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Platelet-derived lysophosphatidic acid supports the progression of osteolytic bone metastases in breast cancer

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The role of lysophosphatidic acid (LPA) in cancer is poorly understood. Here we provide evidence for a role of LPA in the progression of breast cancer bone metastases. LPA receptors LPA1, LPA2, and LPA3 were expressed in human primary breast cancers and a series of human breast cancer cell lines. The inducible overexpression of LPA1 in MDA-BO2 breast cancer cells specifically sensitized these cells to the mitogenic action of LPA in vitro. In vivo, LPA1 overexpression in MDA-BO2 cells enhanced the growth of subcutaneous tumor xenografts and promoted bone metastasis formation in mice by increasing both skeletal tumor growth and bone destruction. This suggested that endogenous LPA was produced in the tumor microenvironment. However, MDA-BO2 cells or transfectants did not produce LPA. Instead, they induced the release of LPA from activated platelets which, in turn, promoted tumor cell proliferation and the LPA1-dependent secretion of IL-6 and IL-8, 2 potent bone resorption stimulators. Moreover, platelet-derived LPA deprivation in mice, achieved by treatment with the platelet antagonist Integrin, inhibited the progression of bone metastases caused by parental and LPA1-overexpressing MDA-BO2 cells and reduced the progression of osteolytic lesions in mice bearing CHO-β3wt ovarian cancer cells. Overall, our data suggest that, at the bone metastatic site, tumor cells stimulate the production of LPA from activated platelets, which enhances both tumor growth and cytokine-mediated bone destruction.

Introduction

Lysophosphatidic acid (LPA) is a naturally occurring bioactive lipid. Three distinct G protein–coupled receptors (GPCRs), namely LPA1, LPA2, and LPA3 (formerly called endothelial differentiation gene receptors 2, 4, and 7, respectively), are specific receptors for LPA (1–3). Recently, a GPCR that is closely related to the purinergic GPCR family (GPR23/P2Y9) but shares no significant identity with other LPA receptors was suggested to be a fourth LPA receptor (LPA4) (4). Additionally, the peroxisome proliferator-activated receptor γ, a transcriptional factor, was recently identified as an intracellular LPA receptor (5). Because the activation of the various G proteins stimulates multiple signal transduction pathways, the cellular responses induced by LPA are remarkably diverse. Among these, LPA modulates proliferation, migration, and survival of many cell types (6).

Despite increasing data from in vitro studies, the pathophysiological role of LPA and its receptors is poorly understood. Their involvement in cancer is, however, emerging (7). LPA is present at elevated levels in ascitic fluid and plasma from patients with ovarian, endometrial, and cervical cancers (7), and could therefore be a potential biomarker or indicator of response to therapy in gynecologic cancers (8, 9). In addition, ovarian cancers frequently show aberrant expression of LPA2 and LPA3 mRNAs compared with the normal ovarian epithelium (9–11).

Human MCF-7 and MDA-MB-435 breast cancer cells express LPA1 and LPA2 and respond to the mitogenic action of LPA in vitro (12). In addition, autotaxin (ATX), which possesses a lysophospholipase D (lyso-PLD) activity allowing the production of LPA from lysophosphatidic choline (LPC), is linked to the invasiveness of breast cancer cells in vitro (13).

Breast cancers frequently metastasize to bone (14). Bone metastases are associated with hypercalcemia due to bone destruction, intractable bone pain, and pathological fractures (14, 15). In bone metastasis, there is a vicious cycle wherein bone-residing tumor cells stimulate osteoclast-mediated bone resorption and bone-derived growth factors released from resorbed bone promote tumor growth (15–17). However, current treatments aimed to inhibit bone resorption (i.e., bisphosphonates) only delay the progression of osteolytic lesions in metastatic patients (18). Therefore, in addition to bone-derived growth factors, other endogenous sources of growth factors are probably involved in promoting skeletal tumor growth. In this respect, aggregation of human blood platelets upon thrombin activation is an important source of LPA (19, 20), and platelet aggregation plays a primordial role in the metastatic spreading of melanoma and Lewis lung carcinoma cells in bone (21) and

Nonstandard abbreviations used: ATX, autotaxin; BS, total trabecular bone surface; BV, bone volume; GPCR, G protein–coupled receptor; HA-LPA, HA-tagged LPA; LPC, lysophosphatidic acid; LPA, lysophosphatidic choline; lyso-PLD, lysophospholipase D; NCBi, National Center for Biotechnology Information; Oc.S, active-osteoclast resorption surface; PLB, phospholipase B; TB, tumor burden; TRAP, tartrate-resistant acid phosphatase; TV, tissue volume; V, tumor volume; W, width.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2004;114:1714–1725 (2004). doi:10.1172/JCI200422123.
lungs (22), respectively. Here we provide experimental evidence for a direct role of LPA in the progression of bone metastasis in breast cancer. We identify platelet-derived LPA as an endogenous source that, in the bone microenvironment, stimulates both tumor growth and bone destruction.

**Results**

The mitogenic action of LPA on breast cancer cells correlates with the presence of LPA receptors. The biological activity of LPA is mediated through its interaction with specific cell-surface receptors (7). As exemplified here by RT-PCR, we detected mRNAs for LPA₁, LPA₂, and LPA₃ receptors in human benign and neoplastic primary breast tumors (Figure 1A). Human MDA-MB-231, MDA-BO2, T-47D, MCF-7, ZR-75-1, Hs 578T, and MDA-MB-435S breast cancer cell lines also expressed all 3 LPA receptors, whereas SK-BR-3 breast cancer cells did not (Figure 1B, insets). Fang et al. (23) also reported the absence of LPA receptors in SK-BR-3 cells. LPA dose-dependently stimulated the proliferation of MDA-MB-231, MDA-BO2, T-47D, MCF-7, ZR-75-1, Hs 578T, and MDA-MB-435S cells (Figure 1B), whereas SK-BR-3 cells did not respond to the mitogenic action of LPA (Figure 1B). These results suggested that LPA-dependent breast cancer cell proliferation was mediated through the activation of LPA receptors LPA₁, LPA₂, and LPA₃.

**Overexpression of LPA₁ sensitizes human MDA-BO2 breast cancer cells to the mitogenic action of LPA in vitro.** A broad range of LPA species can bind and activate LPA₁ (24). Thus, to sensitize tumor cells to LPA stimulation, we developed an LPA₁ receptor overexpression strategy. We used the tet-Off–regulated expression system in which the overexpression of LPA₁ was achieved in the absence of the repressor (doxycycline). MDA-BO2 cells were chosen among the different breast cancer cell lines for transfection experiments with the bidirectional pBiL-HA-tagged LPA₁ (pBiL-HA-LPA₁) vector. In the absence of doxycycline, HA-LPA₁ is overexpressed at the cell surface, whereas luciferase is produced in the cytoplasm. We selected 2 stable clones (MDA-BO2/HA-LPA₁ no. 3 and no. 79) on the basis of their specific and high expression of luciferase.
Real-time RT-PCR indicated that clones no. 3 and no. 79 had a 42- and 13-fold increase, respectively, in LPA1 mRNA expression upon doxycycline withdrawal when compared with parental cells (Figure 2C). In addition, in the presence of the repressor, LPA1 mRNA expression levels in clones and parental MDA-BO2 cells were similar (Figure 2C). Finally, the cell transfection procedure as well as the presence or absence of doxycycline in the culture media did not alter the mRNA expression levels of LPA2 and LPA3 (Figure 2C).

In the presence of doxycycline, the proliferation of clones no. 3 and 79 in response to LPA stimulation was similar to that observed for parental MDA-BO2 cells (Figure 3A). Remarkably, in the absence of doxycycline, the overexpression of HA-LPA1 enhanced the proliferation of clones no. 3 and 79 in response to PDGF, IGF-1, IGF-2, insulin, or EGF (Figure 3B). These results indicated that HA-LPA1 overexpression specifically sensitized MDA-BO2 cells to the mitogenic action of LPA.

LPA1 overexpression in MDA-BO2 cells promotes skeletal tumor growth and breast cancer-induced bone destruction. To examine the role of LPA in the metastatic spreading of breast cancer cells in vivo, we used an experimental model of bone metastasis caused by MDA-BO2 cells that quite closely mimics the conditions likely to occur in naturally arising metastatic human breast cancers (25, 26). Thirty days after intravenous inoculation of each MDA-BO2/HA-LPA1 clone in BALB/C nude mice, radiographic analyses revealed that animals fed with doxycycline-free water exhibited a dramatic increase in bone metastasis formation compared with doxycycline-fed animals and mice inoculated with parental cells (Figure 4A and Table 1). Overexpression of HA-LPA1 in clones no. 3 and 79 induced a 3- and 2-fold increase, respectively, in the extent of
osteolytic lesions (Table 1). Histological examination indicated that when HA-LPA₁ overexpression was turned on by doxycycline withdrawal, the cortical and cancellous bone were almost completely destroyed, and replaced by tumor cells that filled the bone marrow cavity and invaded adjacent tissues (Figure 4A). Trabecular bone was completely destroyed and replaced by tumor cells (T) in tibial metaphysis from untreated animals bearing clone no. 3 cells. Scale bar: 1 mm. (B) Animals fed without (open symbols) or with (filled symbols) doxycycline were inoculated subcutaneously into the flank with MDA-BO2 (triangles), clone no. 3 (circles), or clone no. 79 (squares) cells. Tumors were measured at the indicated time points. V (in mm³) is expressed as the mean ± SD of 9 animals per group. †P < 0.05; ‡P < 0.005 doxycycline-free versus doxycycline-fed tumor-bearing animals. (C) Subcutaneous (upper panels) and skeletal (lower panels) tumor tissue sections immunostained with an antibody against the nuclear Ki-67 antigen. The mitotic index (numbers on each panel) was calculated as the percentage of nuclei positive for Ki-67. Results are the mean ± SD of 6 independent tumor sections. #P < 0.0001 doxycycline-free versus doxycycline-fed tumor-bearing animals. Scale bars: 100 μm.

Figure 4
Effect of LPA₁ overexpression in MDA-BO2 cells on osteolytic lesions and on skeletal and subcutaneous tumor growth. (A) Animals fed without or with doxycycline were inoculated intravenously with MDA-BO2 or clone no. 3 cells. (Upper panels) Representative radiographs of hind limbs from mice bearing MDA-BO2 or clone no. 3 cells 30 days after tumor cell inoculation. In the absence of doxycycline, there was a marked increase in the extent of osteolytic lesions (arrows) in mice bearing clone no. 3 cells. (Lower panels) Representative bone histology of Goldner’s trichrome–stained tibial metaphysis from metastatic animals. Bone is stained in green; bone marrow and tumor cells are stained red. Trabecular bone was completely destroyed and replaced by tumor cells (T) in tibial metaphysis from untreated animals bearing clone no. 3 cells. Scale bar: 1 mm. (B) Animals fed without (open symbols) or with (filled symbols) doxycycline were inoculated subcutaneously into the flank with MDA-BO2 (triangles), clone no. 3 (circles), or clone no. 79 (squares) cells. Tumors were measured at the indicated time points. V (in mm³) is expressed as the mean ± SD of 9 animals per group. †P < 0.05; ‡P < 0.005 doxycycline-free versus doxycycline-fed tumor-bearing animals. (C) Subcutaneous (upper panels) and skeletal (lower panels) tumor tissue sections immunostained with an antibody against the nuclear Ki-67 antigen. The mitotic index (numbers on each panel) was calculated as the percentage of nuclei positive for Ki-67. Results are the mean ± SD of 6 independent tumor sections. #P < 0.0001 doxycycline-free versus doxycycline-fed tumor-bearing animals. Scale bars: 100 μm.

LPA₁ overexpression enhances in vivo MDA-BO2 tumor growth. Tumor cells were implanted subcutaneously into nude mice. The growth rates of MDA-BO2 parental, clone no. 3, and clone no. 79 tumor cells in doxycycline-fed animals were similar (Figure 4B). In contrast, the growth of tumor cells of clones no. 3 and no. 79 was markedly increased upon doxycycline withdrawal (Figure 4B). In agreement with growth curves, in situ immunodetection of the Ki-67 nuclear antigen in tumor sections showed a substantial increase in the proliferation of clone no. 3 overexpressing HA-LPA₁ (Figure 4C). We observed similar results for clone no. 79 (data not shown). In addition, subcutaneous and skeletal tumors overexpressing HA-LPA₁ had a similar increase in proliferation, as judged by Ki-67 nuclear staining (Figure 4C). Therefore, the increase in in situ proliferation of breast cancer cells overexpressing HA-LPA₁ in both subcutaneous and skeletal tumors was strongly suggestive of a local production of bioactive LPA, irrespective of the host tissue.
MDA-BO2 cells do not produce LPA or express ATX. To identify the potential sources of LPA, we first speculated that tumor cells themselves might produce LPA, which could secondarily act as an autocrine factor in vivo. However, using a radioenzymatic assay, we found that MDA-BO2 parental cells and clone no. 3 cells (clone no. 79 cells were not tested) did not produce detectable amount of LPA (less than 0.2 pmol) in their culture media (Figure 5A). These results were in agreement with in vitro cell proliferation experiments showing that cells overexpressing HA-LPA1 grew at the same rate as doxycycline-treated cells when cultured in the control medium (Figure 3A). In addition, MDA-BO2 parental and clone no. 3 cells did not produce detectable amounts of lyso-PLD activity in their culture media (Figure 5B). The absence of ATX mRNA in our cell lines was confirmed by RT-PCR experiments (data not shown). These results strongly suggested that our breast cancer cell lines were unlikely to produce LPA in vivo.

MDA-BO2 cells induce platelet aggregation and the subsequent release of LPA from activated platelets. Platelets present in the blood circulation are an important source of LPA that is released during thrombin-induced platelet aggregation (19, 20). In addition, platelet aggregation induced by tumor cells plays an important role during tumor cell dissemination (21, 22, 27). We therefore studied the platelet-aggregating activity of our breast cancer cell lines (clone no. 79 cells were not tested). MDA-MB-231, MDA-BO2 parental, or HA-LPA1 overexpressing cells (clone no. 3) did induce platelet aggregation (Figure 6A) and stimulated the release of LPA from activated platelets (Table 2). Supernatants of tumor cell–induced platelet aggregation promoted the proliferation of clone no. 3 cells (Figure 6B). Moreover, clone no. 3 cells were sensitized to this mitogenic action in a doxycycline-dependent manner, which was completely abrogated in the presence of phospholipase B (PLB, an LPA-degrading enzyme) (Figure 6B). These results indicated that LPA released from activated platelets upon stimulation by tumor cells was bioactive and promoted breast cancer cell proliferation in vitro.

Platelet-derived LPA deprivation in animals blocks the progression of bone metastases. Pharmacological inhibition of platelet aggregation using a specific integrin αIIbβ3 antagonist prevents the early entry of B16 melanoma cells into bone (21). To examine

Table 1
Quantification of osteolytic lesions and skeletal tumor burden in untreated and doxycycline-treated animals bearing parental MDA-BO2 breast cancer cells or clone no. 3 and clone no. 79 cells overexpressing HA-LPA1

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Osteolytic lesions (mm²)</th>
<th>BV/TV (%)</th>
<th>TB/TV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Dox (n)</td>
<td>−Dox (n)</td>
<td>+Dox (n)</td>
</tr>
<tr>
<td>MDA-BO2</td>
<td>6.3 ± 1.5 (9)</td>
<td>5.7 ± 1.1 (6)</td>
<td>5.8 ± 0.3 (9)</td>
</tr>
<tr>
<td>MDA-BO2/HA-LPA1, no. 3</td>
<td>6.2 ± 1.0 (6)</td>
<td>18.6 ± 2.7 (9)</td>
<td>5.8 ± 0.4 (6)</td>
</tr>
<tr>
<td>MDA-BO2/HA-LPA1, no. 79</td>
<td>5.4 ± 0.3 (6)</td>
<td>10.1 ± 1.04 (6)</td>
<td>5.8 ± 0.4 (6)</td>
</tr>
</tbody>
</table>

Data are the mean ± SD of 2 separate experiments for each cell line using n animals. *P < 0.001 using unpaired Student’s t test when comparing animals fed with and without doxycycline (Dox). MDA-BO2, parental human breast cancer cell line; MDA-BO2/HA-LPA1, no. 3 and no. 79, clones overexpressing HA-LPA1 at the cell surface upon doxycycline withdrawal.

Figure 5
Production of LPA and ATX by MDA-BO2 breast cancer cells. Cell culture media were collected for each indicated cell line placed in the absence or presence of doxycycline (100 ng/ml). (A) Detection of LPA. The production of phosphatidic acid (PA) was due to the transfer of [14C] fatty acyl chain (FA) onto LPA present in the reaction mixture assay. Experiments were carried out in duplicate for each cell line. Purified LPA (50, 100, and 200 pmol) was used as a positive control. DMEM (Cont.) was used as a negative control. (B) Measurement of ATX/lyso-PLD activity. [14C]-LPC was used as the substrate of ATX/lyso-PLD to produce [14C]-LPA. Experiments were carried out in duplicate using culture media from the cell lines described above. Note that MDA-MB-231, MDA-BO2, clone no. 3, and clone no. 79 cell lines did not produce LPA or express ATX.

Figure 6
Platelet-derived LPA deprivation in animals blocks the progression of bone metastases. Pharmacological inhibition of platelet aggregation using a specific integrin αIIbβ3 antagonist prevents the early entry of B16 melanoma cells into bone (21). To examine
the role of platelet aggregation during the progression of established bone metastases, animals bearing MDA-BO2 parental or HA-LPAβ3wt cells, which we have previously shown to induce bone metastasis in animals (26). As exemplified by RT-PCR using CHO-based primers, we detected LPAβ3 mRNA, but not LPAα1 nor LPAα3 mRNAs, in our CHO-β3wt cells (Figure 8A, inset). In agreement with the presence of active LPAα1, the proliferation of these cells was stimulated in response to increasing concentrations of LPA, which was abrogated in the presence of PLB (Figure 8A). CHO-β3wt cells also induced platelet aggregation in vitro (Figure 8B). Thus, Integrin treatment of metastatic animals might also affect the progression of bone metastases caused by CHO-β3wt cells. Indeed, we found that an 11-day treatment of metastatic animals with Integrin, starting at day 10 after cell inoculation, inhibited by 3-fold the extent of bone metastases caused by CHO-β3wt cells (Figure 8C). Overall, these results obtained with 2 different animal models of bone metastasis strongly suggested that blood platelets were the main endogenous source of LPA. Bone-residing tumor cells stimulated the release of LPA by inducing platelet aggregation, which, in turn, promoted the proliferation of tumor cells. The reason that LPAα1 overexpression in MDA-BO2 cells was also associated with a higher bone destruction was, however, unclear.

LPAα1 overexpression in MDA-BO2 cells increases the recruitment of osteoclasts at the bone metastatic site. Tumor cells do not directly destroy bone (21). Instead they mediate the recruitment and stimulate the activity of osteoclasts at the bone metastatic site (15–17). Histological examination showed that the recruitment of osteoclasts on trabecular bone located at the bone/tumor cell interface was increased in metastatic long bones from doxycycline-free water fed animals bearing HA-LPAβ3wt overexpressing MDA-BO2 cells (clone no. 3 and clone no. 79) compared with those from doxycycline-fed animals and from mice bearing parental cells (Figure 9A). Histomorphometric quantification revealed that HA-LPAβ3 overexpression in clones no. 3 and no. 79 induced a 3.5- and 2-fold increase, respectively, in active-osteoclast resorption surface (Oc.S) per trabecular bone surface (BS) (Figure 9B). This observation strongly suggested that the increase in the extent of osteolytic lesions observed in animals bearing cells overexpressing HA-LPAβ3 was due to increased osteoclast bone resorption activity.

LPAα1 overexpression enhances the LPA-dependent production of IL-6 and IL-8 by MDA-BO2 cells. Fang et al. have recently shown that LPA stimulates the production of IL-6 and IL-8 by ovarian and
breast cancer cells (23). Cytokines are important modulators of osteoclast functions (28), and the secretion of IL-6 and IL-8 by tumor cells stimulates osteoclast-mediated bone destruction in vivo (29, 30). Here, LPA stimulated production of both IL-6 and IL-8 (Figure 10A and IL-8 (Figure 10B) by MDA-BO2 cells, and this cytokine production was markedly increased when clone no. 3 cells overexpressed HA-LPA1. Similarly, the supernatant of tumor cell–induced platelet aggregate promoted the production of IL-6 and IL-8 in an LPA-dependent manner (Figure 10, A and B). Thus, in addition to its direct mitogenic effect on tumor cells, LPA indirectly stimulated bone destruction through the increased production of IL-6 and IL-8 by breast cancer cells. Therefore, the increased osteolytic lesions in animals inoculated with tumor cells overexpressing LPA1 might be the result of enhanced LPA-dependent tumor cell proliferation, increased osteoclastogenesis mediated by IL-6 and IL-8, or, more likely, a combination of both mechanisms acting in concert.

Discussion
The role for LPA in ovarian, endometrial, cervical, melanoma, and prostate cancers is currently emerging (7). Despite studies reporting the effects of LPA on breast cancer cells in vitro (12, 23, 31, 32), the role of LPA in breast cancer in vivo was not known. In the present study, we demonstrated that LPA promoted breast cancer progression in vivo. This contention was first supported by the fact that the mitogenic activity of LPA on a series of human breast cancer cell lines correlated with the expression of LPA receptors. We also observed that overexpression of LPA1 specifically sensitized MDA-BO2 breast cancer cells in culture to the mitogenic action of LPA. Secondly, the growth of subcutaneous breast tumor xenografts or skeletal breast tumor metastases was markedly increased when LPA1 overexpression in MDA-BO2 breast cancer cells was turned on by doxycycline withdrawal. Moreover, the mitotic index of MDA-BO2 cells in situ was also increased when LPA1 overexpression was turned on. These results do not preclude the possibility that LPA2 and LPA3 receptors may also play a role in breast cancer progression in vivo. However, because the overexpression of LPA1 did not induce any modification in the expression levels of LPA2 and LPA3 in MDA-BO2 cells, our results strongly suggested that the increased in vivo growth of MDA-BO2 cells upon doxycycline withdrawal was directly related to the overexpression of LPA1. Local production of bioactive LPA in the tumor microenvironment in vivo should therefore support LPA1-dependent breast tumor cell proliferation.

It has been previously reported that MDA-MB-231 breast cancer cells do not directly produce LPA (13). However, ATX in MDA-MB-231 and MDA-MB-43SS breast cancer cells can induce the production of bioactive LPA, which in turn stimulates cell migration, invasion, and proliferation (13, 32, 33). In the present study, MDA-BO2 cells and transfectants did not directly produce LPA or express ATX.

We therefore focused our attention on human blood platelets. Several factors suggested that platelets could locally produce bioactive LPA in vivo in the tumor bed. First, thrombin-activated platelets (19) and lyso-PLDs (20) are major sources of LPA in the serum. Second, platelets play a major role in the metastatic dissemination of tumor cells in vivo (27, 34, 35), and, more recently, platelet aggregation was shown to be essential for successful formation of B16 melanoma bone metastases in animals (21). Third, because of the leaky vasculature of angiogenic tumors (36), platelets are in contact with tumor cells and are therefore able to secrete multiple factors upon activation (37). Fourth, MDA-MB-231 breast cancer cells (as well as other tumor cell lines) interact with platelets and stimulate platelet aggregation in vitro (38), suggesting that the platelet-aggregating activity of breast cancer cells might induce the release of LPA from activated platelets. In agreement with the latter findings (38), we observed here that our MDA-BO2

Table 3
Quantification of LPA in the plasma of metastatic animals

<table>
<thead>
<tr>
<th>Animals</th>
<th>n</th>
<th>LPA (nM)</th>
</tr>
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<tbody>
<tr>
<td>MDA-BO2 cells + vehicle</td>
<td>6</td>
<td>257.6 ± 63.3</td>
</tr>
<tr>
<td>MDA-BO2 cells + Integrilin</td>
<td>9</td>
<td>76.3 ± 7.37A</td>
</tr>
</tbody>
</table>

Animals bearing MDA-BO2 cells were treated with the anti-platelet agent Integrilin or vehicle every 3 days from day 14 to day 30, at which time animals were sacrificed and circulating levels of LPA in the plasma were measured. Results are expressed as the mean ± SE from n animals. *P < 0.01 using unpaired Student’s t test compared with animals not treated with Integrilin.

Figure 7
Effect of in vivo inhibition of platelet aggregation on the LPA-dependent progression of breast cancer bone metastases. (A) Representative radiographs at day 30 of hind limbs from doxycycline-free fed mice bearing MDA-BO2 or clone no. 3 cells that were treated with Integrilin or vehicle from day 14 to day 30. (B) Quantification of osteolytic lesion areas on radiographs in Integrilin-treated (+) and vehicle-treated (−) metastatic animals. Values are the mean ± SE of 6–9 animals per group. *P < 0.01; **P < 0.001, Integrilin-treated versus vehicle-treated animals.
parental and transfected breast cancer cell lines strongly stimulated platelet aggregation and the subsequent release of LPA from activated platelets. In addition, platelet-derived LPA stimulated in vitro the proliferation of parental MDA-BO2 cells, and this activity was further increased upon LPA1 overexpression. In vivo, a highly specific platelet aggregation inhibitor (Integrilin) inhibited by 50% the extent of bone metastases caused by MDA-BO2 cells and markedly blocked the progression of osteolytic lesions and skeletal tumor burden in animals bearing LPA1-overexpressing cells. Moreover, this observation was not restricted to our MDA-BO2 breast cancer bone metastasis model since Integrilin also inhibited the progression of bone metastases caused by CHO-β3wt ovarian cancer cells. Thus, it is most likely that platelet-derived LPA promotes breast cancer growth in vivo. The experiments presented here provide evidence of LPA-dependent tumor promotion in MDA-BO2 and CHO-β3wt models. It is therefore important to test additional tumors to determine whether paracrine signaling by LPA is a general mechanism of metastatic tumor growth.

Because subcutaneous and skeletal MDA-BO2 breast tumors are highly vascularized (39), these data strongly support the idea that platelets from the blood stream come into contact with the tumor bed in vivo, and then aggregate and secrete LPA, which stimulates the proliferation of breast cancer cells. Interestingly, compared with human serum, mouse serum contains relatively little LPA (8); more importantly, in contrast to human platelets, mouse platelets do not aggregate in response to LPA in vitro (40). Therefore, we anticipate that the contribution of LPA to the progression of the bone metastatic disease in patients could be even more important than that observed here in animals.

Besides the fact that LPA and its receptor LPA1 promoted the growth of primary or metastatic breast tumors, we also showed evidence indicating that LPA could indirectly contribute to the bone destruction associated with MDA-BO2 skeletal metastases. LPA was recently shown to stimulate the production of IL-6 and IL-8 by ovarian and breast cancer cells (23). Tumor cells do not directly destroy bone (21). Instead, IL-6 and IL-8 produced by breast cancer cells stimulate osteoclast-mediated bone resorption (41). We showed here that platelet-derived LPA, as well as purified LPA, stimulated the secretion of these cytokines by MDA-BO2 breast cancer cells. In addition, production of IL-6 and IL-8 was markedly increased when tumor cells overexpressed LPA1. It is, therefore, conceivable that beyond its tumor growth–promoting effect, LPA indirectly stimulates osteoclast-mediated bone resorption. Thus, in addition to the vicious cycle related to the direct reciprocal interaction between breast cancer cells and osteoclast-mediated bone resorption (15, 16), we propose that there exists an LPA-dependent cycle wherein bone-residing tumor cells stimulate LPA production by platelets, which in turn enhances tumor growth and cytokine-mediated bone resorption (Figure 11).

Although a role for LPA in cancer was emerging, there was a paucity of experimental evidence to support it. This study is, to the best of our knowledge, the first to demonstrate a role for LPA and its receptor LPA1 in the growth of breast cancer bone metastasis.

Methods

Cell lines and transfection. Hs 578T, MCF-7, MDA-MB-231, MDA-MB-435S, SK-BR-3, T-47D, and ZR-75-1 cells were obtained from the American Type Culture Collection. Characteristics of CHO-β3wt, MDA-MB-231/BO2 (MDA-BO2), and tet-Off–expressing MDA-BO2 breast cancer cells were described elsewhere (26, 39). The cDNA encoding the entire human LPA1 and the antigenic epitope tag (HA) fused at the NH2 terminus of LPA1 was amplified by PCR using the vector pEDG-2 (42) as a template and 2 oligonucleotide primers (5'-CGGCTAGCATGTACCCATACGAC-3').
GTCCCAAGACTGCTAATGGCTGCCATCTCTACT-3′ [HA-edg2N] and 5′-CGCAAGCTTCTAAACACAGATGGTTCT-GC-3′ [Stop-edg2]). The bidirectional vector pBiL/HA-LPA1 was constructed by inserting into the pBiL plasmid (Clontech) the Nhel/HindIII PCR fragment encoding the HA-LPA1 sequence. MDA-MB-231/BO2-tet-Off cells were cotransfected with pBiL/HA-LPA1 together with a vector conferring puromycin resistance (pPur; BD Biosciences — Clontech). Selection of the clones was obtained after growing the cells for 2 weeks in the presence of puromycin (2 μg/ml). Luciferase induction upon doxycycline withdrawal was used to select inducible clones among stable transfectants. Two HA-LPA1-inducible transfectants (clones no. 3 and 79) were used in the present study. Cell lines and inducible transfectants, with the exception of SK-BR-3 cells, were routinely cultured in DMEM/NUT.MIX F-12 W/GLUT-1 medium (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Bio-Media) and 1% penicillin/streptomycin (Life Technologies) at 37°C in a 5% CO2 incubator. SK-BR-3 cells were grown in complete McCoy’s 5a medium (Life Technologies).

Animal studies. All procedures were performed on female BALB/c nu/nu mice of 4 weeks of age (Charles River). 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, Lyon, France. These studies were routinely inspected by the Attending Veterinarian to ensure continued compliance with the proposed protocols. Two days before tumor cell inoculation, animals were provided with drinking water containing 5% (w/v) sucrose supplemented with or without doxycycline (1 mg/ml).

Bone metastasis experiments in animals were conducted as previously described (26, 39). Briefly, doxycycline-treated or untreated MDA-BO2 transfectants (5 × 10⁶ cells in 100 μl of phosphate-buffered saline) were inoculated into the tail vein of anesthetized nude mice treated with doxycycline or left untreated, respectively. Alternatively, CHO-β3wt cells (10⁶ cells in 100 μl of phosphate-buffered saline) were inoculated intravenously into animals. Metastatic animals were also treated every 3 days with the anti-platelet agent epifibatide (Integrilin; Schering-Plough) by intraperitoneal injection (0.5 mg/kg/day). Integrilin treatment began at the time osteolytic lesions were radiographically detectable in animals (at day 10 and day 14 for CHO-β3wt and MDA-BO2 cells, respectively). Radiographs (MIN-R2000 films; Kodak) were then taken 21 (CHO-β3wt cells) or 30 days (MDA-BO2 cells and transfectants) after tumor cell inoculation using a cabinet X-ray system (MX-20; Faxitron X-ray Corporation), and bone metastases were enumerated on each radiograph. The area of osteolytic lesions was measured using the computerized image analysis system Visiolog 2000 (Biocom), and results were expressed in square millimeters.

For tumor xenograft experiments, MDA-BO2 transfectants previously cultured in the presence of doxycycline to block the overexpression of HA-LPA1 were inoculated subcutaneously (10⁶ cells in 100 μl of phosphate-

**Figure 9**

Effect of LPA1 overexpression in MDA-BO2 cells on osteoclast activity in vivo. (A) Animals fed without or with doxycycline were inoculated intravenously with MDA-BO2 or transfectants (clone no. 3 and clone no. 79 cells). Representative histological examination of TRAP-stained proximal tibia section from metastatic animals 30 days after tumor cell inoculation. Lower panels show magnified areas (white squares) from upper panels. Bone is stained in dark blue and osteoclasts are stained in red (arrows). Scale bar: 200 μm. (B) The Oc.S/BS ratio was quantified. Results are the mean ± SD of 4–6 animals per group. *P < 0.001 doxycycline-free versus doxycycline-fed animals.
buffered saline) into the flank of nude mice that had been previously treated for 2 days with doxycycline. Seven days after tumor-cell inoculation, mice were randomized into 2 groups: 1 group received doxycycline for the duration of the experiment, whereas the other group did not. Tumor size was assessed by external measurement of the length (L) and width (W) of the tumors using a Vernier caliper. Tumor volume (V; expressed in mm$^3$) was calculated using the following equation: $V = (L \times W^2)/2$.

**Human studies.** Studies involving primary breast tumors and platelet aggregations were performed according to the principles embodied in the Declaration of Helsinki. All human experiments were approved by the Experimental Review Board from the Laennec School of Medicine.

**Reverse transcription, standard and quantitative PCR.** Total RNA from cell lines and human breast tumors was extracted using Total RNA Isolation System (Promega). cDNA was synthesized using Moloney murine leukemia virus-1 (Promega). Primers for human LPA$_1$, LPA$_2$ (43), GAPDH (10), and ATX (44) were designed as described previously. LPA$_3$ primers were designed from the Lpa3 gene (NCBI accession number AF127138) using nucleotides 738–756 as the forward primer and nucleotides 994–973 as the reverse primer. GAPDH primers recognized GAPDH cDNA from both human and CHO cells. PCR reactions were run using a program consisting of 40 cycles of 95°C for 15 s, 53°C for 30 s, and 72°C for 20 s with a preincubation of 95°C for 2 min. Products from standard PCR were separated by electrophoresis on a 2% agarose gel and then visualized with ethidium bromide under ultraviolet light. Human LPA$_1$, LPA$_2$, and LPA$_3$ mRNAs were quantified by real-time PCR using the Master SYBR Green I kit (Roche Diagnostics). Fluorescence was monitored and analyzed in a Light Cycler (Roche Diagnostics). The fluorescence data were quantitatively analyzed by using serial dilution of control samples included in each reaction to produce a standard curve. GAPDH mRNA expression was analyzed in parallel to confirm the use of equal amount of cDNAs in each reaction. Results are expressed as the percentage of gene expression in each cell line compared with that in the parental MDA-BO2 cells cultured in the absence of doxycycline.

**Luciferase activity assay and flow cytometry.** Luciferase activity was measured on cell lysates in accordance with the manufacturer’s instructions (Promega). Prior to flow cytometry analysis, tumor cells were cultured in medium complemented with or left without doxycycline (100 ng/ml). Expression of HA-LPA$_1$ at the cell surface was detected using a mouse anti-HA monoclonal antibody 12CA5 (Roche Diagnostics). Monoclonal antibody MOPC21 (Santa Cruz) was used as an isotypic negative control antibody.

**Figure 10**

Effect of purified or platelet-derived LPA on the production of IL-6 and IL-8 by breast cancer cells. IL-6 (A) and IL-8 (B) were quantified using culture media from cells pretreated in the presence or absence of LPA, the supernatant of breast cancer cell–induced platelet aggregation, and PLB. Data are expressed as the mean ± SD of 3 replicates and are representative of 2 separate experiments. *P < 0.0001 stimulated versus unstimulated cells.

**Figure 11**

Schematic representation of the LPA effects on progression of osteolytic bone metastases. Breast cancer cells produce factors (PTHrP, cytokines) that stimulate osteoclast-mediated bone resorption. In turn, bone resorption releases growth factors (IGFs, TGF-β) from the bone matrix that stimulate tumor growth and the production of PTHrP by tumor cells (16). This results in a vicious cycle, illustrated by dotted arrows. Bone-residing breast cancer cells also induce platelet aggregation and the release of LPA from activated platelets. Platelet-derived LPA then stimulates both tumor growth and the production of IL-6 and IL-8 by tumor cells (black arrows), which in turn enhance bone resorption.
**Bone histology.** Hind limbs from animals were fixed and embedded in methylmethacrylate. Seven-micrometer sections of undecalcified long bones were stained with Goldner’s trichrome, and histological analyses were performed on longitudinal medial sections of tibial metaphysis using the computerized image analysis system Visiolab 2000 (Biocom), as previously described (26, 39). The in situ detection of osteoclasts was carried out on metastatic bone tissue sections stained for tartrate-resistant acid phosphatase (TRAP) activity using a commercial kit (Sigma-Aldrich). The resorption surface (Oc.S/BS) was calculated as the ratio of the TRAP-positive trabecular bone surface (Oc.S) to the total trabecular bone surface (BS) using a semiautomatic analyzer (Ibas; Leica).

**Immunohistochemistry.** Bone tissue specimens and tumor xenografts were fixed and then embedded as previously described (39). Six-micrometer tissue sections were then subjected to immunohistochemistry using a mouse anti-human Ki67 monoclonal antibody that specifically recognizes proliferative cells (DakoCytomation). The mitotic index was calculated as the ratio of the number of nuclei immunostained for Ki-67 to the total number of nuclei per field and expressed as the percentage of Ki-67-positive nuclei.

**Platelet aggregation.** Blood samples were taken from healthy volunteers. Platelet aggregation experiments were performed using washed platelets from human blood freshly collected in citrate as an anticoagulant, as previously described (38). Breast or ovarian cancer cells (4 × 10⁵ cells) previously cultured in the absence or in the presence of doxycycline were added to washed human platelets (4 × 10⁵ platelets/ml) in a final volume of 400 µl under stirring conditions at 37°C. Platelet aggregation was monitored over the time as the percentage of light transmission.

**Cell proliferation assay.** Experiments were carried out as previously described (45). Briefly, quiescent tumor cells (5 × 10⁴ cells per well) cultured in 96-well tissue culture plates (BD Biosciences) in medium containing 0.1% (w/v) BSA fatty acid-free supplemented with or without doxycycline (100 ng/ml) were treated overnight in the presence or absence of increasing 1-oleoyl LPA concentrations or with the supernatant of platelet aggregates, and, in the presence or absence of PLB (Sigma), and then pulsed with [³H]-thymidine for the last 8 hours.

**Quantification of LPA and lyso-PLD activity.** LPA was butanol-extracted from conditioned medium or citrated mouse plasma and quantified using a radioenzymatic assay as described previously (46). In these conditions, minimal detection of LPA was 0.2 pmol (46). Lyso-PLD activity was measured by conversion of radiolabeled LPC into radiolabeled LPA as described previously (44).

**Measurement of IL-6 and IL-8 production by ELISA.** Conditioned media of cell lines treated with LPA or left untreated or the supernatants of tumor cell-induced platelet aggregates were collected and analyzed for IL-6 and IL-8 production by ELISA using the human IL-6 or IL-8 Module Set Bender MedSystems (TEBU). Concentrations of IL-6 and IL-8 were expressed in pg/ml per 10⁶ cells.

**Statistical analysis.** Data were analyzed with the Stat-View 5.0 software using unpaired Student’s t test. P values less than 0.05 were considered statistically significant.

**Acknowledgments**

This study was supported by grants from the INSERM (to O. Peyruchaud and P. Clerzard), the European Commission (to P. Clerzard, contract LSHTC-CT-2004-503049), and the Comité Départemental de la Loire de la Ligue Nationale contre le Cancer (to O. Peyruchaud). A. Boucharaba is a recipient of a fellowship from the French Ministry for Research.

Received for publication May 11, 2004, and accepted in revised form October 19, 2004.

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