Targeted Anti–Vascular Endothelial Growth Factor Receptor-2 Therapy Leads to Short-term and Long-term Impairment of Vascular Function and Increase in Tumor Hypoxia

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Abstract

Because antiangiogenic therapies inhibit the growth of new tumor-associated blood vessels, as well as prune newly formed vasculature, they would be expected to reduce the supply of oxygen and thus increase tumor hypoxia. However, it is not clear if antiangiogenic treatments lead only to consistent and sustained increases in hypoxia, or transient decreases in tumor hypoxia along with periods of increased hypoxia. We undertook a detailed analysis of an orthotopically transplanted human breast carcinoma (MDA-MB-231) over a 3-week treatment period using DC101, an anti–vascular endothelial growth factor receptor 2 antibody. We observed consistent reductions in microvascular density, blood flow (measured by high-frequency micro-ultrasound), and perfusion. These effects resulted in an increase in the hypoxic tumor fraction, measured with an exogenous marker, pimonidazole, concurrent with an elevation in hypoxia-inducible factor-1α expression, an endogenous marker. The increase in tumor hypoxia was evident within 5 days and remained so throughout the entire course of treatment. Vascular perfusion and flow were impaired at days 2, 5, 7, 8, 14, and 21 after the first injection, but not at 4 hours. A modest increase in the vessel maturation index was detected after the 3-week treatment period, but this was not accompanied by an improvement in vascular function. These results suggest that sustained hypoxia and impairment of vascular function can be two consistent consequences of antiangiogenic drug treatment. The implications of the results are discussed, particularly with respect to how they relate to different theories for the counterintuitive chemosensitizing effects of antiangiogenic drugs, even when hypoxia is increased. (Cancer Res 2006; 66(7): 3639-48)

Introduction

Now that an antiangiogenic drug, bevacizumab (Avastin), the humanized anti–vascular endothelial growth factor (VEGF) monoclonal antibody, has been approved for the treatment of human cancer, the question of what effects such drugs have on the tumor microenvironment, especially hypoxia, has taken on particular significance. The reason for this is that it has been commonly assumed that an agent which inhibits the formation of new blood vessels in tumors, and/or which causes regression of recently formed neovascularure (and hence a drop in the microvessel count in tumors) would be expected to reduce the levels of oxygen and nutrients within tumors and thus inhibit tumor growth by a "tumor starvation" process. However, the resultant increase in tumor hypoxia would be expected to reduce the efficacy of combination radiation therapy or chemotherapy, and as such, would seemingly contraindicate the combined use of an antiangiogenic agent with such standard therapies. In this regard, paradoxically, the addition of antiangiogenic drugs to radiation or chemotherapy usually enhances rather than diminishes the activity of the latter anticancer treatments both preclinically (1, 2) and clinically (3). Indeed, the approval of bevacizumab was based on a pivotal randomized phase III clinical trial in which patients with colorectal carcinoma that were treated with this drug and irinotecan/5-fluorouracil/leucovorin survived significantly longer than patients treated with the same chemotherapy plus a placebo (3).

Several theories have been put forward to explain the aforementioned paradoxical findings, including the following: (a) antiangiogenic drug-induced drops in the high interstitial fluid pressures within tumors and subsequent increased facilitation of intratumoral delivery of chemotherapy as well as transient “normalization” of abnormal tumor blood vessels and hence temporarily decreased levels of tumor hypoxia (2, 4); (b) destruction of activated dividing endothelial cells present in tumor neovasculature by radiation or chemotherapy, the extent of which is amplified by inhibiting survival factors for endothelial cells such as VEGF (5–8); and (c) inhibiting the mobilization or killing of bone marrow–derived proangiogenic circulating endothelial progenitor cells by chemotherapy (9, 10) which, again, may be enhanced by coadministration of an antiangiogenic drug.

Yet another important reason for the need to learn more about the effects of antiangiogenic agents or treatments on tumor hypoxia (and other changes in the tumor microenvironment) is that changes in hypoxia (especially increases) may accelerate tumor progression and facilitate metastasis (11), as well as influence the development of acquired resistance to antiangiogenic drugs by selection of tumor cell subpopulations that are relatively “hypoxia-resistant,” and hence, less dependent on angiogenesis (12). Moreover, if antiangiogenic drugs induce significant and sustained tumor hypoxia, the combination of such drugs with bioreductive “hypoxic cell cytotoxins,” such as tirapazamine, would likely constitute a rational and more effective combination therapy.
strategy to increase the antitumor efficacy of antiangiogenic agents and delay both acquired drug resistance to them and tumor progression (13).

In view of all the abovementioned considerations, it is surprising how few detailed studies of the changes in tumor hypoxia during and after antiangiogenic drug therapy have been reported (2). Moreover, the results of the few studies that have been published are sometimes conflicting, with some showing only increases in tumor hypoxia (2, 14), whereas others show both increases and/or transient decreases in tumor hypoxia (2, 15), when a detailed time course analysis is undertaken. In the cases in which transient decreases are observed, it has been argued that the effects of radiation or chemotherapy may be enhanced, provided the timing and sequencing of the latter therapies are precisely coordinated with the antiangiogenic therapy to take advantage of the vessel normalization "window of opportunity" (2, 4).

Because of the few published studies undertaken in this area, and the conflicting results, along with the major therapeutic implications of antiangiogenic therapy–induced changes in tumor hypoxia, we decided to undertake a detailed time course analysis involving single or multiple injections of a targeted antiangiogenic drug on multiple tumor variables, including microvessel density, vessel maturation, blood flow and perfusion, and tumor hypoxia. The antiangiogenic agent used was DC101, a rat anti–VEGF receptor-2 (VEGFR-2) monoclonal antibody specific for mouse VEGFR-2 (5, 16). We used an orthotopically transplanted human breast cancer (MDA-MB-231) as the tumor model, in part because we have previously reported that DC101 enhances the antitumor effects of both metronomic and maximum tolerated dose chemotherapy in this model (1). Tumor hypoxia was assessed indirectly using the exogenous hypoxia marker pimonidazole as well as hypoxia-inducible factor-1α (HIF-1α) expression (an endogenous marker). High frequency micro-ultrasound imaging (17) was used to estimate blood flow. We report that both acute and sustained increases in tumor hypoxia are induced by DC101 treatment, accompanied by marked and sustained decreases in microvessel density, vascular perfusion, and blood flow. The major implications of these results are discussed, namely, that even in the face of sustained and marked increases in tumor hypoxia induced by an antiangiogenic drug treatment, the efficacy of combined chemotherapy may not be compromised and indeed can still be enhanced.

Materials and Methods

Tumor cell line. The MDA-MB-231 human breast carcinoma cell line was originally obtained from Dr. Jeff Lemontt (formerly of Genzyme Corp.) and was expanded as a monolayer culture by serial passage in tissue culture plates (NUNC, Roskilde, Denmark) in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 5% fetal bovine serum (Gemini BioProducts, Woodland, CA).

Drugs. DC101 (ImClone Systems Inc., New York, NY) is a rat monoclonal antibody specific for mouse VEGFR-2/VEGFR-2 (16, 18). We used an orthotopically transplanted human breast cancer (MDA-MB-231) as the tumor model, in part because we have previously reported that DC101 enhances the antitumor effects of both metronomic and maximum tolerated dose chemotherapy in this model (1). Tumor hypoxia was assessed indirectly using the exogenous hypoxia marker pimonidazole as well as hypoxia-inducible factor-1α (HIF-1α) expression (an endogenous marker). High frequency micro-ultrasound imaging (17) was used to estimate blood flow. We report that both acute and sustained increases in tumor hypoxia are induced by DC101 treatment, accompanied by marked and sustained decreases in microvessel density, vascular perfusion, and blood flow. The major implications of these results are discussed, namely, that even in the face of sustained and marked increases in tumor hypoxia induced by an antiangiogenic drug treatment, the efficacy of combined chemotherapy may not be compromised and indeed can still be enhanced.

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ZD6126 (AstraZeneca, United Kingdom) is a microtubule-inhibiting vascular-disrupting agent known to potently suppress tumor blood flow within hours (19). It was given i.v. (600 μg/mouse) and used as a positive control in the blood flow studies.

In vivo tumor growth assessment and DC101 treatment. CB-17 severe combined immunodeficiency female mice (Charles River, St. Constant, Quebec, Canada) were maintained under pathogen-free conditions in our animal colony according to institutional guidelines. MDA-MB-231 cells were harvested using 1% trypsin-EDTA (Life Technologies), washed twice with fetal bovine serum–free RPMI 1640, and a suspension of 10^7/100 μl was then surgically implanted into the inguinal mammary fat pad of isoflurane-anesthetized 6-week-old mice. Tumor size was assessed twice a week with Vernier calipers by using the formula: width^2 × length × 0.5. Body weights were measured weekly as a surrogate for toxicity. In the first experiment, when the average tumor volume had reached 377 ± 97 mm^3, DC101 or vehicle (PBS) were injected i.p. twice a week over 3 weeks.

In order to study the time course of the effects of DC101, a separate experiment was carried out using 15 DC101-treated and 15 control mice that were sacrificed at 2, 5, or 8 days after the beginning of DC101 treatment (n = 10 mice per time point).

The end of each experiment was always 48 hours after the last DC101 injection.

Blood flow detection using high-frequency micro-ultrasound functional imaging. This was done using the ultrasound "biomicroscopic" system Vevo660 (VisualSonics, Inc., Toronto, ON, Canada). The ultrasound "biomicroscopic" transducer transmits at a central frequency of 40 MHz with a focal length of 6 mm. The lateral and axial resolutions were 68 and 38 μm, respectively (17). Throughout the imaging session, mice were maintained anesthetized with 2% isoﬂurane vaporized in oxygen (Abbott Laboratories, Ltd., Montreal, Quebec, Canada) on a heated stage (THM100, Indus Instruments, Houston TX), with constant monitoring of their body temperature. Ultrasound gel (Aquasonic 100, Parker Laboratories, Fairfield, NJ) was used as a coupling agent on the skin. Real-time ultrasound biomicroscopic imaging at 30 frames per second was done to acquire multiple two-dimensional brightness mode (B mode) image planes and cineloops (300 frames) for each tumor at all time points. Speckle intensity on a B mode image of a stationary tissue remains constant during real-time imaging. As a result, a speckle-variance flow-processing algorithm devised by Yang et al. (20) was used to calculate changes in speckle intensity and as an indication of functional blood flow. It has been shown that speckle-variance can be used to detect flow as low as 0.2 mm/s in flow phantom experiments (20). Frames in each cineloop were obtained using a video editing package (Debabelizer Pro 5, Equilibrium, San Rafael, CA) and processed for speckle-variance. The speckle-variance flow signal in each tumor was quantified using Adobe Photoshop 6.0 software and expressed as "speckle output–positive area (%)" as an index of the percentage of the tumor area covered by vessels with detectable blood flow. Vessels with positive signals but located on the skin were not included in the quantification. Five mice per group were analyzed in each time point and six cineloops per mouse were acquired in the two perpendicular directions.

Evaluation of markers for hypoxia and perfusion. Mice received an i.v. injection of pimonidazole hydrochloride (60 mg/kg; Chemicon International, Inc., Temecula, CA) 90 minutes before euthanasia. This marker was used to assess tumor hypoxia as described elsewhere (21, 22). Pimonidazole hydrochloride is a bioreductive chemical probe activation of which occurs at pO2 levels ≤10 mm Hg (23). For blood vessel perfusion analysis, 1 minute before termination, mice received an i.v. injection of the fluorescent, DNA binding dye, Hoechst 33342 (40 mg/kg; Sigma-Aldrich, St. Louis, MO; refs. 24–26). The combined use of these validated markers for assessment of hypoxia and blood vessel perfusion, respectively, has been previously described elsewhere (25, 27).

Tumors and organs were removed and immediately fixed in 10% buffered formalin or frozen over dry ice in Tissue-Tek optimum cutting temperature compound (Miles Inc., Elkhart, IN) and kept light-protected at −70°C. Snap-frozen samples were collected and immediately placed in liquid nitrogen and kept at −70°C until used for protein extraction.

Tissue processing and immunohistochemistry. Tumor cryosections (5-8 μm) were used for Hoechst 33342 perfusion assay, CD31, α-smooth muscle actin (α-SMA), and pimonidazole immunostaining. Formalin-fixed, paraffin-embedded tumor sections (5-8 μm thick) were stained with H&E or used for pimonidazole and HIF-1α immunohistochemistry.
Francisco, CA) was used and 3,3-

V for pimonidazole and for HIF-1

Cytomation, Carpinteria, CA).

ded tissue. Negative controls were done using mouse IgG 2a (Dako

Jackson ImmunoResearch Laboratories) for formalin-fixed paraffin-embed-

cryosections and biotin-SP-conjugated anti-mouse IgG (dilution, 1:250;

1:200; Jackson ImmunoResearch Laboratories) for pimonidazole staining on

1:250; Sigma-Aldrich).

and a mouse monoclonal anti-HIF-1

mouse monoclonal antibody (dilution, 1:200; Chemicon International),

HIF-1α staining, a FITC-

and a mouse CD31 (dilution, 1:200; PharMingen, San Diego, CA), Hypoxyprobe-1

The following nonconjugated primary antibodies were used: rat anti-

mouse CD31 (dilution, 1:200; Pharmingen, San Diego, CA), Hypoxyprobe-1

body weight measurements. No significant body weight loss was observed
during treatment.

The perfused fraction was analyzed using Adobe Photoshop 6.0 software.
Positive areas (Hoechst 33342-positive) were identified and quantified using
image analysis.

Image acquisition. Tumor sections were visualized under a Carl Zeiss
Axioplan 2 microscope, using bright-field filter or fluorescence filters: 4′6-
diamidino-2-phenylindole (350 nm excitation) for Hoechst 33342; cy3 (540
nm excitation) for CD31 staining and green fluorescent protein (470 nm
excitation) for α-SMA and pimonidazole staining. Images were captured
with a Zeiss AxioCAM camera connected to the microscope using
AxioVision 3.0 software. The number of fields varied between 5 and 20,
depending on the tumor size. Images were collected at a total magnification
of ×100 (×10 objective/×10 eyepiece) for assessment of perfusion and
hypoxia, in which the analysis covered the whole tumor section. A
magnification of ×200 (×20 objective/×10 eyepiece) was used for CD31 and
α-SMA immunostaining to clearly identify vessel structures (n = 5–10 fields).

Image analysis. Analysis of tumor marker pimonidazole was done by calculating
the fraction of the tumor area stained for the hypoxic marker pimonidazole
(“hypoxic fraction”). The staining for pimonidazole was distinct and there
was no difficulty in identifying hypoxic staining from the background. It
always appeared in contiguous cells, facilitating rapid identification and
quantification of the hypoxic areas. For this quantification, Adobe
Photoshop 6.0 software was used and the percentage of hypoxic tissue
was calculated. Necrotic areas were not included in the quantification.

For the analysis of microvascular density (MVD) and the percentage of
α-SMA-positive vessels, the vascular structures (CD31-positive) per field
were counted using Northern Eclipse 6.0 software. Pericytes were identified
as α-SMA-positive cells “wrapping” the vessel structure. The total number
of pericyte-covered vessels and the total number of vessels in all the fields
for each tumor sample were counted and the percentage was calculated
(vessel maturation index). This was done for each tumor sample.

The perfused fraction was analyzed using Adobe Photoshop 6.0 software.
Positive areas (Hoechst 33342-positive) were identified and quantified using
an automatic tool selecting the stained (blue) areas. The perfused fraction
was calculated as the percentage of total tumor area. In all the cases, five
tumor samples corresponding to five mice per group were analyzed.

For each of the analyses, one cross-section was taken from each tumor,
following the longest tumor diameter.

Protein extraction and Western blotting. Nuclear protein extracts
were prepared as described elsewhere with some modifications (28). Briefly,
tumor tissue was mechanically disrupted using lysis buffer [HEPES
10 mmol/L (pH 7.9), MgCl2 1.5 mmol/L, sucrose 300 mmol/L, EDTA
1 mmol/L, DTT 1 mmol/L, NaVO3 0.1 mmol/L, Triton X-100 0.1%, and
protease inhibitors] and a glass homogenizer on ice. The suspension
obtained was washed and resuspended in cold lysis buffer, kept on ice for
1 hour and then centrifuged. The pellet was washed, sonicated, and
ultracentrifuged. Proteins were separated using 8% SDS-PAGE gels. Fifty
milligrams of protein were loaded per lane. Electrophoresis was run at
80 V for the stacking gel and at 150 V for the running gel. The bands
were then transferred using a semi-dry method. Western blotting analysis
was done to detect HIF-1α and HIF-1β. Anti-HIF-1α mouse monoclonal IgG,
(NB100-105; dilution, 1:250; Novus Biologicals) was used as primary
antibody for HIF-1α detection. Anti-HIF-1β (NB 100-110; dilution, 1:500;
Novus Biologicals) was used for HIF-1β detection. Biotinylated anti-mouse
IgG-horseradish peroxidase and anti-rabbit IgG-HRP (Jackson Immuno-
Research Laboratories, Inc., Pennsylvania, PA; dilution, 1:5,000) were used as
secondary antibodies.

Statistical analysis. Differences between control and DC101 group were
assessed using two-tailed Student’s t test. P < 0.05 was considered to be the
cutoff for statistical significance.

Results

Significant effect of DC101 treatment on MDA-MB-231
tumor growth. Female severe combined immunodeficiencymice bearing
MDA-MB-231 breast carcinoma implanted into the inguinal mammary fat pad,
were treated over a period of 3 weeks with 800 μg DC101 injected twice weekly.
This is a rapidly growing tumor, which produces large amounts of VEGF (29). Neither DC101
nor IMC1121, an antihuman VEGFR-2/KDR specific antibody (30)
had a direct effect on the proliferation or survival of these tumors.
cells in vitro (data not shown). Treatment was started on day 27 of tumor growth, when the tumors had reached an average volume of 377 ± 97 mm³, and was continued for 3 weeks. As shown in Fig. 1A, tumor growth was significantly delayed as a consequence of DC101 treatment and the effect could be observed almost immediately. At the end of the experiment, the percentage of tumor volume reduction induced by the DC101 treatment was 65.0 ± 4.7% (P < 0.001). No body weight loss (used as an indicator of possible toxicity) was observed (Fig. 1B). Thus, the treatment seemed to be minimally or non-toxic and showed significant antitumor effects as a monotherapy.

**MVD is decreased by DC101 treatment.** We observed a significant reduction in MVD, based on CD31 immunostaining, in DC101-treated tumors compared with PBS-treated control tumors after the 3-week DC101 treatment (3.3-fold: control, 41.94 ± 15.91 vessels/field; DC101, 12.56 ± 6.16; mean ± SE; P < 0.0001; Fig. 2A). This structural analysis of the tumor microvasculature was then followed by a detailed functional analysis of perfusion and blood flow studies as described below.

**Blood vessel perfusion is decreased in DC101-treated tumors.** The analysis of blood vessel perfusion was carried out using the fluorescent DNA-binding dye Hoechst 33342 injected i.v. 1 minute before euthanasia. The results revealed a significant reduction in the perfused area, measured as the percentage of tumor area covered by perfused vessels, in the DC101-treated tumors with respect to the control group (2.4-fold: control, 9.69 ± 3.84%; DC101, 3.97 ± 1.42%; mean ± SE; P < 0.05; Fig. 2B).

**Functional blood flow measured using speckle-variance analysis of high-frequency ultrasound imaging is decreased in DC101-treated tumors.** Functional blood flow analysis was done once a week during treatment using ultrasound biomicroscopic imaging (17) as explained in Materials and Methods. The speckle-variance analysis of real-time imaging cineloops showed a significant reduction in the percentage of the tumor area containing blood vessels with detectable blood flow as early as 1 week after the beginning of treatment. This difference remained over the entire course of the therapy (tested at 1, 2, and 3 weeks after the first injection; Fig. 3; Supplemental Data online 1).

We also explored the possibility that DC101 might have an acute effect on blood flow as has been observed for vascular disrupting agents. To test this, we measured blood flow using the same methodology, 4 hours after the first DC101 injection and using the vascular disrupting agent, ZD6126, as a positive control (19). We found no evidence of a reduction in blood flow induced by DC101 at this time point [control group, 0.9 ± 0.6%; DC101, 1.3 ± 1.0% (not significant); ZD6126, 0.3 ± 0.2% (P < 0.05); mean ± SE].

**Hypoxia is increased and the distribution pattern of hypoxic areas in tumors changes as a result of DC101 treatment.** In order to determine the overall effect of DC101 on tumor oxygenation and to study possible microregional effects, we did an analysis of tumor hypoxia using the exogenous marker pimonidazole hydrochloride (22, 24, 31). This is a bioreductive chemical probe, activation of which occurs at pO₂ ≤10 mm Hg.

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**Figure 2.** Effect of DC101 on microvessel density counts and vessel perfusion in MDA-MB-231 tumors. A, blood vessel detection using CD31 immunostaining. Representative images of MDA-MB-231 tumor sections for each group (top, control; middle, DC101 treated); red, blood vessel structures immunostained with CD31 and detected with a Cy3-conjugated antibody; blue, counterstaining with 4,6-diamidino-2-phenylindole (magnification, ×200). Bottom, quantification of the MVD (n = 5 mice per group; 5-10 fields per section; ***, P < 0.0005; Student’s t test). B, perfusion was measured after i.v. injection of Hoechst 33342 1 minute before sacrificing the mice. Hoechst was visualized under a fluorescence microscope (blue pixels). Representative images of a MDA-MB-231 tumor section for each group (top, control; middle, DC101 treated; magnification, ×100). Bottom, quantification of the percentage of perfused area (n = 5 mice per group; *, P < 0.05; Student’s t test).
This corresponds to ~1% O_2, which is an accepted borderline between well versus poorly oxygenated tumors (32, 33). The analysis revealed an increase in the extent of the hypoxic tissue in DC101-treated tumors with respect to the control group after a 3-week treatment (4.7-fold; *P < 0.0005). Additionally, a change in the distribution pattern of the hypoxic areas was observed, as shown in Fig. 4. The localization of the hypoxic areas was perinecrotic in both groups; however, whereas a viable rim around a central necrotic core was generally observed in the control group, hypoxic rims in DC101-treated tumors usually appeared in many different locations within the tumor.

**The level of the transcription factor HIF-1α is increased after DC101 treatment.** Because the transcription factor HIF-1 is one of the major regulators of the cellular response to hypoxia (34–36), we analyzed the expression of its oxygen-sensitive regulatory subunit, HIF-1α. Nuclear protein extracts obtained from DC101-treated tumors or control tumors were analyzed by Western blotting. Although the level of the non–oxygen-sensitive subunit, HIF-1β, was not altered by DC101 treatment, the level of HIF-1α was increased (Fig. 4B). These results were confirmed by immunohistochemistry (Fig. 4C).

**Alteration in the ratio of immature versus mature vessels after DC101 treatment.** We anticipated that DC101 treatment might alter the proportion of vessels having a more mature phenotype, by preferentially eliminating new, immature vessels while sparing more established vessels. To test for this possibility, we studied blood vessel pericyte coverage as an indicator of vessel maturity. Pericytes were identified using α-SMA staining whereas endothelial cells were identified using CD31 staining. The vessel maturation index was calculated as the fraction of vessels that were associated with α-SMA-positive periendothelial cells. An increase in the maturation index was observed after 3 weeks of DC101 treatment (control, 1.33 ± 0.26%; DC101, 7.32 ± 0.44; mean ± SE; *P < 0.0005). It is worth noting that the absolute number of pericyte-covered vessels per field did not significantly change (control, 0.77 ± 0.15; DC101, 1.67 ± 0.35; mean ± SE, not significant; Fig. 5A).

**Early effects of DC101 treatment on perfusion, blood flow, MVD, hypoxia, and vascular quality.** In order to evaluate the early effects of DC101 on several vascular variables as well as tumor hypoxia, we did a separate experiment using the same tumor model and treatment, but the time points studied in this case were 2, 5, and 8 days after the beginning of DC101 treatment.

We observed the following sequence (Table 1; Fig. 6): a reduction in perfusion was detected as early as 2 days after the onset of treatment. On day 5, all the variables studied were already affected;
the MVD was reduced, vascular function was impaired—measured both as blood flow and perfusion—and these changes were accompanied by a significant increase in the percentage of the hypoxic area. Perfusion and blood flow remained reduced at day 8.

To test the hypothesis that some subpopulations of blood vessels could be preferentially targeted at these time points, we decided to analyze the quality of the remaining vessels using two approaches. First, we analyzed the level of vessel pericyte-coverage, identifying the pericytes using α-SMA staining. The expectation was that if the less mature vessels are more vulnerable to DC101 treatment, then the proportion of mature vessels (vessel maturation index) in the remaining population after treatment would be increased. However, we did not observe a change in the percentage of pericyte-covered vessels at day 2, although we observed a tendency in this direction at day 5: the percentage of pericyte-covered vessels; control, 0.99 ± 0.29%; DC101, 3.74 ± 1.46% (mean ± SE; n = 5 mice/group; P = 0.07, marginally significant; Fig. 5B). In this regard, the basal level of pericyte-covered vessels in this tumor model at the time points analyzed was very low, and this may account for the small increase. In the second approach, we decided to study the proportion of vessels that were functional, measured by Hoechst/CD31 staining. We hypothesized that if the less functional (non-perfused) vessels were preferentially affected by DC101 treatment, the proportion of the functional (perfused) vessels after treatment would increase. We found no significant change in the percentage of functional vessels, measured as the fraction of total vessels which were perfused or Hoechst 33342–positive (day 5: control, 73.9 ± 6.6% of functional vessels; DC101, 60.2 ± 4.4%; mean ± SE; n = 5 mice/group, not significant; Fig. 5C).

Discussion

By using a battery of molecular and functional imaging markers to quantitate blood vessel numbers, vascular function (including blood flow and perfusion), and changes in tumor hypoxia, we found that treatment with a targeted anti-VEGFR-2 antibody as an antiangiogenic agent consistently increased the extent of tumor hypoxia in the model we studied—an orthotopically growing human breast cancer xenograft. Evidence was also obtained which indicates that the localization or distribution of hypoxic areas in the treated tumors is also altered. The detailed time course study of the alterations in tumor hypoxia and intratumoral vascular function mediated by DC101 treatment showed the following sequence of events: vascular perfusion was significantly suppressed at relatively early time points, e.g., day 2 (but not acutely at 4 hours), whereas the drop in functional blood flow and the increase in tumor hypoxia—which likely occurred as a result of reduction in

Figure 4. Assessment of tumor hypoxia after DC101 treatment. A, hypoxic areas were detected using pimonidazole hydrochloride. Top, representative images of MDA-MB-231 tumor sections from control (left) and DC101 group (right). Brown, areas stained positive for pimonidazole are defined as hypoxic areas (arrows). Counterstaining was done with hematoxilin (magnification, ×100). Note the presence of adipocytes, characteristic of the site of implantation (mammary fat pad). Bottom, quantification of the extension of the hypoxic areas (***, P < 0.005; Student’s t test). B, HIF-1α detection. HIF-1α was detected by Western blotting of nuclear protein extracts from tumors treated with PBS (left) or DC101 (right). Each lane represents one mouse. HIF-1β and β-actin were used as controls. HIF-1α levels were increased in the DC101-treated tumors. C, HIF-1α immunostaining. Representative images of the control (left) and DC101 (right) group; brown, cells positive for HIF-1α (arrows, some representative positive cells). Counterstaining was done with hematoxilin (magnification, ×200).
Although HIF-1α

For example, whereas pimonidazole binding was always found in evaluating the extent and distribution of hypoxic areas in tumors. Comparative analysis of the exogenous marker for hypoxia, tumor hypoxia was indirect, using two well-known markers. The endogenous marker (HIF-1α) was always lower than the exogenous marker pimonidazole. This pattern has also been observed by others (31, 37). There are a number of possible explanations for the lack of complete overlap between these two hypoxia markers, including: (a) the detection method is more sensitive for pimonidazole than for HIF-1α (in part due to the short half-life of this protein as well as its nuclear localization); (b) there may be different kinetics of HIF-1α up-regulation and pimonidazole reduction (38); (c) there is the possibility of different oxygen dependencies between the two processes; (d) it has been shown that both HIF-1α-dependent and HIF-1α-independent mechanisms of the cellular response to hypoxia exist (39–43); and (e) HIF-1α levels are regulated not only by hypoxia but also by other factors (34).

Despite the lack of complete overlap, the expression of these two markers indicated sustained increases in tumor hypoxia during the course of drug treatment. Such changes may have a number of important and therapeutic biological implications. For example, hypoxia can alter the expression of various genes which contribute to invasion and metastasis; thus, in some cases, ultimately increasing the malignant phenotype (11, 34). On the other hand, a potential therapeutic opportunity arises by virtue of the availability of many different drug strategies designed to specifically or preferentially target hypoxic tumor cells, such as bioreductive hypoxic cell cytoxins or other agents which become cytotoxic only under hypoxic conditions (13, 44, 45).

Studies on vascular function. This study is the first to assess the possible acute effects (i.e., within hours) of DC101 on vascular function. We did not detect any changes at such an early time point, suggesting that this type of drug does not have a rapid vascular disrupting agent–like action. Vascular function was impaired after 2 days of treatment, and this is probably a consequence of the primary effect of DC101 on vessel numbers. In this regard, two points should be highlighted. First, we did not observe a change in specific vessel function, i.e., we did not detect an alteration in the percentage of functional vessels as assessed by the double analysis of CD31 immunostaining and Hoechst 33342 perfusion. This suggests the lack of a preferential effect of DC101 on vessels with lower or higher activity. Second, we observed a good correlation between the two variables used to measure vascular function, i.e., perfusion (using Hoechst 33342) and functional blood flow (estimated by using high frequency micro-ultrasound imaging). These two methodologies have different detection limits and characteristics: High frequency micro-ultrasound has the advantage of being a noninvasive, functional imaging tool that allows following individual tumors/mice over the experimental period in a longitudinal manner, and six planes per tumor instead of one could be analyzed, providing a comprehensive assessment of the whole tumor, whereas the Hoechst perfusion has the advantage of being able to detect even smaller vessels. Consequently, the combination of Hoechst perfusion and speckle-variance analysis could provide a real picture of the vascular function status of the tumor before and after therapy. Of note, we are currently carrying out studies on vascular morphology using microcomputed tomography to study the effect of DC101 on tumor vessel morphology (46).

Studies on vascular maturation. We examined the vascular maturation status of the tumors by testing the possibility that DC101 treatment might preferentially target less mature vessels. That

Figure 5. Analysis of changes in immature versus mature tumor blood vessels as a consequence of DC101 treatment. A, pericyte-coverage analysis after 3-week DC101 treatment. Pericytes were detected by α-SMA staining. Representative images of MDA-MB-231 tumor sections from control (left) and DC101 (right) groups; red, CD31 staining used to identify vessel structures; green, pericytes (magnification, ×200). Inset, normal dermal vessels (positive control of the assay). B, pericyte-coverage analysis after a 5-day DC101 treatment. Representative images of MDA-MB-231 tumor sections from control (left) and DC101 (right) groups; red, CD31 staining used to identify vessel structures; green, pericytes (magnification, ×200). C, analysis of the proportion of functional vessels after a 5-day DC101 treatment. Functional vessels were defined as those that were perfused. Representative images of MDA-MB-231 tumor sections from control (left) and DC101 (right); red, CD31 immunostaining; blue, perfusion (Hoechst 33342; magnification, ×200).

Vessel density and function—were first evident after 5 days of treatment, and remained similarly affected over the entire period of treatment and analysis, which involved twice a week injections of the drug for 3 weeks.

An important aspect of our study was the simultaneous measurement of blood vessel density, vascular function, and assessment of different hypoxia markers in the same tumor specimens, as opposed to undertaking separate studies using different samples. Below, we discuss some of the features and implications of our results.

Hypoxia-associated studies. Assessment of the relative state of tumor hypoxia was indirect, using two well-known markers. The comparative analysis of the exogenous marker for hypoxia, pimonidazole, and the endogenous marker (HIF-1α) suggests that although HIF-1α could be valuable for mechanistic studies, pimonidazole seemed to be the better marker of the two for evaluating the extent and distribution of hypoxic areas in tumors. For example, whereas pimonidazole binding was always found in the perinecrotic rims, HIF-1α nuclear staining showed a less uniform pattern and single positive cells were found in regions identified as viable, hypoxic, and even necrotic. Furthermore, the level of HIF-1α was lower than the exogenous marker pimonidazole. This pattern has also been observed by others (31, 37). There are a number of possible explanations for the lack of complete overlap between these two hypoxia markers, including: (a) the detection method is more sensitive for pimonidazole than for HIF-1α (in part due to the short half-life of this protein as well as its nuclear localization); (b) there may be different kinetics of HIF-1α up-regulation and pimonidazole reduction (38); (c) there is the possibility of different oxygen dependencies between the two processes; (d) it has been shown that both HIF-1α-dependent and HIF-1α-independent mechanisms of the cellular response to hypoxia exist (39–43); and (e) HIF-1α levels are regulated not only by hypoxia but also by other factors (34).
would lead to a selection in favor of a more mature vessel phenotype. We found an increase in the percentage of pericyte-covered vessels after the 3-week DC101 treatment, which would suggest that those vessels without pericyte coverage would indeed seem to be more sensitive to this treatment, as also observed by others (47). However, the absolute number of pericyte-covered vessels per field did not significantly change, suggesting that the observed effect would be a consequence of a preferential targeting of the vessels lacking pericyte coverage (47), as opposed to an active recruitment of pericytes (2). In addition, although we observed a tendency towards an increase in the percentage of pericyte-covered vessels at day 5 of treatment, this was not accompanied by an increase in the percentage of functional vessels. The same tendency was also observed in another tumor model we have been studying (MeWo human melanoma xenografts, grown orthotopically; data not shown). Further work is now in progress to explore this important aspect of tumor vascular biology in greater depth and determine if variables such as vessel functionality and maturity may be separable.

**Implications of the results for possible mechanisms to account for the chemosensitizing or radiation-sensitizing effects of antiangiogenic drugs.** Part of the significance of our results lies in the various theories which have been put forward to account for the paradoxical therapy-sensitizing effects of antiangiogenic drugs for both chemotherapy and radiation therapy. A longstanding view has been that a "tumor-starving" antiangiogenic drug or treatment which suppresses blood flow and vascular perfusion of tumor vessels should lead to an increased level of tumor hypoxia, which in turn, would be expected to result in diminished antitumor effects of chemotherapy or radiation. Because the opposite has frequently been observed, several theories have been put forward to account for such unexpected results. One of these is the transient "vessel normalization" hypothesis (4). According to this hypothesis, an antiangiogenic drug such as DC101 could cause not only some regression of immature vessels, but also temporarily "normalize" the abnormal and functionally impaired tumor vasculature that remains, thereby transiently increasing tumor blood flow and perfusion in certain regions of treated tumors. This, in turn, may cause a short burst in the tumor cell proliferative index, increasing the effects of chemotherapy and inducing a drop in hypoxia, which would increase radiation sensitivity as well as chemotherapy (2, 4). A drop in the high interstitial fluid pressures within tumors (as a result of suppression of vascular permeability) may also take place, and such an effect could also enhance the delivery of chemotherapy into tumors, and therefore, antitumor efficacy. In this regard, our results, at least in the model that we have studied, do not provide evidence of a decrease in tumor hypoxia at any of the many time points we examined, despite the fact that there was a trend towards increased vessel maturation. On the contrary, the dominant effect was an increase in tumor hypoxia.

We wish to emphasize that our results do not in any way negate the vessel normalization hypothesis, which clearly could be tumor model–, drug context–, and tumor stage–dependent. Nevertheless, it is noteworthy that we have previously found that combining either standard maximum tolerated dose chemotherapy or low dose “metronomic” chemotherapy with DC101 in the MDA-MB-231 orthotopic breast cancer model leads to significantly enhanced antitumor therapeutic effects when compared with the respective monochemotherapy treatment controls (1). Thus, even in a situation in which there is marked and sustained tumor hypoxia, the effects of chemotherapy can still be significantly enhanced by coadministration of an antiangiogenic drug such as the VEGFR-2-targeting antibody we used. As such, our model and results can be viewed as an approach to try and separate and study mechanisms of chemosensitization by antiangiogenic drugs that are likely independent of significant vessel normalization and transient drops in tumor hypoxia. This clearly highlights the need to consider alternative or complimentary mechanisms, which may account for the chemosensitizing effects of an antiangiogenic drug such as DC101, such as the targeting of dividing endothelial cells present in tumor-associated growing neovasculature, the extent of which would be facilitated by concurrently inhibiting the

### Table 1. Early effects of DC101 on MVD, perfusion, blood flow, and hypoxia

<table>
<thead>
<tr>
<th>Variable assessed</th>
<th>Day 2</th>
<th>Day 5</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MVD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>44.12 ± 5.43</td>
<td>35.84 ± 3.67</td>
<td>48.45 ± 4.21</td>
</tr>
<tr>
<td>DC101</td>
<td>32.37 ± 4.25*</td>
<td>20.73 ± 1.44†</td>
<td>8.72 ± 2.38†</td>
</tr>
<tr>
<td><strong>Perfusion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.27 ± 1.32%</td>
<td>11.60 ± 1.83%</td>
<td>10.57 ± 1.78%</td>
</tr>
<tr>
<td>DC101</td>
<td>3.78 ± 0.93%†</td>
<td>3.88 ± 1.76%†</td>
<td>3.51 ± 0.43%†</td>
</tr>
<tr>
<td><strong>Percentage of area with detectable blood flow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.29 ± 0.29%</td>
<td>1.36 ± 0.33%</td>
<td>0.74 ± 0.16%</td>
</tr>
<tr>
<td>DC101</td>
<td>1.54 ± 0.10%*</td>
<td>0.57 ± 0.17%†</td>
<td>0.12 ± 0.00%†</td>
</tr>
<tr>
<td><strong>Hypoxia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.50 ± 2.40%</td>
<td>4.69 ± 0.82%</td>
<td>1.15 ± 0.23%</td>
</tr>
<tr>
<td>DC101</td>
<td>13.71 ± 10.99%*</td>
<td>13.9 ± 1.79%†</td>
<td>7.89 ± 1.68%†</td>
</tr>
</tbody>
</table>

NOTE: MVD was determined by using CD31 immunostaining on tumor cryosections; perfusion was analyzed by using Hoechst 33342 injected i.v.; blood flow was studied by ultrasound biomicroscopic imaging and the extent of hypoxia was assessed by using pimonidazole hydrochloride detected by specific antibodies (Hypoxiprobe-1) on tumor cryosections. Mean ± SE are shown (n = 5 mice per group).

*Not significant.
†P < 0.05 (Student’s t test).
‡P < 0.005 (Student’s t test).
prosurvival/antiapoptotic function of VEGF for endothelial cells (5, 48) or the destruction of bone marrow–derived circulating proangiogenic cells including endothelial progenitor cells (49), the extent of which may be facilitated by compromising the mobilizing function that VEGF has for such cells (50). A third possibility is that the ability of tumor cell populations to repopulate between successive courses of pulsatile chemotherapy may be significantly impaired by the presence of an antiangiogenic drug treatment during the break periods between successive courses of such chemotherapy (51). This may slow down the rate of drug resistance due to progressively accelerated repopulation kinetics with successive courses of chemotherapy (52).

Finally, with respect to the vessel normalization hypothesis, we wish to emphasize that the model we have studied contains an intrinsically very low proportion of mature blood vessels. Consequently, the opportunity of a small change in this vessel maturation index to be translated into a more functional vasculature after DC101 treatment may be minimal in such a situation. However, we acknowledge that the situation would presumably be quite different in spontaneous, slow-growing, long-established human tumors which would be expected to have a much higher proportion of mature blood vessels compared with most models of rapidly growing transplanted tumors in experimental animals that are analyzed within weeks of tumor cell injection, as we have done. Nevertheless, our results indicate that even in situations of marked and sustained hypoxia induced by an antiangiogenic drug such as DC101, it is still possible that the tumors will show heightened responses to chemotherapy (or radiation), at least for a period of time, such that host survival is prolonged compared with either treatment alone (1).

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Figure 6. Short-term effects of DC101 treatment on MDA-MB-231 tumor xenografts. Tumor-bearing mice were treated with DC101 (800 μg) or PBS i.p. on days 0 and 3. On day 5, mice were imaged, euthanized, and tumor samples were taken. Representative images of several assays are shown. A, CD31 immunostaining; red, CD31-positive vessels. Counterstaining, 4',6-diamidino-2-phenylindole (magnification, ×200). B, perfusion (Hoechst 33342); blue, perfused vessels (magnification, ×100). C, percentage of area with detectable blood flow measured using high-frequency ultrasound biomicroscopy imaging; red, vessels with detectable blood flow. The images were processed and obtained from the acquired cineloops. D, hypoxia detected with pimonidazole hydrochloride and Hypoxiprobe-1 antibody; green, hypoxic areas (pimonidazole); blue, perfused areas (Hoechst 33342; magnification, ×100).
References


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