Blockade of Epidermal Growth Factor Receptor Signaling in Tumor Cells and Tumor-associated Endothelial Cells for Therapy of Androgen-independent Human Prostate Cancer Growing in the Bone of Nude Mice

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ABSTRACT

Purpose: We determined whether blockade of the epidermal growth factor receptor (EGF-R) signaling pathway by oral administration of the EGF-R tyrosine kinase inhibitor (PKI 166) alone or in combination with injectable Taxol inhibits the growth of PC-3MM2 human prostate cancer cells in the bone of nude mice.

Experimental Design: Male nude mice implanted with PC-3MM2 cells in the tibia were treated with oral administrations of PKI 166 or PKI 166 plus injectable Taxol beginning 3 days after implantation. The incidence and size of bone tumors and destruction of bone were determined by digitalized radiography. Expression of epidermal growth factor (EGF), EGF-R, and activated EGF-R in tumor cells and tumor-associated endothelial cells was determined by immunohistochemistry.

Results: Oral administration of PKI 166 or PKI 166 plus injectable Taxol reduced the incidence and size of bone tumors and destruction of bone. Immunohistochemical analysis revealed that PC-3MM2 cells growing adjacent to the bone expressed high levels of EGF and activated EGF-R, whereas tumor cells in the adjacent musculature did not. Moreover, endothelial cells within the bone tumor lesions, but not in uninvolved bone or tumors in the muscle, expressed high levels of activated EGF-R. Treatment with PKI 166 and more so with PKI 166 plus Taxol significantly inhibited phosphorylation of EGF-R on tumor and endothelial cells and induced significant apoptosis and endothelial cells within tumor lesions.

Conclusions: These data indicate that endothelial cells exposed to EGF produced by tumor cells express activated EGF-R and that targeting EGF-R can produce significant therapeutic effects against prostate cancer bone metastasis.

INTRODUCTION

The major cause of death from prostate cancer is metastases that are resistant to conventional therapies (1). The metastases are often located in lymph nodes or bone (1, 2), and the specific organ microenvironment can influence the biological behavior of metastatic cells, including their response to systemic therapy (3). The outcome of organ-specific metastasis depends on multiple interactions between unique subpopulations of prostate cancer cells and specific homeostatic factors in the organ microenvironment (3, 4). More than a century ago, Stephen Paget researched the mechanisms that regulate organ-specific metastasis (i.e., pattern of metastasis by different cancers). His research documented a nonrandom pattern of visceral (and bone) metastasis. This finding suggested to Paget that the process was not because of chance but because certain tumor cells (the seed) had a specific affinity for the milieu of certain organs (the soil). Metastases only resulted when the seed and soil were compatible (5). A prime example of this principle is the contribution of angiogenesis to the growth of neoplasms.

The growth and spread of prostate cancer are dependent on the formation of adequate vasculature, i.e., angiogenesis (4, 6, 7). Angiogenesis consists of multiple, sequential, and interdependent steps. The onset of angiogenesis involves a change in the local equilibrium between positive and negative regulatory molecules. The major proangiogenic factors include bFGF, VEGF/vascular permeability factor, IL-8, EGF, and platelet-derived growth factor (7–12).

PTKs play a key role in the control of cell proliferation (13, 14). A significant number of oncopogenes and proto-oncogenes, including EGF-R, are PTKs (13–16). Under physiological conditions, the binding of EGF to its receptor, EGF-R, leads to receptor tyrosine kinase activity and subsequently to a complex cascade of events culminating in cell proliferation, which is enhanced by

The abbreviations used are: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; IL, interleukin; PCNA, proliferating cell nuclear antigen; TGF, transforming growth factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; VEGF, vascular endothelial growth factor; PTK, protein tyrosine kinase; MAb, monoclonal antibody; IHC, immunohistochemical.
antia apoptotic effects also under the control of EGF (16). PKI 166, a novel EGF-R tyrosine kinase inhibitor of the pyrrolo-pyrimidine class (17), inhibits the intracellular domain of the EGF-R kinase, resulting in inhibition of cell proliferation and stimulation of apoptotic events (18, 19). Because increased expression of EGF, TGF-α, and EGF-R in surgical specimens of human prostate cancers correlates with rapidly progressive disease (20–24), we determined whether administration of PKI 166 to nude mice that had orthotopically implanted human prostate cancer cells in the bone marrow would block the EGF-R signaling pathway and, hence, inhibit progressive growth of experimental bone metastasis.

MATERIALS AND METHODS

PC-3MM2 Metastatic Variant of Human Prostate Cancer Cell Line. The PC-3 human prostate cancer cell line was originally obtained from the American Type Culture Collection (Rockville, MD). The PC-3M cell line was derived from a liver metastasis produced by parental PC-3 cells growing in the spleen of a nude mouse. PC-3M cells were implanted orthotopically into the prostate of nude mice, and after several cycles of in vivo selection, the highly metastatic PC-3-3MM2 line was isolated (25). The PC-3MM2 line was maintained as monolayer cultures in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, a 2-fold vitamin solution (Life Technologies, Inc.), and penicillin-streptomycin (Flow Laboratories, Rockville, MD). Cell cultures were maintained and incubated in 5% CO2/95% air at 37°C. Cultures were free of Mycoplasma and the following murine viruses: reovirus type 3; pneumonia virus; K virus; Thielier’s encephalitis virus; Sendai virus; min virus; mouse hepatitis virus; lymphocytic choriomeningitis virus; ectromelia virus; and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD).

Animals. Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were housed and maintained in specific pathogen-free conditions. The facilities were approved by the American Association for Accreditation of Laboratory Animal Care and met all current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with institutional guidelines when they were 8–12 weeks old.

Intratibial Injection of Tumor Cells. To produce bone tumors, PC-3MM2 cells were harvested from sub confluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and re suspended in Ca2+- and Mg2+-free HBSS (HBSS). Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >95% viability were used to produce tumors in the tibia of mice.

Nude mice were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL). A percutaneous intrasosseal injection was made by drilling a 27-gauge needle into the tibia immediately proximal to the tuberositas tibia. After penetration of the cortical bone, the needle was inserted into the shaft of the tibia, and 20 μl of the cell suspension (2 × 10⁵ cells) were deposited in the bone cortex using a calibrated, push-button-controlled dispensing device (Hamilton Syringe Co., Reno, NV). To prevent leakage of cells into the surrounding muscles, a cotton swab was held for 1 min over the site of injection. The animals tolerated the surgical procedure well, and no anesthesia-related deaths occurred.

Therapy of Human Prostate Cancer Cells Growing in the Tibia of Athymic Nude Mice. PKI 166 (4-[R]-phenethyl-amino-6-[hydroxy]-phenyl-7H-pyrrolo[2,3-d]-pyrimidine), a novel EGF-R tyrosine kinase inhibitor, was synthesized and provided by Novartis Pharma (Basel, Switzerland). For in vivo administration, PKI 166 was dissolved in DMSO/0.5% Tween 80 and then diluted 1:20 in water. Paclitaxel (Taxol), purchased from Bristol-Myers Squibb (Princeton, NJ) and dissolved in water for i.p. injection once per week at 200 μg/mouse.

Three days after the implantation of tumor cells in the tibia, five nude mice were killed, and the presence of actively growing cancer cells was determined by histological examination. The mice were randomized into four groups (n = 10) as follows: (a) three times per week oral administrations of vehicle solution (DMSO containing 0.5% Tween 80 diluted 1:20 in water) and once per week i.p. injection of saline; (b) once per week i.p. injection of 200 μg of Taxol; (c) three times per week oral administrations of 100 mg/kg PKI 166; and (d) three times per week oral administration of 100 mg/kg PKI 166 and once per week i.p. injection of 200 μg of Taxol. The mice were treated for 6 weeks. Tumor size and status of the injected bone (lysis) were evaluated by gross observation and digital radiography as described below.

Digitalized Radiography and Harvest of Tumors. After 3, 4, or 5 weeks of treatment, mice selected randomly from the different treatment groups were anesthetized with Nembutal and placed in a prone position. Digital radiography was carried out using the Faxitron (Faxitron X-Ray Corp., Wheeling, IL). Tumor incidence and size were recorded. The mice were euthanized on week 7 of the study (6 weeks of treatment) and weighed. The leg with tumor and the tumor-free contralateral leg were resected at the head of the femur and weighed. The net tumor weight was calculated by subtracting the weight of the uninjected leg from that of the leg with tumor. The presence of metastatic disease in macroscopically enlarged lymph nodes was confirmed by histological examination.

Reagents for Immunohistochemistry and TUNEL Assay. All antibodies for immunohistochemistry were purchased as follows: (a) rabbit anti-VEGF/vascular permeability factor, rabbit anti-fibroblast growth factor-2 (bFGF), rabbit anti-EGF, and rabbit anti-EGF-R were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); (b) rabbit anti-phospho-EGFR (activated EGF-R; Tyr845) was purchased from Cell Signaling Technology, Inc. (Beverly, MA); (c) rabbit anti-IL-8 was purchased from Biosource International (Camarillo, CA); (d) rat antimonoclonal CD31-PECAM-1 was purchased from Pharmingen (San Diego, CA); (e) mouse anti-PCNA clone PC-10 was purchased from DAKO A/S (Copenhagen, Denmark); (f) peroxidase-conjugated goat anti-rabbit IgG, peroxidase-conjugated goat antirat IgG, Texas Red-conjugated goat antirat IgG, and FITC-conjugated goat antirabbit IgG were purchased from Jackson Research Laboratories (West Grove, CA); (g) peroxidase-conjugated rat antirat IgG2a was purchased from Buretec (Harlan Bioproducts for Science, Inc., Indianapolis, IN); and (h) Alexa Fluor 594-conjugated goat antirabbit IgG was purchased...
CD31/PECAM-1 were sectioned (8–10 μm), mounted on positively charged Plus slides (Fisher Scientific), and air dried for 30 min. Frozen sections were fixed in cold acetone (5 min), 1:1 acetone/chloroform (v/v), 5 min, and acetone (5 min) and washed with PBS. IHC procedures were performed as described previously (18, 19). A positive reaction was visualized by incubating the slides with stable 3,3’-diaminobenzidine for 10–20 min. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 min, and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining. Dilutions of primary antibodies were as follows: (a) EGF-R, 1:100; (b) EGF-R, 1:50; (c) phosphorylated EGF-R, 1:50; (d) VEGF, 1:100; (e) bFGF, 1:100; (f) IL-8, 1:25; (g) PCNA, 1:100; and (h) CD31/PECAM-1, 1:400. Peroxidase-conjugated secondary antibodies were used for immunohistochemistry of EGF, EGF-R, VEGF, bFGF, IL-8, and PCNA.

Alexa Fluor 594-conjugated secondary antibody at 1:400 dilution was used for immunohistochemistry of phosphorylated EGF-R. The sections were rinsed with distilled water and mounted with Vectashield (mounting medium with 4',6-diamidino-2-phenylindole; Vector Laboratories, Inc., Burlingame, CA), which gave nuclear staining of blue fluorescence.

**Immunofluorescence Double Staining for CD31/PECAM-1 (Endothelial Cells) and EGF-R or TUNEL (Apoptotic Cells).** PLP-fixed frozen tissues were sectioned (8–10 μm), mounted on positively charged slides, air dried for 30 min, and fixed in cold acetone for 5 min, in 1:1 acetone/chloroform (v/v) for 5 min, and in acetone for 5 min. The samples were washed three times with PBS, incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min at room temperature, and then incubated with a 1:400 dilution of rat monoclonal antihuman CD31 antibody (human cross-reactive) for 18 h at 4°C. After the samples were rinsed four times with PBS for 3 min each, the slides were incubated with a 1:200 dilution of secondary goat antirabbit antibody conjugated to Texas Red for 1 h at room temperature in the dark. Samples were then washed twice with PBS containing 0.1% Brij and once with PBS for 5 min.

EGF-R immunostaining was performed after CD31 staining. Samples were incubated with protein-blocking solution for 5 min at room temperature and incubated with a 1:50 dilution of rabbit polyclonal antihuman EGF-R antibody (mouse cross-reactive) for 18 h at 4°C. The samples were then rinsed four times with PBS for 3 min each. The slides were incubated with a 1:200 dilution of secondary goat antirabbit antibody conjugated to FITC for 1 h at room temperature. Samples were washed twice with PBS containing 0.1% Brij and once with PBS for 5 min and mounted with Vectashield.

TUNEL was performed using an apoptosis detection kit with the following modifications: samples were fixed with 4% paraformaldehyde (methanol free) for 10 min at room temperature; washed twice with PBS for 5 min; and then incubated with 0.2% Triton X-100 for 15 min at room temperature. After being washed twice with PBS for 5 min, the samples were 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 terminal deoxynucleotidyl transferase enzyme was added to the
tissue sections. The sections were incubated in a humid atmosphere at 37°C for 1 h in the dark. The reaction was terminated by immersing the samples in 2× SSC for 15 min. Samples were washed three times for 5 min to remove unincorporated fluorescein-dUTP. For quantification of endothelial cells, the samples were incubated with 300 μg/ml Hoechst stain for 10 min at room temperature. Fluorescence bleaching was minimized by treating slides with an enhancing reagent (Prolong solution). Immunofluorescence microscopy was performed using a ×40 objective on an epifluorescence microscope equipped with narrow band pass excitation filters mounted on a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured using a Sony 3-chip camera (Sony Corporation of America, Montvale, NJ) mounted on a Zeiss universal microscope (Carl Zeiss, Thornwood, NY) and Optimas Image Analysis software (Bioscan, Edmond, WA) installed on a Compaq computer with Pentium chip, a frame grabber, an optical disk storage system, and a Sony Mavigraph UP-D7000 digital color printer (Tokyo, Japan). To produce prints, images were further processed using Adobe PhotoShop software (Adobe Systems, Mountain View, CA) to make figures. Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nucleus of apoptotic cells. Quantification of apoptotic endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to the total number of endothelial cells in 5–10 random 0.011-mm² fields at ×400 magnification. For the quantification of total TUNEL expression, the number of apoptotic events was counted in 10 random 0.159-mm² fields adjacent to bone and in muscles at ×100 magnification.

Quantification of Mean Vessel Density and PCNA. For the quantification of microvessel density, 10 random 0.159-mm² fields adjacent to the bone and 10 fields of tumor in the muscles at ×100 magnification were captured for each tumor, and mean vessels were quantified according to the method described previously (18, 19, 27).

Statistical Analysis. Tumor incidence (χ² test) and weight (Mann-Whitney U test), incidence of lymph node metastasis (χ² test), and expression of CD31/PECAM-1, CD31/TUNEL, TUNEL, and PCNA (unpaired Student’s t test) were compared.

RESULTS

Inhibition of EGF-R Autophosphorylation in Human Prostate Cancer Cells by PKI 166. In the first set of experiments, we determined whether in vitro treatment of PC-3MM2 cells with PKI 166 could inhibit EGF-stimulated tyrosine phosphorylation of the EGF-R. PC-3MM2 cells incubated for 15 min in medium free of serum but containing EGF exhibited high levels of autophosphorylated EGF-R (Mₚ, 170,000 band) as detected by anti-phosphotyrosine antisera on Western blots of anti-EGF-R-immunoprecipitated cell lysates (Fig. 1). Pretreatment of cells with PKI 166 for 1 h followed by a 15-min treatment with EGF inhibited the autophosphorylation in a dose-dependent manner (0–3.2 μM). The identity of the Mₚ 170,000 band was confirmed by Western blot using anti-EGF-R antisera.

Taxol, at a concentration of 10 μM, did not affect the autophosphorylation of EGF-R (data not shown).

Inhibition of Prostate Cancer Cell Growth and Metastasis. Three days after the implantation of PC-3MM2 tumor cells into the tibia of athymic nude mice, five mice were killed, and the presence of tumor cells was confirmed by histology. The remaining mice were then randomized into four treatment groups of 10 mice each. The first group received three weekly oral administrations of PKI 166 (100 mg/kg/dose). The second group was injected i.p. once per week with 200 μg of Taxol, a third group received three oral doses of PKI 166 (100 mg/kg/dose) per week and one dose of Taxol per week i.p. (200 μg/dose), and the last group received three oral doses per week of vehicle solution for PKI 166 (DMSO/water) and one i.p. injection of water per week. All mice were killed on day 35 (5 weeks of treatment) because the control mice had large tumors in the injected leg. The data of two independent experiments were very similar and therefore are combined in Table 1. All control mice had large tumors in the tibia and surrounding muscles (median weight, 2.7 g), and all mice had lymph node metastasis. Treatment with Taxol (200 μg/dose once per week) did not decrease the incidence or size of the tumors in the bone or the incidence of lymph node metastasis. Three doses of PKI 166 (100 mg/kg) significantly decreased tumor size (median weight, 1.5 g) and the incidence of lymph node metastasis (55%) as compared with control mice. The combination of oral PKI 166 and i.p. Taxol produced highly significant reductions in PC-3MM2 bone lesions. Specifically, the tumor incidence was reduced from 20 of 20 in control mice to 11 of 20 in the treated mice (P < 0.01). The median size of the tumors in the tibia and surrounding muscles was significantly reduced to 0.4 g (P < 0.001), and the incidence of lymph node metastasis was significantly reduced to 35% (P < 0.001).

Digital radiography of representative hind legs of mice from the four treatment groups are shown in Fig. 2. Severe lysis of the tibia was found in control mice and mice treated with Taxol. In mice given oral PKI 166, bone integrity was improved.
The combination of PKI 166 plus Taxol was most successful in preserving the bone structure and preventing bone lysis (Fig. 2).

**Histology and IHC Analyses.** The PC-3MM2-induced lytic lesions in the bone expanded into and grew in the surrounding muscles. Tumor specimens from the bone and muscles were processed for routine histology and IHC analyses. Tumors treated with PKI 166 and Taxol had some necrotic zones. Immunohistochemistry using specific anti-EGF, EGF-R, activated EGF-R, bFGF, VEGF, and IL-8 antibodies demonstrated striking differences in the level of expression (Fig. 3). The tumor cells growing adjacent to bone tissue expressed high levels of EGF, EGF-R, and activated EGF-R, whereas tumor cells growing in the muscle did not. The expression of bFGF, VEGF, and IL-8 also differed between tumor cells growing adjacent to bone (high expression) and those growing in the muscle (low expression; Fig. 3). Immunohistochemistry of bone and muscle lesions from control mice and mice treated with Taxol, PKI 166, or PKI 166 and Taxol did not demonstrate differences in expression of EGF, EGF-R, bFGF, VEGF, and IL-8. These data were expected because PKI 166 inhibits only phosphorylation of the EGF-R, not its expression (18, 19) or the expression of the other cytokines studied here.

The most striking results deal with the activation status of EGF-R in tumors adjacent to the bone or in the muscle of mice treated with PKI 166 alone or with PKI 166 and Taxol (Fig. 4). In all mice, only the tumor cells growing adjacent to bone tissue expressed EGF-R as detected by specific anti-EGF-R antibodies. Immunostaining with antibodies specific against tyrosine-phosphorylated (activated) EGF-R demonstrated that in control mice or mice treated with Taxol, tumor cells adjacent to bone (but not in the muscles) expressed phosphorylated EGF-R. In contrast, tumor cells adjacent to the bones of mice treated with PKI 166 or PKI 166 plus Taxol did not express activated EGF-R (Fig. 4), confirming that PKI 166 inhibits the phosphorylation of EGF-R under both in vitro and in vivo conditions.

The decrease in tumor size in mice treated with PKI 166 or with PKI 166 and Taxol could have been due to inhibition of tumor cell division, increased tumor cell apoptosis, or both. In the next set of IHC analyses, we determined the number of PCNA⁺ and TUNEL⁺ cells in PC-3MM2 tumors harvested from control and treated mice (Table 2). No significant differences in the number of PCNA⁺ cells were found between tumor lesions adjacent to the bone (136 ± 29) and those in the muscle (122 ± 25). Taxol treatment decreased the number of PCNA⁺ cells in both tumors adjacent to the bone and tumors growing in the muscle to 78 ± 33 and 75 ± 29, respectively (P < 0.001). In contrast, PKI 166 treatment decreased the number of PCNA⁺ cells in tumors growing adjacent to the bone (88 ± 20; P < 0.001) but did not decrease the number of PCNA⁺ cells in tumors growing in the muscle (122 ± 20). The decrease in PCNA⁺ cells in tumors of mice treated with Taxol plus PKI 166 differed between tumors growing adjacent to the bone (39 ± 16) and those growing in the muscle (72 ± 25).

Treatment with Taxol or PKI 166 produced a significant increase in apoptosis (TUNEL⁺ cells) in the PC-3MM2 cells growing adjacent to the bone (Table 2). Specifically, the number of TUNEL⁺ cells in control, Taxol-treated, and PKI 166-treated mice was 8 ± 2, 32 ± 15, and 29 ± 14, respectively (P < 0.001). Treatment with PKI 166 and Taxol produced a highly

**Table 1** Therapy of human prostate carcinoma growing in the bone of nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Incidencea</th>
<th>Tumor weight (g) mean ± SD</th>
<th>Lymph node metastasis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20/20</td>
<td>2.8 ± 1/1</td>
<td>100</td>
</tr>
<tr>
<td>Taxol</td>
<td>18/20</td>
<td>2.1 ± 1.5</td>
<td>90</td>
</tr>
<tr>
<td>PKI 166</td>
<td>15/20</td>
<td>1.5 ± 1.4</td>
<td>55a</td>
</tr>
<tr>
<td>PKI 166 + Taxol</td>
<td>11/20</td>
<td>0.8 ± 0.7d</td>
<td>35d</td>
</tr>
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</table>

a Human prostate cancer cells were injected into the tibia of nude mice. Three days later, groups of mice were treated with oral feeding of PKI 166 (100 mg/kg, thrice weekly) alone, weekly i.p. injection of Taxol (200 µg) alone, PKI 166 and Taxol, or water (control).

b Number of positive mice/number of mice receiving injections.
c P < 0.05.
d P < 0.001 versus control.

Fig. 2 Digitalized radiography of PC-3MM2 tumors in hind legs of nude mice. Nude mice received injection into the tibia of human PC-3MM2 prostate cancer cells. After 5 weeks of treatment with PKI 166, Taxol, or PKI 166 and Taxol, the mice were anesthetized with Nembutal and placed in a supine position for digital radiography. The PC-3MM2 cells produced lysis of the tibia in control mice and in mice treated with Taxol. In contrast, treatment with PKI 166 alone, and more so with PKI 166 plus Taxol, significantly prevented lysis of the bone.
Fig. 3 Differential expression of EGF, EGF-R, activated EGF-R, bFGF, VEGF, and IL-8 by PC-3MM2 cells growing in the bone or surrounding muscles. IHC analysis of tumor tissues demonstrates that tumor cells growing adjacent to bone tissue express high levels of EGF, EGF-R, and activated EGF-R, whereas tumor cells growing in the muscles do not. The expression of bFGF, IL-8, and VEGF also differed between tumor cells growing in the bone (high intensity) and those in the muscles (low intensity).
significant induction of apoptosis (71 ± 20 TUNEL$^+$ cells; $P < 0.001$). However, in the PC-3MM2 tumors growing in the muscle, only treatment with Taxol or Taxol plus PKI 166 induced significant levels of apoptosis (29 ± 14 TUNEL$^+$ cells; $P < 0.001$). The combination of PKI 166 and Taxol did not produce additive apoptosis in tumor cells growing in the muscle (34 ± 17 TUNEL$^+$ cells).

**Mean Vessel Density.** In the next set of IHC studies, we determined the mean vessel density in the PC-3MM2 lesions (Table 2). No discernible differences in mean vessel density were found between tumor lesions adjacent to the bone (54 ± 16) and those in the muscle (52 ± 12) of control mice (Table 2). In PC-3MM2 tumors adjacent to the bone, treatment with Taxol or PKI 166 decreased the number of endothelial cells (CD31$^+$/PECAM-1$^+$) to 38 ± 16 and 35 ± 14, respectively. The combination therapy of PKI 166 plus Taxol produced the most significant decrease in mean vessel density to 20 ± 14 ($P < 0.001$). In the PC-3MM2 lesions growing in the muscle, treatment with Taxol or Taxol plus PKI 166 decreased mean vessel density from 52 ± 21 (control mice) to 36 ± 17 and 34 ± 19, respectively ($P < 0.01$). Treatment with PKI 166 alone did not decrease mean vessel density in the tumors.

**Immunofluorescence Double Staining for CD31/PECAM-1 (Endothelial Cells) and EGF-R or TUNEL (Apoptotic Cells).** In the last set of experiments, we determined whether endothelial cells can express the EGF-R and whether treatment with Taxol, PKI 166, or both can induce apoptosis of endothelial cells within the tumors. Endothelial cells within PC-3MM2 tumors in the bone (but not in the muscle) expressed EGF-R on their surface (Fig. 5). We base this conclusion on the CD31$^+$/EGF-R fluorescent double labeling technique, which revealed that endothelial cells in the bone lesions of control mice stained yellow (CD31$^+$/PECAM, Texas Red; EGF-R, FITC green). For studies of apoptosis, the CD31/
TUNEL fluorescent double labeling technique revealed that endothelial cells within bone lesions stained yellow (CD31/PECAM, Texas Red; TUNEL, FITC green). Endothelial cells in the muscle tumors were EGF-R negative. Apoptosis of these endothelial cells was only found in mice treated with Taxol. Endothelial cells in uninvolved bones (contralateral leg) did not express EGF-R on their surface, and apoptosis of endothelial cells was not observed with treatment of PKI 166 plus Taxol.

The median percentage of apoptotic endothelial cells in bone lesions of control mice was 2% (range, 0–7%). In mice treated with PKI 166, it was 9% (range, 0–15%; P < 0.001). Treatment with Taxol induced a median of 5% (range, 0–16%) apoptosis. The combination therapy of PKI 166 and Taxol induced 16% (range, 0–23%) apoptosis (P < 0.001). In the muscle lesions, treatment with Taxol alone or with Taxol plus PKI 166 induced 6% apoptosis in endothelial cells (Table 2). No additional apoptosis of endothelial cells was found in these tumors from mice treated with PKI 166 or PKI 166 plus Taxol. These results suggest that PKI 166 can induce apoptosis only in endothelial cells that express EGF-R.

**DISCUSSION**

Our data show that human prostate cancer cells growing adjacent to mouse bone expressed high levels of EGF, EGF-R, and phosphorylated EGF-R. Moreover, endothelial cells within these tumor lesions also expressed activated EGF-R, whereas endothelial cells in the uninvolved bone marrow or in tumors growing in the muscles did not. Similar to the clinical experience with taxane-based regimens (28, 29), systemic administration of Taxol did not inhibit tumor growth or destruction of the tumor-associated endothelial cells. Because TGF-β has been shown to inhibit the progression of many human carcinomas, including prostate, colon, pancreatic, gastric, ovarian, renal, bladder, breast, and non-small cell lung cancer, has been associated with expression of EGF-R (21, 30–34). Overexpression of EGF-R and one of its ligands has been shown to correlate with rapid progression disease (35). Our present results closely agree with previous studies showing that targeting the EGF-R by the anti-EGF-R C225 antibody in combination with radiation or chemotherapeutic agents can significantly inhibit the growth of human tumors in nude mice (36–42). The present results also agree with our previously published data (18) showing that blockade of EGF-R in a signal of PKI 166 inhibits growth of human pancreatic carcinoma in nude mice and produces apoptosis of tumor-associated endothelial cells.

**Table 2** Immunohistochemical analysis of human prostate carcinoma in the bone of control and treated nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Bone</th>
<th>Muscle</th>
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<tbody>
<tr>
<td></td>
<td>Tumor cells</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td></td>
<td>PCNA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TUNEL&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>136 ± 29</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Taxol</td>
<td>78 ± 33&lt;sup&gt;e&lt;/sup&gt;</td>
<td>32 ± 15&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PKI 166</td>
<td>88 ± 20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>29 ± 14&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PKI 166 + Taxol</td>
<td>39 ± 16&lt;sup&gt;e&lt;/sup&gt;</td>
<td>71 ± 20&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup>Human prostate cancer cells were injected into the tibia of nude mice. Three days later, groups of mice were treated with oral feedings of PKI 166 (100 mg/kg, thrice weekly) alone, weekly i.p. injection of Taxol (200 μg) alone, PKI 166 and Taxol, or water (control).

<sup>b</sup>Mean ± SD.

<sup>c</sup>Median of the ratio of apoptotic endothelial cells to total number of endothelial cells in 5–10 random 0.011-mm<sup>2</sup> fields at ×400.

<sup>d</sup>P < 0.001 as compared with controls.

<sup>e</sup>P < 0.05 as compared with the group treated with Taxol alone.

The oral administration of PKI 166 alone or in combination with Taxol also produced apoptosis in tumor-associated endothelial cells (TUNEL<sup>+</sup>/CD31<sup>+</sup> cells), corresponding to a significant decrease in microvessel density.

The progressive growth of many human carcinomas, including prostate, colon, pancreatic, gastric, ovarian, renal, bladder, breast, and non-small cell lung cancer, has been associated with expression of EGF-R (21, 30–34). Overexpression of EGF-R and one of its ligands has been shown to correlate with rapid progression disease (35). Our present results closely agree with previous studies showing that targeting the EGF-R by the anti-EGF-R C225 antibody in combination with radiation or chemotherapeutic agents can significantly inhibit the growth of human tumors in nude mice (36–42). The present results also agree with our previously published data (18) showing that blockade of EGF-R signaling with PKI 166 inhibits growth of human pancreatic carcinoma in nude mice and produces apoptosis of tumor-associated endothelial cells.

Detailed histological and IHC analyses revealed that robust angiogenesis in these lesions could well be due to two parallel (and additive) mechanisms. First, the major-
and do not divide. In contrast, many endothelial cells within progressive neoplasms are actively dividing, and dividing endothelial cells have been shown to express low levels of EGF-R (47). Mere proliferation, however, is not sufficient to account for high-level expression of EGF-R or its activation. Recent work from our laboratory revealed that murine endothelial cells do indeed express EGF-R when exposed in culture to EGF or TGF-α in a dose-dependent manner. Specifically, the expression of EGF-R and activated EGF-R required incubation of endothelial cells with 20 ng/ml EGF (47). As seen in Fig. 3, PC-3MM2 cells growing adjacent to the bone cortex produce EGF, whereas tumor cells growing at a distance from the bone do not.

Fig. 5 Immunohistochemistry and immunofluorescent double labeling for CD31/PECAM-1 (Texas Red) and EGF-R (FITC green), or TUNEL (FITC green). Images captured for endothelial cells (CD31 red) are overlapped with images captured for EGF-R (green) or apoptosis (TUNEL green) and expression of two markers, CD31/EGF-R or CD31/TUNEL (both cells emitted yellow). Tumor lesions in the bone contained numerous blood vessels (CD31+ structures), and mean vascular densities were significantly reduced in the combined treatment group. Tumor cells growing adjacent to bone tissue expressed EGF and EGF-R. Endothelial cells (red) within the bone lesions also expressed EGF-R (fluorescent yellow). Treatment of mice with PKI 166 plus Taxol induced apoptosis of tumor cells (green) and endothelial cells (yellow) within the bone lesions. Treatment of mice with PKI 166 plus Taxol did not induce apoptosis in the endothelial cells in the tumors growing in the muscles.
The level of expression of EGF-R by endothelial cells within the bone tumor lesions could be related to the concentration of EGF in the microenvironment. The finding that the expression of growth factor receptors (and their activation) on endothelial cells is conditioned by the organ microenvironment is another example that supports the venerable “seed and soil” hypothesis (4, 5, 10). In any event, the expression of activated EGF-R in tumor-associated endothelial cells, but not in endothelial cells within the uninvolved organ, makes EGF-R an attractive target for specific antivascular therapy.

Treatment of mice with PKI 166 and Taxol significantly decreased the number of bone tumor-associated endothelial cells (but not the number of endothelial cells in muscle lesions). The increase of apoptosis in endothelial cells providing vasculature to the bone tumors could be attributed to blockade of EGF-R, which results in cellular arrest at the G1 restriction point (34, 37, 38) and a decrease in expression of proangiogenic molecules that serve as survival factors for immature blood vessel endothelial cells (48–51). The decrease in production of proangiogenic molecules can prevent the recovery of dividing endothelial cells damaged by Taxol and hence lead to increased apoptosis in tumor-associated, dividing endothelial cells. Moreover, EGF-R and its associated PTKs are known to regulate apoptosis (52, 53), and inactivation of EGF-R PTK has been shown to inhibit EGF-induced receptor autophosphorylation, mitogen-activated protein kinase activation, phosphatidylinositol 3’-kinase activity, entry into S phase, and cyclin E-associated kinase activity, all leading to accumulation of cells in the G1 phase of the cell cycle (54). Induction of endothelial cell apoptosis was found only in cells stimulated with EGF (or TGF-α), i.e., cells that expressed the activated EGF-R. Our data agree with a previously published report that inhibition of EGF-R tyrosine kinase by another PTK inhibitor, ZD 1839 (Iressa), can produce antiangiogenic effects (55).

Cancers are biologically heterogeneous, and their phenotype is modified by the organ environment (3, 4). Successful treatment of cancers and their metastases therefore requires multiple agents. The results shown here clearly demonstrate that treatment with PKI 166 and Taxol produces a decrease in tumor size and incidence of metastasis. The combination of PKI 166 plus Taxol, however, produces additive effects demonstrable by induction of apoptosis in tumor cells and, more so, tumor-associated endothelial cells. These results confirm that a heterogeneous disease should be treated by multiple modality therapy.

The outcome of cancer metastasis is controlled by the interaction of specific tumor cells (the seed) with unique host microenvironment (the soil; Ref. 56). Organ-specific angiogenesis is essential for progressive growth of metastases. The demonstration that endothelial cells in experimental prostate cancer bone metastases express growth factor receptor differentially from endothelial cells in uninvolved zones of the same organ offers an exciting new approach to the specific therapy of a devastating disease.

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Blockade of Epidermal Growth Factor Receptor Signaling in Tumor Cells and Tumor-associated Endothelial Cells for Therapy of Androgen-independent Human Prostate Cancer Growing in the Bone of Nude Mice

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