Effects of Blocking Platelet-Derived Growth Factor-Receptor Signaling in a Mouse Model of Experimental Prostate Cancer Bone Metastases

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Background: Expression of platelet-derived growth factor (PDGF) and activation (by autophosphorylation) of its receptor (PDGF-R), a tyrosine kinase, are associated with the growth of metastatic prostate tumor cells in the bone parenchyma. The tyrosine kinase inhibitor STI571 blocks the PDGF signaling pathway by inhibiting PDGF-R autophosphorylation. We examined the effects of STI571, given alone or with paclitaxel (Taxol), on tumor growth in a mouse model of prostate cancer metastasis. Methods: Human prostate cancer PC-3MM2 cells were injected into the tibias of male nude mice. Three days later the mice (20 per group) were randomly assigned to 5 weeks of treatment with oral and injected water (control), daily oral STI571, weekly injected paclitaxel, or STI571 plus paclitaxel. Lesions in bone and the surrounding muscles were then harvested and analyzed by histology, western blotting (for PDGF-R phosphorylation), immunohistochemistry (for expression of proangiogenic molecules), and double immunofluorescence (to identify endothelial cells and apoptotic tumor cells). Growth of bone lesions was monitored by digital radiography. Bone lesions from control mice were used to establish short-term cell cultures for analysis of PDGF-R phosphorylation. All statistical tests were two-sided. Results: PC-3MM2 cells cultured from bone lesions and treated in vitro with STI571 had less phosphorylated PDGF-R than untreated cells. In control mice, bone lesions expressed high levels of PDGF and activated (i.e., phosphorylated) PDGF-R, whereas lesions in the adjacent muscle did not. Activated PDGF-R was present on the surface of endothelial cells within the bone lesions but not in endothelial cells of uninjected bone. Mice treated with STI571 or STI571 plus paclitaxel had a lower tumor incidence, smaller tumors, and less bone lysis and lymph node metastasis than mice treated with water or paclitaxel alone (P<.001 for all). Mice treated with STI571 or STI571 plus paclitaxel had less phosphorylated PDGF-R on tumor cells and tumor-associated endothelial cells, less tumor cell proliferation, statistically significantly more apoptotic tumor cells (all P<.001), and fewer tumor-associated endothelial cells (P<.001) than control mice. Conclusions: Endothelial cells appear to express phosphorylated PDGF-R when they are exposed to tumor cells that express PDGF. Using STI571 to inhibit PDGF-R phosphorylation may, especially in combination with paclitaxel, produce substantial therapeutic effects against prostate cancer bone metastasis. [J Natl Cancer Inst 2003;95:558–70]

Prostate cancer is the most common cancer among men in North America and the second leading cause of cancer-related deaths (1,2). The major cause of mortality from this disease is metastasis of hormone-refractory cancer cells. Prostate cancer metastases are commonly found in bones, where their growth rate exceeds that of primary tumors (3,4). Because taxane-based regimens are not curative and have not even been proven to be associated with substantial survival benefits (5,6), there is a need to identify novel targets for therapy and to develop new regimens for treating this cancer.

To produce a metastasis, tumor cells must complete a series of sequential and highly selective steps whose outcome is determined by homeostatic host mechanisms (7–9). Preferential metastasis of tumor cells to certain organs is independent of vascular anatomy, rate of blood flow, and the number of tumor cells delivered to each organ (7). Paget (10) proposed that some tissues may provide a better environment than others for the growth of certain tumors and suggested that metastasis occurs when certain tumor cells (the seed) are compatible with a particular organ tissue (the soil). Angiogenesis is a prime example of how the organ microenvironment can contribute to the growth and metastasis of cancer (6,11,12). The onset of angiogenesis involves a change in the local equilibrium between proangiogenic and antiangiogenic molecules. Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), basic fibroblast growth factor (bFGF), interleukin 8 (IL-8), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) are among the major proangiogenic molecules (12–18).

The PDGF receptor (PDGF-R), a member of a family of protein tyrosine kinases that includes many oncogenes and proto-oncogenes (19–22), is encoded by two genes (PDGF-Rα and PDGF-Rβ) (22). PDGF itself is a potent mitogen in both normal and tumor cells (23). PDGF is a dimer that consists of AA, BB, and AB proteins (22). PDGF and PDGF-R are co-expressed in many human carcinomas, including those of the stomach, pancreas, lung, and prostate (24). The binding of PDGF to PDGF-R can stimulate cell division (25–27), cell migration (28), and angiogenesis (29). PDGF binding causes PDGF-R activation, which involves dimerization and autophosphorylation (i.e., activation) of specific tyrosines in the cytoplasmic domain of PDGF-R. The phosphoryroses serve as targets for cytoplasmic effector proteins involved in signal transduction. Activation of PDGF-R has also been shown to inhibit some...
We examined the effects of oral STI571 administration, an inhibitor of the tyrosine kinase activities of c-KIT and PDGF-R and in combination with injected paclitaxel because of their experimental bone metastases in nude mice bearing intratibial injections of human prostate cancer cells. We used STI571 alone and in combination with injected paclitaxel because of their potential additive therapeutic effects.

**MATERIALS AND METHODS**

**Cell Culture**

Androgen-independent human prostate cancer PC-3MM2 cells (39) were maintained as monolayer cultures in Dulbecco’s modified Eagle medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, l-glutamine, a two-fold concentrated vitamin solution (Life Technologies), and penicillin–streptomycin (Life Technologies). Cell cultures were 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, Thiel’s encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by BioWhittaker, Walkersville, MD).

**Mice**

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 animal facilities were approved by the American Association for Accreditation of Laboratory Animal Care and met all current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the National Institutes of Health. The animals were used in accordance with institutional guidelines when they were 8–12 weeks old.

**Intratibial Injections of PC-3MM2 Cells**

To produce bone tumors, we first harvested PC-3MM2 cells from subconfluent cultures by briefly exposing them to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped by the addition of medium containing 10% FBS, and the detached cells were collected, washed once in serum-free medium, and resuspended in Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution. Cell viability was determined by trypan blue dye exclusion; only single-cell suspensions with greater than 95% cell viability were used to produce tumors in the tibias of nude mice. Before intratibial injection, the mice were anesthetized with Nembutal (0.5 mg/g body weight) (Abbott Laboratories, North Chicago, IL). A percutaneous intraosseal 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 further inserted into the shaft of the tibia and was deposited to 20 μL of the tumor cell suspension (2 × 10⁵ cells) in the cortex with the use of a calibrated, push button-controlled dispensing device (Hamilton Syringe Co., Reno, NV). To prevent leakage of cell suspensions, a cotton swab was held against the injection site for 1 minute. The animals tolerated this surgical procedure well, and none died during the procedure.

**Treatment of Nude Mice Bearing Tumors Derived From Injected PC-3MM2 Cells**

STI571 (imatinib mesylate, Gleevec) was provided by Novartis Pharma (Basel, Switzerland). For each oral administration, STI571 was dissolved in distilled water at 6.25 mg/mL. For each intraperitoneal injection, paclitaxel (Taxol; Bristol-Myers Squibb, Princeton, NJ) was diluted in distilled water at 1 mg/mL.

Three days after the intratibial PC-3MM2 cell injections were performed, five mice were killed and the injected bones were examined histologically to identify actively growing cancer cells. The remaining mice were randomly assigned to receive one of the following four treatments (10 mice in each treatment group): 1) a daily oral dose of vehicle solution (water) and one intraperitoneal injection per week of distilled water (control group); 2) one intraperitoneal injection per week of paclitaxel at 8 mg/kg and no oral medication (Taxol group); 3) a daily oral dose of STI571 at 50 mg/kg and no intraperitoneal injections (STI571 group); and 4) a daily oral dose of STI571 at 50 mg/kg and one intraperitoneal injection per week of paclitaxel at 8 mg/kg (STI571 plus Taxol group). The mice were treated for 5 weeks. Tumor size and osteolysis of the injected bones were evaluated by gross observation and by digital radiography as described below. This experiment was repeated once.

**Digital Radiography and Harvesting of Bone Tumors and Lymph Nodes**

After 3–4 weeks of treatment, we randomly selected three mice from each treatment group, anesthetized them with Nembutal (0.5 mg/g body weight), placed them in a prone position, and subjected their hind limbs to digital radiography with the use of a Faxitron digital radiography system (Faxitron X-ray Corp., Wheeling, IL) to monitor progression of disease in the bone. After the mice recovered from anesthesia, they resumed their respective treatments. During week 6 of the study (i.e., after 5 weeks of treatment), all mice were killed by injection with Nembutal (1.0 mg/g body weight) and weighed. Digital radiography was carried out on the hind limbs of each mouse, and tumor incidence and size were recorded. The tumor-bearing injected leg and the tumor-free uninjected contralateral leg of each mouse were resected at the head of the femur and weighed. The net tumor weight was calculated by subtracting the weight of the tumor-free leg from that of the tumor-bearing leg. We also harvested macroscopically enlarged lymph nodes and histologically examined them for the presence of metastasis.

**Western Blot Analysis of PDGF-R Autophosphorylation in Cells Cultured From Harvested Bone Tumors**

PC-3MM2 cells were injected into the tibia of six nude mice. Three mice were treated with STI571 (as described above), and three mice received water (control). After 3 weeks of daily oral
treatments, the mice were killed with Nembutal (0.5 mg/g body weight). The tumor-bearing legs of control and STI571-treated mice were rinsed with alcohol and iodine and resected. Tumor tissue confined to the bone was isolated while it was viewed through a dissection microscope and minced. The tissues were dissociated by incubation with collagenase I at 200 U/mL and DNase I at 270 U/mL. (Sigma Chemical Co., St. Louis, MO) for 2 hours at 37 °C in a shaking water bath as described previously (40). Single-cell suspensions were filtered through sterile gauze, washed three times in serum-free medium, and incubated for 72 hours in tissue culture flasks that contained Dulbecco’s modified Eagle medium with 10% FBS. The cells were then washed and cultured for 24 hours in serum-free medium, then incubated in the presence or absence of 1.6 μM STI571 for 60 minutes, followed by an additional 15-minute incubation in the presence or absence of recombinant human PDGF BB at 10 ng/mL (Life Technologies, Rockville, MD). The adherent cells were washed with phosphate-buffered saline (PBS) containing 5 mM EDTA and 1 mM sodium orthovanadate and then scraped into a lysis buffer (20 mM Tris–HCl [pH 8.0], 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, and 0.15 U/mL aprotinin) and incubated for 20 minutes on ice. The lysed cells were centrifuged at 16000g for 15 minutes at 4 °C, and the supernatant was collected. Proteins in the supernatant were quantified by spectrophotometry, and a constant amount of protein was loaded per lane and resolved on 7.5% sodium dodecyl sulfate–polyacrylamide gels and transferred onto 0.45-μm nitrocellulose membranes. The membranes were incubated with 3% bovine serum albumin in Tris-buffered saline (TBS) (20 mM Tris–HCl [pH 7.5], 150 mM NaCl) to block nonspecific binding and then probed with either a sheep polyclonal anti-human PDGF-R antibody (1 : 1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or a mouse monoclonal anti-phosphotyrosine antibody (monoclonal antibody 4G10; 1 : 2000 dilution) (Upstate Biotechnology, Lake Placid, NY) in Tween–TBS (0.1% Tween 20 in TBS) and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-sheep immunoglobulin G (IgG) (1 : 2000 dilution; Sigma Chemical Co.) or sheep anti-mouse IgG (1 : 2000 dilution), respectively, in TTBS. Antibody-reactive protein bands were visualized with the use of an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

Preparation of Tissues

Tumors harvested from the tibia and the surrounding muscles were cut into 2- to 3-mm pieces, fixed in 10% buffered formalin for 24 hours at room temperature, washed with PBS for 30 minutes, decalced by incubation with 15% EDTA (pH 7.4) for 7–10 days at 4 °C, and embedded in paraffin. We also prepared frozen sections of the harvested tumors according to the method described by Mori et al. (42), with the following modifications. Tumors cut into 2- to 3-mm pieces were fixed in 4% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate (PLP) for 24 hours and then washed with PBS containing 10% sucrose for 4 hours, with PBS containing 15% sucrose for 4 hours, and with PBS containing 20% sucrose for 16 hours. All procedures were carried out at 4 °C. The tissues were then embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70 °C.

Immunohistochemistry and Single-Label Immunofluorescence

Paraffin-embedded tissues were sectioned (4- to 6-μm thick) and used to detect expression of PDGF, PDGF-R, activated PDGF-R, VEGF, bFGF, IL-8, and proliferating cell nuclear antigen (PCNA). We used the following primary antibodies for immunohistochemistry and immunofluorescence: rabbit polyclonal anti-VEGF/VPF, anti-FG-2 (which recognizes bFGF), anti-PDGF A, anti-PDGF B, anti-PDGF-Ro, and anti-PDGF-Rβ antibodies (Santa Cruz Biotechnology); goat polyclonal antiphospho-PDGF-Rβ (which recognizes activated PDGF-Rβ) (Santa Cruz Biotechnology); rabbit polyclonal anti-IL-8 (Biogene, Huntsville, AL) was used to visualize antibody reactivity, and all sections were counterstained with Gill’s hematoxylin (Sigma Chemical Co.).

Tissue sections were mounted on positively charged Superfrost slides (Fisher Scientific Co., Houston, TX) and dried overnight. The sections were deparaffinized in xylene, dehydrated in a graded alcohol series (100%, 95%, and 80% ethanol/water [vol/vol]), and rehydrated in PBS (pH 7.5). The sections used to detect PCNA expression were microwaved at 1000W for 5 minutes to improve antigen retrieval. All other paraffin-embedded tissues were treated with pepsin (Biomeda, Foster City, CA) for 15 minutes at 37 °C and then washed with PBS (40, 41). PLP-fixed frozen tissues that were used to detect CD31/PECAM-1 expression were sectioned (8- to 10-μm thick), mounted onto positively charged Plus slides (Fisher Scientific Co.), and air-dried for 30 minutes. Frozen sections were fixed in 4°C acetone for 5 minutes, in 1 : 1 acetone/chloroform (vol/vol) for 5 minutes, and in acetone for 5 minutes and then washed with PBS. Immunohistochemical procedures were performed as described previously (40, 41). Nonspecific binding of the anti-phospho-PDGF-Rβ antibody was blocked by incubating sections with 4% fish gel (Cold Water Fish Skin Gelatin, 40% Aurion; Electron Microscopy Sciences, Fort Washington, PA) in PBS. Positive antibody reactions were visualized by incubating the slides with stable 3,3’-diaminobenzidine (10–20 minutes). The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 minute, and mounted onto slides with the use of Universal Mount (Research Genetics). Control samples, which were exposed to secondary antibody alone, showed no specific staining. Dilutions of primary antibodies were as follows: IL-8 (1 : 25); PDGF A, PDGF-Rβ, phosphorylated PDGF-Rβ, bFGF, PCNA, VEGF (all at 1 : 100); PDGF B (1 : 200); and CD31/PECAM-1 (1 : 400). HRP-conjugated secondary antibodies were used for immunohistochemical detection of the binding of pri-
Three times for 5 minutes each time to remove unincorporated tissue sections, which were then incubated in a dark, humid mix, and terminal deoxynucleotidyltransferase was added to the uterus at room temperature. The equilibration buffer was drained, minutes and then incubated with equilibration buffer for 10 minutes and then mounted onto slides with the use of Vectashield. The samples were washed twice with PBS for 5 minutes, and fixed with 4% paraformaldehyde (methanol-free) for 10 minutes at room temperature, washed twice with PBS containing 0.1% Brij (Fisher PharMingen) for 18 hours at 4 °C. After the samples were rinsed four times with PBS for 3 minutes each, the slides were incubated in the dark with Texas Red-conjugated goat anti-rat antibody (1 : 200 dilution) for 1 hour at room temperature. Samples were then washed twice with PBS containing 0.1% Brij (Fisher Scientific, Pittsburgh, PA) and once with PBS for 5 minutes and then mounted onto slides with the use of Vectashield.

The terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay was performed with the use of a commercial apoptosis detection kit (Promega Corp., Madison, WI) with the following modifications. Tissue samples were fixed with 4% paraformaldehyde (methanol-free) for 10 minutes at room temperature, washed twice with PBS for 5 minutes, and then incubated with 0.2% Triton X-100 for 15 minutes at room temperature. The samples were washed twice with PBS for 5 minutes and then incubated with equilibration buffer for 10 minutes at room temperature. The equilibration buffer was drained, and reaction buffer containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyltransferase was added to the tissue sections, which were then incubated in a dark, humid environment at 37 °C for 1 hour. The reaction was terminated by immersing the samples in 2× SSC (NaCl at 17.5 g/L, citric acid at 8.8 g/L [pH 7.0]) for 15 minutes. The samples were washed three times for 5 minutes each time to remove unincorporated fluorescein-dUTP and then incubated with 300 μg/mL Hoechst stain for 10 minutes at room temperature. Fluorescent bleaching was minimized by treating the slides with an enhancing reagent (Prolong solution, Prolong Antifade Kit; Molecular Probes). Immunofluorescence microscopy was performed with the use of a Zeiss epifluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a ×40 objective and with narrow band-pass excitation filters mounted on a filter wheel (Ludl Electronic Products, Hawthorne, NY). Images were captured with the use of a Sony three-chip camera (Sony Corporation of America, Mountvale, NJ) and Optimas Image Analysis software (Biocscan, Edmond, WA) installed on a Compaq computer with a Pentium chip, a frame grabber, an optical disk storage system, and a Sony Mavigraph UP-D7000 digital color printer (Sony, Tokyo, Japan). Images were further processed with the use of Adobe Photoshop software (Adobe Systems, Mountain View, CA). Endothelial cells were identified by red fluorescence, and DNA fragmentation (i.e., TUNEL-positive apoptotic cells) was detected by localized green fluorescence within cell nuclei. We quantified the total number of TUNEL-positive tumor cells by counting 10 randomly selected 0.159-mm² microscope fields in tumors adjacent to bone and within muscles at ×100 magnification. Quantification of apoptotic endothelial cells (yellow fluorescence) was expressed as the 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.

**Quantification of Microvessel Density and PCNA-Expressing Cells**

For the quantification of microvessel density, we captured the images, at ×100 magnification, of 10 randomly chosen 0.159-mm² microscope fields adjacent to the bone and 10 randomly chosen fields in the muscles for each tumor and used those images to count microvessel-like structures consisting of endothelial cells that were stained with the anti-CD31/PECAM-1 antibody, as described previously (43). We counted the number of cells that stained with the anti-PCNA antibody in the same 10 randomly chosen 0.159-mm² fields at ×100 magnification.

**Statistical Analysis**

The statistical significance of differences between pairs of treatment groups in tumor incidence, tumor weight, incidence of lymph node metastasis, and number of cells positive for PCNA, TUNEL, CD31/PECAM-1, or both CD31 and TUNEL among the treatment groups was compared by using 2 × 2 factorial analysis. The combined data from the STI571 and STI571 + Taxol groups were compared with those from the Taxol and control groups, and data from the Taxol and STI571 + Taxol groups were compared with those from the STI571 and control groups. The Kruskal–Wallis rank sum test was used to determine the statistical significance of differences between two sets of combined experimental groups. For adjustments of unequal variances among the groups, we used the X²/4 transformation (44) for TUNEL-positive cells in bone lesions, the X²/2 transformation for CD31-positive cells in bone lesions, and the X²/1.5 transformation for the percentages of TUNEL-positive cells in bone lesions and PCNA-positive cells in muscle lesions. The log (x) transformation was used to adjust for unequal variances among measurements of the numbers of TUNEL-positive cells in muscle lesions.

**RESULTS**

**Effect of STI571 on PDGF-R Autophosphorylation in Short-Term Cultures of PC-3MM2 Cells Harvested From Bone Lesions**

Preliminary results from our laboratory determined that PC-3MM2 cells growing in vitro do not express detectable levels of the PDGF-R, whereas cells growing in bones of nude mice do. To determine whether exposure of PC-3MM2 cells to STI571 affects autophosphorylation of the PDGF-R, we established cultures of PC-3MM2 cells from cells that were harvested from bone lesions of control mice or mice treated with STI571. After 4 days in culture, the cells were incubated for 15 minutes in serum-free medium containing PDGF BB and then analyzed for levels of autophosphorylated PDGF-R by western blotting using an antibody specific for the phosphorylated PDGF-R (Fig. 1). Whereas these cells exhibited high levels of phosphorylated PDGF-R, PC-3MM2 cells that were pretreated with STI571 for 1 hour before incubation in serum-free medium that contained PDGF BB displayed lower PDGF-R autophosphorylation. The
were therefore combined and analyzed together (Table 1). All 20 only water) had developed large tumors in the legs that were time the mice in the control group (i.e., the group that received mice. All mice in the four treatment groups were killed on day human prostate cancer cells implanted into the bone of nude tumors in the tibias and surrounding muscles (median weight of bone tumors = 2.5 g [interquartile range (IQR) = 1.4–3.3 g]) of the injected leg, and all 20 mice had lymph node metastases. In mice treated with paclitaxel, tumor incidence was 17 of 20, the median weight of the tumors was 1.8 g (IQR = 0.3–2.5 g), and all 17 of the mice with tumors had lymph node metastases. In mice treated with STI571, tumor incidence was 10 of 20, the median weight of the tumors was 1.8 g (IQR = 0.7–2.3 g), and all 10 of the mice with tumors had lymph node metastases. In mice treated with the combination of STI571 and paclitaxel, tumor incidence was 7 of 20, the median weight of the tumors was 0.6 g (IQR = 0.1–1.5 g), and five of the seven mice with tumors had lymph node metastases.

We used 2 × 2 factorial analyses to determine the statistical significance of differences between pairs of groups in tumor incidence, tumor weight, and incidence of lymph node metastasis. Mice treated with paclitaxel (alone and in combination with STI571) had statistically significant differences in tumor weight (P = .0014) and in the incidence of lymph node metastasis (P = .02) compared with control mice and mice treated with STI571. Mice treated with STI571 (alone and in combination with paclitaxel) had statistically significantly lower tumor incidence (P < .001), tumor weight (P < .001), and incidence of lymph node metastasis (P < .001) than control mice and mice treated with paclitaxel. There was no statistically significant interaction (i.e., synergistic effects associated with treatment using the combination of two drugs) with respect to tumor incidence (P = .12), lymph node metastasis (P = .20), or tumor weight (P = .98).

To determine the extent of osteolysis in the different groups, we performed digital radiography on the hind legs of three mice that were randomly selected from each treatment group in each of the two independent experiments on weeks 3 and 4 of treatment. Mice in the control group and mice treated with paclitaxel alone developed lytic bone lesions as early as week 3 of the study. Mice treated with STI571 alone or with STI571 plus paclitaxel also had bone lesions at the same point in time, but those lesions were smaller than lesions in mice in the control or paclitaxel-only groups and displayed a lesser degree of osteoly-

### Table 1. Effect of treatments on human prostate carcinoma cells injected into the bone of nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Incidence†</th>
<th>Median tumor weight, g (interquartile range)</th>
<th>Incidence of lymph node metastasis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20/20</td>
<td>2.5 (1.4–3.3)</td>
<td>20/20</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>17/20</td>
<td>1.8 (0.3–2.5)</td>
<td>17/20</td>
</tr>
<tr>
<td>STI571</td>
<td>10/20</td>
<td>1.8 (0.7–2.3)</td>
<td>10/20</td>
</tr>
<tr>
<td>STI571 + paclitaxel</td>
<td>7/20</td>
<td>0.6 (0.1–1.5)</td>
<td>5/20</td>
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†Human prostate cancer PC-3-MM2 cells were injected into the tibia of nude mice. Three days later, four groups of 10 mice each were treated with water (Control), paclitaxel alone (weekly interperitoneal injection of 8 mg/kg), STI571 alone (oral administration of 50 mg/kg/day), or STI571 plus paclitaxel. The results of two independent experiments were very similar and, therefore, were combined.

†Number of mice that developed bone tumors or lymph node metastases/number of mice receiving PC-3-MM2 cell injections.

Effect of STI571 on Prostate Cancer Cell Growth and Metastasis

We next examined the effects of treatment with STI571 alone or in combination with paclitaxel on the growth and spread of human prostate cancer cells implanted into the bone of nude mice. All mice in the four treatment groups were killed on day 38–40 (i.e., during the 5th week of treatment), because by that time the mice in the control group (i.e., the group that received only water) had developed large tumors in the legs that were injected with PC-3-MM2 cells. The data collected from experiments performed at two different times were very similar and were therefore combined and analyzed together (Table 1). All 20 control mice had large tumors in the tibias and surrounding muscles (median weight of bone tumors = 2.5 g [interquartile range (IQR) = 1.4–3.3 g]) of the injected leg, and all 20 mice had lymph node metastases. In mice treated with paclitaxel, tumor incidence was 17 of 20, the median weight of the tumors was 1.8 g (IQR = 0.3–2.5 g), and all 17 of the mice with tumors had lymph node metastases. In mice treated with STI571, tumor incidence was 10 of 20, the median weight of the tumors was 1.8 g (IQR = 0.7–2.3 g), and all 10 of the mice with tumors had lymph node metastases. In mice treated with the combination of STI571 and paclitaxel, tumor incidence was 7 of 20, the median weight of the tumors was 0.6 g (IQR = 0.1–1.5 g), and five of the seven mice with tumors had lymph node metastases.

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legs of mice from the four treatment groups. We observed severe destruction (lysis) of the injected tibias of control mice and mice treated with paclitaxel alone. By contrast, mice that received oral STI571 or STI571 plus paclitaxel had relatively intact tibias (Fig. 2). Thus, treatment with the combination of STI571 plus paclitaxel was associated with a substantial delay in the development and progression of osteolytic lesions.

**Histologic and Immunohistochemical Analyses of Bone Lesions**

In all 80 mice, PC-3MM2 cells that were implanted in the tibia grew progressively, lysed the bone, and then grew into the surrounding muscles. We collected tumor specimens from the bone and the surrounding muscles of all 80 mice and processed them for histologic and immunohistochemical analysis. Tumors from mice treated with STI571 plus paclitaxel contained prominent necrotic zones. Immunohistochemistry using antibodies specific for PDGF B, PDGF-Rβ, activated PDGF-Rβ, bFGF, VEGF, and IL-8 demonstrated striking differences in the abundance of these proteins between tumor cells growing in the bone and tumor cells growing in the surrounding muscle (Fig. 3). Specifically, tumor cells growing adjacent to bone expressed high levels of PDGF, PDGF-R, activated PDGF-R, and IL-8, whereas tumor cells growing in the muscle expressed low levels of these proteins. These regional differences in the expression of these proteins (high in tumor cells growing adjacent to the bone and low in tumor cells growing in the muscle) were observed in all 54 of the mice that bore tumors. Tumor cells adjacent to the bone also expressed higher levels of VEGF and bFGF than tumor cells in the surrounding muscles, although not to the same extent as was observed for the levels of the other proteins. The abundance of PDGF, PDGF-R, bFGF, VEGF, and IL-8 in tumor cells from mice treated with paclitaxel, STI571, or STI571 plus paclitaxel was similar to the abundance of those proteins in tumor cells from control mice. In all mice, only tumor cells that grew adjacent to bone expressed PDGF-R. Immunostaining with antibodies specific for tyrosine-autophosphorylated PDGF-R demonstrated that PDGF-R expressed on the surface of tumor cells growing adjacent to bone.
cells adjacent to bone was phosphorylated in control mice and in mice treated with paclitaxel, whereas PDGF-R expressed on the surface of tumor cells in the surrounding muscles was not. By contrast, PDGF-R on the tumor cells adjacent to the bones in mice treated with STI571 or STI571 plus paclitaxel did not react with this antibody, even though PDGF-R was expressed by these cells, suggesting that STI571 inhibits the phosphorylation of PDGF-R, not only in vitro but also in vivo (Fig. 4).

**Inhibition of Tumor Cell Proliferation and Induction of Apoptosis**

The mechanism underlying the smaller tumor size among mice treated with STI571 could reflect a decrease in tumor cell division, an increase in tumor cell apoptosis, or both. We therefore determined the number of tumor cells that expressed PCNA, a marker of cell proliferation, and that had DNA strand breaks characteristic of apoptotic cells (i.e., TUNEL-positive cells) in PC-3MM2-derived tumors from all mice with tumors in the four treatment groups (Fig. 5 and Table 2).

The mean numbers of PCNA-positive cells in tumor lesions adjacent to the bone from control mice and mice treated with paclitaxel, STI571, and paclitaxel plus STI571 were 139 cells (95% CI = 5 to 9 cells), 35 cells (95% CI = 25 to 46 cells), 31 cells (95% CI = 22 to 40 cells), and 75 cells (95% CI = 62 to 88 cells), respectively (Table 2). The mean number of TUNEL-positive cells in tumors growing in the muscle of control, paclitaxel-treated, STI571-treated, and paclitaxel plus STI571-treated mice was eight cells (95% CI = 6 to 9 cells), 31 cells (95% CI = 22 to 40 cells), seven cells (95% CI = 5 to 10 cells), and 35 cells (95% CI = 24 to 45 cells), respectively (Table 2). The 2 × 2 factorial analysis revealed that mice treated with paclitaxel (alone and in combination with STI571) had statistically significantly more TUNEL-positive cells in bone (P <.001) and muscle (P <.001) lesions compared with control and STI571-treated mice. By contrast, treatment with STI571 (alone and combination with paclitaxel) was associated with statistically significantly more TUNEL-positive cells only in bone lesions (P <.001) compared with control mice or mice treated with only paclitaxel. The combination therapy, STI571 plus paclitaxel, was not associated with synergistic effects in the bone (P = .21) or muscle (P = .47) lesions.

**Double Immunofluorescence Staining for CD31/PECAM-1 (Endothelial Cells) and PDGF-R or TUNEL (Apoptotic Cells)**

In the next set of experiments, we used double immunofluorescence staining to examine 1) whether tumor-associated en-
dothelial cells express PDGF-R and 2) the effects of the various treatments on the induction of apoptosis in tumor-associated endothelial cells. Endothelial cells within PC-3MM2-derived tumors in bone expressed PDGF-R on their surface, whereas endothelial cells in PC-3MM2-derived tumors in muscle did not (Fig. 6). We base this conclusion on our results from immuno-fluorescence double-labeling for CD31 (red) and PDGF-R (green), which, when colocalized, stained PDGF-R-expressing endothelial cells in the bone lesions of control and treated mice yellow. Immunofluorescence double-labeling of tumors for CD31 expression (red) and TUNEL positivity (green) revealed colocalization (yellow staining) in endothelial cells within bone lesions of mice treated with STI571 plus paclitaxel (Fig. 6, A). Only mice treated with paclitaxel alone or with STI571 displayed TUNEL-positive endothelial cells in muscle tumors (Fig. 6, B). Endothelial cells in uninvolved bones (contralateral leg) of mice treated with STI571 plus paclitaxel did not express PDGF-R on their surface and did not undergo apoptosis in any of the treatment groups (Fig. 6, A, bottom row).

In bone lesions from control mice and mice treated with paclitaxel, STI571, and STI571 plus paclitaxel, the median percentage of TUNEL-positive (apoptotic) endothelial cells (yellow) was 2% (IQR 0–4%), 5% (IQR 0–8%), 7% (IQR 0–10%), and 17% (IQR 7–20%), respectively (Table 2). In muscle lesions from control mice and mice treated with paclitaxel, STI571, or STI571 plus paclitaxel, the median percentage of apoptotic endothelial cells was 3% (IQR 0–5%), 6% (IQR 0–7%), 5% (IQR 0–9%), and 9% (IQR 0–11%), respectively (Table 2). Treatment of mice with either paclitaxel or STI571 was associated with a statistically significantly higher median percentage of TUNEL-positive endothelial cells in bone lesions ($P = .0067$ and $P = .0026$, respectively) but not in tumors growing in the muscle ($P = .44$ and $P = .16$, respectively) than treatment of mice with water.

Table 2. Immunohistochemical analysis of human prostate carcinoma cells growing in the bone and adjacent muscle of control and treated nude mice*

<table>
<thead>
<tr>
<th>Treatment group†</th>
<th>Bone</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of tumor cells</td>
<td>No. of endothelial cells</td>
</tr>
<tr>
<td></td>
<td>PCNA-positive‡</td>
<td>TUNEL-positive‡</td>
</tr>
<tr>
<td>Control</td>
<td>139 (124–154)</td>
<td>7 (5–9)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>76 (57–95)</td>
<td>35 (25–46)</td>
</tr>
<tr>
<td>STI571</td>
<td>85 (70–99)</td>
<td>31 (22–40)</td>
</tr>
<tr>
<td>STI571 + paclitaxel</td>
<td>36 (25–47)</td>
<td>75 (62–88)</td>
</tr>
</tbody>
</table>

*PCNA = proliferating cell nuclear antigen; TUNEL = terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling.
†Human prostate cancer PC3-MM2 cells were injected into the tibia of nude mice. Three days later, four groups of 10 mice each were treated with water (Control), paclitaxel alone (weekly interperitoneal injection of 8 mg/kg), STI571 alone (oral administration of 50 mg/kg/day), or STI571 plus paclitaxel. The results of two independent experiments were very similar and, therefore, were combined.
‡Mean (95% confidence interval).
§Median of the ratio of the number of apoptotic endothelial cells to the total number of endothelial cells in 5–10 random 0.011-mm² microscope fields at ×400 magnification (interquartile range).

Fig. 5. Analyses of cell proliferation by staining for proliferating cell nuclear antigen (PCNA) expression and of apoptosis by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) in human prostate cancer cells growing in the bone (A) and surrounding muscles (B) of nude mice. Human prostate cancer lesions from the bone of nude mice treated for 5 weeks with water (Control), paclitaxel (Taxol), STI571, or STI571 plus paclitaxel (STI571/Taxol) were harvested and processed for histology and immunohistochemical analyses. Tissue sections were stained for the expression of PCNA (brown) and apoptotic cells by TUNEL (green).

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Fig. 6. Immunohistochemistry and immunofluorescence double-labeling of tumors derived from human prostate cancer cells growing in nude mice. Human prostate cancer lesions from the bone (A) and surrounding muscles (B) of nude mice treated for 5 weeks with water (Control), paclitaxel (Taxol), STI571, or STI571 plus paclitaxel (STI571/Taxol) were harvested and processed for immunohistochemical detection (top two rows) of platelet-derived growth factor B (PDGF B) or CD31 expression in endothelial cells of blood vessels (brown) or for double immunofluorescence detection (bottom three rows, A; bottom two rows, B) of CD31 expression (red) and PDGF receptor expression (PDGF-Rα or PDGF-Rβ) (green) or CD31 expression and apoptotic cells by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) (green). Yellow indicates co-expression of PDGF-Rβ and CD31, i.e., PDGF-Rβ expression in endothelial cells. A) In all treatment groups, tumor cells growing adjacent to bone tissue expressed both PDGF B and PDGF-R. Endothelial cells within the bone lesions also expressed PDGF-Rβ (yellow). Mice treated with STI571 plus paclitaxel displayed apoptosis in tumor cells (green) and endothelial cells (yellow) within the bone lesions. Endothelial cells in tumor-free uninjected contralateral leg did not express PDGF-Rβ on their surface and they were not affected by the treatment. B) By contrast, tumor cells growing in the muscle do not express PDGF B or PDGF-Rβ. Endothelial cells (red) within the muscle tumors do not express PDGF-R. Mice treated with STI571 plus paclitaxel did not show apoptosis in endothelial cells in tumors growing in muscles.
Combination treatment with STI571 plus paclitaxel was not associated with a synergistic effect on apoptosis in tumor-associated endothelial cells in bone (P = .29) or muscle (P = .82) lesions.

**Effects of In Vivo Treatments on Microvessel Density**

In the last set of experiments, we examined whether the observed increase in apoptosis of endothelial cells in bone lesions of mice treated with paclitaxel, STI571, or STI571 plus paclitaxel was associated with a decrease in vascularization. To do so, we immunostained tumors excised from mice in the various treatment groups with an antibody to CD31 to detect endothelial cells and then captured the images of 10 randomly chosen 0.159-mm² microscope fields per tumor and used those images to determine the mean number of immunostained microvessel structures per field, which we used as a measure of microvessel density (Table 2). In bone lesions from control mice and mice treated with paclitaxel, STI571, or STI571 plus paclitaxel, microvessel density (number of CD31-positive cells/field) was 56 (95% CI = 42 to 70), 39 (95% CI = 27 to 51), 35 (95% CI = 23 to 46), and 19 (95% CI = 10 to 27), respectively (Table 2). In muscle lesions from control mice, mice treated with paclitaxel, STI571, or STI571 plus paclitaxel, microvessel density (in units of CD31-positive cells/field) was 54 (95% CI = 43 to 64), 41 (95% CI = 30 to 52), 51 (95% CI = 37 to 66), and 38 (95% CI = 26 to 49), respectively (Table 2). The 2 x 2 factorial analysis revealed that mice treated with paclitaxel (alone and in combination with STI571) had statistically significantly lower microvessel density in bone lesions (P = .0018) and muscle lesions (P = .019) than control and STI571-treated mice. Mice treated with STI571 (alone and in combination with paclitaxel) had statistically significantly lower microvessel density in bone lesions (P<.001) but not in muscle lesions (P = .92) than mice treated with water or paclitaxel alone. The combination treatment with paclitaxel plus STI571 was not associated with a synergistic effect on microvessel density in either bone (P = .73) or muscle (P = .91) lesions. These results suggest that STI571 induces apoptosis only in endothelial cells that express PDGF-R and, moreover, that STI571 sensitized the tumor-associated endothelial cells to paclitaxel-mediated cytotoxicity.

**DISCUSSION**

We examined whether oral administration of STI571, with or without intraperitoneal injections of paclitaxel, could inhibit the growth of androgen-independent human prostate cancer PC-3MM2 cells (39) in the bone of nude mice. Our data show that these cells growing in the tibia of nude mice express high levels of PDGF and the PDGF-R, and that the PDGF-R was autophosphorylated. After the bone lysed, tumor cells grew in the surrounding musculature. The tumor cells that grew in the musculature of the leg (distant from bony tissue) expressed low levels of PDGF and PDGF-R. The endothelial cells within the bone lesions expressed activated PDGF-R, whereas the endothelial cells in uninvolved bone or in the PC-3MM2-derived lesions in the muscles did not. Oral administration of STI571 in combination with paclitaxel (administered by intraperitoneal injection) was associated with a lower level of PDGF-R phosphorylation and more apoptotic endothelial cells and tumor cells that expressed PDGF-R than in control mice. Treatment with STI571 plus paclitaxel was also (and, presumably, as a result) associated with a statistically significant inhibition of the growth of bone lesions (tumors) and a preservation of bone structure, as determined by digital radiography and histologic examinations. The finding that PC-3MM2 cells growing adjacent to the bone expressed PDGF and PDGF-R, whereas cells growing in the muscle or in long-term culture did not, provides an example of the “seed and soil” hypothesis (10), which states that the organ microenvironment can influence the phenotype of tumor cells (45).

The progressive growth of many human carcinomas, including those of the prostate (46–49), ovary (50), lung (51), colon (52), stomach (53), esophagus (54), and breast (55,56), and gliomas (57–59), choriocarcinomas (60), melanomas (61), soft tissue tumors (62), and acquired immunodeficiency syndrome-related Kaposi’s sarcomas (63), has been associated with expression of PDGF-R or the PDGF-R and its ligand (49–63). Our results closely agree with those of previous studies that showed that inhibition of PDGF-stimulated tyrosine phosphorylation of PDGF-R6 with the small molecule N-[4-(trifluoromethyl)phenyl]5-methylisoxazole-4-carboxamide is associated with inhibition of the growth of human tumors in nude mice (34,50).

Our detailed histologic and immunohistochemical analyses revealed that robust expression of PDGF and PDGF-R was restricted to PC-3MM2 cells growing adjacent to the mouse bone. The bone microenvironment has high levels of transforming growth factor-beta (TGF-β), which plays an important role in homeostatic processes of the bone, such as resorption and repair (64,65). In response to injury, TGF-β is expressed in bone and regulates expression of other growth factors, such as EGF (66,67), PDGF (68), and their receptors (69). Such associations between the expression of TGF-β and PDGF and PDGF-R could explain our finding that only those PC-3MM2 cells growing adjacent to the bone expressed high levels of PDGF and PDGF-R. It is also possible that PDGF and PDGF-R are preferentially expressed by tumor cells growing near bone tissue because both are constitutive factors in bone marrow; thus, their expression in tumor cells could reflect the responses of those cells to a bone-specific milieu (34–36). In this regard, our finding that PDGF-R was expressed on endothelial cells within tumor lesions in the bone but not in the muscle, was striking. Specifically, we found that autophosphorylated PDGF-R was expressed on tumor-associated endothelial cells within the bone lesions but not on tumor-associated endothelial cells in muscle, which were located only 2–3 mm away from the bone lesions. This differential expression is likely caused by the production of PDGF by tumor cells growing adjacent to the bone but not by those growing at a distance from the bone (Fig. 3). Because mice treated with STI571 plus paclitaxel had increased apoptosis in receptor-positive tumor cells (Fig. 5) and endothelial cells (Fig. 6, A), the expression of activated PDGF-R in tumor-associated endothelial cells, but not in endothelial cells within the uninvolved bone, provides an attractive target for specific antivascular therapy.

The differences in protein expression levels between tumor cells growing adjacent to bone tissue and those growing in the muscle were not limited to PDGF and PDGF-R. We also found that tumor cells adjacent to the bone expressed higher levels of the proangiogenic factors bFGF, IL-8, and VEGF than tumor cells growing in the muscle. These results suggest that angiogenesis in PC-3MM2 cell-derived lesions growing in the musculature could be caused by the presence of sufficient levels of...
proangiogenic factors other than PDGF, such as bFGF, IL-8, VEGF, IL-1, or TNF-α (14,17,18).

Treatment of mice with STI571 plus paclitaxel was associated with a decrease in the incidence and size of tumors in the bone and musculature compared with mice treated with water or paclitaxel alone. We found that mice treated with STI571 plus paclitaxel had statistically significantly fewer dividing tumor cells in bone lesions than mice treated with paclitaxel alone or with water. PDGF directly depolymerizes microtubules during the initiation of DNA synthesis and cell division (70,71). STI571 inhibits PDGF-mediated PDGF-R autophosphorylation and hence stabilizes microtubules in the target cells, a process similar to the mechanism of action of paclitaxel, i.e., lowering the critical concentration of tubulin monomers for polymerization and promoting tubulin assembly into distinct microtubule bundles with stability against depolymerization (72). Thus, combining the two drugs produces additive therapeutic effects.

The progressive growth and metastasis of neoplasms is dependent on the development of vasculature (i.e., angiogenesis) (6,11,12). Endothelial cells can respond to a variety of signals, including those mediated by bFGF, VEGF, EGF, and PDGF (12). Endothelial cell function, proliferation, and survival depend on the expression of specific receptors to these and other factors (14–16), and inhibition of the interactions of these factors with their receptors can lead to endothelial cell apoptosis (73–76). Destruction of vasculature within neoplasms is known to produce necrosis of actively growing tumors (11–16). Hence the induction of apoptosis in tumor-associated endothelial cells (within the bone lesions) produces regression of the tumors.

In summary, we have shown that human prostate cancer cells growing in the bones of nude mice expressed high levels of PDGF and that both tumor cells in the bone and the tumor-associated endothelial cells expressed activated PDGF-R. Systemic treatment with STI571 plus paclitaxel but not with either drug alone was associated with the statistically significant induction of apoptosis in the tumor-associated endothelial cells and in the tumor cells themselves, which was associated with the inhibition of tumor growth and, consequently, the preservation of bone structure. By contrast, endothelial cells in normal bone were not affected by systemic treatment with STI571 plus paclitaxel, presumably because they did not express PDGF-R. The authors of a recent multi-institutional phase II study (35) involving the PDGF-R inhibitor SU101, which was used as a single agent to treat patients with hormone-refractory prostate cancer, recommended further clinical studies with other PDGF-R inhibitors. Our data, however, clearly show that clinically significant therapy of prostate cancer bone metastasis in mice was achieved by the combined administration of STI571 plus paclitaxel. A heterogeneous disease such as prostate cancer requires multimodal therapy, and the translation of these findings to the clinical reality is currently underway.

References


**NOTES**

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