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

Ahmed Boucharaba, Claire-Marie Serre, Sandra Grès, Jean Sébastien Saulnier-Blache, Jean-Claude Bordet, Julien Guglielmi, Philippe Clézardin, Olivier Peyruchaud

J Clin Invest 12/2004; 114(12): 1714-25

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

Ahmed Boucharaba[1], Claire-Marie Serre[1], Sandra Grès[2], Jean Sébastien Saulnier-Blache[2], Jean-Claude Bordet[3], Julien Guglielmi[1], Philippe Clézardin[1] and Olivier Peyruchaud [1].

[1]INSERM Research Unit 403, Université Claude Bernard Lyon 1, Faculté de Médecine Laënnec, Rue Guillaume Paradin, 69372 Lyon Cedex 08, France.

[2]INSERM Research Unit 586, Institut Louis Bugnard, Université Paul Sabatier, CHU Rangueil, Bâtiment L3, 31403 Toulouse cedex 04, France.

[3]EA3735, Laboratoire d'Hémobiologie, Université Claude Bernard Lyon 1, Faculté Laënnec, 69008 Lyon, France

Correspondence should be addressed to: Olivier Peyruchaud, Phone: (33) 4 78 78 57 38; Fax: (33) 4 78 77 86 63 ; Email: peyruchaud@lyon.inserm.fr.

Abstract

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 tumors 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 animals 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, two potent bone resorption stimulators. Moreover, platelet-derived LPA deprivation in animals, using the platelet antagonist Integrilin, inhibited the progression of bone metastases caused by parental and LPA1-overexpressing MDA-BO2 cells, and reduced the progression of osteolytic lesions in animals 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, in turn, 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 (GPCR), namely LPA1, LPA2, LPA3 (formerly Edg-2, Edg-4 and Edg-7) are specific receptors for LPA (1-3). Recent studies suggest that LPA actions are also mediated through LPA4/GPR23, another type of GPCR for LPA (4), and peroxisome proliferator-activated receptor γ, a transcriptional factor identified as an intracellular LPA receptor (5). Since 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 cancer (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, which possesses a lysophospholipase D activity allowing the production of LPA from lysophosphatidic choline, 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 circle 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 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 which, 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 beast cancer cell lines also expressed all of the three 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 sensitises human MDA-BO2 breast cancer cells to the mitogenic action of LPA *in vitro*. LPA1 has a broad ligand specificity for LPA species (24). Thus, in order to sensitize tumor cells to LPA stimulation we developed a 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 bi-directional pBiL-HA-LPA1 vector. In the absence of doxycycline, the HA-tagged LPA1 (HA-LPA1) receptor is overexpressed at the cell surface whereas luciferase is produced in the cytoplasm. We selected two stable clones (MDA-BO2/HA-LPA1#3 and #79) on the basis of their specific and high expression levels of luciferase (Figure 2A) and HA-LPA1 (Figure 2B). Real-time RT-PCR indicated that clones #3 and #79 had a 42- and 13-

fold increase in LPA1 mRNA expression upon doxycycline withdrawal, respectively, 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 culture media did not alter the mRNA expression levels of LPA2 and LPA3 (Figure 2C).

In the presence of doxycycline, the proliferation of #3 and #79 clones in response to LPA stimulation was similar to that observed for parental MDA-BO2 cells (Figure 3A). Remarkably, in absence of doxycycline, the overexpression of HA-LPA1 enhanced the proliferation of #3 and #79 clones in response to LPA stimulation (Figure 3A). In contrast, the proliferation of #3 and #79 clones in response to PDGF, IGF-1, IGF-2, insulin or EGF was similar in the presence or absence of doxycycline (Figure 3B). These results indicated that HA-LPA-1 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 to that observed with doxycycline-fed animals and mice inoculated with parental cells (Figure 4A**,** Table 1). Overexpression of HA-LPA1 in clones #3 and #79 induced a 3- and 2-fold increase in the extent of osteolytic lesions, respectively (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). Histomorphometric analyses confirmed radiographic analyses and showed that the overexpression of HA-LPA1 by breast cancer cells dramatically increased both bone destruction (BV/TV) and skeletal tumor burden (TB/TV) (Table 1). Overall, these results strongly suggested that HA-LPA1 overexpressing cells were sensitized to an endogenous source of LPA produced in the bone microenvironment. We next determined whether this LPA-dependent effect was specific to the bone microenvironment.

LPA1 overexpression enhances *in vivo* MDA-BO2 tumor growth. Tumor cells were implanted subcutaneously into *nude* mice. The growth rates of MDA-BO2, #3 and #79 tumor cells in doxycycline-fed animals were similar (Figure 4B). In contrast, the growth of #3 and #79 tumors were 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 #3 overexpressing HA-LPA1 (Figure 4C). We observed similar results for clone #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 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 nor do they express autotaxin. In order to identify the potential sources of LPA, we first speculated that tumor cells themselves might produce LPA which could secondly act as an autocrine factor *in vivo*. However, MDA-BO2 parental

cells and transfectants did not produce detectable amount of LPA in their culture media (Figure 5). These results were in agreement with *in vitro* cell proliferation experiments showing that HA-LPA1 overexpressing cells grew at the same rate than doxycycline-treated cells when cultured in the presence of the control medium (Figure 3A). In addition, MDA-BO2 and #3 cells did not produce detectable amount of lysoPLD activity in their culture media (Figure 5). The absence of autotoxin mRNA expression in our cell lines was confirmed by RT-PCR experiments (data not shown). Thus, 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). 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. MDA-MB-231, MDA-BO2 and HA-LPA1 overexpressing #3 cells did induce platelet aggregation (Figure 6) and stimulated the release of LPA from activated platelets (Table 2). Supernatants of tumor cell-induced platelet aggregation promoted the proliferation of #3 cells (Figure 6). Moreover, #3 cells were sensitized to this mitogenic action in a doxycycline-dependent manner which was completely abrogated in the presence of phospholipase B (a LPA-degrading enzyme) (Figure 6). These results indicated that LPA released from activated platelets upon stimulation by tumor cells was bioactive and promoted breast cancer cell proliferation.

Platelet-derived LPA deprivation in animals blocks the progression of bone metastasis. 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 or HA-LPA1 overexpressing cells (clone #3) were treated with integrin αIIbβ3 antagonist Integrilin, starting from day 14 after tumor cell inoculation. As expected, Integrilin treatment of metastatic animals induced a severe thrombocytopenia (30+/-9 platelets/nL) when compared to that observed in untreated metastatic animals (210+/-20 platelets/nL). This thrombocytopenia was concomitent with a drastic decrease (70% reduction) of circulating levels of LPA in Integrilin-treated metastatic mice when compared to those observed in untreated metastatic animals (Table 3).These findings strongly suggested that blood platelets were the endogenous source of LPA in metastatic animals. The LPA deprivation upon Integrilin treatment of metastatic animals was associated with a 50% reduction in the extent of osteolytic lesions when compared to that observed with vehicle treated-animals (Figure 7, A and B). Moreover, the dramatic increase of bone destruction observed in animals bearing HA-LPA1 overexpressing cells was completely abolished upon Integrilin treatment (Figure 7, A and B). This inhibition was also associated with a substantial decrease of skeletal tumor burden as judged by histomorphometric analysis (data not shown).

To determine whether the role of LPA was restricted to breast cancer bone metastasis, similar experiments were conducted with hamster CHOβ3-wt ovarian cancer cells for which we have previously shown that they induced bone metastasis in animals (26). Nucleotide sequences for hamster LPA receptors are not available from nucleotide data-bases. Therefore, we were not able to identify which type of LPA receptors were expressed in CHOβ3-wt cells. However, specific hamster LPA receptors might be present in these cells since the proliferation of CHOβ3-wt cells was stimulated in response to increasing concentrations of LPA and the mitogenic action of LPA was abrogated in the presence of phospholipase B, a LPA-degrading enzyme, (Figure 8A). CHOβ3-wt cells also induced platelet aggregation *in*

vitro (Figure 8B). Thus, Integrilin treatment of metastatic animals might also affect the progression of bone metastases caused by CHOβ3-wt cells. Indeed, we found that an 11-day-treatment of metastatic animals, starting from day 10 after cell inoculation, inhibited by 3-fold the extent of bone metastases caused by CHOβ3-wt cells (Figure 8C). Overall, these results obtained with too different animal models of bone metastasis strongly suggested that blood platelets were the main endogenous source of LPA. Bone-residing tumor cells, by inducing platelet aggregation, stimulated the release of LPA which, in turn, promoted the proliferation of tumor cells.

The reason why LPA1 overexpression in MDA-BO2 cells was also associated with a higher bone destruction was however unclear.

LPA1 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 stimulate osteoclast-mediated bone destruction *in vivo* (29, 30). Here, LPA stimulated both IL-6 (Figure 9A) and IL-8 (Figure 9B) production by MDA-BO2 cells and this cytokine production was markedly increased when #3 cells overexpressed HA-LPA1. Similarly, the supernatant of tumor cell-induced platelet aggregation promoted the production of IL-6 and IL-8 in a LPA-dependent manner (Figure 9, 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.

Discussion

The role for LPA in cancer is currently emerging (7). However, direct evidence for the function of LPA and its cognate receptors *in vivo* are missing. The mitogenic activity of LPA *in vitro* on human MCF-7 and MDA-MD453 breast cancer cells has been previously reported (12). In the present study, we showed that this mitogenic activity of LPA on a series of human breast cancer cell lines correlated with the expression of LPA receptors. More importantly, we demonstrated that LPA promoted breast cancer progression *in vivo*, using a strategy in which breast cancer cells were sensitized to LPA stimulation through the overexpression of LPA1 receptor. We showed that the growth of subcutaneous or skeletal breast tumors was markedly increased when conditional LPA1 overexpression in transfected MDA-BO2 breast cancer cells was turned on by doxycycline withdrawal. These results do not preclude the possibility that LPA2 and LPA3 receptors may also play a role in breast cancer progression *in vivo*. However, since 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 increase *in vivo* growth of MDA-BO2 cells upon doxycycline withdrawal was directly related to the overexpression of LPA1. We also observed that the overexpression of LPA1 specifically sensitized MDA-BO2 cells in culture to the mitogenic action of LPA. Moreover, the mitotic index of MDA-BO2 cells *in situ* was also increased when LPA1 overexpression was turned on. Our results were therefore strongly suggestive of a local production of bioactive LPA in the tumor microenvironment *in vivo* that supported LPA1-dependent breast tumor proliferation.

It has been previously reported that MDA-MB-231 breast cancer cells do not directly produce LPA (13). However, autotaxin in MDA-MB-231 and MDA-MB-435S breast cancer cells can induce the production of bioactive LPA which, in turn, stimulates cell migration,

invasion and proliferation (13, 31, 32). In the present study, MDA-BO2 cells and transfectants did not directly produce LPA, nor did they express autotaxin.

We therefore focused our attention on human blood platelets. There are several reasons suggesting that platelets could provide *in vivo*, in the tumor bed, a local production of bioactive LPA. First, platelets are a major source of LPA which is released from activated platelets upon thrombin stimulation (19, 20). Second, platelets play a major role in the metastatic dissemination of tumor cells *in vivo* (27, 33, 34), 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 (35), platelets are in contact with tumor cells and are therefore able to secrete multiple factors upon activation (36). Fourth, MDA-MB-231 breast cancer cells (as well as other tumor cell lines) interact with platelets and stimulate platelet aggregation *in vitro* (37), 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 (37), we observed here that our MDA-BO2 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 the osteolytic lesions in animals bearing LPA1-overexpressing cells. This suggests therefore that platelet-derived LPA deprivation affect the growth of breast cancer cells in bone. Moreover, this observation was not restricted to our MDA-BO2 breast cancer bone metatastasis model since similar findings were observed with a bone metastasis model using CHOβ3-wt ovarian cancer cells.

Because subcutaneous and skeletal MDA-BO2 breast tumors are highly vascularized (38), these data strongly support the idea that platelets from the blood stream come into contact with the tumor bed *in vivo*, then aggregate and secrete LPA which, in turn, stimulates the proliferation of breast cancer cells.

Beside 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 stimulates the production of IL-6 and IL-8 by ovarian and breast cancer cells (23). Tumor cells do not directly destroy bone (22). Instead, IL-6 and IL-8 produced by breast cancer cells stimulate osteoclast-mediated bone resorption (33, 39). 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, IL-6 and IL-8 productions were markedly increased when tumor cells overexpressed LPA1. It is therefore most conceivable that beyond its tumor growth-promoting effect, LPA indirectly stimulates osteoclast-mediated bone resorption. Thus, in addition to this vicious circle related to the direct reciprocal interaction between breast cancer cells and osteoclast-mediated bone resorption (15,16), we propose that there exists a LPA-dependent cycle wherein bone-residing cells stimulate LPA production by platelets which, in turn, enhances tumor growth and cytokine-mediated bone resorption (Figure 10).

In conclusion, our findings provide new insights into the molecular mechanisms of osteolytic bone metastasis development. They reveal LPA and its receptors as potential therapeutic targets which, in combination with the targeting of osteoclasts by bisphosphonates, should improve the treatment of patients with bone metastases.

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 ATCC. Characteristics of CHO-β3wt, MDA-MB-231/BO2 (MDA-BO2) and tet-Off-expressing MDA-BO2 breast cancer cells were described elsewhere (26, 38). The cDNA encoding for the entire human LPA1 and the antigenic epitope tag (hemagglutinin, HA) fused at the NH2-terminus of LPA1 was amplified by PCR using the vector pEDG-2 (40) as a template and two oligonucleotides primers (5'-CCGCTAGCATGTACCCATACGACGTCCCAGACTACGCTATGGCTGCCATCTCTAC T-3' [HA-edg2N] and 5'-CGCAAGCTTCTAAACCACAGAGTGGTCATTGC-3' [Stop-edg2]). The bi-directional vector pBiL/HA-LPA1 was constructed by inserting into the pBiL plasmid (Clontech, Hampshire, United Kingdom) the *Nhe* I/*Hind* III PCR fragment encoding for the HA-LPA1 sequence. MDA-MB-231/BO2-tet-Off cells were cotransfected with the pBiL/HA-LPA1 together with a vector conferring puromycin resistance (pPur, Clontech). Selection of the clones was obtained after growing the cells for 2 weeks in the presence of puromycin (2µg/ml). Luciferase induction upon doxycycline withdrawal was used to select inducible clones among stable transfectants. Two HA-LPA1-inducible transfectants (#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, Boussens, France) and 1% penicillin/streptomycin (Life Technologies) at 37 °C in a 5% CO_2 incubator. SK-BR-3 cells were grown in complete McCoy's 5a medium (Life Technologies, Cergy-Pontoise, France).

Animals studies. All procedures were performed on female Balb/c nu/nu mice of 4 weeks of age (Charles River, St. Germain sur l'Arbresle, 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. These studies were routinely inspected by the Attending Veterinian to ensure continued compliance with the proposed protocols. Two days before tumor cell inoculation animals were treated with or without doxycycline (1 mg/ml) in their drinking water supplemented with 5% (w/v) sucrose.

Bone metastasis experiments in animals were conducted as previously described (26, 38). Briefly, doxycycline-treated or untreated MDA-BO2 transfectants (5 x 10^5 cells in 100 µl of phosphate-buffered saline) were inoculated into the tail vein of anesthetized *nude* mice treated with or without doxycycline, respectively. Alternatively, CHO-β3wt cells (10^6 cells in 100 µl of phosphate-buffered saline) were inoculated intravenously into animals Metastatic animals were also treated every three days with the anti-platelet agent eptifibatide (Integrilin, Schering-Plough, Levaloy Perret, France) by intra-peritoneal injection (0.5 mg/kg/day) starting from day 10 or day 14 after tumor cell inoculation. Radiographs (MIN-R2000 films, Kodak, Rochester, NY) were taken 21 or 30 days after tumor cell injections using a cabinet X-ray system (MX-20, Faxitron X-ray corporation, Wheeling, IL), and bone metastases were enumerated on each radiograph. The area of osteolytic lesions was measured using computerized image analysis system Visiolab 2000 (Biocom, Paris, France) 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 (10^6 cells in 100 µl of phosphate-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 two groups: one group received doxycycline for the duration of the experiment whereas the other group had no doxycycline. Tumor size was assessed by external measurement of the length (L) and width (W) of the tumors using a

Vernier caliper. Tumor volume (TV; expressed in mm^3) was calculated using the following equation: $TV=(LxW^2)/2$.

Reverse transcription, standard and quantitative PCR. Total RNA from cell lines and human breast tumors were extracted using Total RNA Isolation System (Promega, Charbonnière, France). cDNA was synthesized using M-MLV-1 (Promega). Primers for LPA1, LPA2 (41), GAPDH (10) and Autotaxin (42) were designed as described previously. LPA3 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. PCR reactions were run using a program consisting of 40 cycles of 95 °C for 15 sec, 53 °C for 30 sec, and 72 °C for 20 sec with a pre-incubation of 95 °C for 2 min. Products from standard PCR were separated by electrophoresis on a 2% agarose gel, then visualized with ethidium bromide under ultra violet light. The quantification of human LPA1, LPA2 and LPA3 mRNAs were performed by real-time PCR using the Master SYBR Green I kit (Roche Diagnostics, Meylan, France). 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. Analysis of GaPDH mRNA expression was performed in parallel to confirm the use of equal amount of cDNAs in each reaction. Results were expressed as the percentage of gene expression in each cell line compared with that of the parental MDA-BO2 cells cultured in the absence of doxycycline.

Luciferase activity assay and flow cytometry. Luciferase activity was measured on cell lysate following manufacturer's instructions (Promega). Prior to flow cytometry analysis, tumor cells were cultured in media complemented with or without doxycycline (100 ng/ml).

Expression of HA-LPA1 at the cell surface was detected using a mouse anti-HA monoclonal antibody 12CA5 (Roche Diagnostics). Monoclonal antibody MOPC21 (Santa Cruz, San Diego, California) was used as an isotypic negative control antibody.

Bone histology. Hind limbs from animals were fixed and embedded in methylmethacrylate. Seven-µm sections of undecalcified long bones were stained with Goldner's trichrome and histological analyses were performed on longitudinal medial sections of tibial metaphysis by using computerized image analysis system Visiolab 2000 (Biocom, Paris, France) as previously described (26, 38).

Immunohistochemistry. Bone tissue specimens and tumor xenografts were fixed then embedded as previously described (38). Six-µm tissue sections were then subjected to immunohistochemistry using a mouse anti-human Ki67 monoclonal antibody that specifically recognizes proliferative cells (DakoCytomation**,** Trappes, France). The mitotic index was calculated as the percentage of nuclei immunostained for Ki-67.

Platelet aggregation. Platelet aggregation experiments were performed using washed platelets from human blood freshly collected in the anticoagulant acid-citrate dextrose as previously described (37). Breast cancer or CHO-β3wt cells ($4x10^6$ cells) previously cultured in absence of doxycycline were added to washed human platelets ($4x10^5$ platelets/µl) 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 (43). Briefly, quiescent tumor cells (5 x 10^3 cells per well) cultured into 96-well tissue culture plates (BD Biosciences, Le Pont de Claix, France) 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 LPA concentrations or with the supernatant of platelet aggregates, then pulsed with [3H]thymidine for the last 8 hours.

Quantification of LPA and lysophospholipase D activity. LPA was butanol-extracted from conditioned media or citrated mouse plasma and quantified using a radioenzymatic assay as described previously (44). Lysophospholipase D activity was measured by conversion of radiolabeled lysophosphatidyl choline (LPC) into radiolabeled LPA as described previously (42).

Measurement of IL-6 and IL-8 production by ELISA. Conditioned media of cell lines treated without or with LPA 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, Le Perray-En-Yvelines, France). Concentrations of IL-6 and IL-8 were expressed in pg/ml per 10^6 cells.

Statistical analysis. Data were analyzed with the Stat-View 5.0 software using unpaired Student's *t*-test . $P < 0.05$ was considered statistically significant.

Acknowledgements

This study was supported by grants from the INSERM (O.P. and P.C.), and the Comité Départemental de la Loire de la Ligue Nationale contre le Cancer (O.P.). (A.B.) is a recipient of a fellowship from the French Ministry for Research. The authors acknowledge the support of the European Commission through contract LSHC-CT-2004-503049.

Abbreviation list

°C	:	Degrees Celsius
ATX	:	Autotaxin
bp	:	Base pair
BSA	:	Bovine serum albumin
BV/TV	:	Bone volume over tissue volume ratio
cDNA	:	Complementary deoxyribonucleic acid
DMEM	:	Dulbecco modified Eagle's minimum essential medium
Edg	:	Endothelial differenciation gene receptor
EGF	:	Epidermal growth factor
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase
IGF	:	Insulin like growth factor
IL	:	Interleukin (IL-6, IL-8)
LPA	:	Lysophosphatidic acid
LPA1,2,3	:	Lysophosphatidic acid receptor type 1, 2 and 3
LPC	:	Lyophosphatidic choline
M	:	Molar
M-MLV	:	Moloney murine leukemia virus
mRNA	:	Messenger ribonucleic acid
PCR	:	Polymerase chain reaction
PDGF	:	Platelet derived growth factor
PLB	:	Phospholipase B
PLD	:	Phospholipase D
PTHrP	:	Parathyroid hormone related peptide
RNA	:	Ribonucleic acid
RT-PCR	:	Reverse transcription-PCR
SD	:	Standard deviation
SE	:	Standard error
TB/TV	:	Tumor burden over tissue volume ratio
TGFβ	:	Transforming growth factor beta
TV	:	Tumor volume
U	:	Unit

References

1. An, S., Dickens, M.A., Bleu, T., Hallmark, O.G., and Goetzl, E.J. 1997. Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. *Biochem. Biophys. Res. Commun.* **231**:619-622.
2. An, S., Bleu, T., Hallmark, O.G., and Goetzl, E.J. 1998. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J. Biol. Chem.* **273**:7906-7910.
3. Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami-Murofushi, K., Tsujimoto, M., Arai, H., and Inoue, K. 1999. Molecular cloning and characterization of a novel human G-protein-coupled receptor, EDG7, for lysophosphatidic acid. *J. Biol. Chem.* **274**:27776-27785.
4. Noguchi, K., Ishii, S., and Shimizu, T. 2003. Identification of p2y9/GPR23 as a Novel G Protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J. Biol. Chem.* **278**:25600-25606.
5. McIntyre, T.M., Pontsler, A.V., Silva, A.R., St Hilaire, A., Xu, Y., Hinshaw, J.C., Zimmerman, G.A., Hama, K., Aoki, J., Arai, H., et al. 2003. Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARgamma agonist. *Proc. Natl. Acad. Sci. U. S. A.* **100**:131-136.
6. Moolenaar, W.H. 2000. Development of our current understanding of bioactive lysophospholipids. *Ann. N. Y. Acad. Sci.* **905**:1-10.
7. Mills, G.B., and Moolenaar, W.H. 2003. The emerging role of lysophosphatidic acid in cancer. *Nat. Rev. Cancer.* **3**:582-591.
8. Xu, Y., Shen, Z., Wiper, D.W., Wu, M., Morton, R.E., Elson, P., Kennedy, A.W., Belinson, J., Markman, M., and Casey, G. 1998. Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. *Jama.* **280**:719-723.

9. Fang, X., Schummer, M., Mao, M., Yu, S., Tabassam, F.H., Swaby, R., Hasegawa, Y., Tanyi, J.L., LaPushin, R., Eder, A., et al. 2002. Lysophosphatidic acid is a bioactive mediator in ovarian cancer. *Biochim. Biophys. Acta.* **1582**:257-264.

10. Goetzl, E.J., Dolezalova, H., Kong, Y., Hu, Y.-L., Jaffe, R.B., Kalli, K.R., and Conover, C.A. 1999. Distinctive expression and functions of the type 4 endothelial differentiation gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer. *Cancer Res.* **59**:5370-5375.

11. Hu, Y.L., Tee, M.K., Goetzl, E.J., Auersperg, N., Mills, G.B., Ferrara, N., and Jaffe, R.B. 2001. Lysophosphatidic acid induction of vascular endothelial growth factor expression in human ovarian cancer cells. *J. Natl. Cancer Inst.* **93**:762-768.

12. Goetzl, E.J., Dolezalova, H., Kong, Y., and Zeng, L. 1999. Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. *Cancer Res.* **59**:4732-4737.

13. Yang, S.Y., Lee, J., Park, C.G., Kim, S., Hong, S., Chung, H.C., Min, S.K., Han, J.W., Lee, H.W., and Lee, H.Y. 2002. Expression of autotaxin (NPP-2) is closely linked to invasiveness of breast cancer cells. *Clin. Exp. Metastasis.* **19**:603-608.

14. Body, J.J. 1992. Metastatic bone disease: clinical and therapeutic aspects. *Bone* **13**:S57-62.

15. Guise, T.A., and Mundy, G.R. 1998. Cancer and bone. *Endocr. Rev.* **19**:18-54.

16. Mundy, G.R. 2002. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat. Rev. Cancer.* **2**:584-593.

17. Pluijm, G., Lowik, C., and Papapoulos, S. 2000. Tumour progression and angiogenesis in bone metastasis from breast cancer: new approaches to an old problem. *Cancer Treat. Rev.* **26**:11-27.

18. Pickering, L.M., and Mansi, J.L. 2002. The role of bisphosphonates in breast cancer management: review article. *Curr. Med. Res. Opin.* **18**:284-295.

19. Eichholtz, T., Jalink, K., Fahrenfort, I., and Moolenaar, W.H. 1993. The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem. J.* **291**:677-680.

20. Gerrard, J.M., and Robinson, P. 1989. Identification of the molecular species of lysophosphatidic acid produced when platelets are stimulated by thrombin. *Biochim. Biophys. Acta.* **1001**:282-285.

21. Bakewell, S.J., Nestor, P., Prasad, S., Tomasson, M.H., Dowland, N., Mehrotra, M., Scarborough, R., Kanter, J., Abe, K., Phillips, D., et al. 2003. Platelet and osteoclast beta3 integrins are critical for bone metastasis. *Proc. Natl. Acad. Sci. U. S. A.* **100:**14205-14210.

22. Amirkhosravi, A., Mousa, S.A., Amaya, M., Blaydes, S., Desai, H., Meyer, T., and Francis, J.L. 2003. Inhibition of tumor cell-induced platelet aggregation and lung metastasis by the oral GpIIb/IIIa antagonist XV454. *Thromb. Haemost.* **90**:549-554.

23. Fang, X., Yu, S., Bast, R.C., Liu, S., Xu, H.J., Hu, S.X., LaPushin, R., Claret, F.X., Aggarwal, B.B., Lu, Y., et al. 2004. Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells. *J. Biol. Chem.* **279**:9653-9661.

24. Bandoh, K., Aoki, J., Taira, A., Tsujimoto, M., Arai, H., and Inoue, K. 2000. Lysophosphatidic acid (LPA) receptors of the EDG family are differentially activated by LPA species. Structure-activity relationship of cloned LPA receptors. *FEBS Lett.* **478**:159-165.

25. Peyruchaud, O., Winding, B., Pecheur, I., Serre, C.M., Delmas, P., and Clezardin, P. 2001. Early detection of bone metastases in a murine model using fluorescent human breast cancer cells: application to the use of the bisphosphonate zoledronic acid in the treatment of osteolytic lesions. *J. Bone Miner. Res.* **16**:2027-2034.

26. Pecheur, I., Peyruchaud, O., Serre, C.M., Guglielmi, J., Voland, C., Bourre, F., Margue, C., Cohen-Solal, M., Buffet, A., Kieffer, N., et al. 2002. Integrin alpha(v)beta3 expression confers on tumor cells a greater propensity to metastasize to bone. *Faseb J.* **16**:1266-1268.

27. Camerer, E., Qazi, A.A., Duong, D.N., Cornelissen, I., Advincula, R.and Coughlin, S.R. 2004. Platelet, protease-activated receptors, and fibrinogen in hematogenous metastasis. *Blood.* **104**:397-401.

28. Mundy, G., Oyojobi, B., Taraianedes, K., Dallas, S., and Chen, D. 2001. *Cytokines and bone remodeling*. London: Academic Press. 373-403 pp.

29. de la Mata, J., Uy, H.L., Guise, T.A., Story, B., Boyce, B.F., Mundy, G.R., and Roodman, G.D. 1995. Interleukin-6 enhances hypercalcemia and bone resorption mediated by parathyroid hormone-related protein in vivo. *J. Clin. Invest.* **95**:2846-2852.

30. Bendre, M.S., Gaddy-Kurten, D., Mon-Foote, T., Akel, N.S., Skinner, R.A., Nicholas, R.W., and Suva, L.J. 2002. Expression of interleukin 8 and not parathyroid hormone-related protein by human breast cancer cells correlates with bone metastasis in vivo. *Cancer Res.* **62**:5571-5579.

31. Umezu-Goto, M., Kishi, Y., Taira, A., Hama, K., Dohmae, N., Takio, K., Yamori, T., Mills, G.B., Inoue, K., Aoki, J., et al. 2002. Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J. Cell. Biol.* **158**:227-233.

32. Hama, K., Aoki, J., Fukaya, M., Kishi, Y., Sakai, T., Suzuki, R., Ohta, H., Yamori, T., Watanabe, M., Chun, J., et al. 2004. Lysophosphatidic acid and autotaxin stimulate cell motility of neoplastic and non-neoplastic cells through LPA1. *J. Biol. Chem.* **279**:17634-17639.

33. Karpatkin, S., Pearlstein, E., Ambrogio, C., and Coller, B.S. 1988. Role of adhesive proteins in platelet tumor interaction in vitro and metastasis formation in vivo. *J. Clin. Invest.* **81**:1012-1019.

34. Felding-Habermann, B., O'Toole, T.E., Smith, J.W., Fransvea, E., Ruggeri, Z.M., Ginsberg, M.H., Hughes, P.E., Pampori, N., Shattil, S.J., Saven, A., et al. 2001. Integrin activation controls metastasis in human breast cancer. *Proc. Natl. Acad. Sci. U. S. A.* **98**:1853-1858.

35. Carmeliet, P., and Jain, R.K. 2000. Angiogenesis in cancer and other diseases. *Nature.* **407**:249-257.

36. Trikha, M., and Nakada, M.T. 2002. Platelets and cancer: implications for antiangiogenic therapy. *Semin. Thromb. Hemost.* **28**:39-44.

37. Belloc, C., Lu, H., Soria, C., Fridman, R., Legrand, Y., and Menashi, S. 1995. The effect of platelets on invasiveness and protease production of human mammary tumor cells. *Int. J. Cancer.* **60**:413-417.

38. Peyruchaud, O., Serre, C.-M., NicAmhlaoibh, R., Fournier, P., and Clezardin, P. 2003. Angiostatin inhibits bone metastasis formation in nude mice through a direct anti-osteoclastic activity. *J. Biol. Chem.* **278**:45826-45832.

39. Martin, T.J. 2002. Manipulating the environment of cancer cells in bone: a novel therapeutic approach. *J. Clin. Invest.* **110**:1399-1401.

40. Peyruchaud, O., and Mosher, D.F. 2000. Differential stimulation of signaling pathways initiated by Edg-2 in response to lysophosphatidic acid or sphingosine-1-phosphate. *Cell. Mol. Life Sci.* **57**:1109-1116.

41. Zhang, Q., Peyruchaud, O., French, K.J., Magnusson, M.K., and Mosher, D.F. 1999. Sphingosine 1-phosphate stimulates fibronectin matrix assembly through a Rho-dependent signal pathway. *Blood.* **93**:2984-2990.

42. Ferry, G., Tellier, E., Try, A., Gres, S., Naime, I., Simon, M.F., Rodriguez, M., Boucher, J., Tack, I., Gesta, S., et al. 2003. Autotaxin is released from adipocytes, catalyzes lysophosphatidic acid synthesis, and activates preadipocyte proliferation. Up-regulated expression with adipocyte differentiation and obesity. *J. Biol. Chem.* **278**:18162-18169.

43. Abbadia, Z., Clezardin, P., Serre, C.M., Amiral, J., and Delmas, P.D. 1993. Thrombospondin (TSP1) mediates in vitro proliferation of human MG-63 osteoblastic cells induced by alpha-thrombin. *FEBS Lett.* **329**:341-346.

44. Saulnier-Blache, J.S., Girard, A., Simon, M.F., Lafontan, M., and Valet, P. 2000. A simple and highly sensitive radioenzymatic assay for lysophosphatidic acid quantification. *J. Lipid Res.* **41**:1947-1951.

Table 1 : Quantification of osteolytic lesions and skeletal tumor burden in untreated and doxycycline-treated animals bearing parental MDA-BO2 breast cancer cells or #3 and #79 cells overexpressing HA-LPA1.

Cell lines[a]	Osteolytic lesions		BV/TV (%)[b]		TB/TV (%)[c]	
	+ Dox (n)	- Dox (n)	+ Dox (n)	- Dox (n)	+ Dox (n)	- Dox (n)
MDA-BO2	6.3 ± 1.5 (9)	5.7 ±1.1 (6)	5.8 ± 0.3 (9)	5.7 ± 1.5 (6)	32 ± 4.3 (9)	33 ± 3.3 (6)
MDA-BO2/HA-LPA1#3	6.2 ±1.0 (6)	18.6 ± 2.7* (9)	5.8 ± 0.4 (6)	0.03 ± 0.05* (9)	30 ± 3.4 (6)	98 ± 2.0* (9)
MDA-BO2/HA-LPA1#79	5.4 ± 0.3 (6)	10.1 ± 1.0* (6)	5.8 ± 0.4 (6)	2.2 ± 1.5* (6)	34 ± 6.1 (6)	53 ± 8.4* (6)

(a)MDA-BO2 : parental human breast cancer cell line; MDA-BO2/HA-LPA1 #3 and #79 clones overexpressing HA-LPA1 at the cell surface upon doxycycline (Dox) withdrawal.

(b) BV/TV : bone volume to tissue volume ratio

(c) TB/TV : tumor burden to tissue volume ratio

Data are the mean +/- SD of two separate experiments for each cell line using *n* animals.* indicated $P< 0.001$ using unpaired Student's *t* test when comparing animals fed with and without doxycycline.

Table 2 : Quantification of LPA in the supernatant of platelet aggregates.

Platelet stimulating factor	LPA (nM)
none	9 +/- 8.2
Thrombin (0.05 U/ml)	101 +/- 72.6
MDA-MB-231 (-Dox)	97 +/- 39.1
MDA-BO2 (-Dox)	124 +/-30.2
MDA-BO2/HA-LPA1$^{\#}$3 (+Dox)	144 +/- 25.1
MDA-BO2/HA-LPA1$^{\#}$3 (-Dox)	145 +/- 18.5

Results were obtained from 3 to 4 separate donors, and are expressed as the mean +/- SD.

Table 3 : Quantification of LPA in the plasma of metastatic animals.

Animals	n	LPA (nM)
MDA-BO2 cells + vehicle	6	257.6 +/- 63.3
MDA-BO2 cells + Integrilin	9	76.3 +/- 7.37 *

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. * indicated $P < 0.01$ using unpaired Student's *t* test, compared with animals not treated with Integrilin.

Legend to figures

Figure 1: Expression of LPA receptors in breast cancer and mitogenic activity of LPA in breast cancer cell lines. **(A)** RT-PCR experiments using total RNA isolated from human primary tumors: fibroadenomas (F.Ad$^{\#}$1, F.Ad$^{\#}$2), ductal carcinomas (T$^{\#}$1, T$^{\#}$2). Expected size of amplification products for LPA1 (1), LPA2 (2), LPA3 (3) and GaPDH (G) are 428, 352, 256 and 470 bp, respectively. **(B)** Human breast cancer cell lines were stimulated with increasing concentrations of LPA and pulsed with [^{3}H]Thymidine. Cell proliferation was assessed after quantification of [^{3}H]Thymidine incorporation. Data are expressed in cpm as the mean +/- SD of six replicates and are representative of at least three separate experiments. **Insets**: RT-PCR amplification products for LPA1 (1), LPA2 (2), LPA3 (3) and GaPDH (G) using total RNA isolated from each indicated cell line.

Figure 2: Characterization of MDA-BO2 clones stably transfected to conditionally overexpress HA-LPA1. **(A)** Cells transfected with the bi-directional expression vector pBiL-HA-LPA1 were plated with (+) or without (-) Dox. Two stable clones (#3 and #79) were selected using luciferase activity measurement as an endpoint. Data are expressed in relative light unit (rlu). *, P <0.0001 for (-Dox) *versus* (+ Dox). **(B)** Detection of HA-LPA1 cell surface expression in parental MDA-BO2 cells, and clones #3 and #79 by flow cytometry using the anti-HA monoclonal antibody. Black and white histograms referred to cells treated without and with Dox, respectively. The *y* axis depicts the number of cells per channel (events) and the *x* axis the relative fluorescence intensity in arbitrary unit (log scale). **(C)** LPA receptor mRNA expression in parental MDA-BO2 cells, and clones #3 and #79. Cells were cultured in the absence (-) or presence (+) of Dox before total RNA preparation. RT-PCR fragments were separated on a 2% agarose gel, then stained with ethidium bromide. Numbers below the top panel correspond to real-time PCR quantification data of the LPA1 mRNA

copy number for each clone compared with the parental MDA-BO2 cells cultured in the absence of Dox (mean +/- SD; *, $P < 0.001$). No variation of mRNA expression was detected for LPA2, LPA3 and GAPDH in the presence or absence of Dox.

Figure 3: Effect of LPA1 overexpression on the mitogenic action of LPA on MDA-BO2 cells. **(A)** Parental MDA-BO2 cells (triangle) and transfected clones #3 (circle) and #79 (square) were cultured in medium supplemented without (opened symbols) or with (closed symbols) Dox (100 ng/ml) and then treated as described in Figure 1B. Data are expressed in cpm as the mean +/- SD of six replicates and are representative of at least three separate experiments. *, $P < 0.005$; **, $P < 0.001$ untreated *versus* Dox-treated cell lines. **(B)** Cells were cultured in the absence (-) or presence (+) of Dox (100 ng/ml) and stimulated with LPA (0.1 μM) or other indicated growth factors (10 ng/ml). Cell proliferation was measured as described above. Data are expressed as the mean +/- SD of six replicates and are representative of three separate experiments. *, $P < 0.001$ untreated *versus* Dox-treated cell lines.

Figure 4: Effect of LPA1 overexpression in MDA-BO2 cells on osteolytic lesions, and skeletal and subcutaneous tumor growth. **(A)** Animals fed without (-) or with (+) Dox were inoculated intravenously with MDA-BO2 or #3 cells. (Upper panels) Representative radiographs of hindlimbs from mice bearing MDA-BO2 or #3 cells, 30 days after tumor cell inoculation. In the absence of Dox, there was a marked increase in the extent of osteolytic lesions (arrows) in #3 cell-bearing mice. (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 in red. Note that trabecular bone was completely destroyed and replaced by tumor cells (T) in tibial metaphysis from untreated

animals bearing #3 cells. Bar : 1mm. **(B)** Animals fed without (opened symbols) or with (closed symbols) Dox were inoculated subcutaneously into the flank with MDA-BO2 (triangle), #3 (circle) or #79 (square) cells. Tumors were measured at indicated time point. Tumor volume (in mm^3) is expressed as the mean +/- SD of 9 animals per group. *, $P < 0.05$ and **, $P < 0.005$ Dox-free *versus* Dox-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. *, $P < 0.0001$ Dox-free *versus* Dox-fed tumor-bearing animals. Bars : 100 µm.

Figure 5: Production of LPA and autotaxin by MDA-BO2 breast cancer cells. Cell culture media were collected for each indicated cell line placed in the absence (-) or presence (+) of Dox (100 ng/ml). **(A)** Detection of LPA. The production of phosphatidic acid (PA) was due to the transfer of [^{14}C] 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 autotaxin ATX/lysoPLD activity. [^{14}C]LPC was used as the substrate of ATX/lysoPLD to produce [^{14}C]LPA. Experiments were carried out in duplicate using culture media from the cell lines described above. Note that MDA-MB-231, MDA-BO2, $^{\#}$3 and $^{\#}$79 cell lines did not produce LPA nor did they express autotaxin.

Figure 6: Effect of breast tumor cells on platelet aggregation and the release of LPA from activated platelets. **(A)** Indicated tumor cells previously cultured in absence of Dox were added to washed human platelets under stirring conditions. Platelet aggregation was recorded

over the time as the % of light transmission. **(B)** #3 cells were plated without (-) or with (+) Dox and stimulated with DMEM (Cont.), LPA (10^{-7} M) or MDA-BO2-induced platelet aggregation supernatants (Sup.Aggreg), in the presence (+) or absence (-) of phospholipase B (PLB). Cell proliferation was measured as described in the legend of figure 3. Data are expressed as the mean +/- SD of six replicates and are representative of three separate experiments. *, $P < 0.0001$ stimulated *versus* control cells.

Figure 7: Effect of *in vivo* inhibition of platelet aggregation on the LPA-dependent progression of breast cancer bone metastasis. **(A)** Representative radiographs at day 30 of hindlimbs from Dox-free fed mice bearing MDA-BO2 or #3 cells that were treated with Integrilin or the vehicle from day 14 to day 30. **(B)** Quantification of osteolytic lesion areas on radiographs in Integrilin (+) and vehicle (-) treated metastatic animals. Values are the mean ± SE of 6 to 9 animals per group. *, $P <0.01$; **, $P <0.001$ Integrilin *versus* vehicle-treated animals.

Figure 8: Mitogenic effect of LPA on CHO-β3wt cells *in vitro* and effect of *in vivo* inhibition of platelet aggregation on the progression of CHO-β3wt bone metastasis. **(A)** Cell proliferation assay: CHO-β3wt cells were incubated with increasing concentrations of LPA in the absence (closed square) or presence (opened circle) of phospholipase B (PLB), a LPA-degrading enzyme. Cell proliferation was assessed as described in Figure 1B. Data are expressed in cpm as the mean +/- SD of six replicates and are representative of at least three separate experiments. **(B)** CHO-β3wt cell stimulation of platelet aggregation was carried out as described in Figure 6. **(C)** Bone metastasis experiment: Representative radiographs at day 21 of hindlimbs from mice bearing CHO-β3wt cells that were treated with Integrilin or the vehicle from day 10 to day 21. Osteolytic lesions are indicated by arrows. Data represent the

mean +/- SE of osteolytic lesion areas, expressed in mm^2 of 8 to 10 animals per group. *, P <0.001 Integrilin *versus* vehicle-treated animals.

Figure 9: Effect of purified or platelet-derived LPA on the production of IL-6 and IL-8 by breast cancer cells. **(A)** Quantification of IL-6 and **(B)** IL-8 were performed using culture media from cells pre-treated in the presence (+) or absence (-) of LPA, the supernatant of breast cancer cell-induced platelet aggregation (Sup. Aggreg.) and PLB. Data are expressed as the mean +/- SD of three replicates and are representative of two separate experiments. *, $P <$ 0.0001 stimulated *versus* unstimulated cells.

Figure 10: 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, TGFβ) from the bone matrix that stimulate tumor growth and the production of PTHrP by tumor cells (16). This results in a vicious circle as illustrated by black 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 which, in turn, enhance bone resorption (grey arrows).

Figure 1

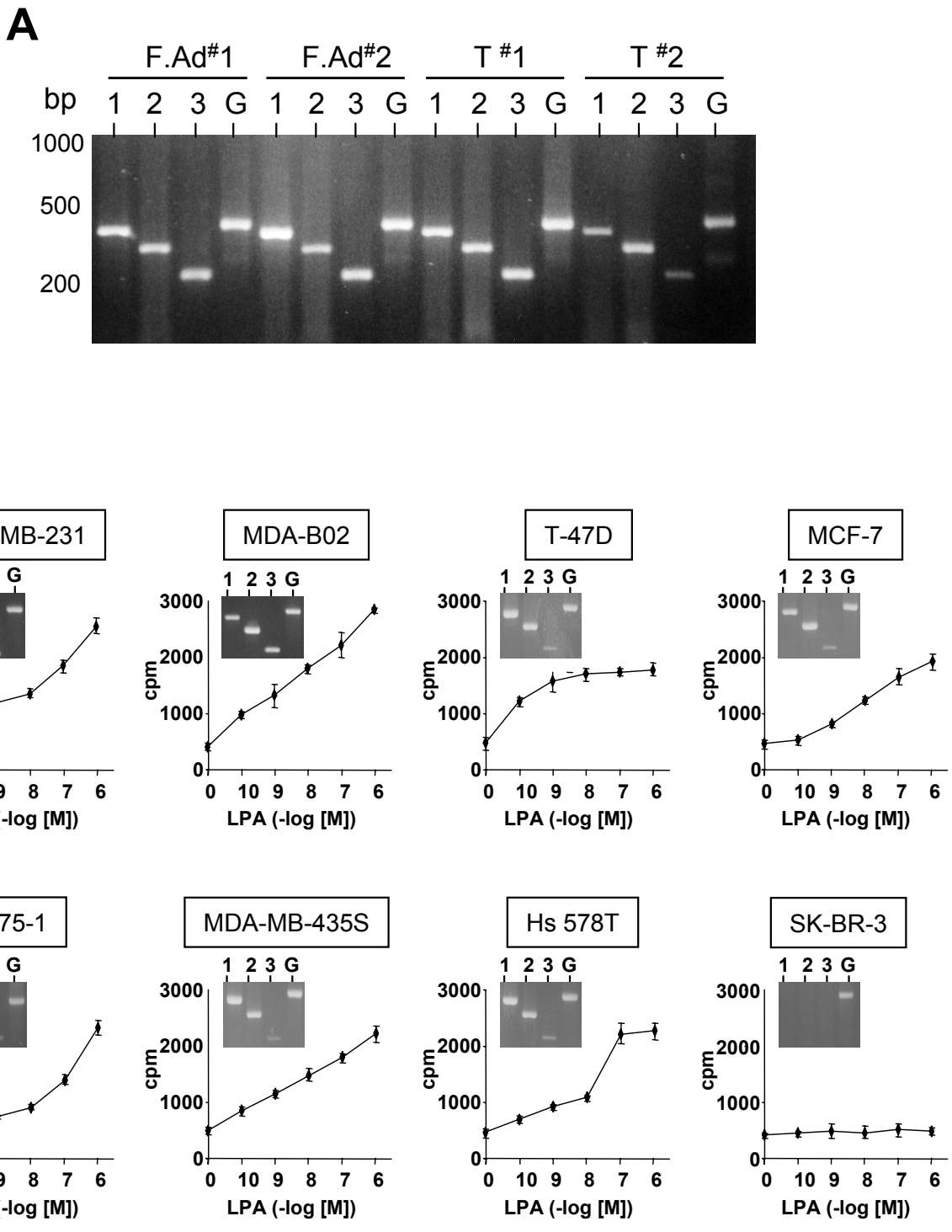


Figure 2

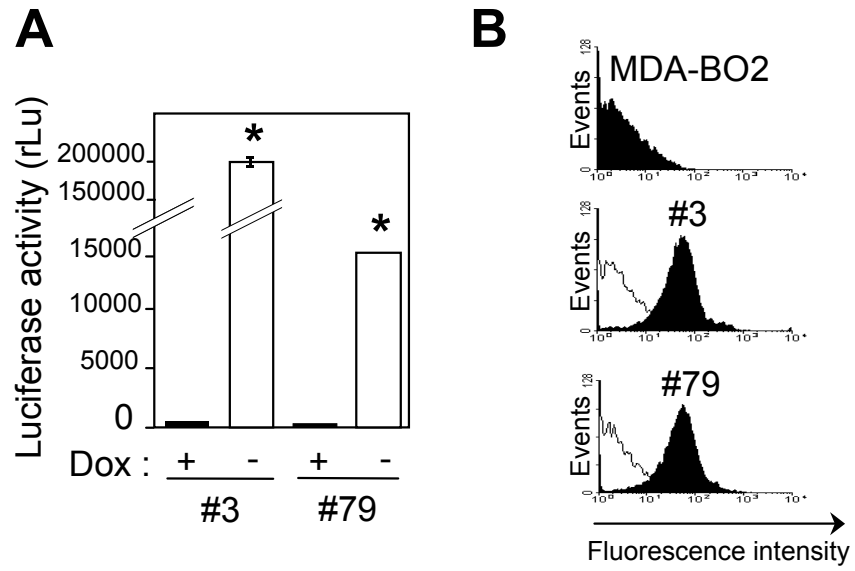


Figure 3

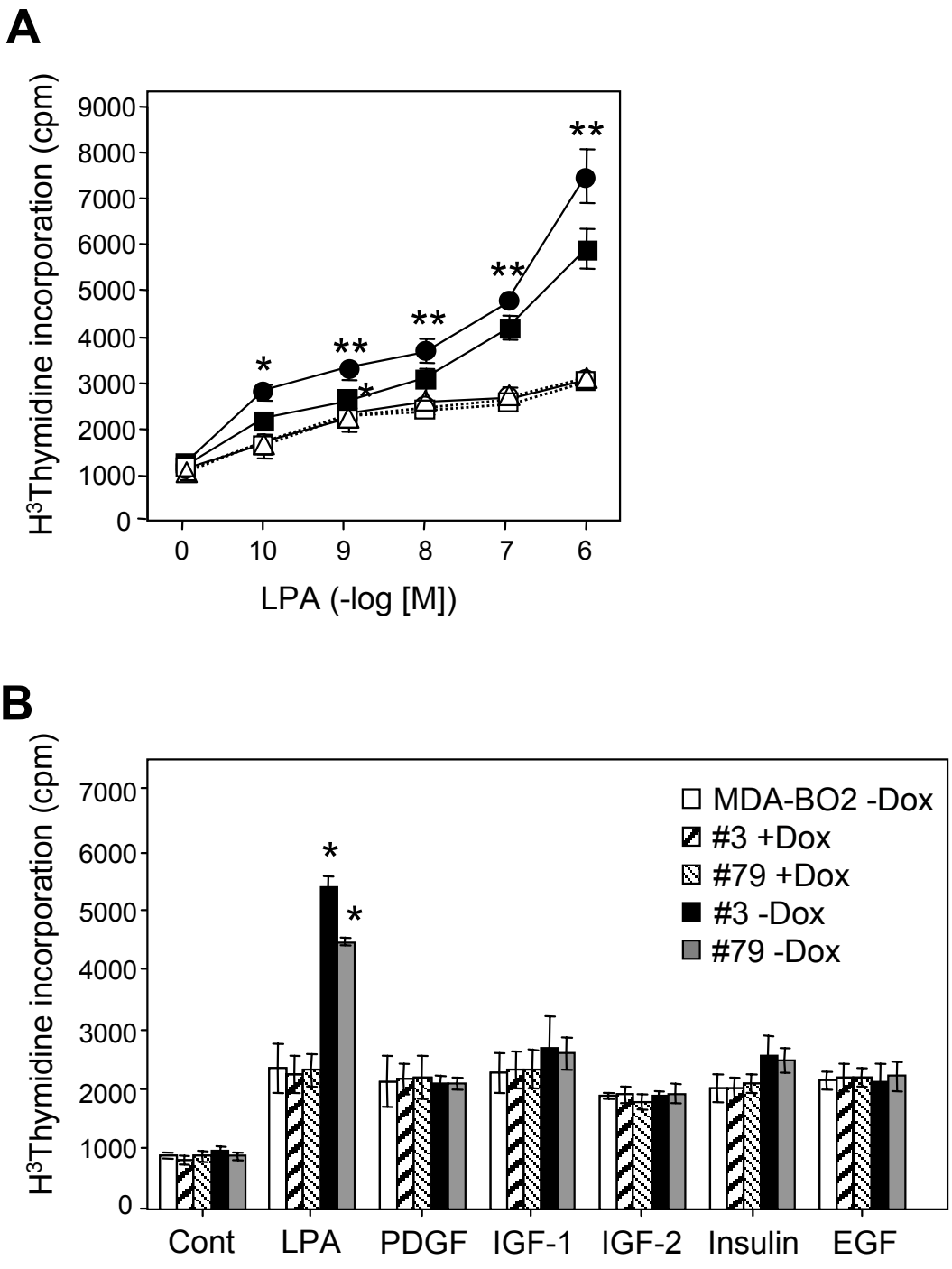


Figure 4

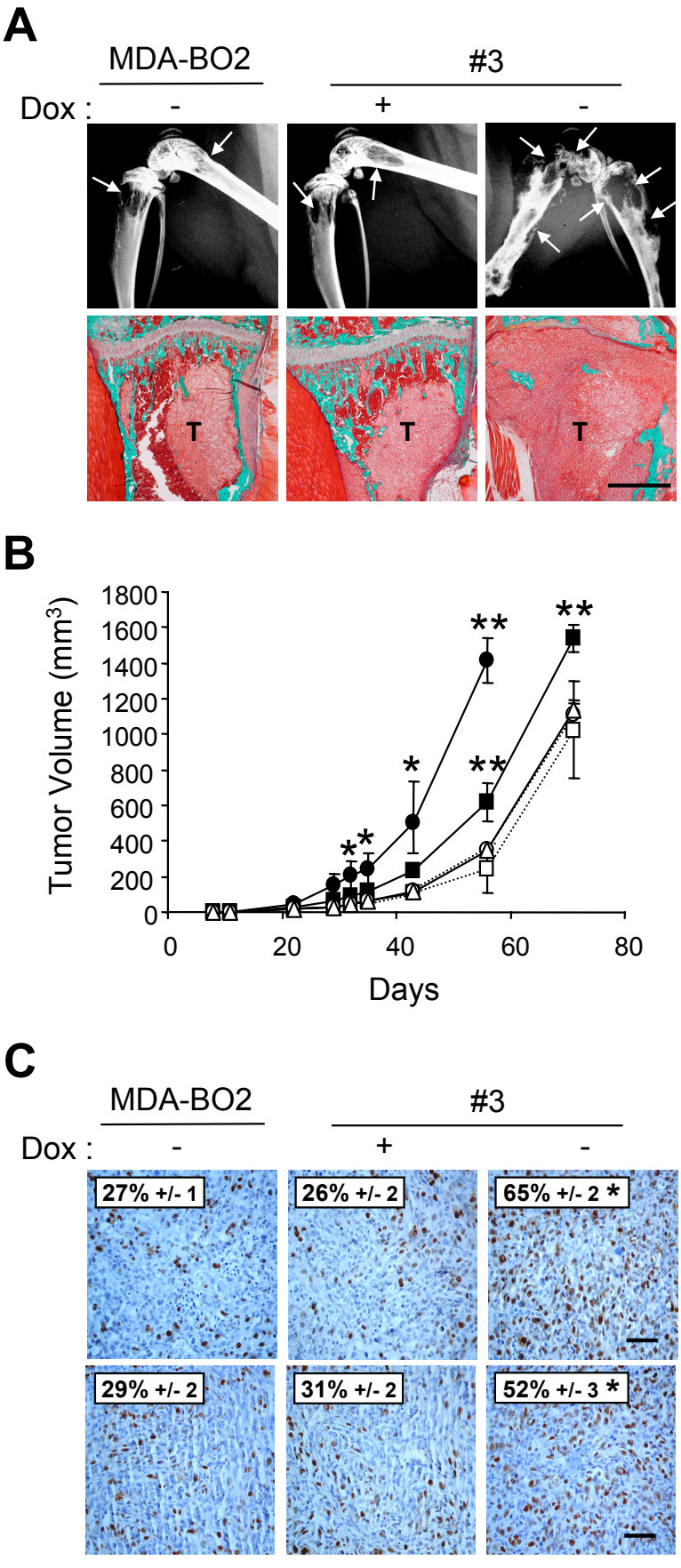


Figure 5

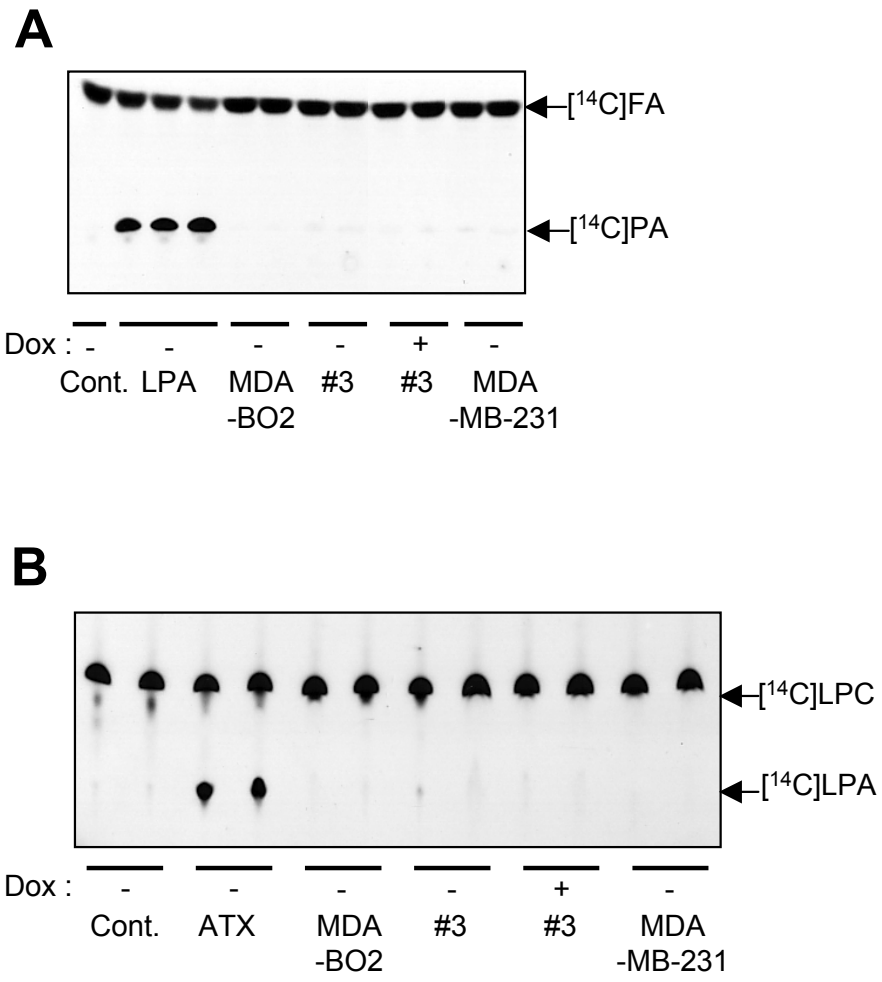


Figure 6

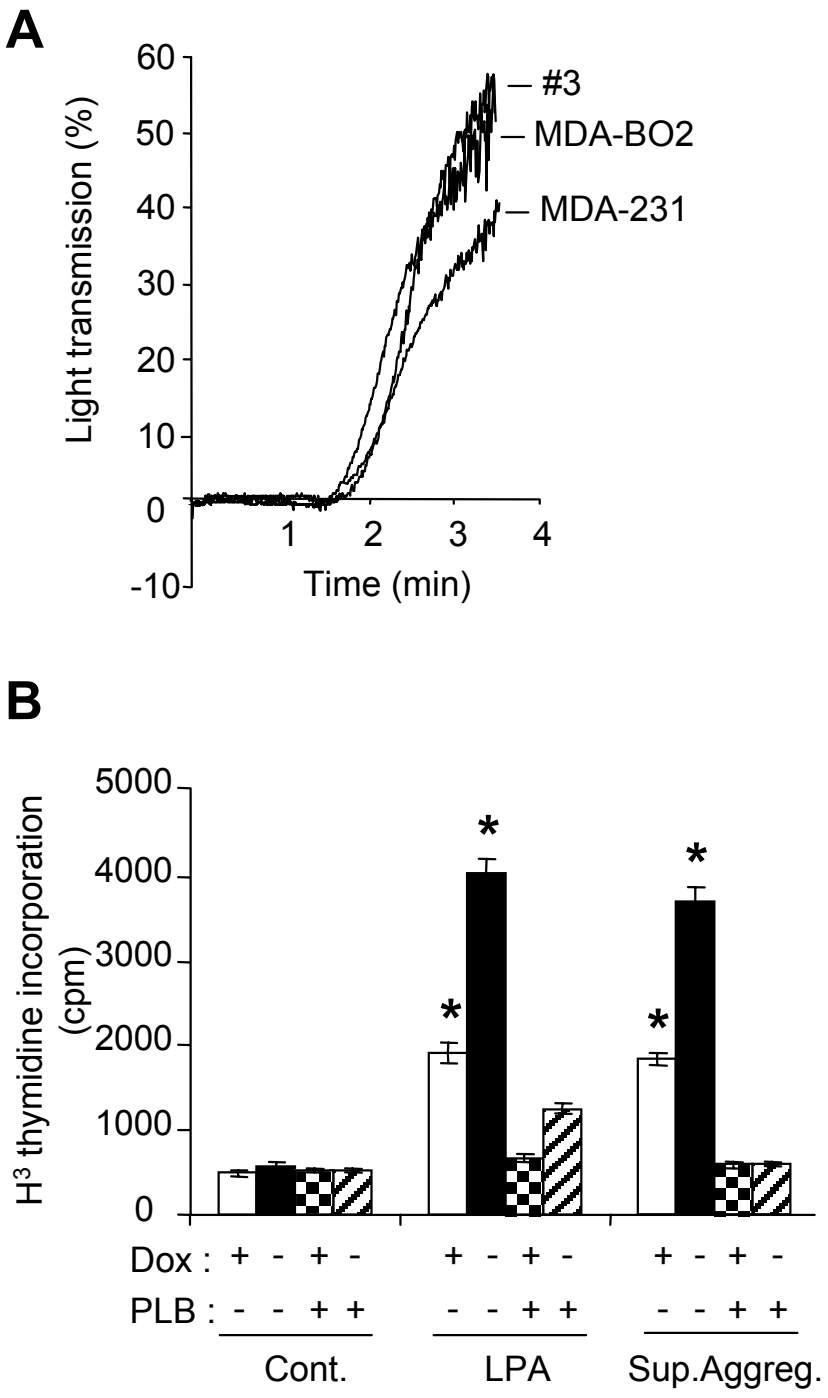


Figure 7

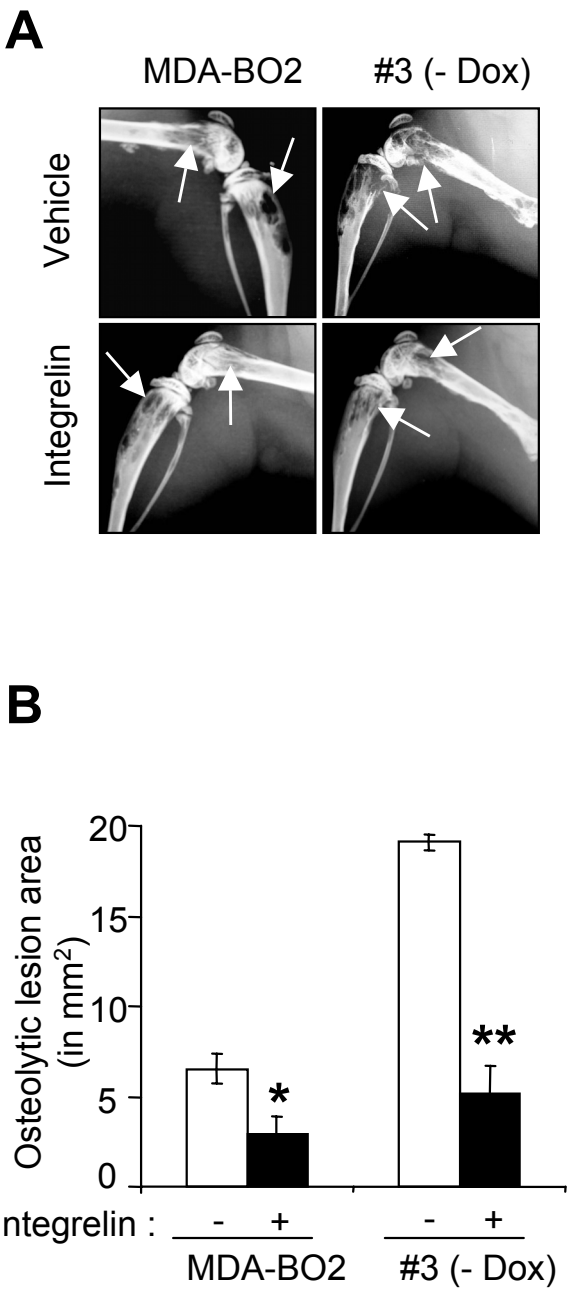
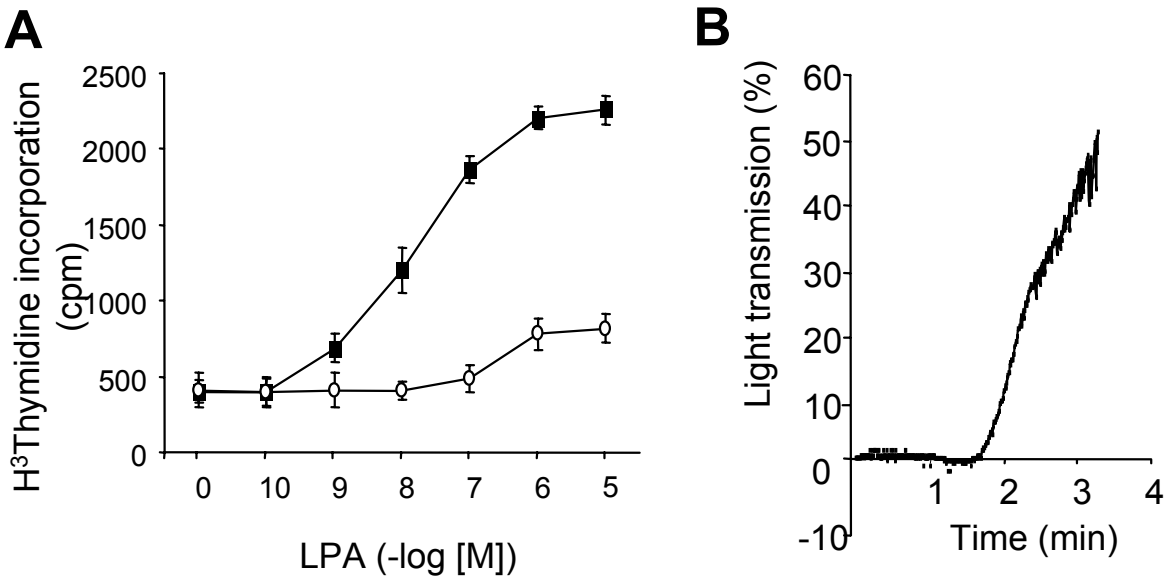


Figure 8



C

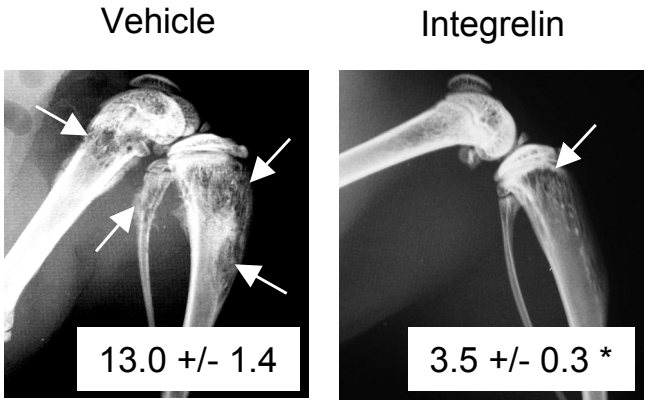


Figure 9

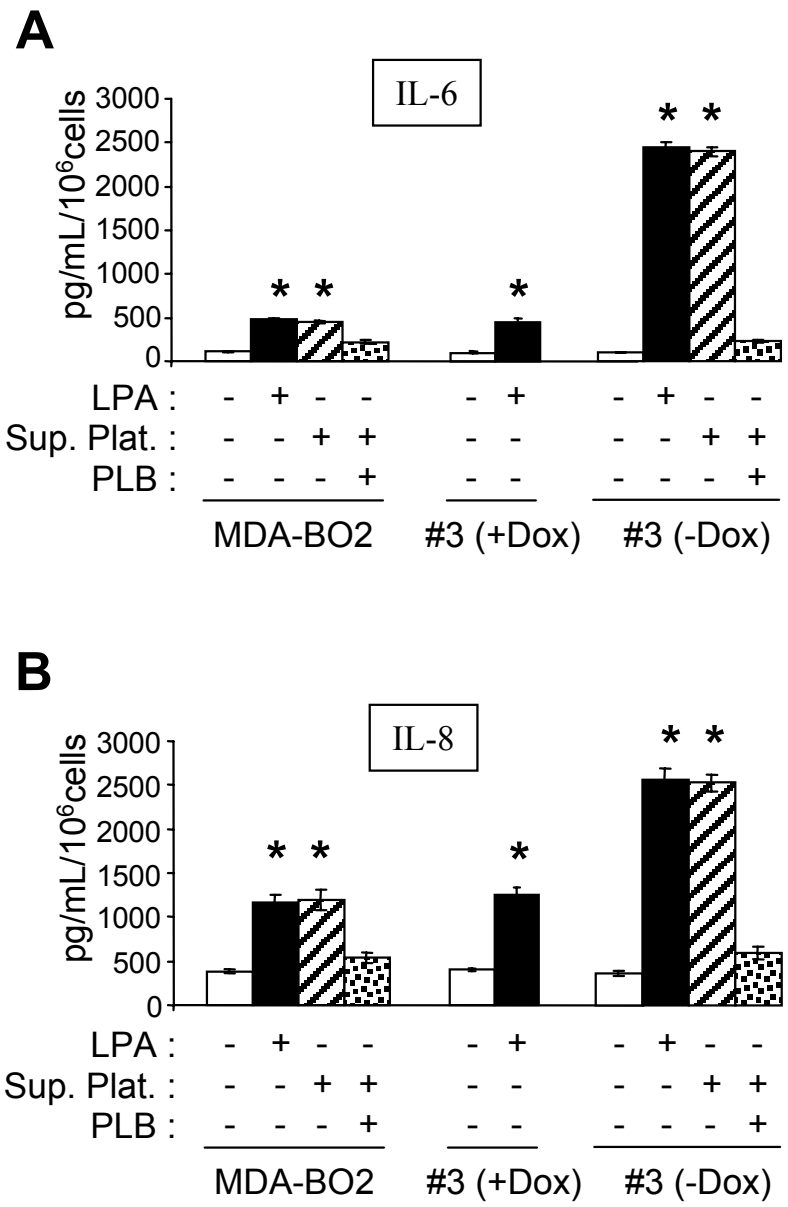


Figure 10