PDGFR Signaling Blockade in Marrow Stroma Impairs Lung Cancer Bone Metastasis

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Abstract

Bone microenvironment and cell–cell interactions are crucial for the initiation and development of metastasis. By means of a pharmacologic approach, using the multitargeted tyrosine kinase inhibitor sunitinib, we tested the relevance of the platelet-derived growth factor receptor (PDGFR) axis in the bone marrow (BM) stromal compartment for the initiation and development of lung cancer metastasis to bone. PDGFRβ was found to be the main tyrosine kinase target of sunitinib expressed in BM stromal ST-2 and MC3T3-E1 preosteoblastic cells. In contrast, no expression of sunitinib-targeted receptors was found in A549M1 and low levels in H460M5 lung cancer metastatic cells. Incubation of ST-2 and human BM endothelial cells with sunitinib led to potent cell growth inhibition and induction of apoptosis in a dose-dependent manner. Similarly, sunitinib induced a robust proapoptotic effect in vivo on BM stromal PDGFRβ+ cells and produced extensive disruption of tissue architecture and vessel leakage in the BM cavity. Pretreatment of ST-2 cells with sunitinib also hindered heterotypic adhesion to lung cancer cell lines. These effects were correlated with changes in cell–cell and cell–matrix molecules in both stromal and tumor cells. Pretreatment of mice with sunitinib before intracardiac inoculation of A549M1 or H460M5 cells caused marked inhibition of tumor cells homing to bone, whereas no effect was found when tumor cells were pretreated before inoculation. Treatment with sunitinib dramatically increased overall survival and prevented tumor colonization but not bone lesions, whereas combination with zoledronic acid resulted in marked reduction of osteolytic lesions and osseous tumor burden. Thus, disruption of the PDGFR axis in the BM stroma alters heterotypic tumor–stromal and tumor–matrix interactions, thereby preventing efficient engagement required for bone homing and osseous colonization. These results support the notion that concomitant targeting of the tumor and stromal compartment is a more effective approach for blocking bone metastasis. Cancer Res; 71(1); 164-74. ©2010 AACR.

Introduction

Metastasis is a multistep process that is characterized by invasion of tumor cells into neighboring tissues, intravasation, survival in circulation, infiltration, and colonization of target organs (1, 2). Bone is a frequent target of dissemination of cells derived from solid tumors, including those of the breast, prostate, kidney, thyroid, and lung (3).

In primary tumors, nontumor cells, such as fibroblasts, immune cells, and endothelial cells (EC), and a variety of bone marrow (BM)–derived cells can induce tumor progression as a response to tumor-mediated signaling (4). A similar paradigm to that which occurs in the primary tumor could also influence metastatic development in a target organ. The long-standing “seed–soil” hypothesis has established the requirement for adaptive compatibility between tumor cells and the host organ (2–5). During early steps of bone metastatic homing, tumor cells infiltrate the osseous fenestrated capillary bed. This offers little mechanical constraint to tumor cells that engage with the host microenvironment, which is mainly formed by ECs of the microvasculature, and the stromal components of the osseous compartment (6). Subsequently, colonization frequently entails the formation of tumor-induced osteolytic lesions and progressive infiltration of malignant cells in the BM compartment. This process is mediated by induction of a subset of genes that endow cells with advantageous functions that lead to increased osteoclastogenic effects. Paracrine loops between bone–matrix-derived factors such as transforming growth factor β (TGF-β) and tumor cells have been shown to increase bone colonization (7–11). In addition, deleterious proteolytic degradation at the...
tumor–stroma interface also contributes to bone destruction (11). During this process, tumor–stroma or tumor–endothelium interactions can exacerbate both mechanisms of bone–matrix degradation.

In tumor cells, several genetic determinants have been described that enhance the propensity to form efficient metastasis in certain organs (12). For instance, CD44, which binds bone collagen and osteopontin, participates in the engagement of tumor cells in the bone microenvironment (13). Similarly, CXCR4 favors chemotaxis toward a cytokine that is highly expressed in bone stroma, SDF-1/CXCL12 (14).

In contrast, the requirements imposed by the host environment for the permissive maintenance and promotion of secondary outgrowth are beginning to be understood for some tumors (15). Crucial signaling pathways in stroma and or endothelium might be required not only for proper cell–cell engagement but also for the release of cytokines or growth factors (16).

Sunitinib is a multitargeted tyrosine kinase receptor with known efficacy in a variety of tumors such as gastrointestinal stromal tumor and metastatic renal cell carcinoma (17). Sunitinib inhibits the tyrosine kinase activity of vascular endothelial growth receptor 1–3 (VEGFR 1–3), platelet-derived growth factor receptor α and β (PDGFRα and β), c-kit, colony-stimulating factor 1 R (CSF-1R), and FLT3 (17). Currently, sunitinib is under intensive investigation in a variety of other cancers including breast, colorectal, and non–small cell lung cancer (18). The major targets VEGFR-1 to -3 are largely expressed in ECs; therefore, sunitinib elicits a potent antiangiogenic effect and might influence BM homeostasis (19–21). PDGFR has been shown to be crucial for pericyte maintenance and its inhibition synergizes with VEGFR blockade to impair tumor growth (22). Furthermore, the PDGFR axis has been shown to be frequently deregulated in a variety of tumors.

Given the relevance of this pathway in stromal and endothelial components of the bone compartment, we hypothesized that altering this axis could derange early steps of tumor metastatic homing and colonization. In this study, using sunitinib in a model of bone metastasis from lung cancer, we showed profound effects on cell proliferation and apoptosis in endothelial and stromal cells both in vitro and in vivo, which led to impaired tumor homing. In addition, sunitinib induced a dramatic delay in bone colonization by tumor cells and prolongation of lifespan. Thus, our findings underscore the validity of targeting the bone microenvironment to increase therapeutic benefits.

Materials and Methods

Cell culture
HBMEC-60 cells (human BM-derived ECs) were a kind gift of Ellen Van der Schoot (University of Amsterdam, Netherlands). Murine stromal ST-2 and MC3T3-E1 cells were a kind gift from Dr. Roberto Civitelli (Washington University, St. Louis, MO). The A549 human adenocarcinoma cell line, H460, and H727 were a kind gift from Dr. Adi F. Gazdar (University of Texas Southwestern, Dallas, TX). Cells were authenticated by sequencing critical mutations in KRAS and P53. A549M1 and H460M5 cells represent subpopulations of cells isolated from long bone after intracardiac (i.c.) inoculation in athymic nude mice (data not shown).

Adhesion assays
Adhesion experiments were performed as described (23). Substrates used were fibronectin, hyaluronic acid, collagen type I, and gelatin, and plastic and bovine serum albumin were used as controls.

For cell–cell monolayer assays, ST-2 cells or HBMECs were seeded at confluence in 6-well plates. Twenty-four hours later, 10^5 luciferase labeled cells/well were seeded onto the monolayers and allowed to drop and adhere for 1 hour. Luminescence was counted using Optimat Luminometer (Berthold Laboratories). Experiments were repeated 3 times.

In vivo treatments
Sunitinib was purchased from LC Laboratories and zoledronic acid from Novartis. Four-week-old athymic nude mice (Harlan) were maintained under specific pathogen-free conditions. Animals were treated with 60 mg/kg/d of sunitinib (by oral gavage) and 3 µg/kg/d of zoledronic acid. Inoculation was performed as previously described (24). All procedures were conducted following the guidelines of our Animal Care and Use Committee (University of Navarra, Spain).

Statistical analysis
Log-rank test was used to compare differences in metastasis survival curves. Data were analyzed by the Kruskal–Wallis test with Dunn’s multiple comparison test. Values were expressed as means ± SEM and statistical significance was defined as P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***) as shown in Figs. 1, 2, and 6.

More detailed information is provided in Supplementary Methods.

Results
Expression of functional tyrosine kinase receptors
We chose A549M1 and H460M5 lung subpopulations that were previously selected by in vivo passage, using previously published protocol (25), and isolated for their propensity to form bone metastatic lesions (data not shown). We also used murine BM stromal cells (ST-2), murine preosteoblastic MC3T3-E1 cells, and HBMECs as representative models of BM stromal and endothelial compartments. Expression levels of different tyrosine receptors targeted by multitargeted tyrosine kinase inhibitor sunitinib were investigated by real-time PCR.

As shown in Fig. 1A, ST-2 and MC3T3-E1 cells expressed high levels of PDGFRβ and PDGFRα, whereas HUVECs and HBMECs (not shown) expressed high levels of VEGFR-1 and VEGFR-2 and residual levels of c-kit. In contrast, receptor levels in A549M1 and H460M5 cells were almost undetectable by quantitative PCR (qPCR) for all the receptors tested. Immunofluorescence analysis confirmed the presence of PDGFRβ in the membrane of ST-2 cells, with the concomitant detection of the stromal marker fibroblast-specific protein 1 (FSP-1; Fig. 1B).
To substantiate this finding and to validate the efficiency of sunitinib, we assessed inhibition of tyrosine kinase receptor levels by Western blot analysis. ST-2 cells expressed PDGFRβ, and the administration of the natural ligand PDGF-A at a dose of 10 ng/mL for 5 or 10 minutes resulted in strong phosphorylation of the receptor. Incubation with sunitinib led to the inhibition of PDGFRβ phosphorylation in these cells (Fig. 1C). Similar results were obtained with MC3T3-E1 cells (data not shown). In contrast, and in agreement with previous findings, no receptors were detected in HBMECs, A549M1 (Fig. 1C), or H460M5 cells (not shown). Similarly, sunitinib effectively blocked the activation of VEGFR-2 in HBMECs (Supplementary Fig. S1).

These data indicate that PDGFRβ is the main target in ST-2 and MC3T3-E1 cells whereas VEGFR-2 is the main tyrosine kinase receptor blocked by sunitinib in HBMECs.

**Effects of sunitinib on cell proliferation and apoptosis**

Next, we tested the effects of sunitinib on cell proliferation by MTT assays. Incubation of ST-2 cells or HBMECs with sunitinib led to decreased cell proliferation in a dose-dependent manner (Fig. 1D). In contrast, growth rate of A549M1, H460M5, or H727 cells was unaffected (Fig. 1D). These data are consistent with the presence of sunitinib-targeted receptors in ST-2 cells and HBMECs.

We also studied the effect of sunitinib on apoptosis. Incubation of ST-2 cells with sunitinib led to increased activity of caspase-3, by flow cytometric analysis, in a dose-dependent manner (Fig. 1E). In contrast, no effect was observed in A549M1 and H460M5 cells treated under identical conditions (data not shown). Thus, blockade of the PDGFR/VEGFR axis by sunitinib decreases proliferation and enhances apoptosis in ST-2 cells and HBMECs.
Figure 2. Sunitinib blocks crucial tumor–stroma interactions in vitro. A, adhesion of A549M1 cells to collagen I (Col I), fibronectin (FN), vitronectin (VN), hyaluronic acid (HA), type II gelatin, and BSA after sunitinib treatment. Sunitinib diminished the adhesion of tumor cells to Col I (P < 0.05), whereas adhesion to fibronectin was enhanced upon sunitinib treatment (P < 0.01). Adhesion to other substrates was not affected upon treatment. B, adhesion of A549M1 and H460M5 cells to monolayers of ST-2 cells was tested under different experimental conditions. Adhesion of A549M1 and H460M5 cells was modified only when ST-2 cells were pretreated for 12 hours with sunitinib (200 nmol/L). C, levels of activity of MMP3/10 (measured by a fluorogenic substrate) in supernatants of ST-2 and A549M1 cells were decreased by sunitinib (P < 0.05), whereas those of HUVEC and H460M5 cells were unaltered. D, increasing doses of sunitinib increased MMP3/10 activity in the supernatants of A549M1 cells was found when they were treated with 20 or 200 nmol/L of sunitinib (P < 0.01). Adhesion to other substrates was not affected upon treatment. E, adhesion of A549M1 cells to HUVEC monolayers was reduced by sunitinib (200 nmol/L) in both cell lines, although it did not reach statistical significance. FN, fibronectin; VN, vitronectin; HA, hyaluronic acid; Gel, type II gelatin; BSA, bovine serum albumin.

**Effects of sunitinib on cell–matrix adhesion**

To study the effects of sunitinib on cell–matrix adhesion, we pretreated A549M1 cells with sunitinib at a dose of 20–200 nmol/L and cultured the cells on different substrates for 6 hours. Sunitinib significantly inhibited the ability of these cells to adhere to collagen type I, the most abundant bone matrix protein, whereas adhesion to fibronectin, an extracellular matrix (ECM) component that is highly abundant in lung parenchyma (26), was increased (Fig. 2A). In contrast, sunitinib had no effect on cell adhesion to other matrix proteins. Slight differences were found when the same experiment was performed with H460M5 cells, indicating that changes in cell adhesion in these cells are cell line specific (Supplementary Fig. S2A).

**Effect of sunitinib on cell–cell adhesion**

To study the adhesive ability of A549M1 and H460M5 cells to monolayers of ST-2 cells and HBMECs, we pretreated these acceptor cells with sunitinib for 12 hours at a dose of 200 nmol/L. A reduction in the ability of A549M1 and H460M5 cells to adhere to ST-2 cells was found when ST-2 cells were pretreated with sunitinib (P < 0.05). In contrast, no effect was seen when the drug and the cells were added simultaneously (Fig. 2B). No changes in adhesion were detected when A549M1 or H460M5 cells were cocultured with HBMECs (Supplementary Fig. S2B). These results indicate that sunitinib alters crucial cell–cell adhesion components in ST-2 stromal cells, which could be required for tumor–cell engagement.

**Effects of sunitinib in migration and invasiveness**

Sunitinib altered cell–matrix adhesion in A549M1 cells; therefore, we investigated whether these changes had functional effects in cell invasion and migration. No effect was found in the migration of A549M1 or H460M5 cells when they were incubated with sunitinib (Supplementary Fig. S2C). Similarly, invasive ability through Matrigel was unaltered in A549M1 or H460M5 cells upon incubation with physiologic doses of sunitinib. However, invasiveness was impaired at high doses (1 μmol/L) in both cell lines, although it did not reach statistical significance in H460M5 cells (Supplementary Fig. S2D). These findings indicate that sunitinib at therapeutic doses did not affect migration and invasiveness of lung cancer cells.

Next, we explored whether sunitinib could modify extracellular matrix metalloproteinase (MMP) activity. To explore this possibility, global MMP activity was assessed by a kinetic fluorescence assay. A significant decrease (P < 0.05) in the MMP3/10 activity in the supernatants of A549M1 cells was found when they were treated with 20 or 200 nmol/L of sunitinib (Fig. 2C). In ST-2 stromal cells, only a high dose (which causes cytotoxicity in these cells) resulted in significant reduction of MMP3/10 activity, whereas no changes were found for ECs (HUVEC) or H460M5 cells (Fig. 2C). MMP2/9
activity was below the level of detection in all the cells tested. These results indicate that sunitinib impairs extracellular MMP activity shown by A549M1 cells.

To examine the effect of sunitinib in osteoclastogenesis, different doses of the drug were incubated with conditioned media from A549M1 cells and added to mouse BM macrophages in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). Sunitinib led to a marked decrease in osteoclast differentiation at high doses (200 nmol/L and 1 μmol/L; \( P < 0.001 \)), whereas low doses significantly affect osteoclast formation to a lower extent (Fig. 2D). Similar results were obtained when the experiment was repeated with H460M5 cells (data not shown). These data indicate that sunitinib impairs osteoclast differentiation.

Transcriptomic effects of sunitinib on ST-2 and A549M1 cells

We used PCR multiplex analysis to study the effects of sunitinib on cell–cell and cell–matrix adhesion molecules in ST-2 and A549M1 cells. We quantified a total of 84 transcripts related to adhesion/invasion.

In ST-2 cells, at least a 2-fold expression change was found in 29 genes (5 upregulated and 24 downregulated). For A549M1 cells, a total of 36 genes were found to be at least 2-fold deregulated. Five of them were upregulated with both doses used, 21 were downregulated, and 10 genes displayed inconsistent behavior depending on the dose administered (Fig. 3).

Furthermore, as shown in Table 1, analysis revealed upregulation of epithelial markers in both cell lines, such as CTNNB1 and CTNNB1, ITGB1, and collagens COLA5A1 and COL6A1. Downregulated genes in both cell lines included mesenchyme-related molecules such as TNC, fibronectin, and integrins ITGA4 and ITGA8. CD44, a known marker of mesenchymal phenotype, was decreased in A549M1 cells after treatment with sunitinib, whereas E-cadherin (CDH1) was markedly increased. Hence, sunitinib alters the pattern of adhesion/invasion molecules in stromal and tumor cells.

Effects of sunitinib on BM in vivo

To study the effects of sunitinib in vivo, we treated mice with 60 mg/kg of sunitinib for 24 and 72 hours. After 24-hour treatment, small areas of hemorrhage in the long bones were observed at this time, even macroscopically. Seventy-two hours posttreatment (Fig. 4A–C), histologic analysis revealed disruption of the BM architecture, with extensive areas of hemorrhage (Fig. 4C). Numerous star-shaped cells undergoing

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**Figure 3.** Transcriptomic analysis by real-time qPCR of cancer-related adhesion and ECM genes in sunitinib-treated ST-2 and A549M1 cells. ST-2 and A549M1 cells were treated with 20 and 200 nmol/L of sunitinib for 24 hours. Real-time qPCR was performed using a commercial panel of primers for adhesion and ECM molecules. Heat maps represent the fold change of those genes that were found to be altered. Results for ST-2 cells treated with 200 nmol/L of sunitinib were omitted because of high levels of apoptosis observed with this dose. As shown in Table 1, upregulated genes included epithelial markers (CDH1, CTNNA1, and CTNNB1) and downregulated genes included previously described mesenchyme-related molecules (CD44, TNC, and FN).
## Table 1. Genes regulated by sunitinib in tumor (A549) and stromal cells (ST-2)

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<th>Gene</th>
<th>A549M1</th>
<th>ST2</th>
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<td>Genes regulated by sunitinib (20 nmol/L) both in A549M1 and ST-2 cells</td>
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<tr>
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<td>Fibroectin</td>
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<tr>
<td>Tenascin C</td>
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<tr>
<td>Genes regulated by sunitinib (20 nmol/L) only in A549M1 cells</td>
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apoptosis in the BM (Fig. 4E, and inset) were detected by active caspase-3 staining. In contrast, at 72 hours, fewer apoptotic cells were detected by active caspase-3 staining than the number observed at 24 hours. Coimmunofluorescence revealed that most of the apoptotic cells coexpressed PDGFRβ (Fig. 4G–I), which suggested that most of these cells belonged to the BM stromal compartment. Of note, bones of mice treated daily for 3 weeks did not display visible abnormalities in the BM, or apoptotic cells or hemorrhagic appearance, which suggested that sunitinib had a transient effect on the BM (Supplementary Fig. S2E). These data indicate that sunitinib induces apoptosis in BM PDGFRβ+ cells for a transient period, which leads to derangement of the bone microenvironment.

**Sunitinib alters tumor homing to bone**

Sunitinib alters BM components in vitro and in vivo; therefore, we sought to investigate the impact of these findings in tumor cells homing to bone. Mice were treated with sunitinib 3 days before i.c. inoculation of tumor cells at 60 mg/kg/d (Fig. 5A). Highly metastatic A549M1 cells were retrovirally transduced with a luciferase reporter gene for in vivo image analysis (27). Bioluminescence image analysis revealed a significant lower intensity in the hind limbs of the sunitinib-treated mice than vehicle-treated controls (P < 0.030; Fig. 5B). At that time point, animals were sacrificed and cells in the hind limbs were isolated by BM flushing. Conspicuous single cell–derived colonies (SCC) that were derived from each animal were counted. Consistent with previous findings, the number of SCCs derived from sunitinib-treated animals was significantly lower than that in the controls (P < 0.021; Fig. 5C). Similar results were obtained with another highly metastatic lung cancer cell line: H460M5 (Supplementary Fig. S3A and B). These data indicate that sunitinib alters tumor homing to bone, presumably by altering the bone microenvironment. However, to discriminate the fact that sunitinib could also affect circulating tumor cells before efficient homing to the bone compartment, we incubated A549M1 cells with vehicle or 200 nmol/L of sunitinib, a dose similar to the levels reached in plasma after administration of 60 mg/kg/d of sunitinib (Fig. 5D). Animals were sacrificed 4 days postinoculation. Bioluminescence image analysis revealed no differences between groups (Fig. 5E). Similarly, isolation of SCCs derived from both groups of animals revealed no differences (Fig. 5F). Similar results were obtained with H460M5 cell line (Supplementary Fig. S3C and D). These findings suggest that incubation of tumor cells with sunitinib in vitro before in vivo inoculation did not affect tumor cells in their ability to efficiently infiltrate and survive in the bone compartment.
Sunitinib effect on bone metastatic activity

Our previous findings suggested that sunitinib alters the BM microenvironment and tumor cell invasive ability. Thus, we tested the extent to which sunitinib could prevent prometastatic activity of lung cancer cells to bone, alone or in combination with ZA, a drug currently used in patients to treat bone metastasis. Highly metastatic A549M1 cells were inoculated i.c. and treated daily with sunitinib alone or in combination with ZA, starting 6 days postinoculation, according to the therapeutic regimen (Fig. 6A). As expected, ZA-treated animals showed a slight increase in overall survival as compared with control mice. Combined drug treatment led to a significant increase in overall survival in animals treated with sunitinib alone or in combination with ZA (Fig. 6B). These findings were correlated with a significant decrease in bone colonization as assessed by bioluminescence imaging (Fig. 6C and D). ZA treatment, alone or in combination with sunitinib, markedly reduced bone metastatic lesions as assessed by X-ray image analysis and micro-CT (Fig. 6E and F). However, despite the decrease in tumor burden observed by bioluminescence, animals treated with sunitinib alone showed prominent bone osteolytic lesions, similar in extent to those in vehicle-treated animals observed by X-ray imaging. These findings were correlated with a decrease in the number of tartrate-resistant acid phosphatase (TRAP)-positive cells, which were counted at the tumor–bone interface in animals treated with ZA alone or in combination with sunitinib (Fig. 6G). Sunitinib-treated animals showed a similar lifespan as the ZA/sunitinib-treated animals, despite the presence of prominent bone lesions, which suggested that other effects related to tumor-induced cachexia might have been prevented by sunitinib. Consistently, the number of TRAP⁺ cells at the tumor–bone interface in sunitinib-treated animals was similar to that in the vehicle-treated control mice. Taken together, these data indicate that the combination of sunitinib and ZA increases animal survival and prevents the development of osteolytic lesions.

Discussion

Compatibility between organ microenvironment and tumor cells is required for proper metastatic colonization (2–5). Although mechanical constraints imposed by the microvascular bed certainly influence cell arrest, in previous studies in a model of lung metastasis to bone, the uniform location of osseous lesions in the metaphysis of long bones suggested that rather than occurring as a result of stochastic events, specific tumor–endothelium/stroma engagement can take place (11). In the present study, we have explored this observation by investigating the contribution of the PDGFR pathway in the microenvironment in early steps of bone homing. We found that perturbed microenvironment caused by sunitinib-induced apoptosis of PDGFR⁺ cells resulted in deranged tumor homing, which led to increased animal survival in a lung cancer metastasis model. Thus, our approach unveiled the relevance of the stromal and endothelial bone environment in early events of homing during tumor cell engagement. Several findings support this contention. First, the PDGFR pathway is required for normal osteostasis, because pharmacologic blockade decreased BM stromal cell proliferation and increased apoptosis in vitro. Second, apoptosis was also observed in PDGFR⁺ cells in vivo. Third, lung cancer cells homing to bone was markedly diminished in pretreated animals. Thus, the PDGFR pathway in the bone

Figure 5. Sunitinib inhibits metastatic cell homing to bone. A, Experimental regimen preconditioning the animals with sunitinib (60 mg/kg) before injection of A549M1 tumor cells. B, Photon flux levels in the metaphysis of hind limbs were quantified. Tumor burden in the sunitinib-treated mice was significantly lower than that found in vehicle-treated mice. C, the number of SCCs isolated after BM “flushing” of hind limbs. After antibiotic selection, lower number of SCCs was obtained from sunitinib-treated mice. D, experimental regimen preincubating the cells with sunitinib (200 nmol/L) before lower than that found in vehicle-treated mice. E, quantification of photon flux levels in the metaphysis of hind limbs showed similar tumor burden in both groups. F, the number of SCCs isolated form BM “flushing” of hind limbs after antibiotic selection was similar in both groups.
Figure 6. Inhibition of lung cancer bone metastatic colonization by sunitinib. A, experimental outline. Mice were treated daily, starting 6 days after i.c. tumor cell inoculation, with sunitinib (60 mg/kg/d), ZA (at 3 μg/kg/d), or a combination of both. B, Kaplan–Meier curves of survival of mice injected with A549M1 cells. Sunitinib, either alone or in combination with ZA, doubled the lifespan of mice (P < 0.001). ZA alone did not improve survival. C and D, photon flux images in the metaphyses of tumor-bearing mice. All treatments decreased tumor burden in the metaphyses of A549 M1-injected mice. The effect was more potent in the case of combined treatment. E, bones were analyzed by X-ray (a–d), micro-computed tomography (e–h), and TRAP (i–m) histochemistry. F, X-ray image analysis of osteolytic lesions in long bones. Treatment of mice with sunitinib and ZA or ZA alone reduced significantly the bone metastatic area as compared with control mice. Sunitinib did not prevent the development of osteolytic lesions. Bones from vehicle and sunitinib-treated mice showed extensive bone destruction in the metaphyses (a, c and e), whereas ZA alone or in combination with sunitinib prevented osteolytic lesions (b, f and d). G, TRAP⁺ multinucleated cells were counted at tumor bone interphase. Bone destruction was observed in sunitinib and vehicle-treated mice, which correlated with the number of TRAP⁺ cells (l and j), whereas ZA, alone or in combination with sunitinib, prevented bone destruction and decreased the number of TRAP⁺ cells (k and m).
microenvironment is crucial for early events in bone metastasis. These findings are in agreement with previous studies that have shown that the antitumor effects of sunitinib are related to the microenvironment rather than a direct effect on tumor cells (28). Similarly, PDGFRα signaling necessary for the recruitment of VEGFR-producing stromal fibroblasts is required for tumor growth and angiogenesis (29, 30).

Other pathways targeted by sunitinib might also be involved in the observed phenotype, including VEGFR, c-kit, or VEGFR-3. In fact, the antiangiogenic effect of sunitinib that is mediated through VEGF pathway blockade has already been demonstrated (17). However, we provide here evidence that the PDGFR axis is highly relevant in tumor–BM stroma interactions. We showed that BM-derived stromal cells (with high expression of PDGFR) were by far more sensitive to sunitinib than BM-derived endothelial and tumor cells. Plasma concentration of sunitinib in mice after administration of 60 mg/kg has been shown to be approximately 100 to 200 nmol/L (31), a dose that severely affects stromal cells but not endothelial or tumor cells.

Rather than an effect on tumor cells, profound changes induced by sunitinib in the BM compartment were most likely impairing tumor-bone homing. In this regard, a large number of adhesion molecules were also deregulated in ST-2 cells. All these changes may promote the decrease in adhesion of A549M1 cells to the bone substrate or BM resident cells in early steps.

In the same line, mononuclear precursors were also targeted by sunitinib, leading to impaired osteoclastic differentiation in vitro. In a model of lung cancer metastasis, previous findings have suggested the requirement of osteoclastic activity in early steps of osseous colonization whereas in late stages tumor-derived proteolytic activity may prevail (11). Indeed, other studies have shown a reduction of bone metastasis by blocking CFS-I R phosphorylation and osteoclast formation in a mouse model of breast cancer upon sunitinib treatment (32). Thus, it is tempting to speculate that in sunitinib-treated animals, tumor-induced osteolytic lesions could be delayed in vivo by impaired osteoclastogenesis, leading to decrease tumor burden and marked delay in osseous colonization.

Even though tumor cell growth was unaffected in vitro, we found that sunitinib also altered cell adhesion and invasion of tumor cells. Slight differences in cell–matrix adhesion suggest that subtle effects of sunitinib on tumor cells were cell line specific. These changes could be mediated by partial inhibition of other kinases and were consistent with the different expression of sunitinib-targeted receptors between A549 and H460 cells. In this line, inhibition of PDGFR/VEGFR-3 or other kinases that present some residual expression in A549M1 cells could be involved in the modulation of relevant matrix adhesion molecules such as integrins, collagen, laminin, TNC, and CD44.

Other in vitro effects such as cell invasiveness and osteoclast activity were consistent between both cell lines. Besides slight differences between tumor cell lines, these changes translate in similar biological effects, as our in vivo findings suggest that tumor cell homing to bone was unaffected when the cells were pretreated before injection at a dosage that caused significant transcriptomic modifications in these cell markers.

Concomitant effects on the microenvironment and tumor cells support the validity of a combinatorial therapeutic approach that targets the stroma. Indeed, bisphosphonates, such as ZA, currently used for the treatment of osteolytic lesions, alter bone-mineralized matrix, thus impairing proper osteoclast attachment required for bone resorption (33). Similarly, the targeting of the stromal/endothelial compartment by sunitinib decreased tumor burden and increased overall survival. Thus, combination of both drugs underscores a new paradigm for the design of novel therapeutic modalities and raises the intriguing possibility of using a similar approach for the maintenance of bone mass in other types of tumors. Besides the impaired tumor homing observed upon sunitinib treatment, the most striking effect of combination of both drugs was the increased lifespan observed in sunitinib-treated animals despite the presence of prominent osteolytic lesions. We speculate that these preventive effects of sunitinib might be related to the blockade of tumor-induced cachexia.

Despite the beneficial effects observed in our model, concerns about the use of sunitinib in vivo have been raised by several recent findings (34, 35). In agreement with the effects of other antiangiogenic therapies, altered BM architecture and vasculature integrity in the BM also require a cautious use of sunitinib in metastasis. This alteration is transient because after continuous administration of the drug (up to 1 month), the appearance of the BM was normal, which suggests that alternative, compensatory pathways might be activated to restore BM homeostasis. However, sunitinib most likely contributes to the release of BM-derived cells into the circulation, as a consequence of BM disruption. Some of these cells, such as VEGFR-1+ cells, could disseminate to distant organs and secrete chemoattractants that favor the development of a prometastatic niche (36), which, in turn, could lead to the recruitment of tumor cells. Although this speculation requires further experimental validation, recent findings have described enhanced metastasis to the lungs after short-term treatment with bevacizumab and sunitinib (34, 35). However, it has been well documented in many types of tumors (both in animal models and in patients) that sustained administration of sunitinib results in potent reduction of tumor growth with minimal toxicity (37). Our findings strongly support the therapeutic benefit of sunitinib in inhibiting tumor burden and bone metastasis.

In summary, our results unveiled the requirement of an intact PDGFR signaling pathway for BM homeostasis and effective engagement of the tumor–bone microenvironment. Host-related effects are likely responsible for the significant impairment in bone homing, bone metastatic colonization, and the dramatic decrease in tumor burden, which significantly extended overall survival. Moreover, this novel mechanism of action offers a new window of opportunity for combination therapy that targets tumor and the stromal microenvironment for effective treatment of bone metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Antón and I. Larzábal were supported by the Basque Government. F. Lecanda is also supported by funds from the I3 Program, "La Caixa Foundation," and is a recipient of the "Ortiz de Landázuri" award (67/2005, Government of Navarre).

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Received May 12, 2010; revised October 8, 2010; accepted November 2, 2010; published OnlineFirst November 19, 2010.

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