Regulation of plasminogen activator activity and expression by cyclic mechanical stress in rat mandibular condylar chondrocytes

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Abstract. To investigate the mechanism of cartilage degradation induced by overloading in the temporomandibular joint (TMJ), the effect of cyclic mechanical compressive stress on the activity of plasminogen activator (PA) and the expression of the predominant components of the PA system were analyzed in cultured mandibular condylar chondrocytes (MCCs) in rats. MCCs were exposed to cyclic mechanical compressive stress (2000, 4000 and 6000 μ strain) at 0.5 Hz by a four-point bending system. The activity of PA was determined by hydrolysis of the chromogenic substrate H-D-Val-Leu-Lys-pNA (S-2251). The mRNA and protein expression levels of urokinase-type PA (uPA), tissue-type PA (tPA), uPA receptor (uPAR) and PA inhibitor 1 (PAI-1) were detected by qPCR and western blot analysis, respectively. Cyclic mechanical stress at 4000 and 6000 μ strain induced the expression of uPA, tPA and uPAR, and increased the activity of PA. Furthermore, cyclic mechanical stress at 6000 μ strain also inhibited the expression of PAI-1. Analysis of pericellular proteolytic activity demonstrated that PA functioned as the active enzyme in excessive mechanical stress responsiveness (e.g., 4000 and 6000 μ strain) largely via uPAR, not PAI-1. Cyclic mechanical stress at 2000 μ strain induced the expression of tPA and PAI-1; however, it did not change the activity of PA. These results suggested that the mechanical induction of uPA, tPA and uPAR upregulated PA activity, which may provide a proteolytic environment of extracellular matrix components and subsequently contribute to the cartilage degradation in TMJ osteoarthritis.

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Introduction

Regular joint loading is required for the maintenance of healthy cartilage tissue. The ability of cartilage to withstand these stress loads predominantly depends on extracellular matrix (ECM), proteoglycans and collagens. Physiological mechanical stress is essential for chondrocyte metabolism, and a pre-requisite for maintaining the normal composition and structure of joint cartilage. Conversely, excessive mechanical stress is recognized as a definite degradation factor for cartilage ECM and as a key factor in osteoarthritis (OA).

OA is a predominant and much neglected cause of disability in the aged. During the development of this multifactorial degenerative disease, joint pain and progressive limitation of motion are commonly accompanied by the gradual loss of joint cartilage, sclerotic and cystic changes in local bone, and osteophyte formation. Although the increased synthetic activity within OA cartilage has been previously demonstrated, the ability of chondrocytes to synthesize novel matrix is exceeded by the rate of cartilage degradation (1). Previous studies have suggested that the elevated proteolytic activities mediated by several proteinases may be involved in the degradation of the cartilage ECM (2). Among these enzymes, the plasminogen activators (PAs) and the matrix metalloproteinases (MMPs) are the most strongly implicated in cartilage degradation (3). Following the demonstration of the involvement of PAs in the endogenous activation of latent collagenases (4), intra-articular PA has been suggested as a possible activator of cartilage destruction in rheumatoid arthritis and OA. Several studies have demonstrated that the PA levels in OA patients were positively correlated with the degradation of cartilage (5-7). Furthermore, the activity of PA was also correlated with the levels of active MMP1 and MMP3, which were directly involved in the degradation of cartilage proteoglycans and collagens during the pathological processes of OA (8-10). However, these studies only determined the involvement of PA in the cartilage destruction of patients with OA, and did not demonstrate that mechanical load results in OA by affecting the expression of PA. In the temporomandibular joint (TMJ), mandibular motions may result in diversely static and/or dynamic loading during talking and chewing. Cyclic mechanical stress is suggested to be the predominant factor resulting

in the etiology of TMJ OA among various types of mechanical stress (11). However, whether cyclic mechanical stress induces the PA-mediated degradation of cartilage remains unclear.

The aim of the present study was to investigate the effects of cyclic mechanical stress on PA activity and expression in cultured mandibular condylar chondrocytes (MCCs). Furthermore, based on the suggestion that PA may be intensified and activated by its cell-bound receptor (uPAR) and inactivated by its specific inhibitor (PAI-1), it was also investigated whether other predominant components of the PA system, uPAR and PAI-1, are involved in the regulation of PA activity during mechanical loading. The results demonstrated that the PA system increased the activity of the MCCs as their response to excessive mechanical stress was dependent upon the mechanical induction of uPA, tPA and uPAR. Thus, PA functions as the active enzyme in the process of mechanical stress responsiveness, largely via uPAR not PAI-1.

Materials and methods

Cell isolation and culture. Mandibular condylar cartilage was isolated from Sprague-Dawley rats (age, 1-2 weeks) and was subsequently minced. Following digestion with 0.25% trypsinase and 0.2% collagenase, the primary MCCs were rinsed with phosphate-buffered saline three times and prepared as a single-cell suspension in a growth medium of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 25 mM HEPES, 1% L-glutamine and 100 mg/ml kanamycin. MCCs were separated from the debris by filtration through a 40-μm mesh nylon sieve. The cells were then washed, suspended in DMEM and counted. Viable cells were seeded at a density of $2x10^5$ cells/cm^2 in a humidified atmosphere at 37˚C and 5% CO_2. Following 5-7 days in culture, third-generation MCCs were validated by type II collagen immunohistochemical staining as described previously (12). The cells were then utilized for the subsequent experiments. This study was approved by the Institutional Animal Care and Use Committee of Sichuan University (Chengdu, Sichuan, China).

Lentiviral short hairpin RNA (shRNA) vectors and transfection. In order to knockout uPAR expression, RNAi-Ready Plvx-shRNA2 vector (Clontech Laboratories, Inc., Palo Alto, CA, USA) was used. This vector was provided prelinearized for ligation with a double-stranded oligonucleotide encoding a shRNA. In addition to expressing shRNAs, plvx-shRNA2 also expressed the fluorescent protein ZsGreen 1 and an ampicillin resistance gene for the selection of stable transfectants. Two sequences of the human uPAR gene Plaur (accession no. NM 017350) were selected as targets for RNA interference, including shuPAR 1 (start 89: CGGTGCATACAGTGCGAAA) and shuPAR 2 (start 174: GGGAATGGGAAGACGCCGA) along with shLuciferase (start 1594: CTGTCTGGCACAAGAAAGTGT). For the majority of the experiments, shuPAR 1 was used. To produce recombinant lentivirus for target cell infection, Lenti-X plasmid vectors were cotransfected into Lenti-X 293T cells, along with a Lenti-X HTX Packaging mix (Clontech Laboratories, Inc.). Empty vector (EV) expressing enhanced green fluorescent protein was used as a control for infection efficiency. At 48 h after transfection, supernatants containing the lentivirus were collected and frozen at -70˚C. Cells were infected with twice-diluted supernatant and 4 μg/ml polybrene for 8 h prior to being washed. Lentiviral vector expressing enhanced green fluorescent protein served as a control for infection efficiency; 90-95% of the cells were fluorescent for 4-6 days following infection and only a fraction of cells (<10%) were destroyed during the 2-day selection process.

Mechanical stress on the cell culture. When the cells had reached confluence, culture plates were transferred to bending dishes filled with 25 ml fresh medium. The cells were then subjected to cyclic uniaxial mechanical compressive stress using the four-point bending system (patent no. 01129166.4 and 01256849.x), which consisted of a computer-controlled, servomotor-driven, linear actuator assembly with an interface controller that controlled vertical displacement and actuator ram speed (displacement rate). Culture plates were loaded with or without cyclic mechanical compressive stress of 2000, 4000, 6000 and 8000 μ strain at 0.5 Hz for 6, 12 and 24 h, separately.

Determination of the rates of DNA, proteoglycan and collagen synthesis. For determining the rate of DNA synthesis, MCC cultures (on day 7) were exposed to 5 μCi/ml [3H] thymidine in DMEM containing 0.5% fetal bovine serum (FBS) for the final 4 h of the application of varying mechanical stress. The radioactive counts of the cell layer were measured by a scintillation counter. To determine the rate of proteoglycan and collagen syntheses, the MCC cultures were exposed to 2.5 μCi/ml [35S] sulfate or 10 μCi/ml [2,3-3H] proline in DMEM containing 0.5% FBS for the final 4 h of the application of various mechanical stressors. The rates of proteoglycan and collagen synthesis were determined by measuring the incorporation of [35S] sulfate or [2,3-3H] proline into the materials precipitated with cetylpyridinium chloride (Nacalai Tesque Inc., Kyoto, Japan) in a scintillation counter.

qPCR. qPCR was used to measure the mRNA expression levels of uPA, tPA, uPAR and PAI-1 in MCCs subjected to different methods of mechanical loading. Total RNA was isolated from cultured rat MCCs, which had or had not been subjected to cyclic mechanical stress, with TRIzol reagent (Invitrogen Life Technologies Inc., Carlsbad, CA, USA) while being treated with RNase-free DNase I (Takara Bio, Inc., Shiga, Japan) to avoid genomic DNA contamination. A single-stranded cDNA was synthesized from 1 μg total RNA using a RevertAid First-Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) with a random hexamer primer. The genes were amplified in a 25-μl reaction mixture containing PreDeveloped TaqMan Assay Reagents (Applied Biosystems, Foster City, CA, USA) using ABI PRISM 7700 (Applied Biosystems). The conditions comprised an initial denaturation step at 94˚C for 1 min, followed by 45 cycles at 94˚C for 10 sec, 55˚C for 30 sec and 72˚C for 1 min, and finally an extension step at 72˚C for 5 min. As an internal control of each sample, the glyceraldehyde 3-phosphatase dehydrogenase (GAPDH) gene was used for standardization. Quantitative results of real-time PCR were assessed with a cycle threshold

Table I. Oligonucleotide primers and probes for qPCR.

Gene	Forward primer 5'→3'	Reverse primer 5'→3'	TaqMan probe 5'→3'
uPA	TCACTGGCTT	TCCAATGTGG	TGCTCGGGAG
	CGGACAAGAGA	GACTGAATCCA	ATTCAGGAGGACCTCTTA
tPA	GGCCAAATGC	CGTGGTATAC	TACTGCAGAA
	CATCAAGCT	TTCCCTGCCTTAAA	ACCCAGACCGAGACGTG
uPAR	GTCCTGTTGG	CACGGTGCTT	TCACCACCTC
	TCTTCTCCTTGTG	CGGGAATG	CAGCTCCTCGGC
PAI-1	CCTCGGTGCT	GTGCCCCTCT	ACCACAGCAG
	GGCTATGCT	CACTGATATTGAA	GGAAAACCCGGC
GAPDH	CAAGTTCAAC	TGGTGAAGAC	TCTTCCAGGA
	GGCACAGTCAA	GCCAGTAGACTC	GCGAGATCCCGCTAAC

uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; uPAR, uPA receptor; PAI-1, plasminogen activator inhibitor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(C*t*) value, which identified a cycle when the fluorescence of a given sample became significantly different from the base signal. The sequences of the primers and probes for uPA, tPA, uPAR, PAI-1, MMP-2, MMP-9 and GAPDH, are listed in Table I. The relative expression levels of the genes were calculated using the ΔΔCt method comparing the results with those of the 0 h control groups.

Western blot analysis. Total proteins were extracted from MCCs that had or had not been subjected to mechanical stress load and were quantified using a Bicinchoninic Protein Assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Total protein (25 μg) from each sample was separated by 10% sodium dodecyl sulfate polyacrylamide gel and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore, Boston, MA, USA) using a Bio-Rad Mini Trans-Blot system (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and were incubated with primary antibodies against uPA (1:400), tPA (1:400), uPAR (1:800), PAI-1 (1:500) and GAPDH (1:1,000) for 4 h at room temperature. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody (1:5,000 diluted in TBST with 2% bovine serum albumin) following 1 h of incubation. The bands were scanned using a densitometer (GS-700; Bio-Rad Laboratories), and quantification was performed using Quantity One 4.6.3 software (Bio-Rad Laboratories). All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, US).

Determination of PA activity. The conversion of plasminogen (PLG) to plasmin by PA in cell lysates was determined by the hydrolysis of the chromogenic substrate H-D-Val-Leu-Lys-pNA (S-2251, KabiVitrum, Stockholm, Sweden) as described previously. Briefly, cells were lysed by repeated freezing and thawing, and were then mixed with PLG (1.3 mM; Sigma-Aldrich, St. Louis, MO, USA), S-2251 (0.7 mM) and TBST (50 mM Tris and 0.05% Tween 80, pH 8.0). The optimal density was measured at 450 nm in a microtest plate spectrophotometer. The PA activity was quantified with a calibration curve using human urokinase (Sigma-Aldrich) as a standard and was presented in units according to international standards.

Statistical analysis. Results are expressed as the mean ± SD. Statistical analysis was performed using analysis of variance followed by Scheffe's test for multiple comparisons and an independent Student's t-test for comparisons of two groups of data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of mechanical stress on cell appearance and mechanical stress condition selection. To mimic the natural living status and select appropriate mechanical stress conditions, cyclic mechanical stresses of 2000, 4000, 6000 and 8000 μ strain at 0.5 Hz were adopted for this investigation. By day 7, the cultures reached confluence (Fig. 1A) and type II collagen staining was positive in the cytoplasm (Fig. 1B). The cell appearance, including morphology and adherence, was unchanged in the cultures with 2000 μ cyclic mechanical strain during the experimental period (Fig. 1C). Subsequent to 6 h of loading, cyclic mechanical strains of 4000 and 6000 μ strain resulted in a marked change in cell appearance of the MCCs, which manifested in the change in morphology from star-shaped to shuttle- or spindle-shaped. Furthermore, the long axes of the MCCs was oriented parallel to the direction of mechanical stress (Fig. 1D and 1E). Increasing the loading magnitude at 8,000 μ strain of stress, markedly decreased cell adhesion, resulting in the detachment of a number of MCCs from the culture plate during stress loading (Fig. 1F). These results indicated that an abnormally high magnitude of mechanical stress (8000 μ strain) as an *in vitro* regulatory factor for mechanical loading may be unsuitable for this study. The appropriate stress magnitude was thus defined at 2000, 4000 and 6000 μ strain.

Furthermore, to investigate the effects of mechanical stimulation on chondrocyte metabolism, the synthesis of DNA,

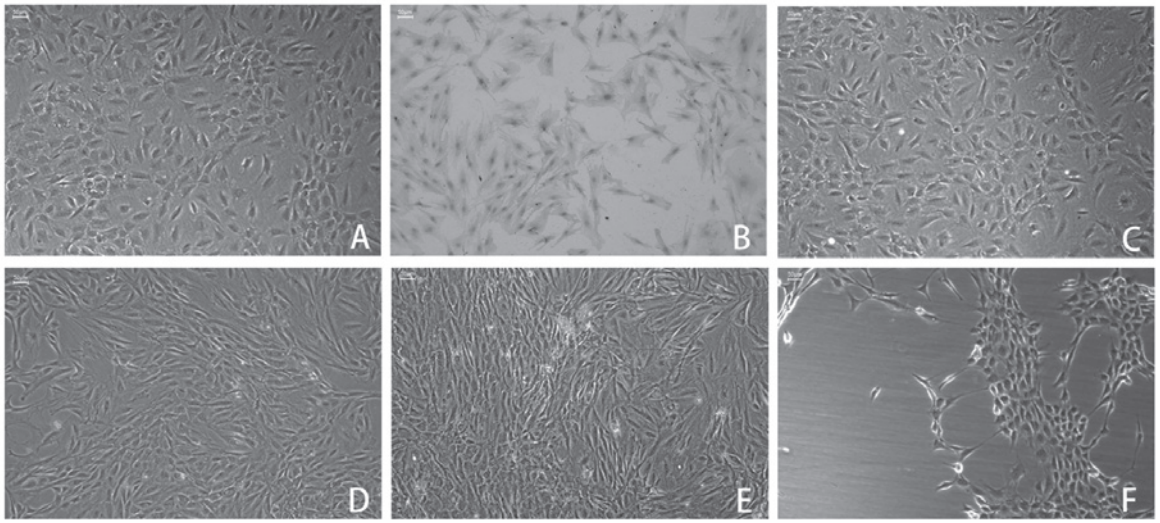

Figure 1. Effects of cyclic mechanical stress on MCC cell appearance. (A) Third-generation MCCs were polygon-shaped, expressed a vivid typical 'pavement stone' appearance. (B) Positive immunoperoxidase staining of type II collagen was observed in the cytoplasm of MCCs. (C) No obvious arrangements of MCC cell appearance at cyclic mechanical stress of 2,000 μ strain. (D and E) Following 6 h of loading, MCCs rearranged at cyclic mechanical stress of 4,000 and 6,000 μ strain and the cell morphology changed from star-shaped to shuttle- or spindle-shaped, and the cells long axes were oriented parallel to the direction of stress loading. (F) Large numbers of MCCs were detached from the plate following 6 h of 8,000 μ strain of mechanical stress. Magnification, x100; scale bar, 50 μm. MCC, mandibular condylar chondrocyte.

collagen and proteoglycans was measured by [3H] thymidine, [35S] sulfate and [2,3-3H] proline incorporation into MCCs at different mechanical stress levels. Compared with the unloaded control, cyclic mechanical stress of 2000 μ strain significantly increased the DNA synthesis of MCCs, whereas cyclic mechanical stress of 4000 or 6000 μ strain decreased the rate of DNA synthesis (Fig. 2A). Similar observations were identified in the synthesis of collagen and proteoglycans. Cyclic mechanical stress of 2000 μ strain also increased the synthesis of collagen and proteoglycans in MCCs, whereas cyclic mechanical stresses of 4000 and 6000 μ strain inhibited the MCC collagen and proteoglycan synthesis during loading periods (Fig. 2B and 2C). Based on these data, the cyclic mechanical stress of 4000 and 6000 μ strain were defined as excessive stress stimuli, whereas cyclic mechanical stress of 2000 μ strain was defined as a moderate stress stimuli, as described previously (14-16).

Effect of mechanical stress on PA activity. The cell-associated PA activity on cell surface and soluble PA activity was determined in the culture supernatant of MCCs with and without subjection to mechanical stress. Compared with the unloaded control, the PA activity on the cell surface was gradually increased following loading of 4000 and 6000 μ strain; however, did not significantly change under 2000 μ strain (Fig. 3A). The soluble PA activity showed no significant change at 2000 and 4000 μ strain loading; however showed a significant increase at 6000 μ strain loading (Fig. 3B). The cell-associated PA activity at 6000 μ strain was significantly higher compared with that of the 4000 μ strain (Fig. 3C).

Effect of mechanical stress on the mRNA expression of uPA, tPA, uPAR and PAI-1. To determine whether mechanical stress induced gene expression of the predominant components of PAs, uPA, tPA, uPAR and PAI-1, qPCR was performed using primer pairs with a probe for each. Compared with the unloaded control, cyclic mechanical stress at 2000 μ strain exhibited no effect on the uPA mRNA levels. However, cyclic mechanical stress at 4000 and 6000 μ strain significantly increased the uPA mRNA levels during the different loading periods (Fig. 4A). Cyclic mechanical stress at 2000, 4000 and 6000 μ strain significantly increased the tPA mRNA levels and these elevated mRNA levels were approximately equivalent among different loading periods (Fig. 4B). Cyclic mechanical stress also resulted in an increase in uPAR mRNA expression at 4000 and 6000 μ strain and the increased mRNA level simultaneously peaked following 12 h of loading. However, cyclic mechanical stress at 2000 μ strain exhibited no effect on uPAR mRNA expression (Fig. 4C). Cyclic mechanical stress at 2000 μ strain increased the PAI-1 mRNA expression as early as 6 h following the application of mechanical loading. Conversely, cyclic mechanical stress at 6000 μ strain decreased the PAI-1 mRNA expression following 6 h of loading. Cyclic mechanical stress at 4000 μ strain showed no effect on the expression of PAI-1 mRNA (Fig. 4D).

Effect of mechanical stress on the protein expression of uPA, tPA, uPAR and PAI-1. To investigate the involvement of PAs, the proteins from MCCs, with and without exposure to cyclic mechanical stress, were analyzed by western blot analysis. Compared with the unloaded control, the expression of uPA protein was significantly increased following the application of 4000 or 6000 μ strain of mechanical loading. The increased uPA protein expression between 4000 and 6000 μ strain of mechanical stress showed no significant difference. No significant change in the uPA protein expression was identified following the application of mechanical stress at 2000 μ strain (Fig. 5A). The expression of tPA protein was significantly increased following the application of loading at 2000, 4000 and 6000 μ strain and these elevated tPA protein levels

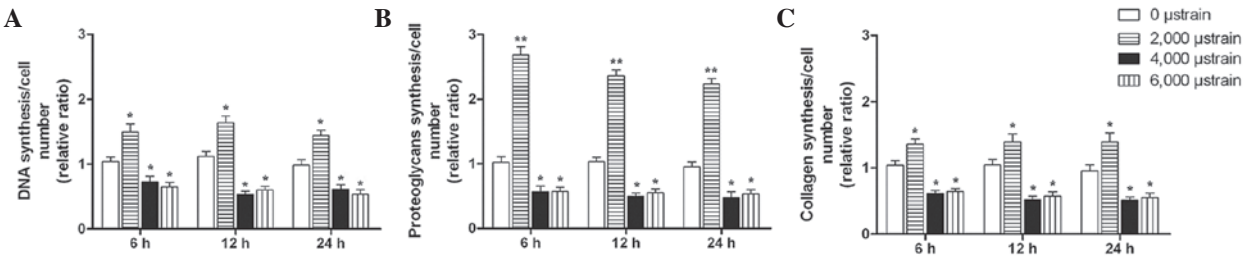

Figure 2. Effect of cyclic mechanical stress on MCC cell metabolism. The syntheses of (A) DNA, (B) proteoglycans and (C) collagen were determined as described in Materials and methods. Rat MCCs were exposed to cyclic mechanical stress of 2000, 4000 and 6000 μ strain for 6, 12 and 24 h, and labeled with [*H] thymidine (5 μ Ci/ml), [35S] sulfate (2.5 μ Ci/ml) and incorporation of [35S] sulfate and [2,3-3H] proline for the final 4 h, respectively. The data obtained from the loaded experimental cultures were normalized to the average of the 0 h group control cultures. *P<0.05 and **P<0.01, vs. unloaded control. Mean ± SD; n=5. MCC, mandibular condylar chondrocyte.

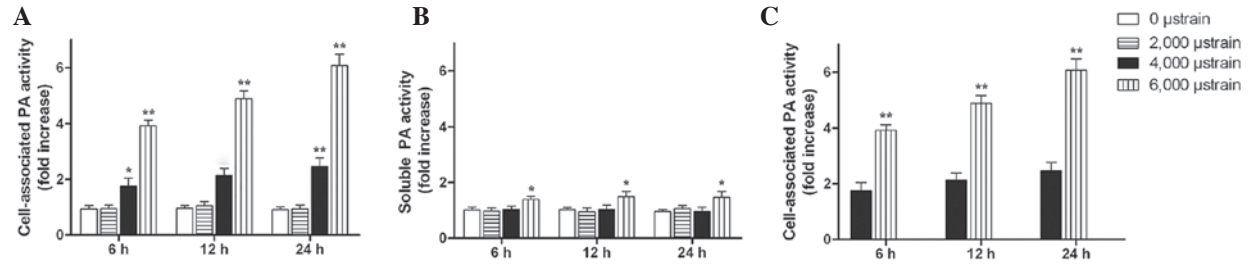

Figure 3. The effect of cyclic mechanical stress on the PA activity. Rat MCCs were exposed to cyclic mechanical stress of 2000, 4000 and 6000 μ strain. The activity of cell-associated PA at (A) cell surface and (B) soluble PA in the culture were detected by hydrolysis of the chromogenic substrate S-2251. (C) The activity of PA at mechanical stress of 6000 μ strain was significantly increased compared with that of the 4000 μ strain. Values are expressed as the fold-increase relative to that in the 0 h group control cultures. *P<0.05 and **P<0.01, vs. unloaded control. Mean ± SD, n=3. PA, plasminogen activator; MCC, mandibular condylar chondrocyte.

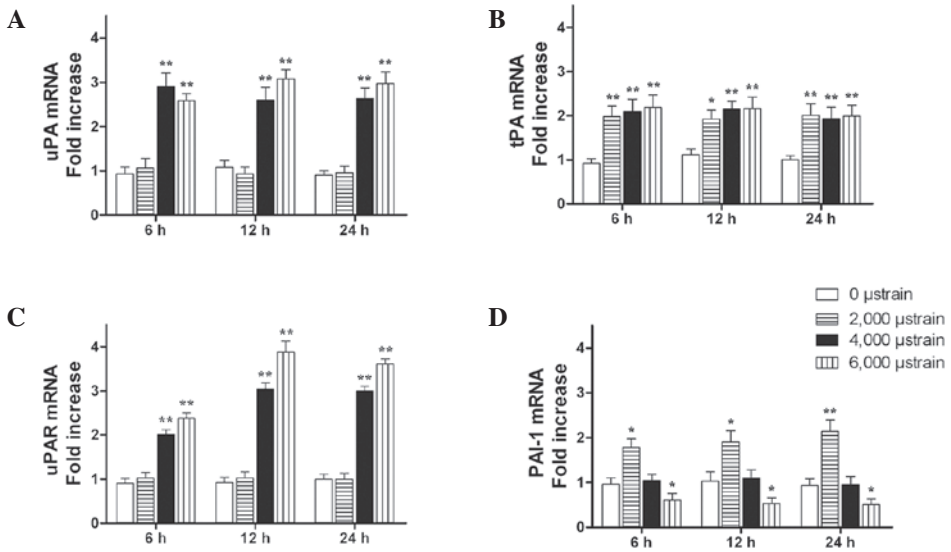

Figure 4. Effect of cyclic mechanical stress on the expression of mRNA. The mRNA level of (A) uPA, (B) tPA, (C) uPAR and (D) PAI-1 were determined using qPCR. Rat MCCs were exposed to cyclic mechanical stress of 2000, 4000 and 6000 μ strain for 6, 12 and 24 h, respectively. Values were normalized with the expression level of glyceraldehyde 3-phosphate dehydrogenase and expressed as the fold-increase of mRNA expression relative to that in 0 h group control cultures. *P<0.05 and **P<0.01, vs. unloaded control. Mean ± SD; n=5. uPA, urokinase-type plasminogen activator; tPA, tissue-type PA; uPAR, uPA receptor; PAI-1, PA inhibitor 1; MCC, mandibular condylar chondrocyte.

were approximately equivalent among the different loading magnitudes (Fig. 5B). The expression of uPAR was unchanged following mechanical stress of 2000 μ strain. In contrast, at cyclic mechanical stress of 4000 and 6000 μ strain, the uPAR protein expression significantly increased following the application of mechanical loading. Furthermore, the increased uPAR protein levels at 6000 μ strain magnitude were higher than that of the 4000 μ strain (Fig. 5C). The expression of PAI-1 protein gradually increased subsequent to the application of mechanical loading of 2000 μ strain; however, decrease following the

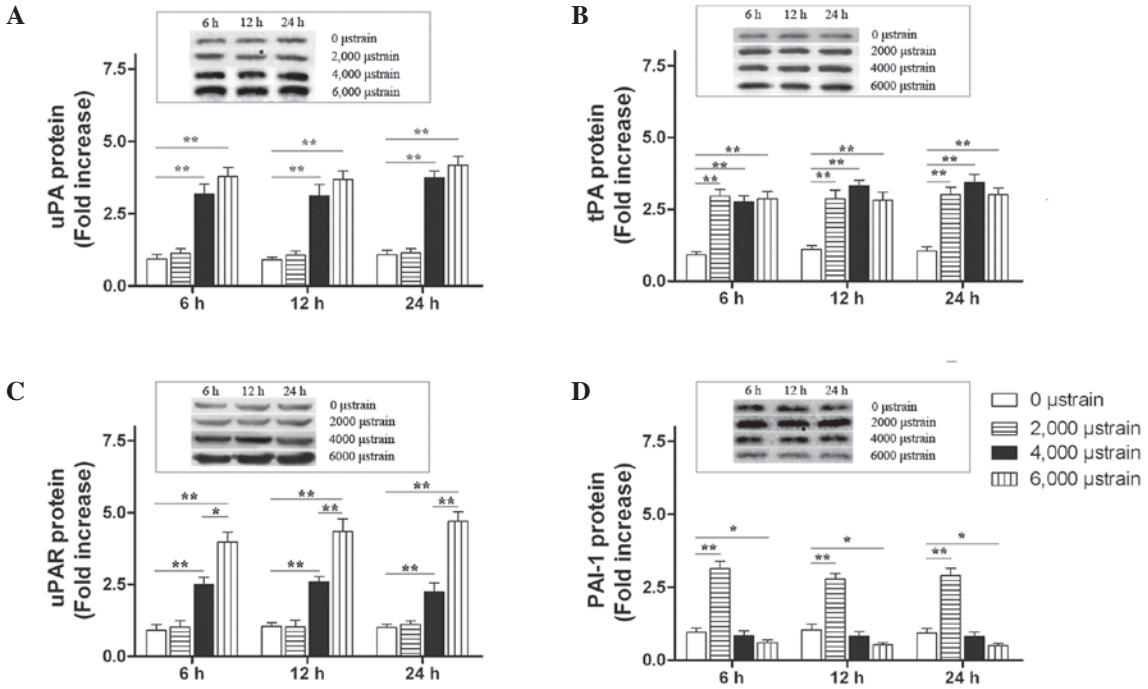

Figure 5. Effect of cyclic mechanical stress on the expression of protein. Rat MCCs were exposed to cyclic mechanical stress of 2000 and 4000 μ strain for 3, 6, 12 and 24 h. The protein level of (A) uPA, (B) tPA, (C) uPAR and (D) PAI-1 were determined by western blot analysis. All values were normalized with the expression level of glyceraldehyde 3-phosphate dehydrogenase and expressed as the fold-increase of protein expression relative to that in 0 h group control cultures. *P<0.05 and **P<0.01, vs. unloaded control were indicated. Mean ± SD; n=3. uPA, urokinase-type plasminogen activator; tPA, tissue-type PA; uPAR, uPA receptor; PAI-1, PA inhibitor 1; MCC, mandibular condylar chondrocyte.

application of mechanical loading at 6000 μ strain (Fig. 5D). No significant change in the PAI-1 expression was observed following the application of mechanical loading of 4000 μ strain. These results suggested that the expression of proteins of the PA system was involved in the regulation of tje effects of mechanical stress on PA activity.

Knockdown of uPAR expression by RNAi inhibits PA activity. As the activity of PA was regulated by its receptor (uPAR) and inhibitor (PAI-1), the potential of shRNA-mediated downregulation of uPAR was determined in the regulation of PA activity on the cell surface of MCCs with and without exposure to cyclic mechanical stress for 24 h. Following knockdown of uPAR, the uPAR expression was significantly reduced to 42% of that of the controls (non-transfected MCCs) at 4000 μ strain mechanical loading and to 38% at 6000 μ strain (Fig. 6A). Compared with the unloaded control group, knockdown of uPAR significantly decreased the expression of uPAR and the cell-associated PA activity, but it exhibited no effect on the expression of uPA, tPA and PAI 1, or the soluble PA activity in the culture supernatant (data not shown). The cell-associated PA activity in shuPAR-transfected MCCs demonstrated no significant difference at 4000 and 6000 μ strain mechanical loading (Fig. 6B). These results suggested that the mechanical induction of the PA activity at the cell surface may be substantially regulated by uPAR (Fig. 6B). Moreover, analysis of the cell-associated PA activity on shu-PAR-transfected MCCs at 4000 and 6000 μ strain mechanical loading showed that PA functions as the active enzyme and is not regulated mainly by its specific inhibitor, PAI 1, alone (Fig. 6B).

Discussion

Several factors (including the position or form of the mandibular condyle and other elements of the joint) affect the load on the condyle, which renders it difficult to determine the actual strain in the TMJ. For this reason, the effect of stress applied to MCCs was determined and the magnitude of the applied mechanical stress was obtained and assessed with reference to the *in vitro* data of previous studies (12,14,17). In the present study, rat MCCs were analyzed in a four-point bending system to investigate the effect of cyclic mechanical tension stress on the cell viability and metabolism. In this system, it was demonstrated that mechanical stress of 8000 μ strain resulted in a decrease in cell viability; whereas, following continuous exposure to mechanical stress at 2,000, 4,000 and 6,000 μ strain, MCCs exhibited no obvious changes in cell viability. These results indicated that the abnormally high magnitude of mechanical stress affected the cell viability and was cytotoxic to MCCs. Notably, cyclic mechanical stress at 4,000 and 6,000 μ strain suppressed the synthesis of the intracellular DNA, proteoglycan and collagen, whereas reducing the magnitude of loading to 2000 μ strain, promoted their synthesis. Fujisawa *et al* (14) and De Witt *et al* (17) also demonstrated that moderate mechanical stress exhibited anabolic effects on chondrocytes, whereas high magnitude mechanical stress markedly suppressed the intracellular anabolism of chondrocytes. Based on these results, cyclic mechanical stresses of 4000 and 6000 μ strain were defined as appropriately excessive mechanical loadings and imitated the mechanical response to different mechanical stimuli.

The possibility that mechanical stimulation may be involved in the regulation of PA activity was initially suggested

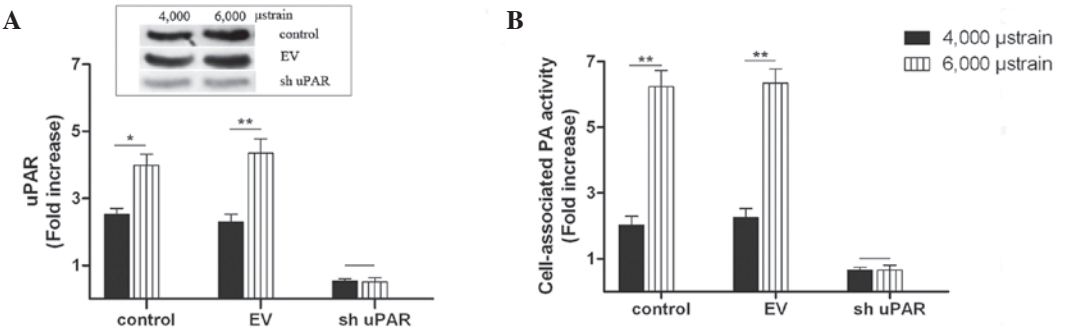

Figure 6. Knockdown of uPAR expression by RNAi inhibited PA activity. (A) Following knockdown of uPAR, the uPAR expression was significantly reduced to 42% of that of the controls (non-transfected cells) at 4000 μ strain mechanical loading and to 38% of that of the controls at 6000 μ strain. The difference between the 4000 and 6000 μ strain group were insignificant. (B) ShRNA-uPAR transfection inhibited the activity of cell-associated PA at cyclic mechanical stress of 4000 and 6000 μ strain, and the downregulated PA activity between mechanical stress of 4000 and 6000 μ strain was not different. All values were normalized to the expression level of glyceraldehyde 3-phosphate dehydrogenase and denoted as the fold-increase of protein expression relative to that in 0 h group control cultures. *P<0.05 and **P<0.01, vs. unloaded control were indicated. Mean ± SD; n=3. uPAR, urokinase-type plasminogen activator receptor; shRNA, short hairpin RNA.

following an *in vitro* study, which demonstrated that excessive shear stress induced the tPA expression in endothelial cells (18). However, whether the mechanical stimulation affected the PA expression and activity in cartilage chondrocytes remains unclear, particularly on MCCs. In the present study, using a direct *in vitro* cell loading method, a marked increase in PA activity in MCCs in response to the excessive mechanical stress was observed. Furthermore, the elevated PA activity identified following excessive mechanical stress appeared to be associated with an increase in PA and a decrease in PAI-1 expression; however, it may be correlated with mechanical induction of uPAR. No change was identified in the PA activity when MCCs were subjected to moderate mechanical stress.

Due to the rupture of surface cartilage following compressive overload in OA pathology (19,20), it is suggested that excessive mechanical stress stimulates the chondrocytes to synthesize PA. This may accelerate the activation of plasminogen and subsequently promote cartilage degradation by providing a proteolytic environment. Furthermore, the *in situ* presence of enhanced PA gene expression has been demonstrated in previous studies, particularly in the clefts and fissures of the cartilage defects with TMJ disk displacement and in knee OA cartilage lesions following anterior cruciate ligament injury. The presence of PA was also concurrent with the increased activity of plasmin and collagenase (21-23). Moreover, Yamaguchi *et al* (24) demonstrated that mechanical stress induced tPA expression and enhanced plasmin activity in human periodontal ligament cells. In this study, the positive correlation between the enhanced plasmin activity and the increased PAs (uPA and tPA) expression indicated that the mechanical induction of PA was involved in the stress-mediated regulatory mechanism of PA activity for excessive mechanical stress responsiveness.

In the present study, the excessive mechanical stimuli induced the mRNA and protein expression of uPAR in MCCs. As demonstrated previously, uPA involved in tissue remodeling was localized and/or modulated on the cell and/or matrix surface, where uPA catalyzed and accelerated the conversion of inactive plasminogen to plasmin through binding with high-affinity cell and/or matrix binding sites (21). uPAR, as a specific receptor of uPA, binds and localizes uPA/pro-uPA to the cell surface through its N-terminal domain 1 structure. Receptor binding not only focalizes the PA activity on the cell surface, but also increases the activation rate of PA (25,26). Thus, if an increased number of cell membrane uPAR receptors are expressed and highly bound, this would suggest that increased active PA would be available at the cell surface (27), whereas the absence of extracellular uPA binding sites may reduce activation of the inactive PA zymogen. In OA, the presence of uPAR in cartilage lesions was often accompanied by an increased fibrinolytic activity, whereas reducing the number of uPAR on chondrocyte surfaces with a diacetylrhein agent resulted in a decrease in the fibrinolytic activity (28,29). The present study indicated that, in response to excessive mechanical stress, the stress-mediated PA activity appeared to be modulated primarily by mechanical induction of uPAR in MCCs. This may also be implicated directly in the degradation of cartilage matrix, for example by its involvement in the degeneration of annulus tissue (30).

PAI-1, as a negative regulator of the PA system, is able to bind to uPA bound receptors at the cell surface, where PAI-1 accumulates in a complex form of PAI-1-uPA-uPAR and undergoes internalization followed by degradation (27). Therefore, it may lead to an imbalance between the quantity of the enzymes and the level of the physiological inhibitor. Yeh *et al* (31) showed that mechanical stimuli upregulated the PAI-1 gene expression in chondrocytes exposed to moderate stress levels. Consistent with this study, a stimulatory effect of mechanical stress was also observed on PAI-1 expression accompanied by an increase in the tPA expression under moderate mechanical stress. Furthermore, the PAs activity remained stable. This indicated that PAI-1 was pivotal in balancing the PAs activity when MCCs were subjected to moderate mechanical stress. Notably, it was identified that, in the presence of mechanical induction of uPA, tPA and uPAR with excessive mechanical responsiveness, the expression of PAI-1 was decreased or remained unchanged. As a result, the equilibrium of PA/PAI-1 may be disturbed in the MCC response to excessive mechanical stimuli, which may subsequently result in an enhancement in the PA activity. This may due to the fact that, the relative activity levels of the PA/PAI-1 often change in OA cartilage lesions in response to exces-

sive stress, where the cartilage chondrocytes bear abnormal loading (22,29).

In conclusion, this study demonstrated that moderate mechanical stress may only induce physiological remodeling of cartilage matrix by simultaneously inducing an increase of tPA and PAI-1 expression. In contrast, excessive mechanical stress resulted in an enhancement in the PA activity by inducing the upregulation of uPA, tPA and uPAR, as well as changes in the PA/PAI-1 repair. This may provide a pericellular proteolytic environment, which subsequently affects the cartilage degradation in TMJ OA.

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References

1. Sandell LJ and Aigner T: Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. Arthritis Res 3: 107-113, 2001.
2. Goldring MB: The role of the chondrocyte in osteoarthritis. Arthritis Rheum 43: 1916-1926, 2000.
3. Saksela O and Rifkin DB: Cell-associated plasminogen activation: regulation and physiological functions. Annu Rev Cell Biol 4: 93-126, 1988.
4. Werb Z, Mainardi CL, Vater CA and Harris ED Jr: Endogenous activiation of latent collagenase by rheumatoid synovial cells. Evidence for a role of plasminogen activator. N Engl J Med 296: 1017-1023, 1977.
5. Hsieh YS, Yang SF, Lue KH, Chu SC and Lu KH: Effects of different molecular weight hyaluronan products on the expression of urokinase plasminogen activator and inhibitor and gelatinases during the early stage of osteoarthritis. J Orthop Res 26: 475-484, 2008.
6. Nonaka T, Kikuchi H, Ikeda T, Okamoto Y, Hamanishi C and Tanaka S: Hyaluronic acid inhibits the expression of u-PA, PAI-1, and u-PAR in human synovial fibroblasts of osteoarthritis and rheumatoid arthritis. J Rheumatol 27: 997-1004, 2000.
7. Sakamaki H, Ogura N, Kujiraoka H, Akiba M, Abiko Y and Nagura H: Activities of plasminogen activator, plasmin and kallikrein in synovial fluid from patients with temporomandibular joint disorders. Int J Oral Maxillofac Surg 30: 323-328, 2001.
8. Milner JM, Elliott SF and Cawston TE: Activation of procollagenases is a key control point in cartilage collagen degradation: interaction of serine and metalloproteinase pathways. Arthritis Rheum 44: 2084-2096, 2001.
9. Pap G, Eberhardt R, Röcken C, Nebelung W, Neumann HW and Roessner A: Expression of stromelysin and urokinase type plasminogen activator protein in resection specimens and biopsies at different stages of osteoarthritis of the knee. Pathol Res Pract 196: 219-226, 2000.
10. Walter H, Kawashima A, Nebelung W, Neumann W and Roessner A: Immunohistochemical analysis of several proteolytic enzymes as parameters of cartilage degradation. Pathol Res Pract 194: 73-81, 1998.
11. Kuroda S, Tanimoto K, Izawa T, Fujihara S, Koolstra JH and Tanaka E: Biomechanical and biochemical characteristics of the mandibular condylar cartilage. Osteoarthritis Cartilage 17: 1408-1415, 2009.
12. Li H, Yang HS, Wu TJ, *et al*: Proteomic analysis of early-response to mechanical stress in neonatal rat mandibular condylar chondrocytes. J Cell Physiol 223: 610-622, 2010.
13. Sadowski T and Steinmeyer J: Differential effects of nonsteroidal antiinflammatory drugs on the IL-1 altered expression of plasminogen activators and plasminogen activator inhibitor-1 by articular chondrocytes. Inflamm Res 51: 427-433, 2002.
14. Fujisawa T, Hattori T, Takahashi K, Kuboki T, Yamashita A and Takigawa M: Cyclic mechanical stress induces extracellular matrix degradation in cultured chondrocytes via gene expression of matrix metalloproteinases and interleukin-1. J Biochem 125: 966-975, 1999.
15. Hall AC, Urban JP and Gehl KA: The effects of hydrostatic pressure on matrix synthesis in articular cartilage. J Orthop Res 9: 1-10, 1991.
16. Takahashi K, Kubo T, Kobayashi K, *et al*: Hydrostatic pressure influences mRNA expression of transforming growth factor-beta 1 and heat shock protein 70 in chondrocyte-like cell line. J Orthop Res 15: 150-158, 1997.
17. De Witt MT, Handley CJ, Oakes BW and Lowther DA: In vitro response of chondrocytes to mechanical loading. The effect of short term mechanical tension. Connect Tissue Res 12: 97-109, 1984.
18. Diamond SL, Eskin SG and McIntire LV: Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. Science 243: 1483-1485, 1989.
19. Flachsmann R, Broom ND and Hardy AE: Deformation and rupture of the articular surface under dynamic and static compression. J Orthop Res 19: 1131-1139, 2001.
20. Morel V, Berutto C and Quinn TM: Effects of damage in the articular surface on the cartilage response to injurious compression in vitro. J Biomech 39: 924-930, 2006.
21. Appella E, Robinson EA, Ullrich SJ, *et al*: The receptor-binding sequence of urokinase. A biological function for the growth-factor module of proteases. J Biol Chem 262: 4437-4440, 1987.
22. Martel-Pelletier J, Faure MP, McCollum R, Mineau F, Cloutier JM and Pelletier JP: Plasmin, plasminogen activators and inhibitor in human osteoarthritic cartilage. J Rheumatol 18: 1863-1871, 1991.
23. Pelletier JP, Mineau F, Faure MP and Martel-Pelletier J: Imbalance between the mechanisms of activation and inhibition of metalloproteases in the early lesions of experimental osteoarthritis. Arthritis Rheum 33: 1466-1476, 1990.
24. Yamaguchi M, Shimizu N, Ozawa Y, *et al*: Effect of tension-force on plasminogen activator activity from human periodontal ligament cells. J Periodontal Res 32: 308-314, 1997.
25. Ellis V, Scully MF and Kakkar VV: Plasminogen activation initiated by single-chain urokinase-type plasminogen activator. Potentiation by U937 monocytes. J Biol Chem 264: 2185-2188, 1989.
26. Vassalli JD, Baccino D and Belin D: A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J Cell Biol 100: 86-92, 1985.
27. Plow EF, Herren T, Redlitz A, Miles LA and Hoover-Plow JL: The cell biology of the plasminogen system. FASEB J 9: 939-945, 1995.
28. Del Rosso M, Fibbi G, Magnelli L, *et al*: Modulation of urokinase receptors on human synovial cells and osteoarthritic chondrocytes by diacetylrhein. Int J Tissue React 12: 91-100, 1990.
29. Fibbi G, Pucci M, Serni U, Cerinic MM and Del Rosso M: Antisense targeting of the urokinase receptor blocks urokinase-dependent proliferation, chemoinvasion, and chemotaxis of human synovial cells and chondrocytes in vitro. Proc Assoc Am Physicians 110: 340-350, 1998.
30. Ford JL and Downes S: Cellularity of human annulus tissue: an investigation into the cellularity of tissue of different pathologies. Histopathology 41: 531-537, 2002.
31. Yeh CC, Chang HI, Chiang JK, *et al*: Regulation of plasminogen activator inhibitor 1 expression in human osteoarthritic chondrocytes by fluid shear stress: role of protein kinase Calpha. Arthritis Rheum 60: 2350-2361, 2009.