Advances in Brief

Treatment of Human Metastatic Transitional Cell Carcinoma of the Bladder in a Murine Model with the Anti-Vascular Endothelial Growth Factor Receptor Monoclonal Antibody DC101 and Paclitaxel

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Abstract

Vascular endothelial growth factor (VEGF) regulates angiogenesis and metastasis of bladder cancer (transitional cell carcinoma, TCC) through binding to VEGF receptor-2 (VEGFR-2). In this study, we evaluated whether the anti-VEGFR monoclonal antibody (Mab) DC101 in combination with paclitaxel inhibited tumorigenesis, angiogenesis, and metastasis of human TCC growing within the bladder of athymic nude mice. In vivo therapy with Mab DC101 and paclitaxel induced significant regression of bladder tumors compared with either agent alone. Median bladder weights were reduced from 601 mg in untreated controls, 422 mg in mice treated with paclitaxel alone (P < 0.005), 361 mg in mice treated with DC101 alone (P < 0.005), and 113 mg in mice that received combination therapy (P < 0.0005). Only one of nine mice developed spontaneous lymph node metastasis after combined treatment, compared with seven of seven untreated controls (P < 0.0005), six of eight after DC101 (P < 0.01), and five of eight mice after paclitaxel (P < 0.05). Combined treatment with both paclitaxel and DC101 inhibited tumor-induced neovascularization compared with all other groups (P < 0.005), without altering the expression of VEGF or flk1. Mab DC101 and paclitaxel combined enhanced apoptosis in the tumor and endothelial cells compared with other treatment (P < 0.005). These studies indicate that Mab DC101, which blocks VEGFR-2 function, has significant efficacy against human TCC, especially when combined with the chemotherapeutic agent paclitaxel. The antitumor effect was mediated by inhibition of angiogenesis and induction of both tumor cell and endothelial cell apoptosis.

Introduction

Tumor growth, invasion, and subsequent metastasis depend upon the establishment of an adequate blood supply through the process of angiogenesis (1). This process is regulated by the balance between stimulatory and inhibitory factors released by both tumor and host cells (2–4). A number of angiogenesis factors are expressed by the highly vascular TCC3 of the bladder (3), including bFGF and acidic FGF (5, 6), IL-8 (7), midkine (8), hepatocyte growth factor (9, 10), thymidine phosphorylase (11, 12), transforming growth factor-α (13, 14), and VEGF (15–18). The level of VEGF expression by human TCC directly correlates with tumor progression (15–18) so that the VEGF signaling pathway is a suitable therapeutic target for TCC. VEGF regulates angiogenesis after binding to its type I (flt) or II (flk1/KDR) receptor, both of which are relatively endothelial cell-specific transmembrane tyrosine kinases. VEGF-mediated signaling pathways mediate endothelial proliferation, migration, morphogenesis, and differentiation (19–30).

Previous studies have found that down-regulation of VEGF expression or VEGFR signaling pathways by neutralizing antibodies to VEGF, antisense VEGF constructs, dominant-negative VEGFRs, or tyrosine kinase inhibitors of VEGFR inhibited the growth and metastasis of human malignancies (31–38). Systemic therapy with Mab DC101, an inhibitor of the murine VEGF-R2, inhibited the growth of human epidermoid, renal, pancreatic, and glioblastoma xenografts growing within the subcutis of athymic nude mice (39).

In the present study, we evaluated the therapeutic effect of the Mab DC101 in combination with paclitaxel against established metastatic TCC growing orthotopically within the bladder of athymic nude mice. Because TCC overexpresses VEGF and tumor endothelial cells overexpressed VEGFR (15–18), we hypothesized that blockade of this receptor in combination with systemic chemotherapy provides a novel approach to the anti-

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3 The abbreviations used are: TCC, transitional cell carcinoma; VEGF, vascular endothelial cell growth factor; VEGFR, vascular endothelial cell growth factor receptor; bFGF, basic fibroblast growth factor; IL, interleukin; Mab, monoclonal antibody; MMP-9, matrix metalloproteinase type 9; IHC, immunohistochemical staining; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PCNA, proliferating cell nuclear antigen; ERK, extracellular signal regulated kinase.
angiogenic treatment of human TCC which, to this point, has not been adequately explored.

**Materials and Methods**

**Cell Lines and Culture.** The highly metastatic human bladder carcinoma cell line 253J-B-V was grown as a monolayer in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, nonessential amino acids, and penicillin-streptomycin (40).

**Reagents.** The monoclonal rat antimouse VEGFR-2 antibody DC101 was generously provided by ImClone Systems, Inc. (New York, NY; Refs. 38 and 39). Paclitaxel was purchased from Bristol-Myers Squibb Co. (Princeton, NJ).

**Animals.** Male athymic BALB/c nude mice were obtained 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.

**Orthotopic Implantation of Tumor Cells.** Cultured 253J-BV cells (60–70% confluent) were prepared for injection as described previously (41). Mice were anesthetized with methoxyflurane. A lower midline incision was then made, and viable tumor cells in HBSS (1 × 10^6/0.05 ml) were orthotopically implanted into the bladder wall on day 0. Formation of a bulla indicated a satisfactory injection. The bladder was returned to the abdominal cavity, and the abdominal wall was closed with a single layer of metal clips.

**In Vivo Therapy of Human TCC Growing in the Bladders of Athymic Nude Mice.** Treatment commenced 21 days after tumor implantation. Tumors were harvested from one group of controls at the time therapy commenced; the remaining control and experimental mice were killed and necropsied 5 weeks later.

![Study design. Treatment commenced 21 days after tumor implantation. Mice were randomly separated into four groups and treated for 4 weeks according to the schedule (paclitaxel: ↓, DC101: ↓)].

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In Situ mRNA Hybridization Analysis. Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts based on published reports of the cDNA sequence: VEGF/vascular permeability factor (42); bFGF (41); IL-8 (43); and MMP-9 (44). The specificity of the oligonucleotide sequence was initially determined by a GenBank European Molecular Biology Library database and was also confirmed by Northern blot analysis (45). A poly d(T)20 oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesized with six biotin molecules and were reconstituted to
1 μg/μl in a stock solution containing 10 mM Tris (pH 7.6) and 1 mM EDTA.

**In situ** mRNA hybridization was performed as described previously, with minor modifications (46, 47). In **in situ** hybridization was performed using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 48). Tissue sections (4 μm) were dewaxed and rehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was performed for a total of 80 min at 45°C, incubated with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed with alkaline phosphatase enhancer for 1 min, and then incubated with a chromogen substrate for 15 min at 45°C.

**Quantification of Color Reaction.** Stained sections were examined using a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip, charge-coupled device color camera (model DXC-969 MD; Sony Corp., Tokyo, Japan). The images were analyzed using Optimas image analysis software (version 4.10; Media Cybernetics, Silver Spring, MD). Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and an image analyzer using the Optimas software program (Media Cybernetics). Three different areas in each sample were quantified to yield an average measurement of intensity of immunostaining. The results are presented relative to the value for the control group, which was set at 100 (49).

**Quantification of Microvessel Density.** Microvessel density was determined by light microscopy after immunostaining of sections with anti-CD31 antibodies using the procedure of Weidner et al. (51). Microvessel density is expressed as the average of the five highest areas identified within a single ×200 field.

**TUNEL assay.** For the TUNEL assay, tissue sections (5 μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized and treated with a 1:500 proteinase K solution (20 μg/ml) for 15 min, and endogenous peroxidase was blocked by using of 3% hydrogen peroxide for 12 min. The samples were incubated for 10 min at room temperature with terminal deoxynucleotidyl transferase and biotin-16-dUTP. After counterstaining with peroxidase-conjugated streptavidin, the slides were incubated with diaminobenzidine, counterstained with Gill’s hematoxylin, and then mounted.

**Quantification of Cell Proliferation and Apoptosis.** Cell proliferation and apoptosis were determined by light microscopy after immunostaining of sections with anti-PCNA and TUNEL assay. The density of proliferative cells and apoptotic cells is expressed as the average of the five highest areas identified within a single ×200 field.

**Immunofluorescence Double Staining of Apoptotic Endothelial Cells.** For immunohistochemical analysis, frozen tissue sections (8 μm thick) were fixed with cold acetone. The samples were washed three times with PBS and incubated for 20 min at room temperature with a protein-blocking solution containing PBS (pH 7.5), 5% normal horse serum, and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with a 1:400 dilution of rat monoclonal anti-CD31 antibody (50, a 1:500 dilution of rabbit polyclonal anti-VEGF/vascular permeability factor antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a 1:500 dilution of rabbit polyclonal anti-bFGF antibody (Sigma Chemical Co., St. Louis, MO), a 1:50 dilution of a rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA), a 1:100 dilution of mouse monoclonal anti-MMP-9 antibody (Oncogene Research Products, Cambridge, MA), a 1:100 dilution of mouse monoclonal anti-PCNA antibody (Dako, Copenhagen, Denmark), or a 1:100 dilution of mouse monoclonal anti-flk1 antibody (Transduction Laboratories, Lexington, KY). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of the secondary antibody. The slides were rinsed with PBS and incubated for 5 min with dianmobilisinde. The sections were then counterstained with Gill’s hematoxylin and mounted. **Quantification of Immunostaining Intensity.** The intensity of the immunostaining of VEGF, bFGF, IL-8, MMP-9, and flk1 was measured in three different areas of each sample by an image analyzer using the Optimas software program (Media Cybernetics). Three different areas in each sample were quantified to yield an average measurement of intensity of immunostaining. The results are presented relative to the value for the control group, which was set at 100 (49).
The sections were then washed two times with PBS. TUNEL assay was performed using a commercial kit according to the manufacturer’s protocol (Promega Corp., Madison, WI). The tissue sections were fixed in 4% paraformaldehyde at room temperature for 15 min. The tissue sections were permeabilized by incubating with 0.5% Triton X-100 in PBS for 5 min at room temperature. The slides were rinsed two times with PBS for 5 min each time and then incubated with equilibration buffer for 10 min. The equilibration buffer was then removed, and reaction buffer containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme was then added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h in the dark. EDTA was added to the slides for 5 min to stop the reaction.

To identify all cell nuclei, the slides were incubated with a 1:2000 dilution of Hoechst (300 μg/ml) at room temperature for 10 min. The slides were examined under a microscope (Inverted System IX70; Olympus, Melville, NY), and images were captured using a digital camera. When this procedure is used, the endothelial cells of CD31-positive microvessels are indicated by localized red fluorescence. All cell nuclei are indicated by blue fluorescence. The proportion of apoptotic endothelial cells is expressed as the ratio of apoptotic endothelial cells to the total number of endothelial cells in 10 random 0.011-mm² fields at 3400.

### Statistical Analysis

The statistical differences for the number of vessels, proliferative cells, and apoptotic cells and for staining intensity for VEGF, bFGF, IL-8, MMP-9, and flk1 within the bladder tumors were analyzed by the Mann-Whitney test. The incidences of tumor and metastasis were analyzed by the χ² test. A value of P < 0.05 was considered significant.

### Results

#### Inhibition of Growth and Metastasis of Established Human TCC

Therapy with DC101 and paclitaxel together was significantly more effective at reducing bladder tumors than was single-agent treatment with either paclitaxel or DC101 (Table 1). Combination therapy also resulted in significantly lower spontaneous lymph node metastasis (only one mouse thus treated had metastasis) compared with treatment with either paclitaxel or DC101 (P < 0.001). This is a representative experiment of two.

#### Table 2 In vivo mRNA and protein expression levels and microvessel density in athymic nude mice treated with paclitaxel alone, Mab DC101 alone, or both after orthotopic implantation of human TCC of the bladder

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>mRNA expression index</th>
<th>Protein expression index</th>
<th>Microvessel density (per ×200 field)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bFGF</td>
<td>VEGF</td>
<td>IL-8</td>
</tr>
<tr>
<td>Control (PBS)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Paclitaxel only</td>
<td>103</td>
<td>103</td>
<td>103</td>
</tr>
<tr>
<td>DC101 only</td>
<td>102</td>
<td>101</td>
<td>97</td>
</tr>
<tr>
<td>Paclitaxel plus DC101</td>
<td>103</td>
<td>103</td>
<td>94</td>
</tr>
</tbody>
</table>

* The intensity of the cytoplasmic color reaction was measured by an image analyzer and compared with the maximal intensity of poly d(T) color reaction in each sample. Results for each cell line are expressed relative to the result for the control group (defined as 100).

* The intensity of the cytoplasmic immunostaining was measured by an image analyzer in three different areas of each sample to yield an average measurement. Results for each cell are expressed relative to the result for the control group (defined as 100).

* Microvessel density was expressed as the average of the five highest areas identified within a single ×200 field.

* P > 0.05 against CTRL and paclitaxel.

* P < 0.005 against CTRL, paclitaxel, and DC101 (Mann-Whitney statistical comparison).

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Fig. 2 Long-term survival after treatment with paclitaxel and DC101. Mice were treated by the schedule shown in Fig. 1, except that treatment was maintained until the mice became moribund. A Kaplan-Meier survival curve demonstrates that survival was significantly enhanced in mice treated with paclitaxel and DC101 (P < 0.001). This is a representative experiment of two.
course of therapy. Seven of 10 animals treated with both paclitaxel and DC101 were alive with both primary tumor and metastases at 132 days after initiation of therapy, whereas all control animals became moribund, and all were necropsied by day 53 (Fig. 2).

**Inhibition of VEGF, bFGF, IL-8, MMP-9, and flk1 Expression and Microvessel Density after flk1/KDR Blockade and Paclitaxel.** The expressions of VEGF, bFGF, IL-8, and MMP-9 mRNA and protein were analyzed by in situ hybridization and IHC, respectively; flk1 was analyzed by IHC (Table 2). Compared with results for the control group, mRNA and protein expressions of VEGF, bFGF, IL-8, and MMP-9 were not significantly altered in any of the three experimental groups.

However, microvessel density was significantly less in tumors of mice treated with Mab DC101 alone or with both Mab DC101 and paclitaxel compared with controls. Paclitaxel alone had no significant effect on microvessel density (Fig. 3). The expression of flk1 was not affected by treatment.

**Enhancement of Apoptosis and Inhibition of Proliferation by Treatment with DC101 and Paclitaxel.** We evaluated the effect of Mab DC101 and paclitaxel on cellular proliferation and apoptosis by PCNA and TUNEL assays, respectively (Table 3; Fig. 3). The number of PCNA-positive cancer cells counted per $\times 200$ field was significantly lower after treatment with either paclitaxel alone or Mab DC101 alone, respectively ($P < 0.005$).

![Fig. 3](image) The expression of proliferation, microvessel density, and apoptosis was analyzed by IHC with anti-PCNA antibody, anti-CD31 antibody, and TUNEL assay. Apoptosis of endothelial cells was determined after double labeling immunofluorescence. The combination of Mab DC101 and paclitaxel significantly inhibited proliferation (PCNA staining), compared with each agent alone. The number of CD-31+ microvessels counted per $\times 200$ field in the bladder tumors was significantly lower in tumors treated with Mab DC101 alone or in combination with paclitaxel than in control tumors. Paclitaxel alone had no significant effect on MVD. The number of apoptotic cancer cells counted per $\times 200$ field was significantly increased after therapy with Mab DC101 and paclitaxel. The combination of Mab DC101 and paclitaxel significantly enhanced apoptosis. The expression of apoptosis of endothelial cells was analyzed by immunofluorescence double staining of endothelial cells (CD-31) and apoptotic cells (TUNEL). Endothelial cells are detected by localized red fluorescence, whereas green fluorescence is detected within the nuclei of apoptotic cells. Double labeling of endothelial cells undergoing apoptosis results in localized yellow fluorescence.
Therapy of Bladder Cancer with DC101 and Paclitaxel

**Table 1** In vivo apoptosis and PCNA indices for implanted 253J B-V cells from athymic nude mice treated with paclitaxel alone, Mab DC101 alone, or both after orthotopic implantation of human TCC of the bladder

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Apoptotic index</th>
<th>PCNA index</th>
<th>Apoptosis: PCNA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>4 ± 2 (2–6)</td>
<td>224 ± 55 (169–299)</td>
<td>1.8</td>
</tr>
<tr>
<td>Paclitaxel only</td>
<td>11 ± 4 (7–15)</td>
<td>84 ± 19a</td>
<td>(66–111) 13.1c</td>
</tr>
<tr>
<td>DC101 only</td>
<td>15 ± 5 (9–22)</td>
<td>65 ± 17c</td>
<td>(42–88) 23.1d</td>
</tr>
<tr>
<td>Paclitaxel plus DC101</td>
<td>26 ± 7 (19–36)</td>
<td>29 ± 9 (22–42)</td>
<td>89.7d</td>
</tr>
</tbody>
</table>

* The density of apoptosis in cancer cells by TUNEL assay and cell proliferation by IHC with PCNA antibody was expressed as the average of the five highest areas identified within a single ×200 field. Values are mean ± SD (with range in parentheses) per ×200 field.

**Discussion**

Although TCC of the bladder is a chemosensitive tumor, most deaths from bladder cancer are caused by metastases that resist conventional chemotherapy (52–54). Most patients with advanced TCC will have an initial response to chemotherapy, but chemoresistant disease rapidly ensues (53–55). Therefore, new chemotherapeutic strategies must be developed if we are to improve the outcome for patients with advanced bladder cancer.

Recently, it has been appreciated that biological agents such as IFN-α, epidermal growth factor, and Her-2/neu receptor antagonists (49, 56, 57) modulate host responses and enhance the efficacy of standard cytoreductive chemotherapy. The anti-EGFR Mab C225 (58–61) has significant antitumor effect against TCC of the bladder when combined with paclitaxel, a chemotherapeutic agent that is active against advanced human TCC (62–65). Paclitaxel inhibits cell replication by enhancing polymerization of tubulin monomers into stabilized microtubule bundles that cannot reorganize into the proper structures for mitosis (62–68). This results in cell cycle blockade in mitosis and subsequent activation of an apoptotic pathway (64, 65). When combined, Mab C225 and paclitaxel enhanced apoptosis within tumor cells and down-regulated tumor-induced neovascularization.

Human TCC is characterized by an overexpression of VEGF relative to normal urothelium (15–18). The blockade of the VEGF/VEGFR signaling pathway by VEGF-neutralizing antibodies or by VEGF antisense or dominant-negative VEGF receptors has been shown repeatedly in all cases to significantly inhibit tumor growth and metastasis (31–33, 36–39). Various antibodies directed to the extracellular domain of the VEGFR type II have been developed (35, 37–39). These agents block the binding of VEGF to its receptor, inhibit VEGF-induced signaling in endothelial cells, and inhibit the growth of human tumors in mice by an antiangiogenic mechanism (35, 37–39).

In the present study, we observed that treatment of human TCC growing within the bladder of athymic nude mice with Mab DC101, which targets the VEGFR-2 in combination with paclitaxel, enhanced apoptosis and inhibited tumorigenesis, angiogenesis, and metastasis. The survival of mice was also prolonged by Mab DC101 and paclitaxel.

The mechanisms responsible for the interaction between Mab DC101 and paclitaxel are unclear but likely involve an increase in apoptosis within endothelial cells coupled with a decrease in proliferation. The enhanced apoptosis represents the summation of effects on separate pathways regulating apoptosis.
(69, 70). Paclitaxel has a significant antitumor effect for TCC of the bladder, either as a single agent or in combination with other cytotoxic agents. Paclitaxel increases microtubule stability by preventing tubulin depolymerization, leading to tubulin bundling (71). These cytoskeleton changes result in cell cycle arrest and apoptosis within 20 h of paclitaxel exposure (72). VEGF signaling inhibits apoptotic cell death in endothelial cells by several mechanisms, including the induction of the antia apoptotic proteins Bcl-2 (73, 74) and MCL1 (75), phosphorylation of phosphatidylinositol 3-kinase (76–78), the phosphorylation of ERK1 and ERK2 (79), and inhibition of stress-activated protein kinase/c-Jun-NH2-kinase (80). The antia apoptotic effect of VEGF was reversed in capillaries by flk1/KDR fusion proteins (36, 81) and by mitogen-activated protein kinase/ERK inhibitors (79, 82).

The results of the present study are in accordance with these previous observations. We observed a significant increase in apoptotic death of both tumor and endothelial cells after treatment with Mab DC101 and paclitaxel. Apoptotic tumor cells were seen clustered around apoptotic endothelial cells, suggesting an interaction between paclitaxel and relative hypoxia within the tumor caused by the regression of the neovascularure. Alternatively, it has been shown that VEGF signaling inhibits apoptosis induced by chemotherapeutics, thereby possibly rendering these agents relatively ineffective (75). Mab DC101 may enhance the effects of paclitaxel by overcoming this VEGF-induced inhibition of apoptosis (83, 84). Simultaneously, cellular proliferation as measured by PCNA was maximally inhibited by therapy with Mab DC101 and paclitaxel. The antiproliferative effect of therapy was maximized by treatment with paclitaxel followed by Mab DC101.

In summary, our experiments demonstrate that paclitaxel enhances the antitumor effect of Mab DC101 against metastatic human TCC growing within the bladder of athymic nude mice and improved the survival of mice with metastatic human TCC growing within their bladders. The improved response to this strategy seems to result from enhancement in the induction of apoptosis by paclitaxel and Mab DC101. The results of this preclinical study justify the clinical application of this novel form of combination therapy as treatment of advanced bladder cancer.

References
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