Effects of low-intensity pulsed ultrasound on osteosarcoma and cancer cells

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Abstract. Metastatic bone tumors cause pain and pathological fractures due to bone destruction. If we could enhance new osteogenic activities and prevent progression of osteolytic change by malignant cells, patients could achieve satisfactory activity of daily living. Low-intensity pulsed ultrasound (LIPUS), which leads to bone formation by osteoblasts, has been used for the treatment of fractures. LIPUS has been reported to enhance the effect of an anticancer drug on lymphoma and liver cancer cells. However, there have been no reports of proliferation, vascularization and migration effects on cancer cells. In this study, we investigated the effects of LIPUS treatment on cancer and osteosarcoma cells and specifically whether it promotes bone formation without accelerating proliferation of tumor cells. We used MC3T3-E1 cells, a mouse osteoblast cell line, LM8, a mouse osteosarcoma cell line, SaOS2, a human osteosarcoma cell line, 786-O, a human renal cancer cell line, PC-3, a human prostate cancer cell line, and A549, a human lung cancer cell line. The expression of extracellular signal-regulated kinase (ERK1/2), Akt, β-catenin, vascular endothelial growth factor (VEGF), and cell migration were analyzed. LIPUS stimulation did not affect proliferation of all the cells examined. The phosphorylation of ERK1/2 and Akt was induced by LIPUS stimulation in MC3T3-E1, LM8, SaOS2 and A549 cells, but not in PC-3 and 786-O cells. LIPUS stimulation did not significantly increase β-catenin. VEGF protein levels and cell migration were significantly increased only in MC3T3-E1 cells. It may be concluded that LIPUS stimulation on metastatic bone tumors induces differentiation of osteoblasts without proliferation of tumor cells. Our study suggests that LIPUS may be a new method of treatment without surgery for metastatic bone tumors.

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Introduction

Many people suffer from metastatic bone tumors caused by a wide variety of cancers. A skeletal-related event (SRE), such as a pathological fracture or spinal cord compression, and the requirement for orthopedic surgery or palliative radiotherapy reduce patients' activity of daily living (ADL) and quality of life (QOL) and worsen prognosis. Treatment for the prevention of an SRE improves patients' ADL and QOL. Osteolytic change with bone metastasis leads to pain and pathological fracture. When surgery, chemotherapy and radiotherapy are not effective, bone destruction progresses. If we could inhibit progression of osteolytic change and enhance osteogenic change more than osteolytic change, it may be possible to prevent fracture and pain and to maintain QOL. In addition, there is a need to develop non-invasive treatment when considering patients' general condition. Low-intensity pulsed ultrasound (LIPUS) stimulation is known as one of the methods for promoting bone formation in orthopedics (1). LIPUS stimulation is an established and widely used intervention for accelerating fracture healing and bone maturation in distraction osteogenesis, and delayed fracture union and nonunion in clinical settings (1-4). To influence bone repair, LIPUS is distinguished by being non-invasive and easy to apply, and the LIPUS signal has a sufficiently low intensity to be considered non-destructive (5). Because tumor cells in metastatic bone tumors coexist with normal osteocytes in impending fractures, osteoblasts and osteoclasts are stimulated by tumor-induced bone destruction all the time. In this condition, we hypothesized that LIPUS stimulates bone formation similar to normal fracture healing and prevents progression of pathological fractures. Effects of LIPUS on cell proliferation, vascularization, and migration in the process of fracture healing have been reported in many cases (6,7). Although the mechanisms involved have not been elucidated, LIPUS stimulation has been reported to affect osteoblast differentiation without increasing cell number of osteoblast (8,9). LIPUS was reported to enhance the effect of an anticancer drug on lymphoma

and liver cancer cells (10). However, there were no reports of proliferation, vascularization and migration effects on cancer cells. Therefore, there was a need to investigate the effects of LIPUS on cancer cells because LIPUS might stimulate tumor proliferation, vascularization, and migration. In this study, we investigated the effects of LIPUS treatment on cell viability, cell proliferation, vascularization, and migration in mouse osteoblast, mouse and human osteosarcoma, human prostate cancer, renal cancer, and lung cancer cell lines under the same conditions as in clinical use.

Materials and methods

Cell culture. We used MC3T3-E1, a mouse osteoblast cell line, LM8, a mouse osteosarcoma cell line, SaOS2, a human osteosarcoma cell line, 786-O, a human renal cancer cell line, PC-3, a human prostate cancer cell line, and A549, a human lung cancer cell line. MC3T3-E1 was maintained in alpha-minimum essential medium (α-MEM) with 10% fetal calf serum (FCS) containing antibiotics (100 U/ml penicillin G, 100 mg/ml streptomycin). LM8, SaOS2, 786-O, and A549 were maintained in Dulbecco's modified Eagle's medium (DMEM) with FCS containing antibiotics. PC-3 was maintained in RPMI-1640 with FCS containing antibiotics. The cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C.

Ultrasound treatment. An ultrasound exposure system, which was made by Medical Engineering Research Laboratories of Teijin Ltd. (Tokyo, Japan), consisted of an array of six 2.5-cm-diameter PZT-4 (lead-zirconate titanate) transducers, specially designed for a 6-well tissue culture plate. This array was placed at the bottom of a water tank, and the culture plate was located above the array. The temporal average intensity was 30 mW/cm^2 and the frequency was 1.5 MHz with a 200-μs tone burst repeated at 1.0 KHz (11,12). This setting is the same as in clinical use. LIPUS was administered for 20 min every day for the duration of this experiment.

Determination of cell number. MC3T3-E1 cells were seeded in a 6-well plate at a density of 2.0x10^4 cells/cm^2. LM8, SaOS2, PC-3, 786-O, and A549 cells were seeded at a density of 1.0x10^4 cells/cm^2. The cells were cultured in the presence or absence of daily LIPUS stimulation for up to 72 h. The cells were detached by gentle trypsinization and counted microscopically using a trypan blue dye exclusion test.

Western blot analysis. MC3T3-E1, LM8, SaOS2, PC-3, 786-O, and A549 cells were plated in a 6-well tissue cell culture plate at a density of 5.0x10^4 cells/cm^2 and were harvested 5, 30 min, 1, 3, 6, 12, 24, and 48 h after LIPUS stimulation. The cells were washed twice with PBS and lysed with RIPA buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 40 mM NaF, and protease inhibitor cocktail (Sigma, St. Louis, MO, USA)]. The lysates were centrifuged at 15,000 rpm for 20 min. The supernatant lysate with sample buffer [0.0625 M Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, 5% 2-ME] was boiled at 95˚C for 5 min. The samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto a nitrocellulose membrane (Amersham Biosciences, Tokyo, Japan). The membranes were saturated with 5% (wt/vol) non-fat dry milk in Tris-buffered saline with Tween-20 (TBST) [25 mM Tris-HCl (pH 7.8), 140 mM NaCl, 0.1% (vol/vol) Tween-20] and then incubated overnight with the following antibodies (diluted 1:1,000 in TBST): extracellular signal-regulated kinase (ERK1/2) (Cell Signaling Technology, Beverly, MA, USA), phosphorylated ERK1/2 (pERK1/2) (Cell Signaling Technology), Akt (Cell Signaling Technology), phosphorylated Akt (p-Akt) (Cell Signaling Technology) and β-catenin (Becton-Dickinson, Franklin Lakes, NJ, USA). The membranes were washed thoroughly with TBST and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or -rabbit IgG (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) (diluted 1:5,000 in TBST). Detection was performed with enhanced chemiluminescence kits (Amersham Biosciences).

Enzyme-linked immunosorbent assay (ELISA) analysis. MC3T3-E1, LM8, SaOS2, PC-3, 786-O, and A549 cells were plated in a 6-well tissue cell culture plate at a density of 5.0x10^4 cells/cm^2. After 48 h, each culture medium was exchanged with 1 ml of serum-free medium. Twenty-four hours after the medium exchange, the cells were treated with LIPUS. The supernatants were harvested 0, 3, 6, 12, 24, and 48 h after LIPUS stimulation. ELISA assays for vascular endothelial growth factor (VEGF) were performed with the human and mouse VEGF ELISA kit (Ray Biotech, Norcross, GA, USA).

Wound healing assay. MC3T3-E1, LM8, PC-3, SaOS2, 786-O, and A549 cells were cultured until confluence. The confluent cell monolayer in a 6-well plate was wounded by manually scraping the cells with a pipette tip. The cells were treated with or without LIPUS stimulation. Cell migration into the wound surface was monitored by microscopy at 0, 6, and 12 h after LIPUS stimulation. Quantitation was carried out in terms of % wounded area filled [% wounded area filled = 100 x (initial width of wounding - final width)/initial] by measuring the distance of the wound edge of the migrating cells from the start point to the migrated point from 3 independent experiments (13).

Statistical analysis. The data are represented as the mean ± standard deviation (SD). Statistical significance was determined using Student's t-test. $P<0.05$ was considered statistically significant.

Results

Cell number. The cell number did not change regardless of the presence or absence of daily LIPUS stimulation, up to 3 days (Fig. 1).

Phosphorylation of ERK and Akt and expression of β-catenin. ERK1/2: immunoreactive bands at 44 kDa (ERK1 and phosphorylated ERK1 protein) and 42 kDa (ERK2 and phosphorylated ERK2 protein) were observed. LIPUS stimulation on MC3T3-E1 and LM8 temporarily induced phosphorylation of ERK1/2 between 5 min and 1 h after LIPUS stimulation. LIPUS stimulation on SaOS2 and A549 bimodally induced phosphorylation of ERK1/2 at 1 and 24 h. LIPUS stimulation on PC-3 and 786-O did not induce phosphorylation of ERK1/2 (Fig. 2A).

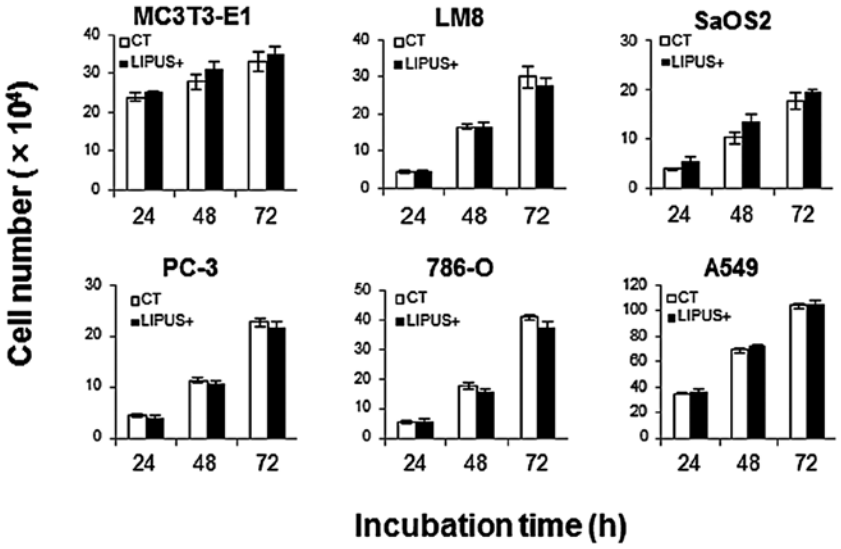

Figure 1. LIPUS did not affect the cell number. Each cell type was cultured in the presence (black bars, LIPUS+) or absence (white bars, CT) of daily LIPUS stimulation for up to 72 h. The cell number was compared with control culture by counting the cells using a trypan blue dye exclusion test. The rate of increase in the cell numbers showed no significant difference. The data are shown as the mean ± SD (bars, ± SD) of at least three independent experiments.

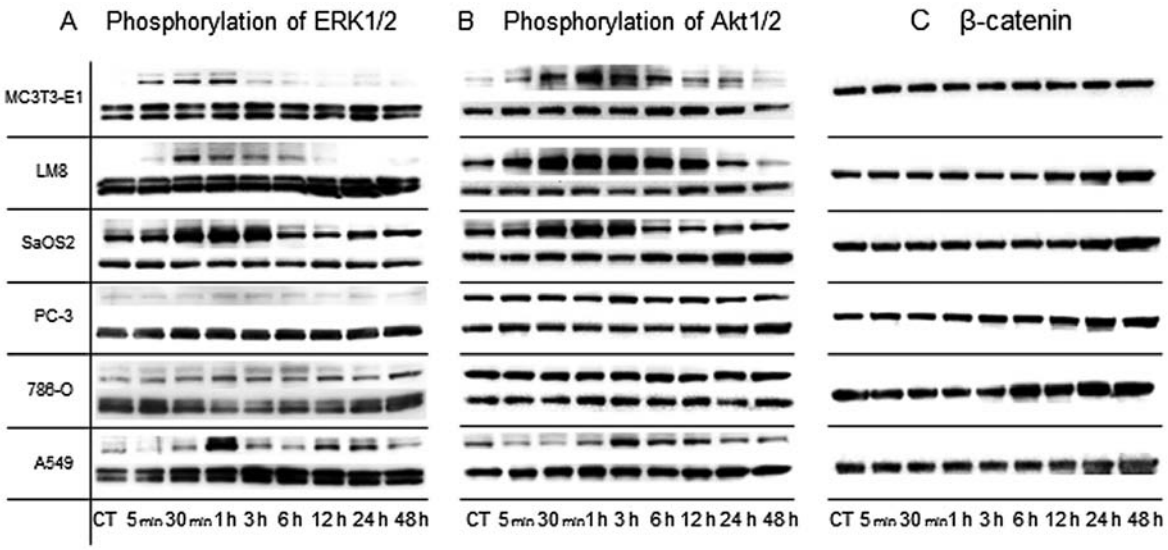

Figure 2. LIPUS stimulation on MC3T3-E1, LM8, SaOS2, and A549 induced phosphorylation of ERK1/2 and Akt. Each cell type was harvested between 5 min and 48 h after LIPUS stimulation. The expressions of pERK1/2, ERK1/2, pAkt, Akt, and β-catenin were analyzed by western blotting. (A) The expressions of ERK1/2 and pERK1/2 were observed (upper, pERK; lower, ERK). Phosphorylation of ERK1/2 on MC3T3-E1, LM8, SaOS2, and A549 was induced by LIPUS stimulation, but not in PC-3 and 786-O. (B) The expressions of Akt and pAkt were observed (upper, pAkt; lower, Akt). Phosphorylations of Akt on MC3T3-E1, LM8, SaOS2, and A549 were induced by LIPUS stimulation, but not in PC-3 and 786-O. (C) The expression of β-catenin was observed. β-catenin expression was not increased by LIPUS stimulation.

Akt: immunoreactive bands at 60 kDa (Akt and phosphorylated Akt protein) were observed. LIPUS stimulation on MC3T3-E1 temporarily induced phosphorylation of Akt between 30 min and 24 h. LIPUS stimulation on LM8 temporarily induced phosphorylation of Akt between 5 min and 12 h. LIPUS stimulation on SaOS2 temporarily induced phosphorylation of Akt between 30 min and 3 h. LIPUS stimulation on A549 temporarily induced phosphorylation of Akt at 3 h. LIPUS stimulation on PC-3 and 786-O did not induce phosphorylation of Akt (Fig. 2B).

β-catenin: immunoreactive bands at 92 kDa (β-catenin protein) were observed. LIPUS stimulation of the cells did not significantly increase β-catenin expression (Fig. 2C).

Expression of VEGF protein. VEGF protein levels were assessed in the collected culture media by sandwich-ELISA. VEGF protein levels in MC3T3-E1 at 12, 24, and 48 h after LIPUS stimulation were significantly increased compared with those of the control, whereas those in LM8, SaOS2, PC-3, 786-O, and A549 were not (Fig. 3).

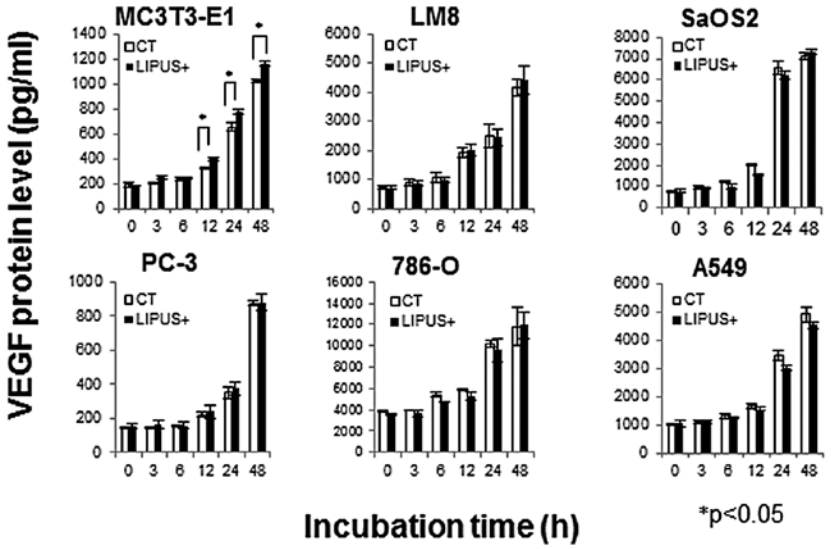

Figure 3. LIPUS stimulation increased VEGF protein levels only in MC3T3-E1. Each cell type was cultured in the presence (black bars, LIPUS+) or absence (white bars, CT) of daily LIPUS stimulation for up to 48 h. The supernatants of MC3T3-E1, PC-3, LM8, SaOS2, 786-O, and A549 cells were harvested between 0, 3, 6, 12, 24, and 48 h after LIPUS stimulation. VEGF protein levels were assessed in the collected culture media by sandwich-ELISA. VEGF protein levels in MC3T3-E1 were significantly increased compared with those of the control and not significantly increased in LM8, SaOS2, PC-3, 786-O, and A549 (*p<0.05). The data are shown as the mean ± SD (bars, SD) of at least three independent experiments.

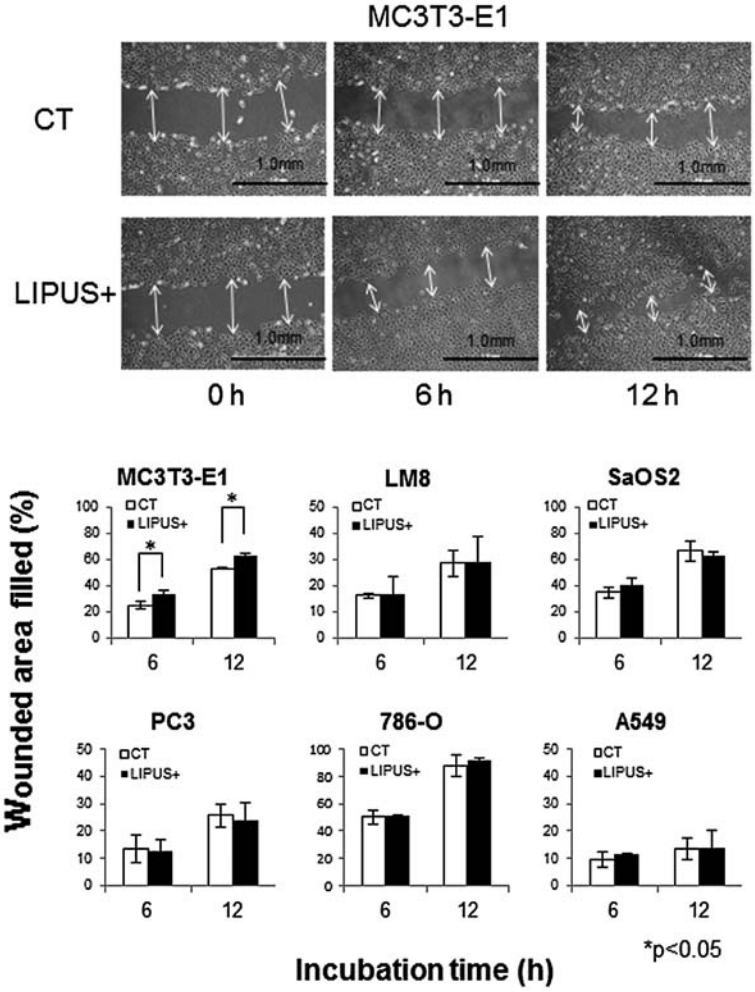

Figure 4. LIPUS stimulation promoted cell migration only in MC3T3-E1. Each cell type was cultured in the presence (black bars, LIPUS+) or absence (white bars, CT) of daily LIPUS stimulation for up to 12 h. The cell migration of MC3T3-E1, LM8, SaOS2, PC-3, 786-O, and A549 cells into the wound surface was monitored by microscopy at 0, 6, and 12 h after LIPUS stimulation. Quantitation was carried out in terms of % wounded area filled [% wounded area filled = 100 x (initial width of wounding - final width)/initial] measuring the distance of the wound edge of the migrating cells from the start point to the migrated point. LIPUS stimulation resulted in significant promotion of MC3T3-E1 cell migration. SaOS2, PC-3, 786-O, and A549 cells were not affected (*p<0.05). The data are shown as the mean ± SD (bars, ± SD) of at least three independent experiments.

Cell migration. In order to investigate the effect of LIPUS stimulation on cell migration, scratch wound healing assays were performed. LIPUS stimulation resulted in significant promotion of MC3T3-E1 cell migration. MC3T3-E1 cell migration increased by 8.7 and 9.4% at 6 and 12 h, respectively, compared with those of control cells. LM8, SaOS2, PC-3, 786-O, and A549 cells were not affected (Fig. 4).

Discussion

Application of adequate mechanical stress to bone is essential for maintaining bone mass and strength. Various types of mechanical loading have been clinically tested for their bone mass-promoting activity in the treatment of bone fractures. Among them, LIPUS was reported to promote healing of bone fracture in 1983 (1). Then it was widely reported that LIPUS reduced the time required for fracture healing (14,15). Furthermore, LIPUS induced bone maturation in distraction osteogenesis, and delayed fracture union and nonunion in animal models as well as in clinical settings (1-4). *In vitro* experiments have demonstrated that LIPUS stimulation affects mouse and rat osteoblast differentiation without influencing proliferation (8,9). Although the mechanisms involved have not been elucidated, LIPUS transmits signals into the cell via an integrin that acts as a mechanoreceptor on the cell membrane (16). The integrin/Ras/MAPK pathway is known to be a general pathway involved in cell proliferation. A previous study demonstrated that ERK phosphorylation increased after LIPUS stimulation on MC3T3-E1, starting at 5 min, reaching a maximum between 15 and 30 min, and then gradually decreasing, and that LIPUS stimulation on human pre-osteoblastic cells progressively increased ERK phosphorylation in 1 h (17,18). The PI3K/Akt pathway, on the other hand, is known to be involved in various functions such as cell survival, proliferation, motility, control of cell size, and metabolism. It was reported that LIPUS exposure in MC3T3-E1 increased Akt phosphorylation in a time-dependent manner and maximal activation was detected 15 min after LIPUS stimulation (17). In the present study, LIPUS stimulation on MC3T3-E1 temporarily induced phosphorylation of ERK1/2 between 5 min and 1 h and phosphorylation of Akt between 30 min and 24 h after LIPUS stimulation. These results are comparable to those of a previous study.

Wnt/β-catenin signaling pathway has been reported to play a crucial role in cell proliferation, differentiation, and possibly apoptosis (19). There have been no reports of studies investigating the effect of LIPUS stimulation on β-catenin expression. Instead, mechanical loading in mouse tibial bone increased expression of canonical Wnt pathway and Wnt/β-catenin target genes (20). Our experiment indicated that LIPUS stimulation did not significantly increase β-catenin expression.

VEGF has a central role in the regulation of vascularization. VEGF expression has been reported to be increased by LIPUS stimulation in human fetal pre-osteoblastic cell and rabbit bone-tendon junction (6,21). Migration of osteoblasts has an important impact on fracture healing. Our experiment indicated that LIPUS stimulation of osteoblasts, MC3T3-E1, promoted cell migration. MC3T3-E1 cell migration increased by 8.7 and 9.4% at 6 and 12 h, respectively, compared with that of control cells. In addition, VEGF protein levels in MC3T3-E1 at 12, 24, and 48 h after LIPUS stimulation were significantly increased compared with those of the control. These changes may help differentiation and formation of bone. However, for malignant cells, these changes observed in osteoblastic cells may favor tumor growth. Therefore, we next studied whether LIPUS also affects malignant cells, viz., osteosarcoma cells, and other visceral cancer cells.

Osteosarcoma is generally characterized by exhibition of osteoblastic differentiation and production of osteoid matrix. LIPUS effects on osteosarcoma cell lines such as an osteoblastic cell line has been reported (9,22). In the present study, LIPUS on LM8 and SaOS2 induced phosphorylation of ERK1/2 and Akt and did not affect cell number, β-catenin expression, VEGF protein expression, or cell migration. Although phosphorylated ERK and Akt in osteosarcoma were generally considered to activate the tumor cell proliferation, Cagnol and Chambard reported that activation of ERK1/2 in osteosarcoma cells induced apoptosis and autophagy (23). From our study, it was concluded that LIPUS stimulation did not affect the cell number of osteosarcoma. Moreover, angiogenic and migration effects were different from those of osteoblast cells. It is tempting to speculate that LIPUS stimulation of osteosarcoma cells might promote bone differentiation and reduce the activity of a tumor, although our results are limited.

Two types of cancer cells, 786-O and PC-3, were not affected by LIPUS stimulation in any of the experiments. It may thus be argued that LIPUS used for bone metastases from renal and prostate cancer induces osteoblastic differentiation without inducing cancer proliferation, vascularization, and migration. On the other hand, the effect of LIPUS stimulation of A549 induced phosphorylation of ERK1/2 and Akt and did not affect cell number, β-catenin expression, VEGF protein expression, or cell migration. Although activations of ERK and Akt were considered to induce proliferation of lung cancer cells, it was reported that activation of ERK is required for apoptosis in A549 lung cancer cells (24,25). In our study, LIPUS stimulation did not enhance proliferative activity of cancer cells, regardless of the tissue of origin.

Generally, a part of intact bone is substituted with malignant cells in metastatic bone tumor and malignant bone tumor. Progression of tumor growth causes pain and bone destruction, resulting in pathological bone fracture. Tumor growth stimulates activation of osteoclasts leading to bone resorption whereby more space is produced adequate for malignant cell proliferation. Stimulation to osteoclasts essentially occurs in combination with stimulation of osteoblasts. Metastatic bone tumor is similar to bone fracture because both osteoclasts and osteoblasts are stimulated. However, proliferative activity of malignant cells is generally greater than that of osteoblasts. If we could enhance new osteogenic activities in spite of malignant cell growth, it would stop progression of the osteolytic change which may prevent bone fracture. Patients could thus achieve relief of pain, prevention of SRE, and finally satisfactory ADL and QOL.

This study model showed limited outcome because *in vitro* experiments are different from *in vivo* environments of metastatic bone tumor. The mechanisms by which metastases are formed are complex, involving many types of cells and steps that include angiogenesis, invasion, and proliferation in the bone microenvironment. Tumor cells in the bone micro-

environment produce a large number of cytokines such as tumor-produced parathyroid hormone-related protein (PTHrP), transforming growth factor β, and interleukin-6 (26).

In conclusion, LIPUS did not significantly increase cellular proliferation, migration or VEGF production of malignant cells compared with that without LIPUS. In contrast, LIPUS induced migration and VEGF production without proliferation in osteoblasts. LIPUS when applied on metastatic bone tumors might be beneficial by inducing osteoblast differentiation without cancer proliferation. In the future, LIPUS stimulation might be one of the treatments of metastatic bone tumor because of its non-invasiveness.

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