Anti-epidermal Growth Factor Receptor Antibody C225 Inhibits Angiogenesis in Human Transitional Cell Carcinoma Growing Orthotopically in Nude Mice

Paul Perrotte, Takashi Matsumoto, Keiji Inoue, Hiroki Kuniyasu, Beryl Y. Eve, Daniel J. Hicklin, Robert Radinsky, and Colin P. N. Dinney

Introduction

Angiogenesis is crucial for the growth and metastasis of human TCC (1–4). Human TCC overexpresses the angiogenic factors bFGF, VEGF, and platelet-derived endothelial growth factor (PDGF), which are important in modulating tumor-host interactions that result in tumor neovascularization. Urine from patients with bladder cancer stimulates endothelial cell proliferation and migration (5, 6). Furthermore, the prognosis of patients with advanced TCC correlates with microvessel density (7). EGFR-mediated signaling mechanisms are also fundamental to the tumorigenicity of human TCC and compose an important pathway regulating cell division. The level of expression of EGFR correlates with stage, grade, and progression of human TCC (7–16).

EGFR signaling and angiogenesis have been independently evaluated as targets for therapy, but the link between has only recently been identified (17–24). Both EGF and TGF-α, which are ligands for EGFR, induce angiogenesis. Therefore, we hypothesized that down-regulating EGFR signaling pathways may inhibit tumor growth by inhibiting tumor-mediated angiogenesis, independent of any direct cytostatic effect on tumor growth. We recently reported that therapy with either protein tyrosine kinase inhibitors (20) or anti-EGFR MAb C225(4) inhibited the growth of established human TCC growing orthotopically in athymic nude mice. We now report that therapy with MAB C225 reduces TCC neovascularization by down-regulating the tumor cell expression of the angiogenic factors VEGF, IL-8, and bFGF, resulting in abrogation of tumor growth and metastasis.

Materials and Methods

Tumor Cell Line. The highly metastatic human TCC cell line 253J B-V was maintained as a monolayer in modified EMEM supplemented with 10% FBS, vitamins, sodium pyruvate, l-glutamine, and nonessential amino acids, as described previously (25).

3 The abbreviations used are: TCC, transitional cell carcinoma; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IL-8, interleukin-8; ISH, in situ hybridization; MAB, monoclonal antibody; EGF, epidermal growth factor; EGFR, EGF receptor; EMEM, Eagle’s MEM; FBS, fetal bovine serum; TGF, transforming growth factor.

Inhibitory effect of MAb C225 on 253J B-V cells grown in vitro

**Fig. 1** In vitro growth inhibition of 253J B-V cells treated with MAb C225 evaluated by [3H]thymidine incorporation assay. There is a dose-dependent inhibition of growth with maximum cytostasis of 55% at 10 µg/ml. Lines represent varying concentrations of FBS.

**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 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 United States Department of Agriculture, Department of Health and Human Services, and NIH standards.

**Antibodies.** Chimeric anti-EGFR MAb C225 was generously provided by ImClone Systems, Inc. (New York, NY). Human IgG (Sigma Biosciences, St. Louis, MO) was used as a control.

**In Vitro Therapy of Tumor Cells.** The in vitro dose-dependent antiproliferative effect of MAb C225 was evaluated by incubating 253J B-V cells for 24 h in serum-free medium, then exchanging the medium for 0%, 1%, or 10% FBS-supplemented EMEM containing increasing concentrations of MAb C225 (0.08–10 µg/ml). Growth inhibition was determined by [3H]thymidine incorporation after a 72-h incubation period. To evaluate expression of VEGF, IL-8, and bFGF after therapy with MAb C225, 5 × 10^5 cells were plated in a 96-well plate in 200 µl of EMEM supplemented with 10% FBS, then treated the next day with 1 µg/ml or 10 µg/ml of MAb C225. Ten percent FBS-supplemented EMEM without MAb C225 and EGFR-irrelevant human IgG (10 µg/ml) served as controls. After treatment, cells were counted, and both the supernatant and the cells were collected and stored at -20°C for protein quantification. To evaluate the influence of EGFR activation on the expression of the angiogenic factors, exogenous EGF (50 ng/ml) was added to stimulate EGFR signaling pathways. Cell-associated bFGF and supernatant VEGF and IL-8 protein levels were measured using the commercially available Quantine ELISA kit (R&D Systems, Inc., Minneapolis, MN). Steady-state mRNA expression was determined by Northern blot analysis.

**Northern Blot Analysis.** Polyadenylated mRNA was extracted from 10^6 cells growing in culture with the FastTrack mRNA isolation kit (Invitrogen Co., San Diego, CA). The mRNA was electrophoresed on 1% denaturing formaldehyde/agarose gel and electrotransferred to Genescreen nylon membrane (DuPont Co., Boston, MA), using a UV Stratagene 1800 cross-linked with 120,000 µJ/cm². Filters were washed at 55°C with 30 mM sodium citrate and 0.1% SDS (w/v). The membranes were then hybridized and probed for VEGF, bFGF, IL-8, and β-actin as a control. The cDNA probes used were: (a) a Jird actin 1.2-kb DNA fragment (26); (b) a 1.4-kb cDNA fragment of bovine bFGF (27); (c) a 204-bp BamHI EcoRI fragment of human VEGF cDNA (28); and (d) a 0.5-kb EcoRI cDNA fragment corresponding to human IL-8 (a gift from Dr. K. Matsushima, Kanazawa, Japan; Ref. 29). The probes were radiolabeled by a random primer technique and [α-32P]dCTP (Amersham Corp., Arlington Heights, IL). Autoradiography of the membrane was performed after washing. Densitometry scanning permitted quantitation of the bands.

**Orthotopic Implantation of Tumor Cells.** For the in vivo portion of the study, cultured 253J B-V cells (70% confluent) were prepared for injection, as previously described (25). Mice were anesthetized with methoxyflurane, a lower midline incision was made, and the bladder was exposed. Viable tumor cells (1 × 10^6/0.05 ml of HBSS) were injected into the wall of the bladder. The formation of a bulla was the sign of a satisfactory injection. The bladder was returned into the abdominal cavity, and the abdominal wall was closed in a single layer with metal clips.

**Therapy of Established Human TCC Tumors Growing in the Bladders of Athymic Nude Mice.** Treatment commenced 28 days after tumor implantation. The presence and volume of tumors were confirmed using ultrasound or palpation. At the time of therapy, tumor weights were between 200 and 400 mg. Mice were randomly separated into two groups. The first group (eight mice) was treated with 1 mg of anti-EGFR chimeric antibody MAB C225 i.p. twice a week for 5 weeks. Eight control mice received an equivalent volume of PBS on the same schedule. Treated mice were closely monitored for any signs of progressive disease and sacrificed if they became moribund. Control mice were sacrificed at or about 5 weeks after tumor implantation because they became moribund. To evaluate the temporal effects of MAB C225 on angiogenic factor expression and microvessel density, mice were sacrificed 1, 3, and 5 weeks after initiation of therapy.

**Necropsy.** Necropsy was performed, and local tumorigenicity and distant metastases (lymph node and lung nodules) were determined. The bladders were harvested, weighed, and either mechanically dissociated for in vitro culture, flash-frozen in liquid nitrogen for mRNA extraction, or cut in two and either embedded in Optimal Cutting Temperature gel (Sokera Inc., Torrence, CA) for frozen sections or fixed in 10% buffer and formalin for paraffin sections. The lymph nodes were fixed in formalin. Lungs were mechanically dissociated for reestablishment in culture to evaluate the presence of micrometastases or fixed in Bouin’s fluid for histological analysis.

**Immunohistochemical Determination of bFGF, VEGF, and IL-8.** The expression of angiogenic factors was detected in cryostat (IL-8) or paraffin sections (bFGF, VEGF) of tumors treated with either MAB C225 or PBS (30). bFGF protein expression was detected using primary rabbit anti-bFGF (Sigma Chemical Co., St. Louis, MO), which reacts with residues 147–153 and shows no cross-reaction with acidic fibroblast growth factor (50 ng/ml) served as a positive control.
factor. VEGF and IL-8 proteins were detected using primary rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1200 dilution. The α-immunoperoxidase technique for immunohistochemical staining was used with a second peroxidase-conjugated goat antirabbit antibody (IgG, F[ab]2 fragment; Jackson ImmunoResearch Laboratory, Inc., West Grove, PA; Ref. 30).

Quantification of Microvessel Density. Cryostat sections of bladder tumor were fixed with acetone and chloroform solutions for 15 min and then washed twice with PBS. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol, and the sections were washed with PBS and incubated overnight in a protein-blocking solution. The excess blocking solution was removed, and the samples were incubated with the rat antimouse CD31 antibody that stains endothelial cells (PharMingen, San Diego, CA). Swine peroxidase conjugated antirabbit antibody was applied for 30 min after the primary antibody was removed. The samples were rinsed with PBS and developed with 3-amino-9-ethylcarbazole at room temperature for 20 min. The sections were counterstained with aqueous hematoxylin. A positive reaction was indicated by a brownish precipitate (30).

Two investigators counted the microvessels independently in a blinded fashion. The tissues were examined at low power (×40), and the three ×200 fields of highest microvessel density were identified for vessel counts. The three selected fields (high-power field, ×20 objective and ×10 ocular, 0.739 mm²/field) were recorded using a computer-linked cooled CCD Optotronics Tec 470 camera (Optotronics Engineering, Goleta, CA) to ensure that both investigators would count the same

Fig. 2 A, in vitro inhibition of VEGF, IL-8, and bFGF protein production by C225 MAb. The 253J B-V cells were cultured in the presence or absence of C225. B, inhibition of VEGF, IL-8, and bFGF protein production by MAb C225 after EGF stimulation (50 ng/ml; P < 0.05). IL-8 and cell-associated bFGF were measured by ELISA. The protein production of these angiogenic factors is expressed in relation to the production of untreated cells, which was assumed to be 100%. Values are the mean ± SD of triplicate cultures and have been normalized to account for cell number.
areas within the specimens. Microvessels were quantitated according to the method described by Weidner et al. (31). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. The results were expressed as the highest number of microvessels identified within a single $3^200$ field.

**In Situ mRNA Hybridization.** Paraffin sections of fixed tissue (3–5 μm) were mounted on ProbOn slides (Fisher Scientific, Pittsburgh, PA). The slides were dewaxed and prepared, and ISH was performed using the Microprobe system (Fisher Scientific) as described previously (32, 33). Slides were rinsed three times in Tris buffer for 30 s; hybridization of the probes was then performed at 45°C for 45 min. The slides were washed with 2 $\times$ SSC three times for 2 min each time at 45°C. The samples were then incubated with avidin labeled with phosphatase for 30 min at 45°C, rinsed in 50 mM Tris buffer (pH 7.6), and then briefly (1 min) rinsed in alkaline phosphatase. The samples were then incubated with chromogen substrate for 20 min at 45°C. If necessary, additional incubation was performed with fresh chromogen to enhance a weak reaction. The samples were then covered with Universal Mount fixative (Research Genetics, Huntsville, AL), heat-dried, and examined. A positive reaction in this assay stained red. To control for endogenous alkaline phosphatase, additional samples were treated in the absence of biotinylated probe. No immunoreactivity was observed in the controls.

**Densitometry Quantification of Immunohistochemical and In Situ mRNA Hybridization.** The intensity of immunohistochemical staining and *in situ* mRNA hybridization was evaluated in five fields representing areas of highest staining intensity. Each field was evaluated using the ImageQuant analyzer and Optimas software program (Bioscan, Edmonds, WA). Immunohistochemical staining intensity of each specimen was compared with the staining intensity of the normal urothelium in the same sample and expressed as a ratio (tumor cells:normal urothelium). *In situ* mRNA hybridization quantification was performed in the same manner on corresponding sections. Normal urothelium served as the internal control for mRNA expression, and poly-dT staining controlled for mRNA preservation. Results were expressed as the ratio of the intensity of tumor ISH staining to normal urothelium staining and normalized for poly-dT expression.

**Statistical Analysis.** Bladder tumor weights were compared by the Mann-Whitney test. Expression of VEGF, IL-8, and bFGF and microvessel density quantification were compared by Student’s *t* test.

**Results**

**In Vitro Cell Growth Inhibition by MAb C225.** *In vitro* treatment of 253J B-V cells with MAb C225 resulted in dose-
dependent cytostasis, as measured by [$^3$H]thymidine incorporation assay. The maximum inhibition observed was 55% after 72 h of continuous exposure at the highest dose (10 μg/ml) in serum-free medium (Fig. 1). Treatment with MAb C225 in 10% serum-containing medium resulted in minimum cytostasis (<20%).

**Down-regulation of bFGF, VEGF, and IL-8 in Human TCC Cells after in Vitro Therapy with MAb C225.** Using an ELISA, we observed dose-dependent down-regulation of VEGF, IL-8, and bFGF after continuous exposure to MAb C225 for 48 h. Secreted VEGF and IL-8 protein production and cell-associated bFGF protein production by the 253J B-V cell line was significantly inhibited by MAb C225 at 10 μg/ml (Fig. 2A). Northern blot analysis directly correlated with the ELISA results (Fig. 3). A decrease in the steady-state gene expression of VEGF, IL-8, and bFGF was observed. After treatment, VEGF steady-state mRNA expression was reduced 2-fold, IL-8 expression was reduced 10-fold, and bFGF expression was reduced 2-fold when cells were treated with the high dose of MAb C225 at 10 μg/ml.
When the cells were treated with EGF (50 ng/ml), increased overexpression of all three angiogenic factors was observed by ELISA (Fig. 2B). Treatment of 253J B-V cells with MAb C225 after pretreatment with EGF resulted in inhibition of the production of VEGF, IL-8, and bFGF (Fig. 2B). These data support that EGFR blockade by MAb C225 in TCC cells results in decreased expression and protein production of VEGF, IL-8, and bFGF.

**Inhibition of Growth and Metastasis of Established Human TCC Tumors in the Bladders of Mice.** Therapy of established 253J B-V tumors commenced 28 days after tumor cells implantation into the bladder wall of athymic nude mice. Therapy of mice with 1 mg of MAb C225 i.p. twice a week for 5 weeks resulted in dramatic tumor regression compared with control with significant reductions in tumor weight and inhibition of metastasis (Table 1). All control mice harbored lymph node metastases, and approximately 40% demonstrated lung metastases at the time of death. In contrast, none of the MAB C225-treated mice harbored lymph node or lung metastasis at necropsy ($P < 0.005$). Median tumor weight was reduced from 834 mg in control animals to 67 mg after therapy with MAb C225. This difference in tumor weight represents tumor regression and inhibition of tumor growth because the treated tumors were smaller after completion of therapy than when therapy was initiated. In contrast, in the control group, all mice showed signs of progressive disease and became moribund and were sacrificed within 2–4 weeks of initiating therapy.

**Inhibition of bFGF, VEGF, and IL-8 Production and Microvessel Density in Vivo after EGFR Blockade.** Because treatment with MAb C225 down-regulated bFGF, VEGF, and IL-8 production by 253J B-V cells in vitro, we analyzed the potential for this agent to inhibit angiogenesis in vivo. The expression of VEGF, IL-8, and bFGF and the microvessel density of 253J B-V tumors growing in the bladders of nude mice was analyzed in both MAB C225-treated and control animals. Protein and mRNA expression of VEGF, IL-8, and bFGF was evaluated by immunohistochemistry and in situ mRNA hybridization, respectively, after 1, 3, and 5 weeks of EGFR blockade therapy and compared with expression by control tumors. Representative tumor sections analyzed for expression of mRNA of VEGF, IL-8, and bFGF using in situ mRNA hybridization are shown in Fig. 5. After completion of therapy of tumors, the intensity of the expression of VEGF, IL-8, and bFGF message as evaluated by in situ mRNA hybridization in the treated tumors was lower than in control tumors (Fig. 4). Computer image analysis of representative sections confirmed a significant reduction in the steady-state mRNA expression for bFGF and IL-8 by week 1 and VEGF by week 3 after initiation of therapy (Fig. 4, Table 2). Using immunohistochemical staining, we observed that the immunoreactivity of VEGF, IL-8, and bFGF protein in 253J B-V tumors growing in the bladders of athymic nude mice followed the same pattern of down-regulation after therapy with MAB 225 (Fig. 5, Table 3).

Microvessel density was significantly lower in tumors treated for 5 weeks with MAB C225 than in control tumors ($84 \pm 4$ versus $185 \pm 30$, $P < 0.005$). This reduction in microvessel density was not observed 1 week ($200 \pm 16$) or 3 weeks ($193 \pm 15$) after initiation of therapy. The down-regulation of VEGF, IL-8, and bFGF mRNA and protein between 1 and 5 weeks of therapy preceded the reduction in microvessel density (Tables 2, 3). This temporal sequence suggests that the inhibition in the expression of VEGF, IL-8, and bFGF is responsible for the reduction in neovascularity observed in the treated tumors. No significant difference in microvessel density or VEGF, IL-8, and bFGF expression was observed over time within the control tumors.

**Discussion**

In the studies described herein, we have identified a new mechanism that contributes to the antitumor effect of EGFR blockade therapy with MAB C225: inhibition of angiogenesis. Our data suggest that the reduction in bladder cancer vascularization is secondary to down-regulation of VEGF, IL-8, and bFGF expression by EGFR blockade therapy with MAB C225. Our data indicate that the observed antiangiogenic effect is not an artifact of the antiproliferation secondary to blockade of EGFR signaling pathways: (a) immunohistochemical staining and in situ mRNA hybridization studies demonstrate that after therapy viable bladder tumors were less vascular than control-treated tumors; and (b) the down-regulation of angiogenic factors precedes the involution of microvessels. We hypothesize that selective down-regulation of VEGF, IL-8, and bFGF by the tumor cells after MAB C225 therapy leads to involution of tumor vessels, contributing to the growth inhibition and regression of the primary tumors and, hence, reduction in spontaneous metastases from these highly metastatic tumors. This premise is strengthened by the observation of Benjamin and Keshet (34), who reported that down-regulation of VEGF in hemangioblastoma directly resulted in eventual endothelial cell apoptosis and blood vessel regression.

Previously, we reported that the EGFR tyrosine kinase inhibitor CGP 54211 inhibited the growth of 253J B-V tumors established in the bladders of athymic nude mice (20). Although the mechanism of this drug was presumed to be cytostatic, we observed fibrosis in the treated tumor consistent with some degree of cytolysis. Similarly, our results suggest that the in vivo effect of C225 is a result of cytotoxic activity as well as antiproliferative activity because in vitro maximum cytostasis was only 55% and in vivo there was actual regression of the tumors. The regression of life-threatening hemangiomas of infancy after antiangiogenic therapy with IFN-α is a clinical
example of the cytotoxic effect of angiogenesis-directed therapy (35).

The mechanisms by which EGFR signaling pathways regulate VEGF, IL-8, and bFGF are unclear, but it is established that up-regulation of these factors follows activation of the EGFR signaling pathways by EGF or TGF-α. Transcription of VEGF is potentiated by activation of the four AP-1 binding sites within its promoter, the bFGF and IL-8 promoter have one AP-1

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Fig. 5 Immunohistochemical staining of human TCC tumors growing in the bladders of athymic nude mice. MAb C225-treated tumors (after 3 weeks and 5 weeks of therapy) and control tumors were stained with CD31 antibodies directed against mouse endothelial cells (A–C). Note the increased vessel density in the control tumors (A) and the 3-week C225-treated tumors (B) compared with that of the 5-week C225-treated specimen (C). Immunostaining for VEGF (D–F), IL-8 (G–I), and bFGF (J–L) is higher in the control tumors (D, G, and J) than in the MAb C225-treated specimens (E, F, H, I, K, and L) at 3 weeks and 5 weeks. Note that the down-regulation of VEGF, IL-8, and bFGF precedes the inhibition of angiogenesis.
Inhibition of Angiogenesis in TCC by EGFR Blockade

The down-regulation of these angiogenic factors produced by the tumor restores the balance between stimulating and inhibitory factors that keeps angiogenesis dormant under normal conditions. The observation that down-regulation of the angiogenic stimulus of the tumor cells inhibits the host angiogenic response emphasizes the complexity of tumor-host interactions. These experiments demonstrate that inhibition of angiogenesis characterizes in part the antitumor effect of therapy directed at inhibiting EGFR signaling pathways in human TCC cells. Furthermore, these experiments indicate that normalization of angiogenesis-related biomarkers such as VEGF, IL-8, bFGF, or microvessel density are clinically useful to demonstrate regression or eradication of cancer after EGFR-directed therapy. Analysis of these biomarkers should be included in clinical trials for evaluating this form of therapy. Finally, combination of this approach with standard chemotherapy may provide increased benefit in patients with advanced TCC.

References


| Table 3 Immunochemical analysis of microvessel density and VEGF, IL-8, and bFGF protein production after therapy with MAb C225 |
|-----------------|----------|----------|----------|
| Microvessel count | VEGF | IL-8 | bFGF |
| Control\(a\) | 185 ± 50 | 259 ± 20 | 155 ± 29 | 90 ± 10 |
| MAb C225 | 200 ± 16 | 235 ± 62 | 83 ± 5 | 76 ± 6\(b\) |
| Week 1 | 193 ± 15 | 174 ± 30 | 21 ± 3\(c\) | 33 ± 14\(c\) |
| Week 2 | 84 ± 4\(d\) | 56 ± 15\(d\) | 34 ± 1\(d\) | 14 ± 9\(d\) |

\(a\) VEGF, IL-8, and bFGF cytoplasmic staining was evaluated by computer-assisted image analysis and is expressed as a ratio of tumor expression to normal urothelium expression.

\(b\) Control mice were sacrificed when they became moribund about 4–6 weeks after tumor implantation. No significant difference in microvessel density or VEGF, IL-8, and bFGF expression was observed in control mice sacrificed between 4 and 6 weeks.

\(c\) P < 0.05.

\(d\) P < 0.005.

\(\) P. Perrotte et al., manuscript in preparation.

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