Ultrasound Stimulation of Different Dental Stem Cell Populations: Role of Mitogen-activated Protein Kinase Signaling

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Abstract

Introduction: Mesenchymal stem cells (MSCs) from dental tissues may respond to low-intensity pulsed ultrasound (LIPUS) treatment, potentially providing a therapeutic approach to promoting dental tissue regeneration. This work aimed to compare LIPUS effects on the proliferation and MAPK signaling in MSCs from rodent dental pulp stem cells (DPSCs) compared with MSCs from periodontal ligament stem cells (PDLSCs) and bone marrow stem cells (BMSCs). **Methods:** Isolated MSCs were treated with 1-MHz LIPUS at an intensity of 250 or 750 mW/cm^2 for 5 or 20 minutes. Cell proliferation was evaluated by 5-bromo-2-deoxyuridine (BrdU) staining after 24 hours of culture following a single LIPUS treatment. Specific ELISAs were used to determine the total and activated p38, ERK1/2, and JNK MAPK signaling proteins up to 4 hours after treatment. Selective MAPK inhibitors PD98059 (ERK1/2), SB203580 (p38), and SP600125 (JNK) were used to determine the role of activation of the particular MAPK pathways. **Results:** The proliferation of all MSC types was significantly increased after LIPUS treatment. LIPUS at a 750-mW/cm^2 dose induced the greatest effects on DPSCs. BMSC proliferation was stimulated in equal measures by both intensities, whereas 250 mW/cm^2 LIPUS exposure exerted maximum effects on PDLSCs. ERK1/2 was activated immediately in DPSCs after treatment. Concomitantly, DPSC proliferation was specifically modulated by ERK1/2 inhibition, whereas p38 and JNK inhibition exerted no effects. In BMSCs, JNK MAPK signaling was LIPUS activated, and the increase in proliferation was blocked by specific inhibition of the JNK pathway. In PDLSCs, JNK MAPK signaling was activated immediately after LIPUS, whereas p-p38 MAPK increased significantly in these cells 4 hours after exposure. Correspondingly, JNK and p38 inhibition modulated LIPUS-stimulated PDLSC proliferation. **Conclusions:** LIPUS promoted MSC proliferation in an intensity and cell-specific dependent manner via activation of distinct MAPK pathways. *(J Endod 2016;42:425–431)*

Key Words

Bone marrow mesenchymal stem cells, cell proliferation, dental pulp stem cells, dental tissue regeneration, low-intensity pulsed ultrasound, mitogen-activated protein kinase signaling, mesenchymal stem cells, periodontal ligament–derived stem cells, ultrasound

Ultrasound equipment has been used in clinical dentistry since the 1950s, and its current applications range from periodontal scaling to endodontic root canal irrigation (1, 2). The acoustic energy from low-frequency kilohertz ultrasound (20–42 kHz) emitted by an oscillating tip of clinical dental scalers is used for surface cleaning by disrupting calculus, debris, and bacteria on the external or internal surfaces of the tooth (2). Our previous work explored the molecular and biological effects of kilohertz ultrasound on dental cells showing distinct changes in gene expression including the expression of growth factors such as transforming growth factor beta 1 and vascular endothelial growth factor (3, 4). Ultrasound was also shown to promote the proliferation and differentiation of odontoblastlike cells (5), overall supporting the notion that low-intensity ultrasound has the therapeutic potential to promote endodontic tissue repair and dentin-pulp complex regeneration (6).

Unlike kilohertz ultrasound, low-intensity pulsed ultrasound (LIPUS) using a frequency in the low megahertz range (1–3 MHz) is a more widely used approach as a therapeutic application for tissue repair and regeneration, in particular for bone fracture healing (7–9). LIPUS delivers more focused, low-intensity acoustic pressure waves that produce small biomechanical interactions with the cells to elicit intracellular biological effects, ultimately resulting in tissue repair and regeneration. Accumulating evidence indicates that LIPUS is effective to stimulate osteoblasts and promote bone formation (7). Moreover, research has suggested that stem cell–based tissue regeneration responds to LIPUS stimulation. LIPUS reportedly enhanced viability, proliferation, and multilineage differentiation in a variety of postnatal mesenchymal stem cells (MSCs) including adipose-derived stem cells, bone marrow mesenchymal stem cells (BMSCs), periodontal ligament–derived stem cells (PDLSCs), and human umbilical cord–derived MSCs (10–14). Several studies have also suggested that LIPUS might be clinically beneficial in promoting dental tissue regeneration. Indeed, *in vitro* and *in vivo* studies indicated that the exposure of dental tissues to LIPUS may promote dentinogenesis, accelerated periodontal tissue healing, and dental implant osseointegration (15–17).

The mechanism by which LIPUS stimulates cells and tissues has not yet been fully elucidated but is in general attributed to its nonthermal biomechanical affects. In particular, through acoustic microstreaming and physical radiation, LIPUS may affect the cell membrane and cytoskeleton to trigger downstream signaling processes. Mitogen-activated protein kinases (MAPKs) (ERK, JNK, and p38 MAPK) have been shown to

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play a role in mechanotransduction in various cell types, such as bone cells (18), periodontal ligament cells (19), and muscle cells (20). ERK and p38 MAPK pathways have been reported to control cell proliferation after mechanical stimulation (9, 21).

The use of MSCs, in particular dental pulp stem cells (DPSCs), BMSCs, and PDLSCs, is increasingly being explored for application in dental tissue engineering strategies. Studies have suggested that MSCs from various tissue sources may show cell-specific responses under biomechanical stimuli via the activation of different intracellular signaling pathways (12). In this study, we investigated the effects of LIPUS on DPSC proliferation and the involvement of MAPKs in comparison with PDLSCs and BMSCs. We used primary cells isolated from rats, which enabled us to establish and compare standardized and consistent cultures of MSCs derived from the different tissue sources from the same donor animal (22). The findings of this study highlight the distinct differences between the responses of MSCs from different tissues and underscore the potential of LIPUS as a therapeutic tool for dental tissue regeneration.

Materials and Methods

Cell Cultures

Six-week-old male Wistar Han rats (weight 120 g) were used as tissue donors (Charles River Laboratories, Margate, UK). Dental pulp tissue was harvested from incisors and bone marrow from femora according to standard procedures (22). PDLSCs were isolated from periodontal ligament tissues dissected from rodent molars following incubation in phosphate-buffered saline solution containing 0.25% trypsin and 1 mmol/L EDTA (Gibco, Paisley, UK) for 45 minutes at 37°C in a rotary incubator (SI20H; Bibby Sterilin, Stone, UK) and centrifugation at 900*g* for 5 minutes (23). DPSCs, PDLSCs, and BMSCs were cultured in alpha minimum essential medium/10% fetal bovine serum (Gibco).

Characterization of Cell Cultures

The expression of CD29, CD90, vimentin (24), Nanog, Klf4, dentin matrix protein 1 (DMP1), osteocalcin (OCN), osteopontin (OPN), and bone sialoprotein (BSP) was analyzed using semiquantitative reverse-transcription polymerase chain reaction (sqRT-PCR). RNA was extracted from cultures at passages 2 and 4 using the RNeasy Mini Kit and reverse transcribed using an Omniscript RT kit according to the manufacturer's instructions (Qiagen, Manchester, UK). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize gene intensity. Primer details are provided in Table 1.

To confirm the differentiation potential, DPSCs, PDLSCs, and BMSCs were cultured in osteogenic medium (50 μg/mL ascorbic acid [Sigma-Aldrich, St Louis, MO], 10 mmol/L β-glycerophosphate [Sigma-Aldrich], and 10^{-9} mol/L dexamethasone [Sigma-Aldrich]) or adipogenic medium (0.5 mmol/L 1-methyl-3-isobutylxantine [Sigma-Aldrich], 60 μmol/L indomethacin [Sigma-Aldrich], and 0.5 μmol/L hydrocortisone [Sigma-Aldrich]). After 3 weeks, the cultures were fixed and stained with alizarin red S or oil red O to show osteogenic and adipogenic differentiation, respectively, using previously reported protocols (23).

LIPUS Stimulation

A DuoSon therapeutic ultrasound device (SRA Developments, Devon, UK) emitting pulsed ultrasound at a frequency of 1 MHz (pulsed at a 63-Hz repetition rate with a pulse duration of 3.2 milliseconds) was used for ultrasound delivery. In brief, the MSCs were seeded into the wells of a 6-well plate (Corning, Flintshire, UK) 1 day before ultrasound treatment (10,000 cells/well, 7 mL medium/well). The following day, the 6-well culture plate was housed in a custom-built silicon antireflection chamber on top of a plate shaker and kept at 37°C (5, 25). The ultrasonic transducer head was then carefully placed into the medium of a culture well for ultrasound irradiation of the cultures. The DuoSon device, calibrated using a radiation force balance, had 2 customized power settings to deliver 250 and 750 mW/cm^2. Both settings were used in all initial studies with 2 single treatment durations of 5 and 20 minutes, respectively.

Cell Proliferation Analysis

Cell proliferation was evaluated by 5-bromo-2-deoxyuridine (BrdU) incorporation after 24 hours of labeling using an immunocytochemical assay according to manufacturer's instructions (No. 11299946001; Roche Biosciences, Burgess Hill, UK). Microscopic

TABLE 1. DNA Sequences, Annealing Temperatures, Cycle Numbers, and Accession Numbers for Primers Used in the Semiquantitative Reverse-transcription Polymerase Chain Reaction*

Gene	Sequences	Annealing temperature (°C)	Cycle no.	Accession no.
GAPDH	F-CCCATCACCATCTTCCAGGAGC R-CCAGTGAGCTTCCCGTTCAGC	60.5	27	NM_017008
CD29	F-AATGGAGTGAATGGGACAGG R-TCTGTGAAGCCCAGAGGTTT	60.5	27	NM_017022.2
CD90	F-AGCTCTTTGATCTGCCGTGT R-CTGCAGGCAATCCAATTTTT	60.5	26–33	NM_012673
Vimentin	F-AGATCGATGTGGACGTTTCC R-GCAGGTCCTGGTATTCACG	60.5	27	NM_031140.1
Nanog	F-TATCGTTTTGAGGGGTGAGG R-CAGCTGGCACTGGTTTATCA	60.5	33	NM_001100781
Klf4	F-ATCATGGTCAAGTTCCCAGC R-ACCAAGCACCATCGTTTAGG	60.5	27	NM_052713
DMP1	F-CGGCTGGTGGTCTCTCTAAG R-CATCACTGTGGTGGTCCTTG	60.5	31–33	NM_203493.3
OCN	F-TCCGCTAGCTCGTCACAATTGG R-CCTGACTGCATTCTGCCTCTCT	60.5	33	NM_013414.1
OPN	F-AAGCCTGACCCATCTCAGAA R-GCAACTGGGATGACCTTGAT	60.5	33	NM_012881.1
BSP	F-ATGGAGATGGCGATAGTTCG R-TCCACTTCTGCTTCTTCGTTC	60.5	27	NM_012587.2

*All primers were designed using Primer Blast software (http://ncbi.nlm.nih.gov/tools/primer-blast/) and manufactured by Invitrogen (Paisley, UK).