Antibody to Vascular Endothelial Growth Factor Slows Growth of an Androgen-independent Xenograft Model of Prostate Cancer

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ABSTRACT

Purpose: Human tumors are dependent on angiogenesis for growth, and vascular endothelial growth factor (VEGF) is a major regulator of this process. We aimed to study clinical utility of a recombinant humanized monoclonal anti-VEGF antibody (rhuVEGF) in the treatment of the CWR22R androgen-independent xenograft model of prostate cancer.

Experimental Design: rhuVEGF has previously shown clinical activity in several xenograft cancer models. We administered 5 mg/kg rhuVEGF i.p. twice weekly as a single agent and together with paclitaxel to established CWR22R xenografts.

Results: rhuVEGF inhibited established tumor growth by 85% (P < 0.01 for trajectories of the average tumor volumes of the groups) at 3 weeks, but after cessation of rhuVEGF treatment, tumor regrowth ensued. A paclitaxel dosage of 6.25 mg/kg s.c. five times/week slowed tumor growth (72% compared with controls at 3 weeks, P = 0.02). The combination of paclitaxel and rhuVEGF resulted in greater inhibition of tumor growth than that observed with either agent alone (98% growth inhibition, P = 0.024 versus rhuVEGF alone and P = 0.02 versus paclitaxel alone). Paclitaxel alone had no antiangiogenic effects at the dosage studied, whereas rhuVEGF had significant inhibition of angiogenesis, noted by microvessel density and CD34 staining.

Conclusions: rhuVEGF has cytostatic clinical activity in this androgen-independent prostate cancer xenograft model, and the addition of paclitaxel demonstrates increased clinical activity.

INTRODUCTION

Angiogenesis is the progressive and structured progress for the creation of new vasculature (1, 2). Angiogenesis is mediated through a mitogenic signaling cascade characterized by a localized increase in protease expression, degradation of the venule extracellular matrix, endothelial chemotaxis and proliferation, proliferation of the nascent vessel with pericytes, a basement membrane, and smooth muscle cells (1, 2). Prominent during normal embryonic development and somatic growth, angiogenesis is restricted in adults to wound healing, certain reproductive functions, and with the pathogenesis of cancer, rheumatoid arthritis, and proliferative retinopathies (3–5). The angiogenic process is regulated by multiple humoral factors, including acid/basic fibroblast growth factor, tumor necrosis factor-α, interleukin 8, and VEGF1 (5).

VEGF is a highly conserved, homodimeric glycoprotein with a dominant isoform of $M_r$ 45,000 (6). Membrane-bound or secreted VEGF is a potent endothelial cell-specific angiogenic mitogen that plays an integral role in organ development and differentiation, tissue repair, and reproductive function (3, 5). VEGF is also involved in several pathological states, being critical for the growth of solid neoplasms beyond 1–3 mm$^3$ and secreted by tumor cells (7). Although the VEGF promoter does not contain a consensus androgen response element (6), in relation to the growth of prostate cancers, evidence suggests that VEGF may be androgen responsive because VEGF expression by normal prostatic tissue is up-regulated in response to exogenous androgen. In addition, androgen withdrawal results in a decrease in VEGF (but not basic fibroblast growth factor) concentrations, followed by a decrease in MVD and size in the prostate cancer tissue (8, 9).

Although they are not considered to be highly vascular tumors, prostate cancer specimens show increased VEGF expression and vascularity when compared with benign prostate hyperplasia and normal prostatic tissue (10). An association does exist between MVD in tumor and basement membrane derangement and Gleason score, metastases, tumor aggressiveness, and progression (11–15). Because the process of angiogenesis is dependent on the cytoskeleton for cell motility, we hypothesized that there may be a synergistic anticancer effect when combining an antiangiogenic with a microtubule stabilizer. Paclitaxel, a diterpene originally extracted from the bark of the western yew tree, preferentially binds to the β subunit of tubulin, preventing depolymerization and arresting cell cycle, and has demonstrated clinical activity in hormone-refractory prostate cancer. In some systems, paclitaxel alone has been

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; rhuVEGF, recombinant humanized monoclonal antibody to VEGF; MVD, microvascular density; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling.
postulated to have antiangiogenic effects in addition to a direct effect on tumor cells, contributing to the overall therapeutic outcome (16).

In the current study, we demonstrate that malignant prostate epithelial cells in radical prostatectomy specimens, as well as prostate cancer xenograft models, express more VEGF than normal prostate epithelial cells. We then treated an androgen-independent prostate cancer xenograft, CWR22R (17–20), with rhuVEGF (21–23), previously demonstrated to have antiangiogenic properties, as a single agent and in combination with paclitaxel. In the xenograft model, rhuVEGF was highly active, whereas the combination of rhuVEGF and paclitaxel was more effective than either therapy alone.

MATERIALS AND METHODS

Animal Studies. Nude athymic BALB/c mice, 4–6 weeks of age, were obtained from the National Cancer Institute-Frederick Cancer Center and maintained in pressurized ventilated cages at the Memorial Sloan-Kettering Cancer Center and the Cedars-Sinai Research Institute. The CWR22R androgen-independent prostate cancer line, provided by Dr. Thomas Pretlow (Case Western Reserve University, Cleveland, OH), was propagated in the animals by the injection of minced tumor tissue from an established tumor into the s.c. tissue of the flanks of athymic nude female mice together with reconstituted basement membrane (Matrigel; Collaborative Research, Bedford, MA; Refs. 7, 8). Three to 4 weeks after inoculation, tumors of 1000 mm3 can be measured. Tumor size was determined by caliper measurements of length and width. Tumor volume was estimated using the formula \( V = \frac{4}{3} \pi r^3 \).

Treatments consisted of rhuVEGF (Genentech, Inc., San Francisco, CA) at 5 mg/kg i.p., twice weekly for 4 weeks, and/or paclitaxel (Taxol; Bristol Myers, Danbury, CT) at 6.25 mg/kg s.c. five times a week for 3 weeks. Control mice were administered saline alone. All cohorts were treated prophylactically with amoxicillin/clavulanate potassium (Augmentin; SmithKline Beecham, Philadelphia, PA) administered in the drinking water for skin infections secondary to excessive handling and chemotherapy. Only animals with established tumors of at least 65 mm3 were included in the study.

Immunohistopathology. After consent was obtained, normal prostate and prostate cancer tissue was taken from prostatectomy samples, and additional prostate tumor tissue was obtained from mouse xenografts. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections of 5 μm were deparaffinized in xylene and rehydrated in increasing concentrations of ethanol. Endogenous peroxidase activity was quenched with 0.01% H2O2 sections were boiled in 0.01 M citric acid (pH 6.0), and nonspecific binding was blocked with 10% normal rabbit serum. After washing, rat antihuman CD34 monoclonal primary antibody (PharMingen, La Jolla, CA) specific to vascular endothelium was applied at 25 μg/ml and incubated overnight at 4°C. After washing in PBS, biotinylated rabbit antimouse antibody, diluted 1:100, was applied, followed by incubation at room temperature for 30 min. After washing again in PBS, avidin-biotin complex (Vector Laboratory, Burlingame, CA) diluted 1:25 was applied and incubated at room temperature for 30 min and washed again in PBS. Tissue sections were developed with diaminobenzidine and peroxide and counterstained with hematoxylin. MVD was assessed by counting the microvessels at \( \times 200 \) in a field that had the highest vascularization by scanning at \( \times 40 \) (12). Stained slides were examined blind and were also scored from one to four with increasing vascularity. Vessel diameter and overall branching of vascular structure were noted.

Western Blot Analysis. Total protein from normal human prostate, human prostate cancer, and prostate cancer mouse xenograft tissues was extracted by the addition of suspension buffer [0.1 M NaCl, 0.01 M Tris-Cl (pH 7.6), 0.001 M EDTA (pH 8), 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 0.7 μg/ml pepstatin, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride]. Human prostate and prostate tumors were acquired from patient samples after review by a pathologist and promptly flash frozen in liquid nitrogen. All patients signed informed consent, and the protocol was approved by the Institutional Review Board. Protein concentration was quantified by the Bradford method using the Bio-Rad Protein Assay kit (Bio-Rad, New York, NY). VEGF was detected by subjecting total protein to 10% SDS-PAGE, followed by semidry transfer onto Hybond-P polyvinylidene difluoride membrane (Amersham Life Science, Buckinghamshire, United Kingdom), which was then incubated in antihuman VEGF antibody (PharMingen, San Diego, CA) at 2 μg/ml. Protein was detected with an antimouse horseradish peroxidase conjugate secondary antibody (Amersham Pharmacia Biotech).

TUNEL Assay. To study the microanatomical distribution of apoptosis, we assayed consecutive sections from the blocks used for immunohistochemistry by a modification of the method of TUNEL described previously (24). Nuclear staining was assessed by immunohistochecmical scoring, counting different fields, and evaluation >500 tumor cells.

Statistical Analysis. To compare differences between treatment groups (x and y) with respect to tumor volumes over time, a permutation test was used. The null hypothesis for this test is that the treatment has no differential effects on the treatment groups with respect to the statistics selected. The statistic used to test the hypothesis was the sum of the squared differences between mean tumor volumes summed over all time points.

\[
SS_{DEV} = \sum_{i=1}^{k} (x_i - y_i)^2
\]

SS_DEV was used to capture average differences between treatment groups at each time point. This statistic reflects the amount by which the two treatment groups are different between the trajectories of average tumor volume of the two groups, the greater the value of the statistic. All possible permutations (NCn) were examined. For each possible permutation, the statistic was calculated and compared with the value of the statistic obtained when we applied it to the data that we observed. The \( P \) generated corresponds to the proportion of the test statistics from the permutation distribution that are as, or more, extreme than the test statistic observed.
RESULTS

Expression of VEGF Is Increased in Human Prostate Tumor Specimens and Xenograft Prostate Tumors When Compared with Normal Human Prostate. Western analysis of tumor specimens demonstrated VEGF expression in cells obtained from radical prostatectomy specimens of patients with prostate cancer (data not shown) as well as in the xenograft tumor samples (Fig. 1). VEGF protein was not detected or detected at low levels in normal prostate samples when compared with tumor samples by Western analysis (Fig. 1).

Effect of rhuVEGF on Growth of an Established Androgen-independent Prostate Cancer Xenograft. We evaluated the efficacy of rhuVEGF in well-established, androgen-independent prostate cancer xenografts. The CWR22R model was used for these experiments because it provides reproducible growth curves. rhuVEGF (5 mg/kg twice weekly) demonstrated significant growth inhibition when compared with control (n = 5 each group, 85% growth inhibition, P < 0.01 for the growth curve trajectories of the average tumor volumes of the groups; Fig. 2A). The histology of the tumors in each group was examined by light microscopy, and no visible difference in necrosis was seen between the groups (data not shown). The rhuVEGF-mediated growth inhibition was no longer present after treatment was discontinued, because the treated tumors began to grow (Fig. 2B).

Effect of rhuVEGF Combined with Paclitaxel on Growth of an Established Androgen-independent Prostate Cancer Xenograft. Graded doses of paclitaxel were administered previously to the CWR22R xenograft-bearing animals to determine a dosage of paclitaxel that inhibited growth but that did not cause complete regression of the tumors (25). Paclitaxel, at a dosage of 6.25 mg/kg s.c. five times/week, was administered to animals with well-established tumors and produced growth inhibition relative to control at the time of sacrifice (72% growth inhibition, P = 0.02; Fig. 2C). When paclitaxel and rhuVEGF were coadministered to animals, there was a marked reduction in tumor volume versus control (98% growth inhibition, P < 0.01; Fig. 2C). The degree of inhibition observed with the combination treatment was significantly greater than that observed with either agent alone at the end of the treatment period (mean tumor volumes, n = 6 each group, 1250 mm³ paclitaxel, 550 mm³ rhuVEGF, and 100 mm³ paclitaxel and rhuVEGF, respectively).

Figure 1: Western blot analysis showing VEGF expression in neoplastic and normal prostate tissue. Representative Western blot analysis showing VEGF expression in CWR22R androgen-independent human prostate xenograft tumor (n = 5) and lack of expression in a normal human prostate specimen (n = 16). Samples were incubated with an antihuman VEGF antibody. Tubulin blot demonstrated equivalent protein loading.

Figure 2: rhuVEGF and rhuVEGF/paclitaxel mediated growth inhibition in an androgen-independent prostate cancer xenograft model. Combination rhuVEGF and paclitaxel has an added inhibitory effect on CWR22R xenografts growth in nude mice. The percentage of growth inhibition was calculated based on tumor volumes on the day of sacrifice. Ps were calculated based on tumor volumes over time. A, rhuVEGF (5 mg/kg i.p. two times/week; □) administered twice weekly (n = 6; ○) demonstrated significant growth inhibition (85%; P < 0.01) when compared with the control-treated group (n = 5; □). B, growth inhibition of xenograft tumors in separate cohort administered rhuVEGF (n = 5; ○) was no longer present once rhuVEGF treatment was discontinued. C, paclitaxel (6.25 mg/kg s.c. five times/week; ○) and rhuVEGF (5 mg/kg i.p. two times/week; □) administered concomitantly (n = 5; ○) resulted in greater growth inhibition (98%; P < 0.01) than with paclitaxel (n = 5; △) or rhuVEGF (n = 5; □) used as a single agent (P = 0.02 and P = 0.024 versus paclitaxel and rhuVEGF, respectively).
rhuVEGF; \( P = 0.024 \) versus rhuVEGF alone and \( P = 0.02 \) versus paclitaxel alone; Fig. 2C). A cohort of animals was not sacrificed after completion of treatment with the combination of rhuVEGF and paclitaxel, and the tumors regrew in these animals (data not shown).

**Effect of rhuVEGF on Microvascular Content and Apoptosis in an Established Androgen-independent Prostate Cancer Xenograft.** The microvascular content of the tumors was evaluated by both visual grading and descriptions of CD34 staining and MVD scoring (Table 1). Control tumors had 2+ microvascular staining with a thick, well-developed and branching vasculature (MVD score, 41; Fig. 3A). The rhuVEGF-treated tumors and the combination rhuVEGF/paclitaxel-treated tumors both scored 1+ with thin, unbranching vessels (MVD scores, 9 and 4, respectively; \( P = 0.02 \) and 0.02 versus control group; Fig. 3, B and C). Tumors that initially responded to rhuVEGF, but which were allowed to grow after treatment was discontinued, showed a vasculature architecture and MVD scores similar to untreated controls (MVD score, 21; \( P = 0.10 \) versus control group; Fig. 3D). The vasculature of the paclitaxel group was similar to the control group (2+, branching thick vessels; MVD score, 51). TUNEL staining demonstrated no difference in apoptotic cells in the rhuVEGF versus control (data not shown).

**DISCUSSION**

We demonstrate, as has been shown previously by other groups (26–30), that VEGF is expressed in human prostate cancer samples as well as the prostate cancer xenograft models used in this study. We also demonstrate that rhuVEGF alone has antitumor activity in the CWR22R androgen-independent human prostate cancer xenograft model. The monoclonal antibody must achieve clinically significant concentrations in the tumor microenvironment, as evidenced by its efficacy in inhibiting tumor growth. Thus, VEGF is an important paracrine growth factor for the growth of the supporting stroma in the xenograft model studied, and its inhibition limited vascular expansion that, in turn, limited tumor growth. There is ample clinical data correlating tumor vascularity with negative prognostic outcomes. Multiple factors have been implicated in tumor vascularity including the ligand VEGF, VEGF receptors, flt, and KDR, but this study demonstrates that by interfering solely with the function of the VEGF ligand alone, the neovascularization process can be affected.

We also demonstrate that rhuVEGF has at least an additive effect with paclitaxel in inhibiting prostate cancer xenograft growth. The inhibition of tumor growth, even in the presence of

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<td>2+</td>
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<tr>
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<td>9</td>
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<tr>
<td>Posttreatment</td>
<td>51</td>
<td>2+</td>
<td>Branching, thick vessels</td>
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Fig. 3 Vascular staining in CWR22R xenograft tumors. Photomicrographs of immunocytochemistry staining of tumors from nude mice with CWR22R androgen-independent human prostate cancer xenografts, stained with an anti-CD34 mAb (×400). Representative areas of sections are from tumors from cohorts receiving PBS as control (A), rhuVEGF (B), rhuVEGF and paclitaxel (C), and rhuVEGF and then allowed to grow 14 days after treatment discontinuation (D).
paclitaxel, did not cure animals of the tumors. Growth inhibition by rhuVEGF appears to be through the antiangiogenic properties of the antibody, as evidenced by the associated profound changes in the anti-CD34 staining and the MVD of the treated tumors. The lack of an apoptotic response may reflect the few cells actually undergoing apoptosis at any one time, the inability to sample tumors repeatedly at different times, or the small overall contribution of apoptotic cell death to tumor growth inhibition. The mechanism of the additive activity of rhuVEGF and paclitaxel is not known. Endothelial cells in microvasculature require microtubule formation for mobility, and thus drugs that target microtubules may act synergistically on the microvasculature (31).

Although it has been postulated that paclitaxel may exert part of its antitumor effects by inhibition of neovascularity, no evidence of this effect was observed, using an end point of MVD, at the dosage used in our studies. This does not, however, exclude an effect on endothelial cell migration, which was not assessed.

Is VEGF a cytokine for prostate cancer, whereby inhibiting this ligand will have a primary growth effect on the tumor cells? Prostate cancer cells express VEGF receptors (26, 32, 33). In a rat prostate cancer model, VEGF induced chemotactic migration only in cancer cells expressing the VEGF receptor, Flt-1 (34). Thus, interfering with the autocrine VEGF loop may have an impact on the growth of the cancer.

Cell stress responses up-regulate VEGF production (35, 36). The administration of rhuVEGF in these studies may block an important cell stress response to the introduction of paclitaxel, thus resulting in increased inhibition of tumor growth by the combination of these two agents.

This is another example showing additive effects of antibodies to important cell signaling molecules (ligands or receptors) and a cytotoxic agent, similar to what has been demonstrated with combinations of C225, 2C4, and Herceptin in model systems and in humans (25, 37–40).

VEGF is an androgen-responsive gene (41, 42). In xenograft models and cell lines, androgen withdrawal results in a marked decrease in both VEGF mRNA and VEGF protein expression (41, 42). VEGF expression levels increase in androgen-independent tumors similar to the increase in serum PSA that is noted with the emergence of androgen-independent tumors in patients (28).

Although the degree of effect in this single animal model was significant, it is unclear whether a similar degree of inhibition would be observed in the clinic. It further suggests that combination studies should be conducted. Clinical trials are currently under way with rhuVEGF alone and in combination with various chemotherapy regimens (43). Studies in androgen-independent prostate cancer with the addition of paclitaxel are warranted.

REFERENCES


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