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13. ABSTRACT (Maximum 200 Words)
The proposal will test the hypothesis that bone represents a unique microenvironment favoring the survival and growth of metastatic breast cancer cells. Further, that cells in breast cancer bone metatases are specialized populations of cancer cells, endowed with properties that promote their growth in bone. The presence of breast cancer cells can disrupt the normal balance of bone turnover and promote osteoclast activatity. Understanding the biology of breast cancer bone metastasis and the contribution of cancer-derived factors, such as platelet-derived growth factor (PDGF) will lead to new approaches for control or prevention of this significant clinical problem. Expression analyses will be performed using cDNA arrays, testing samples from breast cancer cell lines growing in different conditions - *in vitro* and *in vivo* (direct injection into bone or mammary fatpad, and/or metastases from different organs in mice). The arrays will be used to identify cytokines and recepotrs, and genes invovled in specific pathways (Cell cycle regulation, cell death, metastasis, and invasion, signal transduction, angiogenesis). One of the factors known to promote bone resorption is PDGF, and the consequences of release of PDGF by metastatic breast cancer cells will be determined *in vitro* using immortalized osteoblasts.

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Manuscript submitted to *Clinical Cancer Research*, "Inhibition of Platelet-Derived Growth Factor Receptor Signaling Restricts the Growth of Human Breast Cancer in the Bone of Nude Mice ", D. Chelouche Lev, S.J. Kim, A. Onn, V. Stone, D.Y. Nam, S. Yazici, I.J. Fidler, and J. E. Price.

Tumor-host interactions in breast cancer bone metastasis

INTRODUCTION:

Breast cancer is the most common cancer (after non-melanoma skin cancer) of women in the United States of America. Twenty percent of women with early stage, node-negative breast cancer may subsequently develop metastatic disease, while as many as 90% of women with locally advanced, or with extensive lymph node involvement will develop metastases [1]. In addition to the axillary lymph nodes, other sites where breast cancer metastases are found include the liver, lungs and brain. However, the most common site of breast cancer metastasis is the bone [2,3,3,4,4]. Breast cancer metastases in bone predominantly present as osteolytic lesions. Such lesions can have serious complications, including hypercalcemia, pain, pathologic fractures and central nervous compromise (spinal cord or nerve root compression)[5]. Bone metastases are the most common cause of pain for cancer patients, resulting from either mechanical or chemical stimulation of pain receptors in the periosteum or endosteum. Pressure effects, microfractures and cytokine release also contribute to the pain [6]. Patients with bone as the first site of relapse of breast cancer can have a significantly longer survival than patients with liver as the first site (20 months vs. 3 months median survival after relapse) [5]. However, the prolonged course of a disease with such complications as bone pain and pathological fractures severely reduces a patient's quality of life, and can make heavy demands on health care resources. There is increasing support for the idea that certain properties of breast cancer cells contribute to the high incidence of bone metastases of this disease. One observation is the correlation between expression of parathyroid hormone related protein (PTHrP) and breast cancer bone metastasis [7]. PTHrP has been shown to be upregulated in breast cancer cells exposed to TGF-β1[8]. This may serve to enhance PTHrP expression locally, since the bone matrix is a rich source of a variety of growth factors, including insulin-like growth factors I and II (IGF-I, IGF-II) and TGF-β[9,10].

In healthy adult bone there is a continuous process of turnover with a balance maintained between resorption and new bone formation. In post-menopausal women or in conditions of estrogen depletion the balance of normal bone turnover can become uncoupled. Osteoblast function is reduced while osteoclast function is maintained, leading to net bone loss. Multiple cytokines and hormones are involved in osteoblast stimulation of osteoclastogenesis. Many of these, for example PTHrP, IL-6, IL-11 and M-CSF, are expressed by tumor cells [11]. Another key factor is the TNF-like cytokine osteoprotegerin ligand (OPGL), also known as RANKL (receptor activator of NF-κB ligand) or ODF (osteoclast differentiation factor). The receptor RANK is expressed by osteoclasts. The action of OPGL is antagonized by a soluble "decoy" form of the receptor, osteoprotegerin (OPG), which is also expressed by osteoblasts [12] suggesting a finely tuned system for the local control of osteoclast activation. Exposure of osteoblasts to cytokines and other factors (including PTHrP) that promote bone resorption can stimulate expression of OPGL and reduce levels of OPG [13,14,14]. Bone resorption leads to the local release of matrix-bound factors and cytokines that normally stimulate osteoblasts to form new bone [9], yet could also promote the survival and growth of breast cancer cells. As noted above, TGF-β, which is abundant in bone matrix, can increase PTHrP production by metastatic breast cancer cells. In a preliminary study we found that TGF-β treatment of breast cancer cells increased the release of PDGF, another factor which can stimulate bone resorption. Others have shown that the media from cultures of resorbing bone are chemotactic and growth stimulatory for rat and human breast cancer cells [15,16]. Breast cancer bone metastases are commonly found in sites of active bone

remodeling, predominantly in trabecular bone, suggesting that the growth and survival of metastatic cells are promoted in areas of remodeling one.

A report by Kang et al [17] was published in 2003, since the last annual report, describing studies with a very similar goal to those of this award, to identify genes important for bone metastasis of breast cancer. Their study used gene expression array analysis of variants of the MDA-MB-231 breast cancer cell line that had been isolated from bone metastases generated from the left heart injection into nude mice. Among the set of genes identified were CXCR4 and Connective tissue growth factor (CTGF). CXCR4 is a chemokine receptor for CXCL12/Stromal derived factor 1-α (SDF1α), and expression of the receptor has been shown to contribute to the metastatic dissemination of breast cancer cells in SCID mice [18]. CTGF is a reported to act as an angiogenic factor and has been linked to malignant progression of breast cancers [19]. In other studies in the laboratory, CTGF was shown to have increased expression in lung metastasis derived variants of the GI101A breast cancer cell line (Chelouche Lev et al, manuscript submitted for publication). Thus, the increased expression of CTGF may be more associated with metastatic potential, rather than specifically with bone metastasis ability.

The hypothesis tested in this work is that bone represents a unique microenvironment favoring the survival and growth of metastatic breast cancer cells. Further, that cells in breast cancer bone metastases are specialized populations of cancer cells, endowed with properties that promote their growth in bone. The presence of breast cancer cells can disrupt the normal balance of bone turnover and promote osteoclast activity. Understanding the biology of breast cancer bone metastases and the contribution of cancer cell-derived factors, such as platelet derived growth factor (PDGF), will lead to new approaches for control or prevention of this significant clinical problem.

BODY:
Specific Aim #1: Gene expression comparisons of breast cancer cells growing *in vivo* and *in vitro*

Analyses of gene expression were proposed using two breast cancer cell lines, injected into nude mice to generate tumors, either in the mammary fatpad (as previously described [20]), or into the tibia, which is a model for growth of cancer in the bone [21,22]. The cell lines proposed, BBM1 and SUM149, were determined in preliminary data to be able to grow in the tibia of nude mice, and also in the mammary fatpad. However, in our initial experiments, the cell lines have failed to produce a reliable incidence of bone tumors, such that the recovery of tumor tissues for isolation of RNA for expression analyses has not yet been accomplished with these cell lines. At the time of this report, bone tumors of the SUM149 cell line were available, with more being formed in ongoing studies, with which to accomplish the comparisons. The MDA-MB-435 breast cancer cell line, which forms osteolytic tumors, is the other cell line studied so far. Tumor tissues have been recovered from mammary fatpad and bone tumors, and total RNA isolated to begin analyses of gene expression. The delay in obtaining the tumors has resulted in slower progress than anticipated in performing the gene expression arrays. In the interim, the total RNA collected has been used for initial studies of comparisons of gene expression using real time PCR. The genes reported in Table 1 (Appendix) include those that have been implicated in previous reports as involved in the formation of bone metastases, and the activation of osteoclasts.

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Increased expression of PTHrP was seen in RNA isolated from tumors in the mammary fatpad and the bone. PDGF-Rβ expression was not detected in the tissue culture cells, yet was detected in the tumors of both cell lines. Expression of RANKL was not detected in the cultured cells, but found in the SUM 149 bone tumor sample, at 80% of the expression seen in the calibrator sample of osteosarcoma cells. The robust expression of two osteoclast-activating genes, PTHrP and RANKL by the SUM149 bone tumor samples corroborates the observation of high levels of osteoclast activation in the bone tumors, as noted from staining for tartrasine-resistant acid phosphastase (TRAP), Figure 1 (Appendix). Only modest changes in expression of OPG, CXCR4, CTGF and MCSF between the in vitro and in vivo samples were seen. Thus the expression of some genes is responsive to the tissue environment, although there no differences between the two sites of tumor growth were seen, for the one line tested so far (MDA-MB-435).

One factor that we hypothesized to be an important player in tumor-host interactions of breast cancer bone metastasis is the platelet-derived growth factor (PDGF) family of growth factors. PDGFs are among the cytokines and growth factors released by breast cancer cells that have the potential for promoting bone resorption [23,24,24]. The PDGFs form a family of disulfide binding dimeric isoforms; at present there are four known isoforms, A, B, C, and D, and two of these (C and D) require proteolytic activation [25,26]. PDGF A and B can form either homo- or heterodimers, and different cell types differ in expression of the PDGF isoforms. The two specific receptors, PDGF α- and β-receptor are members of the tyrosine kinase receptor super-family. Ligand binding promotes dimerization of receptor subunits, and triggers tyrosine-specific phosphorylation, initiating a signal transduction cascade and ultimately phenotypic changes [27,28]. PDGFs are potent bone mitogens which stimulate proliferation of osteoblasts, and also increase bone resorption, probably by increasing osteoclast number [23,23,29]. Osteoblasts express receptors for PDGF, and respond to the factors with various phenotypic changes, including upregulation of IL-6 [30]. There is less information on the actions of PDGFs directly on osteoclasts, although there is one report that these cells express PDGF receptors [24].

We previously reported the expression of PDGFs, and the induction of PDGF receptors in the tumors of MDA-MB-435 growing in the mammary fatpad and the bone of nude mice, detected by immunohistochemistry. While the ligand and receptor are present in the tumors from both sites, there is substantially more activation of the receptor in the bone tumors than in the mammary fatpad tumors. These data are included in a report submitted for publication (Chelouche Lev et al, *Inhibition of Platelet-Derived Growth Factor Receptor Signaling Restricts the Growth of Human Breast Cancer in the Bone of Nude Mice,* Appendix) that investigated the consequence of blocking the activation of PDGF-receptors with the small molecule inhibitor STI571. Blockade of the PDGF receptor signaling was found to inhibit the development of the osteolytic tumors in nude mice, and reduce bone lysis. The observation that the breast cancer cells were expressing the receptor *in vivo* was an unexpected one, and thus the interpretation of the effects of the tyrosine kinase inhibitor is that of blocking possible autocrine and paracrine interactions initiated by the cancer cell-derived PDGF.

The RT-PCR data presented in Table 1 confirms the immunohistochemistry findings of induction of PDGF-receptors in the tumors, both in the mammary fatpad and the bone, albeit at very low levels of expression, (relative to placenta RNA, the calibrator sample used for the data in Table 1). The mechanism(s) for this induction is not known. TGF-β, a cytokine released from bone matrix by the action of osteoclasts [9] has been reported to increase expression of PDGF in breast cancer cells [31], and we have confirmed this *in vitro* by ELISA measurements of supernatant from breast cancer cells treated with TGF-β, and PDGFs are reported to regulate the

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expression of the cognate receptors in some cells [32], again by unknown mechanism(s). Whether TGF-β contributes to increased PDGF expression in bone tumors remains to be established. How the difference in receptor activation may relate to the expression of other cytokines involved in promotion of osteoclast activation will be determined by immunohistochemistry using the tissue specimens already available, and then using gene expression arrays from RNA isolated from xenograft tumors.

Specific Aim #2 PDGF-mediated regulation of osteoblast expression of osteolytic cytokines

This aim employs a SV40-large T antigen transformed hFOB1.19 human fetal osteoblast cell line. These cells, grow actively at 34°C, and grow slowly and differentiate at the permissive temperature of 39° C [33]. Studies of PDGF receptor phosphorylation have been performed in cells at the permissive temperature, i.e. differentiated phenotype. In the previous report we described the results of PDGF stimulated phosphorylation of receptors in the cells, and stimulation of the receptors by medium collected from breast cancer cells.

Colony-formation assays were attempted to test the effect of PDGF on the hFOB1.19 cells; however, these produced no conclusive results, possibly as the cells grow poorly when plated at very low density used for such assays. As an alternate assay, the cells were plated at a higher density in 6-well plates, in serum-free medium supplemented with 1 or 5 ng/ml PDGF-BB. Over 3 weeks of culture, the numbers of cells surviving in the plates were significantly higher in the wells supplemented with PDGF compared with the control, although the numbers did not increase, suggesting that PDGF may act to promote the survival of osteoclasts (Figure 2, Appendix).

The effect of PDGF on expression of RANKL, OPG, MCSF and IL-6 was measured using quantitative RT-PCR. Total RNA was isolated from hFOB1.19 cells grown in the presence of 1 or 10 ng/ml PDGF BB or PDGF AA, at 8 h incubation with the growth factors, and also the conditioned medium (CM) from the breast cancer cell line BBM1, which was isolated from a bone metastasis. (Figure 3, Appendix). The data show that PDGF BB and PDGF AA, and to a less extent the conditioned medium from cells that express a high level of PDGF (reported in previous annual report) can modulate osteolytic cytokine expression in immortalized osteoblasts. RANKL expression was increased in the PDGF-BB and CM treated cells at 8 h. OPG and MCSF expression was unchanged, or slight reduced. For IL-6 the 8 h treatment with PDGF-BB was reduced at 8h at the higher dose, but at 24 was elevated 4 fold relative to control (not shown) sugesting a time- and dose-dependent regulation by the factor. The data do not unequivocally identify PDGF as the factor in the breast cancer conditioned medium that is responsible for modulating the cytokines in the cells. Experiments using a neutralizing antibody to remove PDGF from the conditioned medium did not give consistent results, and the presence of the antibody alone appeared to stimulate receptor phosphorylation in the osteoblasts (although the antibody had been reported to be capable of PDGF depletion in published reports). An alternative approach being used in current experiments is to use the PDGF receptor inhibitor STI571.

Progress with this aim has been slowed by problems with the culture of the hFOB1.19 cells, which have a long-doubling time (> 48 h) and are very sensitive to culture conditions. For example, if the cultures become over confluent the cells detach and die. Alternative cell lines being considered for use in this part of the study include two human osteosarcoma cell lines (SAOS-2, MG63), both of osteoblastic phenotype, and which express receptors for PDGF (data

not shown). Although these are not normal osteoblast cells, they may provide a model with which to examine regulation of cytokines involved in osteoclast activation by PDGFs. Other alternative cells that have been purchased from the American Type Culture Collection and tested for the initial expression of PDGF receptors are the M3T3-E clones [34], that when cultured in ascorbic acid are reported to express an osteoblastic phenotype. In the initial characterization we found robust expression of PDGF-Rα and PDGF-Rβ. Additional primers for measuring the expression of cytokines in the mouse osteoblast-like cells are being tested, as those used previously were for human samples, and are reported to be species specific.

KEY RESEARCH ACCOMPLISHMENTS
- Demonstration of differential expression of PDGF-Rβ, RANKL, and PTHrP in breast cancer cells growing in vivo compared with cells grown in tissue culture.
- The inhibition of breast cancer bone tumor growth by inhibition of PDGF-receptor signaling, using a small molecule inhibitor.
- Demonstration that PDGF and conditioned medium from breast cancer cells can regulate the expression of RANKL in immortalized osteoblasts.

REPORTABLE OUTCOME
- Thesis, entitled "PDGF expression by breast cancer cells, and its role in regulating osteolytic cytokine expression in osteoblasts", presented by Claudia P. Miller to the faculty of the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences, in partial fulfillment of the requirement for the degree of Master of Science. August 2003.
- Oral presentation at The IVth International Conference on Cancer-Induced Bone Diseases, San Antonio, Dec 7-9, 2003; Title "Inhibition of Platelet-Derived Growth Factor Receptor Restricts the Growth of Breast Cancer Cells in the Bone but not in the Mammary Fatpad", D. Chelouche Lev, S.J. Kim, A. Onn, V. Stone, D.Y. Nam, S. Yazici, I.J. Fidler, and J. E. Price.
- Manuscript submitted to *Clinical Cancer Research*, "Inhibition of Platelet-Derived Growth Factor Receptor Signaling Restricts the Growth of Human Breast Cancer in the Bone of Nude Mice ", D. Chelouche Lev, S.J. Kim, A. Onn, V. Stone, D.Y. Nam, S. Yazici, I.J. Fidler, and J. E. Price.

CONCLUSIONS:
This grant is testing the hypothesis that the bone represents a unique microenvironment favoring the survival and growth of metastatic breast cancer cells. One result of the studies so far is that growth in the bone lead to enhanced activity of the PDGF-receptor in breast cancer cells compared with the same cells growing in the mammary fatpad of mice. This result confirms microenvironmental regulation in the breast cancer cells, which will be examined further in the continuing studies of gene expression patterns of breast cancers growing in the different organs (mammary fatpad or bone). Other than the differential activation of the receptors in the bone tumors, the results at present do not support organ specific gene regulation, i.e. differences between the mammary fatpad and the bone tumors, of factors that have been reported previously to be important for bone metastasis (CTGF, CXCR4, PTHrP). PDGFs may

have multiple actions in the bone microenvironment, acting on the breast cancer cells and also on osteoblasts, by regulating key cytokines (for example RANKL) involved in activation of osteoclasts, the cell responsible for bone destruction seen in lytic metastases. Another gene showing increased expression in breast cancer cells grown *in vivo*, compared with in vitro was PTHrP, another key factor in the promotion of osteoclast activation. The expression of the gene in the mammary fatpad tumors may suggest that in addition to a function(s) in the bone microenvironment, PTHrP may be involved with the growth of the breast cancer cells in other tissue environments, and that expression may be regulated by factors present in the local tumors.

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Figure legends:

Figure 1: A: Radiographic image of nude mouse tibia injected human breast cancer cells (SUM 149, 5×10^5 cells in 0.02 ml), forming an osteolytic tumor. The tumor was removed at necropsy, fixed in formalin and and the tissue decalcified in EDTA. Paraffin embedded tissues were sectioned and stained for the presence of tartrasine-resistant acid phospatase (TRAP), indicating the activated osteoclasts. B and C: Sections of the tumor in the tibia stained for TRAP positive cells, located at the interface between the tumor and bone (B, 100 X mag., C, 200X mag.)

Figure 2: Influence of PDGF on the in vitro survival of hFOB1.19 cells
1×10^3 hFOB1.19 cells were plated per well in 35-mm diameter plates, and after serum depletion for 24 h, the medium was supplemented with 1 ng/ml or 5 ng/ml of PDGF BB for a period of 3 weeks. The medium was replenished weekly. The MTT assay was used to evaluate relative cells numbers (from absorbance readings (OD) of formazan solubilized in DMSO from cells exposed to the MTT solution for 2 h), at weekly intervals. Student's t-tests were used to test the significane of the differences in absorbance values.

Figure 3: Cytokine expression in hFOB1.19 cells in response to PDGF and breast cancer conditioned medium (CM).
Total RNA was isolated from hFOB1.19 cells incubated for 8 h with PDGF (1 or 10 ng/ml) and relative levels of RNA for RANKL and OPG measured by quantitative PCR. Total RNA was reversed transcribed with random primers from the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City,CA). The Gene AMP PCR system 9700 thermal cycler was used to perform the RT step using the following cycle conditions: 25°C for 10 min, 37° C for 120 min. cDNA was amplified in duplicate samples using the ABI 7000 Sequence Detection System for the expression of RANKL, OPG, MCSF, IL-6 and 18S using TaqMan® Assay Reagents (Applied Biosystems) and following the manufacturer's recommended amplification procedure. Results were recorded as mean Ct, and relative expression was determined using the comparative Ct method. The ΔCt was calculated as the difference between the average Ct value of the endogenous control, 18s, from the average Ct value of the cytokine of interest. To compare the relative amount of target gene expression in different samples, human placenta RNA (Promega, Madison, WI) or RNA from the SAOS human osteosarcoma cell line (for RANKL) was used as a calibrator. The ΔΔCt was determined by subtracting the ΔCt of the calibrator from the ΔCt of the test sample. Relative expression of of the target gene is calculated by the formula, $2^{-\Delta\Delta Ct}$, which is the amount of gene product, normalized to the endogenous control and relative to a calibrator.
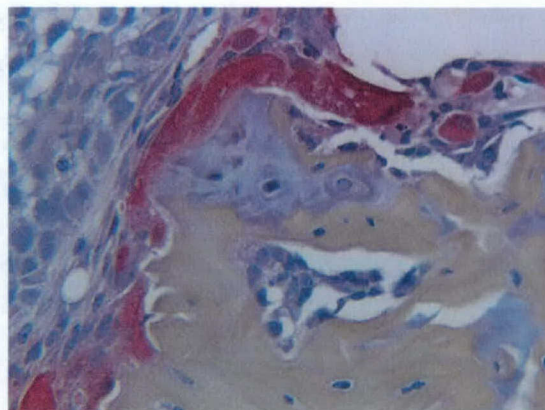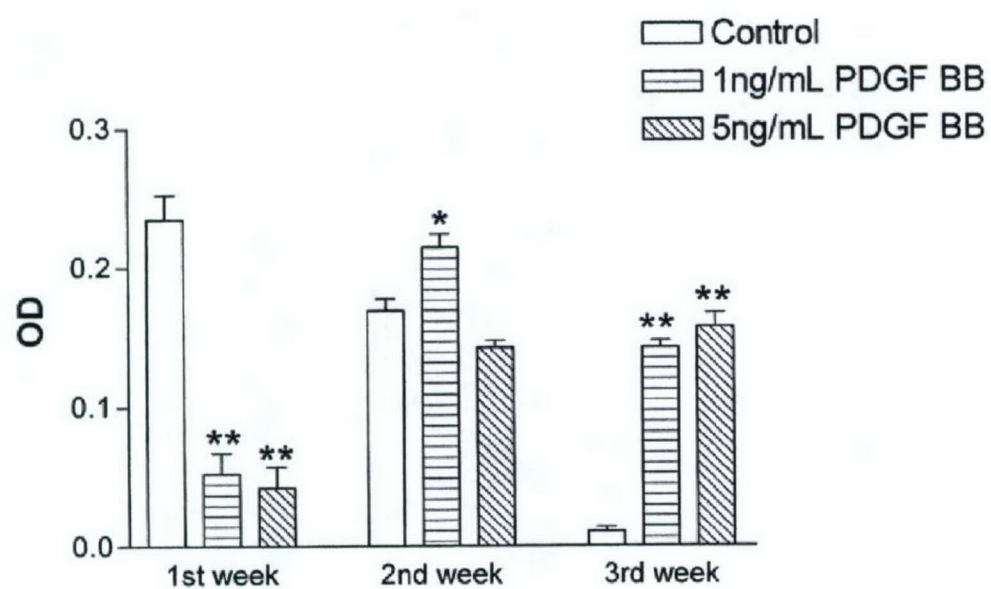
Figure 1:

A

B
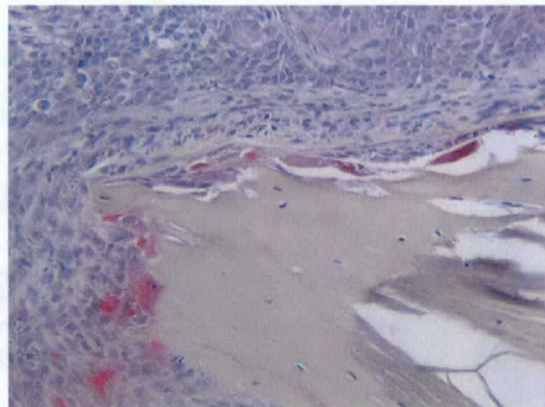
C

Figure 2:

**P<.01 compared to control
*P<.05 compared to 1ng/mL PDGFBB/1st week
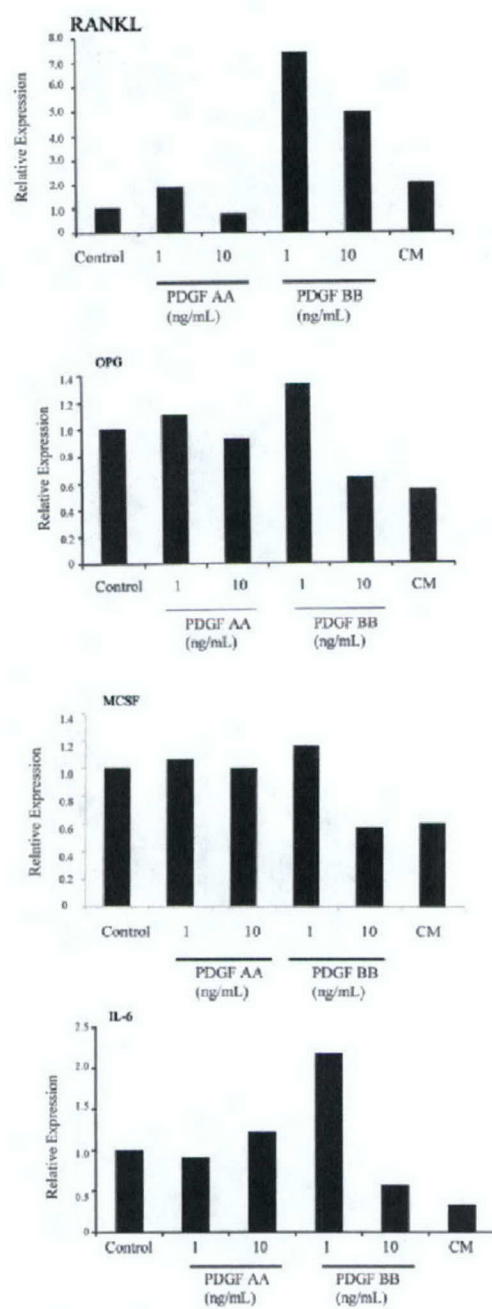
Figure 3:

Table 1: Relative expression of selected genes in MDA-MB-435 and SUM149 breast cancer cells, growing in vitro and in vivo

RNA sample	PTHrP	CTGF	RANKL	OPG	CXCR4	MCSF	PDGF-Rβ
Placenta (calibrator)	1	1	1 (SAOS)	1	1	1	1000
MDA-MB-435	0.0047 (1.0)	0.061 (1.0)	NE	0.177 (1.0)	0.023 (1.0)	0.171 (1.0)	NE
435-bone 1	0.28 (60.7)	0.168 (2.8)	NE	0.316 (1.8)	0.025 (1.1)	0.275 (1.6)	0.21
435-bone 2	0.065 (14)	0.332 (5.4)	NE	0.414 (2.3)	0.035 (1.5)	0.771 (4.5)	0.46
435-bone 3	0.011 (2.46)	0.118 (1.9)	NE	ND	0.029 (1.3)	0.105 (0.6)	0.301
435-mfp 1	0.072 (15.3)	0.259 (4.24)	NE	0.261 (1.5)	0.138 (6.0)	0.176 (1.0)	0.30
435 mfp 2	0.095 (20.3)	0.201 (3.29)	NE	0.135 (0.76)	0.026 (1.1)	0.209 (1.2)	NE
435 mfp 3	0.065 (13.8)	0.159 (2.6)	NE	ND	0.021 (0.9)	0.192 (1.1)	0.2
SUM149	3.58 (1.0)	0.157 (1.0)	NE	ND	0.173 (1.0)	1.23 (1.0)	NE
SUM149 bone	37.4 (11.3)	0.101 (0.64)	0.82	ND	0.378 (2.2)	0.259 (0.21)	0.717

Total RNA was isolated from breast cancer cells in vitro, or tumor samples from the mammary fatpad (mfp) or tibia (bone) tumors from nude mice injected with MDA-MB-435 or SUM 149 breast cancer cells, and relative levels of RNA for selected genes measured by quantitative PCR. Total RNA was reversed transcribed with random primers from the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City,CA). The Gene AMP PCR system 9700 thermal cycler was used to perform the RT step using the following cycle conditions: 25°C for 10 min, 37° C for 120 min. cDNA was amplified in duplicate samples using the ABI 7000 Sequence

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Detection System for the expression of genes shown in the table and 18s using TaqMan® Assay Reagents (Applied Biosystems) and following the manufacturer's recommended amplification procedure. Results were recorded as mean Ct, and relative expression was determined using the comparative Ct method. The ΔCt was calculated as the difference between the average Ct value of the endogenous control, 18s, from the average Ct value of the gene of interest. To compare the relative amount of target gene expression in different samples, human placenta RNA (Promega, Madison, WI) was used as a calibrator; for the RANKL comparisons, cDNA from the osteosarcoma cell line SAOS2 was used as the calibrator sample. The ΔΔCt was determined by subtracting the ΔCt of the calibrator from the ΔCt of the test sample. Relative expression of of the target gene is calculated by the formula, $2^{-\Delta\Delta Ct}$, which is the amount of gene product, normalized to the endogenous control and relative to a calibrator.

The value shown in parentheses is the expression normalized to that of the cell line used to originate the tumor sample grown in vitro.

NE = no expression detected.
ND = not done.

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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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Running title: PDGF-R inhibition blocks breast cancer growth in bone

Keywords: STI571; osteolysis; host-tumor interactions

Abstract

Purpose: Bone is a common site for breast cancer metastasis. Platelet-derived growth factor (PDGF) and PDGF-receptors (PDGF-Rs) are involved in the regulation of bone resorption. This study examined the effects of STI571 (imatinib mesylate), which inhibits PDGF-R 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 oral and injected water (control), daily oral STI571, weekly injection of paclitaxel, or daily STI571, plus weekly paclitaxel, for up to eight 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 and PDGF-Rα and PDGF-Rβ were expressed in the bone tumors. STI571 treatment inhibited PDGF-R phosphorylation in tumor cells and tumor-associated endothelial cells, coincident with increased apoptosis, reduced proliferation and lower microvessel density in the tumors.

Conclusions: Activated PDGF-Rs are expressed by endothelial and tumor cells in breast cancer tumors growing in the bone of nude mice. Interfering with PDGF-R 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 (PDGFs), which are multi-functional 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 (PDGF-Rα and -Rβ) (10). Immunohistochemical studies of breast cancer specimens have demonstrated expression of PDGFs in cancer cells, and expression of the receptors predominantly in stromal cells, notably the alpha smooth muscle-staining cells and vascular endothelial cells in the periepithelial stroma (7;11). This expression

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of PDGF and PDGF receptors suggests a paracrine mechanism for tumor development or maintenance. A key paracrine action of PDGFs that can 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 breast cancer patients (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). Since 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), a derivative of 2-phenylaminopyrimidine, developed as an inhibitor of the Abl protein tyrosine kinase, and a potent inhibitor of PDGF-R 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 PDGF-R signaling can impair the growth of breast cancer in bone.

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MATERIALS AND METHODS

Cell line: The MDA-MB-435 breast cancer cell line was provided by Dr Relda Cailleau (The University of Texas M.D. Anderson Cancer Center). Cells were maintained in monolayer culture in Minimum Essential Medium supplemented with 5% fetal bovine serum, sodium pyruvate, L-glutamine, and vitamin solution (2X-MEM; BRL-GIBCO, Grand Island, NY) in a humidified incubator with 5%-CO_2-95% 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 Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS).

Animals: Female athymic NCr-nu mice were purchased from the NCI-Frederick Cancer Research Facility (Frederick, MD). The mice were housed in a specific pathogen-free facility, and used at 7-8 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 (DHEW, PHS Publ. 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 UT 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 (5×10^5 cells) in the cortex with a calibrated, push button-controlled dispensing

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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) 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. Therapy was initiated two 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: 1) a daily oral dose of vehicle solution and weekly intraperitoneal injection of distilled water (control group); 2) no oral medication and weekly intraperitoneal injection of 8.5 mg/kg paclitaxel (paclitaxel group); 3) a daily oral dose of 50 mg/kg STI571 and weekly intraperitoneal injection of distilled water (STI571 group); and 4) a daily oral dose of 50 mg/kg STI571 and weekly intraperitoneal 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 Faxitron 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

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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.

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 7–10 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 M lysine and 0.01 M sodium periodate (PLP) for 24 hours and then 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 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, 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 and CD-31. The following primary antibodies were used for immunohistochemistry and immunofluorescence: rabbit polyclonal anti-VEGF/VPF, anti-FGF-2 (which recognizes bFGF), anti-PDGF A, anti-PDGF B, anti-PDGF-R, and anti-PDGF-R (Santa Cruz Biotechnology, Santa

Cruz, CA); goat polyclonal anti-phospho-PDGF-R (which recognizes activated PDGF-R) (Santa

Cruz); rabbit polyclonal anti-IL-8 (Biosource International, Camarillo, CA); rat monoclonal anti-

mouse CD-31/platelet–endothelial cell adhesion molecule-1 (PECAM-1) (PharMingen, San

Diego, CA); and monoclonal anti-PCNA, clone PC-10 (DAKO A/S, Copenhagen, Denmark).

The tissue 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.

Secondary antibodies used were: horseradish peroxidase (HRP)-conjugated goat anti-

rabbit IgG, HRP-conjugated goat anti-rat IgG, Texas Red-conjugated goat anti-rat IgG, and

fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson Research 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 IgG (Molecular Probes,

Eugene, OR); and Biogenex multilink and Biogenex label used for enhancing antibody detection

(BioGenex, 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 10–20 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, Huntsville, AL). Control

samples, which were exposed to secondary antibody alone, showed no specific staining. The

sections treated with Alexa Fluor were rinsed with distilled water and mounted with medium

with 4',6-diamidino-2-phenylindole [DAPI] (Vectashield ;Vector Laboratories, Inc., Burlingame,

CA), which produced blue fluorescence in the cell nuclei.

Immunofluorescence Double Staining for CD31/PECAM-1 and PDGF-R or TUNEL:

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 deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using a commercial apoptosis detection kit (Promega Corp., Madison, WI), as described previously (24).

Immunofluorescence microscopy was performed using an epifluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a x40 objective and narrow band-pass 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 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 green fluorescence localized within cell nuclei. The total number of TUNEL-positive tumor cells was determined in tissues at x100 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-mm^2

fields at x400 magnification.

Quantification of Microvessel Density and PCNA-Expressing Cells: To quantify microvessel

density, we captured the images (x100 magnification) of 10 randomly chosen 0.159-mm^2

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-mm^2 fields at x100 magnification.

Statistical analysis: Comparisons of tumor weight, and numbers of TUNEL-positive, PCNA-

positive and CD-31 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 PDGF-R in MDA-MB-435 bone tumors: Preliminary experiments

(unpublished data) had shown that cultured MDA-MB-435 cells release PDGF-A and PDGF-B.

In the work reported here, immunohistochemistry of the tumors in the mouse tibia demonstrated

expression of the ligands PDGF-A and PDGF-B (Fig. 2). Although the cultured cells did not

express detectable levels of PDGF-R (measured by immunoblotting, data not shown), both

PDGF-Rα and PDGF-Rβ 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 P = 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 P = 0.003 in experiments 1 and 2, respectively) and the mice treated with paclitaxel alone (P= 0.03 and P = 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 P = 0.018 in experiments 1 and 2; paclitaxel *versus* combination; P = 0.004 and P = 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 P = 0.54 in experiments 1 and 2, respectively).

We assessed the extent of osteolysis in the different treatment groups using digital radiography (Figure 3), and scoring by three observers using a semi-quantitative 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 PDGF-R in MDA-MB-435 bone tumors and tumor-associated endothelial cells: Specimens of the MDA-MB-435 bone tumors were

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processed and used for histologic and immunohistochemical studies. Hematoxylin and eosin staining of decalcified sections of tumors from mice treated with STI571, with or without 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.

Immunohistochemistry using antibodies specific for PDGF-Rα, PDGF-Rβ and activated receptors was performed to 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 PDGF-R in the tumors from the 4 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 PDGF-R (Fig. 4). Thus, oral administration of STI571 inhibited PDGF-R activity in the MDA-MB-435 tumors.

To investigate whether inhibition of PDGF-R phosphorylation was restricted to the tumor cells, or was also seen in stromal cells within the tumors, we used double immunofluorescence staining to examine PDGF-R activation on tumor-associated endothelial cells. Endothelial cells were identified by staining for CD-31, and co-localization of this marker and phosphorylated PDGF-R 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 PDGF-R. 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 PDGF-R 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 23.85% in tumors from mice treated with STI571 plus paclitaxel (P< 0.001). The difference between the proportions of proliferating cells in the tumors from STI571 treated mice 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 100X 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 CD-31, for measurement of microvessel density, and immunofluorescence double labeling for CD-31 and TUNEL were used to evaluate the effects of STI571 on tumor-associated endothelial cells. Paclitaxel treatment alone had no

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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 CD-31 positive cells (P < 0.001). Immunofluorescence demonstrated CD-31 expression (red fluorescence, Fig. 6) TUNEL-positivity (green fluorescence), and co-localization of the signals (yellow fluorescence) in endothelial cells in the tumors of mice treated with STI571 or STI571 plus paclitaxel. No co-localization 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 of endothelial cells was not due to diminished expression of these pro-angiogenic factors in MDA-MB-435 tumor cells growing in the bone.

DISCUSSION:

As originally described by Stephen Paget in 1889 (26), the characteristic patterns of metastasis seen in breast cancer and other cancer patients 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

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"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 feed-back 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 breast cancer patients have been correlated with a higher incidence of metastasis and hence, shorter survival (13;29). Our study tested whether oral administration of STI571, a small molecule inhibitor of PDGF-R 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 PDGF-Rα and PDGF-Rβ. 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)(unpublished data). The expression level of PDGF-R 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 PDGF-R, whereas endothelial cells in unaffected bone or in tumors growing in muscle did not express PDGF-R (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 significantly inhibited the growth of MDA-MB-435 tumors, and resulted in preservation of bone structure, as evaluated with digital radiography and histologic preparations. These results suggested that signaling through PDGF-R 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 those we previously reported, which demonstrated that STI571 inhibited the growth of human prostate cancer cells in mouse bone (23). In both our previous and current studies, STI571 blocked PDGF-R phosphorylation in tumor cells in the 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 that express phosphorylated PDGF-R, and in the microenvironment of bone metastases these include tumor cells and tumor-associated endothelial cells, osteoblasts (31) 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;32). 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;33). Blocking the interactions between these factors and

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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 anti-vascular 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 pre-clinical models of human cancer and metastasis (25;34;35).

Systemic administration of STI571 has been shown to enhance anti-tumor effects of chemotherapy by reducing interstitial hypertension and increasing drug uptake (36). Although we found evidence of impaired PDGF-R 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 we used was lower than the maximal tolerated dose for mice, and was deliberately chosen to demonstrate a potential additive effect when combined with STI571. In preliminary studies (unpublished data) 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 (37;38), 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 PDGF-R. Systemically administered STI571 inhibited PDGF-R activation, induced apoptosis in the endothelial and breast cancer cells, and significantly decreased tumor size and osteolysis.

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These results suggest that interfering with the PDGF-R 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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Table 1 STI571 inhibits the growth of MDA-MB-435 in the bone of nude mice

Treatment group	Tumor incidence[a]	Mean tumor weight[b] (mg, +/- SD)	Lysis score[c]
Experiment 1			
Control	15/15	281 ± 67	2.1 ± 0.3
Paclitaxel	14/15	282 ± 60	1.7 ± 0.1
STI571	13/15	102 ± 40 [d]	1.2 ± 0.1
STI571 + paclitaxel	13/15	67 ± 24 [e]	0.8 ± 0.1
Experiment 2			
Control	12/12	470 ± 77	2.5 ± 0.2
Paclitaxel	13/13	416 ± 102	2.1 ± 0.2
STI571	10/12	165 ± 37 [f]	1.5 ± 0.2
STI571 + paclitaxel	10/12	211 ± 61 [g]	1.0 ± 0.4

[a] Number of mice with tumors/number of mice injected.

[b] Difference in weight between the tumor-bearing and non-tumor bearing hind legs.

[c] Mean score for degree of lysis seen in radiographs of the tumors, with 0 = no lysis to 4 = extensive bone destruction.

[d] P= 0.044 *versus* control, Student's *t* test.

[e] P= 0.007 *versus* control, Student's *t* test.

[f] P= 0.003 *versus* control, Student's *t* test.

[g] P= 0.018 *versus* control, Student's *t* test.

Table 2 Effects of STI571 and paclitaxel treatment on proliferation, apoptosis and microvessel

density in MDA-MB-435 bone tumors.

Treatment	Mean counts ($\pm$ SD)[a]		
	TUNEL-positive	% PCNA-positive	CD31-positive
Control	5.1 ± 2.8	59.2 ± 6.9	26.9 ± 10.4
Paclitaxel	8.0 ± 4.6 [b]	48.9 ± 7.3 [c]	27.8 ± 9.9 [d]
STI571	34.1 ± 13.6 [c]	27.5 ± 3.4 [c]	9.6 ± 3.9 [c]
STI571 + paclitaxel	42.2 ± 1.7 [c]	23.8 ± 3.9 [c]	10.75 ± 5.8 [c]

[a] 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 CD-31 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.

[b] P= 0.01 *versus* control, Student's *t* test.

[c] P= 0.001 *versus* control, Student's *t* test.

[d] P= 0.8 *versus* control, Student's *t* test.

FIGURE LEGENDS

Fig. 1 Radiologic and histologic appearance of nude mouse tibia two weeks after injection of MDA-MB-435 human breast cancer cells. *A*, digital radiograph shows no evidence of osteolysis. *B*, histologic section shows a representative focus of breast cancer cells growing in the bone marrow space (*arrowhead*), with no evidence of osteolysis. (Hematoxylin and eosin stain, original magnification X 200).

Fig. 2 Immunohistochemical detection of *A,* PDGF-B, *B* PDGF-Rβ (shown in *brown*)and *C,* phosphorylated PDGF-Rβ (*green* fluorescence) in MDA-MB-435 tumors growing in the bone of nude mice. (Original magnification X100).

Fig.3 Digital radiographic and histologic appearance of MDA-MB-435 tumors in the tibias of nude mice at the end of 6 weeks of therapy with paclitaxel, STI571 or STI571 plus paclitaxel. The radiographs (left of each pair of images) were taken at necropsy. In the micrographs (right of each pair of images), substantial areas of necrosis were seen only in tumors of mice treated with STI571, with or without paclitaxel. (Hematoxylin and eosin stain, original magnification X100).

Fig. 4 Immunohistochemical detection of PDGF-B, PDGF-Rβ and phosphorylated PDGF-Rβ in MDA-MB-435 tumors growing in the tibias of nude mice, treated with paclitaxel, STI571 or STI571 plus paclitaxel. The first and second columns show representative staining (*brown*) for PDGF-B and PDGF-Rβ, respectively. The third column shows representative images of fluorescent immunohistochemistry for phosphorylated (*p*)- PDGF-Rβ (*green*). Treatment with STI571, alone or in combination with paclitaxel reduced PDGF-Rβ activity in the tumors. (Original magnification X 100).

Fig. 5 Immunofluorescence histochemical detection of tumor-associated endothelial cells with antibody against CD-31and phosphorylated PDGF-Rβ (p PDGF-Rβ) in MDA-MB-435 tumors growing in the bone of nude mice, treated with paclitaxel, STI571 or STI571 plus paclitaxel. Staining with CD-31 antibodies was detected with Texas Red-conjugated goat-anti-rat antibody, and fluorescein isothiocyanate-conjugated goat anti-rabbit antibody detected phosphorylated PDGF-Rβ staining. Co-localization of the signals (*yellow color*) showed that CD-31 positive cells also expressed pPDGF-Rβ in tumors from control and paclitaxel treated mice, whereas treatment with STI571 with or without paclitaxel inhibited PDGF-Rβ activity. (Original magnification X 200).

Fig. 6 Immunohistochemical detection of CD-31 positive cells and apoptotic cells in MDA-MB-435 tumors growing in the bone of nude mice, treated with paclitaxel, STI571 or STI571 plus paclitaxel. Treatment with STI571 reduced the numbers of CD-31 positive cells (*left*), detecting the primary antibody with horseradish peroxidase-conjugated antibody and increased apoptosis, (*center*), detected with the TUNEL reaction. Co-localization of signals for TUNEL positive cells, and antibody to CD-31 detected with Texas Red-conjugated goat-anti-rat antibody (*right*) was seen only in tumors from mice treated with STI571, with or without paclitaxel. (Original magnification X 200).

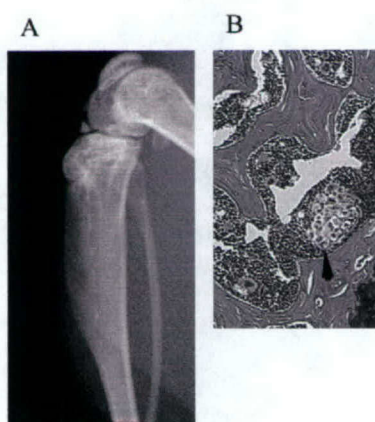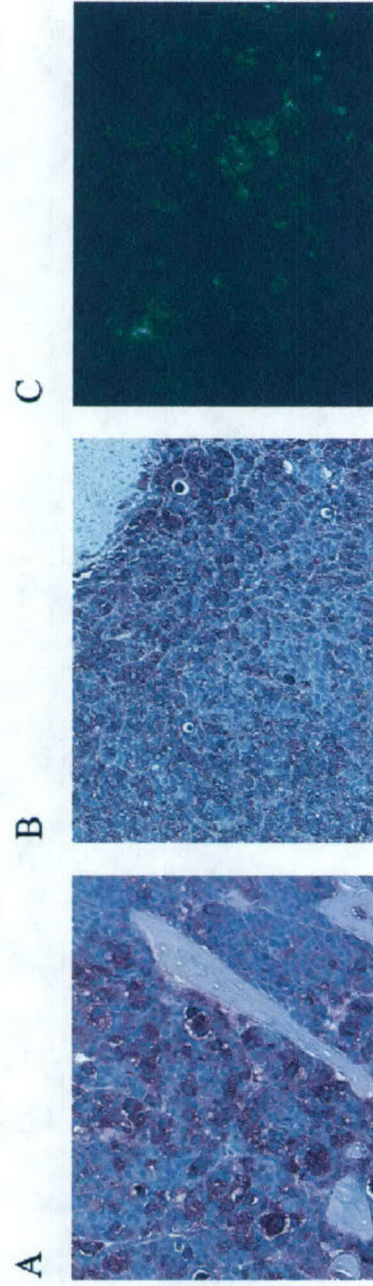28

Figure 1

A   B

Figure 2

Figure 3

Control

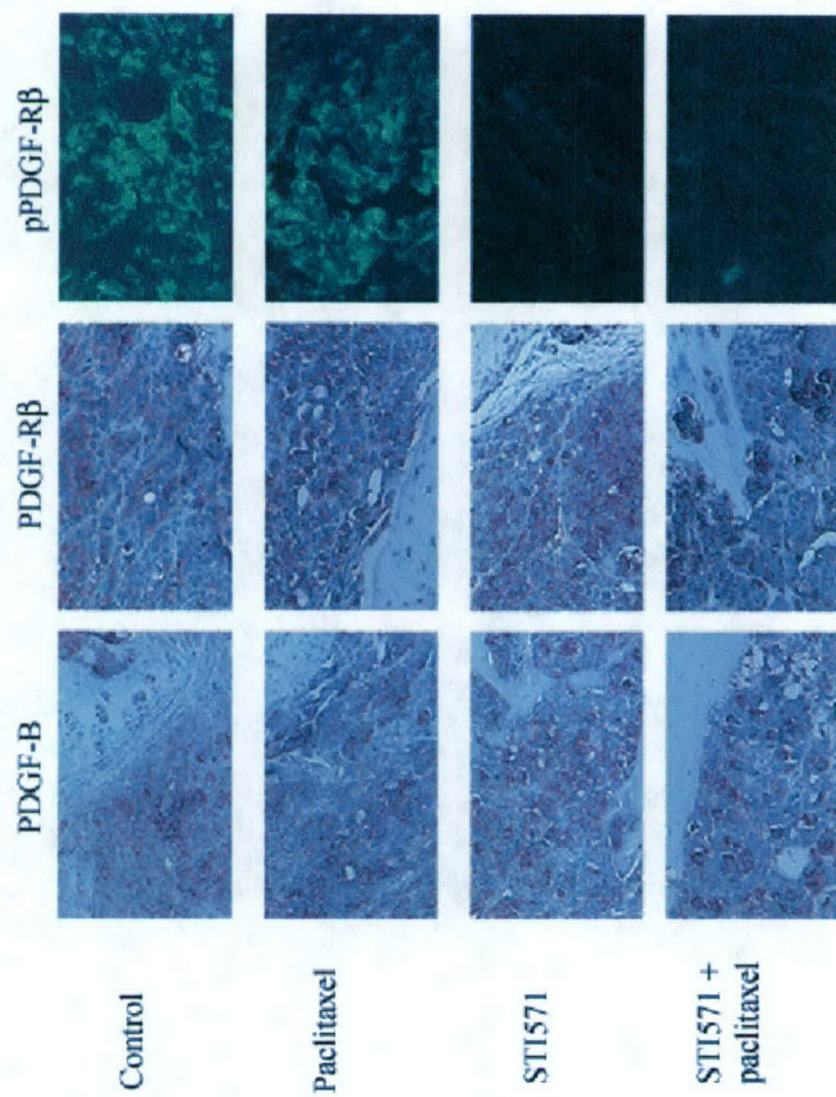Paclitaxel

STI571

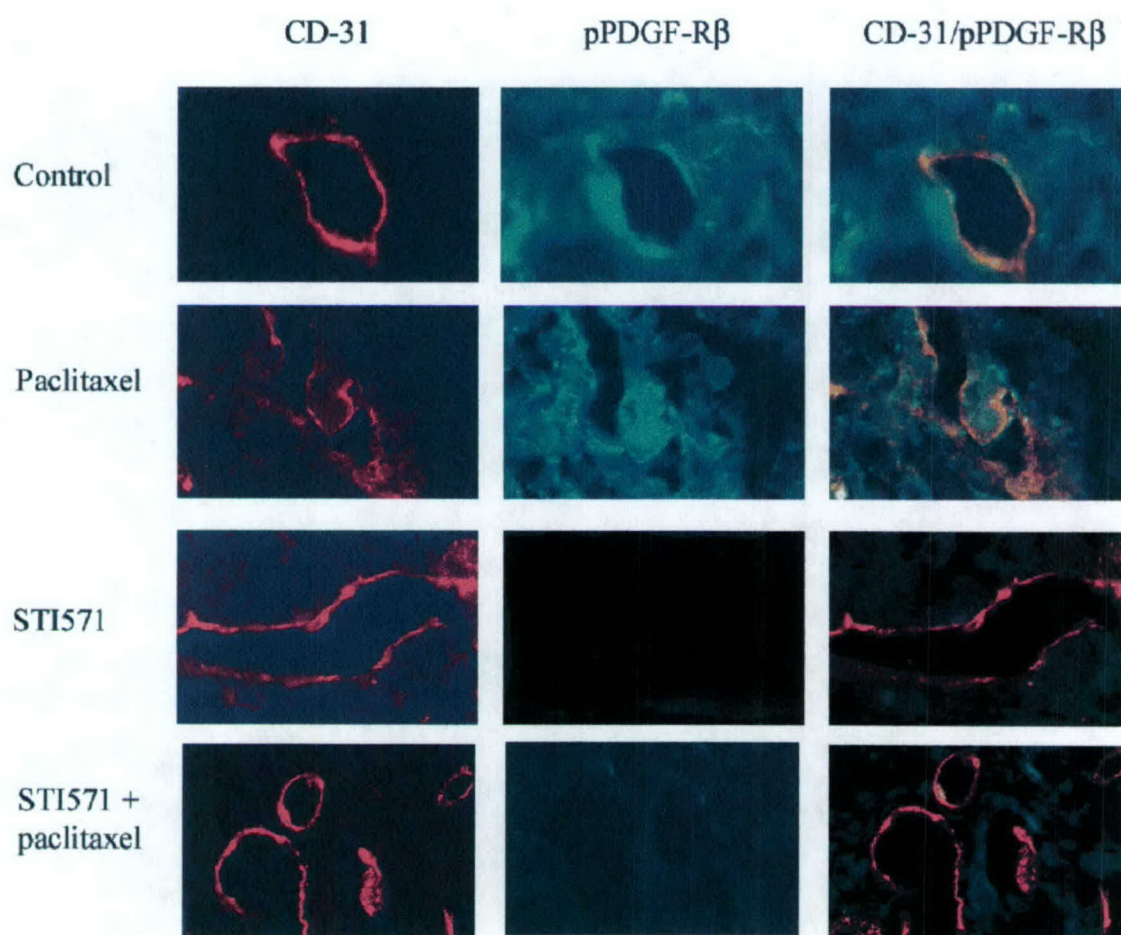STI571+ paclitaxel

Figure 4

Figure 5

	CD-31	pPDGF-Rβ	CD-31/pPDGF-Rβ
Control			
Paclitaxel			
STI571			
STI571 + paclitaxel			

Figure 6