J. Biochem. 125, 966-975 (1999)

Cyclic Mechanical Stress Induces Extracellular Matrix Degradation in Cultured Chondrocytes *via* Gene Expression of Matrix Metalloproteinases and Interleukin-1[1]

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Received November 2, 1998; accepted February 12, 1999

To clarify the mechanism of cartilage degradation induced by mechanical stress, we investigated the influence of cyclic tension force (CTF) on the metabolism of cultured chondrocytes. The chondrocytes were exposed to CTF using a Flexercell strain unit. Five or 15 kPa of high frequency CTF significantly inhibited the syntheses of DNA, proteoglycan, collagen, and protein. Fifteen kPa of high frequency CTF induced the expression of interleukin-1 (IL-1), matrix metalloproteinase (MMP)-2 and -9 mRNA, and increased the production of pro- and active-MMP-9. The degradation of proteoglycan was inhibited by and MMP inhibitor, indicating that MMPs are involved in the degradation of proteoglycans induced by high frequency CTF. Moreover, reducing the frequency of CTF from high to low decreased the inhibition of proteoglycan synthesis. These findings suggest that the CTF frequency is one of the key determinants of chondrocyte metabolism. Low magnitude CTF, whether high or low frequency, did not cause the gene expression of cartilage degradation factors, suggesting that this CTF magnitude causes only minor changes in the cartilage matrix. High magnitude and frequency CTF caused the gene expression of IL-1 and MMP-9, followed by increases in the production of MMP-2 and -9 proteins, suggesting that excessive and continuous cyclic mechanical stress induces the production of IL-1 and MMP-9, resulting in cartilage degradation.

Key words: chondrocyte, interleukin (IL)-1, matrix metalloproteinase (MMP), mechanical stress, proteoglycan.

Articular cartilage is always subjected to various magnitudes and cycles of mechanical stress. The ability to withstand these stresses largely depends on its extracellular matrix (ECM), which consists mainly of proteoglycan and collagen. Under physiological conditions, moderate levels of mechanical stress are an important regulator of chondrocyte metabolism and a pre-requisite for maintaining the ECM properties of normal cartilage (*1*). However, excessive mechanical stress has long been recognized as a degradation factor for the cartilage ECM and as one of the major factors in osteoarthritis (OA).

OA is a major and much neglected cause of disability. In this multifactorial disease process, joint pain and progressive limitation of motion are accompanied by the gradual loss of articular cartilage, sclerotic and cystic changes in local bone, and osteophyte formation. Current experimental evidence strongly suggests that proteinases may be involved in the degradation of the cartilage ECM (*2*). Indeed, the levels of MMPs, tissue inhibitors of MMPs (TIMPs), and inflammatory cytokines such as IL-1 and -6 in the serum or synovial fluid of patients with rheumatoid arthritis (RA) or OA have been shown to increase significantly (*3-9*). The expression of these molecules in cartilage is up regulated in RA and OA patients. However, these findings only indicate that MMPs, TIMPs, and inflammatory cytokines are secreted into the serum or synovial fluid of patients with RA or OA, and do not present direct evidence that mechanical stress causes OA through the induction of these cartilage degradation-factors.

Among several types of mechanical stress, cyclic mechanical stress is thought to be the most relevant to the etiology of OA. Several studies have indicated that exposure of cartilage explants to mechanical stress causes decreases (*10-13*), increases (*10, 12, 14-17*), or no change (*14*) in

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (TK, AY, MT), and a grant from the Health Science Foundation (MT) and the Promotion Award of Bone and Joint Disease Science sponsored by Hokuriku Pharmaceuticals from the Japan Rheumatism Foundation (MT).

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Abbreviations: bFGF, basic fibroblast growth factor; CTF, cyclic tension force; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; IL-1, interleukin-1; IL-6, interleukin-6; MMP, matrix metalloproteinase; OA, osteoarthritis; PBS, phosphate buffered saline; RA, rheumatoid arthritis; TGFβ, transforming growth factor β; TIMP, tissue inhibitor of matrix metalloproteinases; TMJ, temporomandibular joint.

ECM synthesis by chondrocytes. But these experiments concerned only ECM synthesis in chondrocytes. Recently several cytokines and MMPs have been reported to regulate chondrocyte metabolism, including proteoglycan synthesis and cartilage degradation, but the role of mechanical stress in the expression of cytokines and MMPs has not yet been reported. It is, therefore, very important to investigate the expressions of cytokines and MMPs in chondrocytes under mechanical stress, especially cyclic stress.

In the present study, we investigated the effects of cyclic tension force on chondrocyte metabolism *in vitro*. The chondrocyte response was quantified by analyzing DNA, proteoglycan, collagen and protein syntheses and the production of 92 and 72 kDa gelatinase (MMP-2 and MMP-9), and reverse transcriptase-PCR analysis of mRNA signals for IL-1, MMP-2 and -9, and TIMP-1.

MATERIALS AND METHODS

Cell Culture—A human chondrosarcoma-derived chondrocyte cell line (HCS-2/8) was used as an ideal model for normal chondrocytes, because these cells resemble chondrocytes in that they synthesize cartilage-specific matrix components such as collagen types II, IX, and XI and aggrecan (*18-22*) and an angiogenesis inhibitor (*23*), and possess a response profile to various vitamins and growth factors similar to normal chondrocytes (*19, 22*).

Rabbit chondrocytes were isolated from the costal cartilage of young male New Zealand rabbits, 300-500 g in weight, as previously described (*24-26*). Briefly, the cartilage specimens were minced and treated with 0.1% EDTA (w/v) in phosphate-buffered saline (PBS) containing 0.2% glucose (w/v) (PBS-G). Chondrocytes were then released by digestion with 0.15% collagenases (Wako, Osaka) (w/v) in PBS-G and filtered through a 100 μm nylon filter.

HCS-2/8 cells and rabbit chondrocytes were seeded at densities of 6×10^4/cm^2 and 2×10^4/cm^2, respectively, into a flexible or non-flexible bottomed 6-well plate (Flex I and II plates) and grown in DMEM supplemented with 10% fetal bovine serum (FBS: Cansera International, Rexclale, Ont, Canada) and 50 μg/ml kanamycin at 37°C in an atmosphere of 5% CO_2 in air. Cells were used in experiments after reaching confluence.

Mechanical Stress—CTF was applied with a Flexercell strain unit (27) (Flexercell Corp., McKeesport, PA). This unit consists of a computer-controlled vacuum unit and a baseplate to hold the culture dishes. High frequency CTF was applied to the cells using a pressure of 5 kPa or 15 kPa at 30 cycles/min with 1 s duration and a 1 s interval (Fig. 1A), middle frequency was at 1 cycle/2 min with 1 s duration and a 119 s interval (Fig. 1B), and low frequency was at 1 cycle/4 min with 1 s duration and a 239 s interval (Fig. 1C). Experimental dishes were stressed for various periods and control dishes received no stress for the same periods as the experimental dishes.

Determination of DNA Synthesis—DNA synthesis in response to CTF was measured by [^{3}H]thymidine (25 Ci/mmol) (Amersham International, Aylesbury, UK) incorporation into the cells (*28, 29*). When HCS-2/8 cells reached confluence, the medium was replaced with DMEM containing 0.5% FBS and the cells were exposed to 5 or 15 kPa of each frequency CTF for 48 h and labeled with [^{3}H]thymidine (5 μCi/ml) for the final 4 h. After labeling, the cell layer was rinsed three times with PBS, and the cells were collected in 0.2 ml of PBS containing 0.25% trypsin (w/v) and 0.02% EDTA (w/v). The mixture was harvested onto glass-fiber filter paper with a semiautomatic microharvester, and [^{3}H]thymidine incorporation was determined by a scintillation counter.

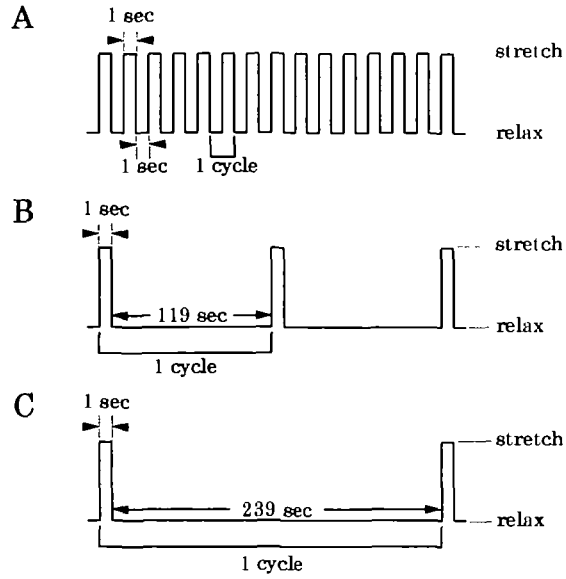

Fig. 1. **Deformation regimen used in this study.** Cyclic tension force was applied using a Flexercell strain unit. High frequency CTF was applied to the cells at a pressure of 5 or 15 kPa at 30 cycles/min with 1 s duration and a 1 s interval (A); middle frequency was at 1 cycle/2 min with 1 s duration and a 119 s interval (B); and low frequency was at 1 cycle/4 min with 1 s duration and a 239 s interval (C).

Determination of Cell Number—When HCS-2/8 cells reached confluence, the medium was replaced with DMEM containing 0.5% FBS and the cells were exposed to 5 or 15 kPa of high frequency CTF for 48 h. After CTF, the cells were rinsed with PBS, dispersed with 0.25% trypsin (w/v), and counted with a Bürker-Türker haemacytometer (Kayagaki Irikakogyo, Tokyo).

Protein and Collagen Syntheses—Protein and collagen syntheses in HCS-2/8 cells were measured by determining the incorporation of [2,3-^{3}H]proline (50 Ci/mmol) (Amersham International, Aylesbury, UK) into the cells. The incorporation of [2,3-^{3}H]proline into collagenase-digestable protein was determined using purified bacterial collagenase (SIGMA CHEMICAL, St. Louis, USA) by the method of Peterkofsky and Diegelmann (*30-32*). When HCS-2/8 cells reached confluence, the medium was replaced with DMEM containing 0.5% FBS and the cells were exposed to 5 or 15 kPa of high frequency CTF for 48 h and labeled with [2,3-^{3}H]proline (10 μCi/ml) for the final 4 h. After labeling, the cell layer was rinsed twice with PBS, solubilized with 1.5 ml of 50 mM Tris-HCl buffer (pH 7.2) containing 0.2% Triton-X 100 and 1 mM PMSF (buffer A), and homogenized with a polytron. Then 0.25 ml of cell homogenate was digested in the presence or absence of 100 U collagenase dissolved in 0.02 ml of 25 mM Tris-HCl buffer (pH 7.4) containing 10 mM $CaCl_2$, and incubated for 4 h at 37°C. After incubation, 0.08 ml of 10 mg/ml gelatin

solution was added followed by the addition of 10% trichloroacetic acid (TCA) containing 0.5% tannic acid. The mixture was centrifuged at 10,000 rpm for 10 min and the precipitates were rinsed three times with 10% TCA/0.5% tannic acid and then ethanol-diethyl ether (3:1, v/v). The precipitates were each finally solubilized in 1 ml of buffer A and radioactivity was measured in a scintillation counter.

Determination of Protein Content—When HCS-2/8 cells reached confluence, the medium was replaced with DMEM containing 0.5% FBS and the cells were exposed to 5 or 15 kPa of high frequency CTF for 48 h. After CTF, the cells were rinsed with PBS, collected in 0.5 ml of 0.9% NaCl containing 0.2% Triton-X 100, homogenized on ice, and centrifuged at 5,000 rpm for 10 min. The resultant supernatants were transferred to test tubes and used for total protein assay. The amount of total protein was measured using a BCA Protein Assay Kit (Pierce, Illinois, USA).

Uronic Acid Content—Uronic acid contents were assayed as a measure of proteoglycan accumulation as described by Bitter and Muir (*33*). When HCS-2/8 cells reached confluence, the medium was replaced with DMEM containing 0.5% FBS and the cells were exposed to 5 or 15 kPa of high frequency CTF for 48 h. The cell layer was rinsed three times with PBS, collected in 0.49 ml of 0.9% NaCl containing 0.2% Triton-X 100, and homogenized on ice. Then, 0.01 ml of 50 mg/ml actinase E (Kaken Pharmaceuticals, Tokyo) was added, and the mixture was incubated for 16 h at 56°C. After incubation, 2 ml of 95% ethanol containing 1.3% potassium acetate was added to the mixture. After 4 h at 4°C, the mixture was centrifuged at 10,000 rpm for 15 min and the precipitate was collected. The precipitate was solubilized with 0.5 ml of distilled water and used for the assay of uronic acid content. An 0.2 ml aliquot of cell layer sample was mixed on ice with 0.5 ml of sulfuric acid containing 0.95% sodium borate and incubated for 15 min at 100°C. Then 0.02 ml of ethanol containing 0.125% carbazole was added, and the mixture was incubated for another 15 min at 100°C. The generated D-glucuronic acid was measured by a spectrophotometric method using carbazole at 530 nm.

Determination of Proteoglycan Synthesis and the Effect of an MMP Inhibitor on Proteoglycan Synthesis—Proteoglycan synthesis was monitored by determining the incorporation of [^{35}S]sulfuric acid (Carrier free; Amersham International, Aylesbury, UK) into cethylpyridinium chloride (CPC)-precipitable materials, as described previously (*25, 26, 34*). When HCS-2/8 cells reached confluence, the medium was replaced with DMEM containing 10% FBS and the cells were exposed to appropriate CTF and labeled with [^{35}S]sulfuric acid for the final 3 h or 24 h. In some experiments, the cells were exposed to 15 kPa of high frequency CTF for 48 h with the addition of [^{35}S]sulfuric acid in the presence or absence of an MMP inhibitor, BAY 12-9566, which suppresses the activity of MMPs, including MMP-2, -3, and -9 (*24*) (mol wt., 410.9; inhibition activity toward MMP-2: $K_i = 11$ nM, MMP-3: $K_i = 134$ nM, and MMP-9: $K_i = 301$ nM) (Bayer Yakuhin, Osaka) (1 μM) in DMEM containing 10% FBS. After labeling, the medium was collected and the cell layer was rinsed twice with PBS. Then, 1 ml of 0.2 M Tris-HCl buffer (pH 7.8) containing 5 mM $CaCl_2$ and 1 mg/ml of actinase E was added to the cell layer, and the samples were incubated for 21 h at 55°C. Then, 2 ml of 0.2 M Tris-HCl (pH 7.8) containing 2 mM $MgSO_4$, 0.2 ml of 0.1 mg/ml chondroitin sulfate, and 2 ml of 1% CPC containing 20 mM NaCl were added to the digested material. One ml of 0.2 M Tris-HCl (pH 7.8) containing 2 mM $MgSO_4$, 0.1 ml of 0.1 mg/ml chondroitin sulfate, and 1 ml of 1% CPC containing 20 mM NaCl were added to 0.1 ml of the medium collected. Both mixtures were kept for 3 h at 37°C, and the precipitates were collected on glass-fiber filter papers and washed three times with 2 ml of 1% CPC containing 20 mM NaCl. The radioactivity of the material precipitated with CPC was measured in a scintillation counter.

Reverse Transcriptase-PCR (RT-PCR) for the Quantitation of mRNA Expression—Total RNA was isolated from HCS-2/8 cells and rabbit chondrocytes cultured for 3, 6, 12, and 24 h with or without 5 or 15 kPa of high frequency CTF using TRIZOL (GIBCO BRL, NY, USA), and the RNA obtained was treated with DNase I (Promega, Madison, WI) for 30 min at 37°C (*24, 35*). RT-PCR was performed using a Gene Amp RNA PCR Kit (Perkin Elmer, Branchburg, NJ). Total RNA (0.2 μg) was reverse transcribed to cDNA using oligo $d(T)_{16}$ primer with M-MLV reverse transcriptase for 30 min at 42°C in the presence of [α-^{32}P]-dCTP (60 μCi/ml) (Amersham International, Aylesbury, UK) (3,000 Ci/mmol). Then, the cDNA, except human MMP-9 cDNA, was amplified with each primer set using 0.5 U of Ampli TaqDNA polymerase (Perkin Elmer); human MMP-9 cDNA was amplified using 0.5 U of AmpliTaq Gold (Perkin Elmer). The sequence of the synthesized primers and the expected size of the PCR products are shown in Table I. The amplification conditions were as follows: for human 18S rRNA: 94°C (2 min) for 1 cycle,

TABLE I. **Nucleotide sequence of the primers used for RT-PCR.**

		Sequence	Expected size of PCR products (bp)
IL-1[c]	(S)[a]	5′-GAACAGAAAGGTTTTTGAGTACG-3′	
	(AS)[b]	5′-GCCCCCTTTGAATAAATTAGACC-3′	343
MMP-9[c]	(S)	5′-GGTCCCCCCACTGCTGGCCCTTCTACGGCC-3′	
	(AS)	5′-GTCCTCAGGGCACTGCAGGATGTCATAGGT-3′	640
MMP-9[d]	(S)	5′-GCAAGGTGCTGCTGTTCGGCGCGCA-3′	
	(AS)	5′-GGAAGGACGCCCTAGTCCTCAGGGC-3′	271
TIMP-1[c,d]	(S)	5′-CACCCACAGACGGCCTTCTGCAAT-3′	
	(AS)	5′-AGTGTAGGTCTTGGTGAAGCC-3′	345
MMP-2[d]	(S)	5′-CTGGCTTCCCTCGGCTCATCGCGGA-3′	
	(AS)	5′-CTGAGATAGTCCCTGTTGGCTCCGA-3′	497
18S rRNA	(S)	5′-GCGAATTCCTGCCAGTAGCATATGCTTG-3′	
	(AS)	5′-GGAAGCTTAGAGGAGCGAGCGACCAAAGG-3′	126

[a]sense primer. [b]antisense primer. [c]primer for human. [d]primer for rabbit.

94°C (1 min)-58°C (1 min)-72°C (2 min) for 22 cycles, and final incubation at 72°C for 5 min; for rabbit 18S rRNA: 94°C (2 min) for 1 cycle, 94°C (1 min)-58°C (1 min)-72°C (2 min) for 19 cycles, and final incubation at 72°C for 5 min; for human IL-1: 94°C (2 min) for 1 cycle, 94°C (1 min)-58°C (1 min)-72°C (2 min) for 32 cycles, and final incubation at 72°C for 5 min; for human MMP-9: 95°C (10 min) for 1 cycle, 94°C (1 min)-65°C (2 min) for 35 cycles, and final incubation at 65°C for 10 min; for human and rabbit TIMP-1: 94°C (2 min) for 1 cycle, 94°C (1 min)-58°C (1 min)-72°C (2 min) for 22 cycles, and final incubation at 72°C for 5 min; for rabbit MMP-2 and -9: 94°C (2 min) for 1 cycle, 94°C (1 min)-57°C (1 min)-72°C (2 min) for 32 cycles, and final incubation at 72°C for 5 min. A 5 μl aliquot of each reaction mixture were electrophoresed in a 3.5% polyacrylamide gel, and the gels were dried. The dried gels were exposed to imaging plates and the amounts of RT-PCR products were analyzed using a Bioimaging analyzer BAS2000 (Fuji Film, Tokyo). The amounts of RT-PCR products were compared to the amount of 18S rRNA as a standard, and relative expression ratios were obtained.

Gelatin Zymography—Gelatinase activity was measured by gelatin-substrate zymography as described (*24, 34, 36*), using the culture medium from HCS-2/8 cells and rabbit chondrocytes treated for appropriate periods with or without 5 or 15 kPa of high frequency CTF in DMEM containing 0.5% FBS. Five milliliters of each culture medium sample from a 6-well plate was lyophilized and reconstituted in 0.5 ml distilled water. Then, 15 μl of each solution was subjected to electrophoresis at 4°C in a 10% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gels were washed with 10 mM Tris-HCl buffer (pH 8.0) containing 2.5% Triton X-100, and incubated in 50 mM Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl and 5 mM $CaCl_2$ at 37°C for 72 h. The gels were then stained with 1% Coomassie Brilliant Blue, and destained. The stained gels were analyzed with a one-dimensional diversity image analyzing system (Toyobo, Tokyo).

Statistical Analysis—Unless otherwise specified, the experiments were repeated at least twice and similar results were obtained in the repeated experiments. Statistical analysis was performed by Student's *t*-test. Data are expressed as means ± standard deviation (SD).

RESULTS

Effects of CTF on DNA Synthesis and Cell Number of HCS-2/8 Cells—High frequency CTF at 5 and 15 kPa resulted in a significant inhibition of DNA synthesis compared with unloaded control cultures of HCS-2/8 cells. However, reducing the load frequency from 30 cycles/min to 1 cycle/2 min or 1 cycle/4 min reduced the inhibition of DNA synthesis (Fig. 2A). On the other hand, high frequency CTF at 5 and 15 kPa had no effect on cell number in confluent cultures of HCS-2/8 cells (Fig. 2B).

Effects of CTF on Protein and Collagen Synthesis, and Protein Accumulation by HCS-2/8 Cells—High frequency CTF at 5 and 15 kPa resulted in a significant inhibition of protein (Fig. 3A) and collagen (Fig. 3B) synthesis compared with unloaded control cultures of HCS-2/8 cells. However, high frequency CTF at 5 and 15 kPa had no effect on the accumulation of total protein by confluent cultures of HCS-2/8 cells (Fig. 3C).

Effects of CTF on the Synthesis and Accumulation of Proteoglycans in HCS-2/8 Cells—Proteoglycans are a major component of the ECM of cartilage, so uronic acid content and/or the incorporation of [^{35}S]sulfate into proteoglycans has been used as a biochemical marker of the differentiated phenotype of chondrocytes. High frequency CTF at 5 and 15 kPa resulted in a significant inhibition of proteoglycan synthesis (Fig. 4A). The inhibition of proteoglycan synthesis was time- and frequency dependent. Fifteen kPa of low frequency CTF only slightly decreased proteoglycan synthesis in HCS-2/8 cells compared with unloaded control cultures, whereas high frequency CTF decreased proteoglycan synthesis in a time dependent manner (Fig. 4B).

Figure 4C shows the effect of CTF on proteoglycan accumulation in HCS-2/8 cells. After the application of CTF to HCS-2/8 cells, the accumulation of uronic acid in the cell layer was significantly suppressed compared to unloaded control cultures at both high frequency levels (Fig. 4C).

Effect of the MMP Inhibitor on the Accumulation of Newly Synthesized Proteoglycan by HCS-2/8 Cells—To investigate whether the decrease in uronic acid content in the CTF-loaded cultures is due solely to the inhibition of proteoglycan synthesis or to both the inhibition of proteo-

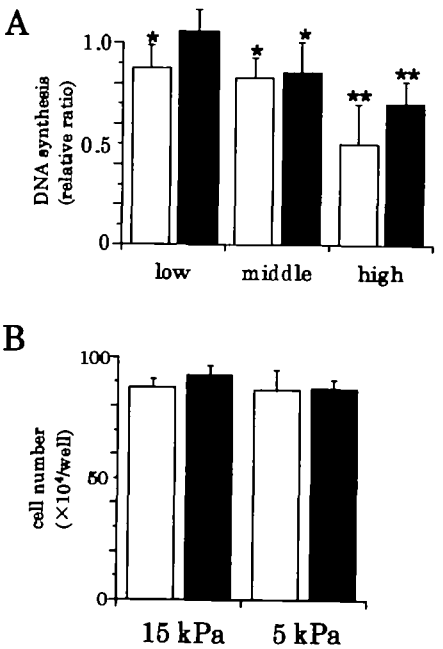

Fig. 2. **Effect of CTF on DNA synthesis (A) and cell number (B) of HCS-2/8 cells.** (A) HCS-2/8 cells were exposed to 15 kPa (closed columns) or 5 kPa (open columns) of high, middle, and low frequency CTF for 48 h and labeled with [^{3}H]thymidine (5 μCi/ml) for the final 4 h. Other experimental procedures are described in "MATERIALS AND METHODS." The data obtained from the loaded experimental cultures were normalized to the average of the unloaded control cultures. Significant differences *versus* control were indicated: $^*p < 0.05$, $^{**}p < 0.01$. The average radioactivities of controls were 3,772.7 ± 1,419.4 and 3,898.4 ± 770.3 cpm at 5 and 15 kPa, respectively. Columns and bars are the means and SD of 6 wells. (B) HCS-2/8 cells were exposed to high frequency CTF at 15 or 5 kPa for 48 h and cell numbers in the loaded experimental cultures (closed columns) and unloaded control cultures (open columns) were determined. Columns and bars are the means and SD of 6 wells.

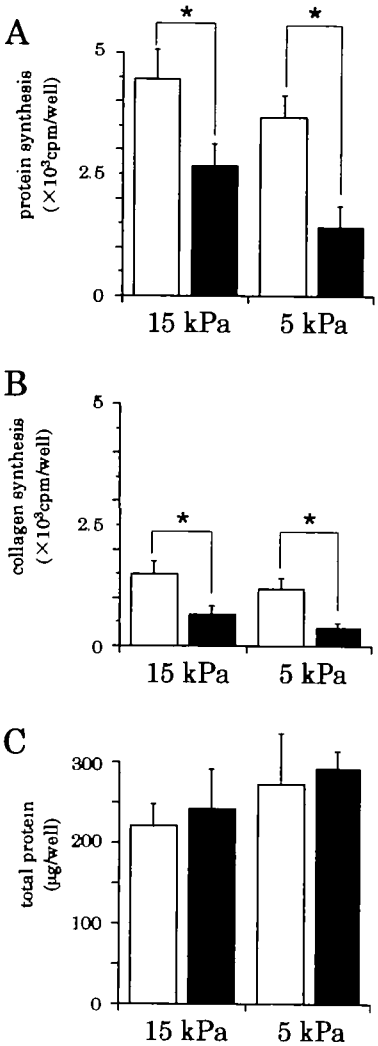

Fig. 3. **Effect of CTF on protein (A) and collagen (B) syntheses, and protein accumulation (C) by HCS-2/8 cells.** HCS-2/8 cells were exposed to high frequency CTF at 15 or 5 kPa for 48 h (closed columns). Control cultures were not exposed to CTF (open columns). (A, B) Protein and collagen syntheses in HCS-2/8 cells were measured by determining the incorporation of [2,3-^{3}H]proline (10 μCi/ml). Other experimental procedures are described in "MATERIALS AND METHODS." (C) The amount of total protein was measured as described in "MATERIALS AND METHODS." Columns and bars are the means and SD of 6 wells. Significant differences are indicated: $^*p<0.01$.

TABLE II. **Effect of MMP inhibitor on proteoglycan synthesis in HCS-2/8 cells.**

Inhibitor		Proteoglycan synthesis ($\times 10^3$ dpm/well)	
		Experiment 1	Experiment 2
(−)	Control	632.4±70.9	1,729.3±108.0
	CTF	182.2±32.6[a]	1,131.7±58.7[a]
(+)	Control	587.6±89.2	1,523.6±176.4
	CTF	340.1±43.8[a,b]	1,291.5±117.6[a,b]

When HCS-2/8 cells reached confluence, the medium was replaced with DMEM containing 10% FBS and [^{35}S]sulfuric acid (6 and 10 μCi/ml, respectively) and the cells were exposed to high frequency CTF at 15 kPa in the presence or absence of 1 μM of the MMP inhibitor, BAY 12-9566. [^{35}S]sulfate incorporation into CPC-precipitable materials was determined after 48 h. Data are expressed as mean±SD of 6 wells. [a]Indicates a significant difference between control and loaded cultures at $p<0.05$. [b]Indicates a significant difference between the absence and presence of the MMP inhibitor in loaded cultures at $p<0.05$.

glycan synthesis and the acceleration of degradation, we continuously labeled HCS-2/8 cells with [^{35}S]sulfate in the presence of the MMP inhibitor under application of high frequency CTF. As shown in Table II, CTF also decreased [^{35}S]sulfate incorporation into proteoglycans after 48-h exposure, and the MMP inhibitor partially restored the decrease caused by CTF (Table II). These findings suggest that CTF not only inhibits proteoglycan synthesis but also causes the degradation of proteoglycans, and MMP is involved in this degradation.

Effects of CTF on the Expression Levels of mRNAs for IL-1, MMP-2, -9, and TIMP-1—To determine whether or not CTF induces the gene expression of cartilage degradation factors such as IL-1, MMP-2 and -9, and TIMP-1, we performed RT-PCR using each primer with [α-^{32}P]dCTP. In HCS-2/8 cells (Fig. 5A), the mRNA levels of IL-1 increased as early as 3-6 h after the application of high frequency CTF at 5 and 15 kPa, and then decreased gradually. The mRNA levels of MMP-9 also increased as early as 3-6 h after the application of high frequency CTF, then decreased gradually after CTF at 5 kPa, but continued to increase after CTF at 15 kPa. The mRNA levels of TIMP-1 showed no remarkable change at either level of high frequency CTF. In rabbit chondrocytes (Fig. 5B), the mRNA levels of MMP-2 increased as early as 3-6 h after the application of high frequency CTF at 5 and 15 kPa, and then decreased gradually. The changes in MMP-9 and TIMP-1 were similar to those in HCS-2/8 cells.

Effects of CTF on the Secretion of Gelatinases into the Medium by HCS-2/8 Cells and Rabbit Chondrocytes—To investigate the involvement of MMPs, the culture medium from HCS-2/8 cells and rabbit chondrocytes with or without exposure to CTF were analyzed for gelatinase activity by a gelatin-substrate zymography technique. In HCS-2/8 cells, 15 kPa of high frequency CTF caused a significant increase in the production of pro- and active-MMP-9, but had little or no effect on the production of pro- and active-MMP-2 (Fig. 6A). High frequency CTF at 5 kPa had no effect on the production of MMPs (Fig. 6B). In rabbit chondrocytes (Fig. 6C), high frequency CTF at 15 kPa resulted in an increase in the production of MMP-9, but had no effect on the production of MMP-2.

DISCUSSION

Although there have been many studies in which the relationship between mechanical stress and ECM synthesis in cartilage explants has been examined, these studies have not shown the effect of mechanical stress on intracellular events in chondrocytes. Because cellular responses, especially early responses, are difficult to investigate in cartilage explants, cultured chondrocytes have been exposed to various mechanical stresses such as direct compressive forces (*37, 38*), tension (*39-41*), shearing stress (*42*), and hydrostatic pressure (*43-47*), but there have been few studies of the effects of cyclic tension force on chondrocyte metabolism. Therefore, in the present study, we investigated the effects of cyclic mechanical stress, which acts on the articular cartilage during such daily behaviors as

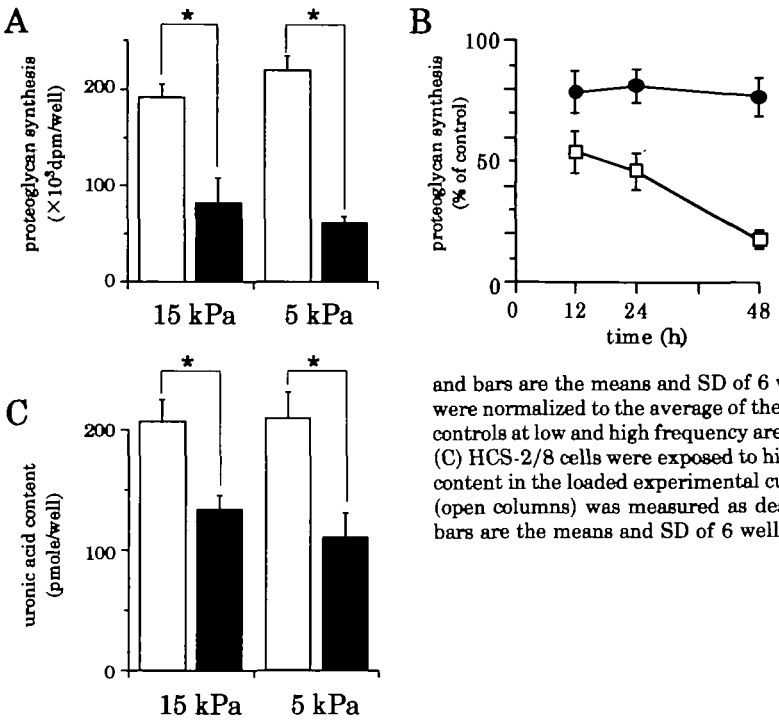

Fig. 4. **Effects of CTF on the synthesis (A, B) and accumulation (C) of proteoglycans in HCS-2/8 cells.** (A) HCS-2/8 cells were exposed to high frequency CTF at 15 or 5 kPa for 48 h and labeled with [^{35}S]sulfate (10 μCi/ml) for the final 24 h. Proteoglycan synthesis in the loaded experimental cultures (closed columns) and in the unloaded control cultures (open columns) was measured as described in "MATERIALS AND METHODS." Columns and bars are the means and SD of 6 wells. Significant differences are indicated: $^*p<0.01$. (B) HCS-2/8 cells were exposed to high (□) or low (●) frequency CTF at 15 kPa for 12, 24, and 48 h and labeled with [^{35}S]sulfate (20 μCi/ml) for the final 3 h. Proteoglycan synthesis was measured as described in "MATERIALS AND METHODS." Points and bars are the means and SD of 6 wells. The data obtained from loaded experimental cultures were normalized to the average of the unloaded control cultures. The average radioactivities of the controls at low and high frequency are 183.1±3.0 and 93.4±19.5 (×10³ dpm/well), respectively. (C) HCS-2/8 cells were exposed to high frequency CTF at 15 or 5 kPa for 48 h and the uronic acid content in the loaded experimental cultures (closed columns) and in the unloaded control cultures (open columns) was measured as described in "MATERIALS AND METHODS." Columns and bars are the means and SD of 6 wells. Significant differences are indicated: $^*p<0.01$.

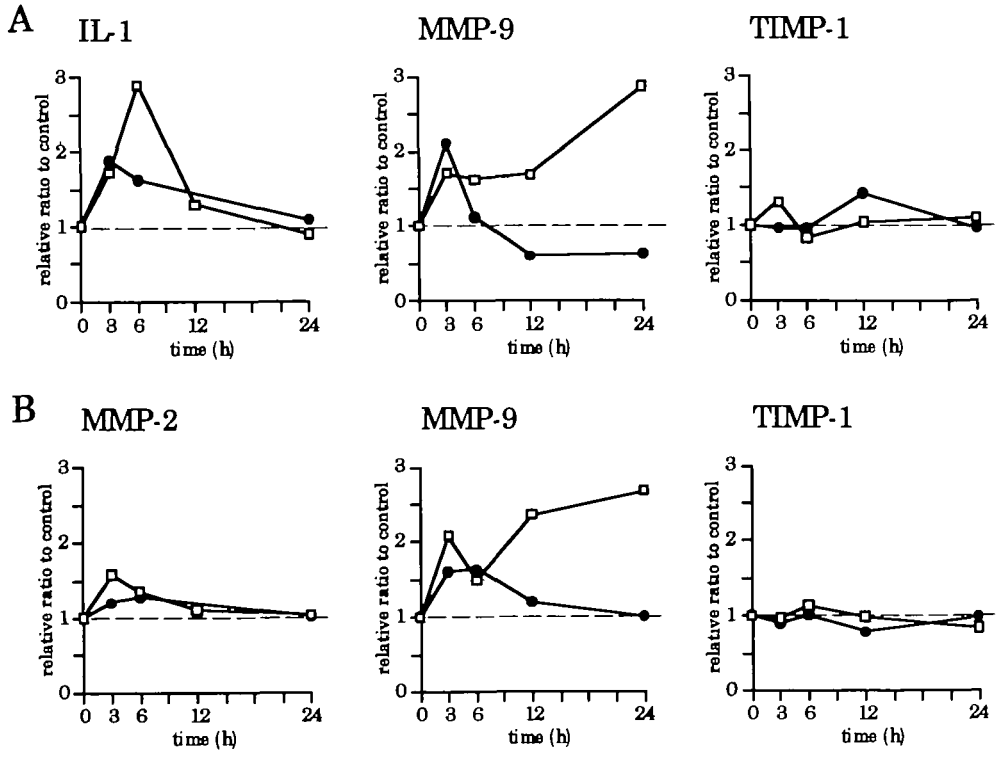

Fig. 5. **Effects of CTF on the expression of mRNA levels of IL-1, MMP-2, -9, and TIMP-1.** HCS-2/8 cells (A) and rabbit chondrocytes (B) were exposed to high frequency CTF at 15 kPa (□) or 5 kPa (●) and RT-PCR was performed. Other experimental procedures are described in "MATERIALS AND METHODS." All data were normalized to the level of 18S rRNA at each time, and all experiments were performed at least three times. Each point shows the average relative level of expression compared with the level in the corresponding unloaded control at each time.

walking and mastication, on rabbit chondrocytes and an extensively characterized human chondrocytic cell line obtained from a human chondrosarcoma (*18-23*).

Articular cartilage is always subjected to various magnitudes and cycles of mechanical stress. Maximum contact pressures in the human hip during walking are reported to range from 3 to 10 MPa (*48*), and the levels of pressure in normal joints can rise to almost 20 MPa during some exercises (*49*). Rubin and Lanyon (*50*) reported that in the limb bone of turkey, horse, and elephant, the peak strain of the bones range from 0.1 to 0.3% elongation during walking. In the temporomandibular joint (TMJ), there are some reports that the condylar head is loaded during molar chewing with about 4.5 Newton in macaca (*51*), and in pig,

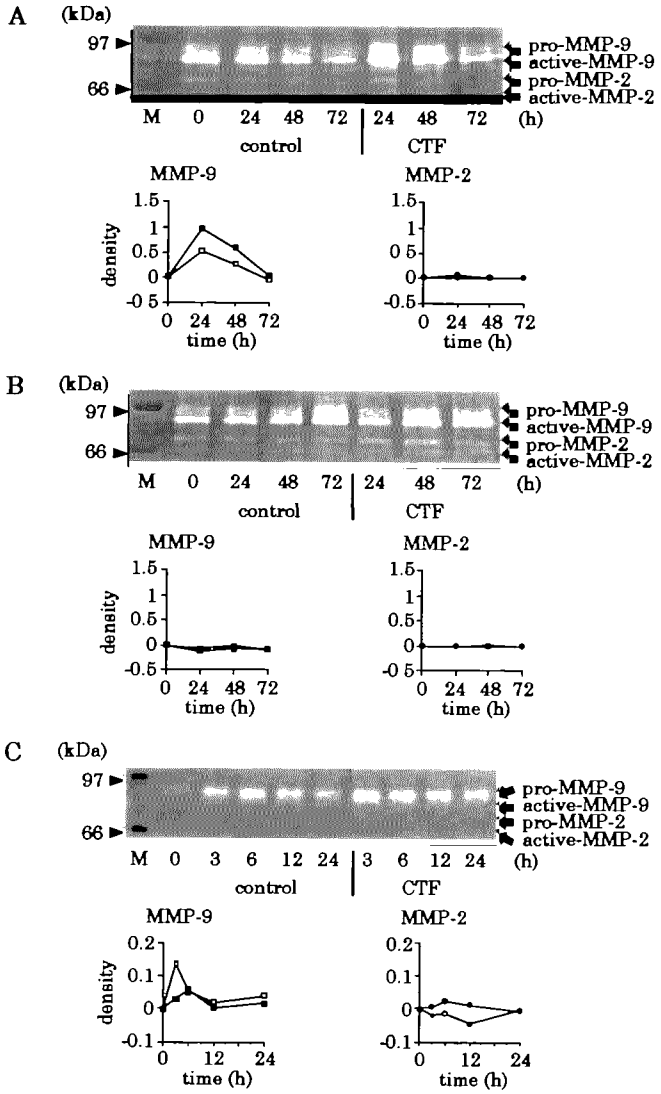

Fig. 6. **Effects of CTF on the secretion of gelatinases into the medium by HCS-2/8 cells (A, B) and rabbit chondrocytes (C).** HCS-2/8 cells were exposed to high frequency CTF at 15 kPa (A) or 5 kPa (B) for 24, 48, and 72 h; rabbit chondrocytes were exposed to high frequency CTF at 15 kPa for 3, 6, 12, and 24 h (C). Other experimental procedures are described in "MATERIALS AND METHODS." The molecular masses of the marker proteins are indicated on the left (×kDa). The ordinate indicates the increase in density caused by CTF above the density of the respective controls. The graphs show the expression levels of pro-MMP-9 (□), active-MMP-9 (■), pro-MMP-2 (○), and active-MMP-2 (●).

the strain on the neck of the condyle during normal mastication is about 0.02% elongation (*52*). Moreover, in the human TMJ, the condylar strain measured *in vitro* is reported to be about 0.04% elongation (*53*). However, because several factors, such as the form of mandibular condyle and other elements of the TMJ, affect the load on the condyle, actual load measurement is difficult, and because of the difficulty of the surgical approach, *in vivo* measurements of condylar strain are limited. Moreover, in these experiments only the strain on the bone was measured and there were no experiments in which the cartilage that covers the condyle was examined. For this reason, we determined the effect of stress application on chondrocytes, and the magnitude of the applied stress was obtained with reference to the *in vitro* data of De Witt *et al.* (*41*). They reported that when the chondrocytes are subjected to 5.5% elongation tension forces, DNA and proteoglycan syntheses are significantly increased. The Flexercell strain unit has been designed to provide regulated strain to cells in culture, but it is well known that the deformation at the bottom of the well does not occur equally throughout the well (*54*). When negative pressure is applied to the well, the well bottom stretches downward in a largely uniaxial gradient, maximal at the well periphery and minimal at the well center. Therefore, not all cells on the well are subjected to an equal applied stress. In fact, Vadiakas and Banes (*55*) reported that the rates of incorporation of calcium into osteoblastic cells after the application of stress by the Flexercell strain unit differ between the periphery and the center of the well, but the tendencies are similar and the overall rate of calcium incorporation throughout the well, reflects these tendencies, so the differences have no effect on the comparison between stress and control cultures. In the Flexercell strain unit, stretching induced by the application of a negative pressure of 5 kPa to the bottom of the well causes about 11% maximum elongation in the periphery of the well, but about 5% average elongation overall, so, in this study, we used this degree of magnitude as a suitable stress to promote matrix synthesis in chondrocytes, and 15 kPa as a stress to induce ECM degradation.

Unexpectedly, we found that continuous exposure for 48 h to not only 15 kPa but also 5 kPa high frequency CTF decreases the levels of DNA, protein, collagen, and proteoglycan synthesis in HCS-2/8 cells (Figs. 2A, 3A, 3B, and 4A). However, neither 5 nor 15 kPa high frequency CTF has any significant effect on cell number (Fig. 2B). Moreover, high frequency CTF has no effect on protein accumulation (Fig. 3C). Furthermore, the trypan blue exclusion test revealed no cell death in CTF-loaded cultures. These findings indicate that the high frequency CTF in this study, with 1 s duration and a 1 s interval, results in some growth inhibition, but is not cytotoxic to HCS-2/8 cells after 48 h. In contrast, proteoglycan accumulation was inhibited under the same conditions (Fig. 4C), suggesting that proteoglycan metabolism is more sensitive to CTF. Although ECM syntheses were inhibited, the mRNA levels of type II collagen and aggrecan showed no remarkable changes at either level of CTF (unpublished), suggesting that ECM syntheses related to stress may be regulated at the post-transcriptional level.

Reducing the load frequency at 5 or 15 kPa CTF from 30 cycles/min to 1 cycle/4 min reduced the inhibition of DNA (Fig. 2A) and proteoglycan (Fig. 4B) synthesis, indicating that not only the load magnitude but also the load frequency affects DNA and proteoglycan synthesis in HCS-2/8 cells, and that high frequency CTF has a more suppressive effect on DNA and proteoglycan synthesis in HCS-2/8 cells.

When HCS-2/8 cells were continuously labeled with [^{35}S]sulfate under high frequency CTF conditions, the accumulation of [^{35}S]sulfate-labeled proteoglycans was significantly inhibited after 48 h and this inhibition was partially, but not fully, restored by the MMP inhibitor (Table II). Because [^{35}S]sulfate incorporation into proteoglycans under these experimental conditions indicates the sum of proteoglycan synthesis and the degradation of newly synthesized proteoglycans, it is unlikely that the MMP

inhibitor fully restores the decrease caused by CTF. In fact, the MMP inhibitor fails to restore the decrease in [^{35}S]-sulfate incorporation into proteoglycans observed after 24-h exposure to CTF (unpublished), suggesting that CTF causes the inhibition of proteoglycan synthesis alone up to 24 h and the inhibition of both proteoglycan synthesis and the degradation of newly synthesized proteoglycans between 24 and 48 h.

It has been proposed that inflammatory cytokines such as IL-1 play an important role in mediating cartilage degradation (*56–58*). For example, increased levels of IL-1 in the synovial fluid of the OA joint have been demonstrated (*59*), and the intra-articular injection of IL-1 causes the production of MMPs (*60*). Moreover, IL-1 induces the synthesis of MMP-9 by chondrocytes *in vitro* (*61*). On the other hand, the effects of cyclic mechanical stress on the expression of MMPs and cytokines have not been investigated in chondrocytes. In other types of stress, however, the expression of cartilage degradation factors following exposure to mechanical stress has been reported. For example, hydrostatic pressure induces the expression of the mRNA for IL-6, tumor necrosis factor α, transforming growth factor β1 (TGFβ1), and heat shock protein 70 (*44, 45*). Fluid-induced shear increases the gene expression of TIMP-1 (*62*). Moreover, hydrostatic pressure affects the production of MMP-3 and TIMP-1 in the human lumbar intervertebral disk (*63*). Our study provides the first demonstration of a change in the expression of the mRNA for MMP-2 and -9 and IL-1 in cultured chondrocytes after exposure to cyclic mechanical stress. The mRNA levels of IL-1 increase as early as 3–6 h after the application of high frequency CTF at both 5 and 15 kPa; especially at 15 kPa CTF, this change is remarkable (Fig. 5A). This result suggests that high frequency CTF at 15 kPa mediates the expression of IL-1 in HCS-2/8 cells and may participate in the expression of MMPs. Moreover, the mRNA level of MMP-9 also increases transiently after CTF at 5 kPa, but increases continuously for up to 24 h after CTF at 15 kPa (Fig. 5, A and B). In contrast, high frequency CTF has no effect on the expression of TIMP-1 mRNA at either 5 or 15 kPa (Fig. 5, A and B). The mRNA level of TIMP-2 also does not change following high frequency CTF (unpublished). In other words, high frequency CTF at 15 kPa produces a contrastive pattern of expression between MMPs and TIMPs in chondrocytes. Moreover, as observed by gelatin zymography, high frequency CTF at 15 kPa, but not 5 kPa significantly increases the production of pro-MMP-9 and active-MMP-9 in HCS-2/8 cells and rabbit chondrocytes (Fig. 6, A and C). Because an imbalance between MMPs and TIMPs might cause cartilage degradation (*64*), we can hypothesize that 15 kPa CTF may cause extensive degradation of the cartilage matrix *via* an increase in expression and production of MMPs. The finding that the MMP inhibitor inhibits the CTF-induced decrease in [^{35}S]sulfate-labeled proteoglycan accumulation (Table II), also supports this hypothesis. On the other hand, 5 kPa CTF might cause only transient changes in the expression of degradation factors for cartilage matrix.

Because 15 kPa causes more stress than 5 kPa in the Flexercell strain unit, we used 15 kPa CTF as a stress level that should inhibit ECM synthesis. However, in our study, 5 kPa CTF seems to be a more inhibitive than 15 kPa CTF for ECM and DNA syntheses in HCS-2/8 cells. Moreover, regardless of the finding that 5 kPa CTF inhibits ECM and DNA syntheses more than 15 kPa CTF, the expression of cartilage degradation factors at 5 kPa were lower than at 15 kPa. In our preliminary experiments, the mRNA levels of basic fibroblast growth factor (bFGF), which stimulates DNA, collagen, and proteoglycan syntheses in chondrocytes (*65*), and TGFβ considered to be the most important cartilage degradation defensive factor (*65*), tended to increase only after the application of 15 kPa CTF. Therefore, bFGF and TGFβ induced by 15 kPa CTF may restore the DNA, collagen, and proteoglycan syntheses inhibited by CTF.

Some of our results conflict with those reported by others concerning the effect of cyclic loading on chondrocyte metabolism. For example, Holmvall *et al.* (*40*) reported that 18 kPa of stress (2 s on/2 s off) increases the mRNA levels of ECM components such as type II collagen and aggrecan. However under our CTF conditions such as 1 s on/1 s off (30 cycles/min), the mRNA levels of type II collagen and aggrecan showed no remarkable changes at either level of CTF (unpublished). Furthermore, Fukuda *et al.* (*39*) reported that 2 kPa of stress (3 s on/357 s off) increases proteoglycan synthesis, while 10 kPa of stress (3 s on/3 s off) decreases proteoglycan synthesis. On the other hand, in our experiments, proteoglycan synthesis was significantly inhibited by CTF, regardless of the CTF magnitude or frequency. These discrepancies may result from the CTF magnitude and frequency, or the type of chondrocytes. Further investigations are needed to explain these discrepancies.

In conclusion, the present study demonstrates a possible mechanism, that is the involvement of MMPs, for CTF-induced cartilage degradation. CTF at 5 kPa does not cause the gene expression of cartilage degradation factors such as IL-1 and MMP-9, suggesting that this magnitude of CTF causes only minor changes in the cartilage matrix. On the other hand, 15 kPa of high frequency CTF causes the expression of the IL-1 and MMP-9 genes, followed by increases in the production of MMP-2 and -9. Moreover, the degradation of proteoglycans induced by high frequency CTF at 15 kPa, is inhibited by the MMP inhibitor. These findings suggest that excessive and continuous cyclic mechanical stress induces the production of IL-1 and MMP-9, resulting in cartilage degradation.

We thank Dr. Kazuhiro Sasaki (Dept. of Orthopedic Surgery, Okayama University Medical School), Dr. Tohru Nakanishi and Dr. Takashi Nishida (Dept. of Biochemistry and Molecular Dentistry, Okayama University Dental School) for valuable discussion. We also thank Ms. Etsuko Fujisawa for secretarial assistance.

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