Effects of Low-Intensity Ultrasound on Chondrogenic Differentiation of Mesenchymal Stem Cells Embedded in Polyglycolic Acid: An in Vivo Study

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ABSTRACT

In this study we investigated the effects of LIUS on chondrogenic differentiation of bone marrow-derived mesenchymal stem cells (BM-MSC). Our hypothesis is that LIUS may be a noninvasively effective stimulant to a biological system in vivo by turning on differentiation of MSCs and promotion of chondrogenesis. MSCs were isolated from the bone marrow of New Zealand white rabbits and cultured in monolayer for 2 weeks. They were then harvested and seeded into polyglycolic acid (PGA) non-woven mesh at a number of $5 \times 10^6$ cells. Cultured with a chondrogenic-defined media for 1 week, the PGA/MSCs constructs ($n = 4$) were implanted subcutaneously in the back of nude mice ($n = 9$, each group). The ultrasound (US) group received US stimulation at a frequency of 0.8 MHz and intensity of 200 mW/cm$^2$ for 10 min every day up to 4 weeks, while the control group had no US stimulation. Analyses of histological, immunohistochemical, biochemical, and mechanical characteristics were made at 1, 2, and 4 weeks post-stimulation, respectively. Total DNA contents showed no significant difference between the two groups. Total collagen and glycosaminoglycan (GAG) increased more significantly in the US-stimulated group than in the control. Histology of Safranin O/Fast green confirmed more intense and spreading extracellular matrix (ECM) at 2 and 4 weeks in the US-stimulated specimens. Mechanical tests exhibited that compressive strengths were also significantly higher in the US-stimulated cells at later times. This study strongly suggests that it may be possible for ultrasound to have some stimulatory effects in vivo on the chondrogenesis of MSCs.

INTRODUCTION

Mesenchymal stem cells (MSC) have been widely studied as an alternative cell source for cartilage tissue engineering, due mostly to their capability to differentiate into chondrogenic lineage and phenotypic stability maintained with multiple passages. Chondrogenesis of MSCs requires specific culture conditions, such as three-dimensional (3D) environment and some growth factors, especially transforming growth factor-beta (TGF-$\beta$). 1-5 Biomechanical stimulations were also found to be effective in directing MSCs into chondrogenesis. Angele et al. 6 showed that cyclic hydrostatic pressure stimulated chondrogenic differentiation of bone marrow derived MSCs (BM-MSC) in pellet culture. Studies of transplanted MSCs reported that application of mechanical stimuli improved cartilage healing in the rabbit knee joint. 7,8 Huang et al. 9 suggested that cyclic compressive loading could promote chondrogenesis of rabbit BM-MSCs by inducing endogenous TGF-$\beta$.

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In utilizing the benefits of mechanical stimulation, especially in human patients, one of the technical barriers is the effective delivery to target sites. In reviewing the alternative sources of mechanical signals, we chose a low-intensity ultrasound (LIUS) and carefully examined its feasibility as an equivalent of mechanical cue. Therapeutic LIUS in clinical settings has proven useful in bone fracture healing. Recent studies of the biological effects of LIUS on chondrocytes and cartilage metabolism have also presented some promising results. LIUS stimulation significantly increased aggregan mRNA and proteoglycan synthesis, indicating a calcium channel involved in the path. US exposure to chondrocytes embedded in Atelocollagen gel promoted synthesis of chondroitin sulfate. It improved healing of osteochondral defects in animal models without surgical intervention. Ebisawa et al. provided evidence of the chondrogenic capability of LIUS with human BM-MSCs.

Despite these up-to-date findings on the promising effects of LIUS, however, the biological mechanisms are still unknown. US is a mechanically vibratory form of energy that travels through a medium. When the displacement of the medium is in the same direction as the US wave propagation, this acoustic wave is called a compression or longitudinal wave. Another type of wave is a shear wave, in which the medium displacement is perpendicular to the US propagation direction. When US passes through soft tissues, the dominant wave is the compression type. It is thus plausible that US may have the capability to generate a deforming environment in the path and, as a result, produce a certain level of stress to the affected area. We assume that these unspecified stresses might produce some stimulatory effects on the cellular level. Our hypothesis in this work is that US may play as positive a role as the conventional mechanical stimuli in the chondrogenesis of MSCs.

To test our hypothesis, we prepared polyglycolic acid (PGA)/rabbit MSC constructs and implanted them in nude mice. The mice received ultrasonic stimulation delivered in the back for 10 min every day. The retrieved specimens in control and test group were analyzed at 1, 2, and 4 weeks postimplantation, respectively, for the effects of LIUS on the chondrogenic differentiation of rabbit MSCs. The results were evaluated by gross and histological examination, synthesis of the extracellular matrix (ECM), and mechanical property.

**MATERIALS AND METHODS**

*Isolation and culture of rabbit MSCs*

MSCs were isolated from 2-week-old female New Zealand white rabbits (Joong-Ang Experimental Animal Center, Seoul, Korea). The bone marrow aspirates from the tibia and femur were suspended in 5% acetic acid and then centrifuged at 1500 rpm for 5 min to remove the red blood cells. The cell pellets were resuspended in minimum essential medium (MEM, eagle-α modification, Sigma-Aldrich, St. Louis, MO) supplemented with antibiotics and 10% newborn calf serum (NCS). Cells of $1.5 \times 10^7$ were seeded in tissue culture plate (150 mm diameter) and cultured in monolayer for 2 weeks at 37°C in 5% CO₂ incubator. The attached cells were then isolated using 0.05% trypsin-EDTA (Gibco, New York, NY) and followed by centrifugation at 1500 rpm for 10 min. The resuspended cells were replated at a number of $1.5 \times 10^6$ cells per plate, and culture medium was changed three times a week. The second passaged cells were used for PGA scaffold seeding.

**Preparation of PGA/MSCs construct**

Biodegradable PGA non-woven mesh (Albany International, Mansfield, MA) was used as a scaffold for 3D MSC culture. The PGA mesh was cut into pieces of equal dimension (6 mm × 6 mm × 3 mm), submerged in 70% alcohol for 10 h for sterilization, and kept at 4°C before MSC seeding. Washed three times with antibiotics-included phosphate-buffered saline (PBS), the PGA scaffolds were held in Dulbecco’s modified eagle medium (DMEM, Gibco) for 12 h at 37°C incubator. The passaged MSCs were then statically seeded with $5 \times 10^5$ cells per scaffold. The culture medium was a chondrogenic-defined medium, DMEM supplemented with ITS, 50 μg/mL ascorbate 2- phosphate, 100 nM dexamethasone, 40 mg/mL proline, 1.25 mg/mL BSA. They were cultured for a week before implantation.

**In vivo implantation and US stimulation**

Five-week-old male nude mice ($n = 9$, each group) were anesthetized with a mixture of ketamine hydrochloride and rumpun. The back skin of the mouse was incised for the implantation of MSC-seeded PGA scaffold into the subcutaneous tissue, a total of four specimens implanted at a time per mouse. Two specimens were placed longitudinally at one side of the back, and another two were positioned at the other side. On the following day, US stimulation was given first to one side for 10 min and then to the other side for another 10 min. An ultrasound device (Fig. 1), Noblelife (Duplogen, Suwon, Korea) was used for the stimulation that was applied every day for 1, 2, and 4 wk, respectively, at a frequency of 0.8 MHz and an intensity of 200 mW/cm² in a continuous wave mode. Control mice were prepared in the same manner and grew without exposure to US.

**Measurement of total DNA and protein**

The implanted specimens were retrieved at the end of US stimulation, weighed for their wet weight, and then immediately frozen in liquid nitrogen before biochemical assays. They were fully digested for 24 h at 60°C in
papain digestion solution (pH 6.2), containing 125 μg/mL papain in 0.1 M sodium phosphate buffer with 5 mM L-cysteine-HCl and 5 mM EDTA. Total DNA content of each specimen was determined using Hoechst 33258 dye method. A standard curve of mouse thymus DNA (Takara, Shiga, Japan) was used in the range of 0–2 μg/mL. Total protein was also measured by bicinchoninic acid (BCA) protein assay method. The digested samples were incubated with BCA reagents for 30 min at room temperature, and the absorbance was measured at 562 nm. A standard curve of BSA was used in the range of 0–1.6 mg/mL.

Measurement of total collagen and GAG

Total collagen of each specimen was measured using the Tullberg-Reinert method. The digested samples, mixed with Sirius red dye solution (pH 3.5), were dried in a 96-well plate, reacting with the dye solution for 1 h. The absorbance of dye-sample complex was then read at 550 nm wavelength. Total collagen was extrapolated using a standard plot of bovine collagen (Sigma-Aldrich) in the range of 0–100 μg/mL. Total GAG in each sample was determined using the 1,9-dimethylmethylene blue (DMB) colorimetric method. The papain-digested samples were mixed with the DMB solution, and the absorbance was measured at a wavelength of 525 nm. Total GAG was extrapolated using a standard plot of shark chondroitin sulfate (Sigma-Aldrich) in the range of 0–50 μg/mL.

Histology and immunohistochemistry

The implanted PGA/MSC constructs were fixed in 4% formaldehyde for 48 h, dehydrated, and then embedded in paraffin wax. Sections of 5 μm thickness were stained with Safranin O/Fast green to identify GAG in the matrix. For immunohistochemical analysis of collagen type II, other sections were rinsed with PBS and treated with 0.15% Triton X-100 to increase tissue permeability. Once nonspecific bindings were blocked with 1% BSA, the section slides were incubated for 1 h with mouse anti-human collagen type II monoclonal antibody (Chemicon, Temecula, CA) at 1:500 dilution and then for another hour with 1:200 diluted biotinylated secondary antibody (DAKO LSAB System, Carpinteria, CA). Washed in PBS, they were incubated with peroxidase-conjugated streptavidin solution (DAKO LSAB System) for 30 min at room temperature. The slides were counterstained with Mayer’s hematoxylin (Sigma-Aldrich) and then mounted with a mount solution before microscopic observation (Nikon E600, Japan).

Mechanical test

The retrieved specimens were subjected to an unconfined compression test using the Universal Testing Ma-
machine (Model H5K-T, HTE, Salfords, England). Placed on a bottom platen of the machine, each sample was compressed at a speed of 1 mm/min. The machine automatically stopped after moving a programmed length in between top and bottom platen. A peak load was obtained from the load-displacement curve and individual compressive strength was calculated. Specimens in each group ($n$/H110053) were tested for different time points (1, 2, and 4 wk), and the data were collected and statistically analyzed.

Statistical analysis

Statistical difference in the mechanical and biochemical assay was sought between the two groups using one-way analysis of variance (ANOVA), a Student Newman-Keuls test, and a two-tailed Student $t$ test. A statistical significance was assigned (*$p < 0.05$ and **$p < 0.01$, respectively).

RESULTS

Gross examination of PGA/MSC constructs

The size and shape of the implanted specimens were continuously changing in both the control and US-stimulated groups (Fig. 2). The overall size was reducing gradually, and the shape became rounded over the 4-week period. A significant turnover in the shape of the retrieved constructs was noticed between 1 and 2 weeks postimplantation. The US-stimulated specimens, especially at 4 weeks, exhibited a cartilaginous tissue formation and were better in size and color. When examined using an image program (NIH Image J 1.33), the difference in dimension was clear at 4 weeks: $41.8 \pm 3.6$ mm$^3$ vs $56.6 \pm 5.3$ mm$^3$ for control and US-stimulated specimens, respectively.

Total DNA and total protein

Measurement of the total DNA showed no significant differences, either between the two groups or within the

FIG. 3. Changes in amounts of total DNA (A) and total protein (B) at different time points between control and US-stimulated specimens. A statistically significant difference was seen (**$p < 0.01$) at 4 weeks.

FIG. 4. Water content (A), total GAG (B), and total collagen (C) between control and US-stimulated specimens at each time point. Statistically significant differences were noted. *$p < 0.05$, **$p < 0.01$, respectively.
same group for all the times (Fig. 3A). While the total proteins were gradually increasing for both groups, statistically significant difference ($p < 0.01$) was present at 4 weeks (Fig. 3B). The total proteins on the fourth week were $2.02 \pm 0.04$ mg and $2.42 \pm 0.07$ mg for control and US groups, respectively.

**Water, GAG, and collagen content**

The water content was not significantly different in the two groups, ranging on average from 78–82% (Fig. 4A). Normalized by the dried weight (mg) of each specimen ($n = 3$, each group), the total GAG contents (mg/mg) sharply increased over the time periods. For the specimens at 2 and 4 weeks, there were even statistically significant differences between the two groups (Fig. 4B). The amounts of total GAG were $8.2 \pm 0.5$ vs $13.5 \pm 1.5$ at 2 weeks and $20.1 \pm 2.8$ vs $25.8 \pm 2.9$ at 4 weeks for control and US-stimulated groups, respectively. The total collagen content (mg/mg) was not significantly different until 4 weeks: $29.8 \pm 2.3$ vs $34.8 \pm 2.7$ for control and US-stimulated groups, respectively (Fig. 4C).

**Histological and immunohistochemical evaluations**

When synthesis of cartilaginous ECM was identified with Safranin O/Fast green at each time point, the difference between the two groups became obvious in terms of intensity and distribution of GAG molecules (Fig. 5). While the characteristic red staining was not apparent in the control group until 4 weeks, it was evident at 2 weeks.
in the US-stimulated specimens and became more intense and spreading widely into the larger area at 4 weeks. Immunohistochemical assay of collagen type II also identified the protein expression gradually increasing for both groups (Fig. 6). The collagen type II was detected positively at 2 weeks, mostly in a small, localized area, but it spread into the larger area by 4 weeks.

**Compressive strength**

The compressive strengths of the retrieved specimens were progressively increasing in both groups while there were statistically significant differences, especially on the second and fourth week post-stimulation (Fig. 7). The average compressive strengths (MPa, n = 3, each group) were $1.06 \pm 0.1$ vs $1.69 \pm 0.16$ at 2 weeks and $1.85 \pm 0.07$ vs $2.25 \pm 0.13$ at 4 weeks for control and US-stimulated groups, respectively.

**DISCUSSION**

The most notable finding in this work is that chondrogenesis of MSCs in vivo was not only possible but significantly upregulated by the aid of US, resulting in the formation of cartilage-like tissue. The present data demonstrate that compared to the control group, US-stimulated specimens have more competitive characteristics of cartilaginous tissue in terms of histology, biochemical assays, and mechanical tests. It has been known that chondrogenic differentiation of MSCs requires specific conditions, including cultural dimension, growth factors, and mechanical cues. Bosnakovski et al. showed that chondrogenesis of bovine MSC occurred in pellet culture without using any bioactive stimulants. Many growth factors, such as TGF-β, insulin-like growth factor, bone morphogenetic protein, and fibroblast growth factor have been evaluated for either single or combined effects on chondrogenesis of MSCs. Various forms of the mechanical loads, either cyclic hydrostatic pressure or cyclic compression, are understood as an effective tool for chondrogenesis of MSCs.

As mentioned previously, our hypothesis is that the compression wave of LIUS may create a “deformation-induced microenvironment” in the path. Although we do not have a direct clue at this time that establishes US as an equivalent of mechanical stimulation, it is plausible that repeated US forces can produce some feedback responses at genetic and cellular levels. Therapeutic implications of US primarily come from nonthermal effects, such as acoustic streaming and cavitation. Once acoustic streaming (a steady circulation of fluid) is set up near the cell or bubble (cavitation), it turns to microstreaming in nature. As a result, viscous (shear) stresses are built, which can affect cell membrane structure and function, either transiently or permanently. Provided that the intensity of US is well-controlled and the mechanical stresses do not damage cells, the outcome can be therapeutic. It should be noted that there is a major difference between ultrasonic and physiological stimulation. One cycle of US wave proceeds into a given length within microseconds so that strain rate can be very high with a much lower strain. In the physiological situation, however, a loaded articular cartilage experiences relatively higher strain with a much lower strain rate under normal conditions.

Previous study of LIUS in our laboratory found that rabbit MSCs cultured in alginate beads produced the chondrogenic markers more significantly than control in response to LIUS stimulation without addition of TGF-β. The current study of in vivo stimulation also exhibited similar effects as shown in the alginate-rMSC construct. In the recent study of Ebisawa et al., they applied LIUS to human MSCs in pellet culture and found that LIUS could drive hMSCs into chondrogenic differentiation when used together with TGF-β. The US alone was barely effective in their study, which is controversial with the present data, because chondrogenic differentiation of rMSCs was possible without supplementing TGF-β. Although it is complicated to pinpoint the exact reasons behind this result, some explanations are conceivable: (1) US protocols used, especially intensities (200 mW/cm² vs 30 mW/cm²), (2) cell source, (3) 3D culture environment, (4) culture system, and (5) other unknown factors.

Upon the gross examination of the retrieved specimens, differences between the two groups were negligible at the first week, while they became visible after the second week. The overall shape and volume of the PGA/rMSC constructs were changing with time, due primarily to the degradation of the PGA mesh and newly synthesized, accumulated cartilaginous ECM. Based on the results of total DNA, US stimuli do not seem to affect cell proliferation, an observation in agreement with other studies. Little effect of LIUS was reported on human MSC proliferation in pellet culture. A cyclic compressive load-
Our preliminary studies suggest that in- channels and subsequently turn on downstream cellular could affect stretch-activated channels and/or unknown early studies indicate that mechanotransductive signals mediated signal pathway. Although stresses imposed on cell are still unclear at this time. One plausible theory is that response to an external US signal and the subsequent events could affect the significantly improved compressive strengths. The US-stimulated specimens could be a major contribu-

This result may be associated with the issues of diffusion and mass transport in the constructs, indicating a further positive contribution of ultrasound.

In reviewing the results of compressive strengths, it is intriguing that the significant differences at 2 and 4 weeks reflect exactly that of the total GAG. Mechanical characteristics of normal cartilage tissue depend largely on the well-organized structural integrity between collagen network and high concentration of aggregan. Aggrecan in cartilage matrix is believed to be responsible for compressive load while collagen controls tensile load. It is reasonable to conclude that more concentrated GAG in the US-stimulated specimens could be a major contribu-

As mentioned previously, the mechanisms of cell re-

sponse to an external US signal and the subsequent events are still unclear at this time. One plausible theory is that ultrasound might be partly involved in cell receptor-mediated signal pathway. Although stresses imposed on cell surface are complicated in nature and immeasurable, early studies indicate that mechanotransductive signals could affect stretch-activated channels and/or unknown channels and subsequently turn on downstream cellular responses. Our preliminary studies suggest that integrin receptors and stretch-activated cation channels might participate in the US-mediated signal pathway (data not shown). Along with the mechanism of LIUS-induced chondrogenesis in MSCs, an optimal protocol of US stimulation needs to be investigated systemically.

This study demonstrates that LIUS has great potential in stimulating the chondrogenic differentiation of MSCs in vivo without using chondrogenic growth factors. With the addition of more supportive data in future studies, our findings could hold a significant implication in the clinical applications of ultrasound.

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REFERENCES


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