Effects of mechanical strain on ANK, ENPP1 and TGF-β1 expression in rat endplate chondrocytes *in vitro*

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Abstract. We investigated the effects of mechanical strain on the progressive ankylosis (ANK) gene and extracellular nucleotide phosphatase/phosphodiesterase (ENPP)1 mRNA expression and TGF-β1 protein expression in rat endplate chondrocytes *in vitro*. Endplate chondrocytes were isolated and cultured *in vitro*. Following identification with toluidine blue and immunocytochemical staining, chondrocytes were subjected to 10% elongation with various frequencies (0.5, 1, 1.5 and 2 Hz) using a Flexercell Tension Plus system at various intervals (3, 6, 12, 24, 36 and 48 h). As a control, cells that had been cultured statically on the same type of plate but were not subjected to stretch were also observed. Real-time reverse transcription-polymerase chain reaction and the enzyme-linked immunosorbent assay were used to study the effects of mechanical strain on ANK and ENPP1 mRNA expression and TGF-β1 concentration in the supernatant, respectively. Following treatment, the shape of the chondrocytes displayed a significant change from the original polygon to a typical spindle cell morphology; and the arrangement of the cells exhibited a change from a haphazard arrangement to an alignment with a certain direction. In the 0.5 Hz, 24-h group, the ANK gene expression was significantly increased compared to the control group ($P<0.05$); whereas in the other groups, the ANK and ENPP1 expression levels were reduced. With the increased frequencies in the 24-h group, the ANK gene expression gradually reduced. Changes in the expression of ANK and ENPP1 followed similar trends. TGF-β1 in the supernatant increased gradually in each frequency group, with a clear increase in the 0.5 Hz group. We conclude that various frequencies of mechanical strain can affect the expression of ANK, ENPP1 and endogenous TGF-β1 in endplate chondrocytes. Our results indicate that 0.5 Hz, 24 h may be the optimal stimulation condition to prevent calcification occurrence and to maintain the function of endplate chondrocytes.

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Introduction

Intervertebral disc degeneration is one of the main causes of back leg pain and has a serious impact on human health. However, the exact cause of this is unclear. Studies have found that disc degeneration begins in the cartilage endplate (1). Previous studies have shown that the process of cartilage disc degeneration in the vertebral endplate occurs with different degrees of calcification and that the degree of disc degeneration is positively correlated with calcification. A study on the molecular mechanism of calcification in cartilage endplate is therefore required. Currently, chondrocytes of the endplate are mainly in static culture, which does not reflect the effects of mechanical factors on cartilage endplate chondrocytes. In this study, we used different frequencies of cyclic strain to simulate the mechanical environment that endplate chondrocytes encounter in the body, and studied the expression of calcification-related genes and endogenous TGF-β1. This study provides evidence for the functional regulation of the cartilage endplate, and therefore offers new targets for the prevention and treatment of disc degeneration.

Materials and methods

Chondrocyte isolation and culture. The lumbar vertebrae were obtained from 160 to 180 g male Sprague-Dawley rats euthanized by intraperitoneal injection with 3% sodium pentobarbital (40 mg/kg). On a sterile bench, the lumbar vertebrae were washed twice with phosphate-buffered saline (PBS; containing penicillin 100 million U/l and streptomycin 1 g/l). The fiber annulus was incised, the nucleus pulposus was removed with a blade under a dissecting microscope, and the shallow dish-like translucent endplate cartilage, which is thin in the center and thick at the edge, was revealed. The endplate cartilage was cut with ophthalmic scissors into pieces of approximately 1 mm^3 in size. Endplate cartilage pieces were collected aseptically, and chondrocytes were obtained by sequential digestion with Trypsin (Gibco) and Collagenase II (Gibco) as described previously (1). Cells were washed twice with PBS and cultured in Petri dishes at 37˚C in a humidi-

fied incubator containing 5% CO_2. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium supplemented with 10% fetal bovine serum (FBS; Gibco), 100 million U/l penicillin and 1 g/l streptomycin. The cells from confluent primary cultures were digested with trypsin/EDTA and reseeded in DMEM/10% FBS. The identity of the chondrocytes was verified by positive immunocytochemical testing with type II collagen monoclonal antibody (Abcam company) and toluidine blue staining. The second passage was used for experiments.

Application of cyclic strain. Chondrocytes (the second passage) were seeded on an elastic silicone membrane coated with collagen I (BioFlex, Flexcell International) at a density of $1x10^5$ cells/well (diameter 35 mm) for culture. When reaching confluence, the cultured cells were pretreated with serum-free DMEM for 24 h for synchronization and stretched using a Flexercell Tension Plus system (FX-4000T; Flexercell International). The silicone membranes with cultured cells were then placed on a vacuum manifold situated in an incubator. When a precise vacuum was applied to the bottom, controlled by a computer, the silicone membranes were deformed to a pre-arranged elongation percentage, and returned to their original conformation once the vacuum was released. During this procedure, the cells remained adherent, and the deformation of the membrane was directly transmitted to the chondrocytes. The Flexercell Tension Plus system is capable of achieving a maximal effective frequency of 2 Hz. Therefore, the loading frequencies on the chondrocytes were 0.5, 1, 1.5 and 2 Hz, and the loading time-points were 3, 6, 12, 24, 36 and 48 h. In the experiments the membranes were set by computer to deform to 10% of physiological elongation. As a control, cells cultured statically on the same type of plates and without receiving stretching were also observed (S group). Following treatment, cells were photographed under a microscope. Quantitative polymerase chain reaction (Q-PCR) and the enzyme-linked immunosorbent assay (ELISA) were performed to study the effects of mechanical strain on the progressive ankylosis (ANK) gene and extracellular nucleotide phosphatase/phosphodiesterase (ENPP)1 mRNA expression and TGF-β1 concentration in the supernatant, respectively.

RNA isolation and Q-PCR. Total RNA from cultured chondrocytes was isolated by the acidified guanidinium isothiocyanate method, using TRIzol reagent (Invitrogen Corp.). First-strand cDNA was synthesized from total RNA (1 μg) by reverse transcription according to the manufacturer's instructions. Q-PCR was performed in a 10 μl reaction that contained 1 μl of the cDNA preparation and 1X SYBR-Green Supermix (Takara), using the following PCR parameters: 95˚C for 10 sec followed by 40 cycles of 95˚C for 5 sec, 55˚C for 34 sec, and 60˚C for 34 sec. The fluorescence threshold value (Ct) was calculated using the Roche 480 system software. The absence of nonspecific products was confirmed by the analysis of melt curves. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal standard of mRNA expression. This gene was selected to serve as the control, as it did not exhibit any significant difference in expression in the array analysis. Real-time Q-PCR was performed using Lightcycler (Roche Diagnostics). The primers used for Q-PCR amplification were as follows: ANK forward, 5'-CAAGAGAGACAGGGCCAAAG-3' and reverse, 5'-AAGGCAGCGAGATACAGGAA-3'; ENPP1 forward, 5'-GCTAATCATCAGGAGGTCAAG-3' and reverse, 5'-CTGGTAGAATCCCGTCAATC-3'; GAPDH forward, 5'-CTCAACTACATGGTCTACATGTCCA-3' and reverse, 5'-CTTCCCATTCTCAGCCTTGACT-3'. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai).

Statistical analysis. SPSS 11.0 statistical software was used for statistical analysis. Results were presented as the means ± standard deviation of at least three independent experiments. Comparisons of overall differences between groups were made by analysis of variance, and the control group and treatment groups were compared by the least signifiant difference (LSD) method. A P-value of <0.05 was considered significant.

Results

Cell culture. Primary cells in culture medium are spherical, with refraction under an inverted phase contrast microscope. Cells adhered to the growth surface gradually over 24 h and extended to polygons, which possessed one or two large and round nuclei and abundant cytoplasm containing secretory granules. Cells resembled cobblestones when at a high density. Following passage, it took less time for the cells to adhere, and the cells proliferated more quickly. Cells completely adhered within 24 h, and finished monolayer fusion in 3-5 days. These cells maintained the polygonal shape for three passages. However, as the passage number increased, cell proliferation began to slow down. After four or five passages, cells became degenerative. The majority of the cells displayed morphological changes, becoming spindle-shaped, showing a tendency to fibrosis.

Phenotypic identification. Positive expression of Type II collagen in immunocytochemical staining revealed brown coloration in the cytoplasm, with no positive staining in the nucleus. Toluidine blue staining revealed purple or red metachromatic glycoprotein secretion around the cell and in the cytoplasm (Fig. 1).

Effects of various frequencies of cyclic strain on shape and arrangement of chondrocytes. Compared to the control group, following treatment, the morphology and arrangement of the chondrocytes demonstrated a significant change from the original polygonal shape into a typical spindle cell morphology, and the cells changed from a haphazard arrangement into an alignment with a certain direction, in which most of the endplate chondrocytes were perpendicular to the direction of the strain (Fig. 2).

Effects of mechanical strain on mRNA expression of ANK and ENPP1. In the 0.5 Hz, 24-h group, ANK gene expression was significantly increased compared to the control group ($P<0.05$), whereas in the other groups, ANK and ENPP1 expression were reduced. With increased frequencies in the 24-h group, ANK gene expression gradually decreased. ANK and ENPP1 expression levels exhibited a near-identical trend (Fig. 3).

Change in TGF-β1 concentration in cell culture supernatant. In the groups treated with various frequencies, TGF-β1 in the

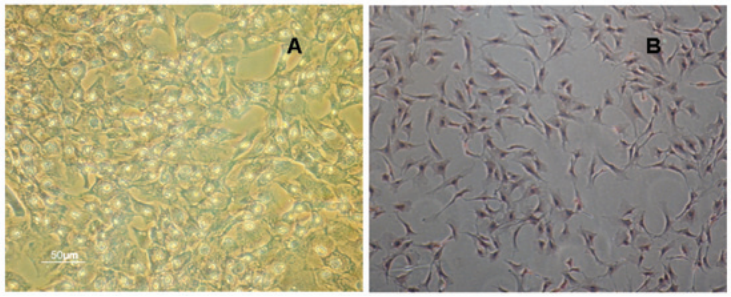

Figure 1. Phenotypic identification. (A) Toluidine blue staining (magnification, x100). (B) Type II collagen immunocytochemical staining (magnification, x100).

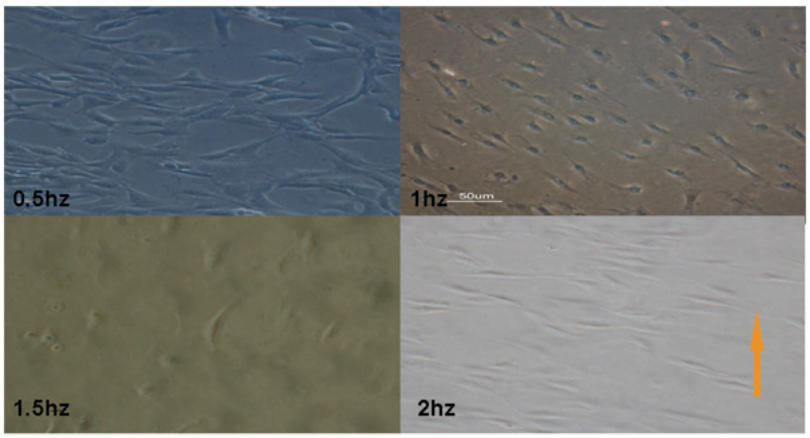

Figure 2. Chondrocyte morphology with treatment of cyclic strain (magnification, x100).

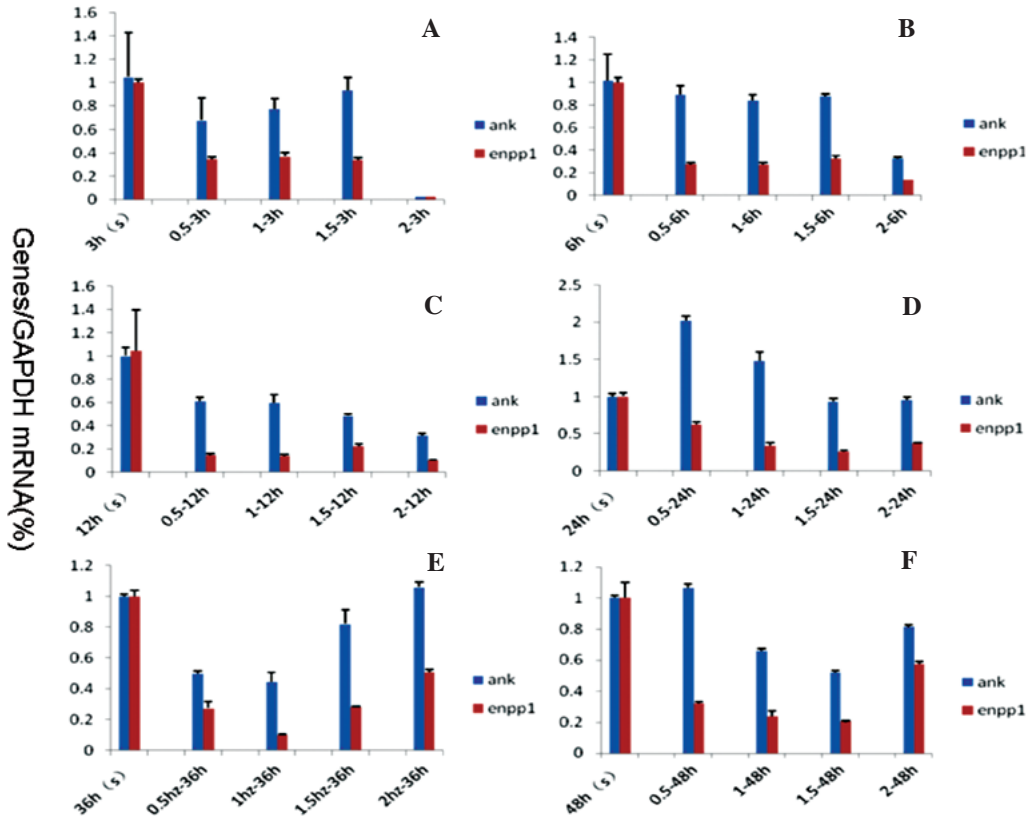

Figure 3. ANK and ENPP1 mRNA expression under cyclic strain treatment. (A-F) ANK and ENPP1 expression at various times and strain frequencies.

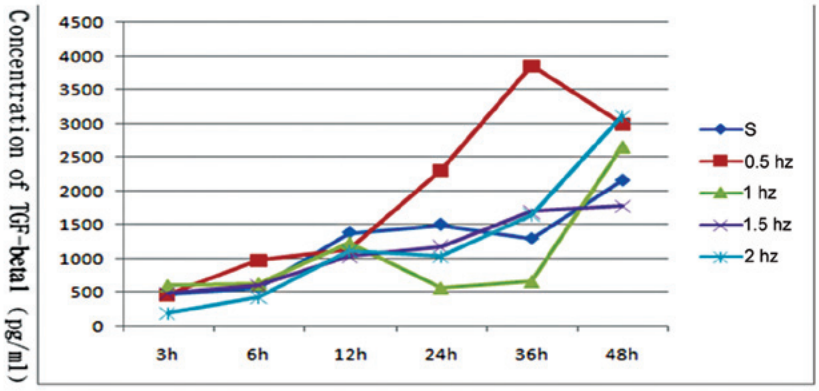

Figure 4. TGF-β1 concentration in cell culture supernatant.

supernatant increased gradually in a time-dependent manner. This was particularly marked in the 0.5 Hz group, in which TGF-β1 reached a peak at 36 h and then decreased. The down-regulation of TGF-β1 was not observed in other groups within 48 h (Fig. 4).

For all frequencies, cells exhibited a significant change from the original polygon shape to a typical spindle morphology. Cells changed from the chaotic arrangement into an alignment with a certain direction. The arrow indicates the direction of the radius (Fig.2).

Discussion

Disc degeneration is a significant cause of lower back pain. However, the exact mechanism involved is still unclear. Previous studies have revealed that nutritional disturbance of the intervertebral disc is one of the main causes of disc degeneration (3). Currently, it is believed that nutrient supply to the intervertebral disc mainly occurs through diffusion from the vascular buds to the cartilage endplate. Previous studies have confirmed that cartilage endplate calcification is the initiating factor of intervertebral disc degeneration and plays a crucial role in the occurrence and development of this disease (2). Cartilage endplate sclerosis, calcification and thickening may lead to decrease of oxygen in the blood supply, inhibition of metabolic clearance, increase in lactic acid concentration and lowering of pH, and therefore results in the nutritional disturbance of the intervertebral disc and subsequent disc degeneration. Peng *et al* and Aringa *et al* (4,5) noted that, following a serious degree of disc degeneration, the calcified layer of vertebral cartilage endplate was markedly thickened, with a highly positive correlation. The phenomenon of cartilage endplate calcification has long been observed. However, few studies have been performed to identify the molecular mechanism of endplate calcification. Endplate chondrocytes are the basic structural and functional units of the cartilage endplate, and play significant roles in maintaining the integrity of the extracellular matrix, adapting to peripheral stimulation, and recognizing and transducing mechanical signals.

This study employed a cell mechanical loading device. The cells adhering to the membrane were imposed with cyclic stretch stimulation through stretching the membrane. Traction force, time and frequency can be quantified and repeated. This device has been widely used in the field of cell mechanics internationally, and is recognized as the best choice. After loading, the arrangement of microscopically observed chondrocytes displayed a significant change from a haphazard arrangement into an alignment perpendicular to the direction of the strain, which is in agreement with previous results, published by Liu *et al*, in vascular smooth muscle cells with stimulation of cyclic stretch (6). They found that with 1.25-Hz stimulation, the cell rearrangement phenomenon became clearer, and the ratio of integrin β1 (integrin-β1) and F/G actin also changed. With the appropriate inhibitors, mechanical stimulation did not cause changes in cell arrangement. Therefore, the frequency of mechanical stimulation played a major role, and integrin β1 and P38MAPK may be involved in mechanical signal transduction. Under our experimental conditions, we observed that with 1 Hz mechanical stimulus, cell rearrangement phenomenon was marked.

The progressive ankylosis (ANK/ANKH) gene is located on human chromosome 5p, close to the D5S1954 region. It is one of the key genes and its product is a 492-amino acid transmembrane protein that regulates intracellular inorganic pyrophosphate (PPi) steady-state concentration. It may function as a transporter that transports PPi to the extracellular environment (7,8). The hydrophilic profile of the ANK protein reveals that it has ten transmembrane segments. The normal ANK protein has a strong function on anti-calcification. Gene mutation or the genetic defect of ANK leads to the inorganic pyrophosphate levels inside and outside of the cell undergoing a change, with elevated intracellular PPi and decreased extracellular PPi. PPi normally presents in synovial fluid, blood and urine. A certain level of PPi can inhibit the occurrence of mineralization. In the extracellular body fluid, PPi is considered to be a natural inhibitor of hydroxyapatite formation (9). Mice with a deficient ANK gene exhibit progressive stiffness of the limbs, joints and spine, joint space narrowing, and ossification of the spinal ligaments and intervertebral disc, which are similar to the symptoms of human ankylosing spondylitis. The ANK gene is so-named for this reason. *In vitro* cell culture experiments have revealed that the overexpression of ANK leads to increased extracellular total PPi, and on the contrary, loss of function of ANK leads to decreased extracellular total PPi (10).

The extracellular nucleotide phosphatase/phosphodiesterases (ecto-nucleotide pyrophosphatases/phosphodiesterases;

ENPPs) are a family of outer membrane proteins with a conserved structure. ENPPs widely exist in mammals, being detected in almost all tissues. To date, seven members of this family have been identified, and each member is limited to a specific sub-structure and/or to specific cell types (11). ENPP1 plays a significant role in regulating physiological bone mineralization and pathological cartilage calcification. ENPP1 catalyzes ATP into AMP + PPi, and PPi is an effective hydroxyapatite crystal inhibitor. Deficiency of extracellular PPi can lead to spontaneous excessive calcification. Rutsch *et al* found that dysfunction of the ENPP1 gene may lead to many human diseases, such as idiopathic infantile arterial calcinosis (12). As the main nutritional pathway of the intervertebral disc, the cartilage endplate plays an essential role in disc degeneration. Studies on the mechanism of endplate cartilage calcification are insufficient, and the correlation between cartilage endplate calcification and the expression of ANK and ENPP1 is unclear. In this study, we used mechanical loading devices to simulate *in vitro* the environment that endplate chondrocytes suffer in the body, by administering cyclic stretch stimulation at various frequencies and for various times. We observed that compared to the control group, ANK was up-regulated in the 24-h group, which was more obvious in the 0.5 Hz, 24-h group (2.025 times higher compared to the control group, $p<0.01$). In the other groups, ANK and ENPP1 expression levels were reduced and ANK and ENPP1 expressions almost showed the same trend. Therefore, the 0.5 Hz frequency, 24 h stimulation time, was the best stimulus.

TGF-β1 is a polypeptide homodimer of 25 kDa. It plays a significant role in cell proliferation, differentiation, migration, apoptosis and extracellular matrix synthesis. Previous research has shown that exogenous TGF-β1 positively regulates chondrocytes, and can promote chondrocyte proliferation, maintain chondrocyte phenotype stability and inhibit cell terminal differentiation and calcification. It was revealed that the expression of TGF-β1 in degenerative endplate chondrocytes was significantly lower than in normal chondrocytes, indicating that when cartilage endplate degeneration occurs, the ability of chondrocytes to secrete TGF-β1 declines, leading to a decreased ability to synthesize the extracellular matrix, which physiologically and pathologically results in a negative feedback that further promotes endplate degeneration. It has been reported that TGF-β1 has a positive regulatory effect on the expression of ANK (13).

Caillotto *et al* found that ANK expression increases in articular chondrocytes cultured *in vitro* by TGF-β1, leading to increased PPi levels, and that ANK is more sensitive to TGF-β1 than ENPP1 (14). It is thought that TGF-β1 regulates ANK gene expression through the Ras/Raf-1/ERK and ca^{2+}-dependent PKC pathways. The present study shows that with the extension of time, TGF-β1 in the supernatant increased gradually in each frequency group, with a marked increase in the 0.5 Hz group. The ANK expression was also increased at the same time, whereas ENPP1 expression was lower than the control group, indicating that this condition is disadvantageous to calcification occurrence, and that this is conducive to the function of endplate chondrocytes. This study only considered single mechanical stimulation to endplate chondrocytes. However, the environment to which endplate chondrocytes are exposed *in vivo* is complicated and many factors are involved in calcification, such as alkaline phosphatase, extracellular enzyme PC-1, osteopontin and cytokines. Therefore, the mechanism of cartilage endplate calcification requires further study.

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