

Low-Intensity Pulsed Ultrasound Activates Integrin-Mediated Mechanotransduction Pathway in Synovial Cells

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Abstract—Low-intensity pulsed ultrasound (LIPUS) suppresses synovial hyperplasia and synovial cell proliferation characterized for rheumatoid arthritis, but the molecular mechanisms remain unknown. The purpose of this study was to examine the mechanotransduction pathway *via* the integrin/mitogen-activated protein kinase (MAPK) pathway in LIPUS exposure on the synovial membrane cells. Rabbit knee synovial membrane cell line, HIG-82, was cultured with or without FAK phosphorylation inhibitor, PF-573228. One hour after stimulation with PF-573228, the cells exposed to LIPUS for 20 min or sham exposure. A possible integrin/MAPK pathway was examined by immunofluorescence and Western blotting analysis with antibodies targeting specific phosphorylation sites on intracellular signaling proteins. LIPUS exposure increased phosphorylation of FAK, JNK, ERK, and p38, but the phosphorylation was inhibited by PF-573228. In conclusion, LIPUS exposure might be involved in cell apoptosis and survival of synovial membrane cells *via* integrin/FAK/MAPK pathway.

Keywords—Low-intensity pulsed ultrasound, Synovitis, Synovial cells.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic, chronic inflammatory disease of the joints that is characterized by synovial hyperplasia, cartilage destruction, and infiltration of inflammatory cells into synovial tissue.²³ Recently, there has been a general consensus that synovitis plays an essential role in the pathophysiology

of RA.²⁴ It is widely accepted that synovitis is associated with clinical symptoms and reflects joint degradation in RA,²⁷ and several studies have focused attention on synovial hyperplasia.³

Synovial hyperplasia is a major pathophysiologic feature of RA and appears to be associated with pro-inflammatory cytokines, notably TNF- α and IL-1 β .²⁶ Therefore, the importance of synovitis in RA joints has been increasingly recognized, particularly in early stages of the disease. Furthermore, synovial fibroblasts in the synovial intimal lining play a key role in producing cytokines and proteases.¹³ Since targeting synovial fibroblasts may improve clinical outcomes in inflammatory arthritis, it is thought that the control of metabolism of synovial fibroblasts is an important consideration for treatment strategies.

Low-intensity pulsed ultrasound (LIPUS) is an acoustic radiation with frequencies above the limit of human hearing and has been used clinically as an accelerator of fracture healing.^{5,11} Furthermore, various cell types have been reported to be sensitive to US exposure, including periodontal ligament cells,¹⁴ cementoblasts,⁸ chondrocytes,²⁹ and osteoblasts.²² We previously demonstrated that the increased expression of Cox-2 in IL-1 β -stimulated synovial membrane cells was significantly inhibited by LIPUS exposure *in vitro*.²⁰ In addition, we showed that LIPUS affected apoptosis and proliferation of the rabbit synovial cells (HIG-82), and the expression of Cox-2 in the knee joints of MRL/*lpr* mice was markedly reduced by daily LIPUS exposure.²¹ Furthermore, inhibition of Cox-2 reduced proliferation and induced apoptosis of human cholangiocarcinoma QBC939 cells through the inhibition of PGE₂ production.³¹ Therefore, it can be hypothesized that the inhibition of Cox-2 expression

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by LIPUS exposure inhibits cell proliferation in synovial tissue as a secondary effect. However, the molecular mechanisms by which LIPUS suppresses synovial hyperplasia and synovial cell proliferation remain unknown.

Since LIPUS provides a mechanical stimulation to the cellular system,⁵ the anabolic effect of LIPUS exposure on the synovial membrane cells may be involved in the mechanotransduction pathway *via* the integrin/mitogen-activated protein kinase (MAPK) pathway. In the present study, we examined a possible integrin/MAPK pathway using Western blotting with antibodies targeting specific phosphorylation sites on intracellular signaling proteins.

MATERIALS AND METHODS

Cell Culture

Rabbit knee synovial membrane cell line, HIG-82, was purchased from American Type Culture Collection (Manassas, VA). The cells were cultured on 100-mm culture dishes (Corning, Corning, NY). The cultures were maintained in 10 mL α -minimum essential medium (α -MEM; Invitrogen, Carlsbad, CA), 50 U/mL penicillin G (Meiji, Tokyo, Japan), and 10% fetal bovine serum (Japan Bioserum, Tokyo, Japan) under an atmosphere of 5% CO₂ in a humidified incubator. The medium was changed every other day.

LIPUS Exposure

A LIPUS exposure system which was a modification of the clinical device (ST-Sonic; Ito, Tokyo, Japan) was employed. This system consisted of a 5.0-cm² circular surface transducer and a culture flask. The ultrasound head of this device has a beam non-uniformity average of 2.7 and an effective radiating area of 4.1 cm².

A pulsed ultrasound signal was transmitted at a frequency = 3 MHz with a spatial-average intensity = 30 mW/cm², and pulsed 1:4 (2 ms on and 8 ms off). A six-well plate was held in place, with the top above water level, in a foam-fronted plastic sliding assembly containing an aperture of matching dimensions to the monolayer (Fig. 1). The distance between the transducer and the cells was less than 4 mm. The cell culture received 20 min of single ultrasound exposure. The tank water was maintained at 37 ± 0.5 °C. The LIPUS exposure assembly was maintained at humidified atmosphere of 5% CO₂ at 37 °C during all experiments. The machines have an electronic control panel, allowing for an electronic check whenever it is switched on, and an alert signal that is triggered if the coupling gel or liquid has been

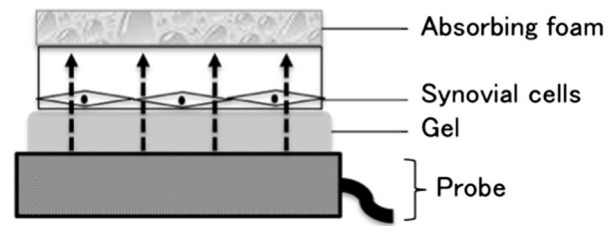


FIGURE 1. Schematic representation of the LIPUS exposure assembly. The culture flask filled with medium was placed in the LIPUS field at a distance of less than 4 mm, which optimized beam uniformity across the target cell region.

depleted. Control samples were also subjected to the same operations in the same conditions without LIPUS stimulation.

Immunofluorescence

Synovial cells seeded on a cover slip were treated with LIPUS. At 5 and 15 min after LIPUS exposure, these cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X (Sigma-Aldrich, St. Louis, MO). To investigate Integrin β 1 phosphorylation, the cells were incubated with monoclonal antibody to p-Integrin β 1 (#17783; Sigma-Aldrich, St. Louis, MO; diluted 1:100) or negative control antibody at 4 °C overnight and with Alexa Flour[®] 594-conjugated (#8889; Cell Signaling Technology, Boston, MA; diluted 1:500) secondary antibody at room temperature for 40 min in the dark. To investigate FAK phosphorylation, the cells were also incubated with monoclonal antibody to p-FAK (#3281; Cell Signaling Technology; diluted 1:200) or negative control antibody at 4 °C overnight and with Alexa Flour[®] 488-conjugated (#4412; Cell Signaling Technology; diluted 1:1000) secondary antibody at room temperature for 40 min in the dark. The nuclear DNA was stained with 4',6-diamidino-2'-phenylindole, dihydrochloride (DAPI; KPL, Gaithersburg, MD). After washing off excess antibody, the coverslips were mounted by Fluorescence Mounting Medium (Dako, Glostrup, Denmark) and observed by a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan).

Inhibition of FAK Phosphorylation

The effect of FAK phosphorylation inhibitor (PF-573228; R&D Systems, Minneapolis, MN) on synovial cells was examined by Western blot analysis. In each well of 6-well plates, a total of 5.0×10^4 cells were seeded onto a 6-well plate in α -MEM, and treated with PF-573228 at doses of 0, 1 and 10 μ M. After 1 h, the cells received LIPUS exposure for 20 min. At 5 min after LIPUS exposure, the cultured cells were collected

and used as the sample for Western blotting analysis described below.

Western Blot Analysis

The cultured cells precipitated were lysed with M-PER[®] Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) and the supernatant was used as samples after centrifuge. Protein concentration was measured using BCA Protein Assay Kit (Thermo Fisher Scientific), Microplate Reader (Corona Electric, Hitachinaka, Japan), and SDS–polyacrylamide gel electrophoresis, which was performed for 20 μ g of each protein. After SDS–PAGE, proteins were transferred onto a PVDF membrane (Millipore, Bedford, MA). The membrane was blocked for 1 h at room temperature with 3% skim milk in TBS 0.1% Tween 20 (Bio-Rad, Hercules, CA) and incubated overnight at 4 °C with primary antibodies (Cell Signaling Technology; diluted 1:500), i.e., phospho-focal adhesion kinase (p-FAK; #3281), FAK (#3285), p-JNK (#9251), JNK (#9252), p-p38 (#9211), p38 (#9212), p-extracellular-regulated kinase (ERK; #9101) and ERK (#9102), with 10% bovine serum albumin (Sigma-Aldrich). β -Actin rabbit antibody (#4967; Cell Signaling Technology; diluted 1:1000) was used as a loading control. After incubation with

HRP-linked anti-rabbit IgG (Cell Signaling Technology) for 1 h at room temperature, Phototope-HRP Western Blot Detection System (Cell Signaling Technology) was used according to the manufacturer's instructions. The original images were quantitated by densitometric analysis of Western blots using an image analysis software (CS Analyzer; ATTO, Tokyo, Japan).

Statistical Analysis

Means and standard deviations were calculated from the data obtained. All data from experiments ($n = 4$) were subjected to Mann–Whitney U test for two-group comparison and Kruskal–Wallis test followed by Steel–Dwass test as a *post hoc* test for multiple comparison to examine mean differences at the 5% level of significance.

RESULTS

LIPUS Induced Integrin β 1 Phosphorylation

We used immunofluorescence to investigate the effect of LIPUS on Integrin β 1 phosphorylation in synovial cells. The p-Integrin β 1 immunopositive cells were detected at 5 min, and markedly increase at 15 min after LIPUS exposure (Fig. 2).

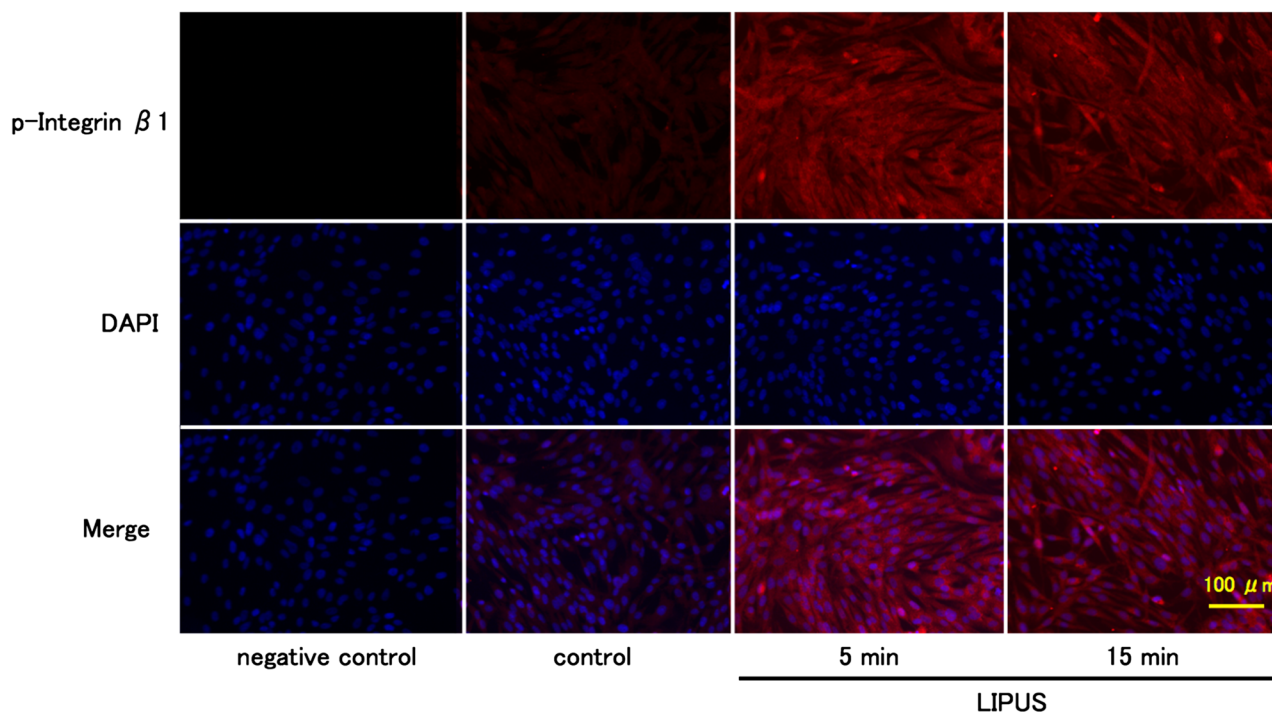


FIGURE 2. Immunofluorescence of phosphorylated Integrin β 1 in the synovial cells after LIPUS exposure. Synovial cells were immunostained with the anti-p-Integrin β 1 antibody (red) and DAPI (blue). The intensity of immunostaining was detected at 5 min and markedly at 15 min after LIPUS exposure. In the negative control, no apparent staining was observed. ($n = 4$).

LIPUS Induced FAK Phosphorylation

We used immunofluorescence and Western blotting to investigate the effect of LIPUS on FAK phosphorylation in synovial cells. The immunofluorescence showed an increase in FAK phosphorylation in cells at 5 and 15 min after LIPUS exposure (Fig. 3a). Furthermore, LIPUS significantly ($p < 0.05$) up-regulated p-FAK in cells at 5 and 15 min after LIPUS exposure (Fig. 3b).

Determination of FAK Phosphorylation Inhibitor Concentration

Western blotting analysis showed that FAK phosphorylation induced by LIPUS exposure was mostly inhibited by addition of 10 μ M PF-573228. Therefore, 10 μ M PF-573228 was used in the following experiments (Fig. 4).

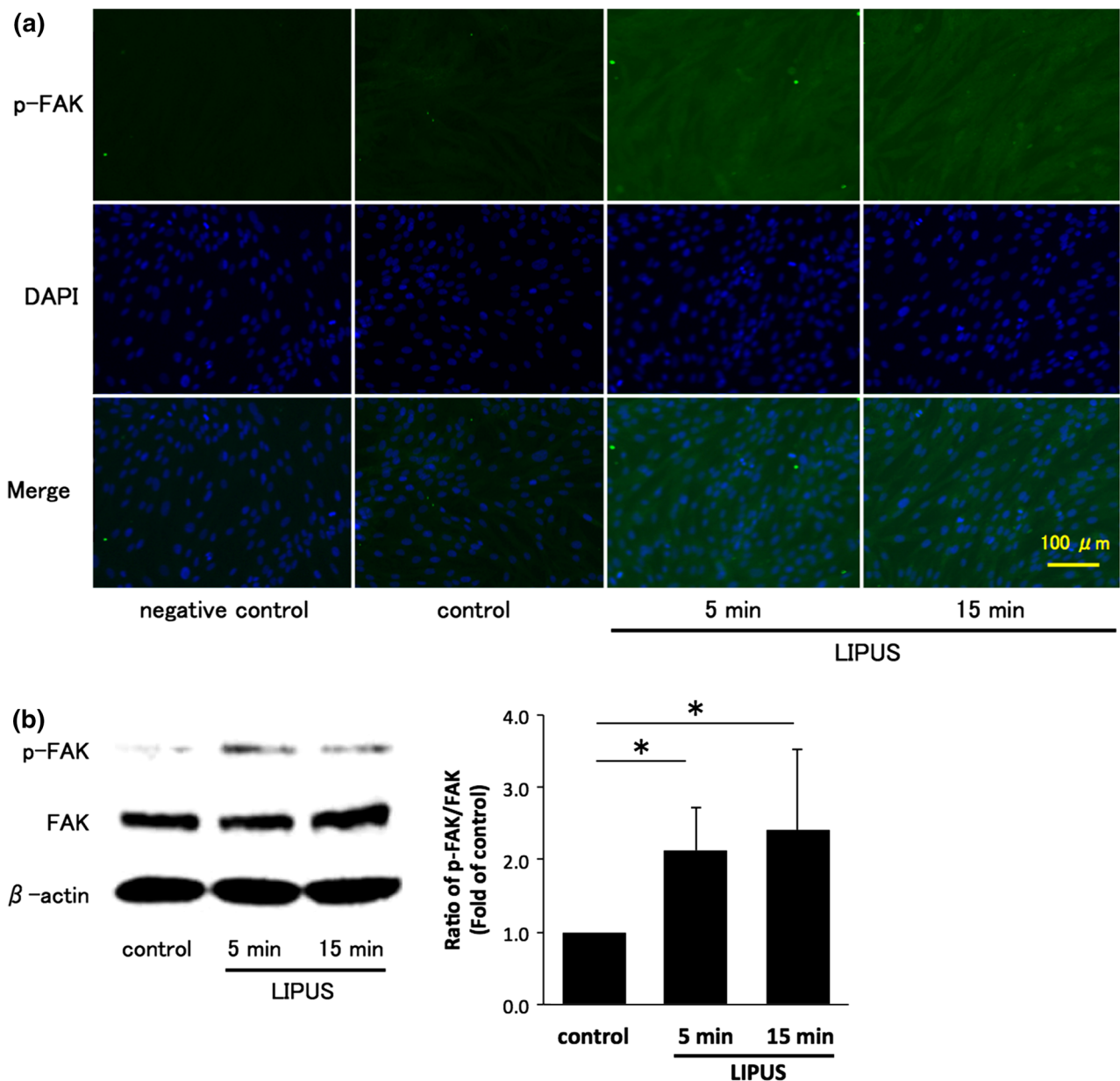


FIGURE 3. Immunofluorescence (a) and immunoblot analyses (b) of phosphorylated FAK in the synovial cells after LIPUS exposure. (a) Synovial cells were immunostained with the anti-p-FAK antibody (green), and the intensity of immunostaining was increased 5 min after LIPUS exposure. In the negative control, no apparent staining was observed. (b) Western blot analysis was performed with antibody against p-FAK and β -actin. Quantitative data expressing the corresponding protein levels was assessed using densitometry and is expressed in relative intensity. The protein level of p-FAK was significantly ($p < 0.05$) increased 5 and 15 min after LIPUS exposure, compared to the untreated control. ($n = 4$).

Inhibitor of FAK Phosphorylation Interfered with JNK, ERK, and P38 Phosphorylation by LIPUS

To understand the mechanism that underlies involvement of FAK and MAPK in synovial cell metabolism, we investigated the effect of FAK phosphorylation inhibition on phosphorylation of proteins involved in cell apoptosis and survival. Western blotting analysis showed JNK, ERK, and p38 phosphorylation were significantly ($p < 0.05$) induced by LIPUS exposure, however, the addition of PF-573228 significantly ($p < 0.05$) inhibited the phosphorylation of MAPKs even if LIPUS exposure (Fig. 5).

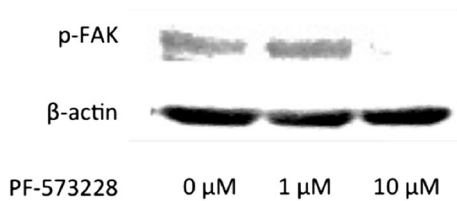


FIGURE 4. Immunoblot analysis of phosphorylated FAK in the synovial cells cultured with FAK phosphorylation inhibitor. The expression of p-FAK markedly decreased in the synovial cells cultured with 10 μM FAK phosphorylation inhibitor. ($n = 4$).

DISCUSSION

LIPUS has been used extensively as a therapeutic, operative, and diagnostic tool in medicine. *In vivo* studies have demonstrated that LIPUS can promote tissue repair and regeneration, accelerate bone fracture healing, prevent root resorption during orthodontic tooth movement.^{2,10,15} It is generally accepted that LIPUS has no deleterious or carcinogenic effects. In addition, LIPUS exposure has no thermal effects to produce biological changes in living tissues. Therefore, LIPUS is accepted as a non-invasive and safe therapeutic tool for the treatment of bone fracture and osteoarthritis.^{4,19}

Previous studies provided evidence that synovial membrane cells are sensitive to ultrasound stimulation. Chung *et al.*⁶ reported that LIPUS showed a potent anti-inflammatory effect in animal arthritis model with reduced infiltration of inflammatory cells into the synovium. Nakamura *et al.*^{20,21} also revealed that the expression of Cox-2 in the knee joints of MRL/lpr mice was markedly reduced by LIPUS exposure *in vivo* as well as the cultured synovial cells *in vitro*. These indicate that LIPUS may prevent synovitis in the initiation and progression of RA and OA through the mediation of synovial cell metabolism.

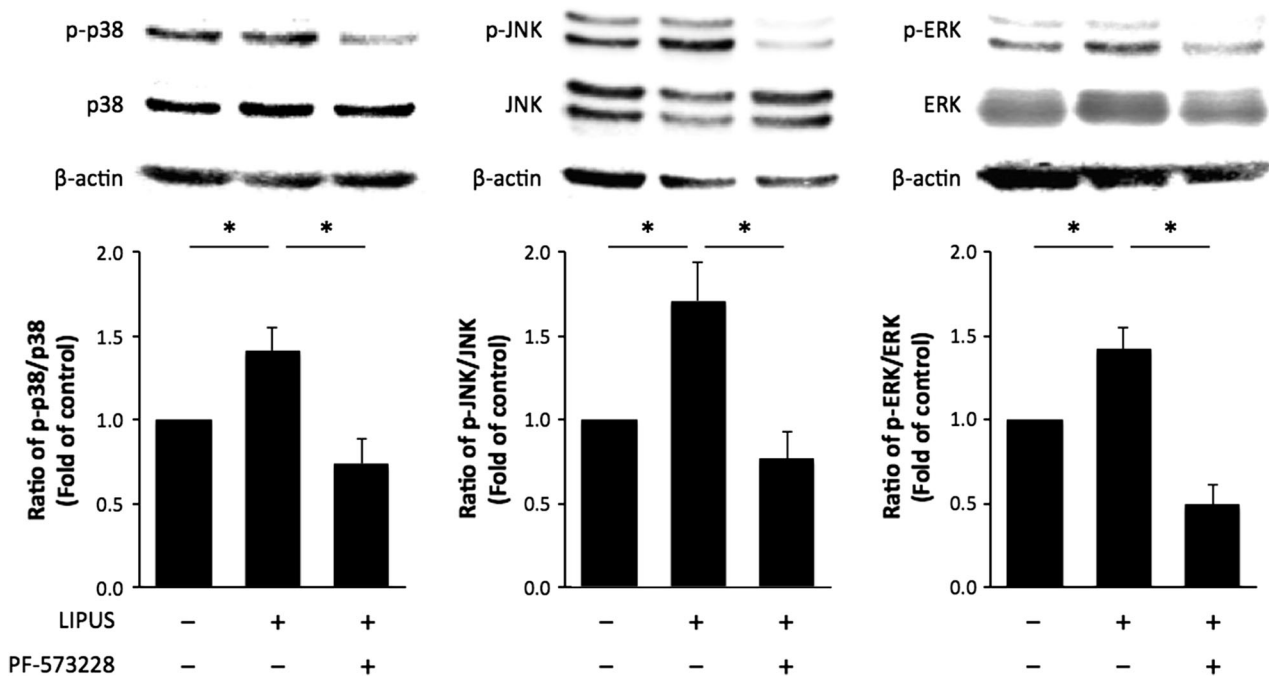


FIGURE 5. Immunoblot analyses of p-38, JNK, and ERK phosphorylation in the synovial cells cultured with FAK phosphorylation inhibitor. Western blot analyses were performed with antibody against phosphorylated p-38, JNK and ERK, and β -actin. The expression of phosphorylated MAPKs were induced in the synovial cells by LIPUS exposure, but the addition of FAK phosphorylation inhibitor significantly ($p < 0.05$) downregulated the expression of phosphorylated p-38, JNK, and ERK induced by LIPUS exposure. ($n = 4$).

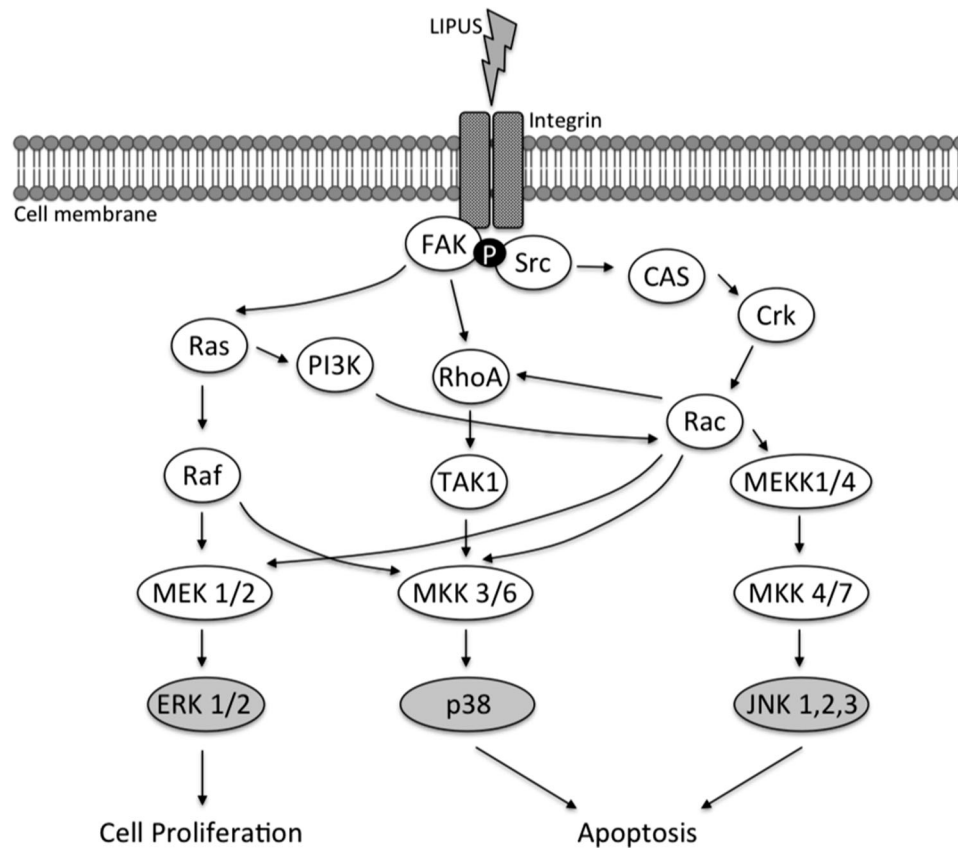


FIGURE 6. Schematic illustration of mechanisms of signal transduction pathways enhanced by LIPUS. LIPUS may regulate synovial cell metabolism *via* integrin/FAK/MAPK pathway particularly.

It is very probable that LIPUS transmits signals into the cell *via* an integrin that acts as a mechanoreceptor on the cell membrane.¹⁸ When LIPUS is transmitted to integrin molecules, this promotes the attachment of various focal adhesion adaptor proteins. FAK is in turn phosphorylated as a result of LIPUS exposure initiating this signal transduction. The present study showed that LIPUS induced a significant up-regulation of phosphorylated FAK in the synovial cells and also that FAK phosphorylation inhibition led significant downregulation of MAPK phosphorylation. Activation of integrins and the downstream signaling pathway have also been reported based on an *in vitro* study of LIPUS using human skin fibroblasts.³² Integrins act as a link between extracellular matrix, cytoskeletal proteins, and actin filaments, and Hsu *et al.*¹² reported treatment with anti-Integrin $\beta 1$ and $\beta 3$ antibodies or transfection with siRNA against Integrin $\beta 1$ and $\beta 3$ antagonized the potentiating action of US stimulation on COX-2 expression, indicating that Integrin $\beta 1$ and $\beta 3$ are very important to mediate the action of US in chondrocytes. Furthermore, we previously reported that LIPUS exposure to cementoblasts mediated cell metabolism through MAPK pathway because LIPUS

enhanced the protein expression of ERK1/2 and also based on the evidence that MEK1/2 inhibitor treatment suppressed the up-regulation of Cox-2 mRNA expression induced by LIPUS.²⁵ The integrin/Ras/MAPK pathway is considered a general pathway involved in cell proliferation. Taken together, the bioeffect of LIPUS exposure to synovial cells might be promoted *via* integrin/FAK/MAPK pathway particularly (Fig. 6).

The proliferation of several cells including synovial cell is mediated by growth factor or cytokine-induced MAPK, a family of serine-threonine proteins.⁷ Although the three MAPK modules, JNK, ERK and p38, run in parallel, there is a considerable degree of cross-talk between them, which creates multiple opportunities for modulating or fine-tuning responses to different signals.⁷ Generally, activation of an ERK signaling pathway has a role in mediating cell division, migration and survival.³⁰ Activation of the JNK signaling cascade generally results in apoptosis, although it has also been shown to promote cell survival under certain conditions.⁹ The p38 subfamily is also involved in affecting cell motility, transcription and chromatin remodeling.¹⁷ It has been reported that cyclic

mechanical stretching of human patellar tendon fibroblasts activates JNK and modulate apoptosis.²⁸ Kanbe *et al.*¹⁶ found ERK and JNK expression without p38 in the synovium of the rotator interval with positive β 1-integrin. Interestingly, JNK expression around vascular tissue with epithelial cells extended more widely than ERK and β 1-integrin expression. Thus, ERK is specific for expression in epithelial cells, and fibroblastic cells and epithelial cells expressed JNK. The present results showed that inhibitor of FAK phosphorylation interfered equally with JNK, ERK, and p38 phosphorylation by LIPUS. It is well known that apoptosis is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding region. Both apoptosis and proliferation play an essential role in controlling cell number and viability.¹ The downregulation of apoptosis can lead to the survival and hyperproliferation of synovial membrane cells in RA and OA joints.¹ Therefore, the results shown in this study may be useful for not only therapies that affect apoptosis, but also for therapies that affect cell proliferation, based on the mechanical effects induced by LIPUS.

In conclusion, it was suggested that LIPUS up-regulates phosphorylated FAK in the synovial cells and that FAK phosphorylation inhibition led significant downregulation of ERK, JNK, and p38 phosphorylation. LIPUS exposure might be involved in cell apoptosis and survival of synovial membrane cells *via* integrin/FAK/MAPK pathway.

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