods

Tumor models and cell lines

Primary ectopic tumors. 4T1 murine-breast carcinoma cells (5 × 10^5) or CT26 murine-colorectal carcinoma cells (2 × 10^5) obtained from the American Type Culture Collection (ATCC) were subcutaneously injected into the flank of 5- to 6-week-old female BALB/c mice (Harlan). Tumor size was assessed regularly with Vernier calipers using the formula width^2 × length × 0.5. When tumors reached 200 mm^3, treatment was initiated unless indicated otherwise. Mice were randomly grouped before therapy (n = 4–5 per group).

Spontaneous lung metastases. 4T1 cells (5 × 10^5) were orthotopically injected into the mammary fat pad of 6-week-old female BALB/c mice. CT26 cells (2 × 10^5) were subcutaneously injected into the flank of 6-week-old female BALB/c mice. When tumors reached 200 to 300 mm^3, they were surgically removed and treatment was initiated when metastases were observed in the lungs of untreated mice (7 and 16 days following tumor resection, respectively; Supplementary Fig. S1).

Experimental lung metastases. 4T1 cells (3 × 10^5) or CT26 cells (5 × 10^5) were injected intravenously to the tail vein of 6-week-old female BALB/c mice. Lung metastatic lesions were observed on day 28 following tumor cell inoculation, at which point treatment was initiated for both tumor types (Supplementary Fig. S2).

All animal studies were approved by the Technion’s (Haifa) institutional committee. In some in vitro experiments, HCT116 human colon carcinoma cells (ATCC) were used. HT1080 fibrosarcoma and LM2-4 (a metastatic variant of the MDA-MB-231) breast carcinoma cell lines, used in some experiments, were cultured in RPMI and 10% fetal calf serum (FCS). All cells were passed in culture for no more than 4 months after being thawed from authentic stocks.

Drugs and their concentration

FOLFOX chemotherapy comprising folic acid (3 mg/kg), 5-FU (5 mg/kg), and oxaliplatin (1.4 mg/kg) was administered intraperitoneally as a single bolus injection in a 14-day cycle. GEM/CDDP chemotherapy, a combination of gemcitabine (33 mg/kg) and cisplatin (0.6 mg/kg), was administered intraperitoneally as a single bolus injection in a 14-day cycle. B20, a...
murine and human VEGF-A antibody (5 mg/kg; kindly provided by Genentech Inc.), was administered intraperitoneally concomitantly with chemotherapy or as monotherapy. The doses of FOLFOX and GEM/CDDP were determined on the basis of mouse survival and body weight loss (Supplementary Table S1) and as indicated elsewhere (27, 28). The B20 dose was used as previously determined (29).

Flow cytometric acquisition and analysis
Blood or cell suspensions were quantitated for viable CEPs, endothelial cells, and MDSCs, using flow cytometry as previously described (23). Details are described in Supplementary Material (30, 31).

Quantitation and visualization of tissue hypoxia, vessel perfusion, and microvessel density
Tissue processing and immunohistochemistry were conducted as described previously (32). Details are described in Supplementary Material.

Evaluation of murine and human VEGF-A protein levels in the plasma
For the determination of VEGF-A levels in the plasma of mice and patients with cancer, blood was collected in EDTA tubes to prevent clotting. Plasma was obtained after cells were removed by centrifugation for 10 minutes at 2,000 × g and immediately stored at −20°C. Thawed plasma was applied onto a specific mouse or human VEGF-A ELISA, respectively, according to the manufacturer’s instructions (R&D systems). Results were analyzed in triplicates.

Evaluation of MMP9 expression using gelatin zymography
MMP9 expression was assessed by gelatin zymography using previously described method (25). Details are described in Supplementary Material.

Blood samples obtained from cancer patients
Patients with cancer who underwent colorectal cancer surgical resection (n = 11) and were subsequently treated with FOLFOX chemotherapy (as part of an adjuvant treatment) at Ha’Emek Medical Center, Afula, Israel, were enrolled in the study. Blood was collected in EDTA tubes at baseline and 24 hours after the first cycle of FOLFOX chemotherapy. Plasma was separated and immediately stored at −20°C. Plasma samples were used to assess the invasion of HCT116 cells using a Boyden chamber as described below and to determine VEGF-A levels using an ELISA. The study was approved by Ha’Emek Medical Center’s ethics committee, and written informed consent was obtained from all the patients before experimentation.

Invasion assay
The invasive properties of CT26 and HCT116 cells in response to plasma of mice and humans, respectively, were evaluated in Matrigel-coated Boyden chambers, using a previously described protocol (25). Details are described in Supplementary Materials.

Quantification of the expression levels of angiogenesis-related factors in plasma
A protein array kit (RayBio biotin label–based mouse Antibody array 1, RayBiotech, Inc.) was used to evaluate the expression levels of angiogenesis-related factors in plasma from control or FOLFOX-treated mice in accordance with the manufacturer’s instruction. The results were analyzed using an online database for annotation, visualization and integrated discovery (DAVID, NIT) bioinformatics resources version 6.7, with categorization for angiogenesis. The fold change between the expression levels of factors in plasma of control untreated or FOLFOX-treated mice was calculated.

Statistical analysis
Data are expressed as mean ± SD. The statistical significance of differences was assessed by one-way ANOVA, followed by Newman–Keuls ad hoc statistical test using GraphPad Prism 4 software. Differences between all groups were compared with each other and were considered significant at values below 0.05.

Results
FOLFOX, but not GEM/CDDP, induced viable CEP mobilization, an effect that was blocked by anti-VEGF-A antibodies
In our previous studies, we evaluated systemic angiogenesis rebound by means of a substantial increase in viable CEP levels using single-agent chemotherapies (11, 12). Here, we sought to determine viable CEP levels in mice treated with drug combinations commonly used as first- and second-line treatments of colon and breast carcinomas (33, 34). Levels of viable CEPs were analyzed in non–tumor-bearing mice treated with the maximum tolerated dose (MTD) of FOLFOX and GEM/CDDP. We also investigated whether coadministration of an anti–VEGF-A antibody (B20) with these chemotherapies can affect viable CEP levels, as previously described for vascular disrupting agents (VDA) and several chemotherapy drugs (11, 32). For these purposes, blood was drawn 24 hours after the mice were treated with FOLFOX, FOLFOX+B20, GEM/CDDP, or GEM/CDDP+B20, and viable CEP levels were analyzed by flow cytometry. As shown in Fig. 1, FOLFOX chemotherapy induced a significant increase (~4-fold) in the mobilization of viable CEPs, whereas GEM/CDDP did not have a significant effect on the levels of these cells. No delayed CEP mobilization effects were documented in both FOLFOX and GEM/CDDP-treated mice in the first 96 hours (Supplementary Fig. S3). The B20 antibody blocked FOLFOX-induced viable CEP mobilization, and as expected, did not affect CEP levels following GEM/CDDP therapy.
treated with FOLFOX and metastases of either CT26 or 4T1 tumors survived longer than those treated with GEM/CDDP. In addition, mice bearing experimental lung metastasis, or spontaneous metastasis following primary tumor resection. The mice were then treated with FOLFOX or GEM/CDDP administered at the maximum tolerated dose with or without 5 mg/kg B20 antibody administered once. Blood was drawn at baseline and 24 hours after therapy. Viable CEP levels were evaluated as described in Materials and Methods. n = 4–5 mice/group; data are presented as mean ± SD. ***P < 0.001.

Coadministration of FOLFOX and anti–VEGF-A antibody enhanced the treatment efficacy of the primary tumor and experimental metastasis but did not affect the survival of mice bearing spontaneous metastasis

To assess whether the treatment outcome can be affected by blocking viable CEP mobilization induced by FOLFOX, 2 tumor models, CT26 murine colon carcinoma and 4T1 murine mammary carcinoma, were implanted in BALB/c mice. The cells were inoculated into mice to generate tumor models often used for preclinical drug evaluation, namely, ectopic primary tumors, experimental metastasis, or spontaneous metastasis following primary tumor resection. The mice were then treated with FOLFOX, FOLFOX + B20, GEM/CDDP, or GEM/CDDP + B20, and tumor volumes and mouse survival (in the cases of metastasis) were monitored until the endpoint. The primary tumor growth of CT26 was suppressed in mice treated with FOLFOX + B20 compared with FOLFOX monotherapy, whereas no differences were observed in the tumor growth curves of CT26 and 4T1 tumor types between the mice group treated with GEM/CDDP and those treated with GEM/CDDP + B20 (Fig. 2A and D). Of note, it appears that GEM/CDDP treatment efficacy in both 4T1 breast and CT26 colon cancers was superior to that of FOLFOX, and that anti–VEGF-A did not significantly enhance the antitumor activity of FOLFOX in 4T1 tumors. In addition, mice bearing experimental lung metastases of either CT26 or 4T1 tumors survived longer when treated with FOLFOX + B20 compared with mice treated with FOLFOX monotherapy (median survival: 57 vs. 44 days and 34 vs. 20 days, respectively). Again, no significant differences in survival curves were observed between the GEM/CDDP and GEM/CDDP + B20 treatment groups (Fig. 2C and F). However, in mice bearing spontaneous metastatic disease, no differences in survival curves were observed for either tumor type in any of the treatment groups (median survival: 55–57 days for FOLFOX-treated groups, 77 days for GEM/CDDP-treated groups in 4T1 tumors, and 85–95 days for all chemotherapy-treated groups in CT26 tumors). Yet, the mortality rate of untreated mice bearing CT26 lung metastasis was faster than any of the treatment groups (Fig. 2B and E). In addition, no significant changes in body weight were documented during the therapy, excluding the possibility of overt toxicity in surviving mice (Supplementary Fig. S4). Taken together, these results suggest that anti–VEGF-A enhance treatment efficacy of FOLFOX but not of GEM/CDDP chemotherapy when the tumors grown as an ectopic primary tumor and as an experimental lung metastasis but fail to do so to the same extent, in the spontaneous lung metastases tumor model.

Treatment with FOLFOX + anti–VEGF-A increased antiangiogenic activity in primary and experimental lung metastases but not in spontaneous lung metastases

The induction of viable CEPs following FOLFOX chemotherapy, and a blockade of this mobilization by anti–VEGF-A, has led us to further investigate whether this drug combination affects angiogenesis. To test this, mice bearing ectopic primary CT26 or 4T1 tumors were treated with FOLFOX or GEM/CDDP, with or without a single dose of the B20 antibody, to block viable CEP mobilization and to minimally affect the local anti-angiogenic activity of the drug, as previously shown (35, 36). Tumors were removed at end point, and microvessel density, perfusion, and hypoxia were evaluated. Anti–VEGF-A therapy did not significantly affect perfusion and hypoxia in both 4T1 and CT26 primary ectopic tumors, although a slight (nonsignificant) reduction in microvessel density was observed. Coadministration of FOLFOX + B20 led to a significant decrease in microvessel density and perfusion and to increased hypoxia compared with the administration of FOLFOX alone in CT26 tumors, whereas only a significant increase in hypoxia was observed in 4T1 tumors from mice treated with FOLFOX + B20 therapy when compared with control. Importantly, no significant differences in antiangiogenic activity in either tumor type were observed in mice treated with GEM/CDDP or with GEM/CDDP + B20 (Fig. 3). In addition, we also tested levels of MDSCs colonizing these tumors as another cell type, which is known to contribute to angiogenesis and tumor refractoriness following antiangiogenic therapy (19). The results in Supplementary Fig. S5 show that while the combination of FOLFOX + B20 antibody significantly and markedly suppressed the number of MDSCs colonizing both 4T1 and CT26 tumors, there were no significant differences in the number of MDSCs in tumors treated with GEM/CDDP with or without the B20 antibody. These results further suggest that a VEGF-A blockade concomitantly with FOLFOX chemotherapy inhibits...
angiogenesis in primary ectopic tumors and may also delay tumor refractoriness to anti–VEGF-A therapy.

Next, to assess the therapy’s impact on angiogenesis of metastatic tumor models, a spontaneous lung metastases model and an experimental lung metastases model for both 4T1 and CT26 tumors were treated with FOLFOX or GEM/CDDP with or without an anti–VEGF-A antibody, at the time points indicated in Materials and Methods. Three days later, the mice were sacrificed and the lungs, in which metastatic lesions are usually observed (see Supplementary Figs. S1 and S22), were removed to evaluate the percentage of endothelial cells in the entire lungs. The results in Fig. 4 show that while anti–VEGF-A therapy reduced the percentage of endothelial cells found in the lungs of mice treated with FOLFOX in the experimental lung metastases, in the spontaneous lung metastases, the percentage of endothelial cells found in the lungs was similar in all treatment groups regardless of whether anti–VEGF-A was added to the treatment. Furthermore, the number of metastatic lesions in the lungs of mice bearing 4T1 and CT26 experimental metastatic models was lower in mice treated with FOLFOX + B20 than in mice treated with FOLFOX monotherapy, and no significant differences in the number of lung metastases were observed in mice treated with either GEM/CDDP or GEM/CDDP + B20. In addition, no significant differences in the

Figure 2. Differential antitumor activity of anti–VEGF-A therapy in primary ectopic tumor, spontaneous lung metastases, and experimental lung metastases. Mice bearing either 4T1 (A–C) or CT26 (D–F) tumors were treated with FOLFOX, FOLFOX + B20, GEM/CDDP, or GEM/CDDP + B20. A and D, primary ectopic tumor: treatment was initiated when tumors reached a size of 200 mm³, and tumor volume was assessed by caliper. Data are presented as mean ± SD; n = 4–5 mice/group. B and E, spontaneous lung metastases: treatment was initiated on day 7 (4T1) and day 14 (CT26) following primary tumor resection, as described in Materials and Methods. Mouse survival was analyzed using the Kaplan–Meier survival curve (n = 6–8 mice/group). C and F, experimental lung metastases: treatment was initiated on day 28 for both 4T1 and CT26 tumors. Mouse survival was analyzed using the Kaplan–Meier survival curve (n = 6–8 mice/group).
number of lesions in the lungs of mice bearing spontaneous metastatic models of both 4T1 and CT26 were observed in any of the treatment groups (Supplementary Fig. S6). These results further suggest that anti–VEGF-A inhibits angiogenesis in both primary tumors and experimental lung metastases by reducing the number of endothelial cells in the established tumor lesions, leading to improved therapy outcomes, yet it fails to do so in mice bearing spontaneous lung metastases.

**Anti–VEGF-A inhibits invasion of tumor cells in response to plasma from mice or patients treated with FOLFOX**

Recent studies have shown that antiangiogenic therapy can promote tumor cell invasion and accelerate metastasis (14, 15). We have also recently shown that several chemotherapy drugs promote a host reaction that induces tumor cell invasion and metastasis (25). We therefore sought to evaluate the invasion properties of tumor cells following treatment with anti–VEGF-A and/or FOLFOX chemotherapy. We focused on FOLFOX-treated mice bearing spontaneous lung metastases of a CT26 colon carcinoma. Plasma samples were obtained from the mice 24 hours after they were treated with FOLFOX or FOLFOX + B20. The plasma was evaluated for its potential to induce tumor cell invasion using the modified Boyden chamber assay. The plasma from FOLFOX-treated mice markedly induced the invasive properties of CT26 tumor cells placed at the upper Boyden chamber compared with plasma from untreated mice. However, these invasive effects were absent in plasma from mice treated with FOLFOX + B20 (Fig. 5A). Similar results were obtained when plasma from non–tumor-bearing mice treated with FOLFOX were used (data not shown). We should note that the exogenous addition of escalating doses of VEGF-A to both 4T1 and CT26 did not increase the invasion.

Figure 3. VEGF-A blockade following treatment with FOLFOX but not GEM/CDDP inhibits angiogenesis in primary ectopic tumors. Eight- to 10-week-old BALB/c mice bearing 500 mm³ primary 4T1 (A and B) or CT26 (C and D) tumors were treated with FOLFOX, FOLFOX + B20, GEM/CDDP, or GEM/CDDP + B20. At end point, tumors were removed and evaluated for microvessel density using CD31 as an endothelial cell marker (in red; bar, 80 μm; A and C), and perfusion (in blue) and hypoxia (in green; bar, 200 μm; B and D). Graphs represent a summary of the quantization (mean ± SD) of microvessel density, and mean percentage (±SD) of perfusion and hypoxia per field (n > 20 fields/group). *, 0.05 > P > 0.01; **, 0.01 > P > 0.001.
properties of these cells, and neither of these cells express the 3 main receptors for VEGF-A: VEGFR1, VEGFR2, and VEGFR3 (Supplementary Fig. S7).

We next investigated whether parallel effects can be found in plasma from patients with colorectal cancer treated with FOLFOX at the adjuvant setting. After the primary tumor was resected, plasma was collected at baseline and 24 hours after FOLFOX therapy ($n = 11$), and HCT116 human colon cancer cells were used to test invasion using the Boyden chamber assay. Bevacizumab (as an anti-VEGF-A antibody) was added exogenously ($5 \mu g/mL$) to the plasma because this drug was not
included in the patients’ treatment plan. Similar to mice, plasma from patients treated with FOLFOX induced invasion of tumor cells, an effect that was abrogated by the exogenous addition of bevacizumab (Fig. 5B). We also examined the impact of FOLFOX + B20 therapy on mice bearing LM2-4 metastatic breast carcinoma tumors and found that FOLFOX-treated mice had a substantial number of liver metastases at the endpoint compared with FOLFOX + B20–treated mice that did not have any metastatic lesions in the liver (Supplementary Fig. S8). Overall, these results suggest that anti–VEGF-A blocks tumor cell invasion induced by host effects following FOLFOX therapy.

A decrease in VEGF-A and changes in MMP9 expression in the plasma of FOLFOX-treated mice

To determine whether neutralization of VEGF-A in the plasma of treated mice or patients with cancer may account for the inhibition of tumor cell invasion, ELISAs for murine and human VEGF-A were conducted on plasma obtained from mice or patients with cancer at baseline and 24 hours after treatment with FOLFOX. The results in Fig. 6A and B show that circulating plasma VEGF-A levels of FOLFOX-treated mice were significantly lower than those of control mice, and plasma VEGF-A levels of patients with cancer did not significantly change between baseline and following FOLFOX chemotherapy. In addition, plasma from FOLFOX-treated or untreated mice was also applied on protein array to assess the changes in the levels of additional pro-angiogenic factors besides VEGF-A. Using DAVID analysis for angiogenic factors, we found a marked increase in several angiogenic factors, among those are factors associated with VEGF and VEGF receptor pathways (Supplementary Table S2). Taken together, these results indicate that although low levels of VEGF-A detected by ELISA following FOLFOX therapy did not correlate with the increased tumor cell invasion properties, other pro-angiogenic factors were upregulated in the plasma of FOLFOX-treated mice and anti–VEGF-A was able to block therapy-induced tumor cell invasion.

As our previous study showed that MMP9 secreted by BMDCs may account for the invasive properties of tumor cells following chemotherapy (25), we sought to determine the levels of MMP9 in the plasma of FOLFOX- or FOLFOX + B20–treated mice. Plasma from treated mice was applied onto gel zymography and levels of MMP9 were evaluated. The results in Fig. 6C show that while FOLFOX induced MMP9 activity in the plasma of treated mice, FOLFOX + B20 antibody markedly reduced its activity. MMP9 activity was not different between GEM/CDDP and GEM/CDDP + B20–treated groups. In addition, no significant differences in the level of MMP9
were observed in primary tumors of both 4T1 and CT26 removed at endpoint in any of the treatment groups (data not shown). These results suggest that anti–VEGF-A may inhibit the activity of MMP9 induced by the host in the plasma of FOLFOX-treated mice but may not affect the levels of MMP9 in the treated tumor site.

Discussion

One of the current problems facing antiangiogenic drug therapy is the lack of marked and significant antitumor activity in several randomized phase III clinical studies for several malignancies despite the remarkable therapeutic benefits such drugs showed in preclinical models. These discrepancies have recently been accounted for by showing that the animal models used did not appropriately mimic the clinical scenario (37). A growing number of preclinical studies have shown antiangiogenic drug efficacy by using preclinical models such as GEMMs or patient-derived xenografts. However, these models require high maintenance and need large numbers of mice for each treatment group mainly because of the high variation and timing of the tumor take (37, 38). One question raised is whether the differences in treatment outcome of antiangiogenic drugs in the various preclinical models stem from a spectrum of tumor models tested, including primary ectopic tumors, small or large tumors, established metastases, and micrometastasis among others. Clinically, anti–VEGF-A therapy, that is, bevacizumab, has been tested and was found to be beneficial in several advanced metastatic malignancies (2–4). It has also shown promising benefits in limited numbers of neoadjuvant treatment setting trials, that is, before primary tumor resection (6, 7). However, in the adjuvant setting, after tumor resection, bevacizumab did not show any significant benefit at least not in the treatment of colorectal cancer (9). In this study, coadministration of anti–VEGF-A and chemotherapy improved the treatment outcomes of primary ectopic tumors and experimental lung metastases with established colon and breast carcinoma tumor lesions. However, no survival benefit was seen in mice bearing spontaneous lung metastasis in which only a microscopic disease of either tumor type was observed in the lungs. Guerin and colleagues have recently shown that the antitumor activity of antiangiogenic drugs is effective in the treatment of primary tumors but was either ineffective or showed modest therapeutic benefit in a postsurgical advanced metastatic disease model (39). In addition, a recent study by Rovida and colleagues showed that the combination of various chemotherapy drugs, including doxorubicin, topotecan, and gemcitabine but not paclitaxel and cisplatin, inhibited the prometastatic effects of sunitinib (40). Importantly, as opposed to our study, which focuses on the impact of the therapy on the host leading to metastasis spread using various tumor models and tumor stages, in Rovida and colleagues study, the authors suggested that increased tumor hypoxia following antiangiogenic therapy is
minimized when chemotherapy is added at the neo-
adjuvant treatment setting (40). Overall, the tumor model used for drug testing, and the impact of the therapy on the host and on the tumor may be critical for the assessment of the therapeutic benefit of any particular anti-
cancer drug.

In our study, we used different tumor models to elucidate the antiangiogenic effects of anti–VEGF-A therapy when coadministered with chemotherapy. We found that the endothelial cell number or microvessel density and perfusion were significantly decreased, whereas hypoxia was significantly increased in primary tumors or established tumors lesions in the lungs of mice treated with FOLFOX + B20 when compared with FOLFOX alone. Such antiangiogenic effects were absent in mice treated with GEM/CDDP in either tumor type. These results emphasize 2 important points. First they suggest that, like in other studies, the efficacy of antiangiogenic therapy does depend not only on the type of tumor being treated but also on the type of chemotherapy that the anti–VEGF-A agent is coad-
ministered with (11, 12). Second our results emphasize, once again, the use of a reproducible cancer model for drug testing, a problem raised in recent reports (37, 38).

The host’s response to several chemotherapy drugs has recently been investigated in various studies (11, 25, 26, 41–43). The first study reported that adminis-
tration of cytotoxic-like vascular disrupting agents to non–tumor-bearing mice induced a rapid CEP mobiliza-
tion (32). On the basis of this study, other studies also reported that various cytotoxic drugs may induce the mobilization of different types of BMDCs in non–tumor-bearing mice, suggesting that these effects are solely related to the host and not to the tumor. Welford and colleagues have recently shown that as opposed to CEPs, Tie2-expressing macrophages were found to highly colonize in tumors treated with a vascular disrupting agent (44). DeNardo and colleagues have also reported that in response to chemotherapy, tumor immune cells home to the tumor microenvironment, especially macrophages, which highly infiltrate mammary adenocarcinomas (42). In a previous study, we reported that the chemotherapy drug, paclitaxel, which induces a rapid mobilization of CEPs, administered in combination with an antiangiogenic drug therapy had an enhanced treat-
ment outcome and the progression of the established tumors decreased (11). In the current study, we show that similarly to paclitaxel, FOLFOX chemotherapy, a drug combination used to clinically treat colon cancer among other malignancies (45), induces CEP mobilization. Con-
sequently, the concomitant administration of anti–VEGF-
A and FOLFOX resulted in a significant inhibition of CEP mobilization, as previously documented for paclitaxel (11). Importantly, when the same experiments were car-
ried out in mice treated with GEM/CDDP, in which CEP mobilization was not induced, the addition of B20 to the chemotherapy did not result in an additional therapeutic benefit. Overall, our results suggest that anti–VEGF-A blocks systemic angiogenesis, which can be induced in response to certain chemotherapies and therefore can improve therapy outcome.

An intriguing question arising from this study is that while treatment with FOLFOX led to reduced levels of VEGF-A in the plasma of mice and, to some extent, in the plasma of patients with cancer, anti–VEGF-A therapy could still inhibit host effects promoting tumor cell inva-
sion. Although we did not address these counterintuitive results by carrying out possible experiments, we speculate that the reduced levels of plasma VEGF-A detected by commercially available ELISA kits do not necessarily detect all VEGF-A isoforms, which in fact could still be active. Under such circumstances, anti–VEGF-A therapy (B20) could block the undetectable isoforms of VEGF-A and therefore inhibit metastasis-promoting host effects despite the low VEGF-A levels detected. This speculation coincides with several studies showing that VEGF-A levels in patients with cancer do not correlate with the antiangiogenic treatment efficacy and do not provide a predictive biomarker for the treatment success of anti-
angiogenic drugs (46, 47). Furthermore, new ELISA kits for VEGF-A, which are not commercially available and therefore, were not available to us for this study, can detect the low-molecular-weight isoforms of VEGF-A. Using such ELISA kits, it has been shown that the low-mole-
cular-weight VEGF-A levels correlate with the efficacy of anti–VEGF-A therapy (S.J. Scherer, unpublished observa-
tions). It is therefore plausible that FOLFOX chemother-
apy upregulates undetected low-molecular-weight VEGF-A isoforms, which are then neutralized by anti–VEGF-A therapy, thus blocking the invasive properties of tumor cells. Overall, it would be of interest to further determine the levels of low-molecular-weight VEGF-A isoforms in the plasma of mice and patients with cancer following cytotoxic drug therapy.

In summary, this study suggests that anti–VEGF-A, in combination with chemotherapy, may have additional therapeutic activities besides antiangiogenesis that are related to blocking host protumorigenic and prometa-
static effects that exist in response to certain chemother-
apy drugs and can explain why antiangiogenic therapy in this context may improve therapy outcome, depending on the preclinical tumor models used.

Disclosure of Potential Conflicts of Interest

Y. Shaked has received commercial research grant from and is a consultant/advisory board member of Hoffmann La Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D. Alishekevitz, D. Loven, S.J. Scherer, Y. Shaked
Development of methodology: D. Alishekevitz, S. Gingis-Velitski
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Alishekevitz, D. Loven, V. Miller, E. Fremder
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Alishekevitz, E. Fremder, S.J. Scherer, Y. Shaked
Writing, review, and/or revision of the manuscript: D. Loven, S.J. Scherer, Y. Shaked
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Bril, T. Voloshin, S. Gingis-Velitski
Study supervision: Y. Shaked

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Dror Alishkevitz, Rotem Bril, David Loven, et al.