Low-intensity pulsed ultrasound induces collagen matrix synthesis and aggrecan remodeling in chondrocytes

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Contents

Abstract

Low-intensity pulsed ultrasound (LIPUS) is used for bone healing in orthopedics. LIPUS at 30 mW/cm^2 can accelerate fracture repair by about 38% faster compared with placebo-treated fractures. In previous *in vivo* and *in vitro* studies, LIPUS has been shown to have promising effects on cellular elements in articular cartilage, particularly chondrocytes in patients with osteoarthritis. However, the effects of LIPUS intensity and the cellular mechanisms through which LIPUS alters extracellular matrix (ECM) synthesis in chondrocytes are unclear. Thus, in this study, I investigated the effects of the optimal intensity and cellular mechanisms of LIPUS on the regeneration of cartilage matrix in ATDC5 mouse chondroprogenitor cells.

Cells were cultured with differentiation medium containing insulin-transferrin-selenium for 14 days and then stimulated with 30 and 60 mW/cm^2 LIPUS for 20 min/day for up to 14 days. The expression levels of phospho-extracellular signal-regulated kinase (p-ERK) 1/2, sox9, aggrecan, collagen type II (Col II), collagen type X (Col X), alkaline phosphatase (ALP), aggrecanase-5 (ADAMST-5) and matrix metalloproteinase-13 (MMP13) were determined using real-time polymerase chain reaction and/or western blotting. The production of cartilage-specific aggrecan was determined by Alcian blue staining.

LIPUS induced collagen synthesis and the remodeling of aggrecan via the activation. In

contrast, MMP13 expression was decreased in chondrocytes. Additionally, chondrocytes responded optimally to LIPUS at an intensity higher than the clinical setting for bone fracture healing (30 mW/cm^2).

These results suggested that LIPUS induced ECM regeneration via increases in hypertrophic chondrocytes and delayed endochondral ossification in chondrocytes. Chondrocytes responded to LIPUS at an intensity higher than that required to activate osteoblasts. Taken together, LIPUS induces Col II synthesis and aggrecan remodeling by chondrocytes.

Introduction

Skeletal development involves intramembranous and endochondral ossification; intramembranous ossification occurs via direct differentiation of mesenchymal precursors into osteoblasts and is confined to the clavicle and the bones of the skull, whereas endochondral ossification involves early chondrogenesis and several stages of chondrocyte differentiation and maturation. Chondrogenesis in endochondral ossification on growth plate cartilage is accompanied by changes in the structure and composition of extracellular matrix (ECM) molecules produced by the differentiated chondrocytes in each step [1, 2].

Low-intensity pulsed ultrasound (LIPUS) has been examined extensively at intensities of less than 100 mW/cm^2, oscillatory frequency at 1.5 MHz $\pm$ 10% or pulse frequency at 1 kHz, with pulsed exposure for 20 min/day [3]. The effects of LIPUS involve acoustic radiation force, acoustic streaming, and propagation of surface waves, which promote fluid flow-induced circulation and redistribution of nutrients, oxygen, and signaling molecules. However, high-intensity ultrasound can damage biological molecules, such as DNA [4]. Notably, LIPUS does not cause the cavitation effect related to pressure levels and does not transform acoustic wave energy into heat. LIPUS is a mechanical stimulus that accelerates healing and regeneration of bone fractures [5-9]. In a previous clinical study, the index of healing was shortened by 12 days/cm with continuous LIPUS stimulation, and the fixator time was reduced

to 95 days compared with healing in the absence of LIPUS stimulation [10]

Joint tissues, including cartilage, are constantly exposed to mechanical stimuli and are exquisitely sensitive to the mechanical environment. Mechanical loading is an important exogenous factor regulating the development and long-term maintenance of joint tissues. Optimal mechanical loading maintains articular cartilage, whereas both disuse and overuse can occur in cartilage degradation [11]. Ito et al. have reported that LIPUS at an intensity of more than 200 mW/cm^2 can cause cell death, whereas that at an intensity of less than 120 mW/cm^2 promotes viability in chondrocytes [12]. In many relevant previous *in vivo* or *in vitro* studies, LIPUS has a promising effect on the cellular elements in articular cartilage, specifically on chondrocytes in knee osteoarthritis [13-19]. However, the effects of the intensity of LIPUS on ECM synthesis in chondrocytes are unclear.

ATDC5 chondrogenic cell line were isolated from mouse embryonal carcinoma and are capable of mesenchymal condensation, establishing cartilage nodule-like structures and differentiated into chondrocytes with insulin-transferrin-sodium selenite (ITS) treatment. ATDC5 cells are suitable for *in vitro* analysis of the molecular mechanisms underlying the regulation of endochondral ossification because they reflect the chronological observation of bone development for the long axis *in vivo* [20-22].

In this study, I investigated the effects of the optimal intensity of LIPUS on the expression

of collagens, proteogrican, matrix metalloproteinase-13 (MMP13), aggrecanase-5 (ADAMTS-5) and alkaline phosphatase (ALP), which are required for the regeneration of cartilage matrix in ATDC5 mouse chondrogenic cells.

Material and Methods

1. Cell culture

The mouse chondroprogenitor cell line ATDC5 was maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS; Japan Bioserum Co., Ltd., Hiroshima, Japan), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 95% humidified atmosphere and 5% CO_2 in air. When cells reached confluence, ITS (Gibco) was added for the indicated times. The medium was replaced every 3 days.

2. Application of LIPUS

Cells were seeded in 6-well culture plates (Iwaki, Tokyo, Japan) at a density of 2×10^4 cells/cm^2 and incubated for 14 days with culture medium containing ITS. In a previous study, LIPUS had stimulatory effects on differentiated chondrocytes which were stimulated by ITS for 14 days. Thus, cells were stimulated with LIPUS for 20 min/day (oscillatory frequency at 1.5 MHz ± 10%, pulse frequency at 1 kHz, transducer characteristics: ERA 3.9 cm ± 20% and BNR 3.5 ± 30%) and incubated for up to 14 days because LIPUS have stimulatory effects on differentiated chondrocytes which were stimulated by ITS for 14 days. LIPUS was applied using a sterilized transducer (OSTEOTRON D_2; Ito Co. Ltd., Tokyo, Japan) placed on the

surface of the culture medium. The distance between the transducer and cells was approximately 3–4 mm. Untreated cells were seeded at the same density but were not stimulated with LIPUS.

3. Real-time polymerase chain reaction (PCR)

Total RNA was isolated on days 3, 5, and 7 of LIPUS stimulation using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and the RNA concentration was measured with a Nano Drop 1000 (ND-1000; Thermo Fisher Scientific, Wilmington, DE, USA). cDNA was synthesized from 0.5 μg DNase-treated total RNA using a Prime Script RT reagent kit (Takara Bio, Shiga, Japan), and the resulting cDNA was analyzed by real-time PCR using a SYBR Green kit (Takara Bio). The primer sequences are shown in Table 1. PCR was performed with a Smart Cycler II instrument (Cepheid, Sunnyvale, CA, USA), and the data were analyzed using Smart Cycler software. The cycling conditions included 35 cycles at 95°C for 5 s and 60°C for 20 s. All real-time PCR experiments were performed in triplicate, and the specificity of the amplified products was verified by melting curve analysis. The calculated values of the target gene expression were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control [23].

4. Western blotting

Cells were cultured in serum-free medium after LIPUS treatment and harvested 24 h later. The total protein concentrations in cell lysates were quantified, and 20 μg of protein from each sample was resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polvinylidene fluoride membranes. The membranes were then treated with blocking reagent (1% [v/v] bovine serum albumin [BSA]) in Tris-buffered saline (TBS: 10 mM Tris, 145 mM NaCl, pH 7.4) for 18 h at 4°C, washed in Tween 20-containing TBS (TBS-Tween), and incubated with rabbit polyclonal IgG antibodies or mouse monoclonal IgG antibodies specific for extracellular signal-regulated kinase (ERK1/2: Cell Signaling Technology, Danvers MA, USA), phospho-ERK1/2 (p-ERK1/2: Cell Signaling Technology, Danvers MA, USA), sox9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), type II collagen (Col II: Santa Cruz Biotechnology, Santa Cruz, CA, USA), aggrecan (Santa Cruz Biotechnology, Santa Cruz, CA, USA), type X collagen (Col X: Santa Cruz Biotechnology, Santa Cruz, CA, USA), MMP13 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ADAMTS-5 (ABGENT, San Diego, CA ,USA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 or 1:1000 in blocking reagent (1% [v/v] BSA) for 1 h at room temperature. β-Actin was used as an internal loading control. Membranes were then washed in TBS-Tween and incubated with horseradish peroxidase-conjugated secondary antibodies

diluted 1:5000 in 1% blocking agent for 2 h at room temperature. Immunoreactive proteins were visualized using Image J software provided by the National Institutes of Health.

5. Histochemistry

ATDC5 cells were plated in 6-well plates and cultured for the indicated periods. Cells were then fixed with 95% methanol for 20 min. For Alcian blue staining, cells were stained with Alcian blue stain solution (pH 2.5; Muto Pure chemicals, Tokyo, Japan) overnight and rinsed with distilled water. The Alcian blue dye was extracted with 6 M guanidine HCl overnight. Total optical density of the extracted dye was measured at 620 nm, as previously described [22].

6. Statistical analysis

Data represent the results of three independent experiments with samples that were prepared in triplicate. Each value represents the mean ± standard error (SE). Differences between groups were evaluated with the one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test or two-way ANOVA with Bonferroni's multiple comparisons test. Differences with P values of less than 0.05 were considered statistically significant.

Results

1. LIPUS increased the expression of p-ERK1/2 in ATDC5

To clarify the first response to the downstream pathway of the LIPUS in ATDC5, I determined the protein expression of p-ERK1/2. p-ERK1/2 was increased in the intensity-dependent manner of LIPUS on 180 min after LIPUS stimulation (by 1.27-, and 1.62-fold, respectively; Fig. 1(a)).

2. The high intensity of LIPUS affected the expression of sox9

To clarify the effects of the intensity of LIPUS on the expression of the chondrocyte differentiation factor sox9 in ATDC5 cells, the expression of the sox9 gene and its protein was determined using real-time PCR and western blotting, respectively. Sox9 mRNA levels were increased by 60 mW/cm^2 LIPUS on day 5 of LIPUS stimulation (by 2.87-fold) compared with that in the untreated control. In contrast, LIPUS decreased sox9 mRNA levels on days 3 and 7 of stimulation compared with that in untreated control cells (by 0.73-, 0.52-, 0.44-, and 0.48-fold, respectively; Fig. 1(b)). Sox9 protein levels were also increased in an intensity-dependent manner on day 7 of LIPUS stimulation (by 1.67- and 2.22-fold, respectively) compared with that in untreated control cells (Fig. 1(c)).

3. Effects of the intensity of LIPUS on the mRNA expression of components of the ECM

Next, the effects of the intensity of LIPUS stimulation on the expression of ECM components were studied. Stimulation with 60 mW/cm^2 LIPUS increased mRNA levels of Col II by day 5 (by 3.12 -fold) compared with that in untreated control cells (Fig. 2(a)). Moreover, mRNA level of aggrecan were increased by LIPUS in an intensity-dependent manner on days 5 or 7 of LIPUS stimulation (by 2.06-, 3.13-, and 1.47-fold, respectively) compared with that in untreated control cells (Fig. 2(b)). Col X mRNA levels were increased by 60 mW/cm^2 LIPUS on day 7 of stimulation (by 1.23-fold) compared with that in the untreated control (Fig. 2(c)).

4. Effects of the intensity of LIPUS on the expression of ECM proteins

Then, the effects of the intensity of LIPUS stimulation on the expression of ECM proteins were studied. Stimulation with 60 mW/cm^2 LIPUS increased protein levels of Col II by day 5 of LIPUS stimulation (by 1.21-fold) compared with that in the untreated control (Fig. 3(a)). The protein expression of aggrecan was also increased in an intensity-dependent manner on day 5 of LIPUS stimulation (by 1.13- and 1.42-fold, respectively) compared with that in the untreated control (Fig. 3(b)). Col X protein levels were increased by LIPUS in an

intensity-dependent manner on day 7 (by 1.61- and 2.11-fold, respectively) compared with that in untreated control (Fig. 3(c)).

5. Effects of the intensity of LIPUS on the expression of MMP13, ADAMTS-5. and ALP and on Alcian blue staining

I next determined the effects of the intensity of LIPUS on MMP13 and ALP expression as markers of the differentiation of hypertrophic chondrocytes and investigated the mRNA expression of aggrecanase-5 (ADAMTS-5). The mRNA and protein levels of MMP13 were reduced by LIPUS at intensities of 30 and 60 mW/cm^2 on day 3 and 7 of stimulation (by 0.15-, 0.15-, 0.53-, 0.27-, 0.88-, and 0.86-fold, respectively) compared with that of the untreated control (Fig. 4(a) and (b)). In contrast, the proteinase of aggrecan ADAMTS-5 mRNA levels was remarkably increased on day 7 of 60 mW/cm^2 LIPUS stimulation (by 2.27-fold) compared to untreated control (Fig. 4(c)). ADAMTS-5 protein levels were also significantly increased by LIPUS in an intensity-dependent manner on day 7 (by 1.13- and 1.21-fold, respectively) compared to untreated control (Fig. 4(d)). However, the mRNA expression of ALP was not affected by LIPUS (Fig. 4(e)). LIPUS also did not affect Alcian blue staining, which stains chondrocyte-specific aggrecan, in chondrocytes on day 14 of stimulation (Fig. 4(f)).

Discussion

Chondrogenesis involves several stages, including differentiation of chondroprogenitors into chondrocytes and maturation of chondrocytes [2]. In the first stages of chondrogenesis, mesenchymal progenitor cells accumulate to form cartilage nodules and act as the chondroprogenitor cell lineage [24]. Chondrocyte progenitor cells are then differentiated into chondrocytes by chondrocyte lineage-specific transcription factors, including sox9, sox5, and sox6, which play critical roles in the proliferation and differentiation of chondroprogenitor cells [16, 25]. Differentiated chondrocyte progenitor cells are characterized by accumulation of cartilage matrix containing Col II and aggrecan [2]. In growth plate cartilage chondrocytes differentiate into a hypertrophic phenotype and express both Col X and ALP during the differentiation and maturation stages. Moreover, the final stage of chondrocyte differentiation is endochondral ossification, which involves cartilage matrix remodeling and vascular and bone cell invasion [2].

Mechanical stimuli act as regulators of musculoskeletal tissues and are important environmental factors responsible for joint homeostasis. Mechanical loading involves multiple joint components, including bone, muscles, articular cartilage, ligaments, and tendons. These components are sensitive to the magnitude, duration, and nature of mechanical stimuli [11]. In many previous studies, the mechanical stimulus of LIPUS increases ECM

synthesis and decreased MMP13 in chondrocyte which isolated from rat and rabbit [12, 17-19, 26]. These results demonstrated that LIPUS affected cartilage matrix synthesis, particularly by increasing collagen production in chondrocytes involved in osteoarthritis. Nishida et al. reported that LIPUS increases the expression of Col II and aggrecan via CCN family protein 2 (CCN2)-phosphorylation of ERK1/2 pathway in chondrocytes [27]. In the present study, LIPUS enhanced p-ERK1/2 levels after the stimulation for 180 min (Fig 1(a)). LIPUS also affected the mRNA and protein expression of Col II, Col X, and aggrecan, with high-intensity LIPUS increasing the expression levels of these targets. Moreover, LIPUS increased the mRNA and protein expression of sox9, which is a main factor affecting the differentiation chondrocytes. However, LIPUS also decreased the mRNA expression of sox9 on days 3 and 7 of LIPUS stimulation. According to previous reports, the chondrocyte lineage-specific transcription factor sox9 is expressed during the proliferation and differentiation of chondroprogenitor cells [16][25]. Thus, LIPUS affected the expression of sox9 during the proliferation and differentiation phases in ATDC5 cells. These results showed that LIPUS enhanced chondrocyte differentiation and ECM synthesis, that implicates ERK1/2 activation. Interestingly, chondrocytes responded to LIPUS stimulation with more dramatic changes in ECM synthesis and aggrecan expression than osteoblasts, as demonstrated in a previous study [28].

MMP13 is a member of the matrix metalloproteinase family of neutral endopeptidases and is expressed in the skeletal tissue during embryonic development. Chondrocytes and synovial cells, which are involved in rheumatoid arthritis, osteoarthritis, and human carcinoma, overexpress MMP13 [29]. Various proteinases, such as MMPs, have been implicated in the remodeling of ECM components. MMPs cleave aggrecan and collagen, which are the two most abundant ECM components in skeletal tissue [1]. Col II is secreted by resting and proliferating chondrocytes and is the primary fibrillar ECM component in the growth plate. Moreover, Coll II also plays a crucial role in establishing correct temporal and spatial organizational relationships with other ECM components, such as proteoglycans [1]. Aggrecan is a major proteoglycan found in the developing growth plate [1]. Aggerecan and Col II are also degraded during the very last stages of chondrocyte differentiation before vascular invasion [30].

MMP13-null mice show marked increases in the hypertrophic domain and exhibit delayed endochondral ossification. In addition, MMP13-null mice show significant interstitial collagen accumulation [29]. However, MMP13-null mice do not exhibit positive staining with Alcian blue, which marks chondrocyte-specific aggrecan [1, 29]. In the present study, LIPUS increased Col II and Col X expression, but decreased MMP13 expression in ATDC5 cells. Interestingly, LIPUS did not affect aggrecan production, as measured by Alcian blue staining,

compared with that in untreated control cells; however, LIPUS increased aggrecan mRNA and protein expression. To clarify why LIPUS did not increase aggrecan production in chondrocytes, I investigated the effects of LIPUS on the expression of aggrecanase-5 (ADAMTS-5), a major proteinase targeting aggrecan [11]. LIPUS remarkably increased the expression of ADAMTS-5 mRNA and protein on day 7 of culture (Fig. 4(c) and (d)). As a possible mechanism, LIPUS may induce the remodeling of aggrecan, even though aggrecan production was not affected. Thus, in future studies, I will aim to clarify the effects of LIPUS on aggrecan remodeling via ADAMTS-5 in chondrocytes. Taken together, these results were consistent with the phenotypes of MMP13-null mice.

In summary, LIPUS induced collagen synthesis, aggrecan remodeling, and increased the activation of ERK1/2, but decreased MMP13 expression in chondrocytes. Furthermore, the response to chondrocytes to the intensity of LIPUS was higher than that in osteoblasts.

Conclusion

These results suggested that LIPUS could induce ECM regeneration by increasing hypertrophic chondrocytes and by delaying endochondral ossification in chondrocytes. These findings suggest that LIPUS uses not only the tool of bone fracture treatment, but also may have a possibility to treat patients who have various diseases (such as osteoarthritis, temporomandibular disorder and knee joint dysfunction) requiring ECM regeneration in

cartilage.

Acknowledgements

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Table 1. PCR primers used in the experiments

| Target	Primers	GenBank Acc.
sox9	5'- GGACAACACATGCCTCTGCAA-3' 5'-TCTCCAGCCACAGCAGTGAGTAA -3'	NM_01148.4
aggrecan	5'-AGTGGATCGGTCTGAATGACAGG -3' 5'-AGAAGTTGTCAGGCTGGTTTGGA -3'	NM_007424.2
Col ll	5'-CATCCAGGGCTCCAATGATGTA -3' 5'- ATGTCCATGGGTGCGATGTC-3'	NM_031163.3
Col X	5'-ATAAGAACGGCACGCCTACGA -3' 5'-CAATTGGAGCCATACCTGGTCA -3'	NM_009925.4
MMP13	5'- TCCCTGGAATTGGCAACAAAG-3' 5'- GCATGACTCTCACAATGCGATTAC-3'	NM_008607.2
ADAMTS-5	5'-GAGACTGTGCGGTCCAGGTCTA -3' 5'-CAGGGCTTGACCCAATGACTTA -3'	NM_011782.2
ALP	5'-GTGGCCGCAAGTTCATGTTTC -3' 5'-AGCTCTGAGCGGTTCCAAACAT -3'	NM_007433.3
GAPDH	5'-AAATGGTGAAGGTCGGTGTG-3' 5'-TGAAGGGGTCGTTGATGG-3'	NM_008084.2

GAPDH, glyceraldehyde-3-phosphate dehydrogenase
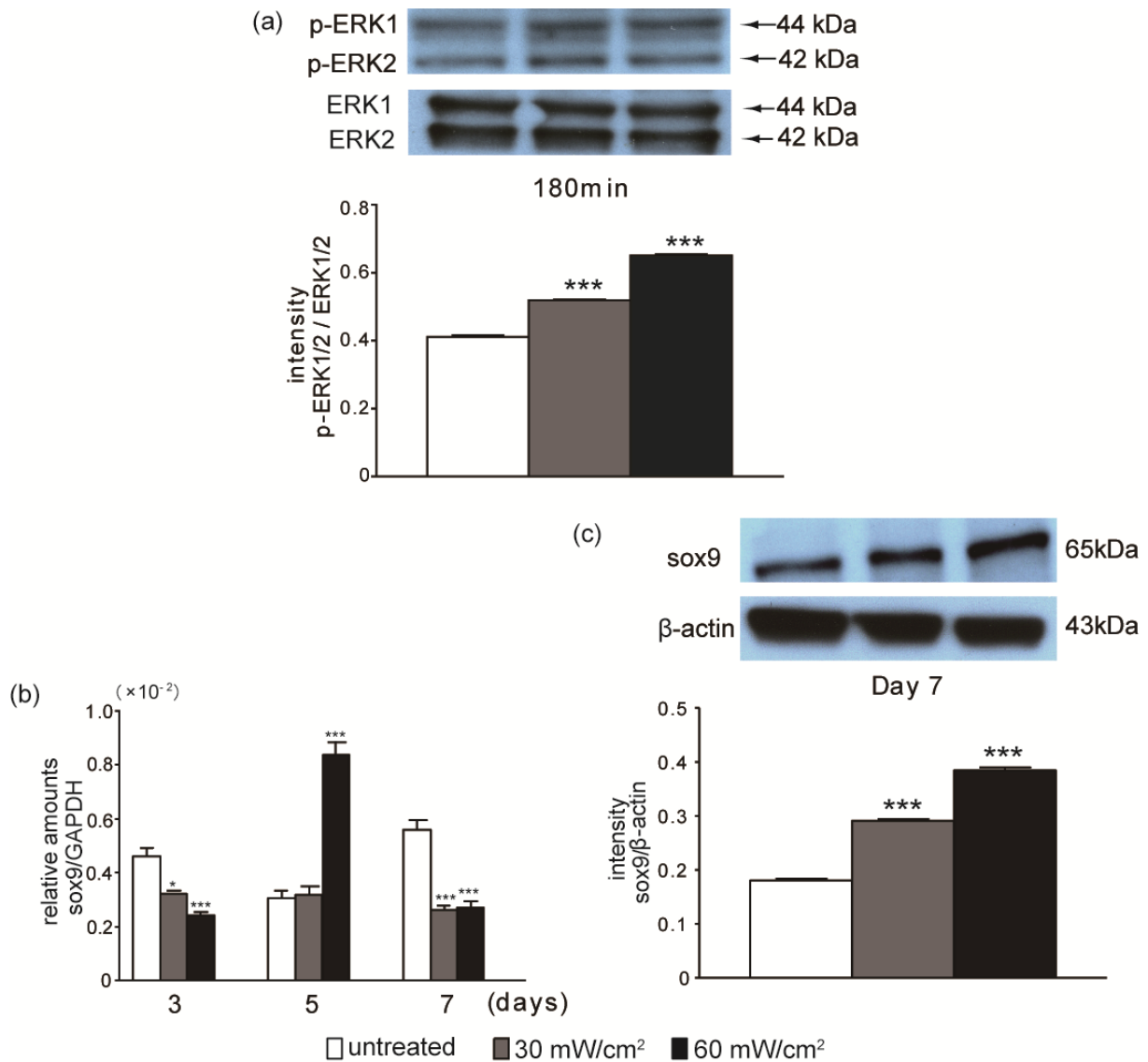
Fig. 1. Effects of LIPUS and the intensity of LIPUS on the expression of phospho-ERK1/2 (p-ERK1/2) and sox9 expression

ATDC5 cells were incubated for 14 days with culture medium containing ITS. Cells were then stimulated with LIPUS for 20 min (intensities of 30 and 60 mW/cm^2). Samples were collected after LIPUS stimulation for 180 min. The levels of ERK1/2 and p-ERK1/2 were determined by western blotting (a).

Cells were treated as described in (a). Cells were then stimulated with LIPUS for 20 min/day and incubated for up to 7 days. The expression of sox9 mRNA was determined after 3, 5, and 7 days of LIPUS stimulation using real-time PCR (b). *$p < 0.05$, ***$p < 0.001$ for LIPUS stimulation versus the untreated control. Cells were treated as described in (b), and protein expression of sox9 was determined by western blotting on day 7 of LIPUS stimulation (c). Histograms show the intensity of western blotting bands for each condition. ***$p < 0.001$ for LIPUS versus the untreated control.
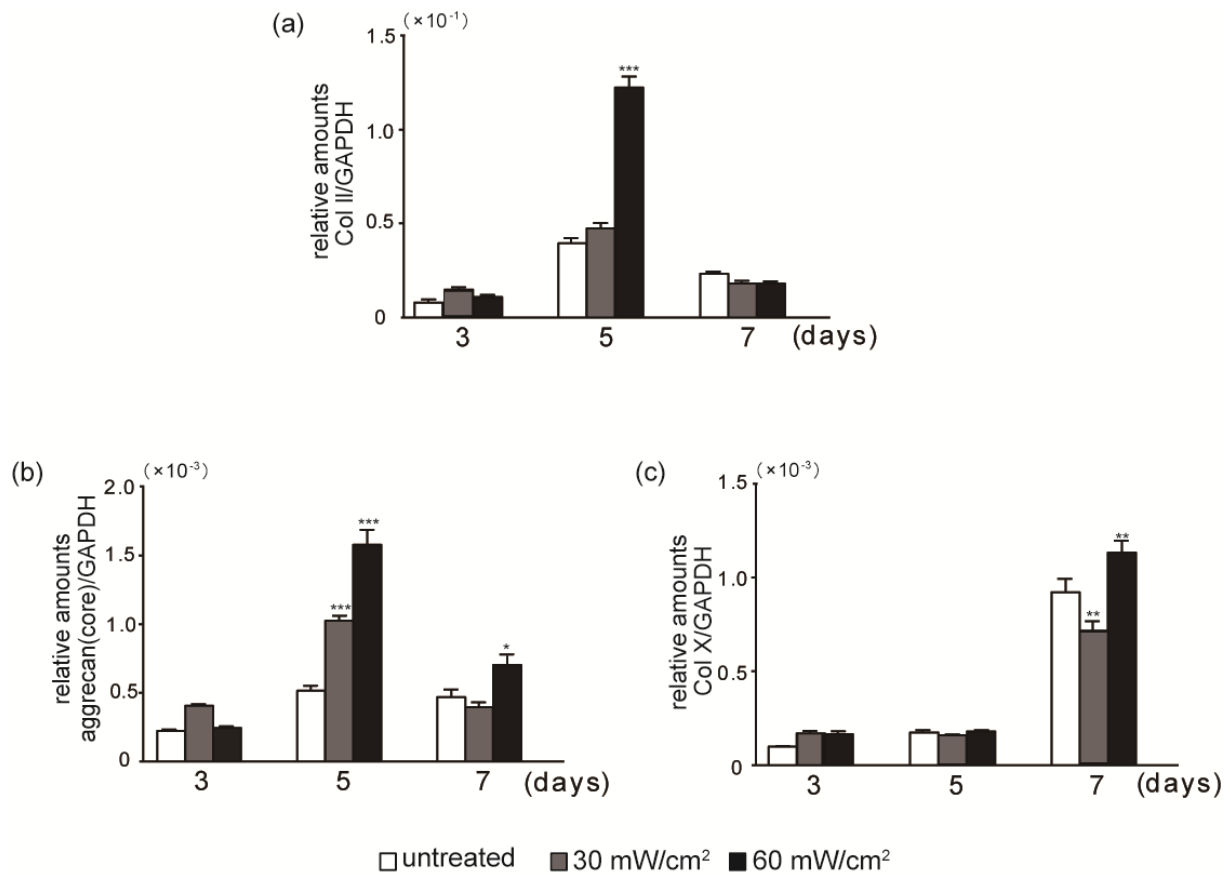
Fig. 2. Effects of the intensity of LIPUS on the mRNA expression of ECM components

ATDC5 cells were incubated for 14 days with culture medium containing ITS. Cells were then stimulated with LIPUS for up to 7 days (intensities of 30 and 60 mW/cm^2), and the expression levels of Col II (a), aggrecan (b), and Col X (c) mRNAs were determined at 3, 5, and 7 days of stimulation using real-time PCR. *p < 0.05, **p < 0.01 and ***p < 0.001 for LIPUS stimulation versus the untreated control.
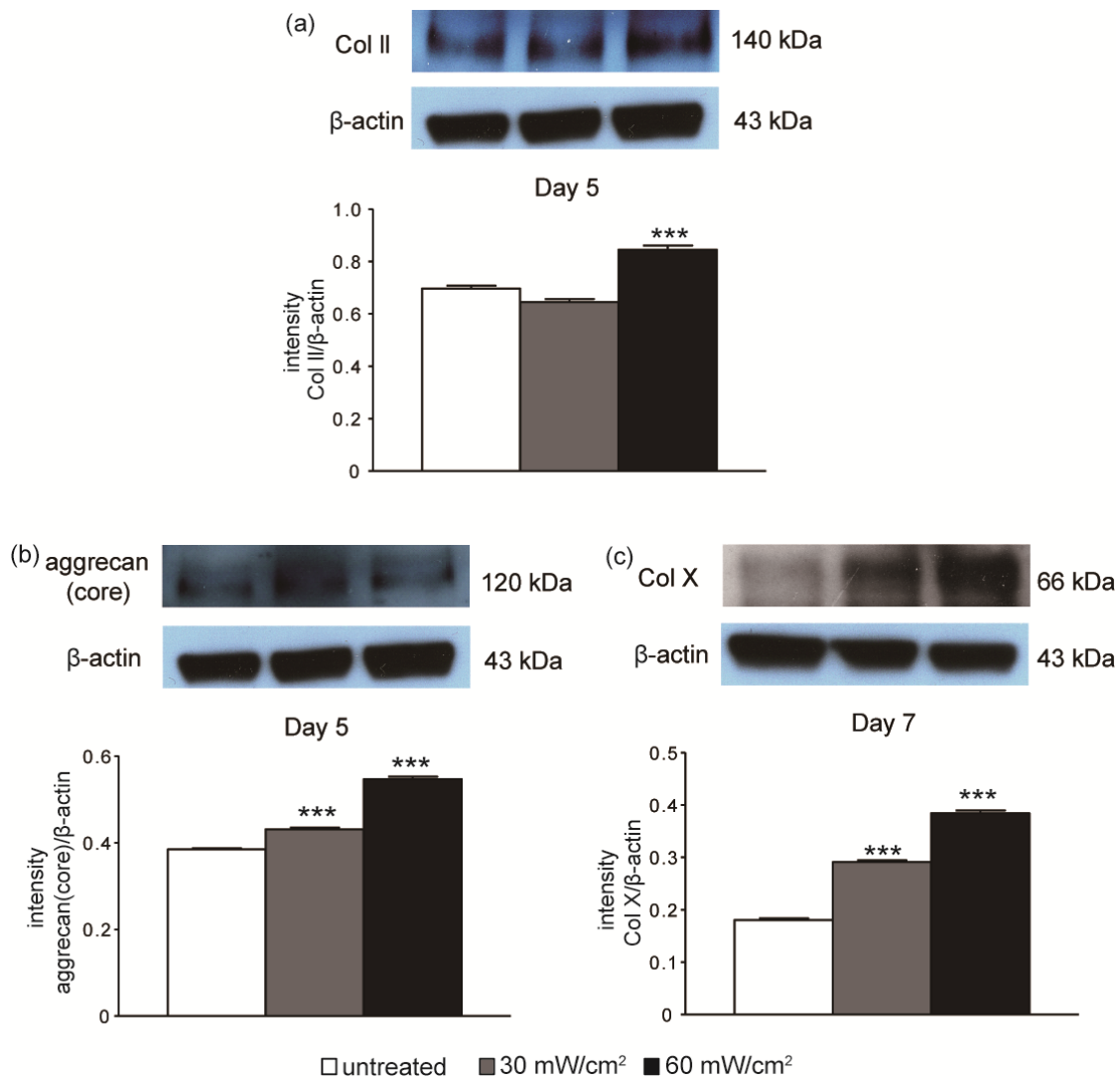
Fig. 3. Effects of the intensity of LIPUS on the protein expression of ECM components

ATDC5 cells were incubated for 14 days with culture medium containing ITS. Cells were then stimulated with LIPUS for 20 min/day (intensities of 30 and 60 mW/cm^2) and incubated for up to 7 days. Protein expression levels of aggrecan (a), Col II (b), and Col X (c) were determined by western blotting on days 5 or 7 of LIPUS stimulation. Histograms show the intensity of western blotting bands for each condition. ***$p < 0.001$ for LIPUS stimulation versus the untreated control.
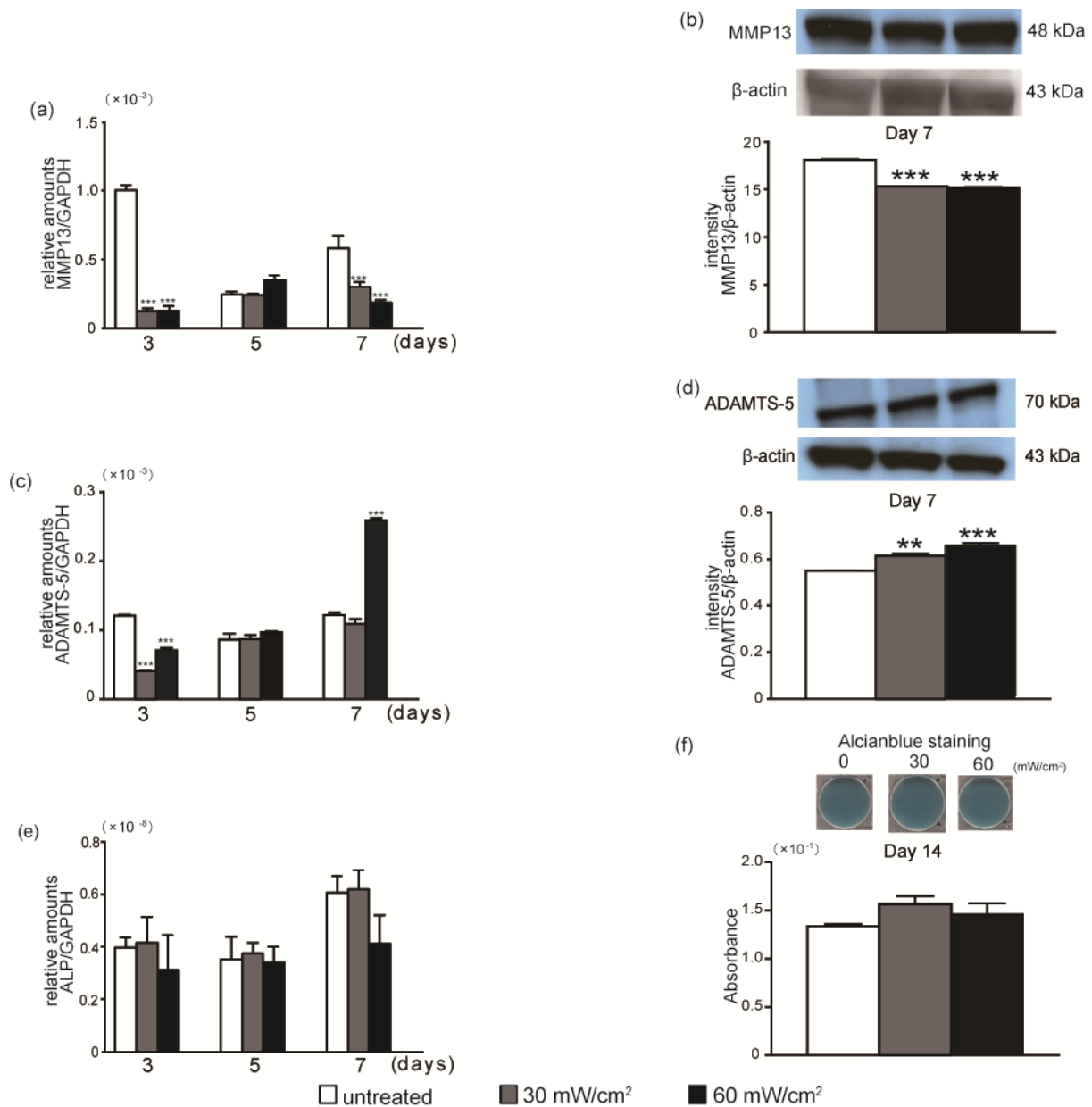
Fig. 4. Effects of LIPUS on the expression of MMP13, ADAMTS-5, ALPase and Alcian blue staining

ATDC5 cells were incubated for 14 days with culture medium containing ITS. Cells were then stimulated with LIPUS for 20 min/day (intensities of 30 and 60 mW/cm^2) and incubated for up to 7 days. The mRNA expression levels of MMP13 (a), ADAMTS-5 (c) and ALPase

(e) were then determined at 3, 5, and 7 days of LIPUS stimulation using real-time PCR, and the protein expression of MMP13 (b) and ADAMTS-5 (d) was determined by western blotting on day 7 of LIPUS stimulation. ***$p < 0.001$ for LIPUS stimulation versus the untreated control. Histograms show the intensity of western blotting bands for each condition. **$p < 0.01$, ***$p < 0.001$ for LIPUS stimulation versus the untreated control. Cells were stained with Alcian blue (upper images). Histograms show Alcian blue dye intensity, measured at 620 nm (f).