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Research article

EFFECTS OF HIGH-IMPACT MECHANICAL LOADING ON SYNOVIAL CELL CULTURES

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ABSTRACT

Cartilage metabolism in response to mechanical loading is an important subject in sports science and medicine. In animal studies high-impact exercise is known to stimulate bone adaptation and increase bone mass. However, mechanical impacts potentially induce tissue swelling and occasionally degradation of connective tissues in synovium and articular cartilage. These detrimental outcomes should be properly evaluated clinically and biochemically. Using two synovial cell cultures derived from normal and rheumatic tissues, we examined the biochemical effects of impulsive mechanical loads on expression and activities of influential proteolytic enzymes in joints, matrix metalloproteinases (MMPs), and their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs). The molecular analysis demonstrates that an impact factor (I_m), the ratio of the maximum force to weight, served as a good indicator for assessment of the inflammatory responses. The results showed that high impact above I_m = 40 to 80 elevated not only expression but also enzymatic activities of MMPs.

KEY WORDS: Impulsive factor, rheumatoid arthritis, synovium, MMP

INTRODUCTION

Inflammation and destruction of joint tissues such as articular cartilage and synovium is a serious health issue in sports science and medicine (Oddis, 1996). Exercise has long been studied as a means of improving bone mass density and stimulating bone growth, and animal studies show enhancement of new bone formation with varying mechanical loading (Turner, 1998). However, osteogenic efficacy of high-impact loads should not jeopardize a process of cartilage metabolism. Previous studies suggest that high levels of mechanical stress are linked to progression of arthritis including cartilage damage, apoptosis of chondrocytes, and matrix degradation (Fujisawa et al., 1999; Lane Smith et al., 2000). A thin layer of synovial membrane in a joint is subjected to varying mechanical stress during running and cutting maneuvers including shear stress and impulsive shock (Armstrong, 1986; Besier, 2000).

In the current study, we addressed a question about whether impulsive shocks would affect expression and enzyme activities of matrix metalloproteinases (MMPs) (Shingleton et al., 1996), a class of tissue-degrading proteases, as well as their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Brew et al., 2000). MMPs are considered influential mediators of joint destruction in diseases such as rheumatoid arthritis, an autoimmune disease that causes the joint to lose its shape and alignment (Konttinen et al., 1999). The role of TIMPs is to reduce proteolytic activities of MMPs. We focused on two independent cultures of

Table 1. PCR primers for MMPs, TIMPs, and GAPDH

gene	sense and antisense primers	cDNA size (bp)
MMP-1	5'-CACAGCTTTCCTCCACTGCTGCTGC-3' 5'-GGCATGGTCCACATCTGCTCTTGGC-3'	396
MMP-2	5'-GACAAGAACCAGATCAATAC-3' 5'-GCCATGCTCCCAGCGGCCAAA-3'	181
MMP-13	5'-TGGTGGTGATGAAGATGAT TTGTCT-3' 5'-AGTTACATCGGACCAAACTTTGAAG-3'	366
TIMP-1	5'-CCTGGCTTCTGGCATCCTGTT-3' 5'-GGGACCTGTGGAAGTATCCG C-3'	280
TIMP-2	5'-CAGTGAGAAGGAAGTGGACTC-3' 5'-CATCTGGTACCTGTGGTTCAG-3'	300
TIMP-3	5'-ACGCCTTCTGCAACTCCGACA-3' 5'-CCTCTCAGCAGGTACTGGTAC-3'	221
GAPDH	5'-CCACCCATGGCAAATTCCA TGGCA-3' 5'-TCTAGACGGCAGGTCAGGTCCACC-3'	600

synoviocytes, one isolated from normal tissue and the other from the tissue with rheumatoid arthritis. These synovial cells cover osseous surfaces and intracapsular ligaments, and bear mechanical loads by deforming their shape (Schett et al., 2001). Synovial cells are the main supply source of MMPs and they show the first signs of inflammatory response.

We defined an impact factor to examine quantitatively the effects of the high-impact mechanical loads on cellular responses. Messenger RNA levels of MMPs and TIMPs of synovial cells subjected to high-impact loading were determined by reverse-transcription polymerase chain reaction (RT-PCR) at varying strength of impacts. Two specific MMP activities, collagenase and gelatinase activities, were assayed with fluorescent markers. The results support significant elevation of proteolytic responses by high-impact loads above a threshold.

METHODS

Cell culture

Two synovial cell cultures, NS101 and MH7A, were used in the study. NS101 cells were derived from a normal human synovial membrane (Khalkhali-Ellis et al., 1997), and MH7A cells were isolated from the intra-articular soft tissue of the knee joint of a patient with rheumatoid arthritis (RA) (Miyazawa et al., 1998). Cells were grown to a confluency of ~ 70% in a 35 mm culture plate in RPMI-1640 media (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, U.S.A.) supplemented by 10% fetal calf serum (Atlanta Biologicals, Norcross, GA, U.S.A.) and antibiotics (1 U/ml penicillin, and 1 μg/ml streptomycin) at 37˚ C. Before applying high-impact mechanical loads, cells were mildly starved for 12 hours in the media with 1% fetal calf serum.

Application of impulsive shock

A single impulsive shock was applied to synovial cells by dropping a culture dish onto a concrete pad ~ 5 cm thick that was suspended by three elastic supporting ropes. The culture dish was filled with the media, and the applied impulsive shock was quantified through the impact factor, I_m (the ratio of the maximum force to weight):

$$I_m = \sqrt{1 + \frac{2h}{d_{st}}} \tag{1}$$

where h = drop height, and d_{st} = static displacement. The value of d_{st}, 0.031 cm in this experiment, was calculated from a spring constant of the extendable supports (Hamrock et al., 1999). The impact factors of 20, 40, and 80 were chosen and achieved by the drop heights of 6 cm, 24 cm, and 97 cm, respectively. The piezoelectric sensor was used to evaluate the impact intensity as a function of the selected drop heights (Figure 1). Note that although the three selected impact factors may simulate varying impulsive shocks with cell cultures, exercise regimens are different from *in vitro* experiments in terms of many factors including stress spectrum, duty cycles, and interactions with surrounding tissues.

RT-PCR

RT-PCR was employed to determine the mRNA levels of MMP-1, MMP-2, MMP-13, TIMP-1, TIMP-2, and TIMP-3. Total RNA was isolated using

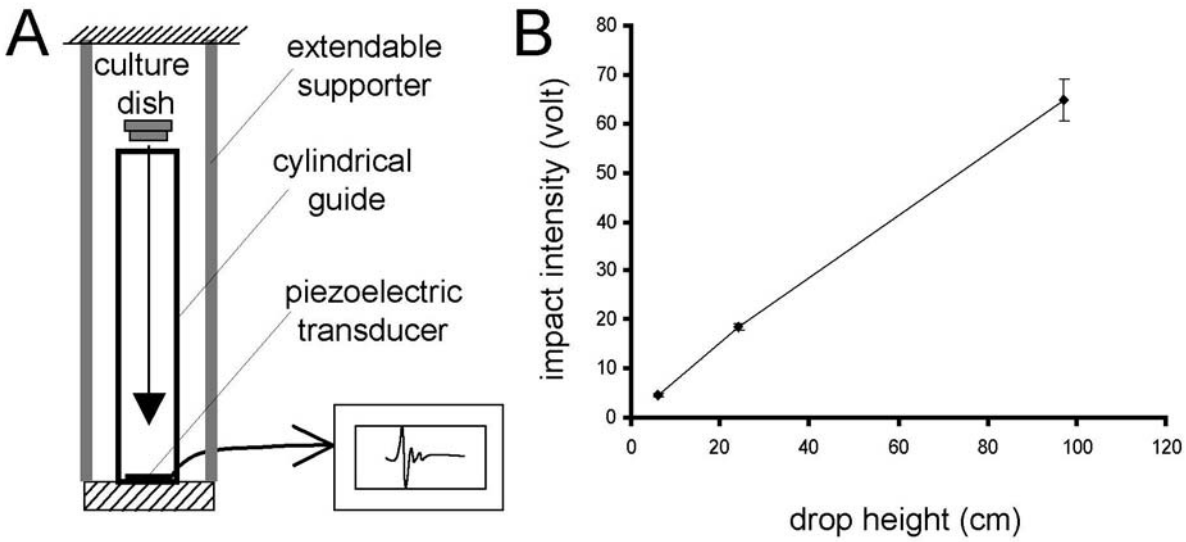

Figure 1. Characterization of impulsive shocks. (A) Schematic diagram of the impulsive shock-loading apparatus with the piezoelectric sensor. Cells were dropped onto the piezoelectric sensor whose output voltage was used to estimate high-impact mechanical stimuli. (B) Impact intensity at varying drop heights.

RNeasy minikits (Qiagen Inc., Stanford Valencia, CA, U.S.A.), and the isolated RNA was reverse-transcribed by MMMLV reverse transcriptase (Applied Biosystems, Branchburg, NJ, U.S.A.) using random primers. The PCR primers are listed in Table 1. The PCR procedure included a hot start at 94˚C for 5 min, followed by 32 cycles at 94˚C for denaturation (45 sec), 55-62˚ C for annealing (90 sec), and 72˚C for extension (30-90 sec). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control. The mRNA level in response to high impacts was normalized by the control level without high-impact stimuli.

Fibril Degradation Assay

Collagenase and gelatinase activities were determined by a fibril degradation assay using an EnzChek gelatinase/collagenase assay kit (Molecular Probes Inc., Eugene, OR, USA) (Sun and Yokota, 2002). Total proteins in the culture medium at 30, 60, and 90 min after application of the impulsive shock were used. The medium was incubated with the fluorescent substrate at room temperature for 2 hours in the reaction buffer. Fluorescent intensity at an absorption/emission wavelength of 495/515 nm was measured with a FluoroMax-2 spectrofluorometer (Instruments S.A., Inc., Edison, NJ, USA).

Statistics

All experiments were performed three times, and the mean and standard error of mean were calculated. The MMP mRNA level and the MMP activity level of loaded cells were normalized by the level of control cells (no mechanical loads). A t-test was conducted between the loaded cells and control cells using StatView (SAS Institute, Inc., Cary, NC, USA), and the p-value < 0.01 was considered statistically significant.

RESULTS

Alteration in MMP mRNA expression by impulsive shock

In both normal synovial cells (NS101) and RA synovial cells (MH7A), the RT-PCR results showed clearly an increase in the mRNA level of MMP-1, MMP-2, and MMP-13 in response to impulsive shocks. The response was dependent on the incubation time after the shock, the impact factors, and MMPs (Figure 2). First, regardless of the impact factor the incubation for 30 min was insufficient to stimulate mRNA expression of any MMPs. It required 60 min for MMP-1 and 120 min for MMP-2 and MMP-13 to elevate their mRNA level. Second, the effects of the shock with $I_m = 20$ was insignificant for all cases except for MMP-1 with a 120-min incubation. The shocks with $I_m = 40$ and 80 were required to alter the expression of MMP-2 and MMP-13.

Alteration in TIMP mRNA expression by impulsive shock

The effects of impulsive shocks on the mRNA level of TIMP-1, TIMP-2, and TIMP-3 were also dependent on the incubation time after the shock, the impact factors, and TIMPs in NS101 (normal) and MH7A (RA) cells (Figure 3). Among three TIMPs studied here only the TIMP-2 mRNA level was elevated after 30-min incubation. The longer incubation for 60 min and 120 min elevated all three TIMPs. The shock with $I_m = 20$ was effective to

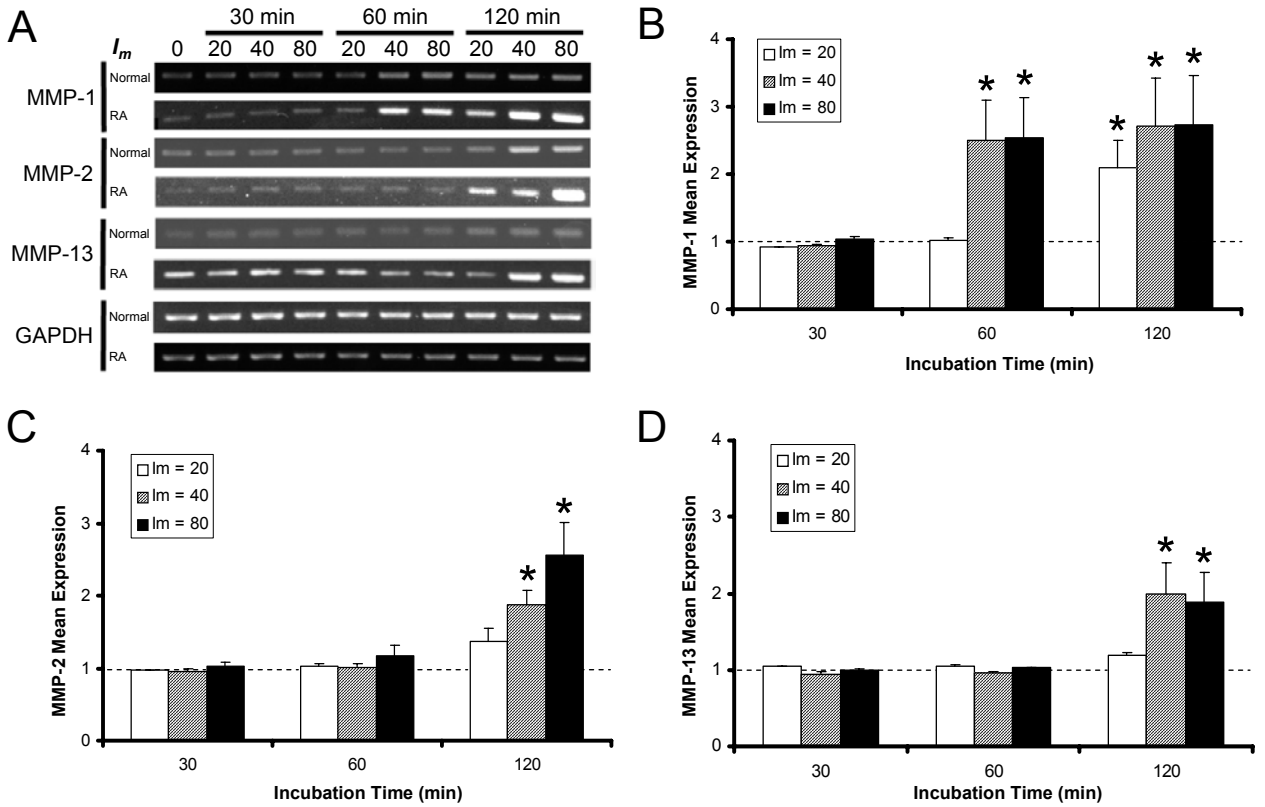

Figure 2. Messenger RNA expression of MMP-1, MMP-2, and MMP-13 in response to high-impact mechanical stimuli. NS101 (normal) and MH7A (RA) cells were harvested 30, 60, and 120 min after the impulsive shock with I_m = 0 (control), 20, 40, and 80. GAPDH was used as control. In (B)-(D), the asterisk denotes statistically significant changes ($p < 0.01$) and the bar represents the standard error of mean. (A) Representative gel images for NS101 (normal) and MH7A cells. (B) MMP-1 mRNA level of NS101 and MH7A cells. (C) MMP-2 mRNA level of NS101 and MH7A cells. (D) MMP-13 mRNA level of NS101 and MH7A cells.

increase the mRNA level of TIMP-3 alone. The shocks with I_m = 40 and 80 elevated the mRNA level of all TIMPs.

Increase in collagenase and gelatinase activities by impulsive shock

Enzyme activities of collagenases and gelatinases increased in response to impulsive shocks in both NS101 cells and MH7A cells. The impact factor was directly correlated to both collagenase and gelatinase activities (Figure 4A). Particularly, collagenase activities, which would initiate degradation of intact collagen fibers, were significantly affected by impact factors.

The proteolytic activity in MH7A cells with rheumatoid arthritis matched or exceeded the activity in NS101 normal cells (Figure 4B). MMP activities in both cells were at the similar level in response to the shock with I_m = 20. In response to I_m = 40 and 80, however, collagenase activities in MH7A cells were higher than those of NS101 cells by as much as 73% (I_m = 80 at 120 min). The difference between NS101 cells and MH7A cells was less pronounced in gelatinase activities, which differed significantly at only one point (I_m = 80 at 120 min).

DISCUSSION

The current study demonstrated that the expression of the selected MMPs as well as their proteolytic activities was elevated by high-impact mechanical loads in normal and rheumatic synovial cells. MMP expression was dependent on the impact factor, and incubation time after application of mechanical loads *in vitro*. Although the expression of TIMPs was also increased, their increase was not sufficient to suppress collagenase and gelatinase activities. After application of high-impact loads with I_m = 80, collagenase and gelatinase activities increased an average of 50.3%. Thus, our *in vitro* study using synovial cells provides evidence that high-impact loads can potentially damage joint tissues by inducing MMP activities. Our results were consistent to the previous studies using chondrocyte

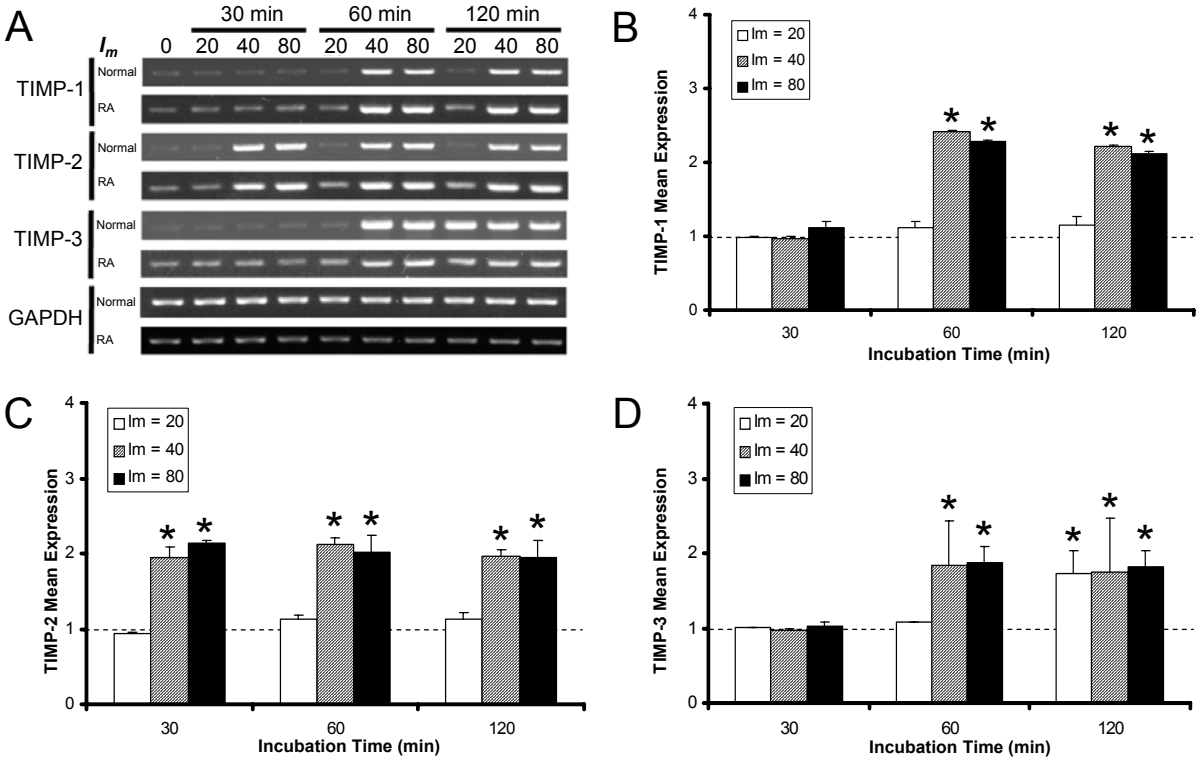

Figure 3. Messenger RNA expression of TIMP-1, TIMP-2, and TIMP-3 in response to impulsive shock. The loading conditions were identical to the analysis of MMPs in Figure 2. (A) Representative gel images for NS101 (normal) and MH7A (RA) cells. (B) TIMP-1 mRNA level of NS101 and MH7A cells. (C) TIMP-2 mRNA level of NS101 and MH7A cells. (D) TIMP-3 mRNA level of NS101 and MH7A cells.

cells, where MMPs were upregulated by shear stress and cyclic loads (Honda et al., 2000; Jin et al., 2000).

The biochemical results also suggested that rheumatoid cells were more sensitive to high-impact loads. We observed higher MMP activities in MH7A rheumatoid cells than normal cells in response to impulsive shock. The findings suggest that normal cells may have superior protective ability to impulsive shock, although the current data are limited with two cell lines and examination of other cell cultures is necessary to derive statistically significant interpretation.

Linking the impact factor in the current biochemical study to loads involved in high-impact exercise is crucial. The ground reactions are not transmitted directly to the knee joint surface. During

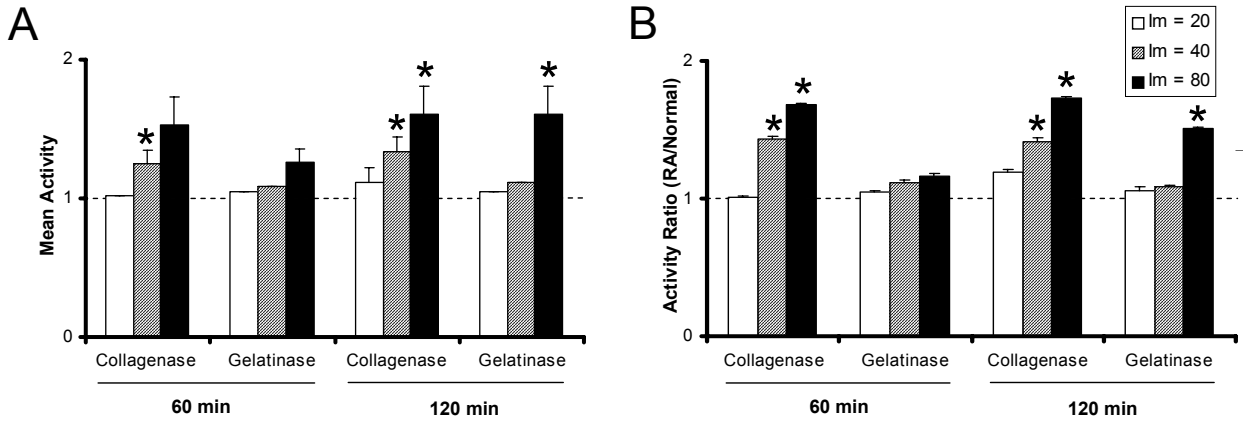

Figure 4. Collagenase and gelatinase activities in response to impulsive shock. The asterisk denotes statistically significant change ($p < 0.01$), and the bar represents a standard error of mean. (A) Collagenase and gelatinase activities in NS101 (normal) and MH7A (RA) cells. (B) Ratio of MMP activities in MH7A cells to MMP activities in NS101 cells.

typical walking, the ratio of maximum loads to body weight, which approximates the impact factor *in vivo*, reaches approximately 4 in a hip joint (Unsworth, 1991). It is of our future interest to determine the impact factor in joints during exercise and evaluate expression and activities of MMPs.

Regular physical activity has numerous health benefits for persons of all ages (NIH, 2001). Animal studies indicate enhancement of bone formation with impact loading (Turner, 1998), and it is reported that high-impact exercise promotes bone gain in well-trained female athletes (Taaffe et al., 1997). We provided unique *in vitro* evidence from a view point of proteolytic activities that excessive high-impact exercise potentially induces joint inflammation and degradation. An allowable level of high-impact loads may differ depending on inflammatory states of joint tissues as well as loading conditions such as duty cycles and rest periods.

CONCLUSION

In conclusion, the current *in vitro* study suggests that expression and enzymatic activities of MMPs can be elevated in the synovium by high-impact exercise.

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KEY POINTS

- High-impact loading elevated expression and activities of MMPs in synovial cell cultures.
- The impact factor was used to define *in vitro* intensity of high-impact loading.

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