Abstract. Background: Lysophosphatidic acid (LPA) production in osteoblasts has multiple effects on osteoclast formation and function and raises the possibility that LPA may serve as a signaling molecule for the reciprocal conversation of both osteoblasts and osteoclasts within the tumor–bone microenvironment for bone resorption. However, little is known on the effect of LPA in regulating the function of both cancer cells and osteoclasts in the bone microenvironment. Materials and Methods: PC-3 tumor growth and bone destruction upon LPA administration were observed in a mouse calvarium xenograft. The osteoclastogenic cytokines produced by LPA-stimulated prostate cancer cells were also defined. Results: LPA administration was found to increase PC-3 tumor growth and bone destruction in a mouse calvarium xenograft. Using a cytokine antibody array, LPA highly stimulated the expression and release of osteoclastogenic cytokines from PC-3 cells. Conditioned medium from LPA-stimulated PC-3 cells containing enhanced levels of osteoclastogenic cytokines facilitated osteoclast formation. Histopathologically, LPA administration supports the erosive type of bone destruction by PC-3 prostate cancer cells. Conclusion: LPA is a critical regulator in the tumor–bone microenvironment and may be a therapeutic target for patients with prostate cancer. In addition, LPA-enhanced osteoclastogenic cytokines are critical to therapeutic strategies targeting osteolytic prostate cancer.

The bone is one of the most frequent sites of metastatic cancer. More than 80% of all men dying of prostate cancer have metastatic disease within their bones (1). Prostate cancer tends to induce osteoblastic bone metastases (2, 3). Therefore there is increased bone density primarily in bone metastases from prostate cancer, although bone resorption is steadily expanded by the osteolytic component at metastatic sites (2-4). Patients with prostate cancer usually exhibit marked osteolytic components in bone lesions (5). The progression of osteolytic bone metastases requires the reciprocal interaction between metastatic cancer cells and bone cells which are mediated by factors crucial for osteoclast differentiation and activity (6). Thus, crosstalk between cancer cells and the bone microenvironment plays a critical role in cancer-mediated bone loss (7). Bone-residing tumor cells release osteolytic factors such as interleukins (ILs) and parathyroid hormone-related protein (PTHrP) (6). These osteolytic factors increase the expression of receptor activator of the nuclear factor-κB ligand (RANKL) on osteoblasts, thereby promoting osteoclast-mediated bone resorption. Growth factors are released from the bone matrix during bone resorption, consequently accelerating cancer progression. Therefore, a control of the function and behavior of tumor cells and bone cells in the bone microenvironment will be an important approach to the inhibition of bone metastasis.

Lysophosphatidic acid (LPA) is an endogenous phospholipid that acts through G protein-coupled receptors (GPCRs). Physiologically, activated platelets and osteoblasts are important sources of LPA (8-10). Among bone cells, LPA was shown to be a chemotactic agent for osteoblast MC3T3-E1 cells leading to stimulation of osteoblast motility (11). In addition, LPA indirectly stimulates osteoclasts through changes in local IL levels, and directly stimulate osteoclast differentiation (8, 12). LPA supports osteoclast fusion through a compensation of the RANKL effect under RANKL-limiting conditions (13). Recently, the possibility was raised that LPA may be a promising molecule for the reciprocal conversation of both osteoblasts and osteoclasts within the tumor–bone microenvironment. However, little is known on which signaling molecules are induced from tumor cells by LPA stimulation, support osteoclast formation and promote bone resorption within the bone microenvironment.
In the present study, we investigated the effect of LPA on PC-3 tumor growth and bone destruction in a mouse xenograft model and identified cytokines secreted from PC-3 prostate cancer cells in response to LPA.

Materials and Methods

Materials and reagents. RPMI-1640, Hank’s balanced salt solution (HBSS), fetal bovine serum (FBS), antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin sulfate and 2 mM L-glutamine), phosphate-buffered saline (PBS), and 0.25% trypsin-EDTA were purchased from Gibco BRL Co. (Rockville, MD, USA). Oleoyl-2-hydroxy-sn-3-glycerol-3-phosphate-Na (lyso phosphatidic acid, LPA) was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). Ki16425 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Dimethyl sulfoxide (DMSO), phenylmethylsulfonylflouride (PMSF), and an Acid Phosphatase Leukocyte kit for tartrate-resistant acid phosphatase (TRAP) staining were purchased from Sigma-Aldrich (St. Louis, MO, USA). BioCoat Osteologic Multitest slides were purchased from BD Biosciences (San Diego, CA, USA). Recombinant mouse RANKL was purchased from Koma Biotech Inc. (Seoul, Korea). Recombinant mouse macrophage-colony stimulating factor (M-CSF) was purchased from R&D System Inc. (Minneapolis, MN, USA).

Cell culture. PC-3 human prostate cancer cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics in a humidified atmosphere of 5% CO2 at 37˚C. Mouse bone marrow-derived macrophages (BMMs) were isolated from the tibiae of 4-week-old ICR mice by Histopaque density gradient centrifugation. BMMs were cultured in α-minimum essential medium (MEM) containing 10% FBS and 30 ng/ml M-CSF in a humidified atmosphere of 5% CO2 at 37˚C.

Nude mouse xenograft model. All animal studies were performed in accordance with experimental protocols approved by the Animal Ethics Committee of Eulji University College of Dentistry. Male BALB/C athymic nude mice (6 weeks of age; Central Animal Lab, Seoul, Korea) were maintained at 20-22˚C on a 12 h light/dark cycle. PC-3 cells (3×10^6 cells/0.1 ml HBSS) were injected subcutaneously into the mouse calvarium under anesthesia using a 0.5 ml insulin syringe (n=10 mice/group). LPA (200 μmol/l) in 100 μl HBSS was intraperitoneally injected every 2 days. The length and width of the tumors were measured by caliper and the tumor size was calculated by the following formula: width^2 × length ÷ 2. Five weeks after transplantation, the calvarium region was monitored using a Skyscan 1076 high-resolution in vivo micro-computed tomography (micro-CT) system (SKYSCAN, Antwerpen, Belgium). NRecon and CT analyzer (CTAn) software (SkyScan) were used to analyze the sample structure. Two-dimensional images were used to reconstruct three-dimensional images with the software.

Hematoxylin and eosin (H&E) staining. Paraffin-embedded tissues (4 μm-thick) were serial sectioned using a Leica 2135 microtome, de-paraffinized in xylene, dehydrated through an alcohol series, and stained with H&E for histopathology. The tissues were then dehydrated and mounted.

Preparation of conditioned medium (CM). PC-3 cells (2×10^6 cells) were plated at 100 mm dish and incubated to adhere overnight. Culture medium was changed to serum-free RPMI-1640 and the cells were cultured for 48 h with or without LPA (1 μM). Ki16425 was pretreated for 2 h as an LPA receptor antagonist. Culture medium was collected and centrifuged at 1,000×g for 10 min. The supernatant was used as CM.

Cytokine antibody array. The human cytokine array C2000 (RayBio C-Series) was purchased from RayBiotech Inc. (Norcross, GA, USA). PC-3 cells were plated in a dish overnight, and the medium was changed to serum-free medium after washing with the same medium. Cells were treated for 24 h with LPA (1 μM) and then the supernatant was collected. Before application to the antibody array, the protein concentration of the supernatant was normalized by dilution with serum-free medium. The relative expression level of each cytokine was determined by comparing signal intensities with Quantity One software and the Gel Doc 2000 system (Bio-Rad Laboratories, Hercules, CA, USA).

Osteoclast formation assay. Isoleted BMMs (3×10^4 cells/well) were cultured on a 96-well plate with cMEM containing M-CSF (30 ng/ml) and RANKL (30 ng/ml) for 5 days in the presence or absence of CM from LPA-treated PC-3 cells. Cultures were fed with fresh medium every day and multinucleated osteoclasts derived from the BMMs were observed. Cells were fixed and stained for TRAP with the Acid Phosphatase Leukocyte kit to detect osteoclast formation. The total number of TRAP-positive multinucleate (≥3 nuclei) cells as osteoclasts per well was counted under a light microscope.

Pit formation assay. The bone resorptive activity of osteoclasts was measured with Biocat Osteologic Multitest slides (BD Biosciences, San Jose, CA, USA). Cells (3×10^4 cells/well) were seeded onto the Biocat Osteologic Multitest slides and cultured in medium containing M-CSF (30 ng/ml) and RANKL (10 ng/ml) with or without CM from LPA-treated PC-3 cells for 15 days. Cultures were fed with fresh medium every other day. The cells were then lysed with 5% sodium hypochlorite solution. Images of the resorbed pits were taken with a light microscope.

Cathespin K assay. Secreted cathespin K was detected in culture media with SensiZyme Cathespin K Activity Assay Kit (Sigma-Aldrich) according to the manufacturer’s protocol. Briefly, concentrated culture media were added to each well of a 96-well plate coated with cathespin K antibody and incubated for 1 h at room temperature. After a brief wash, the kit reaction mixture was added to each well and the plate further incubated for 4 h. The optical density at 405 nm was measured with a microplate reader (BIO-Rad, Richmond, CA, USA). The cathespin K level was determined using a cathespin K standard curve and was expressed as pg/mg protein.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Total RNA from BMMs or PC-3 cells was isolated with TRIzol® reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed using GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA, USA) and was performed on an ABI 7500 real-time PCR system (Applied Biosystems) with 2 μg of complementary DNA in a total reaction volume of 20 μl using the SYBR Green PCR mix (Eurogentec, Seraing, Belgium). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was
used as the housekeeping gene for normalization. Primer sequences are given in Table I. The specificity of the products was confirmed by melting curve analysis. No primer dimer was obtained when the samples were assessed by melting curve analysis.

Statistical analysis. Statistical analysis was performed with InStat GraphPad Prism version 5.01 statistical software (GraphPad Software, Inc., San Diego, CA, USA). Data are expressed as means±standard deviation (SD). Asterisks are used to graphically indicate statistical significances. p-Values of less than 0.05 were considered statistically significant.

Results

LPA administration increases tumor growth and bone destruction. LPA is involved in reciprocal crosstalk between osteoblasts and osteoclasts to accomplish bone resorption (13, 14). Evidence for the tumorigenesis effect of exogenous LPA has been reported by other research groups (15-17). To determine the involvement of LPA in tumor growth and bone destruction, human prostate cancer PC-3 cells were xenografted over calvarium in BALB/c nude mice, vehicle (HBSS) or LPA was intraperitoneally injected and the tumor growth was monitored. Administration of LPA (200 μmol/l in 100 μl HBSS) over 5 weeks increased the tumor growth compared with animals treated with vehicle (Figure 1A). On micro-CT imaging, extensive osteolytic lesions were observed in the calvarium of LPA-administered athymic mice when compared to vehicle-treated mice (Figure 1B). A significant difference in calvarium bone volume was observed (Figure 1C).

CM from LPA-stimulated PC-3 cells supports functional osteoclast formation. To determine the effects of LPA on osteoclast formation, PC-3 cells were treated with LPA for 48 hours.
h and the CM from the LPA-stimulated PC-3 cells was added to cultures of isolated BMMs. As shown in Figure 2A, TRAP-positive multinucleate cells were observed on TRAP staining. The TRAP-positive multinucleate cells also exhibited bone resorptive activity. Osteoclast formation and resorption pits were significantly increased in the BMMs exposed to the CM from the LPA-stimulated PC-3 cells, compared to control BMMs exposed to the CM from the HBSS-stimulated PC-3 cells. Ki16425, an LPA receptor antagonist, notably suppressed osteoclast formation and resorption pit formation induced by the CM from LPA-stimulated PC-3 cells, suggesting that molecules released from LPA-stimulated PC-3 cells may contribute to enhanced osteoclast formation and bone resorption. Cathepsin K is known to be released by functional osteoclasts and mainly contributes to the initial process of bone resorption in an acidic environment (18). An increased level of cathepsin K was detected in the BMMs exposed to the CM from the LPA-stimulated PC-3 cells; this effect was abolished by Ki16425 (Figure 2B).

Next, we performed qRT-PCR to define the osteoclastogenesis-related genes induced during LPA-stimulated osteoclast formation (Table II). Cathepsin K (CTSK) mRNA was significantly induced by the CM from the LPA-stimulated PC-3 cells as shown in Figure 2B. TRAP, CSK, CAR2, DCSTAMP, ATP6v0d2 and NFATC1 mRNA were also increased. However, ADAM12, AQP1, and E-caderin (CDH1) mRNA were weakly induced. Increased osteoclastogenesis-related genes were thoroughly inhibited by Ki16425 during LPA-stimulated osteoclast formation.

**LPA stimulates the expression and release of osteoclastogenic cytokines from prostate cancer cells.** To assess the
cytokines which are produced by LPA-stimulated PC-3 cells, a cytokine antibody array was performed. The CM collected from LPA-stimulated PC-3 cells was used to evaluate the levels of cytokines. As shown in Figure 3, several cytokines were up-regulated in CM from LPA-stimulated PC-3 cells (black rectangle). In particular, IL6, granulocyte-M-CSF, monocyte chemoattractant protein 1 (MCP1), M-CSF, MCP2, and insulin-like growth factor-binding protein (IGFBP) were significantly increased by LPA stimulation (Table III). Interestingly, these cytokines are known to be correlated with osteoclastogenesis. Elevated cytokine levels were also found in LPA-treated PC-3 cells through qRT-PCR (Table IV). These result collectively suggest that LPA stimulates the release of osteoclastogenic cytokines in PC-3 cells for functional osteoclast formation, thereby encouraging tumor growth and calvarium bone destruction.
LPA administration supports erosive-type bone resorption by PC-3 prostate cancer cells in a mouse calvarium xenograft. Histopathologically, the pattern of bone invasion by oral squamous cell carcinoma can be divided into two types: the erosive and the infiltrative type (19). The erosive type appears as a neat interface between the tumor front and the bone, while the infiltrative type appears as a diffuse irregular margin. The calvarium region from mice bearing PC-3 cell xenografts was H&E stained, as shown in Figure 1B, and histological characteristics of bone invasion were observed. PC-3 cells invaded the calvarium bone through erosive-type resorption (Figure 4). PC-3 tumor cells were not directly in contact with nearby bones. Hence a well-organized tumor–bone interface was apparent. Numerous osteoclasts (black arrows in Figure 4) were present in resorption lacunae on the bone surface.

Discussion

Bone has a suitable microenvironment for the growth of prostate cancer because the bone matrix includes high levels of growth factors and cytokines. The pathophysiology of cancer bone metastasis is multifactorial but skeletal responses to tumor are mediated by osteoblasts and osteoclasts (20). The reciprocal crosstalk between tumor cells and the bone microenvironment, including during osteoblast and osteoclast activity, were identified as the ‘vicious cycle’ of the osteolytic out-growth phase of metastasis. However, the molecular mechanism of tumor-induced bone destruction remains poorly understood.

Metastasis of prostate cancer to bone is a common complication of invasive prostate cancer. Although frequently characterized as osteoblastic, prostate cancer bone metastases usually have an osteoclastic component. The RANKL is one of the essential proteins inducing osteoclastogenesis through

Figure 3. Lysophosphatidic acid administration (LPA) stimulates the release of osteoclastogenic cytokines from PC-3 cells. Culture medium collected from LPA-treated PC-3 cells was analyzed by Cytokine Antibody Array C2000 in order to determine the soluble factors released. The analysis of the culture medium collected from Hanks’ balanced salt solution (HBSS)-treated PC-3 cells served as a control. Altered soluble factors are indicated with rectangles and circled numbers. The list of altered factors is given in Table III.

Figure 4. Lysophosphatidic acid administration supports erosive osteoclastic bone destruction by PC-3 prostate cancer cells in mouse calvarium. Hematoxylin and eosin staining of the calvarium bone excised from mice is shown in Figure 1B. The area of erosive bone destruction by cancer cells exhibits an irregular cancer front due to cancer invasion into the cancerous bone. Osteoclasts are indicated with arrows in areas of the bone. Original magnification, ×200. Bo: Bone, T: tumor.
osteoclast activation. Both soluble and membrane-bound RANKL forms are detected in human prostate cancer cells and are intimately involved in osteoclastogenesis (4, 21). PTHrP and IL6 are also produced by prostate cancer cells and can promote osteoclast formation (22). In addition, matrix metalloproteinases are secreted by prostate cancer cells and promote osteolysis through degradation of the bone matrix. Thus, many factors derived from prostate cancer metastases are cooperatively involved in the development of osteolysis, and these factors may serve as diagnostic and therapeutic targets.

Table II. Gene expression of osteoclast-differentiation marker, fusion-related marker, and bone-resorptive factors during osteoclast formation supported with conditioned medium (CM) from lysophosphatidic acid (LPA)-stimulated PC-3 cells. Data are expressed as fold change in LPA-treated or Ki16425/LPA-treated PC-3 cells compared with those treated with Hank’s balanced salt solution (HBSS).

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Gene name</th>
<th>HBSS-treated</th>
<th>LPA-treated</th>
<th>Ki16425/LPA-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTSK</td>
<td>Cathepsin K</td>
<td>1.03±0.15</td>
<td>54.23±0.07*</td>
<td>1.69±0.16</td>
</tr>
<tr>
<td>TRAP</td>
<td>Acid phosphatase, tartrate resistant</td>
<td>1.05±0.03</td>
<td>72.6±0.17*</td>
<td>1.8±0.29</td>
</tr>
<tr>
<td>ADAM12</td>
<td>Meltrin alpha</td>
<td>1.01±0.13</td>
<td>4.32±0.19</td>
<td>1.61±0.15</td>
</tr>
<tr>
<td>CSK</td>
<td>c-src Tyrosine kinase</td>
<td>1.03±0.15</td>
<td>29.69±0.03*</td>
<td>1.83±0.34</td>
</tr>
<tr>
<td>Car2</td>
<td>Carbonic anhydrase II</td>
<td>1.00±0.07</td>
<td>49.57±0.21*</td>
<td>1.04±0.11</td>
</tr>
<tr>
<td>DCSTAMP</td>
<td>Dendrocyte expressed seven transmembrane protein</td>
<td>1.06±0.08</td>
<td>88.21±0.03*</td>
<td>1.13±0.21</td>
</tr>
<tr>
<td>AQP1</td>
<td>Aquaporin 1</td>
<td>1.02±0.19</td>
<td>2.97±0.18</td>
<td>0.91±0.03</td>
</tr>
<tr>
<td>CDH1</td>
<td>E-Cadherin</td>
<td>1.00±0.27</td>
<td>3.39±0.79</td>
<td>1.78±0.43</td>
</tr>
<tr>
<td>ATP6v0d2</td>
<td>ATPase, H+ transporting, lysosomal 38 kDa, V0 subunit d2</td>
<td>1.01±0.08</td>
<td>30.01±0.07*</td>
<td>1.87±0.23</td>
</tr>
<tr>
<td>NFATc1</td>
<td>Nuclear factor of activated T-cells</td>
<td>1.04±0.39</td>
<td>25.65±0.11*</td>
<td>0.95±0.41</td>
</tr>
</tbody>
</table>

Ki16425: LPA receptor antagonist. *Significantly different at p<0.01 compared to HBSS-treated cells.

Table III. Altered expression of human cytokines with RayBio C-Series antibody arrays in LPA-stimulated PC-3 cells.

<table>
<thead>
<tr>
<th>Antibody array result</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold change*</th>
</tr>
</thead>
<tbody>
<tr>
<td>①</td>
<td>IL6</td>
<td>Interleukin 6</td>
<td>8.63</td>
</tr>
<tr>
<td>②</td>
<td>GMCSF</td>
<td>Granulocyte-macrophage colony-stimulating factor 2</td>
<td>7.42</td>
</tr>
<tr>
<td>③</td>
<td>MCP2</td>
<td>Monocyte chemoattractant protein 2</td>
<td>5.12</td>
</tr>
<tr>
<td>④</td>
<td>MCSF</td>
<td>Macrophage colony-stimulating factor</td>
<td>4.53</td>
</tr>
<tr>
<td>⑤</td>
<td>GCP2</td>
<td>Granulocyte chemotactic protein 2</td>
<td>4.51</td>
</tr>
<tr>
<td>⑥</td>
<td>MCP1</td>
<td>Monocyte Chemotactic protein 1</td>
<td>3.29</td>
</tr>
<tr>
<td>⑦</td>
<td>IGF1</td>
<td>Insulin-like growth factor-binding protein</td>
<td>2.87</td>
</tr>
</tbody>
</table>

*Refer to Figure 3. Gene expression levels are shown as fold change in lysophosphatidic acid (LPA)-treated PC-3 cells (*p<0.05 compared with those treated with Hank’s balanced salt solution).

Table IV. Osteoclastogenic cytokine genes expressed by PC-3 prostate cancer cells treated with lysophosphatidic acid (LPA) or Hank’s balanced salt solution (HBSS).

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Gene name</th>
<th>HBSS-treated</th>
<th>LPA-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
<td>1.00±0.11</td>
<td>86.65±0.07*b</td>
</tr>
<tr>
<td>GMCSF</td>
<td>Colony stimulating factor 2 (granulocyte-macrophage)</td>
<td>1.00±0.13</td>
<td>61.3±0.19*b</td>
</tr>
<tr>
<td>MCP2</td>
<td>Monocyte chemoattractant protein 2</td>
<td>1.00±0.07</td>
<td>41.95±0.21*a</td>
</tr>
<tr>
<td>MCSF</td>
<td>Macrophage colony-stimulating factor</td>
<td>1.02±0.14</td>
<td>39.52±0.03*a</td>
</tr>
<tr>
<td>GCP2</td>
<td>Granulocyte chemotactic protein 2</td>
<td>1.01±0.04</td>
<td>32.33±0.03*a</td>
</tr>
<tr>
<td>MCP1</td>
<td>Chemokine (C-C motif) ligand 2 (CCL 2)</td>
<td>1.01±0.03</td>
<td>25.9±0.37*a</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor-binding protein</td>
<td>1.02±0.10</td>
<td>23.17±0.18*a</td>
</tr>
</tbody>
</table>

Significantly different at a p<0.05 and b p<0.01 compared with HBSS-treated cells.
and 100 ng/ml RANKL for 5 days for osteoclast formation assay and cultured for 15 days for pit formation assay for osteoclastic bone-resorbing cells \textit{in vitro}. In this study, we adjusted the concentration of RANKL to 30 ng/ml for validation of the LPA effect on osteoclast formation and bone resorption. Significant differences were observed between CM from control and LPA-stimulated PC-3 cells in osteoclast formation and bone resorptive activity. In addition, effects of LPA stimulation were suppressed by LPA receptor antagonist Ki16425. This evidence indicates that increased osteoclastogenic cytokines from LPA-stimulated PC-3 cells may contribute to osteoclast formation and bone resorption, thereby facilitating the formation of osteoclastic bone-resorbing cells.

Tumor-released cytokines induced osteoblast and osteoclast activity. The release of bone-derived cytokines and growth factors may in turn stimulate tumor cell proliferation. These interactions accelerate cancer progression and bone destruction. We subsequently elucidated the cytokines produced by LPA-stimulated PC-3 cells for osteoclast formation and bone resorption. IL6 (23, 24), M-CSF, GM-CSF (25), granulocyte chemotactic protein 2 (GCP2), MCP1 (26), and MCP2 (27) are well-known cytokines of osteoclastogenesis and are factors essential for osteoclast differentiation (28). An elevated level of IL6 has been reported to induce osteoclast formation and bone resorption (29, 30). IGFBP has been also suggested to be required for fully differentiated and functional osteoclasts (31). Therefore, an increase of osteoclastogenic cytokines from tumor by LPA stimulation may facilitate osteoclast formation and bone-resorptive activity. In addition, these factors may be useful for the determination of therapeutic targets for suppression of tumor-induced osteolysis and the development of effective therapies.

Multiple biological functions of LPA have been defined, including cell proliferation (32), cell survival and drug resistance (33, 34), and cell migration and invasion (35, 36). LPA is detected in plasma at concentrations of 0.1 μM, but increased levels of LPA are found in ascitic fluid and plasma of patients with ovarian, endometrial and cervical cancers (up to 80 μM) (37). Previous research has demonstrated that endogenous LPA promotes the progression of osteolytic bone metastases of cancer cells (38). Autotaxin, which catalyzes the production of LPA from lysophosphatidylcholine \textit{in vivo}, induces cancer invasion via the GPCR LPA4 and metastatic growth of lung cancer cells was reduced upon LPA4 depletion in a mouse model (39). In addition, bone metastases of breast cancer were markedly hindered by LPA1 depletion or LPA receptor antagonist Ki16425 in a mouse hind limb model. In this study, we directly injected LPA intraperitoneally in a mouse calvarium model and investigated osteolytic bone invasion of PC-3 cells. Intraperitoneal injections of LPA accelerated tumor growth in the mouse calvarium, thereby extended osteolytic lesions were validated on micro-CT imaging. In contrast, non-osteolytic cancer cell lines did not undergo osteolysis on mouse calvarium in repeated \textit{in vivo} experiments, even though cancer cells formed a tumor mass. Low-invasive cancer cells resorbed negligible bone during parallel experiments with highly-invasive cancer cells showing further extended bone destruction (unpublished data). These results indicate that exogenous LPA also promotes prostate tumor growth and osteolytic bone invasion. Evidence for the tumorigenesis effect of exogenous LPA has been reported by other research groups. Direct intraperitoneal injection of LPA induces tumorigenesis and metastasis of ID8 murine ovarian cancer cells (40). In immunocompetent mice homozygously lacking the gene for group VIA phospholipase A2, which participates in cell migration and invasion, administration of LPA encouraged tumorigenesis and metastasis of human epithelial ovarian cancer IB8 cells (15). Recently, Rai et al., demonstrated that LPA administration enhances tumor growth of implanted ID8 ovarian cancer cells in the receptor for advanced glycation end-products (RAGE) expression mice, thereby RAGE has been suggested as a functional LPA receptor (17). LPA is physiologically produced in osteoblasts by P2X7 receptor activation, which is involved osteoblast differentiation. LPA also stimulates osteoclast differentiation from macrophage and increases osteoclast survival and bone-resorptive activity (10, 14, 41). We have also reported that osteoclast fusion is promoted by LPA, and osteoclast stimulatory transmembrane protein and P2X7 receptor are involved in LPA-induced osteoclast bone resorption (13).

Histological patterns of tumor bone invasion were assessed as erosive or infiltrative types (42-44). The erosive type leads to a well-defined border between the tumor and the bone, while the infiltrative type displays an irregular margin at the tumor–bone junction. Bone destruction is mediated by osteoclasts rather than by cancer cells directly (45). On calvarium xenograft with PC-3 cells in this study, enlarged areas of bone destruction as a result of LPA administration were observed on micro-CT and 3D images analysis compared to the control. In H&E analysis, numerous osteoclasts were found in regions with bone resorption pits, a leading cause of erosive-type bone destruction in mice administered LPA.

In conclusion, administration of LPA was found to support prostate tumor growth and bone destruction in a mouse calvarium xenograft model. LPA enhanced the production of osteoclastogenic cytokines from prostate cancer cells \textit{in vitro}, thereby facilitating osteoclastogenesis and bone resorption. Consequently, investigation on the molecular mechanism of LPA-inducible genes encoding osteoclastogenic cytokines responsible for bone destruction might be essential in finding an effective therapy for osteolytic prostate cancer.
Funding

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Conflicts of Interest

No conflicts of interest exist with regard to the present study.

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