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Low-Intensity Ultrasound Inhibits Apoptosis and Enhances Viability of Human Mesenchymal Stem Cells in Three-Dimensional Alginate Culture During Chondrogenic Differentiation

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ABSTRACT

Many studies have investigated optimal chondrogenic conditions, but only a few of them have addressed their effects on cell viability or the methods to enhance it. This study investigated the effect of low-intensity ultrasound (LIUS), a well-known chondrogenic inducer, on the viability of human mesenchymal stem cells (hMSCs) during chondrogenic differentiation in three-dimensional (3-D) alginate culture. The hMSCs/alginate layer was cultured in a chondrogenic defined medium and treated with transforming growth factor-beta1 (TGF-β1) and/or LIUS for 2 weeks. Along with chondrogenic differentiation for 2 weeks, the 3-D alginate culture and TGF-β1 treatment resulted in the decrease of cell viability, which appeared to be mediated by apoptosis. In contrast, co-treatment with LIUS clearly enhanced cell viability and inhibited apoptosis under the same conditions. The effect of LIUS on the apoptotic event was further demonstrated by changes in the expression of apoptosis/viability related genes of p53, bax, bcl-2, and PCNA. These results suggest that the LIUS treatment could be a valuable tool in cartilage tissue engineering using MSCs as it enhances cell viability and directs the chondrogenic differentiation process, its well-known activity.

INTRODUCTION

MESENCHYMAL STEM CELLS (MSCs) have been widely studied as an alternative cell source for cartilage tissue engineering, due mostly to their ability to differentiate into chondrogenic lineage and the phenotypic stability maintained for multiple passages.[1] MSCs require specific culture conditions, such as three-dimensional (3-D) environment and growth factors, particularly transforming growth factor-beta (TGF-β).[2,3] However, the 3-D culture condition does not support well cell proliferation and even supply of nutrients.[4] The TGF-β treatment also inhibits cell proliferation and induces in some cases apoptosis of cells.[5] It could be particularly a problem considering that cartilage tissue engineering mainly aims at the repair of damaged tissues from old patients suffering, for example, from osteoarthritis (OA). Autologous MSCs obtained from aged patients have limited capacity for cell proliferation and viability when compared to young and fresh MSCs.[6] Therefore, well maintaining the viability and activity of MSCs would be very important for their efficient chondrogenic differentiation in cartilage tissue engineering.

Recently, biomechanical stimulations such as cyclic hydrostatic pressure and low-intensity ultrasound (LIUS) were also found to be effective tools in directing MSCs into chondrogenesis.[7–13] LIUS was already known to improve repair of damaged cartilage in animal studies[14–17] and enhance the synthesis of matrix proteins such as collagen type II

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and proteoglycans in chondrocyte cultures.[14,15,18–20] However, the effect of LIUS on the chondrogenic differentiation of MSCs was reported only recently. Ebisawa *et al*. showed that LIUS treatment enhanced TGF-β-mediated aggrecan deposition in pellet culture of human MSCs (hMSCs).[10] We also showed that LIUS stimulated chondrogenic differentiation of rabbit MSCs both in alginate culture *in vitro*[11] and in polyglycolic acid (PGA) as a scaffold *in vivo*,[12,13] by enhancing the matrix formation and expression of chondrogenic markers such as aggrecan, type II collagen, and Sox-9.

Although LIUS is shown to have protective effects on chondrocytes and enhance healing of damaged cartilage in animal studies, direct effect of LIUS on cell viability in chondrocytes and MSCs is not yet well understood. We recently demonstrated that LIUS treatment enhanced the viability of human articular chondrocytes obtained from OA patients in 3-D alginate culture system.[15] The present study was performed to investigate the effect of LIUS treatment on the viability of hMSCs under chondrogenic differentiation in 3-D alginate culture, particularly in association with an apoptotic event.

MATERIALS AND METHODS

Isolation and culture of human bone marrow MSCs

Bone marrow samples were obtained from five hematologically normal patients undergoing routine total hip replacement surgery with informed consent. The patients aged 64 on average, ranging from 55 to 73. Primary culture of bone marrow MSCs was established as previously described. In brief, marrow cells were harvested in Dulbecco's phosphate-buffered saline (DPBS; Gibco BRL, Carlsbad, CA) from trabecular bone marrow samples and pelleted by centrifugation at 500 *g* for 5 min at room temperature. The cell pellet was resuspended in 10 mL alpha-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and passed through nylon mesh (90 μm pore size; Lockertex, Warrington, England). Cell suspensions were stained with 0.4% (wt/vol) trypan blue to determine the number and viability of cells.[21] Cells were then plated (1.5×10^7 cells) on a 150 mm culture plate and incubated at 37°C under 5% carbon dioxide (CO_2). After 6 days, nonadherent hematopoietic cells were removed and MSCs on the surface of culture plates were replenished with fresh medium supplemented with 10% FBS. MSCs were expanded in a monolayer culture with the culture medium changed twice per week and passaged every 1–2 weeks until passage 3, when they were moved to 3-D alginate cultures for chondrogenic differentiation.

Chondrogenic differentiation of hMSCs in alginate layer culture

The alginate layer culture of hMSCs was performed as described previously.[11,22] The hMSCs were suspended in 2% alginate (Sigma, St. Louis, MO) solution at the density of 2×10^6 cells/mL. The cell/alginate mixture was added slowly into a 12-well transwell insert and spread evenly on the surface. The transwell was then immersed in 1 mL of sterile $CaCl_2$ solution (102 mM) for 10 min. The alginate layer was then washed twice with 0.15 M NaCl for 10 min each and once with serum-free chondrogenic defined medium containing high glucose Dulbecco's modified Eagle's medium (DMEM-HG), ITS supplement (1.0 mg/mL insulin from bovine pancreas, 0.55 mg/mL human transferrin, and 0.5 μg/mL sodium selenite), 50 μg/mL ascorbic acid 2-phosphate, 100 nM dexamethasone, 40 μg/mL proline, 1.25 mg/mL bovine serum albumin (BSA), and 100 μg/mL sodium pyruvate (all from Sigma).[4] Alginate layers were then moved to 35 mm culture dishes and overlaid with serum-free chondrogenic defined medium for treatment.

Treatment with ultrasound and TGF-β1

The alginate layer cultures were treated with 10 ng/mL TGF-β1 (PeproTech, Rehovot, Israel) and LIUS as described previously.[11] Briefly said, LIUS was applied for 20 min everyday until 1 or 2 weeks at a frequency of 1 MHz and an intensity of 200 mW/cm^2 in a continuous wave fashion. The equipment used for ultrasound stimulation was Noblelife™ (Duplogen, Suwon, Korea) that has three transducers of 35 mm in diameter and controllers for intensity and time. During the LIUS treatment, the cultures were placed in a humidified incubator at 37°C under 5% CO_2 for 1 or 2 weeks with the culture medium changed every other day.

Examination of cell viability

After treatment, alginate layers were washed twice with 0.15 M NaCl and incubated in three volumes of 55 mM sodium citrate solution (Sigma) at 37°C for 15 min to dissolve the layers for cell recovery. The viability of cells was determined by staining them with 0.4% trypan blue.

Lactate dehydrogenase (LDH) activity assay

The cytotoxicity of cells was assessed using the cytotoxicity detection kit plus (Roche, Mannheim, Germany) by measuring the activity of lactate dehydrogenase (LDH) released into the culture medium from damaged cells. The culture supernatant of hMSCs/alginate layer was collected directly and incubated with the reaction mixture from the kit according to the manufacturer's instructions. Absorbance at 492 nm was measured using an ELISA reader.

Live/dead cytotoxicity assay

The percentage of viable and dead cells was measured using a Live/Dead Viability/Cytotoxicity assay (Molecular Probe, Eugene, OR) according to the manufacturer's instructions. The hMSCs/alginate layer was incubated in a solution containing 2 mM ethidium homodimer-1 and 4 mM

calcein AM. After washing in PBS, the construct was placed on a slide glass and pressed flat carefully using a cover glass. The fluorescence image was visualized using a laser-scanning confocal microscope (Zeiss LSM510 Meta; Carl Zeiss, Jena, Germany). The number of viable (green fluorescence) or dead (red fluorescence) cells was quantified by Image-Pro Plus 4.0 image analysis software (Media Cybernetics, Silver Spring, MD). The viability of cells was determined by dividing the number of green cells (viable cells) with the total cells (green cells + red cells).

TUNEL assay

The hMSCs/alginate layers were washed in PBS and fixed for 1 h in 4% formaldehyde, then dehydrated and embedded in paraffin wax. Sections of 5 μm in thickness were prepared. Apoptosis was examined using a colorimetric FragEL DNA Fragmentation Detection kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. The color was developed with a DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA) using 0.1% methyl green as a counter stain. Stained images were captured using a digital camera (DP 50; Olympus, Shibuya-ku, Tokyo) and the positive signals were quantified by Image-Pro Plus 4.0 image analysis software (Media Cybernetics).

Bax/bcl-2 expression by immunohistochemistry

The paraffin sections of hMSCs/alginate were prepared as described above and processed for immunohistochemical staining according to the standard procedure described previously.[12,15] Monoclonal antibodies to bax (1:500; Calbiochem, CA) and bcl-2 (1:200; Calbiochem, CA) were treated for 1 h at room temperature. The signals were detected using biotinylated antimouse secondary antibody and peroxidase-conjugated avidin system according to the manufacturer's instructions (Vector Laboratories). The immunostained sections were counterstained with Mayer's hematoxylin (Sigma) before microscopic examination (Nikon E600; Nikon Co., Tokyo, Japan).

Western blot analysis

Cell extracts were prepared using lysis buffer (40 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40, 100 μg/mL phenylmethylsulphonylfluoride [PMSF], and 2 μg/mL each of aprotinin, pepstatin, and leupetin). The protein concentration was determined by the BCA assay (Sigma). The proteins were separated on an 8% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Protran, Dassel, Germany). After blocking with 5% nonfat dried milk, the blots were incubated overnight at 4°C with each of primary antibodies to p53 (53 kDa, 1:2000; Oncogene, Cambridge, MA), bax (21 kDa, 1:1000; Calbiochem, San Diego, CA), bcl-2 (24–26 kDa, 1:500; Calbiochem, San Diego, CA), PCNA (36 kDa, 1:2000; Upstate Biotech, Charlottesville, VA), and actin (1:5000; Calbiochem). The blots were then incubated with sheep antimouse IgG conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. The immunoreactive signals were detected using enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Buckinghamshire, UK).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from hMSCs in alginate layers using Trizol reagent (Invitrogen, Carlsbad, CA). A total RNA of 1 μg was used for the first strand cDNA synthesis using Superscript First Strand Synthesis System (Roche, Indianapolis, IN), and 2 μg of the synthesized cDNA was used for PCR. The PCR reactions were performed using an AccuPower PCR Premix (Bioneer, Daejeon, Korea) under the following conditions: initial incubation (10 min at 94°C), 30 cycles of annealing (30 s at 55°C), extension (60 s at 72°C) and denaturation (60 s at 94°C), and termination reaction (10 min at 72°C). Sequences of specific primers were as follows: Sox9 (sense: 5′-cacacagctcactcgaccttg-3′, antisense: 5′-ttcggttatttttaggatcatctcg-3′), type II collagen (sense: 5′-gat attgcacctttggacat-3′, antisense: 5′-cccacaatttaagcaagaag-3′), and aggrecan (sense: 5′-gaaaggtgttgtgttccact-3′, antisense: 5′-gtcataggtctcgttggtgt-3′). GAPDH (sense: 5′-ggacatgagtccttc cacgat-3′, antisense: 5′-ggtgaaggtcggagtcaacgg-3′) was used as control. To exclude possible genomic DNA contamination, RT-PCR was also carried out using total RNA without reverse transcriptase.

Statistical analysis

The data are presented as mean ± standard deviation (SD) from five independent experiments ($n = 5$). The results were analyzed by one-way analysis of variance (ANOVA) and Newman-Keuls test using SPSS software. Statistical significance was given as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

LIUS treatment enhanced viability of hMSCs during chondrogenic differentiation

The effect of chondrogenic differentiation condition (chondrogenic medium, 3-D alginate culture, and TGF-β1 treatment) and LIUS on the viability of hMSCs was examined by trypan blue exclusion, LDH assay, and LIVE/DEAD assay (Figs. 1 and 2). The viability of hMSCs in the 3-D alginate culture (US0 group) decreased with time, resulting in the viability of 50–60% remaining at 2 weeks. The TGF-β1 treatment further decreased cell viability to approximately 40% and 30% after 1 and 2 weeks, respectively (US0 + T group). The LIUS treatment alone slightly enhanced cell viability at 1 week (US200 group) and clearly inhibited the effects of TGF-β1 by 28% and 23% at 1 and 2 weeks,

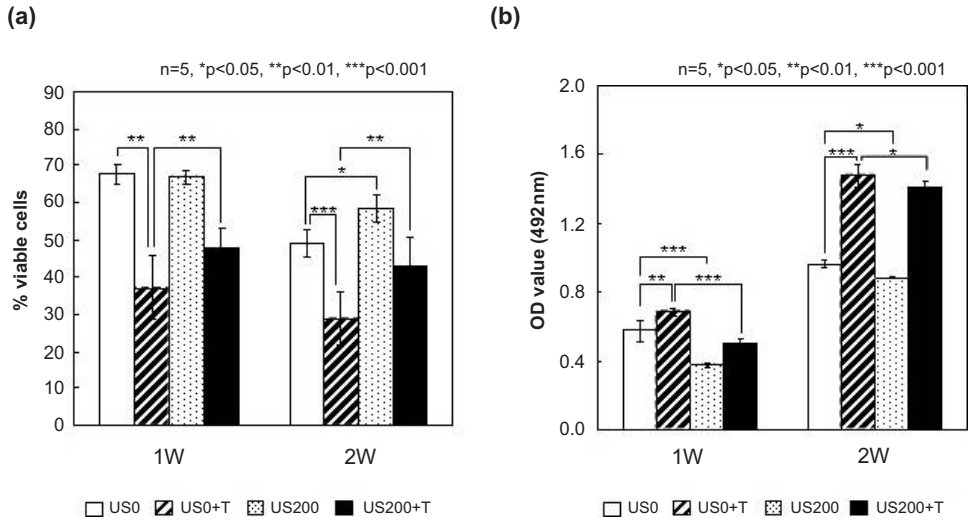

FIG. 1. Effects of LIUS on the viability of hMSCs in alginate layer. (**A**) hMSCs in alginate culture were induced to chondrogenic differentiation as described in Materials and Methods. Cells were recovered from the alginate layer at 1 or 2 weeks and their viability was measured by staining with 4% trypan blue. The percentage of viable cells was presented in the histogram from five independent experiments ($n = 5$). (**B**) Cell damage after chondrogenic differentiation of hMSCs was determined by LDH cytotoxicity assay in the same experiment. The amount of LDH released from damaged cells into the medium was measured at 492 nm ($n = 5$). The data were presented as mean ± SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

respectively (US200 + T group) (Fig. 1A). The cytotoxicity assay, which measured the amount of LDH in the culture medium, also showed that LIUS suppressed the cytotoxicity of TGF-β1 by about 26% at 1 week (Fig. 1B). The effect of LIUS was not clearly seen at 2 weeks when the cytotoxicity of TGF-β1 was very high (a 50% increase compared to the untreated control). The LIUS effect was finally confirmed by the LIVE/DEAD cytotoxicity assay, which showed quite similar results in the stained images (Fig. 2A) and in the histogram (Fig. 2B) to those of the trypan blue exclusion.

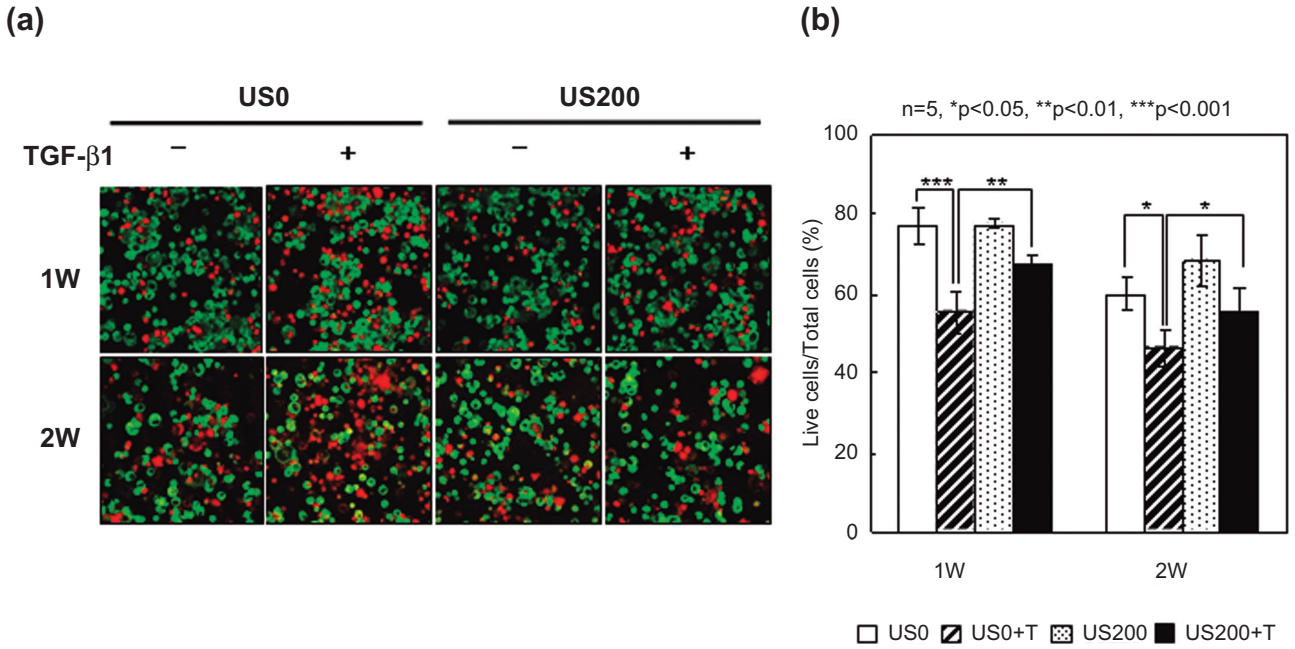

FIG. 2. Effect of LIUS on the hMSCs viability by Live/Dead Viability/Cytotoxicity assay. hMSCs in chondrogenic differentiation were first recovered from the alginate layer at 1 and 2 weeks, and stained with Live/Dead assay system. (**A**) The green color indicates viable cells and red color indicates dead cells in the stained images. (**B**) The percentage of viable cells was presented from five independent experiments ($n = 5$). The data were presented as mean ± SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Color images available online at www.liebertpub.com/ten.

LIUS treatment inhibited apoptosis of hMSCs during chondrogenesis

We investigated if an apoptotic process was involved in the decrease of cell viability and the LIUS effects. When the same set of experiments as above was examined by TUNEL staining, the 3-D alginate culture alone resulted in the apoptosis of hMSCs in a time-dependent manner and the TGF-β1 treatment further aggravated it by approximately 1.5-fold (Fig. 3). However, LIUS treatment clearly reduced the apoptotic events in both cases, with or without TGF-β1 treatment. The LIUS effect on the apoptosis of hMSCs was also confirmed by the bax/bcl-2 ratio examined by immunohistochemical staining at 1 week (Fig. 4). The TGF-β1 treatment enhanced the number of cells positive for bax expression, an apoptotic marker, while inhibiting that of the bcl-2 positive cells. The LIUS treatment clearly inhibited the TGF-β1 effects, leading to the decrease in the bax/bcl-2 ratio. Interestingly, the bax/bcl-2 ratio in the co-treatment group (US200 + TGF-β1) was even lower than that of the control group. This result suggests that the LIUS treatment inhibited apoptosis of hMSCs induced by the 3-D alginate culture and/or TGF-β1 treatment during chondrogenesis.

LIUS treatment inhibited expression of apoptosis-related genes

The LIUS effect was further confirmed on the expression of apoptosis and cell viability related genes such as p53, bax, bcl-2, and PCNA by RT-PCR or Western blot analysis at 1 week after treatment (Fig. 5). The TGF-β1 treatment induced mRNA level of bax but reduced that of bcl-2, which was inhibited by LIUS treatment (Fig. 5A). The protein levels of bax and bcl-2 were correlated with their mRNA levels, reproducing well the TGF-β1 and LIUS effects (Fig. 5B). The protein level of p53 tumor suppressor, a well-known mediator of apoptosis, was also induced by TGF-β1 treatment, which was reduced again by the LIUS treatment (Fig. 5B). In contrast, the protein level of PCNA, a marker for cell proliferation, was marginally induced by TGF-β1 while it was significantly induced by LIUS treatment.

LIUS treatment enhanced chondrogenic differentiation of hMSCs

The effect of LIUS treatment on the chondrogenesis of hMSCs was examined by RT-PCR analysis. As shown in Figure 6, the LIUS treatment alone induced the expression of Sox-9, aggrecan, and type II collagen as much as TGF-β1 treatment alone did, which was similar to our previous results for rabbit MSCs.[11,12] The co-treatment of LIUS and TGF-β1 resulted in the additive effects on their expression, depending on the time points and genes, with the most significant effects in type II collagen expression at 1 week (Fig. 6B). The aggrecan expression was already high enough at 2 weeks in the control group, and significant induction was observed only in the LIUS treated groups. The Sox-9 expression was detected, though at low levels, from

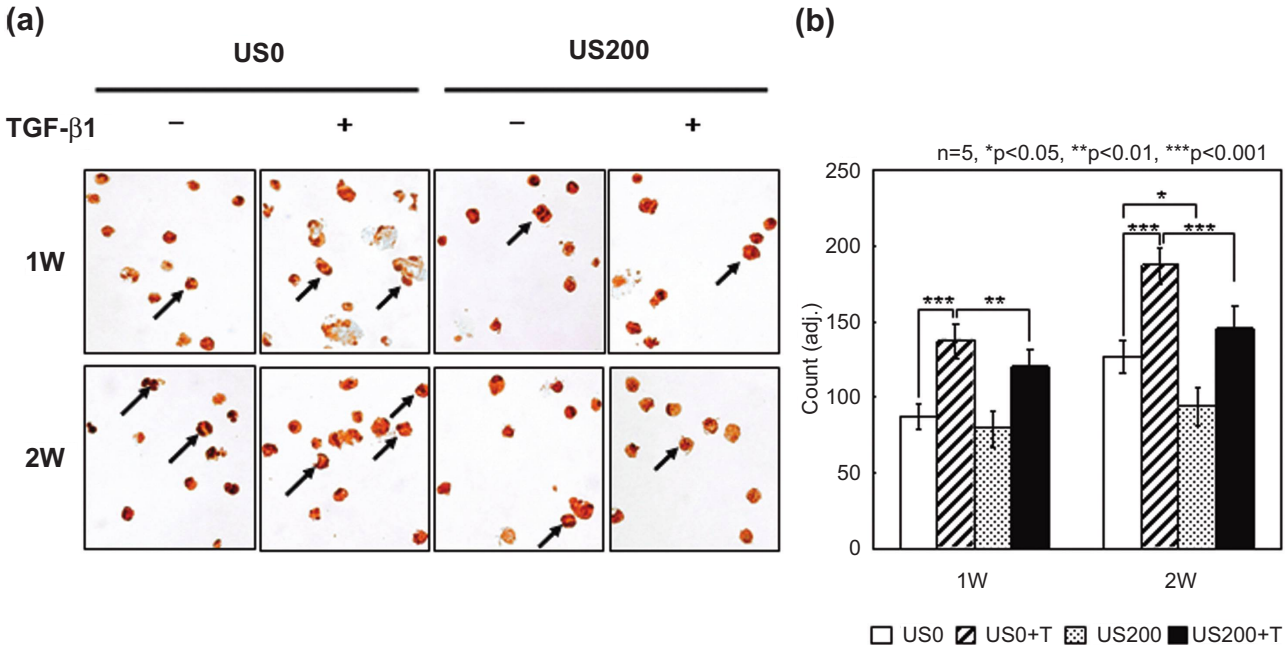

FIG. 3. Effects of LIUS on the apoptosis of hMSCs during chondrogenic differentiation. Thin sections of hMSCs/alginate layer were stained with FragEL DNA Fragmentation Detection kit at 1 and 2 weeks after chondrogenic differentiation. (**A**) The fragmented DNA of apoptotic cells was visualized in the images (arrows). (**B**) The number of apoptotic cells was counted and the results from five independent experiments were presented in the histogram. The data were presented as mean ± SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Color images available online at www.liebertpub.com/ten.

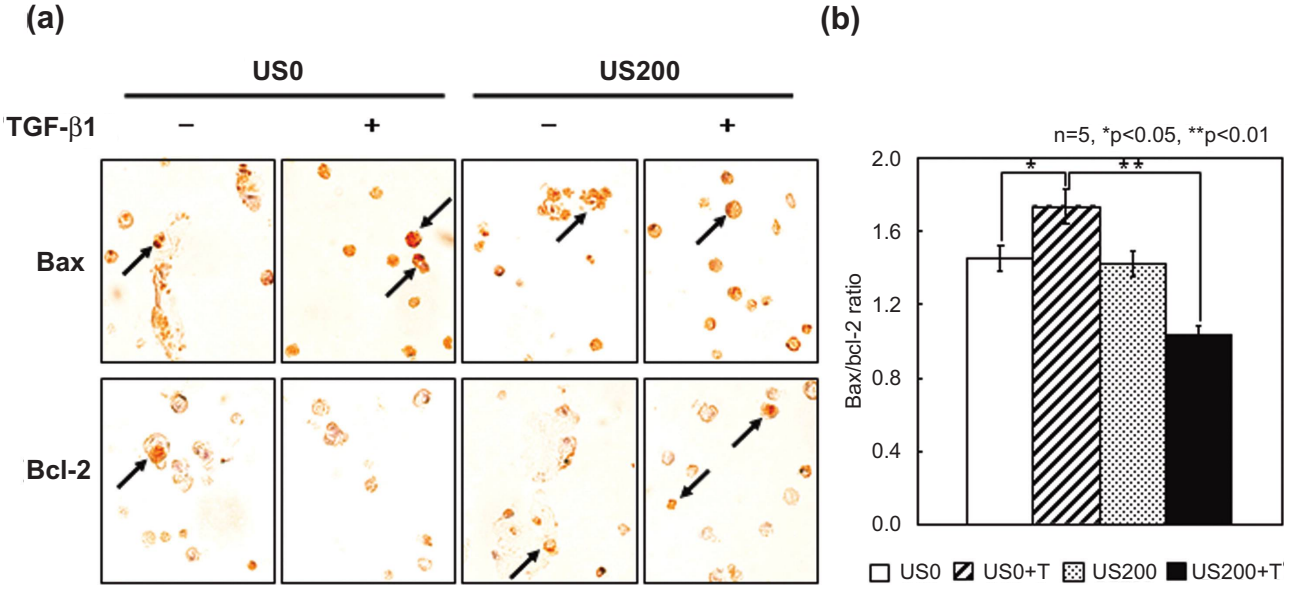

FIG. 4. Effects of LIUS on the bax/bcl-2 ratio in hMSCs under chondrogenic differentiation. Thin sections of hMSCs/alginate layer were immunostained for bax or bcl-2 at 1 week after chondrogenic differentiation. (**A**) Stained images show positive signals for bax or bcl-2 expression (arrows). (**B**) The ratio of bax/bcl-2 positive cell numbers was presented. The results were presented as mean ± SD from five independent experiments. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Color images available online at www.liebertpub.com/ten.

1 week in the control group without any treatment. Although it was further induced by TGF-β1 and/or LIUS treatments, no time-dependent increase was observed. These results suggest that LIUS induced chondrogenic differentiation of hMSCs even without TGF-β1 treatment.

DISCUSSION

In this report, we showed that LIUS enhanced the viability of hMSCs cultured in the 3-D alginate layers (Figs. 1 and 2). The 3-D alginate culture itself resulted in the decrease of cell viability, and TGF-β1 treatment further reduced it with increase in culture time. The decrease in cell viability appeared to be mediated by apoptosis, which was inhibited by the LIUS treatment (Fig. 3). The LIUS effect on the apoptotic events was further supported by the bax/bcl-2 ratio and the expression of apoptosis/viability related genes of p53, bax, bcl-2, and PCNA (Figs. 4 and 5). These results suggest that the LIUS treatment could exert a beneficial effect on the chondrogenic differentiation of hMSCs by inhibiting apoptosis and enhancing cell viability.

Mechanical stimuli are important factors that maintain the normal structure and function of articular cartilage and

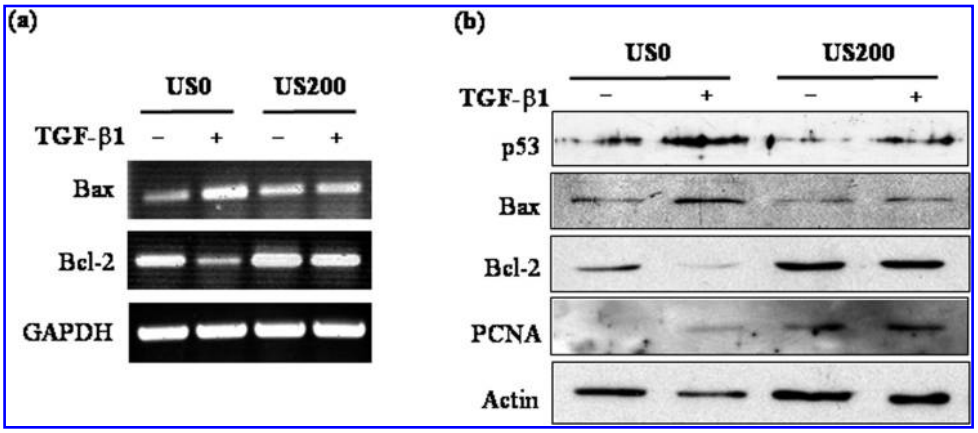

FIG. 5. Effects of LIUS on the expression of apoptosis and cell viability related genes. hMSCs under chondrogenic differentiation were recovered at 1 week for gene expression analysis. (**A**) Total RNAs were extracted from the recovered hMSCs, and mRNA levels of bax (181 bp) and bcl-2 (248 bp) were measured by RT-PCR analysis. The mRNA level of GAPDH (495 bp) was measured as an internal control. (**B**) Total proteins were extracted and the levels of p53 (53 kDa), bax (21 kDa), bcl-2 (24–26 kDa), and PCNA (36 kDa) were measured by Western blot analysis using specific antibodies. The level of actin was measured as an internal control.

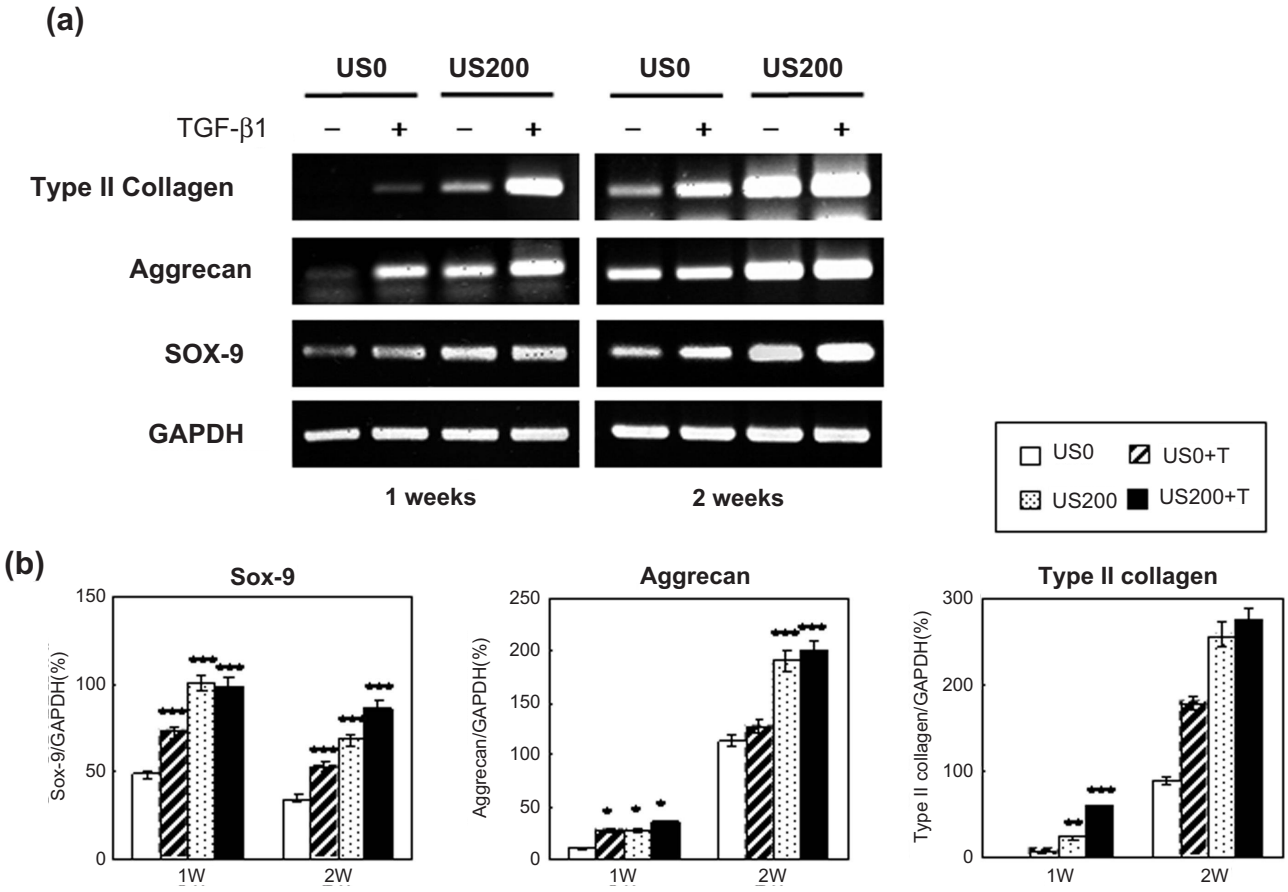

FIG. 6. Effects of LIUS on the chondrogenic differentiation of hMSCs in alginate layer culture. The expression of chondrogenic markers was measured at 1 and 2 weeks by RT-PCR analysis. (**A**) RT-PCR analysis was performed for Sox-9 (185 bp), aggrecan (312 bp), and type II collagen (215 bp). (**B**) Relative band intensities normalized against those of GAPDH were presented from five independent experiments. The statistical significance was evaluated against the control. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

chondrocytes.[7,8,23,24] LIUS was found to have positive effects on the viability, proliferation, and matrix production of cultured chondrocytes, and on the repair of damaged cartilages.[10,14,19,20,25,26] LIUS was also shown to enhance the chondrogenic differentiation of rabbit MSCs *in vitro* and *in vivo* in our previous studies,[11–13,15,18] and human MSCs *in vitro* in this study (Fig. 6). In our studies both *in vitro* and *in vivo*, the LIUS effect was shown to induce chondrogenic differentiation of MSCs independent of TGF-β1.

In this study, TGF-β, a key regulatory cytokine for chondrocyte differentiation, was shown to cause apoptosis of hMSCs and reduce cell viability. Components of the cartilage matrix were deposited by differentiating MSCs exposed to TGF-β, including aggrecan, type II collagen, and type IX collagen.[3,4,27] However, the role of TGF-β in the pivotal stages such as growth, condensation, and apoptosis is poorly understood[28,29] and are sometimes controversial in the chondrogenic process of MSCs.[30,31] In addition, TGF-β was shown to be unnecessary when mechanical stimulations, such as LIUS in this report and cyclic hydrostatic pressure, were applied.[8,9,11]

The 3-D culture in alginate system offers a model that allows the building of a cell matrix and the stability of chondrogenic phenotype for long culture periods *in vitro*.[2,4,11,22] However, the 3-D environment itself is not a favorable culture condition for hMSCs, and alginate might also cause a decrease in cell viability during a long-time culture.[32,33] Therefore, it could be helpful to use more biocompatible scaffolds such as collagen and fibrin/hyaluronic acid for cartilage tissue engineering using MSCs.[34]

Apoptosis is conserved in animal species and represents critical steps in the regulated development of multicellular organisms.[35–39] Apoptosis is a tightly regulated process mediated by a series of proteins involved in its execution and antiapoptotic events.[40] The p53 tumor suppressor is a key regulator of the apoptotic process, being activated by various stress signals at the protein level and transactivating a number of downstream genes including $p21^{WAF1/CIP1}$, Gadd45 (growth arrest and DNA damage inducible gene 45), and Bax (Bcl-2 associated X protein).[41–43] Bcl-2 is an antiapoptotic protein that forms heterodimers with Bax and inhibits its function. The ratio of Bax to Bcl-2 is thought

to represent relative susceptibility of cells to apoptosis.[44–46] Our results showed that the expression of apoptotic (p53 and bax) and antiapoptotic (bcl-2 and PCNA) proteins was balanced by TGF-β and LIUS treatments. The LIUS effects on the apoptosis of hMSCs during chondrogenic differentiation suggest its possible effects on the hypertrophic or osteoarthritic chondrocytes, both of which undergo apoptosis.[47]

In conclusion, our results might demonstrate a possible function of the LIUS in inhibiting apoptosis of hMSCs and enhancing their viability during chondrogenic differentiation. These results suggest that the LIUS treatment could be a valuable tool in cartilage tissue engineering using MSCs by enhancing cell viability and directing the chondrogenic differentiation process itself.

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