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Bevacizumab-Induced Alterations in Vascular Permeability and Drug Delivery: A Novel Approach to Augment Regional Chemotherapy for In-Transit Melanoma

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Abstract

Purpose—To investigate whether the systemically administered anti-VEGF monoclonal antibody bevacizumab could improve regional chemotherapy treatment of advanced extremity melanoma by enhancing delivery and tumor uptake of regionally infused melphalan (LPAM).

Experimental Design—After treatment with systemic bevacizumab or saline, changes in vascular permeability were determined by spectrophotometric analysis of tumors infused with Evan's blue dye. Changes in vascular structure and tumor hemoglobin-oxygen saturation HbO₂ were determined by intravital microscopy and diffuse reflectance spectroscopy, respectively. Rats bearing the low-VEGF secreting DM738 and the high-VEGF secreting DM443 melanoma xenografts underwent isolated limb infusion (ILI) with melphalan (LPAM) or saline via the femoral vessels. The effect of bevacizumab on terminal drug delivery was determined by immunohistochemical analysis of LPAM-DNA adducts in tumor tissues.

Results—Single-dose bevacizumab given three days before ILI with LPAM significantly decreased vascular permeability (50.3% in DM443, $P < 0.01$ and 35% in DM738, $P < 0.01$) and interstitial fluid pressure (57% in DM443, $P < 0.01$ and 50% in DM738, $P = 0.01$). HbO₂ decreased from baseline in mice following treatment with bevacizumab. Systemic bevacizumab significantly enhanced tumor response to ILI with LPAM in two melanoma xenografts, DM443 and DM738, increasing quadrupling time 37% and 113%, respectively ($P = 0.03$). Immunohistochemical analyses of tumor specimens showed that pretreatment with systemic bevacizumab markedly increased LPAM-DNA adduct formation.

Conclusions—Systemic treatment with bevacizumab before regional chemotherapy increases delivery of LPAM to tumor cells and represents a novel way to augment response to regional therapy for advanced extremity melanoma.

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Disclosure of Potential Conflicts of Interest

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Introduction

While the incidence of several other cancers declines, the incidence of melanoma continues to rise and is now the most common fatal malignancy of young adults and the sixth most common cancer among Americans. In 2009, there were an estimated 68,720 people newly diagnosed with invasive melanoma, and more than 8,650 people died of melanoma in the United States (1). Unfortunately, mortality rates for metastatic melanoma have remained high in part because of its high resistance to standard cytotoxic agents (2). A major breakthrough for the treatment of metastatic melanoma has been the recent U.S. Food and Drug Administration (FDA)-approval for the anti-CTLA4 antibody (ipilimumab) and a specific inhibitor targeting the V600E-activating BRAF mutation (vemurafenib) found in approximately 50% of patients with melanoma (3). For patients who recur after treatment with these new therapies, response to other systemic therapy is poor, with standard agents such as dacarbazine, temozolomide, and paclitaxel having complete response rates of <15% (2).

A significant fraction of patients with melanoma will recur with in-transit disease which represents multifocal metastases that have spread through the lymphatic system occurring between the site of the primary lesion and the regional draining lymph node basin (4). This pattern of recurrence is associated with an unfavorable prognosis, having 5-year survival rates ranging from 25% to 30% (5). Historically, standard systemic chemotherapy or immunotherapy has provided little benefit for in-transit disease (6). However, for patients with in-transit disease confined to the extremities, regional chemotherapy delivered by isolated limb perfusion (ILP) or isolated limb infusion (ILI) is a viable treatment option.

Briefly, ILP is conducted by surgically exposing the femoral or subclavian vessels and then cannulating them at the root of the limb. Next, an esmarch tourniquet is placed proximal to the cannulated vessels and the limb is perfused with a high-flow, melphalan-based perfusate using a membrane oxygenator to maintain physiologic oxygenation and pH (5). ILI is a generally less-invasive surgical technique and is conducted by percutaneous catheterization of the involved limb followed by infusion of melphalan in a low-flow circuit without an oxygenator such that tissue becomes hypoxic and acidotic (7).

Using these techniques, complete response rates ranging from 30% to 60% have been reported in larger series (5). Although these initial response rates represent an improvement over systemic therapy, at least 40% of patients with intransit melanoma will eventually recur after regional chemotherapy (8). Thus, novel strategies are needed to improve durable responses rates of locally advanced melanoma to regional therapy.

Strategies to optimize delivery of cytotoxic agents to melanoma during regional chemotherapy may enhance not only the initial response to treatment but also the durability of this response. Melanoma induces angiogenesis that results in tumor vasculature which is anatomically and functionally distinct from that seen in normal tissue (9, 10). The vasculature of solid tumors has been characterized as tortuous, dilated, saccular, and irregular in its pattern of interconnection (11). This erratic tumor vasculature can pose a barrier to optimal drug delivery thus limiting the efficacy of cytotoxic therapy. An important mediator of tumor angiogenesis is VEGF, a cytokine which stimulates angiogenesis and is normally seen during embryonic development as well as in postinjury angiogenesis (12). VEGF is a multifunctional cytokine capable of stimulating endothelial cell proliferation, migration, and survival (13). VEGF is also a potent stimulator of vessel permeability (14). Commonly overexpressed in metastatic melanoma, high serum levels of VEGF have been correlated with adverse prognosis and implicated as an important mediator in creating a dysfunctional vascular network antagonizing optimal drug penetration (15).

Bevacizumab is an FDA-approved monoclonal antibody to VEGF that neutralizes all isoforms of VEGF. It has been used in combination with standard chemotherapies in patients with metastatic colorectal, brain, and lung cancers and is being investigated in combination with other chemotherapy agents for melanoma in multiple clinical trials across the United States (16–18). Bevacizumab also offers a unique tool to temporally restore the balance between angiogenic stimulators and angiogenic inhibitors (11). Emerging evidence shows that vascular targeting agents such as bevacizumab can transiently “normalize” tumor microvasculature toward that seen in normal tissues thereby creating an optimal window for chemotherapy delivery (19). By effectively neutralizing VEGF, bevacizumab is theorized to prune immature and leaky vessels characteristic of solid tumors, leading to functional changes that include increased tumor oxygenation and drug penetration (11). Although beneficial when used in a well circumscribed window, sustained antiangiogenic treatment may lead to severe tumor vessel regression that blocks penetration of therapeutic drugs into the tumor antagonizing, rather than augmenting, cytotoxic therapy. Thus, systemic therapies, which are often administered in multiple doses, may initially benefit from the antiangiogenic therapy but will be hindered at later time points as tumor vessels wither and become inadequate for drug delivery (19).

Because regional chemotherapy is administered as a single infusion or perfusion of high-dose cytotoxic therapy (5), it is an ideal modality to test whether bevacizumab can enhance drug delivery and subsequently improve tumor response. In this report, we provide preclinical evidence that systemically administered bevacizumab decreases tumor vascular permeability and interstitial fluid pressure (IFP) leading to increased drug delivery and improved efficacy of regionally infused melphalan. Pretreatment with systemic bevacizumab before arterial infusion of melphalan improves drug penetration and tumor responses, supporting bevacizumab as a beneficial adjunct to regional therapy for melanoma.

Materials and Methods

Melanoma cell lines and reagents

Tumor cell lines derived from human melanomas (DM lines) were courtesy of Dr. H. Seigler (Duke University Medical Center, Durham, NC). Cells were cultured in Isocove's Modified Dulbecco's Medium with 10% FBS, 2 mmol/L glutamine, 1,000 IU/mL penicillin, and 100 mg/mL streptomycin and grown at 37°C and 5% CO₂. Bevacizumab (Avastin by Genentech) was purchased from the Duke University Pharmacy.

Quantification of melanoma cell VEGF secretion and its modulation with bevacizumab

Human VEGF Quantikine ELISA Kits (R&D Systems) were used according to the directions of the manufacturer. Two-hundred microliters of conditioned media were collected from triplicate samples.

VEGF neutralization and its effect on melphalan sensitivity of melanoma cells

Using cell survival as an endpoint, DM443 and DM738 cells were plated at 1×10^3 per well in a 96-well microtiter plate and after 24 hours, treated with 0 to 2 mg/mL bevacizumab and refed with fresh medium containing 0 to 150 μ mol/L melphalan. Cell survival was measured after 72 hours, using the Cell Titer-Blue assay (Promega), as per the manufacturer's instructions, with excitation and emission wavelengths of 560 and 590 nm, respectively. Surviving fractions, SF, were computed using the equation: $SF = F_t/F_c$, where F_t and F_c are the fluorescence of treated and untreated cells, respectively, normalized against background fluorescence.

Hyperspectral analysis of tumor vasculature after treatment with bevacizumab

The preparation of the dorsal skin-fold window chamber has been described elsewhere (20). All procedures used in this study were approved by the Duke University Institutional Animal Care and Use Committee. Briefly, a 12-mm diameter flap of skin was dissected away from opposing surfaces of the dorsal skin flap of anesthetized nude mice, leaving a fascial plane with associated vasculature. The hole was held vertically away from the body with a titanium saddle that was sutured to both sides of the flap. A total of 3×10^6 DM443 or DM738 cells suspended in 30 μL normal growth medium were injected in the middle of the fascial plane. Glass windows were attached to the center of the saddle to cover the surgical site. BALB/c mice were obtained from Duke Cancer Center Isolation Facility.

For imaging of tumor microvasculature, animals were anesthetized with isoflurane (1.5% with oxygen) and maintained at body temperature. Imaging began when tumors reached 1 to 2 mm in diameter, approximately 5 days after injection. All images were collected with a 2.5 $\times$ objective. Hyperspectral (hemoglobin-oxygen saturation) imaging was collected using an upright microscope (Carl Zeiss Axiovert 200). Hyperspectral images were collected with a 100-W halogen lamp for transillumination, and detection was achieved with a 10-nm bandwidth liquid crystal tunable filter (LCTF; CRI Incorporated) placed in front of a DVC 1412 CCD camera (DVC Company). Custom software was used to tune the filter and acquire images at 10-nm optical wavelength increments between 500 and 620 nm. The spectral characteristics of the illumination source itself were corrected for by measuring the transmission of the source light through a neutral density filter at each wavelength. After the system response was compensated for, hemoglobin saturation was calculated at each pixel location by applying the principles of the Beer–Lambert law in a wavelength-dependent manner. Assuming oxygenated and deoxygenated hemoglobin as the primary absorbers, along with a tissue scattering parameter, these factors were decomposed from the total absorption spectrum using linear nonnegative least-squares regression, yielding hemoglobin saturation values at every observable pixel location. Vascular maps derived from total hemoglobin measurements were used to measure vascular tortuosity using a user-guided algorithm for vessel identification. Tortuosity is represented as the ratio of the vessel centerline distance to the straight line distance between branch points. Vascular length density (VLD) was calculated by dividing the total tumor-associated vessel length by the tumor area (mm/mm^2). The hyperspectral system, software, and analysis techniques have previously been validated on liquid phantoms with an accuracy of approximately 1% and *in vivo* (21).

Diffuse reflectance spectroscopy for quantitative tumor hemoglobin concentration and oxygen saturation

A total of 20 nude mice were inoculated subcutaneously in the right flank with 3×10^6 DM443 melanoma cells suspended in 100 μL of normal growth medium. Three mice did not grow tumors. Once tumor diameters reached 8 to 10 mm (~ 2 –3 weeks after inoculation), mice ($n = 8$) were injected with intraperitoneal bevacizumab (5 mg/kg) or equivalent volumes of intraperitoneal normal saline ($n = 9$). All tumors were monitored optically before treatment and then daily for 6 days after treatment. Briefly, animals were anesthetized with inhaled 1.5% isoflurane gas mixed with oxygen. A bifurcated fiber optic probe using separate illumination and collection paths was noninvasively applied in direct contact with DM443 xenografts and secured with a clamp to prevent movement. Diffuse reflectance was measured in the spectral range of 480 and 650 nm. The acquired diffuse reflectance spectra were calibrated and analyzed using an inverse scalable Monte Carlo model to obtain wavelength-dependent scattering and absorption spectra (22). The concentration of oxygenated hemoglobin (HbO_2) and deoxygenated hemoglobin (dHb) was derived using the Beer–Lambert equation. Total hemoglobin (Hb) was calculated by the sum of HbO_2 and

dHb. Differences in % Δ Hb and % Δ HbO₂ were compared between treatments groups with a Mann–Whitney *U* test using the commercially available software GraphPad Prism.

Rat xenograft studies

Female nude rats (6-week-old; Charles River Laboratories International, Inc.) were injected subcutaneously in the right hind limb with 5×10^6 DM738 or 7×10^6 DM443 cells. Tumor volume was calculated as: $\frac{1}{2} \times [(\text{length}) \times (\text{width})^2]$. Treatment began when tumor volume was 100 mm³. The rat protocol was approved by the Duke University and Durham VA Medical Center Institutional Animal Care and Use Committees.

ILI was conducted as described previously (23). The femoral artery and vein were cannulated. The arterial catheter was attached to a peristaltic pump while venous drainage flowed by gravity into a reservoir. A tourniquet was placed around the thigh. For survival studies, a 15-minute infusion (1.5 mL/min) of melphalan (90 mg/kg) or saline was followed by a 1-minute saline washout (3.0 mL/min).

Tumor vascular permeability

ILI was conducted as described earlier. Rats received an injection of bevacizumab 5 mg/kg intraperitoneally (i.p.) or saline 3 days before a 15-minute infusion of Evan's Blue dye solution (50 mg/kg dissolved in normal saline and infused at 1.5 mL/min) followed by a 2-minute saline washout (3.0 mL/min). Animals were euthanized and tumors were excised followed by incubation in formamide solution for 72 hours at 37°C to extract the Evan's blue dye. To quantify the Evan's blue, absorbance of the formamide solution was measured at 595 and 655 nm and normalized to tumor volume.

Interstitial fluid pressure

Tumor IFP measurements were made with a needle probe pressure monitor (Intra-Compartmental Pressure Monitor System, Stryker), fitted with an 18-gauge side-ported needle (Stryker) and connected to a syringe filled with 0.9% saline. Using isoflurane anesthesia, the needle probe was inserted into the center of the tumor on the hind limb of the rat. IFP was recorded in mm Hg when the measurement stabilized.

Immunohistochemistry of tumor vessel density

Ten DM738 ($n = 5$, saline; $n = 5$, 5 mg/kg i.p. bevacizumab) and 13 DM443 ($n = 6$, saline; 7 5 mg/kg bevacizumab) xenografts were flash-frozen using liquid nitrogen. They were then cut into 10 μ m cross-sections using a LEICA CM 1850 cryotome (Meyer Instruments Incorporated) and maintained at -24°C to -28°C . Sections were then fixed in acetone for 30 minutes, allowed to dry for 1 hour, and then blocked for 30 minutes at room temperature with 5% donkey serum (Jackson ImmunoResearch) in PBS. Sections were incubated with primary antibody (1:200 mouse anti-rat CD31, BD bioscience) at room temperature for 1 hour, followed by a 30-minute incubation with a fluorescent-conjugated secondary antibody (1:100; Jackson ImmunoResearch) at room temperature. Samples were rinsed with PBS 3 times for 5 minutes each between every consecutive step.

Immunohistochemical staining for CD31 was assessed with a Zeiss AxioScop II microscope with a scanning stage. Images of whole tumors were recorded at $\times 5$ magnification and subsequently segmented and evaluated in ImageJ. Distinct immunoreactive endothelial cells were counted as individual microvessels, and nonspecific staining of nonendothelial structures was disregarded in microvessel counts. Mean microvessel density for CD31 was calculated over the total area of the tumor.

Immunohistochemistry of DNA-melphalan adduct

Rats were treated with bevacizumab (5 mg/kg i.p.) or saline 3 days before ILI. Rats were then euthanized 24 hours after LPAM- or saline-ILI. Tumors were then fixed with formalin, paraffin-embedded, and stained using immunohistochemistry as previously described (23, 24). Treated and nontreated DM443 cells were used as positive and negative controls for the MP5/73 antibody which was kindly provided by Dr. M.J. Tilby, Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, United Kingdom (25).

Sections were viewed and digitally photographed with an Olympus Vanox inverted light microscope coupled to an Olympus DT-70 camera. Images were taken at $\times 2$, $\times 10$, and $\times 60$ magnification and saved as JPEG or TIFF files for further processing in Adobe Photoshop (Adobe Systems, Inc.). Positive staining was quantified as the ratio of brown to total pixels using the commercially available software MATLAB (Mathworks). Differences in staining among treatment groups were compared using a one-way ANOVA.

In vivo tumor growth studies

Animals were treated with a single dose of bevacizumab (5 mg/kg i.p.) 3 days before ILI, as based on previous studies showing peak changes tumor vasculature occurred 3 days after bevacizumab (19, 26). Response to drug treatment was followed until tumor volume reached 5,000 mm³ or 60 days for regional studies. Response was evaluated by the percentage of increase in tumor volume and tumor quadrupling time.

Results

Melanoma cell lines secrete VEGF

To evaluate the therapeutic potential of anti-VEGF therapy for the treatment of in-transit melanoma, we measured VEGF secretion across 11 melanoma cell lines capable of forming tumor xenografts in our animal model of extremity melanoma. VEGF secretion was variable across these cell lines, with A2058, DM792, DM751, and DM738, an LPAM-resistant cell line, showing little to no VEGF secretion whereas DM443, a moderately LPAM-sensitive cell line, showing high levels of VEGF secretion (Supplementary Fig. S1A). To confirm bevacizumab can effectively neutralize melanoma VEGF secretion *in vitro*, we treated DM443 and DM738 cells, grown to 80% confluence, with 0 to 2.0 mg/mL bevacizumab for 24 to 72 hours. Bevacizumab at all concentrations tested effectively prevented detection of VEGF by ELISA (Supplementary Fig. S1B and S1C).

Bevacizumab does not alter *in vitro* LPAM chemosensitivity

VEGF possesses multiple cytokine functions and can act as a potent mitogen for endothelial cell proliferation. Given the possibility that VEGF might act as an autocrine growth factor for melanoma (27), we tested whether VEGF neutralization in itself could augment chemosensitivity of melanoma cells to melphalan in an endothelial cell-free system using a low-VEGF secreting, melanoma cell line (LPAM-resistant DM738) and a high-VEGF secreting, melanoma cell line (LPAM-sensitive DM443). VEGF neutralization in an endothelial cell-free system had no effect on the LPAM chemosensitivity for either DM443 or DM738 (Supplementary Fig. S2). On the basis of these data and others (19, 28), we focused our studies on evaluating 2 potential mechanisms by which bevacizumab could augment tumor cytotoxicity to regionally delivered chemotherapy through 2 separate temporal effects: (i) neutralization of VEGF would lead to normalization of tumor microvasculature resulting in a more optimal vascular anatomy for delivering cytotoxic therapy and (ii) impairment of the requisite angiogenesis needed for further tumor growth.

Bevacizumab decreases vascular permeability and interstitial pressure of melanoma xenografts

Previous studies have shown bevacizumab to significantly decrease tumor vascular permeability and the peak functional effects of bevacizumab to occur 3 days after treatment (14, 19, 26, 29). On the basis of this evidence, we hypothesized that a single dose of bevacizumab, given 3 days before ILI would decrease vessel permeability. To test this, ILI was conducted on tumor-bearing rats using Evan's blue dye as the infusate. Absorbance of Evan's blue dye was measured and used as a surrogate of vascular permeability in the xenograft. As shown in Fig. 1A and B, bevacizumab (5 mg/kg) given 3 days before ILI significantly decreased vascular permeability of Evan's blue dye by 70% in DM443 xenografts and 30% in DM738 xenografts.

Increased vascular permeability seen in tumor vasculature may lead to increased IFP which can hinder effective drug delivery to tumor cells (30). Thus, we also measured IFP in melanoma xenografts 3 days after treatment with i.p. bevacizumab (5 mg/kg). Mean IFP measurements in bevacizumab-treated animals were 57% (14.9 ± 0.9 vs. 8.3 ± 0.8 mm Hg, $P < 0.01$) and 50% (18.0 ± 2.9 vs. 9.1 ± 1.2 mm Hg, $P = 0.01$) lower than saline-treated controls for both DM443 and DM738 xenografts, respectively (Fig. 1C and D).

Bevacizumab decreases hemoglobin oxygen saturation in melanoma tumors

Tumor vascular normalization is hypothesized to induce a transient increase in tumor tissue oxygenation after treatments with antiangiogenic agents (11). To test the effect of bevacizumab on tumor tissue oxygenation, we measured tumor hemoglobin saturation HbO₂ using diffuse reflectance spectroscopy; a method which has been previously validated as a surrogate for tissue oxygenation (31). Nude mice bearing DM443 melanoma xenografts were treated with 5 mg/kg of i.p. bevacizumab ($n = 8$) or saline ($n = 9$). The effects of systemic bevacizumab on tumor total hemoglobin (Hb) and HbO₂ levels, Hb and HbO₂ were measured at baseline before treatment and daily for 6 days using diffuse reflectance spectroscopy. Daily HbO₂ levels were shown to be consistently lower than baseline measurements in the bevacizumab-treated mice than in saline-treated controls ($P = 0.004$). Hb levels in both groups decreased as much as 30% from baseline measurements without discernible differences between treatment groups ($P = 0.38$; Fig. 2).

Bevacizumab does not produce changes in vascular structure consistent with tumor vascular normalization in melanoma xenografts

To explore vascular structure alterations associated with decreased permeability, a murine model of dorsal flank melanoma was used in combination with a window chamber model (32) to obtain real-time *in vivo* images of melanoma microvasculature following treatment with bevacizumab. Five days after tumor inoculation and establishment of the window chamber (tumor size was ~3–4 mm and clearly visible; see Materials and Methods), mice were treated with either a single injection bevacizumab (5 mg/kg i.p.) or saline control. Mice were imaged daily for 7 days using a hyperspectral microscope. Reconstructed images of the tumor microvasculature from both DM443 and DM738 xenografts did not reveal qualitative visual evidence of tumor vascular normalization in animals treated with bevacizumab. (Fig. 3A). To quantify tumor vasculature remodeling in response to bevacizumab treatment, we used the acquired hyperspectral images to quantify relative vascular tortuosity for each tumor by normalizing tortuosity at each time point to the measurement on day 0 as previously described (33). As compared with mice treated with saline control, no discernible differences were found in vascular tortuosity or VLD after treatment with systemic bevacizumab (Fig. 3B and C).

To confirm the results of the intravital microscopy experiments, we compared tumor vascular density in bevacizumab- and saline-treated xenografts as determined from immunohistochemical staining for the vascular marker CD31. Representative cross-sections for both xenografts are shown in Fig. 4A and B. Overall, no significant differences in tumor vascular densities were found in either xenograft after treatment with bevacizumab (Fig. 4C and D).

Systemic bevacizumab increases delivery of regionally administered melphalan

To better understand the effects of systemic bevacizumab on the delivery of regional melphalan during ILI, short-term survival surgeries were carried out on nude rats bearing the DM443 and DM738 xenografts. Three days before surgery rats were treated with a single dose of bevacizumab (5 mg/kg). Twenty-four hours after regional infusion tumor tissue was harvested for analysis of LPAM-DNA adduct formation using the specific antibody MP5 as previously described. Animals treated with bevacizumab before infusion of regional melphalan had significantly higher levels of LPAM-DNA adducts than animals infused with melphalan alone. DM443 and DM738 xenografts showed an approximate 2- and 6-fold increase, respectively, in LPAM-DNA adduct reactivity for animals pretreated with bevacizumab before regional infusion as compared with those treated with systemic saline followed by LPAM infusion (Fig. 5). Tumors from animals receiving saline infusion (either alone or following bevacizumab treatment) showed no staining for LPAM-DNA adducts. Taken together, these results show that systemic bevacizumab induced microvasculature changes as early as 3 days after treatment which led to both a decrease in vascular permeability and an increase in the delivery of LPAM through the ILI circuit.

Bevacizumab significantly increases response of melanoma xenografts to regional melphalan

Having showed that regionally infused melphalan given 3 days after systemic bevacizumab treatment led to improved drug delivery into the tumor, we evaluated tumor growth after systemic bevacizumab using our preclinical animal model of ILI with melphalan. Tumor-bearing nude rats were treated with bevacizumab or saline control at day 0 and infused with melphalan or saline at day 3 based on preliminary functional data as well as previous reports (19, 26). Growth curves for both DM738 and DM443 xenografts after systemic saline injection and regional saline infusion are depicted by the black line and show baseline *in vivo* growth characteristics for both melanoma cell lines. DM443 baseline growth kinetics were significantly slower than DM738 (quadrupling time of 34.0 vs. 13.7 days, $P < 0.0001$ *t* test), which is consistent with our previous experience using these xenografts (supplementary Table S1; refs. 23, 34). Bevacizumab showed significant tumor growth inhibition 30 days after treatment when used in combination with regionally delivered melphalan in both the low-VEGF secreting, fast growing DM738 xenograft ($P = 0.0007$, one-way ANOVA) and the high-VEGF secreting, slower growing DM443 xenograft ($P = 0.0335$, one-way ANOVA). Systemically administered bevacizumab alone showed similar efficacy to regional melphalan infusion alone for the low-VEGF secreting DM738 xenograft but not for the high-VEGF secreting DM443 xenograft (Fig. 6A and B). Combination therapy of systemic bevacizumab followed by regional melphalan infusion had the most robust effect on tumor growth inhibition for both xenografts, with a 37% and 113% increase in quadrupling time for DM443 and DM738, respectively, as compared with melphalan infusion alone (Supplementary Table S1).

Discussion

This study shows that systemic bevacizumab given before ILI with melphalan augments response by increasing delivery of melphalan to the tumor cells. This is first study to

evaluate the use of bevacizumab as a part of a regional therapy strategy with the specific intent of augmenting the delivery of chemotherapy to a tumor. Our results, in the context of melanoma, clearly support the emerging concept that the efficacy of antiangiogenic therapy in combination with chemotherapy is not simply an additive effect of long-term hypoxia resulting from tumor vasculature destruction and cytotoxicity from the chemotherapy. Rather it is a synergistic effect resulting from modulation of tumor blood supply leading to improved delivery and penetration of conventional chemotherapeutic agents (11). Rats undergoing limb infusions of high-dose melphalan 3 days after administration of bevacizumab had a robust and significantly greater response than either chemotherapy or bevacizumab alone.

Antiangiogenic therapies such as bevacizumab were developed with the goal of starving tumors of their blood supply. Whereas this effect was observed in preclinical models (35) antivascular agents have failed to control tumor growth through vascular effects alone in the clinical setting (36). Although ineffective as a monotherapy, bevacizumab in combination with conventional chemotherapies has improved overall survival in previously untreated patients with colorectal and lung cancer (17, 36). The mechanisms by which antiangiogenic therapies such as bevacizumab augment cytotoxic therapy are controversial. One theory is that bevacizumab in combination with cytotoxic agents acts synergistically by targeting both tumor cells with cytotoxic therapy and vascular stroma with bevacizumab (37). A second theory revolves around the concept of tumor vascular “normalization,” where tumor vessels are transiently remodeled structurally and functionally to resemble normal blood vessels (11). This normalization may synergistically enhance cytotoxic agent efficacy by overcoming physiologic barriers to drug delivery created by aberrant tumor vasculature (11, 19).

The findings presented in this study are in agreement with reports which show that targeting VEGF with compounds such as bevacizumab can manipulate tumor vasculature to augment cytotoxic therapy (19, 26, 38–40). In this study, we build on the current body of literature by reporting that bevacizumab can augment regional chemotherapy delivery by decreasing tumor vascular permeability and IFP.

In general, criteria for tumor vascular normalization would include functional decreases in vessel permeability and IFP, improved tissue oxygenation, and augmented drug delivery to tumor cells in the context of structural changes such as decreased tumor vessel density, tortuosity, and VLD (11, 26). In contrast to previous reports, the results of our studies failed to show a change in tumor microvasculature structure or improvement in tumor oxygenation after treatment with antiangiogenic therapy. In our murine model engrafted with a human melanoma xenograft, bevacizumab did not lessen tumor vessel tortuosity or VLD, and it did not significantly increase tumor hemoglobin saturation. These discrepancies may, in part, be due to limitations of our preclinical model, which used a neutralizing human-VEGF antibody with weak affinity for murine-VEGF. Although these results may have been influenced by a partial VEGF blockade, in which only VEGF produced from the human melanoma xenograft was neutralized, bevacizumab-induced changes in tumor oxygenation and microvasculature have been previously reported in the syngeneic B16 mouse melanoma models (26). It is also possible that other structural alterations such as increases in interendothelial cell gaps or pericyte coverage could have occurred but not been measured in this report. Finally, the results of our experimental models may have been measured during a more persistent window of functional vascular normalization, which could have occurred after a more transient window of structural normalization had past (41). Taken together, we cannot conclude with certainty that structural vascular normalization did not occur in bevacizumab-treated animals.

Despite a lack of conclusive evidence supporting structural vascular normalization, our results are in support of functional vascular normalization as evidenced by enhanced drug delivery in the setting of decreased tumor vessel permeability and IFP. These results support an important role for IFP in the dynamics of blood supply to the tumor. It has long been recognized that VEGF acts by increasing blood vessel permeability (14, 29). This, in turn, can lead to a loss of normal oncotic and hydrostatic pressure gradients (42) which eventually will cause IFP levels to rise. The decreased permeability seen with antiangiogenic agents such as bevacizumab, therefore, could lead to decreased IFP levels creating a pressure gradient across blood vessel walls and tumor interstitium that is conducive to drug penetration into tumor cells (30). Overcoming this pressure gradient is critically important for delivery of small molecules such as LPAM, for which 60% to 90% of the drug is bound to plasma proteins (43).

The efficacy of bevacizumab in augmenting systemically administered chemotherapy for treatment of melanoma has been investigated recently in multiple phase I and phase II trials (44–46). One of the most publicized is the phase II BEAM study funded by Roche, which investigated the efficacy of bevacizumab in combination with carboplatin and paclitaxel versus chemotherapy alone in 214 patients with melanoma. Although initially reported to show meaningful improvement in overall survival in a late breaking abstract at the ECCO-ESMO cancer congress in Berlin, the statistical review of the data failed to show a significant difference (47). Bevacizumab in combination with chemotherapy was well tolerated with overall survival (12.3 vs. 9.2 months in control arm, $P = 0.19$) and progression-free survival (median 5.6 vs. 4.2 months in control arm, $P = 0.14$) trending toward clinical benefit but not reaching statistical significance. Bevacizumab likely can only augment delivery of cytotoxic therapy when tumors are large enough to have the characteristic leaky, saccular, and aberrant neovasculature characteristic of malignancies. This concept may explain the recent results of the phase III National Surgical Adjuvant Breast and Bowel Project (NSABP) C-08 trial which showed no improvement in disease-free survival (DFS) for stage II and III colon patients given bevacizumab in addition to adjuvant-modified FOL-FOX6 (infusional/bolus fluorouracil, leucovorin, and oxaliplatin) therapy (48). It is possible that patients in this study had tumors that were developing resistance to bevacizumab or the small foci of disease present in some of the patients after resection did not have sufficient tumor neovasculature to fully benefit from the addition of bevacizumab to their treatment course.

The results reported in this study lend support to the use of bevacizumab to augment drug delivery in the setting of regional chemotherapy. In many respects, regional chemotherapy is the ideal platform to test the efficacy of vascular targeting agents in augmenting cytotoxic therapy. In accordance to the preclinical data presented here, patients with in-transit melanoma confined to an extremity could receive a single dose of bevacizumab 3 days before the one-time delivery of cytotoxic therapy. In this setting of regional chemotherapy delivery, any later effects of bevacizumab which could hinder cytotoxic agent delivery would be inconsequential.

Our preclinical data suggest that systemically administered bevacizumab synergistically improves response to regionally delivered melphalan by decreasing blood vessel permeability and improving drug delivery to the tumor as evidenced by the increased formation of DNA-LPAM adducts. Given these preclinical results, a phase I clinical study has been proposed and is currently under institutional review. A precursor study, examining the distribution of hypoxic regions within in-transit lesions using the drug EF5 and how hypoxia affects melphalan drug delivery is currently Institutional Review Board approved and accruing patients. ILI has become a popular technique for delivering regional chemotherapy due to its simple setup and lower major toxicity rate. However, its complete

response rates of 30% to 35% are lower than are seen in HILP (50%; ref. 5). Strategies using targeted agents given systemically around the time of ILI may be an effective way to improve response rates of ILI up into the range seen with HILP. The feasibility of this type of approach has recently been showed with an N-cadherin–targeting agent (ADH-1) which when given approximately 6 to 8 hours before a melphalan-based ILI led to 50% and 38% complete response rates in phase I and II trials, respectively (49). Care must be taken, however, as targeted agents which interfere with VEGF signaling, can increase the toxicity of ILI as was seen in a study of systemic sorafenib given for 1 week before and after melphalan-based ILI in patients with advanced extremity melanoma (50).

Clinical trials in the setting of in-transit disease of the extremity while providing important tools toward improving regional response rates can, in addition, provide tremendous insight into how to optimally use targeted agents in conjunction with chemotherapeutic agents because of the ease of obtaining sequential tissue biopsies to evaluate the effect of the targeted agent on the tumor concurrently with the ability to obtain drug pharmacokinetic data. Studies like the one proposed in this article using systemic bevacizumab and regional melphalan can have application to other tumor types where regional therapy is widely used such as peritoneal perfusion for abdominal carcinomatosis or liver infusion/perfusion for isolated liver metastasis. Furthermore, they can also provide important insight on how to develop rational strategies of incorporating targeted agents into systemic chemotherapeutic protocols for metastatic melanoma for which few effective treatment options currently exist.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

This is the first study to evaluate the use of bevacizumab as a part of a regional therapy strategy for the treatment of advanced extremity melanoma with the specific intent of augmenting delivery of chemotherapy to a tumor. We show that a single dose of bevacizumab before isolated limb infusion improves the delivery of chemotherapy and enhances melanoma tumor responses by decreasing tumor vascular permeability and interstitial fluid pressure. On the basis of these results, a phase I clinical study examining the safety of single-dose bevacizumab before regional infusion of melphalan has been proposed.

Figure 1.

Bevacizumab decreases permeability and IFP of melanoma xenografts. Three days after i.p. injection of 5 mg/kg of bevacizumab, ILI was carried out on tumor-bearing rats using Evan's blue dye as the infusate. After infusion, animals were sacrificed and tumors were excised and incubated in formamide for 72 hours at 37°C to extract the Evan's blue dye into solution. The absorbance of Evan's blue dye was then measured in the formamide at 595 and 655 nm and normalized to tumor volume. Error bars represent the SEM of 4 to 6 rats. A, DM443 tumor permeability decreased by approximately 70% from saline control in DM443 xenografts after treatment with bevacizumab. B, DM738 tumor permeability decreased approximately 30% after treatment with bevacizumab. C, mean IFP measured approximately 43% lower (8.3 ± 0.8 vs. 14.9 ± 0.9 mm Hg) in DM443 tumors treated with bevacizumab than with saline controls. D, mean IFP was also approximately 50% lower (9.1 ± 1.2 vs. 18.0 ± 2.9 mm Hg) in bevacizumab-treated DM738 xenografts. *, 2-tailed *t* test.

Figure 2.

Bevacizumab decreases hemoglobin-oxygen saturation in melanoma xenografts. Nude mice bearing DM443 xenografts were treated with 5 mg/kg i.p. bevacizumab ($n = 8$) or saline control ($n = 9$), and (A) tumor oxygenated hemoglobin (HbO₂) and (B) total Hb (Hb) and were measured daily for 6 days after treatment. Systemic bevacizumab had no effect on tumor Hb and decreased HbO₂. *, Mann-Whitney U test.

Figure 3.

Bevacizumab (Bev.) does not significantly alter tumor microvasculature structure or hemoglobin saturation in melanoma xenografts. The effect of bevacizumab in altering tumor microvasculature was determined using a murine model of dorsal flank melanoma in combination with a window chamber model (see Materials and Methods). After surgical excision of a 12-mm diameter flap of skin, mice were inoculated in the exposed fascia with 3×10^6 DM443 or DM738 cells. The exposed fascia was surgically secured with a window chamber saddle providing access for subsequent imaging. Five days after inoculation, mice were injected with 5 mg/kg i.p. bevacizumab or saline control. Melanoma tumors were then imaged daily using hyperspectral microscopy to observe changes in tumor microvasculature. Two to 4 mice were used for each treatment arm for both cell lines. Representative images are shown earlier. A, in general, mice harboring DM443 and DM738 melanoma xenografts showed no significant alterations in tumor microvasculature after treatment with bevacizumab. Using hyperspectral images acquired from the window chamber model, vascular tortuosity (B) and VLD (C) were calculated as surrogate marker for tumor vascular normalization. In both cell lines, no significant differences in tortuosity or VLD were showed.

Figure 4.

Tumor vessel density is unchanged 3 days after bevacizumab treatment. Mean tumor vessel density measured by immunohistochemistry using an anti-rat CD31 antibody on tumor specimen acquired 3 days after treatment with bevacizumab (5 mg/kg i.p.) or saline control. Five to 7 xenografts were analyzed for each treatment group. Cross-sectional images were reconstructed images acquired from a scanned series of 40 to 80 images acquired at $\times 5$ magnification. Representative cross-sectional images are shown for (A) DM443 and (B) DM738. Mean tumor vessel density was similar between treatment bevacizumab and saline-treated xenografts for (C) DM443 (87.6 ± 12.8 vs. 71.1 ± 7.8 mm⁻², $P = 0.26$) and (D) DM738 (77.9 ± 9.4 vs. 73.0 ± 6.3 mm⁻², $P = 0.63$). *, 2-tailed t test.

Figure 5.

Systemic bevacizumab (Bev.) increases delivery of regionally administered melphalan. Immunohistochemistry of tissue slices taken from saline-, melphalan (LPAM)-, bevacizumab-, and melphalan plus bevacizumab-treated samples. LPAM-DNA adduct formation were measured using the antibody MP5/73. Representative images are shown from rats used in each treatment arm for both DM443 and DM738 tumors. For both DM443 and DM738 xenografts, LPAM-DNA adduct formation was higher (brown stain) in the bevacizumab plus melphalan-treated samples compared with the melphalan alone-treated samples. As expected, no LPAM-DNA adduct staining was seen in samples that were not exposed to LPAM. Average staining intensity for each treatment group for both xenografts is plotted with error bars representing SEM. *, one-way ANOVA. Abbreviations: intra-arterial.

Figure 6.

Bevacizumab significantly increases response of melanoma xenografts to regional melphalan. Three days after a single administration of bevacizumab (5 mg/kg i.p.) or saline control, ILI was conducted on tumor-bearing rats with either saline or melphalan (90 mg/kg) as the infusate. Tumor volume was measured every other day for 60 days after ILI. A, fold increase in tumor volume is plotted as a function of time after ILI with melphalan or saline. In this study, the high-VEGF secreting, slower growing DM443 xenografts was studied. Control (black); bevacizumab following by saline infusion (light blue); melphalan infusion alone (yellow); melphalan plus bevacizumab (dark blue). Points, mean fold change in tumor volume for 4 to 5 animals at each time point; bars, SE. B, fold change in tumor volume plotted as a function of time. In this study, the low-VEGF secreting, fast growing melanoma, DM738, was studied. Control (black); bevacizumab following by saline infusion (light blue); melphalan infusion alone (yellow); melphalan plus bevacizumab (dark blue). Points mean fold change in tumor volume for 4 to 5 animals at each time point; bars, SE. LPAM, melphalan. *, one-way ANOVA at 30 days after ILI.