Osteogenic differentiation of adipose-derived stem cells prompted by low-intensity pulsed ultrasound

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Received 18 November 2012; revision accepted 6 February 2013

Abstract

Objectives: Based on *in vivo* studies, low-intensity pulsed ultrasound (LIPUS) stimulation has been widely used in the clinic for advancing bone growth during healing of non-union alignment, fractures and other osseous defects. In this study, we have investigated osteogenic differentiation of adipose stem cells (ASCs) regulated by LIPUS, and also in a preliminarily manner, we have discussed diverse effects of different duty ratio parameters.

Materials and methods: Mouse adipose stem cells were isolated and osteogenically induced. Then they were treated with LIPUS for 10 min/day for 3 days, 5 days and 7 days, respectively. Finally, effects of LIPUS on osteogenic differentiation of the ASCs were analysed by real-time PCR, western blotting and immunofluorescence.

Results: Our data indicated that LIPUS promoted mRNA levels of runt-related transcription factor 2, osteopontin and osterix in the presence of osteo-induction medium; moreover, protein levels of runt-related transcription factor 2 and osteopontin were upregulated.

Conclusions: We successfully demonstrated that LIPUS enhanced osteogenesis of ASCs, specially at the duty ratio of 20%.

Introduction

Bone has the inherent capacity for self-repair; however, its regeneration takes time, and patients may suffer pain and discomfort during the process. Thus, promoting fracture healing and shortening healing time are of research interest.

In recent years, increasing attention has been focused on adipose tissue as a stem-cell source, due to multilineage potential of the cells, their self-renewal capacity and long-term viability. Adipose-derived stem cells (ASCs) can differentiate into cells of adipogenic (1), chondrogenic (2), myogenic (3), osteogenic (4), hepatic (5) and neurogenic (6) lineages. The relatively high frequency of clonogenic cells and their acquisition from adipose tissue (7), have made them an appealing cell source for tissue engineering (8). Many studies have indicated that ASCs can be osteogenically induced by mechanical stress (9–12) and results from our previous studies also have shown that cyclic tensile mechanical loading can promote their osteogenic differentiation in osteogenic medium (13,14).

Many different mechanical stimuli to induce osteogenic differentiation have been actively studied (for example, tensile strain, shock wave and fluid shear stress), among which is low-intensity pulsed ultrasound (LIPUS). This has proved to be a clinically established, widely used and Food and Drug Administration-approved process. Based on *in vivo* studies (15–18) it has been shown to enhance bone growth during healing of non-union alignment, fractures and other osseous defects.

Therapeutic ultrasound with frequencies between 0.5 and 1.5 MHz and intensities between 30 and 200 mW/cm² is known to facilitate fracture healing, bone deposition and growth (19–22). LIPUS is a pulsed longitudinal wave with compressions and

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rarefactions; duty ratio as a parameter shows its periodic characteristics. Duty ratio is ratio of pulse duration and pulse total cycle, in a string of a square wave. Jung *et al.* found that application of LIPUS after injection of ASCs during implantation of titanium into tibias of diabetes rats provided positive effects on bone regeneration at the early stages (23). In addition, Angle *et al.* found that various intensities of LIPUS could stimulate endochondral ossification of rat bone marrow stromal cells *in vitro* (20).

Although many studies have shown acceleration of bone healing by application of low-intensity ultrasound, and osteogenic differentiation of rat bone marrow stromal cells by various intensities of LIPUS, they do not mention effects of its duty ratio. Also, previous studies have focused on bone marrow mesenchymal stem cells (BMSCs) treated with ultrasound (20), while reports on ASCs are very few.

In this study, we have investigated osteogenic potential of ASCs treated by ultrasound at different duty ratios and time points.

Materials and methods

Isolation and culture of adipose stem cells

All animal procedures were reviewed and approved by the Sichuan University Animal Care and Use Committee. Mouse ASCs (mASCs) were obtained from subcutaneous fat of groins and backs of female, three week old mice (Kunming, Experimental Animal Center of Sichuan University, China). Subsequently, the adipose tissue was finely minced and digested in type I collagenase (0.075% type I collagenase in α -MEM medium without foetal bovine serum (FBS) for half an hour. Then, cells were seeded in culture flasks with α -MEM supplemented with 10% FBS, 1% penicillin/streptomycin and incubated at 37 °C in a humidified atmosphere 95% air with 5% CO₂. After 3–5 days, adherent cells were cultured in a monolayer with non-adherent cells removed. Medium was changed every 3–4 days, and cells were subcultured when they reached 75–80% confluence. Passage 3 cells were used for the following experiments.

Flow cytometry analysis of mASC phenotype

Third passage mASCs processed as single-cell suspensions were prepared in 10% FBS in phosphate-buffered saline (PBS). One set of test tubes had cells stained with fluorescence marked antibodies to Sca-1, CD34, CD44 or CD146, and further tubes without fluorescent antibodies were used as controls. We used purified anti-mouse CD34 and CD146 (both purchased from Biolegend, San

Diego, USA), fluorescence labelled FITC-conjugated Sca-1 antibody (Abcam, Cambridge, MA, USA), and alexa fluor 488 anti-mouse CD44 (Biolegend). Tubes with Sca-1 and CD44 were then incubated in the dark at room temperature for 30 min; simultaneously, tubes with CD34 and CD146 were incubated at room temperature for 1.5 h. Cells with direct antibodies including CD44 and Sca-1 were then washed in PBS (containing 10% FBS) and retained with other samples at 4 °C. Subsequently, tubes with CD34 and CD146 were incubated in the dark together with the secondary antibodies (FITC – goat anti-mouse IgG; Zhongshanjinqiao, China) at room temperature for 30 min, and cells were washed again afterwards. Finally, PBS containing 10% FBS was added into all tubes separately, and cells were mixed with PBS. Cells were analysed using a fluorescence-activated cell sorter (FACS Calibur; BD Biosciences, San Jose, CA, USA) and data analysis was performed using WinMDI2.8 software (The Scripps Institute, West Lafayette, IN, USA).

Ultrasound exposure

After culture for 24 h, third passage mASCs were transferred into osteogenic medium consisting of 10 nM dexamethasone, 50 μ M ascorbate 2-phosphate and 10 mM β -glycerolphosphate (β GP) in 100 ml α -MEM, at 10 000–30 000 cells/3 cm dish; 30 min later, they were treated by exposure to ultrasound. Mechanical loading of LIPUS began on 1st, 3rd and 5th days after osteogenic induction. Loading groups were exposed for 10 min/day and lasted 3 days, 5 days and 7 days, respectively, and each group was treated with two different duty ratios, 20% and 50%, referring to ratio of pulse duration and pulse total cycle of the pulsed longitudinal wave, separately 1:5 and 1:2. All cultures were completed on the eighth day. Cells were incubated for half an hour and RNA and protein were extracted, or they were fixed in paraformaldehyde after the last ultrasound treatment. The experimental setup is illustrated in Fig. 1. The LIPUS device consisted of an array of two transducers, each 30 mm in diameter; LIPUS signal consisted of 1.0 kHz and 100 mW/cm² at duty ratio of 20% and 50%. Culture dishes were seated on the ultrasound transducer array with a thin layer of coupling gel. All LIPUS treatments were performed with the culture plates or dishes inside a tissue culture incubator (37 °C, 5% CO₂, 95% humidity).

RNA isolation and real-time RT-PCR

We investigated mRNA levels of osteogenic genes including runt-related transcription factor 2 (*Runx2*), osterix (*Osx*) and osteopontin (*OPN*) in the mASCs by

Figure 1. Experimental designs to test RT-PCR, western blotting and immunofluorescence. A, B and C represent 7 days, 5 days and 3 days groups separately, and each group contains duty ratio of 20% and 50% and control. A₁, B₁ and C₁ respectively indicate cells of passage three culture dishes or plates with appropriate cell density. A₂, B₂ and C₂ represent medium replaced with osteogenically-defined medium after 24 h cell plating. Groups A, B and C were cultured for 7 days, 5 days and 3 days. All groups were treated with single low-intensity pulsed ultrasound exposure 24 h after first medium change to osteogenic-defined medium and incubated for 30 min after 10-min low-intensity pulsed ultrasound treatment. Finally, on the 8th day, we harvested cells for all methods and purposes.

Table 1. Primer sequences of target genes and *GAPDH* for RT-PCR assay

Genes	Sequence (5'→3')
<i>GAPDH</i>	F:GACGGCCGCATCTTCTGTGC R:TGCAAAATGGCAGCCCTGGTGA
<i>Runx2</i>	F:CCGAAGTGGTCCGCACCGAC R:CTTGAAGGCCACGGGCAGGG
<i>OPN</i>	F:GTGGTGATCTAGTGGTGCCAAGAGT R:AGGCACCGCCATGTGGCTAT
<i>Osx</i>	F:GTCCTATGGCGGGGAGGACTGG R:TGGCAGCTGCAAGCTCTCTGTA

real-time PCR assay. Total RNA was extracted using the Simply P Total Tissue/cell RNA Extraction Kit (Bioflux, Zhejiang, China) according to the manufacturer's protocol. cDNA synthesis was performed using transcriptor reverse transcriptase (Takara Biotechnology, Shiga, Japan) according to the manufacturer's protocol; PCR oligonucleotide primers are listed in Table 1. Expression of genes was quantified using real-time PCR, with SYBR Premix ExTaq™ (Tli RNaseH Plus, Takara, Japan).

Real-time PCR was run in the ABI PRISM 7300 Sequence Detection system (Applied Biosystems, USA) using hot-start DNA Master SYBR Green I Kit (Takara Biotechnology Co., Ltd, Japan) with the following program: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by melting curve analysis. Specificities of PCR products were verified by melting

curve analyses between 60 °C and 95 °C. Expressions of *GAPDH* were used for coherence of real-time PCR results, to compare transcription level of target genes in different quantities of sample. For each reaction, a melting curve was generated to test primer dimer formation and false priming. Then, relative quantification of mRNA levels was carried out by means of a double standard curve method. cDNA of each sample from products of PCR, was examined using agarose electrophoresis.

Western blotting

Protein samples were obtained after treatments for 3 days, 5 days and 7 days. Cells were incubated on ice for 30 min and cultures were washed in PBS. Protein concentrations were determined using BCA protein assay kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Equal amounts of protein extract were fractionated on 10% SDS-polyacrylamide gels, transferred at 80 V for 20 min and 120 V for 40 min, respectively, then transferred to PVDF membranes. These were incubated with anti-Runx2 (Abcam) or anti-GAPDH antibodies (Abcam) then anti-rabbit IgG secondary antibody (Zhongshanjinqiao). Membranes were washed three times and immunocomplexes were visualized using enhanced chemiluminescence reagent (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's instructions.

Immunofluorescence

To demonstrate distribution of OPN proteins, cells were seeded into 12-well plates at 1×10^3 cells/well and cultured in control medium or osteogenic medium for immunofluorescence (IF) staining. After treatment in osteogenic medium by ultrasound exposure and incubation for 30 min, both mechanical loaded and the control cells were washed briefly in PBS, three times. Cells were then fixed in cold 4% paraformaldehyde for 15 min at room temperature, and blocked in goat serum albumin for 15 min. Plates were subsequently incubated overnight at 4 °C with mouse polyclonal antibody to OPN (Abcam). Sequentially, plates were incubated with secondary antibodies conjugated to FITC (Invitrogen, Carlsbad, CA, USA), for one hour at room temperature, and nuclei were stained with DAPI (Molecular Probes, Eugene, OR, USA) for 10 min. After being rinsed in PBS, cells were observed and imaged using a DMI 6000-B fluorescence microscope (Leica, Brunswick, Germany). Images were analysed with Image-Pro Plus 6.0.0.260 (MediaCybernetics, Rockville, MD, USA) and integral optical density (IOD) was measured to evaluate OPN concentration.

Figure 2. Flow cytometry histogram of mouse adipose stem cells. Red shaded area represents control, black curve represents cells positive for CD markers. A, B, C, D separately indicate markers of CD34 ($78.42 \pm 0.17\%$), CD146 ($73.66 \pm 3.22\%$), Sca-1 ($83.42 \pm 1.14\%$) and CD44, respectively ($16.77 \pm 3.87\%$).

Statistical analysis

We performed three or more independent sets of experiments, and each experiment was run at least 3 times. Data are provided as mean values $\pm$ SD. *P* values were calculated using Student's *t*-test, with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). *P* < 0.05 was considered statistically significant.

Results

ASC characterization

Whole adipose tissue has mature adipocytes, vascular smooth muscle cells, fibroblasts, endothelial cells, monocytes, and lymphocytes. Thus, there was need to confirm proportions of ASCs in the mixtures. A representative flow histogram is shown in Fig. 2. mASCs were positive for CD34 ($78.42 \pm 0.17\%$), CD146 ($73.66 \pm 3.22\%$) and Sca-1 ($83.42 \pm 1.14\%$) and negative for CD44 ($16.77 \pm 3.87\%$) (Fig. 2).

Ultrasound exposure promoted Runx2, OPN and Osx transcription after osteoinduction

After 3 days, 5 days, and 7 days ultrasound exposure with osteo-induction, mRNA levels of Runx2, OPN and Osx were detected, using real-time RT-PCR (Fig. 3). mRNA levels of Runx2 in duty ratio 20% and 50% groups were significantly higher than those in corresponding control groups, at day 5 and day 7; at day 5 they were distinctly significantly higher than those on

other time points. In addition, mRNA levels of Runx2 in duty ratio 20% groups revealed clear increase compared to duty ratio 50% groups, on day 3 and day 5.

Expression *OPN* gene in LIPUS-treated groups revealed strong increase compared to control groups on day 3 and day 5. At the same time, mRNA levels of OPN in 20% duty ratio groups were significantly higher than those of 50% duty ratio groups at each time point. In addition, for 20% duty ratio groups, values on day 5 were 4-fold greater, with respect to both day 3 and day 7.

Transcript levels of the gene coding for *Osx* 20% duty ratio groups were significantly higher than those of control groups and 50% groups, at each time point. Additionally, 50% groups were significantly higher after 5 days loading. For 20% duty ratio groups, mRNA levels of *Osx* at day 5 were highest of the three groups (day 3, day 5 and day 7). Meanwhile, mRNA levels of *Osx* on day 3 were significantly higher than on day 7.

Determination of Runx2 protein expression

Western blotting showed that protein levels of Runx2 in 20% duty ratio groups were significantly higher than those of 50% groups, at each time point. Runx2 protein expression 20% duty ratio groups was significantly higher than of control groups at each time point, and protein levels of Runx2 of 50% groups were significantly higher compared to control groups on day 5 and day 7. For duty ratio of 20% and 50% groups, expressions of Runx2 protein on day 5 were significantly higher in contrast to days 3 and 7 (Fig. 4).

Figure 3. A, B and C show RT-PCR analysis of *Runx2*, *OPN* and *Osx* at different time points; ultrasound exposure up-regulated expression of all three genes compared to untreated control cultures. # and *represent significant difference between control and treated groups, respectively ($P < 0.05$ by LSD *t*-test). D show results of agarose gel electrophoresis of RT-PCR products.

Ultrasound exposure promoted *OPN* transcription after osteo-induction

Osteopontin, a cytokine secreted by osteoblasts, is commonly activated in cell cytoplasm. IF staining was performed with anti-OPN antibodies at time points of 3 days, 5 days and 7 days, ultrasound exposure (Fig. 5). We observed that OPN expression was significantly higher in duty ratio 20% groups ($4.42 \pm 0.37\%$) and 50% groups ($3.67 \pm 0.33\%$) compared to controls ($3.04 \pm 0.41\%$) at 3 days. OPN had significantly higher integrated IOD in 20% treated groups ($6.43 \pm 0.045\%$) at 5 days than controls ($3.86 \pm 0.48\%$). Similar results were found at 7 days, and protein level of OPN was sig-

Figure 4. Expression of *Runx2* analysed by western blotting at different time points (A). Ultrasound exposure promoted expression of *Runx2* compared to control cultures at both duty ratios (B). SPSS software used for all statistical analyses. # and *represent significantly difference between control and treated groups, respectively ($P < 0.05$ by LSD *t*-test).

nificantly higher in 20% treated groups ($3.94 \pm 0.19\%$) than controls ($2.98 \pm 0.36\%$). In 50% duty ratio groups of at 7 days, vibration strength was such that many cells detached from bottoms of culture dishes; thus, results were compromised and are not shown in Fig. 5.

Discussion

Mouse ASC populations obtained by current isolation methods inevitably include heterogeneous mixtures of several cell types, and thus cells (for example, fibroblasts or others) other than stem cells might previously have been transplanted in error. Many studies have reported surface markers used to characterize ASCs by flow cytometry, such as CD34, CD44, CD146 and Sca-1, and data from our experiments are in accord with previous results (24–26).

The process of osteogenic differentiation from stem cells to osteoblasts involves a multitude of factors including genetic, metabolic and physical inputs to co-ordinate appropriate adaptive responses. In recent years, increasing studies have been performed on effects of mechanical loading on osteogenic differentiation of ASCs (27). Results from Prè *et al.* demonstrated that high-frequency vibration treatment can promote early osteogenic gene expression as well as related protein translation, and can increase levels of calcium deposition, after 3 weeks (11); also, it has been reported that electric fields enhance healing in bone fractures and

Figure 5. A, B and C show expression of OPN antibody staining; immunofluorescence at different time points. Ultrasound exposure promoted expression of OPN compared to control cultures at duty ratio of 20% (d). SPSS software used for all statistical analyses. # and * represent significant differences between control and treated groups, respectively ($P < 0.05$ by LSD t -test, $n = 4$).

defects (9). Thus, Hammerick *et al.* focused on ability of electric fields to drive osteogenic differentiation in mASCs, and their results confirmed such fields to be candidate enhancers of ASC osteogenesis (10). Also, research has focussed on ultrasound, and results have demonstrated that ultrasound too can induce their osteogenic differentiation of (20). In the study of Marvel *et al.*, effects of different pulse repetition frequencies (PRF) including 1 Hz, 100 Hz and 1000 Hz on differentiation of adult stem cells were investigated, and results revealed that all three different PRFs of LIPUS can induce osteogenic differentiation (28).

Over the past few years, we have carried out a number of studies on effects of mechanical stress on osteogenic and/or adipogenic differentiation of ASCs. We have indicated that these cells might 'sense' mechanical loading in a time-dependent manner, and that cyclic tensile stretch might modulate their osteogenic differentiation *via* the BMP-2 signalling pathway (13). In addition it was shown that, mechanical stretch inhibited adipogenesis but stimulated osteogenesis of these ASCs in the presence of adipogenic medium (14).

Here, we investigated mechanical contribution to osteogenic differentiation of mASCs and results indicated that LIPUS can increase their osteogenic differentiation, specially of 20% duty ratio groups.

Runx2 (also called Cbfa1 in some quarters), is a master regulatory transcription factor for osteogenesis (29,30); it is essential for osteoblastic differentiation and early upregulation in the osteoblast differentiation process (31). To determine whether ASCs differentiate into osteoblasts after any sorts of stimuli, their osteogenic potential under control of certain osteoinductive genes must be evaluated.

Osterix is a zinc finger-containing transcription factor of the *Sp* gene family, located downstream of Runx2, which plays important roles in bone development and mineralization. Nakashima's group was responsible for discovery that Osx (highly specific to osteoblasts), induced expression of genes in pluripotent mesenchymal cells. Also, this team indicated that no deposits of bone matrix could be formed, either intramembranously or by endochondral ossification, in the absence of Osx (32).

Osteopontin, a highly acidic secreted phosphoprotein, is coded for by a bone differentiation marker gene, and promotes bone remodelling (33,34). OPN is related to the osteoblast phenotype and exists widely in them. Generally, researchers have believed that OPN could induce differentiation of osteoblasts and promote reconstruction of bone mineralization tissue (35,36).

In our study, PCR results concluded that 20% duty ratio treatment up-regulated expression of all the three

genes types (*Runx2*, *Osx* and *OPN*) on day 3, day 5 and day 7. Both IF and western blotting results showed that ultrasound loading up-regulated *Runx2* and *OPN* proteins on day 3 and day 5 at 20% duty ratio. Also, expression of *Osx* gene was elevated, as shown by PCR, at 20% duty ratio. In 50% duty ratio groups on day 5, only *OPN* IF result had no significant increase compared to control groups. This phenomenon might be due to vibration strength being over high; many cells dropped from bottoms of culture dishes; this also happened at for 50% duty ratio groups on day 7; for this reason, no IF image was obtained on day 7. Although several results were not significant, there was an increased trend of expression of genes and proteins by PCR, western blotting and IF. Thus, in this study, LIPUS was demonstrated to promote osteogenic differentiation of mASCs, being more obvious with 20% duty ratio of than 50% duty ratio. This was perhaps because ultrasound exposure at 50% duty ratio radiated considerably more heat than the 20% groups, which might damage some cells proteins.

Our study indicated that day 5 groups had more predominant effects of osteogenic differentiation than day 3 and day 7. *Runx2* is an early expression gene, for processes of osteogenesis; *Osx* located downstream of *Runx2* is expressed at the same time followed by *OPN*. However, in our study, expressions of all three genes were most predominant at time point of day 5.

Here, we have demonstrated that LIPUS advanced osteogenic differentiation of mASCs, and its effects at 20% duty ratio were stronger than 50% duty ratio.

Thus, findings of the work showed parameters 20% and 50% duty ratios of the ultrasound system; no further groups of duty ratio were studied due to design limitations. Parameters of ultrasound are crucial to ultrasound dose in LIPUS, such as PRF, intensity and duration time, not only duty ratio. In future experiments based on this work, the range of parameters will expand duty ratio, FRP and frequency densities. In addition, we will advance capacity of the ultrasound system to run multiple parallel experiments and perform additional studies to optimize parameters for increasing osteogenic differentiation of mASCs.

In conclusion, we have demonstrated that LIPUS enhanced osteogenesis of mASCs, specially at the duty ratio of 20%. Upregulation of genes including *Runx2*, *Osx* and *OPN* also occurred at duty ratios of 20% and 50%; at the same time, degree of upregulation by duty ratio of 20% was higher than of 50%.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (81200810, 81201211), and Spe-

cialized Research Fund for the Doctoral Program of Higher Education (20100181110059).

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