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

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Received February 19, 2013; Accepted July 29, 2013

DOI: 10.3892/mmr.2013.1654

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.

Key words: mechanical stress, plasminogen activator, plasminogen activator system, mandibular condylar chondrocytes

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% trypsin 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 1594: CTGTCTGGCACAAGAAAGTGT) and shuPAR 2 (start 174: GGGAATGGGAAGACGCCGA) along with shLu-ciferase (start 1594: CTGTCTGGCACAAGAAGATGT). 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 twicediluted 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 plates 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 synthesis, 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 1 (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.
(Ct) 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,
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 defined as a moderate 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 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 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
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; however, decrease following the
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 the 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...
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 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.

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.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant nos. 30300391, 81172580 and 81272961) and the Fundamental Research Funds for the Central Universities of China (2011).

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