The type 1 lysophosphatidic acid receptor is a target for therapy in bone metastases

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Platelet-derived lysophosphatidic acid (LPA) supports the progression of breast and ovarian cancer metastasis to bone. The mechanisms through which LPA promotes bone metastasis formation are, however, unknown. Here we report that silencing of the type 1 LPA receptor (LPA1) in cancer cells blocks the production of tumor-derived cytokines that are potent activators of osteoclast-mediated bone destruction and significantly reduces the progression of osteolytic bone metastases. Moreover, functional blockade of LPA action on its cognate receptor LPA1 using a pharmacological antagonist mimics the effects of silencing LPA1 in tumor cells in vitro and substantially reduces bone metastasis progression in animals. Overall, these results suggest that inhibition of platelet-derived LPA action on LPA1 expressed by tumor cells may be a promising therapeutic target for patients with bone metastases.

Breast cancer | platelet | treatment

Bone is a frequent metastatic site for many cancers (1). Bone metastasis formation is associated with a high morbidity rate because of intractable bone pain, pathological fractures, hypercalcemia, and nerve compression (1). Bone-residing metastatic cells are not directly able to destroy bone. Instead, they secrete paracrine factors [IL-6, IL-8, and parathyroid hormone-related peptide (PTHrP)] that stimulate osteoclast-mediated bone resorption, leading to osteolysis (1). In this respect, bisphosphonates (as inhibitors of osteoclast activity) are the standard of care in the treatment of patients with bone metastases. Unfortunately, these treatments are only palliative and do not provide a life-prolonging benefit to metastatic patients (2), indicating that more efficacious therapies are required.

We have recently demonstrated that the naturally occurring bioactive lipid, lysophosphatidic acid (LPA), produced by activated blood platelets (3), is coopted by breast and ovarian cancer cells as a tumor mitogen and an inducer of tumor-derived cytokine (IL-6 and IL-8) that, altogether, promote the progression of bone metastases (4). Endogenous receptors through which LPA promotes breast cancer progression and bone metastasis are, however, unknown. LPA binds to three GTP-binding protein-coupled receptors, LPA1 (5), LPA2 (6), and LPA3 (7), that mediate the growth factor-like activity of LPA in a large variety of cell types in culture, including cancer cells (8). Interestingly, mRNA levels for LPA receptors are up-regulated in various cancers (9–11). Yet, the clinical significance of such observations remains to be determined. Using both genetic and pharmacological approaches in vitro and in vivo, we demonstrate here that inhibition of LPA action on its receptor LPA1 is a promising therapeutic target in cancer, especially for metastasis to bone.

Results

Silencing of LPA1 Expression in Cancer Cells Markedly Impairs Bone Metastasis Progression. We have shown previously that CHOβwt ovarian cancer cells express only LPA1 (4). To analyze the role of LPA1 in bone metastasis formation, we generated a CHOβwt cell line in which the expression of LPA1 was stably down-regulated with specific small interference RNAs (siRNAs; see Fig. 1A). In vivo experiments indicated that the down-regulation of LPA1 did not alter the propensity of these cells to induce bone metastases in nude mice (Fig. 1B and Table 1). However, the silencing of LPA1 expression markedly reduced (77% inhibition) the progression of osteolytic bone lesions in animals at the time of death (Fig. 1B and Table 1). The extent of osteolytic lesions was associated with an increase of the bone volume (BV)/tissue volume (TV) ratio, which indicated a prevention of bone loss, and with a decrease of the tumor burden (TB)/TV ratio, which indicated a decrease in skeletal TB (Fig. 1B and Table 1). We next focused our attention on the human MDA-BO2 breast cancer cell line, which endogenously expresses all three LPA receptors (LPA1, LPA2, and LPA3) and induces bone metastases in nude mice (4). Human MDA-BO2 cells have been previously stably transfected to express GFP (MDA-BO2/GFP) to detect fluorescent bone metastases in animals (12). The silencing of LPA1 expression in MDA-BO2/GFP cells was achieved by using a siRNA strategy similar to that described for CHOβwt cells (Fig. 1A). Radiographic analysis indicated that all MDA-BO2/GFP transfectants had the same bone metastasis incidence in animals (Table 1). However, animals injected with MDA-BO2/GFP-SiLPA1 cells displayed an 80% decrease in the extent of osteolytic lesions (Fig. 1C and Table 1). Moreover, there was a 50% decrease in the extent of fluorescent lesions in metastatic animals inoculated with MDA-BO2/GFP-SiLPA1 cells (Fig. 1C and Table 1). Histological examination revealed that the silencing of LPA1 expression in MDA-BO2/GFP cells was associated with a decrease of bone destruction (increased BV/TV ratio) and reduced skeletal TB (decreased TB/TV ratio; see Fig. 1C and Table 1).

LPA1 Controls Tumor Cell Proliferation in Vitro and in Vivo. To assess whether LPA1 played a role in tumor cell proliferation, we introduced wild-type cDNA for LPA1 into the LPA-insensitive human breast cancer SKBr-3 cells (Fig. 2A). As opposed to parental cells, LPA1-expressing SKBr-3 cells (clones #3.1 and #4.1) dose-dependently responded to the mitogenic activity of LPA (Fig. 2B). Conversely, the silencing of LPA1 expression in CHOβwt cells almost totally abolished the mitogenic activity of LPA on these cells (Fig. 2B). As opposed to parental CHOβwt cells, GFP-SiLPA1 cells displayed an 80% decrease in the extent of fluorescent lesions in metastatic animals (Table 1). However, animals inoculated with MDA-BO2/GFP-SiLPA1 cells displayed an 80% decrease in the extent of osteolytic lesions (Fig. 1C and Table 1). Moreover, there was a 50% decrease in the extent of fluorescent lesions in metastatic animals inoculated with MDA-BO2/GFP-SiLPA1 cells (Fig. 1C and Table 1). Histological examination revealed that the silencing of LPA1 expression in MDA-BO2/GFP cells was associated with a decrease of bone destruction (increased BV/TV ratio) and reduced skeletal TB (decreased TB/TV ratio; see Fig. 1C and Table 1).

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Abbreviations: LPA, lysophosphatidic acid; LPA1–3, LPA receptor type 1–3; GM-CSF, granulocyte/macrophage colony-stimulating factor; Gro, growth-related oncogene; BV, bone volume; TV, tissue volume; TB, tumor burden; TRAP, tartrate-resistant acid phosphatase; sbl, scrambled; K16425, 3-(4-[4-([1-(2-chlorophenyl)ethoxy]carbonyl amino)-3-methyl-5-isoxazolyl][benzylsulfonyl]propanoic acid).

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BO2 investigated the role of LPA1 on the LPA-dependent activation after tumor cell injection. Osteolytic lesions were detected by radiography (x-rays), and bone destruction and TB were examined by histology (Histo), as described.

Table 1. Effect of LPA1 expression on CHO and MDA-BO2/GFP cells in metastatic animals

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Osteolytic lesions</th>
<th>Fluorescent lesions</th>
<th>Histomorphometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHOβ3wt</td>
<td>Incidence</td>
<td>Area, mm²</td>
<td>Incidence</td>
</tr>
<tr>
<td>CHOβ3wt-Sb#1</td>
<td>6/6</td>
<td>15.5 ± 1.3</td>
<td>N.A.</td>
</tr>
<tr>
<td>CHOβ3wt-SiLPA#6</td>
<td>5/6</td>
<td>15.6 ± 2.5</td>
<td>N.A.</td>
</tr>
<tr>
<td>BO2/GFP</td>
<td>4/4</td>
<td>23.6 ± 2.3</td>
<td>4/4</td>
</tr>
<tr>
<td>BO2/GFP-SiLPA#9 and #10</td>
<td>5/5, 8/8</td>
<td>20.3 ± 1.2</td>
<td>5/5, 8/8</td>
</tr>
<tr>
<td>BO2/GFP-SiLPA#122, #123, and #132</td>
<td>7/7, 6/6, 8/8</td>
<td>4.1 ± 0.5*</td>
<td>2/7, 2/7, 3/8</td>
</tr>
</tbody>
</table>

Animals were analyzed by noninvasive radiography and fluorescence imaging 21 or 30 days after intravenous inoculation of CHOβ3wt or MDA-BO2/GFP cell lines, respectively. Metastatic hind limbs were analyzed by histomorphometry. Incidence indicates the number of metastatic animals over the total number of animals used in each experiment. Data of osteolytic and fluorescent lesions are expressed as the mean ± SE (in mm²) of n metastatic mice. N.A., not applicable. *, P < 0.0001; ***, P < 0.01 using unpaired Student’s t test when comparing animals injected with SiLPA1 transfectants with animals injected with Sb1 transfectants.

Fig. 1. Effect of silencing of LPA1 expression in CHOβ3wt and MDA-BO2/GFP cells on osteolytic lesions and skeletal TB. (A) Expression levels of LPA1 mRNA expression were quantified by real-time RT-PCR. Data were presented as the mean ± SD of LPA1/GAPDH mRNA ratio of three independent experiments (Upper). *, P < 0.01, parental cells vs. SiLPA1 transfectant. Lysates of tumor cells were resolved by SDS/PAGE and immunoblotted with an anti-LPA1 polyclonal antibody (Lower). (B) CHOβ3wt cells and stable transfectants (clones Sb1#1 and SiLPA#6) were assayed for their ability to induce osteolytic bone metastases. Representative radiographs (x-rays) of hind limbs from mice, 21 days after tumor cell inoculation. There was a marked reduction in the extent of osteolytic lesions (arrows) in CHO-SiLPA1 cell-bearing mice. Representative bone histology (Histo) of Goldner’s trichrome stained tibial metaphysis from metastatic animals. Bone is stained in green; bone marrow and tumor cells (T) are stained in red. Trabecular bone was almost completely preserved in tibial metaphysis from animals bearing CHO-SiLPA1 cells. (C) MDA-BO2/GFP cells and stable transfectants were assayed for their ability to induce bone metastases. Animals were analyzed 30 days after tumor cell injection. Osteolytic lesions were detected by radiography (x-rays), and bone destruction and TB were examined by histology (Histo), as described in B. Fluorescent tumor lesions were identified by fluorescence scanning (Fluo). There was a marked reduction in the extent of osteolytic (arrows) and fluorescent lesions, and trabecular bone was almost completely preserved in tibial metaphysis from animals bearing MDA-BO2/GFP-SiLPA1 cells.
plates were incubated overnight with 1-oleoyl LPA (0–1 µM) in proliferation assays. Quiescent tumor cells plated in 96-well tissue culture plates were incubated overnight with 1-oleoyl LPA (0–1 µM) in serum-free medium containing 0.1% (wt/vol) BSA fatty acid-free then pulsed with [H]thymidine for 8 h. Data of [H]thymidine incorporation were expressed in cpm, are the mean ± SD of six replicates, and are representative of at least three separate experiments. *P < 0.001, parental cells vs. transfectants.

demonstrated that LPA1 mediates the proliferation of cancer cells both in vitro and in vivo.

LPA1 Mediates Tumor Cell-Induced Osteoclast Activity Through the Production of Specific Cytokines. LPA has recently been shown to stimulate the production of IL-6 and IL-8 in ovarian and breast cancer cells because of the activation of each LPA receptor (LPA1, LPA2, and LPA3; see ref. 13). Artificial overexpression of LPA1 in MDA-BO2 cells increases IL-6 and IL-8 production in response to LPA (4). Among the cytokines, chemokines, and growth factors naturally secreted by MDA-BO2 cells, IL-6, IL-8, GM-CSF, Groα, and MCP-1 were detected by RayBio (Norcross, GA) human cytokine antibody detection kit. Data of [H]thymidine incorporation were expressed in cpm, are the mean ± SD of six replicates, and are representative of at least three separate experiments. *P < 0.001, parental cells vs. transfectants.

Table 2. Effect of LPA1 expression by tumor cells on LPA-induced cytokine secretion

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IL-6</th>
<th>IL-8</th>
<th>GM-CSF</th>
<th>Groα</th>
<th>MCP-1</th>
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<tbody>
<tr>
<td>SKBr-3</td>
<td>4 ± 0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>SKBr/LPA1#3.1</td>
<td>454 ± 64</td>
<td>572 ± 42</td>
<td>1,033 ± 133</td>
<td>577 ± 62</td>
<td>281 ± 40</td>
</tr>
<tr>
<td>SKBr/LPA1#4.1</td>
<td>491 ± 54</td>
<td>578 ± 91</td>
<td>1,013 ± 144</td>
<td>576 ± 72</td>
<td>282 ± 38</td>
</tr>
<tr>
<td>BO2/GFP</td>
<td>467 ± 71</td>
<td>987 ± 10</td>
<td>2,313 ± 267</td>
<td>1,161 ± 55</td>
<td>407 ± 45</td>
</tr>
<tr>
<td>BO2/GFP-Sb1#9</td>
<td>524 ± 47</td>
<td>1,048 ± 182</td>
<td>2,241 ± 228</td>
<td>1,142 ± 124</td>
<td>382 ± 113</td>
</tr>
<tr>
<td>BO2/GFP-Sb1#10</td>
<td>570 ± 25</td>
<td>1,079 ± 68</td>
<td>2,219 ± 368</td>
<td>1,117 ± 72</td>
<td>380 ± 132</td>
</tr>
<tr>
<td>BO2/GFP-SILPA1#122</td>
<td>236 ± 45</td>
<td>491 ± 7</td>
<td>665 ± 143</td>
<td>10 ± 17</td>
<td>6 ± 4</td>
</tr>
<tr>
<td>BO2/GFP-SILPA1#123</td>
<td>217 ± 13</td>
<td>446 ± 16</td>
<td>597 ± 160</td>
<td>14 ± 4</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>BO2/GFP-SILPA1#132</td>
<td>209 ± 6</td>
<td>439 ± 11</td>
<td>679 ± 139</td>
<td>9 ± 4</td>
<td>7 ± 4</td>
</tr>
</tbody>
</table>

The specific production of cytokines secreted in the culture media from cells treated with 1-oleoyl LPA (1 µM) for 48 h was quantified by ELISA. Data are expressed as mean ± SD (in pg/ml per 10^6 cells) of three replicates and are representative of two separate experiments.
conditioned medium collected from LPA-treated MDA-BO2/GFP-SiLPA1 cells did not stimulate osteoclastogenesis nor did the conditioned medium from LPA-treated SKBr-3 cells that lack LPA1 (Fig. 4A). These data do not show the functional role of each individual cytokine. However, consistent with an important role of the secretion of IL-6 and IL-8 by tumor cells in osteoclast-mediated bone destruction in vivo (15, 16), the recruitment of mature osteoclasts located at the bone/tumor interface of metastatic long bones in animals bearing tumor cells (MDA-BO2/GFP-SiLPA1 and CHOβ3wt/SiLPA1 cells) that lack LPA1 was also markedly decreased, as judged by tartrate-resistant acid phosphatase (TRAP) staining of bone resorption surfaces (Fig. 4B). Altogether, these results indicate that LPA1 expressed by tumor cells mediates LPA activity that leads to osteoclast activation in vitro and in vivo.

Pharmacological Blockade of LPA1 Activity Inhibits Bone Metastasis Progression. L-((4)-[1-(2-chlorophenyl)ethoxy]carbonylamino)-3-methyl-5-isoxazolyl[benzylsulfanyl]propanoic acid (Ki16425) is a potent antagonist of LPA activity on LPA1 and LPA3 (17). Ki16425 blocks the LPA-induced motility of various LPA1-expressing carcinoma cell lines in vitro (18). Ki16425 also dose-dependently inhibited LPA1-induced proliferation of LPA1-expressing MDA-BO2/GFP and CHOβ3wt cell lines in vitro (Fig. 5) and specifically blocked the LPA-mediated secretion of cytokines (IL-6, IL-8, GM-CSF, Groα, and MCP-1) in LPA1-expressing breast cancer cell lines (data not shown). We therefore examined the effect of Ki16425 on the progression of established bone metastases using our fluorescent animal model. Ki16425 inhibited by 90% the progression of osteolytic lesions and totally blocked the formation of fluorescent tumor lesions (Fig. 6). Histological examination of bone tissue sections showed that bone destruction and skeletal TB were markedly decreased upon Ki16425 treatment (Fig. 6). Unlike in vitro observations, Ki16425 treatment substantially inhibited the recruitment of mature osteoclasts at the bone/tumor interface and also decreased the in vivo proliferation of tumor cells (80% decrease of the Ki67 cell mitotic index; see Fig. 6). Plasma of vehicle-treated metastatic mice was as equipotent as purified 1-oleoyl LPA (0.1 μM) to stimulate proliferation of tumor cells (Fig. 7B). In contrast, the mitogenic activity of plasma from Ki16425-treated animals was markedly reduced (80% inhibition; see Fig. 7A). As indicated in Fig. 7A, the circulating concentration of Ki16425 was 0.1 μM in animals treated for 16 days with a daily dose of 20 mg/kg. In addition, Ki16425 did not affect platelet count after a 16-day treatment of metastatic animals, because the mean ± SD of platelet number per nanoliter was 253.8 ± 32.7 and 261.7 ± 40.7 for vehicle- and Ki16425-treated animals, respectively. Moreover, platelets isolated from Ki16425-treated animals aggregated in response to collagen with the same kinetic and magnitude as platelets isolated from vehicle-treated animals (Fig. 7B). This rules out a possible antiplatelet activity of this compound. In addition to an absence of side effect of Ki16425.
on hemostasis, no alterations of animal weight and behavior have been observed along with treatment. Altogether, these results strongly suggest that the inhibitory activity of Ki16425 on bone metastasis progression is associated with a blockade of platelet-derived LPA action on bone residing-tumor cells.

Discussion

Recent studies suggest that LPA plays a significant role in the development of cancers (8). In breast and ovarian cancer, we have recently demonstrated that LPA produced by blood platelets is a major factor involved in the progression of bone metastases (4). The signaling pathways through which platelet-derived LPA promotes bone metastasis are, however, unknown. LPA mediates its activity by binding to G protein-coupled receptors (LPA1, LPA2, and LPA3; see refs. 5–8), and these receptors share signaling pathways, suggesting that their activities might be redundant (19). The silencing of LPA1 expression in breast and ovarian cancer cells markedly altered the progression of bone metastases because of inhibition of TB and bone resorption. Our results do not exclude a possible contribution of LPA2 and LPA3 in mediating LPA action on bone metastasis progression. Although an artificial overexpression of LPA1 in human MDA-BO2 cells enhances bone metastasis progression (4), such experimental strategy remains inconclusive on the physiopathological role of endogenous LPA1. This LPA receptor was consistently detected at primary and secondary sites of human breast cancers but with no significant variation of expression (9–11). Overexpression of LPA2 and/or LPA3 was observed in several cancers (9–11), and these receptors are involved in the LPA-dependent proliferation of colon cancer HCT116 and LS174T cells in vitro (20), which would suggest that up-regulation of these receptors could play a role in carcinogenesis. However, as shown here, using both genetic and pharmacological approaches, most of the LPA activities on human breast cancer MDA-BO2 cell proliferation and production of proosteoclastic cytokines were LPA1-dependent. Silencing of LPA1 expression decreased TB both in bone and soft tissue, suggesting that the LPA1-dependent promoting effect of LPA on tumor growth was independent of the metastatic site. However, bone destruction is mediated by osteoclasts under the control of growth factors and cytokines. Therefore, factors such as LPA that increased tumor cell cytokine secretion might have a more marked influence on the progression of bone metastases than that of metastases located in other organs.

Clinical trials using antiplatelet agents such as aspirin or heparin have yielded inconclusive results (21, 22). Moreover, bleedings that are frequently encountered in cancer patients treated with cytotoxic chemotherapies because of platelet toxicity often require life-saving transfusions with platelets from healthy donors (23). We demonstrated here that a specific LPA1 antagonist exhibited specificity for tumor cell–platelet interaction without abrogating normal platelet functions. Ki16425 blocked in vivo tumor cell proliferation and inhibited the production by tumor cells of proosteoclastic cytokines, whereas normal platelet functions were unaffected. Different antagonists targeting LPA1 and, to a lesser extent, LPA2 have been described (17, 24–26). In vitro, the concentration of Ki16425 (10 μM) used in this study was suitable with a complete inhibition of LPA1 (Ki, 0.35 μM) and LPA2 (Ki, 0.93 μM; see ref. 17). In contrast, the concentration of Ki16425 (0.1 μM) in the plasma of animals treated for 16 days with a daily dose of 20 mg/kg suggested that the main target in vivo was LPA1. In accordance with an involvement of LPA receptors in pathophysiological processes, the treatment of mice with Ki16425 had no obvious deleterious effects, because the animals behaved normally and had normal platelet functions. Altogether, our results demonstrate that LPA1 expressed by tumor cells plays an essential role in bone metastasis progression and indicate that antagonists blocking LPA1-dependent effects of LPA may be promising in the treatment of bone metastasis.

Methods

Cell Culture. Characteristics of SKBr-3, MDA-MB-231/BO2 (MDA-BO2), MDA-BO2/GFP, MDA-BO2/HA-LPA1, and CHOβ3wt cell lines were described previously (4, 12, 27). SKBr-3/HA-LPA1 cell lines were established as followed. The cDNA encoding the HA-LPA1 was removed from the pBIL/HA-LPA1 (4) using NheI and HindIII restriction enzymes. The 5′ end of the cDNA was blunted by using the T4 DNA polymerase before digestion with HindIII. The vector pCI/HA-LPA1 was constructed by inserting the blunt/HindIII fragment encoding for HA-LPA1 into the pCI-neo plasmid (Promega), previously digested with SmaI and HindIII restriction enzymes. SKBr-3 cells were transfected with pCI/HA-LPA1 using Lipofectamine 2000 (Invitrogen). Cells were cultured for 4 weeks in the presence of G418 (1 mg/ml), and stable SKBr-3/HA-LPA1 clones were isolated by using cloning cylinders. LPA1 expression was assessed at the mRNA level by real-time quantitative RT-PCR, as described (4), and at the protein level by Western blot analysis with a polyclonal antibody to LPA1 (Abgent, San Diego). Oleoyl C18:1 LPA (Avanti Polar Lipids) proliferation assays and production of cell conditioned media, as described (4). Ki16425 is a competitive inhibitor of in vitro LPA-dependent signaling pathways through LPA1 (Ki, 0.35 μM), and to a lesser extent, LPA2 (Ki, 0.93 μM) and LPA3 (Ki, 6.5 μM; see ref. 23). Increasing concentrations of Ki16425 (from 10–10 to 10–4 M) were used in cell proliferation assay in the presence of LPA (10–7 M).

Stable Silencing of LPA1 mRNA Expression in Hamster Ovarian and Human Breast Cancer Cells. We designed small hairpin RNAs (siRNA) and corresponding shRNA sequences directed to LPA1 mRNA target sites based on the human and hamster sequences (GenBank accession nos., NM.001401 and AY522544, respectively), using the online siRNA TARGET DESIGNER 1.51 software (Promega). Pairwise oligonucleotides for hamster siRNA-LPA1, 5′-ACCGCTCTGTCGCAATCTCTG-TAAGTTCCTACAGATGCGAGAAGGCTTTCCTC-3′, 5′-TGCAGAAAAGGCTCGTGGACAACTCTGTA-
sbl clones were isolated by using cloning cylinders. LPA1 expression was assessed by real-time RT-PCR and Western blot analysis, as described above.

Cytokine Quantification. Cytokines produced in cell-culture-conditioned media were quantified by ELISA following the manufacturer's instructions (Bender MedSystems, Vienna).

Animal Studies. All procedures were performed on female NMRI nu/nu mice 4 weeks of age (Janvier, Le Genest St.-Isle, France). Studies involving animals, including housing and care, method of euthanasia, and experimental protocols, were conducted in accordance with a code of practice established by the Experimentation Review Board from the Laennec School of Medicine. Studies involving animals, including housing and care, method of euthanasia, and experimental protocols, were conducted in accordance with a code of practice established by the Experimentation Review Board from the Laennec School of Medicine.

Bone Histology and Immunohistochemistry. Quantification of BV/TV, BT/TV, and in situ detection of osteoclasts (TRAP cells) was carried out on undecalcified bone tissue sections, as described (4). The mitotic index of tumor cells in vivo was quantified by immunohistochemistry by using a mouse anti-human Ki67 monoclonal antibody (Dakocytomation, Dako), as described (4).

Osteoclastogenesis Assay in Vitro. Bone marrow cells from mice were collected, and mononuclear cells were isolated by using lymphocyte separation media (ICN), then seeded in a 48-well tissue culture plate at a density of 2.5 × 10^3 cells per well in α-MEM medium (Invitrogen) supplemented with FCS, macrophage-CSF (PreproTech, Rocky Hill, NJ) and receptor-activated nuclear receptor factor xβ ligand (RANK-L, generous gift of Pr. Juridic, CNRS, Lyon, France). After incubation for 6 days, differentiated osteoclasts were enumerated under a light microscope as a function of multinucleation (more than three nuclei) and TRAP activity. Results were expressed as the number of osteoclasts per mm².

Platelet Experiments. Blood samples were collected from metastatic mice in the presence of ACD-A as an anticoagulant. Platelets were counted, and aggregation experiments were performed by using washed platelets under stirring conditions at 37°C in the absence or presence of collagen (2.5 µg/ml). Platelet aggregation was monitored over time as the percentage of light transmission, as described (4).

Statistical Analysis. Unpaired Student’s t-test analyses were carried out by using STAT-VIEW 5.0 software. P < 0.05 was considered statistically significant.

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