Effect of low intensity pulsed ultrasound in activating the mitogen-activated protein kinase signaling pathway and inhibition inflammation cytokine synthesis in chondrocytes

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Objective: Low intensity pulsed ultrasound (LIPUS) has been shown to accelerate cell proliferation and tissue healing in both animal models and clinical trials. However, details of the clinical effects of LIPUS have not been well characterized. The aim of this study was to investigate the effect of LIPUS on mitogen-activated protein kinase (MAPK) activation in rat articular chondrocytes.

Design: Cross-sectional study.

Methods: Chondrocyte were cultured in six well cell culture plates for 72 hours at 37°C with 5% CO₂, and then exposed to LIPUS at 1.5 MHz frequency and 30-mW/cm² power. Changes in chondrocyte activities were evaluated in response to oxydative stress in dose-dependent (0 and 300 uM) and time-dependent (0-24 hr) manner. The cell viability were analyzed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide]. The expression of p38 MAPK was measured using western blotting.

Results: Oxidative stress was induced in rat chondrocytes using hydrogen peroxide (H₂O₂). The cell viability was decreased in chondrocytes after the H₂O₂ dose and time-dependent treatment. The p38 MAPK phosphorylation occurred at a significantly increased rate after H₂O₂ treated ($p < 0.05$). Expression of p38 MAPK was decreased in the p38 inhibitor groups compared with the oxidative stress-induced chondrocyte damage via the p38 MAPK signaling pathways ($p < 0.05$).

Conclusions: It could be concluded that LIPUS can inhibit oxidative stress-induced chondrocyte damage via the p38 MAPK signaling pathways.

Key Words: Chondrocytes, MAPK, Ultrasound

Introduction

Osteoarthritis (OA), the most common type of degenerative arthritis, is characterized by marked alterations in the composition factor, structure, and function of joints in the lower extremities [1]. OA is a painful and inflammatory disease that affects an estimated 12%-15% of the population aged 25-74 years in the United States (US) [2]. From the initial stages of OA, pathogenesis involves increased chondrocyte proliferation and the synthesis of matrix proteins, proteinases, cytokines, and other inflammatory cytokines by chondrocytes [3]. Moreover, articular cartilage is difficult to

regenerate once damaged.

According to these histological features of cartilage, the first treatment for OA is aimed at reducing pain and stiffness to allow patients to maintain or recover their mobility and improve their quality of life. The second treatment aims to prevent the progression of cartilage degradation and regenerate damaged articular tissue. OA therapy can be pharmacological, non-pharmacological, or physical, and mechanical stimulation, but it is more commonly a combination of therapies, depending on the patients' state [4-6]. Various methods of mechanical stimulation of chondrocytes have been reported, such as loading with hydrostatic pres-

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sure [7], oscillation using a vibrator [8], tensile strain [9], and low-intensity pulsed ultrasound (LIPUS) [10-12]. Mechanical stimulation is an essential factor that promotes the differentiation and proliferation of intact chondrocytes. Many attempts have been made to achieve regeneration of damaged cartilage tissue. The acoustic pressure waves produced by US can be considered high-frequency micro-mechanical perturbations that may have a direct mechanical effect on exposed cells. LIPUS has been demonstrated in many studies to accelerate cell proliferation and differentiation, the healing process, and fracture repair [13-15]. Chondrocytes are probably the most important cells in the development of osteoarthritic progression [3]. Many studies have reported that chondrocytes are stimulated by LIPUS, which has been widely used in the clinical setting to accelerate the anti-inflammatory and pain relief processes. However, it remains unclear how LIPUS influences the exact mechanism leading to delayed cell degradation during chondrocyte damage in arthritis.

Accordingly, the aim of this study was to elucidate the mechanisms underlying the mechanical activation of chondrocytes and intracellular signaling through the mitogen-activated protein kinase (MAPK) pathways in LIPUS-treated chondrocytes exposed to oxidative stress.

Methods

Cell culture

A modified method for harvesting chondrocytes was used as previously described [16]. Chondrocytes were isolated from articular cartilage of 3 week old male Sprague-Dawley rats. Cartilage was removed from animals that were subsequently euthanized by an overdose of anesthesia. The cartilage was cut into thin slices, washed with sterilized phosphate-buffered saline (PBS), and soaked in 5% penicillin-streptomycin (Sigma, St. Louis, MO, USA) for 15 min. The cartilage slices were washed with PBS to remove residual antibiotic solution and digested with 0.02% type II collagenase (Sigma) in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, UT, USA) for 2 hours in a 37°C water bath. The digested cartilage was collected and centrifuged. The pellet was resuspended in DMEM and filtered through 70- μ m nylon mesh. The resultant chondrocytes were cultured in DMEM supplement with 10% fetal bovine serum and 1% penicillin-streptomycin in a 5% CO₂/95% air at 37°C. The cultured medium was replaced with fresh DMEM containing 10% fetal bovine serum and

1% penicillin-streptomycin to three times weekly. All experiments were performed when cells reached confluence within the first passage.

Low intensity pulsed ultrasound stimulation

LIPUS (Exogen 4000+; Smith & Nephew Inc., Memphis, TN, USA) at a frequency of 1.5 MHz was applied to the chondrocytes after being cultured for 72 hours. The US parameters included a spatial-average temporal-average output intensity of 30 mW/cm². The frequency was 1.5 MHz with a 200 μ s tone burst repeated at 1.0 kHz. Each 6-well plate of the LIPUS group was placed on an ultrasonic transducer [17]. After the plate cover had been removed, an anti-reflection chamber was placed in each well while taking care to avoid producing air bubbles. LIPUS was applied to the chondrocytes after 24 hours in culture through the bottom of the culture dish that had been placed between the LIPUS transducer and the dish. LIPUS was administered every day during the span of this experiment. The control plates were handled in the same manner without LIPUS. Thereafter, the cultured cell was harvested after 3 days.

Cell proliferation

Cell viability was analyzed using a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Tetrazolium salts such as MTT are metabolic by mitochondrial dehydrogenases to form a blue formazan dye and are, therefore, useful for the measurement of cell viability. The cells were gently washed with Hanks' balanced salt solution (Sigma), and exposed to rat primary chondrocyte. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. The cells were incubated for 2 hours at 37°C, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 110 μ l of dimethyl sulfoxide. A 100 μ l aliquot of each sample was then translated to 96 well plates and the absorbance of each well was measured at 550 nm with the enzyme-linked immunosorbent reader (Bio-Rad Instrument, Hercules, CA, USA). The data were expressed as a percentage of control measure in the absence of rat primary chondrocyte [18].

Western blotting analysis

For the western blotting analysis of the dish cultured for 1 week, cartilage tissues specimens were harvested 2 hours after the last LIPUS and were cut into smaller pieces. Chondrocytes were pretreated with the p38 inhibitor

(SB203580; Cell Signaling Technology, Lakewood, NJ, USA), followed by stimulation with LIPUS. Each sample was then incubated for about 8 hours in the DMEM (HyClone) supplemented with 0.1% type-II collagenase (Worthington Biochemical Co., Lakewood, NJ, USA), 10%-fetal bovine serum (Equitech-Bio Inc., Lakewood, NJ, USA) and antibiotics. The chondrocytes were then isolated, washed with culture medium, and collected by centrifugation (2,000 rpm, 37°C, 5 min). After the supernatant medium was removed, the cells were rinsed with 200 μ l PBS, filtered by centrifugation, and added to a 200 μ l aliquot of 2 $\times$ sample buffer (62.5 mmol/l Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 50 mmol/l dithiothreitol, 0.01% bromophenol blue). The cell lysates were then boiled for 10 minutes at 75°C. Equal amounts of the protein were distributed on a 10% SDS-polyacrylamide gel at 200 V, 25 mA for 80 minutes and were transblotted to nitrocellulose membranes (Millipore, Billerica, MA, USA) using a wet transfer system (BIO-RAD, Hercules, CA, USA) at 200 V, 150 mA for 60 minutes. The membranes were blocked with blocking buffer (5% skimmed milk in tris-buffered saline and 0.05% Tween

20 and Blocking One-P; Nacalai Tesque Inc., Kyoto, Japan) and were incubated with the anti-phospho-p38 MAPK (Santa Cruz Biotechnology, Dallas, TX, USA). After incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies (1/5,000), membranes were finally incubated with a chemiluminescent reagent (Perkin Elmer Life Science, CA, USA) and the signals produced were recorded on X-ray film (BIOMAX XAR Film, Rochester, NY, USA) for a densitometric analysis.

Data analysis

Data analysis was performed with PASW Statistics 18.0 (IBM Co., Armonk, NJ, USA). All of the data were expressed as mean $\pm$ standard deviation. The difference between the two mean values was analyzed by the Student's t-test. Values of $p < 0.05$ were considered as significantly different.

Results

To examine the effect of LIPUS on rat chondrocytes, the cell viability of the chondrocytes in the presence of H₂O₂ was determined. The dependence of the inhibitory effect of oxidative stress on the H₂O₂ concentration (0 to 300 μ M) and time (0 to 24 hours) was examined. Figure 1 shows that H₂O₂ added at levels > 50 μ M and 3 hours for significantly inhibited cell viability ($p < 0.05$). Figure 2 shows that the

Figure 1. Time and dose response of H₂O₂ on cell proliferation. Values are mean $\pm$ standard error of three independent experiments with triplicate dishes. * $p < 0.05$ vs. control. ** $p < 0.01$ vs. control.

Figure 2. Dose-dependent effects of H₂O₂ phosphorylation of p38 mitogen-activated protein kinase. Values are mean $\pm$ standard error of three independent experiments with triplicate dishes. ** $p < 0.05$ vs. control. *** $p < 0.01$ vs. control.

Figure 3. Effect of SB203580 on H₂O₂ and low-intensity pulsed ultrasound (LIPUS) induced phosphorylation of p38 mitogen-activated protein kinase. * $p < 0.05$ vs. control, ** $p < 0.05$ vs. LIPUS or H₂O₂ alone. # $p < 0.05$ vs. LIPUS and H₂O₂.

p38 MAPK phosphorylation occurred at a significantly increased rate after H₂O₂ treatment ($p < 0.05$). Western blot analysis results showed increase phosphorylation of p38-MAPK according to H₂O₂ concentration dependent compared with the control group ($p < 0.05$). Moreover, the phosphorylation of p38-MAPK by H₂O₂ and LIPUS was almost completely attenuated by the p38-MAPK inhibitor, SB203580 ($p < 0.05$; Figure 3).

Discussion

Half of the world's population aged ≥ 65 years have arthritis [19,20]. OA is commonly described as a non-inflammatory disease that distinguishes it from other inflammatory arthritis types such as rheumatoid arthritis [3]. These joint tissue changes impair the ability of chondrocytes to synthesize the surrounding extracellular matrix. Accordingly, the ability to maintain cartilage homeostasis is decreased in aged chondrocytes, and matrix proteins are smaller and more irregular [21]. The pathological mechanism of OA is closely associated with excessive mechanical and oxidative stress, but its underlying mechanisms have not been fully clarified. The present study investigated the effect of LIPUS in cell proliferation and related signal mechanisms that are implicated in OA using rat chondrocytes subjected

to H₂O₂ induced oxidative stress.

Mechanisms of the pathogenesis of OA such as oxidative stress and free radical generation, have been suggested to be important factors involved in OA [22]. Suitable amounts of oxidative stress have a variety of biological effects on various cell types. Free radical exposure is known to promote cellular senescence and apoptosis. However, the presence of H₂O₂ may produce reactive oxygen species and nitrous oxygen species in addition to inducing the apoptosis of chondrocytes [23,24]. Free radicals are related to many chronic inflammatory diseases [25]. Thus, H₂O₂ may be an important factor involved in the pathological mechanism of OA. In our study, cell proliferation showed significantly decreased viability after H₂O₂ treatment. In general the adult articular cartilage shows slow cellular turnover. In this turnover, new optimal tissue bearing mechanical stress is built and damaged cells subjected to oxidative stress are removed. Thus, one of the most important therapeutic significance in OA research is that adequate amounts of mechanical stress may prompt articular cartilage repair [26,27].

The MAPK family consists of proline-directed serine/threonine kinases and can be divided into two basic groups. Extracellular regulated kinases 1 and 2 are stimulated by mitogens and involved in cell growth and differentiation. In contrast, Jun N-terminal kinases and p38 MAPK are stimulated by cellular stress [28]. The p38 MAPK pathways are known signal transducers that regulate proliferation and differentiation. The role of p38 MAPK signaling in cartilage development has been reported to be involved in the maturation of growth plate chondrocytes [29]. Our results showed that activation of p38-MAPK pathways in cell subjected to oxidative stress after LIPUS stimulation was decreased by the SB203580 treatment. However, the literature is ambiguous regarding the influence of LIPUS on articular chondrocytes and the possible acceleration of the chondrogenic pathways. Some experimental studies have shown evidence that LIPUS results in enhanced cartilage repair during fracture healing and callus distraction [30,31].

Our results suggest the involvement of p38 MAPK in the regulation of apoptosis and survival after LIPUS. However, further experiments should be performed to precisely determine the involvement of the different pathways.

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