Simultaneous Blockade of Platelet-Derived Growth Factor-Receptor and Epidermal Growth Factor-Receptor Signaling and Systemic Administration of Paclitaxel as Therapy for Human Prostate Cancer Metastasis in Bone of Nude Mice

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ABSTRACT

Once prostate cancer metastasizes to bone, conventional chemotherapy is largely ineffective. We hypothesized that inhibition of phosphorylation of the epidermal growth factor receptor (EGF-R) and platelet-derived growth factor receptor (PDGF-R) expressed on tumor cells and tumor-associated endothelial cells, which is associated with tumor progression, in combination with paclitaxel would inhibit experimental prostate cancer bone metastasis and preserve bone structure. We tested this hypothesis in nude mice, using human PC-3-MM2 prostate cancer cells. PC-3-MM2 cells growing adjacent to bone tissue and endothelial cells within these lesions expressed phosphorylated EGF-R and PDGF-R. Combination therapy using oral PKI166 and STI571 with i.p. injections of paclitaxel induced a high level of apoptosis in tumor vascular endothelial cells and tumor cells in parallel with inhibition of tumor growth in the bone, preservation of bone structure, and reduction of lymph node metastasis. Collectively, these data demonstrate that blockade of phosphorylation of EGF-R and PDGF-R coupled with administration of paclitaxel significantly suppresses experimental human prostate cancer bone metastasis.

INTRODUCTION

Prostate cancer is the second greatest cause of cancer-related death among men in North America (1, 2). Metastasis to the bone, which is resistant to conventional therapies, causes devastating symptoms such as intractable bone pain, nerve compression, and pathological fractures (3, 4). Despite progress in the detection of prostate cancer, ~24% of patients have metastatic lesions at initial diagnosis, and in an additional 30% of the patients, metastases are discovered during surgical staging. A significant number of patients with clinically localized disease who are treated with radical prostatectomy may also develop metastasis (5–8). Clearly then, the development of new treatment modalities for metastatic prostate cancer is important to the many men who will have metastatic prostate cancer.

Developing such a treatment requires an understanding of the progression of prostate cancer. To produce metastasis in the bone, prostate cancer cells must complete a series of sequential and highly selective steps (5, 9, 10). We recently confirmed the “seed and soil” hypothesis of Stephen Paget (11) that certain tumor cells have a specific affinity for the milieu of certain organs. Specifically, we reported that the expression of various cytokines, growth factors, and their receptors on prostate cancer cells and endothelial cells differ in different organ microenvironments (12, 13). Moreover, cell response to protein tyrosine kinase receptor inhibitors, such as epidermal growth factor receptor (EGF-R; Ref. 12) and platelet-derived growth factor receptor (PDGF-R; Ref. 13), depends on the host organ microenvironment. EGF and EGF-R pathways have been reported to be related to cell proliferation (14, 15), activation of antiapoptotic effects (16), and disease progression (17–21). The PDGF and PDGF-R pathways are involved in cell division (22–24), cell migration (25), angiogenesis (26), and cell survival (27).

In our animal model, endothelial cells in tumors growing in the bone expressed phosphorylated EGF-R or PDGF-R on their surfaces, whereas endothelial cells in tumors growing in the muscle or endothelial cells in unaffected tissues did not (12, 13). Oral administration of the protein tyrosine kinase inhibitors PKI166 (28) or STI571 (29, 30) has been found to inhibit phosphorylation of EGF-R and PDGF-R, respectively, and when each was combined with i.p. injection of paclitaxel, these inhibitors significantly inhibited tumor growth (12, 13). Because human prostate cancer cells growing adjacent to bone tissue express both EGF and PDGF and both tumor cells and tumor-associated endothelial cells express phosphorylated EGF-R and PDGF-R, we hypothesized that simultaneous targeting of both receptors combined with systemic injections of paclitaxel would produce additive therapeutic results. We report here the therapeutic effects of combining PKI166 with STI571 and paclitaxel on experimental human prostate cancer bone metastases in the tibias of nude mice.

MATERIALS AND METHODS

PC-3-MM2 Metastatic Variant of Human Prostate Cancer Cell Line. The highly metastatic cell line PC-3-MM2 (31) was maintained as monolayer cultures in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, t-glutamine, a 2-fold vitamin solution (Life Technologies, Inc.), and penicillin-streptomycin (Flow Laboratories, Rockville, MD) in 5% CO₂ at 95% air at 37°C. The cultures were free of Mycoplasma and the following murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler’s encephalitis virus, ectomelia 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 6 to 8 weeks of age.

Intratibial Injection of Tumor Cells. To produce bone tumors, PC-3-MM2 cells were harvested from subconfluent cultures by 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 resuspended in Ca⁺²⁺- and Mg⁺²⁺-free HBSS. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions with >95% viability were used to produce tumors in the tibias of mice.

Nude mice were anesthetized with Nembutal (0.5 mg/g of body weight;
Abbott Laboratories, Chicago, IL). A percutaneous intraosseous 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 × 10^6 cells) were deposited in the bone cortex by use of 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 for Human Prostate Cancer Cells Growing in the Tibias of Athymic Nude Mice.** PK1166 (4-phenethylaminio-6-[hydroxyl]phenyl-7H-pyrrolo[2,3-d]-pyrimidine), a novel EGF-R tyrosine kinase inhibitor, was synthesized and provided by Novartis Pharma (Basel, Switzerland). For oral administration (three times per week), PK1166 was dissolved in DMSO–0.5% Tween 80 and then diluted 1:20 in water (12). The PDGF-R tyrosine kinase inhibitor STI571 (imatinib meslate, Gleevec), synthesized and provided by Novartis Pharma (Basel, Switzerland), was dissolved in distilled water at 0.25 mg/ml for daily oral administration (13). Paclitaxel (Taxol) purchased from Bristol-Myers Squibb (Princeton, NJ) was diluted in water for once per week i.p. injection.

Three days after the implantation of tumor cells into the tibias, five mice were killed, and the presence of actively growing cancer cells was confirmed by histology. The remaining mice were randomized into eight treatment groups (n = 10) as follows: (a) control mice receiving daily administration of distilled water and one i.p. injection per week of distilled water (n = 5) or oral administration three times per week of water diluted 1:20 with DMSO–0.5% Tween 80 and a once-per-week i.p. injection of distilled water (n = 5); (b) once-per-week i.p. injection of paclitaxel at 8 mg/kg and daily oral administration of distilled water (n = 5) or oral administration three times per week of water diluted 1:20 with DMSO–0.5% Tween 80 (n = 5); (c) oral administration three times per week of 100 mg/kg PK1166 and once per week i.p. injection of distilled water; (d) daily administration of 50 mg/kg STI571 and once per week i.p. injection of distilled water; (e) oral administration three times per week of 100 mg/kg PK1166, a daily oral administration of STI571 at 50 mg/kg, and once per week i.p. injection of distilled water; (f) oral administration three times per week of 100 mg/kg PK1166 and once per week i.p. injection of paclitaxel at 8 mg/kg; (g) a daily oral administration of STI571 at 50 mg/kg and once per week i.p. injection of paclitaxel at 8 mg/kg; and (h) oral administration three times per week of 100 mg/kg PK1166, daily oral administrations of STI571 at 50 mg/kg, and once per week i.p. injection of paclitaxel at 8 mg/kg. All mice were treated for 5 weeks. Tumor size and status of injected bone (lysis) were evaluated by gross observation and digital radiography as described below.

**Digital Radiography and Harvest of Tumors.** After 3 and 4 weeks of treatment, mice selected randomly from each of the different treatment groups were anesthetized with Nembutal (0.5 mg/g body weight) and placed in a prone position. Digital radiography was carried out with a Faxitron (Faxitron X-Ray Corporation, Wheeling, IL). On week 6 of the study (5 weeks of treatment), all mice were euthanized by injection of Nembutal (1.0 mg/g) and weighed. Digital radiography was carried out on the hind limbs of each mouse, and tumor incidence and size were recorded. The tumor-bearing leg and the tumor-free contralateral leg of each mouse were resected at the head of the femur and weighed. Digital radiography was carried out with a Faxitron (Faxitron X-Ray Corporation, Wheeling, IL). On week 6 of the study (5 weeks of treatment), all mice were euthanized by injection of Nembutal (1.0 mg/g) and weighed. Digital radiography was carried out on the hind limbs of each mouse, and tumor incidence and size were recorded. The tumor-bearing leg and the tumor-free contralateral leg of each mouse were resected at the head of the femur and weighed. The net tumor weight was calculated by subtraction. Macroscopically enlarged lymph nodes were harvested and prepared for histological examination to determine the presence of metastasis.

**Reagents for Immunohistochemical and Terminal Deoxynucleotidyltransferase-Mediated Nick End Labeling (TUNEL) Assay.** All antibodies for immunohistochemistry were purchased as follows: rabbit anti-EGF-R (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-PDGFRα and -β (Santa Cruz Biotechnology); rabbit anti-CD31 (PharMingen, San Diego, CA); mouse anti-proliferative cellular nuclear antigen (PCNA) clone PC-10 (DAKO A/S, Copenhagen, Denmark); peroxidase-conjugated goat anti-rabbit IgG, peroxidase-conjugated goat antirat IgG, Texas Red-conjugated goat antirat IgG, and FITC-conjugated goat anti-rabbit IgG (Jackson Research Laboratories, West Grove, PA); rabbit anti-CD31-PECAM-1 (Endothelial Cells) and EGF-R, PDGF-R, or TUNEL. Microarray analysis was performed in six fields of each slide and analyzed statistically.

**Immunofluorescence Double Staining for CD31/PECAM-1 (Endothelial Cells) and EGF-R, PDGF-R, or TUNEL.** Pyridoxal phosphate-fixed tissues were sectioned (8–10 μm), mounted on positively charged Plus slides (Fisher Scientific), and air-dried for 30 min. Frozen sections were washed three times with PBS. IHC procedures were performed as described previously (12, 13). Control samples exposed to secondary antibody alone showed no specific staining. Dilutions of primary antibodies were as follows: EGF-R (1:50), phosphorylated EGF-R (1:50), PDGF-Rα and -β (1:100), phosphorylated PDGF-Rβ (1:100), PCNA (1:100), and CD31/PECAM-1 (1:400). Samples for EGF-R, activated EGF-R, PDGF-Rα and -β, CD31/PECAM-1, and PCNA were incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum for 20 min, and the sample for activated PDGF-Rβ was incubated with protein-blocking solution containing 4% fish gel (Cold Water Fish Skin Gelatin, 40%, Aurion; Electron Microscopy Science, Fort Washington, PA) in PBS for 20 min. We used stable 3,3′-diaminobenzidine (Research Genetics) and Gill’s hematoxylin (Gill’s hematoxylin, Santa Cruz, CA) for visualization of immunohistochemical staining with EGF-R or PDGF-Rα, PDGF-Rβ, PCNA, and CD31/PECAM-1 and counterstaining or Alexa Fluor 594-conjugated secondary antibody for immunohistochemistry of EGF-R, PDGF-R, phosphorylated EGF-R, and phosphorylated PDGF-Rβ. The sections stained with immunofluorescence-tagged secondary antibody were rinsed with distilled water and mounted with Vectashield (mounting medium with 4',6-diamidino-2-phenylindole; Vector Laboratories, Inc., Burlingame, CA), which gave blue fluorescence nuclear staining. Immunofluorescence microscopy was performed with an ×20 or ×40 objective on an epifluorescence microscope equipped with narrow band-pass excitation filters mounted on a filter wheel (Ludl Electronic Products, Hawthorne, NY).

Distance from bone fragments was marked, and cells were counted in ×100 fields (×10 objective and ×10 ocular; 0.14 mm²/field) to calculate the percentage of cells stained positively for EGF-R or PDGF-Rα and -β (% positive cells in number of positive cells in 0.14 mm²/total number of cells in 0.14 mm² × 100). The intensity of staining was measured in the same fields where positivity of cells was calculated by use of ImageQuant analyzer and Optima software program (Bioscan, Edmonds, WA). Cell counting and intensity measurements were performed in six fields of each slide and analyzed statistically.
incubated with a 1:400 dilution of rat monoclonal antimouse CD31 antibody (human cross-reactive) over 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, PDGFR-α, and PDGFR-β 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 anti-EGF-R antibody or 1:100 dilution of anti-PDGFR-α or -PDGFR-β antibody for 18 h at 4°C. The samples were then rinsed four times with PBS for 3 min each, and 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.

Endothelial cells were identified by red fluorescence, and EGF-R, PDGFR-α, and PDGFR-β were identified by green fluorescence. Colocalization of endothelial cells/EGF-R or endothelial cells/PDGFR-α, PDGFR-β (endothelial cells, red + EGF-R, PDGFR-α, or PDGFR-β green = yellow) was obtained by superimposing two images. We measured the distance of endothelial cells expressing EGF-R or PDGFR-α and -β (yellow) from the cells expressing EGF-R or PDGFR-α and -β (green) with the Euclidian distance map (33, 34).

TUNEL was performed using an apoptosis detection kit with the following modification: 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 the kit) for 10 min at room temperature. The equilibration buffer was drained, and reaction buffer containing equilibration buffer, nucleotide mixture, and terminal deoxynucleotidyl transferase enzyme was added to the tissue sections. The sections were incubated in a humidified atmosphere of 37°C for 1 h in the dark. The reaction was terminated by immersing the samples in 2× SSC for 15 min, and 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 the slides with an enhancing reagent (Prolong Solution, Prolong Anfade Kit; Molecular Probes). Immunofluorescence microscopy was performed with a ×40 objective on a Zeiss epifluorescence microscope equipped with narrow band-pass excitation filters mounted on a filter wheel (Ludl Electronic Products) to individually select for green, red, and blue fluorescence. Images were captured by use of a Sony 3-chip color camera (Sony, Tokyo, Japan). To produce prints, images were further processed by use of Adobe PhotoShop software (Adobe Systems, Mountain View, CA). Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nuclei of apoptotic cells. Apoptosis in endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to total number of endothelial cells in 5–10 random 0.011-mm² fields at ×400 magnification.

Quantification of Mean Vessel Density and PCNA. For quantification of microvessel density, 10 random 0.159-mm² fields in the bone at ×100 magnification were captured for each tumor, and the vessel fields were counted according to the method described previously (35). Cells positively stained with anti-PCNA antibody were counted in the same fields.

Statistical Analysis. Tumor incidence (x² test), weight, positivity and intensity of EGF-R and PDGFR-α and -β (Mann–Whitney U test), incidence of lymph node metastasis (x² test), and PCNA-positive cells, mean vessel density (CD31/PECAM-1), and ratio of CD31/PECAM-1/TUNEL localized cells (unpaired Student’s t test) were compared.

RESULTS
Inhibition of Prostate Cancer Cell Growth and Metastasis. We determined the therapeutic efficacy of PKI166, STI571, and paclitaxel alone or in different combinations on the growth and metastasis of human prostate cancer cells implanted in the tibias of nude mice and the bone structure. The data from two independent experiments were very similar and were therefore combined and analyzed together (Table 1). All control mice had large tumors in the tibias and surrounding muscles (median weight, 4.0 g; range, 2.5–6.4 g) with 100% incidence of lymph node metastasis. All mice receiving only paclitaxel also developed large tumors (median weight, 3.4 g; range, 1.2–5.0 g) with 100% incidence of lymph node metastasis. Similar to our previous reports (12, 13), oral administrations of PKI166 (three times per week) significantly (P < 0.05) decreased tumor incidence, tumor size, and lymph node metastasis. The daily oral administration of STI571 also significantly (P < 0.05) decreased tumor incidence, tumor size, and lymph node metastasis. Tumor incidence, size of tumor, and lymph node metastasis were decreased even more (P < 0.01) in mice receiving the combination of PKI166 and paclitaxel. Similarly, therapy with STI571 and paclitaxel significantly decreased (P < 0.01) tumor incidence, median tumor weight, and lymph node metastasis. Combination treatment with PKI166 and STI571 did not produce any additive effects over either PKI166 or STI571 administered as single agents; however, combination therapy with PKI166, STI571, and paclitaxel produced the greatest improvement (P < 0.001). Specifically, tumor incidence, median tumor weight, and incidence of lymph node metastasis were all further reduced.

Digital radiographs of representative hind legs of mice from the eight treatment groups are shown in Fig. 1. Control mice and mice treated with paclitaxel alone had severe lysis of the tibia. In mice given oral PKI166 or STI571, lysis of the bone was less pronounced. In mice treated with oral PKI166 and STI571, the lysis of bone was reduced. Mice treated with oral PKI166 and injectable Taxol and mice treated with oral STI571 and injectable Taxol exhibited a significant decrease in bone lysis. In mice given the combination of PKI166, STI571, and paclitaxel, the bone structure was remarkably preserved (Fig. 1).

Site-Specific Expression of Growth Factors and Receptors. PC-3MM2 prostate cancer cells can produce lysis of bone and invade the surrounding musculature where they proliferate into large lesions. In this study as well as our previous reports using PKI166 plus Taxol (12) or STI571 plus Taxol (13), PC-3MM2 tumor cells growing in the muscle did not respond to therapy. We therefore investigated the reason that PC-3MM2 cells growing in the muscle did not respond to therapy with either PKI166 or STI571. Because targeted therapy requires that tumor cells (or endothelial cells) express a target, i.e., phosphorylated protein tyrosine kinase (34), we determined whether the level and expression pattern of EGF-R and PDGFR-β on tumor

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Bone lesion</th>
<th>Tumor incidence</th>
<th>Tumor weight (g)</th>
<th>Incidence of lymph node metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5/20</td>
<td>3.5 ± 1.2</td>
<td>20/20</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>3/20</td>
<td>3.8 ± 1.2</td>
<td>20/20</td>
<td></td>
</tr>
<tr>
<td>PKI166</td>
<td>2/20</td>
<td>4.0 ± 1.2</td>
<td>18/20</td>
<td></td>
</tr>
<tr>
<td>STI571</td>
<td>1/20</td>
<td>4.2 ± 1.2</td>
<td>18/20</td>
<td></td>
</tr>
<tr>
<td>PKI166 + paclitaxel</td>
<td>2/20</td>
<td>4.0 ± 1.2</td>
<td>18/20</td>
<td></td>
</tr>
<tr>
<td>STI571 + paclitaxel</td>
<td>1/20</td>
<td>4.2 ± 1.2</td>
<td>18/20</td>
<td></td>
</tr>
<tr>
<td>PKI166 + STI571</td>
<td>1/20</td>
<td>4.2 ± 1.2</td>
<td>18/20</td>
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<tr>
<td>PKI166 + STI571 + paclitaxel</td>
<td>1/20</td>
<td>4.2 ± 1.2</td>
<td>18/20</td>
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* * * Compared with control group: * P < 0.05, ** P < 0.01, *** P < 0.001.
of staining for EGF-R was significantly decreased in cells growing more than 1.0 mm away from the nearest bone fragment ($P < 0.001$, Mann-Whitney $U$ test). The percentage of positive cells stained with PDGF-R also correlated with distance from nearest bone fragment-tissue (Table 3). The percentage of positive tumor cells growing more than 2 mm away from the nearest bone tissue was significantly decreased ($P < 0.001$, Mann-Whitney $U$ test). Staining intensity also inversely correlated with distance from the bone tissue (Table 3).

We next examined whether the expression of EGF-R, PDGF-R$, and PDGF-R$ on endothelial cell surfaces correlated with expression on tumor cells. Endothelial cells expressing EGF-R on their surfaces were mostly located within 20 $\mu$m ($19.6 \pm 8.0 \mu$m) of tumor cells expressing EGF-R on their surfaces (Fig. 3). Endothelial cells expressing PDGF-R$ or PDGF-R$ were located within 36–37 $\mu$m ($36.6 \pm 11.4$ and $37.4 \pm 10.6 \mu$m, respectively) of tumor cells positive for this receptor.

**Specificity of Tyrosine Kinase Inhibitors.** As shown in Fig. 4, PKI166 and STI571 treatments were specific for cells that expressed

<table>
<thead>
<tr>
<th>Distance from bone (mm)</th>
<th>Positive cells$^{a,b}$ (%)</th>
<th>Intensity$^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0–0.5</td>
<td>84 ± 10</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>0.5–1.0</td>
<td>40 ± 10</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>1.0–1.5</td>
<td>14 ± 8$^{c}$</td>
<td>0.7 ± 0.1$^{c}$</td>
</tr>
<tr>
<td>1.5–2.0</td>
<td>6 ± 2$^{c}$</td>
<td>0.5 ± 0.1$^{c}$</td>
</tr>
<tr>
<td>2.0–2.5</td>
<td>4 ± 2$^{c}$</td>
<td>0.3 ± 0.2$^{c}$</td>
</tr>
</tbody>
</table>

$^{a}$ Number of positive cells in 0.14 mm$^{2}$/number of cells in 0.14 mm$^{2} \times 100%$.
$^{b}$ Mean ± SD.
$^{c}$ $P < 0.001$, Mann-Whitney $U$ test, compared with control group.

Fig. 2. Immunohistochemical analyses to determine expression of epidermal growth factor receptor (EGF-R) and platelet-derived growth factor receptor $\alpha$ (PDGF-R$\alpha$) and $\beta$ (PDGF-R$\beta$) in PC-3MM2 tumor cells growing in the tibias of nude mice. Samples were stained with anti-EGF-R or anti-PDGF-R$\alpha$ or $\beta$ antibodies. The distance from bone was marked, and the staining intensity in several 0.14-mm$^{2}$ fields was analyzed by ImageQuant analyzer and Optima software program. The percentage of positively stained cells was calculated using the formula: $\%$ positive cells = number of positive cells/total number of cells $\times 100%$.

Table 2. Epidermal growth factor receptor expression on PC-3MM2 cells in bone lesions

Distance from the bone was marked, and cells were counted in multiple $\times$ 100 fields ($\times$ 10 objective and $\times$ 10 ocular; 0.14-mm$^{2}$/field), and the percentage of positive cells stained with epidermal growth factor receptor was calculated. The intensity of staining was measured in the same fields by use of ImageQuant analyzer and Optima software program (Bioscan). Cell counting and intensity measurements were performed in six fields of each slide and analyzed statistically.

Fig. 1. Digital radiographs of hind legs of nude mice. Nude mice implanted with human PC-3MM2 cells in the tibia received 5 weeks of treatment with single or various combinations of vehicle solution (Control), paclitaxel (8 mg/kg dose; i.p. injection once per week), PKI166 (100 mg/kg; oral administration three times per week), and STI571 (50 mg/kg; daily oral administration). The mice were then euthanized with 1 mg/g Nembutal and analyzed by digital radiography in a prone position. Tibias of mice treated with PKI166 and/or STI571 with paclitaxel were well preserved. Combination therapy of PKI166, STI571, and Taxol produced the most significant inhibition of tumor growth and the best preservation of the bone structure.

cells growing adjacent to and distant from bone tissue can provide an explanation (Fig. 2). The percentage of cells positive for EGF-R decreased with distance from the nearest bone tissue, from $84 \pm 10\%$ (mean ± SD) in tumors within 0.5 mm from the nearest bone fragment to $4\%$ ± $2\%$ in tumors 2.0–2.5 mm from nearest bone fragment ($P < 0.001$, Mann–Whitney $U$ test; Table 2). The intensity
Table 3 Platelet-derived growth factor receptor (PDGF-R) expression on PC-3MM2 cells in bone lesions

Distance from the bone was marked, and cells were counted in multiple ×100 fields (10×10 objective and ×10 ocular; 0.14-mm²/field), and the percentage positive cells stained with PDGF-Rα or PDGF-Rβ was calculated. The intensity of staining was measured in the same fields by use of an ImageQuant analyzer and Optima software program (Bio-scan). Cell counting and intensity measurements were performed in six fields of each slide and analyzed statistically.

<table>
<thead>
<tr>
<th>Distance from bone (mm)</th>
<th>PDGF-Rα Positive cells (%)</th>
<th>Intensity</th>
<th>PDGF-Rβ Positive cells (%)</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1</td>
<td>79 ± 12</td>
<td>2.0 ± 0.5</td>
<td>84 ± 14</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>1–2</td>
<td>51 ± 5</td>
<td>1.3 ± 0.3</td>
<td>52 ± 23</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>2–3</td>
<td>43 ± 3°</td>
<td>0.8 ± 0.2°</td>
<td>34 ± 19°</td>
<td>0.8 ± 0.3°</td>
</tr>
<tr>
<td>3–4</td>
<td>26 ± 8°</td>
<td>0.9 ± 0.1°</td>
<td>33 ± 13°</td>
<td>0.5 ± 0.2°</td>
</tr>
<tr>
<td>4–5</td>
<td>4 ± 2°</td>
<td>0.3 ± 0.2°</td>
<td>4 ± 1°</td>
<td>0.2 ± 0.2°</td>
</tr>
</tbody>
</table>

a Number of positive cells in 0.14 mm²/total number of cells in 0.14 mm² × 100%.

b Mean ± SD.

c P < 0.001, Mann–Whitney U test, compared with control group.

the corresponding receptor. Control treatments did not inhibit phosphorylation (activation) of EGF-R or PDGF-Rβ. Treatment with PKI166 inhibited phosphorylation only of EGF-R, treatment with STI571 inhibited phosphorylation only of PDGF-Rβ, and the combination of PKI166 and STI571 inhibited phosphorylation of both EGF-R and PDGF-Rβ (Fig. 4). Similar data were obtained with PDGF-Rα (data not shown).

Cell Proliferation and Apoptosis. We next determined the number of PCNA+ and TUNEL+ cells in PC-3MM2 tumors harvested from the tibias of control and treated mice (Table 4). Tumors from control mice had 130 ± 14 CD31+ endothelial cells in control mice. All of the various treatments produced significant reductions, with the largest decrease (to 12 ± 6) in CD31+ cells occurring in mice given a combination of all three agents (P < 0.001).

Immunofluorescence Double Staining for CD31/PECAM-1 (Endothelial Cells) and TUNEL (Apoptotic Cells). Using the results of our previous study, we determined whether the combination of all three agents would significantly increase apoptosis in endothelial cells. The CD31/TUNEL fluorescent double-labeling technique (colocalization) revealed a median percentage of apoptotic endothelial cells in control mice of 2% (range, 0–7%). Mice in the treatment groups had median percentages of 7% (range, 0–15%) to 19% (range, 5–26%) apoptotic endothelial cells (Table 4).

DISCUSSION

The progressive growth of metastases is dependent on the presence of an adequate blood supply and the development of vasculature, i.e., angiogenesis is a rate-limiting factor for this process (36). In previous studies, we reported that human prostate cancer cells growing adjacent to bone tissue express high levels of EGF and PDGF and that the tumor cells and tumor-associated endothelial cells, but not endothelial cells in uninvolved bone, expressed EGF-R and PDGF-R (12, 13). These data confirmed that targeting the tumor cells and the organ microenvironment can produce significant therapeutic benefits (37). Because prostate cancer cells growing adjacent to bone tissue and endothelial cells within these lesions expressed both EGF-R and PDGF-R, it raised the question of whether simultaneous inhibition of phosphorylation of both receptors combined with systemic adminis-
and PDGF can up-regulate (and activate) the expression of their respective receptors in osteoblasts and osteoclasts. The production of EGF in response to bone tissue is associated with expression of transforming growth factor receptor β (PDGF-Rβ) in PC-3MM2 human prostate cancer cells growing in the tibias of nude mice. Samples were stained with anti-phospho-EGF-R or anti-phospho-PDGF-Rβ.

In the first set of experiments, we determined the relationship of the expression of EGF or PDGF and their receptors. The production of EGF and PDGF can up-regulate (and activate) the expression of their respective receptors in an autocrine (tumor cells) and paracrine manner (tumor-associated endothelial cells). Indeed, our present study demonstrates that endothelial cells expressing EGF-R on their surface are located within 20 μm of tumor cells expressing EGF/EGF-R. Endothelial cells expressing PDGF-Rα or -β are located within 36–37 μm of tumor cells expressing PDGF/PDGF-R. These slight differences may well be due to either the concentration of the ligands or to differences in their diffusion coefficients within solid tissues.

The expression of EGF or PDGF and their receptors has been shown to correlate with progressive growth of human carcinomas of the prostate (43, 44), ovary (45, 46), lung (47, 48), colon (49, 50), stomach (51, 52), and esophagus (53, 54), breast (55, 56) as well as gliomas (57, 58) and melanomas (59, 60); therefore, blockade of their signaling pathways has been developed as a new therapeutic strategy (61–65). Specifically, blockade of EGF-R signaling has been shown to produce arrest at the G1 restriction point (66, 67) and to increase apoptosis (68, 69). Administration of STI571, which inhibits phosphorylation of PDGF-R, has been shown to inhibit depolymerization of microtubules (70, 71).

Whether protein tyrosine kinase inhibitors have specific, restricted activity has been controversial (61). Our present data directly addressed this question. We report that the systemic administration of PKI166 inhibited phosphorylation of EGF-R but not PDGF-R in tumor cells and endothelial cells, whereas systemic administration of STI571 inhibited phosphorylation of PDGF-R but not EGF-R in tumor cells and endothelial cells. Systemic administration of both agents blocked the phosphorylation of both receptors.

Blockade of EGF-R or PDGF-R can alter phosphatidylinositol 3′-kinase activity, leading to a change in interstitial fluid homeostasis, which results in a reduction of interstitial hypertension with an increase in transcapillary transport (72–74). The roles of EGF and PDGF in angiogenesis have been reported for several human tumors (75–78), and destruction of the vasculature within neoplasms can produce necrosis of actively growing tumors (79, 80). The induction of apoptosis in tumor-associated endothelial cells suggests that this regimen of therapy is directed against tumor vasculature.

Activation of the PDGF-R has been shown to increase expression of PCNA, proliferative cellular nuclear antigen; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling.

Table 4 Immunohistochemical analysis of human prostate carcinoma in bones of control and treated nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>PCNA (%)</th>
<th>TUNEL (%)</th>
<th>CD31 (%)</th>
<th>% TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>130 ± 21</td>
<td>7 ± 2</td>
<td>55 ± 14</td>
<td>0.7</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>80 ± 16e</td>
<td>28 ± 9f</td>
<td>32 ± 1f</td>
<td>0.78</td>
</tr>
<tr>
<td>PKI166</td>
<td>82 ± 13d</td>
<td>27 ± 7d</td>
<td>32 ± 1d</td>
<td>0.66</td>
</tr>
<tr>
<td>STI571</td>
<td>80 ± 16d</td>
<td>28 ± 9e</td>
<td>32 ± 1f</td>
<td>0.78</td>
</tr>
<tr>
<td>PKI166 + paclitaxel</td>
<td>53 ± 16e</td>
<td>42 ± 12f</td>
<td>28 ± 13c</td>
<td>0.78</td>
</tr>
<tr>
<td>STI571 + paclitaxel</td>
<td>53 ± 16e</td>
<td>42 ± 12f</td>
<td>28 ± 13c</td>
<td>0.78</td>
</tr>
<tr>
<td>PKI166 + STI571 + paclitaxel</td>
<td>53 ± 16e</td>
<td>42 ± 12f</td>
<td>28 ± 13c</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* PCNA, proliferative cellular nuclear antigen; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling.
* Mean ± SD.
* Ratio of median number of apoptotic endothelial cells to total number of endothelial cells in 10 random 0.11-mm² fields × 400×.
* Compared with the control group (unpaired Student’s t test): *p < 0.05; **p < 0.01; ***p < 0.001.
of Bel2 and P13K/Akt and to decrease the level of caspase in cells, i.e., increase resistance to apoptosis (81). Because treatment of cells with STI571 increases their sensitivity to an anticancer drug, e.g., Taxol, the results support the hypothesis that PDGF could be a survival factor for tumor cells and endothelial cells. The proliferation rate of endothelial cells within the vasculature of normal organs is <0.01%, whereas 2–9% of endothelial cells in tumor-associated vessels divide daily (82). Because the activation of the PDGF-R on tumor-associated endothelial cells can enhance their resistance to anticancer drugs (36), the administration of protein tyrosine kinase inhibitors such as STI571 can inhibit the resistance of dividing endothelial cells to anticancer drugs, such as paclitaxel. Indeed, the oral administration of PKI166 and STI571 without Taxol completely blocked the phosphorylation of EGF-R and PDGF-R but did not produce therapeutic effects, whereas the combination of PKI166, STI571, and paclitaxel produced the best therapeutic outcome. These results support the rationale that a heterogeneous disease such as cancer should be treated with combined modalities that target both tumor cells and host microenvironment factors, such as unique vasculature and thus support the rationale that cancer (5) should be treated by combination therapy targeting both tumor cells and factors of the host microenvironment, such as the tumor vasculature.

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Simultaneous Blockade of Platelet-Derived Growth Factor-Receptor and Epidermal Growth Factor-Receptor Signaling and Systemic Administration of Paclitaxel as Therapy for Human Prostate Cancer Metastasis in Bone of Nude Mice

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