Regional Effects of an Antivascular Endothelial Growth Factor Receptor Monoclonal Antibody on Receptor Phosphorylation and Apoptosis in Human 253J B-V Bladder Cancer Xenografts

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a key angiogenic factor in a variety of solid tumors, making it one of the most attractive therapeutic targets. VEGF promotes the proliferation, survival, and differentiation of vascular endothelial cells by stimulating autophosphorylation and activation of VEGF receptor-2 (VEGFR-2, fetal liver kinase-1, and kinase insert domain-containing receptor). We developed fluorescence-based, quantitative methods to measure total VEGF-2, VEGF-2 phosphorylation, apoptosis, and microvessel density and size within whole tumor cross-sections using a laser scanning cytometer. Using these methods, we characterized the effects of DC101, a blocking antibody specific for murine VEGF-2, on orthotopic human 253J-BV bladder tumors growing in nude mice. Basal levels of receptor phosphorylation were heterogeneous, with approximately 50% of endothelial cells positive for phosphorylated VEGF-2 at baseline. DC101 therapy resulted in a 50% decrease in overall VEGF-2 phosphorylation and a 15-fold and 8-fold increase in endothelial cell (CD31-positive) and tumor cell apoptosis, respectively. DC101 also decreased overall tumor microvessel density, but it mostly affected smaller CD105-negative microvessels located in the periphery of the tumor. Intriguingly, anti-VEGFR-2 therapy resulted in increased mean vessel size and an increase in overall VEGF-2 levels. Increases in total VEGF-2 levels were localized to the tumor core and were associated with increased expression of the oxygen-sensitive transcription factor, hypoxia inducible factor-1α. These data suggest that VEGFR inhibitors preferentially target discrete populations of tumor endothelial cells associated with the smaller peripheral blood vessels. Thus, agents that target a single receptor (e.g., VEGF-2) may not be sufficient to completely inhibit tumor angiogenesis.

INTRODUCTION

The growth of solid tumors beyond the diffusion limit of oxygen (1–2 mm) is dependent on the formation of a new vascular network within the tumor (1–4). Tumors appear to initiate angiogenesis by disrupting normal homeostatic control of the pro- and antiangiogenic factors that regulate blood vessel density in normal tissues (5, 6). Prominent among these factors is vascular endothelial growth factor (VEGF; Refs. 7 and 8), a cytokine that plays an obligate role in the angiogenesis-related markers and laser scanning cytometry (LSC) to quantitatively assess the effects of VEGF-2 target inhibition in orthotopic 253J B-V tumors. We initially focused on using the LSC to determine the extent of target expression (activated VEGF-2) at baseline and after anti-VEGFR-2 therapy. Innovative use of the LSC allowed us to capture information for whole-tumor sections, as opposed to the analyses of "hot spots" or high-power fields conventionally evaluated.
in studies dealing with solid tumors growing in vivo. We also adapted the contouring feature of the LSC to allow for quantification of microvessel densities and relative vessel sizes, as well as endothelial cell death, a downstream consequence of VEGFR-2 inhibition. The quantitative results demonstrate that inhibition of VEGFR-2 phosphorylation increases endothelial cell apoptosis and reduces MVD but also increases VEGFR-2 expression. More important, the results also reveal that VEGF inhibitors preferentially target small, CD105-negative vessels in the tumor periphery but do not significantly affect larger, established vessels within the tumor core.

MATERIALS AND METHODS

**Orthotopic Model of Human Transitional cell carcinoma and DC101 Therapy.** We obtained male athymic BALB/c nude mice from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in a laminar air flow cabinet under specific pathogen-free conditions and used at 8–12 weeks of age. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the NIH. The highly metastatic human bladder carcinoma cell line 253J-BV (60–70% confluent) was prepared for injection as described previously (25). Mice were anesthetized with methoxyflurane, and viable tumor cells in HBSS (100 μl) were orthopedically implanted into the bladder wall. Formation of a bulla indicated a satisfactory injection.

**Immunofluorescent Detection of CD31/CD105 (endothelial cells) and Terminal Deoxynucleotyl Transferase-Mediated Nick End Labeling (TUNEL).** For frozen tissues, sections were fixed with cold acetone for 5 min. Tissues were washed with PBS for 3 min and incubated with 0.2% Triton X-100 for 5 min. Tissues were washed three times for 3 min with PBS. Next, tissues were incubated with protein block (5% normal horse serum in PBS) for 15 min at room temperature. Protein block was drained, and tissues were incubated with a 1:400 dilution of rabbit monoclonal anti-CD31 antibody (PharMingen, San Diego, CA) or anti-CD105 antibody (PharMingen) in protein block overnight at 4°C. Tissues were washed with PBS three times for 3 min. Tissues were incubated in the dark with a 1:400 dilution of secondary goat antirabbit IgG conjugated to Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA) in protein block for 4 h at 4°C. Tissues were washed with PBS containing 0.1% Brij for 3 min and washed twice with PBS for 3 min. TUNEL (Promega, Madison, WI) was performed with the following modifications: Tissues were fixed with 4% paraformaldehyde for 10 min at room temperature and washed with PBS twice for 3 min. Tissues were incubated in the dark with a reaction mixture (from kit) in a humid atmosphere at 37°C for 1 h. Tissues were washed three times with PBS for 3 min to remove unincorporated fluorescein-dUTP. Cell nuclei were counterstained with 1 μg/ml propidium iodide for LSC analysis or a 1:2000 dilution of Hoechst (300 μg/ml) for 5 min for visualization using an epifluorescence microscope. Tissues were washed with PBS three times for 3 min and Prolong (Molecular Probes, Eugene, OR) was used to mount coverslips. All images were captured using a Zeiss Plan-Neofluar objective on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel to select for green or red or blue fluorescence. Images were processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

**Immunofluorescent Detection of CD31, Phosphorylated-VEGFR-2 or Total VEGFR-2, and Hypoxia-Inducible Factor-1α (HIF-1α).** For two-color immunofluorescent detection of CD31 and phosphorylated-VEGFR-2, CD31 staining was performed according to the previous section. After incubation with secondary antibody and washing, tissues were incubated with a 1:1000 dilution of rabbit antiphospho-VEGFR-2 antibody (Ab-1; Oncogene, La Jolla, CA) in protein block overnight at 4°C. Tissues were washed with PBS containing 0.1% Brij for 3 min twice and with PBS for 3 min. Tissues were incubated with biotinylated goat antirabbit IgG (Biocare Medical, Walnut Creek, CA) for 2 h at room temperature. Tissues were washed with PBS containing 0.1% Brij for 3 min twice and with PBS for 3 min. A 1:1000 dilution of Avidin-FITC (PharMingen) was added to the tissues for 30 min at room temperature. Tissues were washed with PBS three times for 3 min and incubated with 1 μg/ml propidium iodide for LSC analysis or a 1:2000 dilution of Hoechst (300 μg/ml) at room temperature for 5 min for visualization on an epifluorescence microscope.

For detection of total VEGFR-2 or HIF-1α, tissues were incubated with a 1:400 dilution of rabbit anti-fetal liver kinase-1 antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) or HIF-1α (clone H1α67, Novus Biologicals, Littleton, CO) overnight at 4°C. Tissues were washed with PBS containing 0.1% Brij for 3 min twice and with PBS for 3 min. Tissues were incubated with a 1:400 dilution of secondary goat antirabbit IgG conjugated to Cy5 for 4 h at room temperature. Tissues were washed with PBS twice for 3 min and counterstained with 1 μg/ml propidium iodide for LSC analysis or a 1:1000 dilution of Sytox (Molecular Probes) for 3 min for visualization on an epifluorescence microscope. Tissues were washed with PBS three times for 3 min, and Prolong was used to mount coverslips.

**LSC Quantification of Apoptosis in Endothelial Cells and Tumor Cells.** Percentages of apoptotic endothelial cells and tumor cells were determined by LSC as described previously (26, 27). All xenografts were stained with H&E to pathologically confirm the presence and location of tumor cells (Fig. 4C). Desired tumor areas were visually located using the epifluorescence microscope of the LSC to detect cell nuclei counterstained with propidium iodide, and the scan area was set using WinCyte software (Compucyte Corp., Boston, MA). Lasers were selected to detect red (cell nuclei), green (TUNEL), and long-red (CD31 or CD105) fluorescence and detector gain voltages were set so that a maximum of 50% saturation was achieved for the maximum pixel event scanned. Slides were scanned using an ×200 objective, and the minimum-area threshold was set to optimize single cell contours. Analytical gates (Fig. 6C) were set based on the fluorescent properties of the negative staining controls. The gates defined four quadrants that determined the total number of cells within each population (quadrant 2, CD311/TUNEL1; quadrant 1, CD311/TUNEL1; and so forth). The relocation feature was used to identify and confirm TUNEL-positive and CD31-positive cells.

**LSC Analysis of Microvessels, Phosphorylated VEGFR-2, and Total VEGFR-2.** MVD is usually quantified by counting CD31-positive structures in several high-powered microscopic fields (×200), focusing on the areas displaying the greatest staining intensity (“hot spots”; Refs. 22–24). Data obtained in this fashion may not, however, be representative of more global effects of the drug within the tumor.

We used the LSC, therefore, to quantify changes in MVD, including vessel size, and changes in other biomarkers in whole-tumor cross-sections to more directly measure locoregional effects of drug treatment. Using the scan area of the LSC, we quantifying approximately 100 cells, we performed a second scan by contouring long-red fluorescence (CD31 or CD105) to count microvessels within whole tumor sections. Slides were scanned using an ×200 objective, and microvessels were contoured using the long-red fluorescence detector with the perimeter option selected (to determine vessel size). The minimum-area threshold was set to optimize contours of individual vessels. Thus, the total number of positive-events counted was equal to the total number of microvessels within the scanned region. Percent MVD was determined by (vessels/total cells × 100). The formula (control-treated/control × 100) was used to calculate changes in MVD. To measure the size of each vessel, the data file was replayed with the area/perimeter ratio selected on the X axis of the histogram window. The histograms represent the mean vessel size within each tumor section.

To determine the levels of phosphorylated VEGFR-2 within each endothelial cell, we selected lasers to detect red (cell nuclei), long-red (CD31), and green (p-VEGFR-2) fluorescence. Slides were scanned using an objective (×200) and detector gain voltages were set so that a maximum of 50% saturation was achieved for the brightest maximum pixel event scanned. The minimum area threshold was set to optimize single cell nuclei contour events. Analytical gates were set based on the fluorescent properties of the negative staining controls. The gates were used to define the quadrants that determined which cells were positive for CD311/p-VEGFR-21, and others. The relocation feature was used to visually confirm positive-stained cells. The formula (control-treated/control × 100) was used to calculate changes in endothelial cell expression of phosphorylated-VEGFR-2.
To measure total VEGFR-2 levels, we selected lasers that detect red (cell nuclei) and long-red fluorescence (VEGFR-2). Slides were scanned using a ×200 objective, and cell nuclei were contoured by setting the minimum area threshold. Total VEGFR-2 levels were quantified by taking the integral of the long-red fluorescence. Cells were scored as either positive or negative for VEGFR-2 based on the fluorescent properties of the negative control sample with appropriate gating. Relocation was used to visually confirm positive marker expression.

**Analysis of Intratumoral Biomarker Expression.** After acquisition of the data for each biomarker as described above, we determined the differential expression of biomarkers in the periphery versus the core of each tumor (Table 2). First, an X and Y coordinate position map was generated using the analytical software of the LSC. Each acquired data file containing total cell nuclei detection (using propidium iodide) was replayed to generate a 2-dimensional tumor cross-section tissue map for each tumor as shown in Fig. 6B. Each black pixel represents a single cell within the tumor cross-section. Next, an elliptical draw tool was used to create a boundary that defined the tumor core from the periphery as shown in Fig. 4B. The size and placement of the elliptical boundary was based on morphological evaluation of each specimen (Fig. 4C). Each acquired biomarker data file (e.g., microvessels) was then replayed to determine the number of events within each tumor region as shown in Table 2. For example, Fig. 4B illustrates the distribution of microvessels (represented by each pixel) within each tumor compartment (red = core; black = periphery). Importantly, replay of the data file included only positive events that were based on gating of negative staining controls for each biomarker. For example, in Fig. 6C quadrants 3 and 4 were excluded from the differential analysis. Thus, each quadrant or cell population can be manually selected to determine quantitative distribution within each compartment displayed on the X and Y coordinate position map (Fig. 4B). These steps were repeated to determine differential expression for each biomarker in each tumor (Table 2).

**Statistics.** All statistical analyses were performed with SPSS software (SPSS, Chicago, IL). Experiments subjected to statistical analysis included an independent sample t test. Differences between values were considered significant for \( P < 0.05 \). The results included three-four animals in each group.

**RESULTS**

**DC101 Inhibits Phosphorylation of VEGFR-2 and Increases Total VEGFR-2 Expression In Vivo.** Tumor angiogenesis is dependent on VEGFR-2, but the effects on VEGFR-2 expression after...
LSC-MEDIATED ANALYSIS OF ANGIOGENESIS INHIBITION

Table 1  Laser scanning cytometry-mediated analysis of total cells, microvessels, apoptotic tumor endothelial and tumor cells, and cells positive for phosphorylated VEGFR-2 in whole sections of human tumors (transition cell carcinoma) of the bladder treated with DC101

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total cells</th>
<th>CD31+ vessels</th>
<th>CD105+ vessels</th>
<th>CD31+/TUNEL+</th>
<th>CD31−/TUNEL+</th>
<th>CD105+/TUNEL+</th>
<th>CD31+/p-VEGFR-2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>150,495 ± 10,185</td>
<td>13.212 ± 1,178</td>
<td>5.325 ± 977</td>
<td>0.25% ± 0.07</td>
<td>1% ± 0.28</td>
<td>13.55% ± 0.91</td>
<td>49.8% ± 3.39</td>
</tr>
<tr>
<td>DC101</td>
<td>69,221 ± 7,000a</td>
<td>3.707 ± 178a</td>
<td>3.083 ± 1,361</td>
<td>3.7% ± 0.42a</td>
<td>7.75% ± 1.9a</td>
<td>5.85% ± 0.21a</td>
<td>22.05% ± 2.6a</td>
</tr>
</tbody>
</table>

VEGFR-2, vascular endothelial growth factor receptor-2; TUNEL, TdT-mediated nick end labeling.

We next compared total VEGFR-2 levels in control with DC101-treated tumors by immunofluorescence staining (Fig. 2A). LSC analysis of whole-tumor cross-sections revealed that DC101 induced a significant increase in expression of total VEGFR-2 (Fig. 2B, note peak shift to the right), demonstrating that the observed decreases in phosphorylated VEGFR-2 were not attributable to reductions in total receptor levels.

Previous studies have suggested that tumor angiogenesis is more active at the tumor periphery than the tumor core (28–30). LSC-generated tumor tissue maps revealed that baseline levels of phosphorylated VEGFR-2 were highest in the tumor periphery and that DC101 produced the most dramatic inhibition of VEGFR-2 phosphorylation within these regions (Fig. 3; Table 2). In contrast, total VEGFR-2 was localized throughout the tumor at baseline, and the DC101-induced increase in VEGF receptor expression occurred primarily within the tumor core (Fig. 3; Table 2).

Effects of DC101 on the Tumor Vasculature. The patterns of baseline VEGFR-2 phosphorylation observed in the 253J-BV tumors suggested to us that DC101 might preferentially target tumor microvessels located in the tumor periphery. To directly test this possi-
Fig. 3. Effects of DC101 on locoregional expression of phosphorylated and total VEGFR-2. Tissues stained in Figs. 1 and 2 were subsequently scanned by LSC to generate an X and Y coordinate position of endothelial cells positive for either phosphorylated VEGFR-2 (top row) or total VEGFR-2 (bottom row) within whole tumor cross-sections. Note that constitutive expression of phosphorylated VEGFR-2 appears most intense at the periphery of untreated tumors. The number of cells positive for phosphorylated-VEGFR-2 decreases after DC101 therapy compared with untreated tumors, indicated by the decrease in the density of pixels. In contrast, DC101-induced increase expression of total VEGFR-2 occurred within the tumor core. Laser scanning cytometry-generated tissue maps are representative of observations made with three independent tumors. VEGFR-2, vascular endothelial growth factor receptor-2.

Table 1. Loco-regional effects of DC101 on angiogenesis-related marker expression.

<table>
<thead>
<tr>
<th>Treatment group/region</th>
<th>HIF-1α</th>
<th>VEGFR-2</th>
<th>p-VEGFR-2</th>
<th>CD31</th>
<th>CD105</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Periphery</td>
<td>92.95% ± 0.63</td>
<td>92.58% ± 1.0</td>
<td>89.92% ± 0.8</td>
<td>82.19% ± 0.6</td>
<td>87.03% ± 0.1</td>
</tr>
<tr>
<td>Core</td>
<td>7.49% ± 0.44</td>
<td>7.42% ± 0.7</td>
<td>10.08% ± 0.5</td>
<td>17.81% ± 0.4</td>
<td>12.97% ± 0.1</td>
</tr>
<tr>
<td>DC101 Periphery</td>
<td>79.89% ± 1.0</td>
<td>79.22% ± 6.0</td>
<td>73.94% ± 0.2</td>
<td>66.69% ± 2.0</td>
<td>79.55% ± 6.5</td>
</tr>
<tr>
<td>Core</td>
<td>20.11% ± 1.00</td>
<td>20.78% ± 4.00</td>
<td>26.06% ± 0.24</td>
<td>33.31 ± 2.0</td>
<td>23.36% ± 1.7</td>
</tr>
</tbody>
</table>

VEGFR-2, vascular endothelial growth factor receptor-2; HIF-1α, hypoxia-inducible factor-1α.

The table summarizes the expression levels of HIF-1α, VEGFR-2, p-VEGFR-2, CD31, and CD105 in the periphery and core of control and DC101-treated tumors. The data are expressed as percentages of positive cells located within each region. DC101 treatment significantly decreased overall MVD by 39% compared with controls when microvessel densities were normalized for reductions in total cells (P < 0.001, Table 1). Furthermore, the LSC-generated MVD contour map revealed that the vascular targeting was most prominent in the tumor periphery (Fig. 4B). We then used the MVD contour map to compare the changes in vessel density with tumor morphology (detected by H&E staining; Fig. 4C, I and II correspond with the panels in Fig. 4B). Total cellular density appeared to be reduced in the tumor stroma, most notably in the tumor periphery of DC101-treated tumors.

Visual inspection of the anti-CD31-stained sections suggested that DC101 preferentially affected smaller blood vessels, sparing the blood vessels that were larger and more elongated (note vessel structures in Figs. 1B and 4A). To directly measure this effect, we used the LSC to measure the area and perimeter of the microvessels within whole-tumor sections. A rightward shift in the mean area/perimeter ratio of microvessels indicated that DC101 treatment inhibited the formation of small vessels and did not affect larger vessels (Fig. 4D).

It has been suggested that the marker endoglin (CD105) can be used to distinguish angiogenic tumor blood vessels from already established vessels (19). We therefore stained tumor sections with an antibody specific for CD105 to measure the effects of DC101 therapy on these vessels. Contour map analyses revealed that CD105-positive vessels were concentrated around the tumor periphery in control tumors (Fig. 5; Table 2). Strikingly, however, the CD105-positive vessels largely persisted in the extreme periphery of tumors harvested from DC101-treated mice, and a new concentration of CD105-positive vessels accumulated within the tumor core (Fig. 5; Table 2). Thus, DC101 therapy did not result in a significant overall decrease in MVD within the CD105-positive subset when microvessel densities were normalized for reductions in total cells (Table 1).

Locoregional Effects of DC101 on Apoptosis. To obtain more precise information about the effects of anti-VEGFR-2-targeted therapy on endothelial cell apoptosis, we stained tumor sections with antibodies to either CD31 or CD105 and fluorescent TUNEL to
Fig. 4. Effects of DC101 on tumor vasculature. Tissues were stained with anti-CD31 or anti-CD105 antibody, and microvessels were identified by contouring secondary antigen fluorescence by LSC. A, representative LSC-generated scanned images of control (left) and DC101-treated (right) whole tumor cross-sections (×200). The LSC-guided contour appears as a red perimeter surrounding each microvessel. B, whole tumor cross-section contour maps of microvessel locoregional distribution within representative control and DC101-treated tumors. Each microvessel detected by LSC was plotted on an x and y coordinate position map as a single pixel. The red gate within the center of each tumor separates the tumor core from the periphery. Note the overall significant decrease in microvessel density (39%), notably in the periphery, of DC101-treated tumors compared with controls. Roman numerals correspond to regions visualized by H&E staining in Fig. 4C. C, representative histological and morphological characteristics of control and DC101-treated tumors as assessed by H&E staining. Note the decreased cellular density in the periphery of DC101-treated tumors. D, Effects of DC101 on individual tumor microvessel size. The mean area/perimeter ratios (X axis) of all vessels (Y axis) were calculated by LSC as a measure of vessel size. Note rightward shift in DC101-treated tumors (red) compared with controls (black), indicating increased mean microvessel size after therapy. LSC, laser scanning cytometry.
DC101 (Fig. 6A) did not increase in the CD105-positive subset after therapy with cell death in the CD105-positive vessels (13.55%) than in the CD31-negative subset of endothelial cells revealed much higher baseline levels of HIF-1α. The methods performed on three independent tumors.

Effects of DC101 on Expression of HIF-1α. The transcription factor HIF-1α is an oxygen-sensitive protein that is stabilized and accumulates when cells are exposed to hypoxia (31, 32). We, therefore, measured locoregional changes in HIF-1α expression using LSC analysis to obtain a surrogate measure of relative hypoxia in the 253J-BV tumor sections. At baseline, expression of HIF-1α was low and confined to patches in the tumor core and a thin rim around the tumor periphery (Fig. 7A). After DC101 therapy, HIF-1α levels increased dramatically throughout the tumors but especially within the tumor cores (Fig. 7B; Table 2). Closer inspection of HIF-1α staining suggested that protein localization was heterogeneous with some cells displaying prominent nuclear localization and others displaying primarily cytoplasmic localization (Fig. 7C). LSC-based quantitative analyses revealed that DC101 induced a 3-fold increase in HIF-1α protein levels (Fig. 7D).

DISCUSSION

The VEGF/VEGFR-2 pathway is required for angiogenesis and neovascularization in many solid tumors (8, 10) including bladder cancer (24). These observations have prompted strong interest in developing blocking antibodies and small molecules specific for the VEGF/VEGFR-2 pathway as candidate anticancer therapeutic agents. Recent studies have shown that these inhibitors attenuate the growth and metastasis of human tumors in mice (16, 17, 33, 34). However, these studies did not characterize the effects of anti-VEGFR-2 agents on the dynamics of VEGFR-2 activation, VEGF receptor modulation, tumor cell growth, and angiogenesis within the entire tumor.

In this study, we used an innovative approach to characterize the locoregional effects of VEGFR-2 blockade on VEGFR-2 phosphorylation, MVD and vessel size, apoptosis, and the expression of the hypoxia-associated transcription factor HIF-1α. The results demonstrated that constitutive phosphorylated-VEGFR-2 levels in endothelial cells were highest around the tumor periphery and that anti-VEGFR-2 therapy preferentially reduced the number of smaller CD31-positive blood vessels located within the same region. These effects were associated with increased expression of HIF-1α and VEGFR-2, both of which were concentrated within the tumor core. Increases in apoptosis within tumor-associated endothelial cells occurred throughout the tumors but appeared to largely correlate with inhibition in VEGFR-2 phosphorylation. Together, our results strongly suggest that the primary effects of VEGFR-2 inhibition are localized to the tumor periphery but that they cause secondary changes, including hypoxia, increased VEGFR-2 expression, and tumor cell death, most notably within the tumor core. Our data are consistent with recent studies demonstrating increases in blood volume in tumors treated with these agents (35) and a larger body of work suggesting that angiogenesis is most active in the leading edge (i.e., periphery) of tumors (28–30).

Although we expected that DC101 would reduce levels of phosphorylated VEGFR-2 in the 253J-BV tumors, the observation that total VEGFR-2 levels increased after therapy was unanticipated. A trivial explanation for the effect could be that the antibody we used to detect total VEGFR-2 reacts more strongly with the unphosphorylated receptor than it does with the phosphorylated receptor, perhaps because one of the phosphorylation sites interferes with the epitope of the antibody. However, we observed the strongest increases in VEGFR-2 within the tumor core, whereas levels of baseline phosphorylated-VEGFR-2 were highest in the periphery, arguing against this explanation. Alternatively, the increases in VEGFR-2 could represent an adaptive response to the central hypoxia stimulated by DC101 therapy. Growth regulatory pathways are often controlled by feedback loops that reduce the expression of pathway components when signal transduction is active and increase levels when signaling is inactive. The DC101-induced increases in VEGFR-2 expression were associated with strong up-regulation of HIF-1α, indicating that induction of VEGFR-2 occurred preferentially within regions of relative hypoxia (i.e., in the tumor core). Previous work has demonstrated that VEGF plays a role in the paracrine stimulation of angiogenesis (36, 37) but may also act via an autoregulatory pathway activated when VEGF signal transduction is interrupted (38). In the orthotopic 253J-BV tumor model, up-regulation of VEGFR-2 could serve to rapidly reestablish the vascular network once therapy is terminated.

The recognition that tumor microvessels display marked structural and functional heterogeneity has stimulated a search for proteins that might serve to identify and target specific subsets of tumor-associated endothelial cells. Endoglin (CD105) is a 180-kDa hypoxia-induced, proliferation-associated cell surface glycoprotein, expressed exclusively by vascular endothelial cells, that has recently been advanced as one such marker (19). The protein binds to and regulates the signaling activity of transforming growth factor-β (39), a cytokine that has been broadly implicated in angiogenesis (40). Endoglin-deficient mice die during embryonic development because of defects in angiogenesis (41, 42), confirming the central role of the molecule in the process. Other recent studies suggest that CD105 is a more informative marker for tumor angiogenesis than are pan endothelial markers such as CD31 or CD34 (18, 22, 43).
Analysis of CD105 expression in control 253J-BV tumors confirmed that high-level expression was concentrated in the tumor periphery, a region that also contained the highest concentration of phosphorylated-VEGFR-2 and smaller blood vessels, presumably because of active angiogenesis at the leading edge of the tumor. Therefore, we expected that DC101 therapy would produce stronger effects in the CD105-positive vessels than in CD105-negative/CD31-positive vessels. Surprisingly, we found the opposite to be true, in that MVD as measured by CD105-positive staining did not decrease, and CD105-positive cells did not display increases in apoptosis. Further-

Fig. 6. Effects of DC101 on apoptosis. A, representative images of immunofluorescence anti-CD31 or anti-CD105 (red) and TUNEL (green) staining in DC101-treated tumors. Note that more CD31-positive endothelial cells are apoptotic compared with the CD105-positive subset (CD31 or CD105 (red) + TUNEL (green) = yellow). TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling. B, tissues were scanned by LSC to generate contour maps of the intratumoral distribution of apoptotic endothelial cells (CD31+/TUNEL+, blue pixels) and apoptotic tumor cells (CD31+/TUNEL+, yellow pixels) within whole tumor cross-sections (black pixels represent total cell nuclei). Superimposing CD31+/TUNEL+ cells and CD31+/TUNEL+ cells reveals that DC101-induced apoptosis is heterogeneous, with clusters of dead CD31-positive cells in the tumor periphery. Note the increase in blue and yellow pixels in the DC101-treated tumors (bottom panel) compared with the controls (top panel). Tissue maps are representative of observations in three independent tumors. LSC, laser scanning cytometry. C, LSC-mediated quantification of apoptotic cells in whole tumor sections. Tissues stained for anti-CD31 or anti-CD105 and TUNEL were subsequently scanned by LSC to generate scattergrams. Each cell nucleus was analyzed by LSC and plotted on a scattergram relative to the fluorescent intensity of each antigen (CD31 or CD105/red or TUNEL/green). Gates were set based on the intensity of negative staining controls. Note the increase in apoptotic tumor cells (quadrant 1, yellow) and endothelial cells (quadrant 2, blue) after DC101 therapy compared with controls. Scattergrams are representative of observations in three independent tumors.
more, CD105-positive vessels appeared to accumulate within tumor cores after therapy in parallel with increased VEGF-R-2 expression. The most straightforward explanation for these observations is that both events were driven by increased hypoxia produced by therapy. That CD105-positive vessels are relatively refractory to VEGF-R-2-directed therapy suggests that they may arise and persist via a VEGF-insensitive mechanism. Additional effort is required to functionally characterize these and other vessel subsets in tumors treated with antivascular agents.

Recent studies have used two-color immunofluorescence detection of CD31 staining and DNA fragmentation (by TUNEL) to identify dying endothelial cells in tumors treated with antangiogenic agents (16, 17, 44–46). Accurate manual quantification of these events may be impossible in a practical sense. Baseline levels of endothelial and tumor cell apoptosis in the 253J B-V tumors were 0.3 and 1.1%, respectively (Table 1), and these cells were scattered heterogeneously throughout the tumor (Fig. 6B). Therefore, even a large (5–10-fold) increase in cell death would be difficult to detect using conventional strategies. Not only is the LSC able to detect smaller increases in fluorescence, it is also capable of rapidly analyzing many events, allowing for the detection of even modest changes in target levels (47, 48). Our ongoing efforts are aimed at determining whether the assays described here provide useful information about biological response in biopsies obtained before and after anticancer therapy (27, 47–49).

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