Inhibition of Angiogenesis by the Antiepidermal Growth Factor Receptor Antibody ImClone C225 in Androgen-independent Prostate Cancer Growing Orthotopically in Nude Mice

Takashi Karashima, Paul Sweeney, Joel W. Slaton, Sun J. Kim, Daniel Kedar, Jonathan I. Izawa, Zhen Fan, Curtis Pettaway, Daniel J. Hicklin, Taro Shuin, and Colin P. N. Dinney

INTRODUCTION

In human androgen-independent prostate cancer (PCa), epidermal growth factor receptor (EGFR) regulates angiogenesis, tumor growth, and progression. In this study, we evaluated whether the blockade of EGFR by the anti-EGFR antibody ImClone C225 (IMC-C225) inhibited tumor growth and metastasis by inhibiting angiogenesis, and whether paclitaxel enhanced the results of therapy in androgen-independent PCa. PC-3M-LN4 PCa cells were implanted orthotopically in athymic nude mice and treated with i.p. IMC-C225 (1 mg twice a week) and/or paclitaxel (200 μg once a week). In vitro treatment of PC-3M-LN4 with IMC-C225 inhibited EGFR autophosphorylation without any significant antiproliferative effect. In contrast, in vivo therapy with IMC-C225 alone (P < 0.05) or in combination with paclitaxel (P < 0.005) significantly inhibited PCa growth and metastasis. Serum levels of interleukin (IL) 8 were lower after therapy, and IL-8 mRNA expression was down-regulated within the tumors after therapy. The down-regulation of IL-8 correlated with reduced microvessel density. IMC-C225 reduced tumor cell proliferation, enhanced p27kip1 expression, and induced tumor and endothelial cell apoptosis. These studies indicate that IMC-C225 has significant antitumor effect in this murine model, mediated in part by inhibition of cellular proliferation and angiogenesis, and by enhancement of apoptosis. The simultaneous administration of paclitaxel enhanced this effect.

Received 8/31/01; revised 2/12/02; accepted 2/13/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by NIH Grant CA67914, National Cancer Institute Cancer Center Core Grant CA16671, NIH Prostate Specialized Programs of Research Excellence Grant P50-CA90270-01, and a grant from ImClone Systems.

2 To whom requests for reprints should be addressed, at Department of Urology, Box 446, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3250; Fax: (713) 792-4824. E-mail: cdinney@mdanderson.org.

3 The abbreviations used are: PCa, prostate cancer; MAb, monoclonal antibody; EGFR, epidermal growth factor; IGF, insulin-like growth factor; EGFR, epidermal growth factor receptor; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial cell growth factor; IL-8, interleukin-8; MMP-9, matrix metalloproteinase type 9; CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling; TGF, transforming growth factor; MAPK, mitogen-activated protein kinase; poly(dtT), polydeoxythymidylic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MVD, microvessel density.
of a variety of tumor cells and human xenografts growing in athymic nude mice (26–32). We reported recently that treatment of human transitional cell carcinoma with IMC-C225 inhibited tumor growth and metastasis in a murine model by inhibiting angiogenesis (33). IMC-C225 inhibited tumor-induced neovascularization by down-regulating the expression of the angiogenic factors VEGF, IL-8, bFGF, and MMP-9 (33, 34). Moreover, paclitaxel synergistically enhanced the antiangiogenic effect of IMC-C225 (34).

EGFR blockade has been evaluated as therapy for PCa (32, 35, 36). IMC-C225 inhibited the proliferation of the androgen-independent PCa cell lines DU145 and PC3 (35–40). The anti-body-induced growth inhibition of DU145 was associated with induction of the CDK inhibitor, p27kip1 (37). Treatment of DU145 xenografts, which express relatively high levels of the EGFR, with IMC-C225 and doxorubicin induced the regression of established s.c. tumors (32). Recently, multiagent chemotherapy regimens including paclitaxel have effectively treated human PCa (3, 9, 41, 42). Because PCa growth and metastasis are regulated by tumor host interactions within the prostate, we evaluated the therapeutic effect of IMC-C225 and paclitaxel against highly metastatic androgen-independent PCa growing within the prostate of athymic nude mice (43).

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Cells of the highly metastatic human androgen-independent prostate carcinoma line PC-3M-LN4 were grown as monolayer cultures in RPMI 1640 supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, nonessential amino acids, and penicillin-streptomycin (complete RPMI) as described previously (43).

Reagents. Chimeric anti-EGFR IMC-C225 was generously provided by ImClone Systems, Inc. (New York, NY). Paclitaxel was purchased from Bristol-Myers Squibb Co. (Princeton, NJ).

In Vitro Growth Inhibition. In vitro antiproliferation was determined after incubating 1 × 10⁴ PC-3M-LN4 cells for 24 h in 10% fetal bovine serum-supplemented MEM, then exchanging the medium for serum-free medium containing EGF (40 ng/ml; R & D Systems Inc., Minneapolis, MN) and up to 100 µg/ml IMC-C225 with or without paclitaxel. Viable cells were counted by MTT assay (44).

Western Immunoblotting and Immunoprecipitation. Cells were lysed in NP40 lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptin, and 25 µg/ml aprotinin] at 4°C. The supernatants were cleared by centrifugation. Protein concentrations were measured by the Bradford method. Equal amounts of protein were subjected to immunoprecipitation in the presence of murine anti-human EGFR antibody (Oncogene Research Products, Boston, MA) for 2 h at 4°C, followed by incubation with immobilized protein G plus/ protein A-agarose beads (Oncogene Research Products) overnight at 4°C. For Western immunoblotting, the immunoprecipitates or equal amounts of crude extract were boiled in Laemmli SDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with mouse antiphosphorylation primary antibody (Upstate Biotechnology, Lake Placid, NY), rabbit anti-EGFR primary antibody (Upstate Biotechnology), or rabbit anti p27kip1 primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C overnight. After the blots were incubated for another 1 h at room temperature with horseradish peroxidase-labeled antimouse secondary antibody (Amersham Life Science Inc., Arlington Heights, IL), signals were detected by using the enhanced chemiluminescence assay (Amersham Life Science Inc.) according to the manufacturer’s instructions (40).

Animals. Male athymic nude BALB/c 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 airflow cabinet under specific pathogen-free conditions and used at 8–12 weeks of age. All of the 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 PC-3M-LN4 cells (60–70% confluent) were prepared for injection as described previously (43). Mice were anesthetized with i.p. nembutol. For orthotopic implantation, a lower midline incision was made, and viable tumor cells (1 × 10⁴/0.04 ml) in HBSS were injected beneath the capsule of the prostate. The formation of a bulla indicated a satisfactory injection. The prostate was returned to the abdominal cavity, and the abdominal wall was closed with a single layer of metal clips.

In Vivo Therapy of Established Human PCa Growing in the Prostate of Athymic Nude Mice. Treatment commenced 3 days after tumor implantation. Mice were randomly separated into four groups and treated for 5 weeks. One group was treated with PBS (200 µl i.p.), a second with IMC-C225 (1 mg i.p twice weekly), a third with paclitaxel (10 µg/g i.p weekly), and a fourth with a combination of IMC-C225 and paclitaxel. Tumors were harvested 5 weeks after initiation of therapy. The primary tumors were removed and weighed, and the presence of lymph node metastases was determined grossly and microscopically. The prostetes were then either fixed in 10% buffered formalin, placed in OCT compound (Miles Laboratories, Elkhart, IN), or mechanically dissociated and put into tissue culture. The lymph nodes were either fixed in 10% buffered formalin or mechanically dissociated and put into tissue culture.

Enzyme-linked Immunosorbent Assay. Blood was collected from the tail vein of each mouse at necropsy, and the serum was separated by centrifugation. Levels of human bFGF, IL-8, and VEGF proteins were determined using the commercial Quantikine ELISA kit (R & D Systems, Inc.). This kit has minimum cross-reaction with other species.

In Situ mRNA Hybridization Analysis. Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts based on published reports of the cDNA sequences for MMP-9, VEGF, IL-8, bFGF, and EGFR (45, 46). The specificity of the oligonucleotide sequence was initially determined by a Gene Bank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (GCG, Madison, WI) based on the FastA algorithm; these sequences showed 100%
homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each sequence was also confirmed by Northern blot analysis (45). A poly(dT)$_{20}$ oligonucleotide was used to verify the integrity and lack of degradation of the mRNA in each sample. All of the DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3’ end via direct coupling with the use of standard phosphoramidite chemistry (Research Genetics, Huntsville, AL). The lyophilized probes were reconstituted to a stock solution at 1 $\mu$g/$\mu$L in 10 mmol/liter Tris (pH 7.6) and 1 mmol/liter EDTA. Immediately before use, the stock solution was diluted with probe dilution (Research Genetics).

In situ mRNA hybridization was performed as described previously with minor modifications (46) using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Refs. 45, 46). Tissue sections (4 mm) of formalin-fixed, paraffin-embedded specimens were mounted on ProbeOn slides (Fisher Scientific). The slides were placed in the Microprobe slide holder, dewaxed, and rehydrated with Auto dewaxer and Auto alcohol (Research Genetics) followed by enzymatic digestion with pepsin. Hybridization of the probe was performed for 45 min at 45°C, and the samples were then washed three times with 2× SSC for 2 min at 45°C. The samples were incubated with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed in 50 mm Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer for 1 min, and incubated with a chromogen substrate for 15 min at 45°C. Additional incubation with fresh chromogen substrate was done if it was necessary to enhance a weak reaction. A red stain indicated a positive reaction in this assay. To check the specificity of the hybridization signal, the following controls were performed: (a) RNase pretreatment of sections; and (b) substituting a biotin-labeled sense probe for the antisense probe. No hybridization signal was observed under either of these conditions. Control for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and the use of chromogen alone (45, 46).

Quantification of Color Reaction. Stained sections were examined in 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 the Optimas image analysis software (version 4.10; Bothell, WA). The slides were prescreened by one of the investigators to determine the range in staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue mode of the color camera. All of the subsequent images were quantified based on this threshold. The integrated absorbance of the selected fields was determined based on its equivalence to the mean log inverse gray value multiplied by the area of the field. The samples were not counterstained, so the absorbance was attributable solely to the product of the in situ hybridization reaction. Three different fields in each sample were quantified to derive an average value. The intensity of staining was determined by comparison with the integrated absorbance of poly(dT)$_{20}$. The results were presented as the ratio of the expression level of each gene compared with controls (defined as 100; Ref. 34).

Immunohistochemical Analysis. For immunohistochemical analysis, frozen tissue sections (8-µm thick) were fixed with cold acetone. Tissue sections (5-µm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with PBS, and appropriate antigen retrieval was performed; endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The samples were washed three times with PBS and incubated for 20 min at room temperature with a protein-blocking solution of PBS (pH 7.5) containing 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 (1:25) dilution of a rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA), a 1:100 dilution of rat monoclonal anti-CD31 antibody (PharMingen, San Diego, CA; 47), a 1:100 dilution of mouse monoclonal anti-PCNA antibody (DAKO, Glostrup, Denmark), or a 1:250 dilution of mouse polyclonal anti p27kip1 antibody (Transduction Laboratory, Kingston, MA). 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: antirabbit IgG, Fab fragment (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA), peroxidase-conjugated antirat IgG (IgG; H+L; Jackson ImmunoResearch Laboratory, Inc.), or antimouse IgG1 (PharMingen). The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill’s hematoxylin (Biogenex Laboratories, San Ramon, CA), and washed three times with PBS. The slides were mounted with mounting medium after dehydration with alcohol and xylene (Universal Mount; Research Genetics).

Quantification of Intensity of Immunostaining. The intensity of immunostaining of IL-8 was quantified in each sample by an image analyzer using the Optimas software program (Bioscan, Edmonds, WA). Five different areas in each sample were evaluated to yield an average measurement of intensity of immunostaining. The results were presented as a ratio between the expression by the tumor and normal mucosa (which was set at 100; Ref. 34).

Quantification of MVD. MVD was determined by light microscopy after immunostaining of sections with anti-CD31 antibodies according to the procedure of Weidner et al. (48). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. Tissue images were recorded using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering, Goletha, CA) linked to a computer and digital printer (Sony Corporation). The MVD was expressed as the average number of five highest areas identified within a single ×100 field.

Quantification of Cell Proliferation, p27kip1, and Apoptosis. Cell proliferation, p27kip1 expression, and apoptosis were determined by immunohistochemical staining of tissue sections with anti-PCNA and anti-p27kip1 antibodies and TUNEL assay. Tissue images were recorded using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering) as before. Densities of proliferative cells and apoptotic cells were expressed as the mean for the five highest areas identified within a single ×100 field (34).
TUNEL Assay. For the TUNEL assay, tissue sections (5-μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with distilled water with BRIJ and treated with proteinase K (20 μg/ml) for 15 min; endogenous peroxidase was blocked by use of 3% hydrogen peroxide in PBS for 12 min. The samples were washed three times with distilled water with BRIJ and incubated for 10 min at room temperature with Tdt buffer. Excess Tdt buffer was drained, and the samples were incubated for 18 h at 4°C with terminal transferase and biotin-16-dUTP. The samples were then rinsed four times with termination buffer (300 mM sodium chloride, 30 mM sodium citrate) and incubated for 30 min at 37°C with a 1:400 dilution of peroxidase-conjugated streptavidin. The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill’s hematoxylin (Biogenex Laboratories), washed three times with PBS, and mounted with a mounting medium (Universal Mount; Research Genetics) after dehydration with alcohol and xylene (34).

Immunofluorescence Double Staining of Tumor and Endothelial Cells. Frozen tissue sections (8 μm) were fixed with cold acetone for 5 min, with acetone plus chloroform (1:1) for 5 min, and with acetone for 5 min. The samples were washed three times with PBS and incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min. The blocking solution was drained, and the samples were incubated with a 1:400 dilution of rat monoclonal anti-CD31 antibody (PharMingen; Ref. 47) for 24 h at 4°C. The samples were then rinsed with PBS three times for 3 min and incubated with protein-blocking solution for 10 min at room temperature. The blocking solution was drained, and the samples were incubated with a 1:200 dilution of secondary goat antirat conjugated to Texas Red (Jackson ImmunoResearch Laboratory) for 1 h at room temperature. The samples were then washed two times with PBS containing 0.1% BRIJ and then washed with PBS for 5 min. TUNEL was performed using a commercial kit (Promega, Madison, WI) with the following modifications. The samples were fixed with 4% paraformaldehyde for 10 min at room temperature. The samples were washed with PBS two times for 5 min and then incubated with 0.2% Triton X-100 for 15 min at room temperature. The samples were then washed with PBS two times for 5 min each and incubated with equilibration buffer (from kit) for 10 min at room temperature. The equilibration buffer was drained, and reaction buffer containing equilibration buffer, nucleotide mix, and Tdt enzyme was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h avoiding exposure to light. To terminate the reaction, the samples were immersed in 2× SSC for 15 min. The samples were then washed three times for 5 min to remove unincorporated fluorescein-dUTP.

To quantify endothelial cells, the samples were incubated with 300 g/ml of Hoechst stain for 10 min at room temperature. This medium stains all of the cell nuclei blue. The samples were then washed with PBS two times for 5 min each. To preserve fluorescence and reduce bleaching, the Prolong antifade kit (Molecular Probes, Inc., Eugene, OR) was used to mount coverslips. The slides were examined using a fluorescent microscope (Inverted System IX70; Olympus, Melville, NY). Endothelial cells were identified by red fluorescence at 600 nm, and DNA fragmentation was detected by localized green fluorescence within the nucleus of apoptotic cells at 520 nm, and colocalized yellow fluorescence within the nucleus indicated apoptotic endothelial cells. Results were expressed as an average of the ratio of apoptotic endothelial cells (yellow nuclei) to the total number of nucleus of endothelial cells (red with blue nuclei) in 10 random fields at ×100 magnification (34).

Statistical Analysis. Tumor weights and staining intensities are expressed as median ± SD. Differences among the treatment groups in the number of vessels, proliferative cells, and apoptotic cells, and in the staining intensity for EGFR and bFGF, VEGF, IL-8, and MMP-9 within the prostate tumors were statistically analyzed using the Mann-Whitney test. Incidences of tumors and metastases were analyzed by the χ² test. Differences with P < 0.05 were considered significant.

RESULTS

Inhibition of Ligand-stimulated EGFR Tyrosine Phosphorylation in PC-3M-LN4. We first determined EGFR expression level of PCa cells (PC-3, PC-3M-LN4, LNCaP, and DU145) and A431 as positive control. PC-3M-LN4 cells expressed the lowest level of EGFR compared with other cells (Fig. 1A). Next, we determined whether IMC-C225 inhibits
EGF-stimulated tyrosine phosphorylation of the EGFR in PC-3M-LN4 cells. Under basal conditions in serum-free medium, PC-3M-LN4 cells demonstrated a low level of EGF tyrosine autophosphorylation, which was enhanced after exposure to EGF for 15 min. IMC-C225 (10 μg/ml) significantly inhibited the EGFR tyrosine phosphorylation (M, 170,000 band) by PC-3M-LN4 cells in response to exogenous EGF stimulation (Fig. 1B). Fig. 1C shows induction of CDK inhibitor p27kip1 in DU145 and PC-3M-LN4 treated with IMC-C225 for 16 and 24 h. As reported previously, IMC-C225 increased p27kip1 expression in DU145 cells after 16-h exposure to IMC-C225 (49). However, the p27kip1 expression in PC-3M-LN4 cells was unaltered by IMC-C225 treatment.

Inhibition of in Vitro Proliferation by IMC-C225 and Paclitaxel. We next evaluated the in vitro antiproliferative effects of IMC-C225 treatment of PC-3M-LN4 cells. After exposure to EGF, treatment with up to 100 μg of IMC-C225 failed to significantly inhibit the growth of PC-3M-LN4 cells as measured by the MTT assay (Ref. 44; Fig. 2). Moreover, IMC-C225 did not enhance the in vitro antiproliferative effects of paclitaxel.

Inhibition of Growth and Metastasis of Human PCa. The purpose of these experiments was to determine whether IMC-C225 would inhibit the growth and metastasis of PC-3M-LN4 tumor cells growing within the prostate of athymic nude mice and whether this effect was enhanced by paclitaxel. Therapy commenced 3 days after tumor implantation and continued for 5 weeks, at which point all of the control mice had become moribund and had to be sacrificed. Treated mice were closely monitored for any signs of progressive disease and were euthanized if they became moribund before 5 weeks of therapy was completed. The results of therapy are summarized in Table 1.

Enhancement of Apoptosis, Up-Regulation of p27kip1 Expression, and Inhibition of Proliferation and MVD by Treatment with IMC-C225 and Paclitaxel. We evaluated the effect of therapy with IMC-C225 and paclitaxel on cellular proliferation, MVD, and apoptosis of tumor and endothelial cells (Table 4; Fig. 4). The number of PCNA-positive cancer cells counted per ×100 field increased from 15 ± 4 in controls to 24 ± 9, 29 ± 8, and 30 ± 9 after therapy with IMC-C225,
paclitaxel, and IMC-C225 plus paclitaxel, respectively \((P < 0.05; \text{Table 4}); 4). We also observed an increase in \(p27^\text{kip1}\) expression after therapy with IMC-C225 alone \((18 \pm 5)\) or in combination with paclitaxel \((23 \pm 11)\), compared with paclitaxel \((10 \pm 5)\) or saline \((9 \pm 4)\) \((P < 0.05)\).

Because therapy with IMC-C225 inhibited IL-8 expression, we determined whether IMC-C225 alone or in combination with paclitaxel inhibits angiogenesis in vivo. MVD was significantly decreased in mice treated with IMC-C225 alone or in combination with paclitaxel compared with control group \((P < 0.05; \text{Fig. 4}; \text{Table 4}); 4). Paclitaxel alone had no effect on MVD and did not enhance the reduction in MVD observed after IMC-C225 therapy.

Endothelial cell apoptosis was determined by double-labeled immunofluorescence. By this technique, endothelial cells fluoresce red, whereas green fluorescence is detected within the nuclei of apoptotic cells. Double labeling of endothelial cells undergoing apoptosis results in localized yellow fluorescence. The endothelial cell apoptotic index was calculated as the ratio of double labeled to total nuclei of endothelial cells counted per \(100 \text{ field}\). Treatment with either IMC-C225 alone or IMC-C225 plus paclitaxel significantly increased the apoptotic index of endothelial cells to \(0.88 \pm 0.18\) and \(1.36 \pm 0.57\), respectively, compared with treatment with paclitaxel alone \((0.28 \pm 0.21)\) or saline \((0.13 \pm 0.26; P < 0.05; \text{Table 4}; \text{Fig. 4}); 4).

**DISCUSSION**

Whereas advanced PCa initially responds to hormone deprivation therapy, most patients will eventually develop androgen-independent cancer, rendering additional hormonal manipulation relatively ineffective \((2, 3); 4). This hormone-independent disease state is characterized by an inhibition of apoptosis within the tumor after androgen deprivation \((47); 4). The progression from androgen-dependent to androgen-independent disease is associated with the up-regulation of autocrine or paracrine loops involving several polypeptide growth factors including EGF, TGF-\(\alpha\), and IGF \((5, 6, 8, 11); 4). In androgen-insensitive DU145 cells, for instance, there is an elevated basal activity of MAPK \((35); 4\), which is a downstream target of the EGFR-mediated signal transduction pathway. The elevated basal level of MAPK can be abrogated by blockade of the EGFR receptor kinase with the selective EGFR tyrosine kinase inhibitor tyrphostin AG1478 \((35); 4\), and tumor growth can be inhibited by the anti-EGFR antibody IMC-C225 or by anti-TGF-\(\alpha\) oligonucleotides \((37–40, 50); 4\). A recent clinical study indicated that PCa progression is characterized by a transition from a paracrine to an autocrine loop between the EGFR and TGF-\(\alpha\); in primary tumors, the neoplastic cells express EGF and the surrounding stromal cells express TGF-\(\alpha\), whereas in advanced disease, the neoplastic cells coexpress the EGFR and TGF-\(\alpha\) \((51, 52); 4\). Another recent clinical study \((53); 4\) examined the activation status of MAPK in primary and metastatic human prostate tumor specimens and reported that non-neoplastic prostate tissue has little or no MAPK activity. However, in prostate tumors, the level of activated MAPK increased with increasing Gleason score and tumor stage. Two patients in their study originally showed no activation of MAPK in tumor samples before androgen ablation therapy but exhibited high levels of activated MAPK in their recurrent tumors after androgen ablation treatment \((53); 4\). Taken together, these results indicate that during PCa progression after androgen ablation therapy, there may be a microenvironment in which peptide growth factors activate signal transduction pathways that help to drive PCa cells to an androgen-independent state.

Even in the androgen-responsive state of PCa, androgens may mediate their stimulatory effects on prostate cells partially through an autocrine or paracrine loop involving TGF-\(\alpha\) and the EGF \((54–56); 4\). Exposure of the androgen-responsive prostate carcinoma cell line ALVA101 to testosterone or to its active metabolite dihydrotestosterone up-regulated both TGF-\(\alpha\) and the EGF receptor at the mRNA level and increased cellular proliferation. An anti-EGFR MAB, 528, blocked the cell proliferation induced by dihydrotestosterone \((55); 4\). On the other hand, growth factors such as EGF or IGF-1 may stimulate androgen receptor-mediated gene transcription in the absence of androgen
Treatment of the androgen-responsive MDA PCa 2a and MDA PCa 2b cell lines with flutamide and IMC-C225 decreased CDK2 activity and increased p27kip1 to levels greater than that observed with IMC-C225 treatment alone (40). These studies suggest that the androgen-signaling pathway and growth factor-mediated pathway may coexist in the regulation of proliferation of PCa. In early stages of the progression of PCa, androgen is a dominant factor. After castration, growth factors...
Therapy of Prostate Cancer with IMC-C225 and Paclitaxel

Table 4  Apoptosis, proliferation, and MVD after therapy with IMC-C225 and/or paclitaxel

All therapy significantly inhibited proliferation and induced apoptosis of tumor cells compared with controls (P < 0.05). In particular, cell proliferation in the tumors treated with combination therapy with IMC-C225 and paclitaxel was significantly lower than in the tumor treated with paclitaxel alone (P < 0.05). The therapy with IMC-C225 significantly increased p27Kip1-positive cells compared with controls or paclitaxel alone (P < 0.05). Apoptosis of endothelial cells was significantly induced by IMC-C225 alone and combination therapy with IMC-C225 and paclitaxel compared with either controls or paclitaxel alone (P < 0.05).

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Proliferation indexa</th>
<th>p27Kip1 indexa</th>
<th>Apoptosis indexa</th>
<th>MVDb</th>
<th>Apoptosis index of endothelial cellsc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Control (saline)</td>
<td>244 ± 55</td>
<td>9 ± 4</td>
<td>15 ± 4</td>
<td>76 ± 14</td>
<td>0.13 ± 0.26</td>
</tr>
<tr>
<td>IMC-C225</td>
<td>164 ± 35d</td>
<td>18 ± 5d/</td>
<td>24 ± 9d</td>
<td>39 ± 17d/f</td>
<td>0.88 ± 0.18/e</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>174 ± 43e</td>
<td>10 ± 5</td>
<td>29 ± 8d</td>
<td>74 ± 13</td>
<td>0.28 ± 0.21</td>
</tr>
<tr>
<td>IMC-C225/paclitaxel</td>
<td>139 ± 33d/f</td>
<td>23 ± 11d</td>
<td>30 ± 9d</td>
<td>38 ± 5d/f</td>
<td>1.36 ± 0.57e/f</td>
</tr>
</tbody>
</table>

a The density of cell proliferation and p27Kip1 by immunohistochemistry with anti PCNA and p27Kip1 antibody, and tumor cell apoptosis by TUNEL assay was expressed as an average number of five highest areas identified within a single 100× field.

b Microvessel density was expressed as an average number of CD-31+ cells in the five highest areas identified within a single 100× field.

c The density of endothelial cell apoptosis by immunofluorescent CD-31 and TUNEL double labeling was expressed as an average of the ratio of apoptosis in the nucleus of endothelial cells to the total number of nuclei of endothelial cells taken from the areas with the highest MVD identified within a single 100× field.

d P < 0.05 compared to control.

e P < 0.05 compared to IMC-C225.

f P < 0.05 compared to paclitaxel (P: Mann-Whitney U test).

may become a dominant factor to refuel the growth of PCs in an androgen-independent mechanism.

The anti-EGFR mAb IMC-225, which binds to the EGFR with a greater affinity than the natural ligands EGF or TGF-α, blocks the binding of EGF/TGF-α, and prevents activation of receptor tyrosine kinase (21–25). This antibody can inhibit the proliferation of a variety of malignant cell lines stimulated by either exogenous EGF or endogenous TGF-α (26–30) and has potent antitumor activity against a variety of cultured and human xenografts, principally through the induction of apoptosis and inhibition of invasion and tumor-induced angiogenesis (33–35, 38, 56). We reported previously that the in vitro treatment of bladder cancer cell IMC-C225 resulted in only a modest antitumor effect, whereas in vivo therapy had profound effects on tumor growth, and both down-regulated the expression of the angiogenic factors VEGF, bFGF, IL-8, and MMP-9, and induced apoptosis of tumor and endothelial cells (33, 34). Previous reports indicate that IMC-C225 also inhibits the proliferation of the androgen-independent PCa cell lines DU145 and PC3 (37, 38, 40). In DU145 cells, the antibody-induced growth inhibition was associated with a decrease of the CDK inhibitor p27Kip1 (37), and regression of established DU145 xenografts, which overexpress the EGFR relative to PC-3M-LN4, was observed after therapy with IMC-C225 and doxorubicin (32). Furthermore, the direct injection of antisense oligonucleotides to TGF-α induced the regression of PC-3 xenografts in athymic nude mice (48).

The present study confirms that EGFR blockade with IMC-C225 inhibits the growth of the highly metastatic human PCa cell line PC-3M-LN4, which expresses relatively low levels of the EGFR compared with other PCa cell lines (32, 37, 40, 57). Therapy with IMC-C225 did not cause regression of PC-3M-LN4 as was reported previously for DU145 or A431 xenografts, which are profoundly dependent on EGFR signaling for their growth (31, 32). In the case of A431, therapy with EGFR inhibitors results in the complete regression of established tumors. However, when therapy is halted tumor regrowth occurs, which is resistant to additional EGFR-directed therapy. This acquired resistance is characterized by constitutive overexpression of VEGF (58). The PC-3M-LN4 tumor model is not ideal for these types of survival studies, because control animals succumb to tumor burden effects after 5–6 weeks, and the tumors in the treated animals do not regress completely.

Despite a minimal antiproliferative effect and lack of induction of p27Kip1 expression in vitro, therapy with IMC-C225 in vivo inhibited tumor cell proliferation, up-regulated p27Kip1 expression, enhanced tumor cell apoptosis, and down-regulated the expression of IL-8 (33, 34, 56). This activity was associated with endothelial cell apoptosis and the inhibition of tumor-induced angiogenesis, resulting in decreased tumor growth and metastatic tumor burden (33, 34, 56, 59). The antitumor effect observed with PC-3M-LN4 xenografts, which express low levels of EGFR (Fig. 1A), emphasizes that overexpression of the EGFR is not necessary for successful EGFR-directed therapy. Thus, any cell expressing even low levels of the EGFR represents a valid target. Indeed some of the therapeutic effects observed with IMC-C225 may be mediated by perturbations in the stromal-tumor interactions, perhaps via down-regulation of IL-8 as observed in the current study.

We hypothesize that IMC-C225 exerts its in vivo antitumor effect in androgen-independent PCs in part by down-regulating the expression of IL-8, which in turn induces endothelial cell apoptosis leading to the regression of tumor neovascularity. Inoue et al. (60) reported that metastatic PCs is characterized by an enhanced angiogenic response in the host microenvironment. Inoue et al. (60) also identified IL-8 as an important mediator of tumor-induced angiogenesis by the PC-3M-LN4 cell line, whereas VEGF was expressed at only low levels by this cell line (61). Down-regulation of IL-8 expression after stable transfection of PC-3M-LN4 with antisense IL-8 transcripts resulted in decreased tumor induced angiogenesis, tumorigenicity, and metastasis after implantation of the transplanted cells into the pros-
The enhanced induction of tumor and endothelial cell apoptosis after therapy with IMC-C225 and paclitaxel combination. This is consistent with the role of IL-8 as survival factors in ovarian cancer after therapy with paclitaxel or other chemotherapeutic agents (62, 63). The present study demonstrates that inhibition of angiogenesis characterizes, in part, the antitumor effect of EGFR blockade in PCa, and confirms the importance of IL-8 as a mediator of PCa angiogenesis and metastasis (18, 60, 64, 65). Therefore, therapy such as anti-EGFR therapy, which targets IL-8 expression may be effective against androgen-independent PCa. Similar observations were made after the successful treatment of human bladder and pancreatic cancer xenografts in nude mice (33, 34, 56). Collectively, these studies demonstrate that down-regulation of the angiogenic stimulus provided by the tumor cells inhibits the host angiogenic response and emphasize the complexity of tumor-host interactions. The enhanced induction of tumor and endothelial cell apoptosis after therapy with IMC-C225 and paclitaxel may be because of the dependence these cells on IL-8 as a survival factor after cellular damage from paclitaxel therapy, tipping the balance toward drug-induced apoptosis. This is consistent with the role of IL-8 as survival factors in ovarian cancer after therapy with paclitaxel or other chemotherapeutic agents (62, 63). The present study demonstrates that inhibition of angiogenesis characterizes, in part, the antitumor effect of EGFR blockade in PCa, and confirms the importance of IL-8 as a mediator of PCa angiogenesis and metastasis (18, 60, 64, 65).
of paclitaxel might be that the development of drug resistance by exposure to paclitaxel was accompanied with alterations of gene expression including IL-8 (63). Paclitaxel increases microtubule stability by preventing tubulin polymerization, which results in tubulin bundling (66, 67). These cytoskeleton changes lead to cell cycle arrest and apoptosis within 20 h of paclitaxel exposure (45, 46). On the other hand, the induction of G1 arrest by IMC-C225 is associated with the inhibition of CDK-2 activity and increased levels of the CDK inhibitor p27kip1 (40). In fact, IMC-C225 augments the antitumor activity of several chemotherapeutic agents and irradiation in human xenograft models (30, 32, 34, 56, 59). The capacity of IMC-C225 to modulate cell cycle distribution may also play a central role in regulating cell proliferation and prostate cancer risk: a prospective study. Science (Wash. DC), 279: 563–566, 1998.


Inhibition of Angiogenesis by the Antiepidermal Growth Factor Receptor Antibody ImClone C225 in Androgen-independent Prostate Cancer Growing Orthotopically in Nude Mice

Takashi Karashima, Paul Sweeney, Joel W. Slaton, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/8/5/1253

Cited articles
This article cites 71 articles, 35 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/8/5/1253.full.html#ref-list-1

Citing articles
This article has been cited by 23 HighWire-hosted articles. Access the articles at:
/content/8/5/1253.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.