Inhibition of Platelet-Derived Growth Factor Receptor Signaling Restricts the Growth of Human Breast Cancer in the Bone of Nude Mice

Dina Chelouche Lev, Sun Jin Kim, Amir Onn, Valerie Stone, Do-Hyun Nam, Sertac Yazici, Isaiah J. Fidler and Janet E. Price
Department of Cancer Biology, University of Texas M.D. Anderson Cancer Center and the Program in Cancer Biology, University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas

ABSTRACT

Purpose: Bone is a common site for breast cancer metastasis. Platelet-derived growth factor (PDGF) and PDGF receptors (PDGFR) are involved in the regulation of bone resorption. This study examined the effects of STI571 (imatinib mesylate), which inhibits PDGFR tyrosine kinase signaling, on the growth of human breast cancer cells in the bone of nude mice with consequent osteolysis.

Experimental Design: Human breast cancer MDA-MB-435 cells were injected into the tibia of female nude mice. Two weeks later the mice were treated with p.o. and injected water (control), daily p.o. STI571, weekly injection of paclitaxel, or daily STI571, plus weekly paclitaxel, for up to 8 weeks. Growth of tumors in bones and osteolysis were monitored by digital radiography and tumors were collected for histochemical analysis.

Results: Mice treated with STI571 or STI571 plus paclitaxel had smaller bone tumors with less lytic bone destruction than did mice treated with water or paclitaxel alone. The results of treatment with paclitaxel plus STI571 did not differ from those with STI571 alone. Immunohistochemistry showed that PDGF-A, PDGF-B, PDGFRα, and PDGFRβ were expressed in the bone tumors. STI571 treatment inhibited PDGFR phosphorylation in tumor cells and tumor-associated endothelial cells, coincident with increased apoptosis, reduced proliferation, and lower microvessel density in the tumors.

Conclusions: Activated PDGFRs are expressed by endothelial and tumor cells in breast cancer tumors growing in the bone of nude mice. Interfering with PDGFR signaling may be an approach to control the progressive growth of breast cancer cells and thus reduce bone lysis.

INTRODUCTION

The skeleton is the most common site of breast cancer metastasis, with bone lesions found in approximately 70% of patients with metastatic disease (1). Although patients who have only bone metastases generally have a better prognosis and longer median survival time than patients with metastases in lung, liver, or brain do, they tend to suffer from long-term skeletal morbidity, leading to considerable reduction in quality of life (2). The complications of bone metastasis include pain, pathologic fractures, spinal cord compression, and hypercalcemia. Currently, no curative therapy exists for bone metastasis, and clinical management is generally palliative. Treatment options include surgery or radiation to prevent or repair fractures and the use of bisphosphonates and analgesics to reduce osteolysis and pain (1, 3).

Research is gradually leading to a better understanding of the molecular biology of breast cancer and the genotypic and phenotypic processes underlying the progression to metastasis (4–6). Identification of key molecules controlling the growth of breast cancer cells in the primary and metastatic sites can lead to the development of improved and potentially specific therapeutic strategies. Breast cancer cells produce various growth factors and cytokines that may contribute to malignant progression, through autocrine or paracrine mechanisms (7). One example is the family of platelet-derived growth factors (PDGF), which are multifunctional cytokines involved in the growth, survival, and differentiation of connective tissues (8, 9). The A and B isoforms of PDGF can form either homodimers or heterodimers that bind to and activate the protein tyrosine kinase PDGF receptors (PDGFRα and PDGFRβ; ref. 10). Immunohistochemical studies of breast cancer specimens have shown expression of PDGFs in cancer cells and expression of the receptors predominantly in stromal cells, notably the α-smooth muscle–staining cells and vascular endothelial cells in the periepithelial stroma (7, 11). This expression of PDGF and PDGFRs suggests a paracrine mechanism for tumor development or maintenance. A key paracrine action of PDGFs that may affect the malignant phenotype is the promotion of tumor stroma and angiogenesis (8, 9). Elevated levels of PDGF in plasma and increased expression of PDGF in tumor tissues correlate with increased incidence of metastasis, lower response to chemotherapy, and shorter survival time of patients with breast cancer (12, 13).

In the bone microenvironment, osteoblasts both produce and respond to PDGF, which can promote proliferation, bone resorption, collagen degradation, and collagenase expression (14, 15). The presence of cancer cells in the bone microenvironment may shift the balance of bone homeostasis toward osteolysis (16). Because PDGF has been reported to stimulate...
bone resorption, by regulating expression of cytokines such as interleukin (IL)-6 by osteoblasts, or by direct action on osteoclasts (17), the release of PDGF by metastatic breast cancer cells may influence the development and progressive growth of bone metastases (18–20).

Identification of molecules responsible for paracrine interactions involved in promoting growth of metastases presents an opportunity to interfere with this process. Several small-molecule inhibitors of different signaling pathways, notably tyrosine kinase inhibitors, have shown therapeutic efficacy and are undergoing clinical trials (21). We previously reported that STI571 (imatinib mesylate, Gleevec, Novartis Pharma, Basel, Switzerland), a derivative of 2-phenylaminopyrimidine, developed as an inhibitor of the Abl protein tyrosine kinase, and a potent inhibitor of PDGFR and C-Kit tyrosine kinases (22), can slow the progressive growth of experimental bone metastases of a human prostate cancer (23). In this study we used the same strategy we used in that previous study to test the therapeutic effect of STI571, both alone and in combination with paclitaxel, against human breast cancer cells growing in the tibias of nude mice, to test the hypothesis that inhibiting PDGFR signaling can impair the growth of breast cancer in bone.

**MATERIALS AND METHODS**

**Cell Line.** The MDA-MB-435 breast cancer cell line was provided by Dr. Relda Cailleau (University of Texas M.D. Anderson Cancer Center, Houston, TX). Cells were maintained in monolayer culture in MEM supplemented with 5% fetal bovine serum, sodium pyruvate, l-glutamine, and vitamin solution (2× MEM; Life Technologies, Inc., Grand Island, NY) in a humidified incubator with 5% CO2-95% air. For all in vivo experiments, tumor cells in exponential growth phase were harvested by brief exposure to 0.25% trypsin in 0.02% EDTA, then washed and resuspended in Ca2+- and Mg2+-free PBS.

**Animals.** Female athymic NCr-nu mice were purchased from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were housed in a specific pathogen-free facility and used at 7 to 8 weeks of age. The care and use of laboratory animals was in accordance with the principles and standards set forth in the Principles for Use of Animals (NIH Guide for Grants and Contracts), the Guide for the Care and Use of Laboratory Animals (DHFW, Public Health Service Publication 80-23, Rev. 1978), the provisions of the Animal Welfare Acts (P.L. 89-544 and its amendments). The study was approved by the Institutional Animal Care and Use Committee of the University of Texas M.D Anderson Cancer Center.

**Intratibial Injections of MDA-MB-435 Cells.** To establish bone tumors, 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 tibial tuberosity (24). After penetration of the cortical bone, the needle was inserted farther into the tibial shaft to deposit 20 μL of the MDA-MB-435 cell suspension (5 × 106 cells) in the cortex with a calibrated, push button–controlled dispensing device (Hamilton Syringe Co., Reno, NV). A cotton swab was then held against the injection site for 1 minute to prevent leakage of the inoculum. The animals tolerated this procedure well.

**Experimental Design.** STI571 (imatinib mesylate, Gleevec). For each p.o. administration, STI571 was dissolved in distilled water (dH2O) at 6.25 mg/mL. For each i.p. injection, paclitaxel (Taxol; Bristol-Myers Squibb, Princeton, NJ) was diluted in dH2O at 1 mg/mL. Therapy was initiated 2 weeks after injection of the tumor cells, according to preliminary results showing that at this point the mice had tumors confined within the marrow space (Fig. 1). Mice (12–15 mice per treatment group) were randomly assigned to receive one of the following four treatments: (a) a daily p.o. dose of vehicle solution and weekly i.p. injection of dH2O (control group); (b) no p.o. medication and weekly i.p. injection of 8.5 mg/kg paclitaxel (paclitaxel group); (c) a daily p.o. dose of 50 mg/kg STI571 and weekly i.p. injection of dH2O (STI571 group); and (d) a daily p.o. dose of 50 mg/kg STI571 and weekly i.p. injection of 8.5 mg/kg paclitaxel (STI571 + paclitaxel). In the first experiment, the mice were treated for 6 weeks, and in the second, the treatment was extended to 8 weeks. Tumor size and osteolysis of the injected bone were evaluated by gross observation and by digital radiography as described below.

**Digital Radiography and Harvesting of Bone Tumors.** Progression of disease in the bone was monitored by digital radiography, starting 2 weeks after initiation of treatment and every second week thereafter. Mice were anesthetized and placed in a prone position, and their hind limbs were imaged using a digital radiography system (Faxitron X-ray Corp., Wheeling, IL). At the end of the study, the mice were euthanized and the hind limbs were imaged and then resected and weighed. The tumor weight was calculated as the difference between the weights of the tumor-bearing and tumor-free legs. A semiquantitative grading system of osteolysis, with numeric values ranging from 0 to +4+, was used to assess the extent of bone destruction (24). A grade of 0 represented no lysis, 1+ was minimal but visible bone lysis within the medullary canal, 2+ was moderate osteolysis in the medullary canal with preservation of the cortex, 3+ was severe osteolysis with cortical disruption, and 4+ was massive destruction with extension of the tumor into the soft tissue.

**Fig 1** Radiologic and histologic appearance of nude mouse tibia 2 weeks after injection of MDA-MB-435 human breast cancer cells. Digital radiograph of a noninjected tibia for reference (A) and of an injected tibia (B) shows no evidence of osteolysis. C, a histologic section of the tibia shown in B shows a representative focus of breast cancer cells growing in the bone marrow space (arrowhead), with no evidence of osteolysis. H&E stain, original magnification ×200.
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, decalcified by incubation with 15% EDTA (pH 7.4) for 7 to 10 days at 4°C, and embedded in paraffin. Frozen sections of the tumors were prepared following the method described previously (23). Tumors cut into 2- to 3-mm pieces were fixed in 4% paraformaldehyde containing 0.075 mol/L lysine and 0.01 mol/L sodium periodate for 24 hours, washed with PBS containing 10% sucrose for 4 hours, then with PBS containing 15% sucrose for 4 hours, and finally with PBS containing 20% sucrose for 16 hours. All procedures were carried out at 4°C. The tissues were then embedded in omithine carbamyl transferase compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C.

Immunohistochemistry and Single-Label Immunofluorescence. Paraffin-embedded sections were sectioned (4 to 6 μm) and used to detect expression of PDGF, PDGFR, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), IL-8, and proliferating cell nuclear antigen (PCNA). Frozen sections were used for detecting activated PDGFR, CD31, and phosphorylated Akt. The following primary antibodies were used for immunohistochemistry and immunofluorescence: rabbit polyclonal anti-VEGF/VF, anti-FGF-2 (which recognizes bFGF), anti-PDGFA, anti-PDGFB, anti-PDGFRα, and anti-PDGFRβ (Santa Cruz Biotechnology, Santa Cruz, CA); goat polyclonal anti-phospho-PDGFR (which recognizes activated PDGFR, Santa Cruz); rabbit polyclonal anti-IL-8 (Biosource International, Camarillo, CA); rat monoclonal anti-mouse CD31/platelet-endothelial cell adhesion molecule-1 (PECAM-1) (PharMingen, San Diego, CA); monoclonal anti-PCNA, clone PC-10 (Dako A/S, Copenhagen, Denmark), and rabbit polyclonal anti-phospho-Akt (Ser473, Cell Signaling Technology, Beverly, MA). The tissue sections used to detect PCNA expression were microwaved at 1,000 W for 5 minutes to improve antigen retrieval. All other paraffin-embedded tissues were treated with pepsin (Biomed, Foster City, CA) for 15 minutes at 37°C and then washed with PBS.

Secondary antibodies used were horseradish peroxidase (HRP)–conjugated goat anti-rabbit immunoglobulin G, HRP–conjugated goat anti-rodent immunoglobulin G, Texas Red–conjugated goat anti-rat immunoglobulin G, and FITC–conjugated goat anti-rabbit immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA); HRP–conjugated rat anti-mouse IgG2a (Serotec, Harlan Bioproducts for Science, Inc., Indianapolis, IN); Alexa Fluor 594–conjugated goat anti-rabbit immunoglobulin G (Molecular Probes, Eugene, OR); and Biogenex multilink and Biogenex label used for enhancing antibody detection (San Ramon, CA).

Immunohistochemical procedures were performed as described previously (24). Positive antibody reactions in sections reacted with HRP-labeled antibodies were visualized by incubating the slides with stable 3,3′-diaminobenzidine for 10 to 20 minutes. The sections were rinsed with dH2O, counterstained with Gill’s hematoxylin for 1 minute, and mounted onto slides with the use of Universal Mount (Research Genetics, Huntsville, AL). Control samples, which were exposed to secondary antibody alone, showed no specific staining. The sections treated with Alexa Fluor were rinsed with dH2O and mounted with medium with 4′,6-diamidino-2-phenylindole (Vectorshield, Vector Laboratories, Inc., Burlington, CA), which produced blue fluorescence in the cell nuclei.

Immunofluorescence Double Staining for CD31/PECAM-1 and PDGFR or Terminal Deoxynucleotidyl Transferase–Mediated Nick End Labeling. Frozen sections were incubated with a protein-blocking solution (5% normal horse serum and 1% normal goat serum in PBS) for 20 minutes at room temperature and then incubated for 18 hours at 4°C with a 1:400 dilution of rat monoclonal anti-mouse CD31/PECAM-1 antibody, which recognizes human and mouse PECAM-1. The samples were then rinsed four times with PBS for 3 minutes each, and the slides were incubated in the dark for 1 hour at room temperature with a 1:200 dilution of Texas Red–conjugated goat anti-rat antibody. Samples were then washed twice with PBS containing 0.1% Brij (Fisher Scientific, Pittsburgh, PA) and once for 5 minutes with PBS and then mounted onto slides with the use of Vectashield. The terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay was done using a commercial apoptosis detection kit (Promega Corp., Madison, WI), as described previously (24).

Immunofluorescence microscopy was done using an epifluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a 40× objective and narrow-bandpass excitation filters mounted on a filter wheel (Ludl Electronic Products, Hawthorne, NY). Images were captured with the use of a three-chip camera (Sony Corporation of America, Montvale, NJ) and Optimas Image Analysis software (Bioscan, Edmond, WA). Images were further processed with the use of 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 green fluorescence localized within cell nuclei. The total number of TUNEL-positive tumor cells was determined in tissues 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 to 10 random 0.011-mm² fields at ×400 magnification.

Quantification of Microvessel Density and PCNA-Expressing Cells. To quantify microvessel density, we captured the images (magnification ×100) of 10 randomly chosen 0.159-mm² microscope fields 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 (25). We also 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. Comparisons of tumor weight and numbers of TUNEL-positive, PCNA-positive, and CD31-positive cells were analyzed by Student’s t tests. Differences between groups were considered statistically significant at P < 0.05.

RESULTS

Expression of PDGF and PDGFR in MDA-MB-435 Bone Tumors. Preliminary experiments2 had shown that cultured MDA-MB-435 cells release PDGF-A and PDGF-B.

2 D. Chelouche Lev and J. E. Price, Unpublished data.
In the work reported here, immunohistochemistry of the tumors in the mouse tibia showed expression of the ligands PDGF-A and PDGF-B (Fig. 2). Although the cultured cells did not express detectable levels of PDGFR (measured by immunoblotting, data not shown), both PDGFRα and PDGFRβ were detected in the MDA-MB-435 cells growing in bone (Fig. 2), suggesting that expression of the receptors can be regulated by the organ microenvironment.

Effect of STI571 on the Growth of MDA-MB-435 Tumors in the Bone of Nude Mice. We evaluated the effects of treatment with STI571 alone and in combination with paclitaxel on the growth of the tumors in the bone of nude mice in two experiments; the results were similar in the two experiments (Table 1). No significant differences in tumor incidence occurred between the groups of mice receiving the different treatments. The control mice had the largest bone tumors, and the weights of tumors in mice receiving paclitaxel alone did not differ significantly from tumors of the control animals (P = 0.99 and 0.7 in experiments 1 and 2, respectively). Mice treated with STI571 alone had significantly smaller tumors than the control animals (P = 0.04 and 0.003 in experiments 1 and 2, respectively) and the mice treated with paclitaxel alone (P = 0.03 and 0.045 in experiments 1 and 2, respectively). Mice receiving the combination of STI571 and paclitaxel had significantly smaller tumors than the control or paclitaxel-treated mice (control versus combination, P = 0.007 and 0.018 in experiments 1 and 2, respectively; paclitaxel versus combination; P = 0.004 and 0.10 in experiments 1 and 2, respectively). However, the tumor sizes in the mice treated with the combined agents did not differ significantly from those of mice treated with STI571 alone (P = 0.44 and 0.54 in experiments 1 and 2, respectively).

We assessed the extent of osteolysis in the different treatment groups using digital radiography (Fig. 3), and scoring by three observers using a semiquantitative scale already described. Control mice and those treated with paclitaxel alone developed obvious osteolytic lesions by week 4 of the experiments, whereas in those treated with STI571 or STI571 plus paclitaxel the appearance of osteolytic lesions was delayed by 2 or more weeks. At the end of experiment 1 (6 weeks of treatments) the average osteolysis scores for the control and paclitaxel groups were 2.1 and 1.7, respectively; in contrast, the scores for mice treated with STI571 or STI571 and paclitaxel were 1.2 and 0.8, respectively. Similar results were found in experiment 2. Thus, the use of STI571, either alone or in combination with paclitaxel, was associated with a substantial delay in the development and progression of osteolytic MDA-MB-435 tumors.

STI571 Treatment Inhibits the Phosphorylation of PDGFR in MDA-MB-435 Bone Tumors and Tumor-Associated Endothelial Cells. Specimens of the MDA-MB-435 bone tumors were processed and used for histologic and immunohistochemical studies. H&E staining of decalcified sections of tumors from mice treated with STI571 or STI571 and paclitaxel, revealed prominent necrotic zones, notably within tumor lesions in the marrow cavity and, to a lesser extent, in tumor extending into the surrounding muscles (Fig. 3). On the other hand, the tumor samples from control and paclitaxel-treated mice revealed minimal or no necrosis.

### Table 1

STI571 inhibits the growth of MDA-MB-435 in the bone of nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumor incidence*</th>
<th>Mean tumor weight† ± SD (mg)</th>
<th>Lysis score‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15/15</td>
<td>281 ± 67</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>14/15</td>
<td>282 ± 60</td>
<td>1.7 ± 0.1†</td>
</tr>
<tr>
<td>STI571</td>
<td>13/15</td>
<td>102 ± 40‡</td>
<td>1.2 ± 0.1‡</td>
</tr>
<tr>
<td>STI571 + paclitaxel</td>
<td>13/15</td>
<td>67 ± 24**</td>
<td>0.8 ± 0.1‡</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12/12</td>
<td>470 ± 77</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>13/13</td>
<td>416 ± 102</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>STI571</td>
<td>10/12</td>
<td>165 ± 37†</td>
<td>1.5 ± 0.2‡</td>
</tr>
<tr>
<td>STI571 + paclitaxel</td>
<td>10/12</td>
<td>211 ± 61††</td>
<td>1.0 ± 0.4‡</td>
</tr>
</tbody>
</table>

*Number of mice with tumors/number of mice given injections.
†Difference in weight between the tumor-bearing and non–tumor-bearing hind legs.
‡Mean score for degree of lysis seen in radiographs of the tumors, with 0 = no lysis to 4 = extensive bone destruction.
**P = 0.004 versus control, Student’s t test.
*P = 0.008 versus control, Mann-Whitney test.
|P = 0.03 versus control, Student’s t test.
**P = 0.007 versus control, Student’s t test.
††P = 0.003 versus control, Student’s t test.
†††P = 0.018 versus control, Student’s t test.

Fig. 2 Immunohistochemical detection of PDGF-A, PDGF-B, PDGFRα, and PDGFRβ (brown) and phosphorylated PDGFRα and PDGFRβ (green fluorescence) in MDA-MB-435 tumors growing in the bone of nude mice. (Original magnification ×100).
determine the effect of STI571 on phosphorylation of the receptors in the tumors. No differences were found in the abundance of PDGF-A or PDGF-B or of the two forms of PDGFR in the tumors from the four treatment groups, suggesting that neither STI571 nor paclitaxel affected the expression of these proteins in the MDA-MB-435 tumors. However, treatment with STI571 alone or in combination with paclitaxel greatly reduced the expression of phosphorylated PDGFR (Fig. 4). Thus, p.o. administration of STI571 inhibited PDGFR activity in the MDA-MB-435 tumors. The activation of Akt, a signaling molecule downstream of PDGFR was also substantially reduced in the tumors of mice treated with STI571.
To investigate whether inhibition of PDGFR phosphorylation was restricted to the tumor cells or was also seen in stromal cells within the tumors, we used double-immunofluorescence staining to examine PDGFR activation on tumor-associated endothelial cells. Endothelial cells were identified by staining for CD31, and colocalization of this marker and phosphorylated PDGFR was apparent in tumor specimens from control and paclitaxel-treated animals (Fig. 5). In contrast, in tumors from mice treated with STI571 or STI571 plus paclitaxel, endothelial cells did not express phosphorylated PDGFR. The effects of STI571 in inhibiting the growth of MDA-MB-435 bone tumors may therefore be through a direct effect on the tumor cells and also through inhibition of PDGFR signaling in the tumor-associated endothelial cells.

**STI571 Inhibits Tumor Cell Proliferation and Induces Apoptosis.** Inhibition of tumor growth in the STI571-treated mice could be the consequence of decreased tumor cell division, increased tumor cell death, or both. The proportion of cells expressing PCNA, a marker of proliferating cells, and the number of apoptotic cells indicated by the TUNEL reaction were determined in the bone tumors from the different treatment groups (Table 2). The mean percentage of PCNA-positive cells was 59.2% in control tumors and 48.9% in paclitaxel-treated tumors ($P = 0.01$; Table 2). More substantial reductions in the proportion of proliferating cells was found in tumors from STI571-treated mice, with 27.5% PCNA positive cells in the STI571-treated tumors ($P < 0.001$ versus control) and 23.8% in tumors from mice treated with STI571 plus paclitaxel ($P < 0.001$). The difference between the proportions of proliferating cells in the tumors from mice treated with STI571 and those from mice treated with STI571 plus paclitaxel was significant ($P = 0.0037$), suggesting that the combination treatment had an additive effect in inhibiting cellular proliferation.

Few TUNEL-positive cells were detected in the tumors from control mice (mean number 5.1 per $100 \times$ field), with a modest increase in those from the paclitaxel-treated mice ($8.0, P = 0.01$; Table 2; Fig. 6). The tumors from mice treated with STI571, alone or in combination with paclitaxel, had significantly more TUNEL-positive cells than did those from control and paclitaxel-treated mice ($P < 0.001$). The combination of the two agents produced an additive induction of apoptosis ($P = 0.039$, STI571 versus STI571 + paclitaxel).

**STI571 Induces Apoptosis in Tumor-Associated Endothelial Cells and Reduces Tumor Microvessel Density.** Immunohistochemical testing for CD31 (for measurement of microvessel density) and immunofluorescence double labeling for CD31 and TUNEL were used to evaluate the effects of STI571 on tumor-associated endothelial cells. Paclitaxel treatment alone had no effect on microvessel density in the tumors (Table 2), whereas treatment with STI571, alone and in combination with paclitaxel, resulted in a significant reduction in the number of CD31 positive cells ($P < 0.001$). Immunofluorescence showed CD31 expression (red fluorescence, Fig. 6) TUNEL positivity (green fluorescence), and colocalization of the signals (yellow fluorescence) in endothelial cells in the tumors of mice treated with STI571 or STI571 plus paclitaxel. No colocalization of the red and green fluorescence was detected in tumors from the control or paclitaxel-treated mice. These results suggested that STI571 can induce apoptosis in both MDA-MB-435 and endothelial cells. Immunohistochemical staining of the bone tumors for VEGF, IL-8, and bFGF did not reveal differences between the four treatment groups (data not shown), suggesting that the STI571-mediated apoptosis...
DISCUSSION

As originally described by Stephen Paget in 1889 (26), the characteristic patterns of metastasis seen in patients with breast cancer and other cancers are the result of multiple interactions between the metastasizing cancer cells (the “seeds”) and the compatible organ environment (the “soil”). The mediators of interactions between tumor and normal cells include cytokines and growth factors, which act in an autocrine or paracrine manner (4, 27). Identifying the mechanisms of these tumor-host interactions, notably those involved in the promotion of tumor angiogenesis, offers opportunities for therapeutic intervention.

Metastasis to the bone is a common complication for patients with breast cancer. The predominantly lytic nature of breast cancer bone metastases is thought to be a consequence of the “vicious cycle” described by Chirgwin and Guise (16), in which metastatic cells in the bone microenvironment release factors and cytokines that promote osteoclast activation and bone destruction. In turn, this liberates factors from the bone matrix, notably transforming growth factor-β, which provide feedback that further enhances the osteolysis-promoting phenotype in the breast cancer cells (16, 28). Among the cytokines and growth factors thought to contribute to the regulation of bone turnover are the PDGFs (14), which are expressed by many types of cancer, including breast cancers (7, 11). High levels of PDGF in plasma or tumor tissues from patients with breast cancer have been correlated with a higher incidence of metastasis and, hence,

of endothelial cells was not due to diminished expression of these proangiogenic factors in MDA-MB-435 tumor cells growing in the bone.

Table 2  Effects of STI571 and paclitaxel treatment on proliferation, apoptosis, and microvessel density in MDA-MB-435 bone tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean counts (± SD)*</th>
<th>TUNEL positive %</th>
<th>PCNA positive</th>
<th>CD31 positive</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>5.1 ± 2.8</td>
<td>59.2 ± 6.9</td>
<td>26.9 ± 10.4</td>
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</tr>
<tr>
<td>Paclitaxel</td>
<td>8.0 ± 4.6†</td>
<td>48.9 ± 7.3†</td>
<td>27.8 ± 9.9</td>
<td></td>
</tr>
<tr>
<td>STI571</td>
<td>34.1 ± 13.6†</td>
<td>27.5 ± 3.4†</td>
<td>9.6 ± 3.9†</td>
<td></td>
</tr>
<tr>
<td>STI571 + paclitaxel</td>
<td>42.2 ± 1.7†</td>
<td>23.8 ± 3.9†</td>
<td>10.75 ± 5.8†</td>
<td></td>
</tr>
</tbody>
</table>

*Mean values of positively stained cells counted in 10 randomly selected fields of 3 tumor samples from each treatment group. Values for TUNEL positive and CD31 positive cells are the numbers of stained cells per field. Values for PCNA positive cells are expressed as the percentage of PCNA positive cells counted per field.

†P = 0.01 versus control, Student’s t test.
†P = 0.001 versus control, Student’s t test.
shorter survival (13, 29). Our study tested whether p.o. administration of STI571, a small-molecule inhibitor of PDGFR tyrosine kinase, would inhibit the growth of human breast cancer cells implanted into the tibias of nude mice as an experimental model of cancer growing in the bone environment. The data from immunohistochemistry showed that the breast cancer cells growing in the bone of mice expressed PDGF-A and PDGF-B as well as PDGFRα and PDGFRβ. Because the MDA-MB-435 cells do not express detectable levels of these receptors when grown in tissue culture, these in vivo findings suggest up-regulation by factors present in the tissue environment. Transforming growth factor β, which is found in abundance in bone matrix, can promote PDGF expression in breast cancer cells in vitro (30). The expression level of PDGFR can be modulated by various conditions and factors in the tissue microenvironment (9). We previously reported that endothelial cells in prostate cancer bone lesions express high levels of PDGFR, whereas endothelial cells in unaffected bone or in tumors growing in muscle did not express PDGFR (23). Similarly, endothelial cells present in the MDA-MB-435 bone tumors expressed the receptors for PDGF, and the receptors were phosphorylated.

Treatment with STI571 blocked activation of PDGFR and also substantially reduced Akt activation in the tumors, thus blocking a signal transduction pathway that is important for tumor growth and survival (31). Growth of the MDA-MB-435 tumors was significantly inhibited and bone structure was preserved in the STI571-treated mice. These results suggested that signaling through PDGFR is important for the development of osteolytic breast cancer lesions and that inhibiting this pathway may be an effective method of controlling the progression of skeletal metastasis. Our current findings are similar to a previous report, demonstrating that STI571 inhibited the growth of human prostate cancer cells in mouse bone (23). In both these studies, STI571 blocked PDGFR phosphorylation in tumor cells in bone lesions and in tumor-associated endothelial cells, coincident with the appearance of apoptotic cells and reduced microvessel density within the tumors. STI571 targets cells expressing phosphorylated PDGFR, and in the microenvironment of bone metastases, these include tumor cells and tumor-associated endothelial cells, osteoblasts (32) and osteoclasts (17). Our study documented a significant reduction in proliferation and an increase in apoptosis in the breast cancer cells, and apoptosis in tumor-associated endothelial cells in STI571-treated mice. The study did not determine whether STI571 interfered with the actions of tumor-derived PDGF on osteoblasts or osteoclasts. STI571 treatment reduced the extent of osteolysis, but the current study cannot distinguish whether this effect was primarily by inhibiting the growth and survival of the breast cancer cells or whether STI571 inhibited the release of osteolytic factors by MDA-MB-435 cells.

The progressive growth of primary tumors and metastases depends on the development and maintenance of vasculature (4, 33). The function, proliferation, and survival of endothelial cells depend on expression of receptors responding to various factors, including bFGF, VEGF, epithelial growth factor, and PDGF (9, 34). Blocking the interactions between these factors and their receptors or inhibiting the receptor function can lead to endothelial cell apoptosis, resulting in the loss of vasculature and leading to tumor necrosis. Our study provides another example of a potential antivascular action from blocking PDGF-mediated signaling in the MDA-MB-435 tumors in the tibias of nude mice. The inhibition of different receptor tyrosine kinases in tumor-associated endothelial cells has been shown to be an effective therapeutic strategy in several preclinical models of human cancer and metastasis (25, 35, 36).

Systemic administration of STI571 has been shown to enhance antitumor effects of chemotherapy by reducing interstitial hypertension and increasing drug uptake (37). Although we found evidence of impaired PDGFR signaling in the bone tumors of STI571-treated mice, our results did not demonstrate any enhancement of paclitaxel’s antitumor in the tumors. The dose of paclitaxel used was lower than the maximal tolerated dose for mice, and was deliberately chosen to show a potential additive effect when combined with STI571. In preliminary studies, we found that the same dose and administration schedule of paclitaxel used in this work significantly inhibited the growth of MDA-MB-435 tumors in the mammary fat pads of nude mice, yet our data from this study showed a minimal effect of the paclitaxel alone on the MDA-MB-435 tumor cells growing in the bone. This may be an example of the effect of different organ environments on modulation of drug sensitivity of cancer cells (38, 39), and the possibility that the organ microenvironment can regulate P glycoprotein levels in tumor cells is currently being investigated.

In summary, we found that human breast cancer cells growing in the bone of nude mice express PDGF and that both tumor cells and tumor-associated endothelial cells express activated PDGFR. Systemically administered STI571 inhibited PDGFR activation, induced apoptosis in the endothelial and breast cancer cells, and significantly decreased tumor size and osteolysis. These results suggest that interfering with the PDGFR signaling pathway may be a useful approach for controlling the progressive growth of breast cancer cells within the bone microenvironment. The data reported here are potentially significant for developing additional therapeutic strategies for breast cancer that has metastasized to bone.

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PDGFR Inhibitions Blocks Breast Cancer Growth in Bone

Inhibition of Platelet-Derived Growth Factor Receptor Signaling Restricts the Growth of Human Breast Cancer in the Bone of Nude Mice


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