Original

Establishment of a Mechanical Stress Load Arthritis Model in a Human Synovial Sarcoma Cell Line

Yoshito Kobayashi [1,2], Yuko Udaka *[1], Haruna Shirako [1,2],
Miki Kato [3], Yoshiko Kudo [1], Nana Ichimura [1],
Mayumi Tsuji [1] and Yuji Kiuchi [1]

Abstract : Osteoarthritis (OA) is a degenerative disease that occurs in joints throughout the body and includes various concomitant pathologies due to possible mechanical stress, such as destruction of cartilage, hyperplasic changes, and synovial inflammation. However, there have been few studies on the mechanical stress that is the basic cause of OA. Our goal was to establish an OA model at the cellular level, by measuring inflammatory cytokines and cartilage destruction markers that are induced after a mechanical stress load. Using a human synovial sarcoma cell line (SW982 cells), we provided two types of mechanical stress load for 48 hr : shaking stress (amplitude 2 mm, speed range 1,000 rpm), and the addition of hydroxyapatite (5 μg/ml) into the culture medium. Then we measured the phosphorylation activity of nuclear factor (NF)-κB transcription factor in the cell lysate, and the levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 as inflammatory cytokines, and the level of matrix metalloproteinase (MMP)-3 as a cartilage destruction marker, released in the medium. Shaking stress significantly induced phosphorylation of NF-κB and production of TNF-α, compared with untreated controls. On the other hand, hydroxyapatite stress only increased production of TNF-α. Both stresses together significantly induced phosphorylation of NF-κB and production of TNF-α, IL-6 and MMP-3 rather than a single stress load. In this study, markers related to inflammation and cartilage destruction (IL-6, TNF-α, and MMP-3) significantly increased. Therefore, we suggest that the mechanical stress load conditions used in this study might be useful as an OA model.

Key words : osteoarthritis (OA) model, mechanical stress, shaking, hydroxyapatite, inflammation

Introduction

  As Japan's population is aging with every passing year, degenerative diseases based on age-related changes are consistently increasing. In 2009 it was estimated that 47,000,000 people had at least one of either lumbar spondylosis, osteoarthritis (OA) of the knee or osteoporosis, and

[1] *Department of Pharmacology, Showa University School of Medicine*, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan.
[2] *Department of Orthopedics, Showa University School of Medicine.*
[3] *Showa University School of Pharmacy.*
* To whom corresponding should be addressed.

as many as 5,400,000 had all three[1]. OA is an orthopedic disease that is encountered most frequently in outpatient clinics. It is a degenerative disease of the bones and cartilage that occurs with age in various joints throughout the body, such as the knees, hips, shoulders, and spine, and affects approximately 10 times as many patients as does rheumatoid arthritis (RA).

A joint is enclosed by a joint capsule, with the inner layer being the synovial membrane. The synovial membrane secretes synovial fluid, which functions as a nutrient and lubricating agent for the joint and cartilage. Collagen fibers form the structure of cartilage, and they spread out into a network in which proteoglycans are embedded. Proteoglycans bond to hydrophilic hyaluronic acid, thereby forming a sponge-like cushion that contains water, protecting the joint from shock. However, when the cartilage is injured by weight gain or external injury, then the collagen fiber structure is destroyed. Consequently, this causes the proteoglycan content to decrease, thus disturbing cartilage homeostasis, leading to cartilage deformation and destruction[2].

Chronic inflammation can be caused by biomolecules [damage-associated molecular pattern molecules (DAMPs)] that are emitted from tissues and cells that have been injured[3]. Approximately 70% of the bone consists of hydroxyapatite (HA) which is a type of calcium phosphate. In OA, the microscopic bone chips that are generated by the destruction of cartilage and bone become DAMPs, and the friction within the joint gradually increases. They induce symptoms similar to those of chronic inflammation, and promote synovitis and destruction of cartilage[4]. Exceptionally advanced research has been reported on RA, in which inflammatory cytokines are excessively produced because of an abnormal immune response that induces synovitis[5]. It is also well known that many remarkable drugs for RA, such as biological preparations, have been developed. On the other hand, in OA, pathological alterations caused by age-related changes in cartilage cells, mechanical stress, inflammatory cytokines, and genetic factors interact in complicated ways[6]. It has been suggested that the cells of the innate immune system within the synovial fluid, such as macrophages, fibroblasts, dendritic cells, and neutrophils, become activated by DAMPs floating in the synovial fluid, and inflammation is induced[7]. However, it is difficult to define the pathological conditions, and the details of the pathogenic processes are still unclear. Based on the levels of cytokines found in the synovial fluid of OA patients, interleukin (IL)-6 and tumor necrosis factor (TNF)-α also are considered to exhibit significant increases, depending on OA severity[8]. It is therefore thought that these factors could be OA pathogenesis markers.

Treatment of an OA-related cell line with IL-1β *in vitro* increases various cytokines and inflammation markers[9]. However, there have been no reports of experiments that induce inflammation with mechanical stress, which is the basic cause of OA. Therefore, in this study, we investigated an *in vitro* OA model by applying two types of mechanical stress, a shaking stress and the addition of HA, the main inorganic component of bone tissue as DAMPs, to a human synovial sarcoma cell line (SW982), and measured the inflammatory cytokines and cartilage destruction markers.

Methods

Materials and cell cultures

We purchased the human synovial sarcoma cell line (SW982 cells)[10] from ATCC (Manassas,

VA, USA), Dulbecco's modified eagle medium high glucose from Wako Pure Chemical Industries, Ltd., (Osaka, Japan), and fetal bovine serum from Sigma Aldrich Co. (Tokyo, Japan). We purchased HA as MicroSHAp (IHM-10010) from SofSera Corporation (Tokyo, Japan), and a mini-shaker (PSU-2T) for microplates from Waken Btech Co., Ltd. (Kyoto, Japan). We cultured the SW982 cells in Dulbecco's modified eagle medium high glucose containing 10% fetal bovine serum under conditions of 37°C and 5% CO_2[11].

Mechanical stress exposure

We applied two types of mechanical stress loads to the SW982 cells. One was a shaking stress, with a shaking amplitude of 2 mm and a shaking speed range of 1,000 rpm. The second type of stress load was the stress of adding HA into the culture medium, which is assumed to generate microscopic bone fragments (DAMPs) created by bone destruction (HA concentration: 1, 5, 10, 25, or 50 μg/ml). We combined these two types of stress in the four shake patterns: shake (−) HA (−), shake (−) HA (+), shake (+) HA (−), and shake (+) HA (+). Then we cultured them for 24, 48, or 72 hr, and investigated the cell survival rate.

Measurement of cell survival rate

Cells were adjusted to $2×10^4$ cells/ml, seeded in 96-well plates, and cultured for 24 hr. Then, the medium was replaced with medium containing various concentrations of HA, and the cells were exposed, or not exposed, to shaking stress. For quantifying cell survival and growth rate at each time period of 24, 48 and 72 hr, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method with the MTT yellow tetrazole assay kit, CellTiter 96® AQueous One Solution Assay (Promega, Madison, WI, USA), and measured the absorbance at 570 nm wavelength with a microplate reader.

Measurement of nuclear factor (NF)-κB phosphorylation activity

Cells were adjusted to $3×10^5$ cells/ml in 6-well plates for 24 hr. Then, the medium was replaced with medium containing 0 or 5 μg/ml HA, and the cells were exposed to no shaking or shaking stress. After 48 hr, we collected the cell lysate by centrifuging the samples at 3,000×g for 5 min, and using the isolated residual cells, then followed the protocol for the NF-κB p65 InstantOne™ ELISA kit (eBioscience, San Diego, CA, USA). The protocol included sampling the cell lysate, and measuring the absorbance at 450 nm wavelength with a microplate reader to measure the protein content of total NF-κB p65/phospho NF-κB.

Measurement of TNF-α levels

Cells were adjusted to $3×10^5$ cells/ml in 6-well plates for 24 hr. Then, the medium was replaced with medium containing 0 or 5 μg/ml HA, and cells were exposed to no shaking or shaking stress. After 48 hr, we collected the supernatant by centrifuging the samples at 3,000×g for 5 min. We then followed the protocol for the Human TNF-α Quantikine ELISA kit (HSTA00E; R&D Systems, Minneapolis, MN, USA), and measured the absorbance at 450 nm

wavelength with a microplate reader to determine TNF-α production.

Measurement of IL-6 levels

Cells were adjusted to 3×10^5 cells/ml in 6-well plates for 24 hr. Then, the medium was replaced with medium containing 0 or 5 μg/ml HA, and cells were exposed to no shaking or shaking stress. After 48 hr, we collected the supernatant by centrifuging the samples at 3,000×g for 5 min. We then followed the protocol for the Human IL-6 Quantikine ELISA kit (D6050; R&D Systems), and measured the absorbance at 450 nm wavelength with a microplate reader to determine IL-6 production.

Measurement of matrix metalloproteinase (MMP)-3 levels

Cells were adjusted to 3×10^5 cells/ml in 6-well plates for 24 hr. Then, the medium was replaced with medium containing 0 or 5 μg/ml HA, and cells were exposed to no shaking or shaking stress. After 48 hr, we collected the supernatant by centrifuging the samples at 3,000×g for 5 min. We then followed the protocol for the Human MMP-3 Total Quantikine ELISA kit (DMP300; R&D Systems) and measured the absorbance at 450 nm wavelength with a microplate reader to determine MMP-3 production.

Statistical analysis

Experimental results are shown as mean ± standard error (n = 5 to 10). The statistical software Ystat 2010 (Igaku Tosho Shuppan Co., Ltd., Tokyo, Japan) was used to perform analysis of variance, followed by the Student-Newman-Keulis test for repeated measurements; $P < 0.05$ was considered to indicate a significant difference.

Results

Effect of HA addition and/or shaking stress on SW982 cell survival rate

Figure 1 shows the cell survival rate of SW982 cells, calculated by the MTT method, with shaking stress for 24, 48 or 72 hr, and the addition of 1, 5, 10, 25 or 50 μg/ml HA. For both the shake (−) and shake (+) cohorts, the live cells decreased in a dose-dependent manner with increasing HA concentration, but no large difference was found between the two cohorts. In contrast, for each HA concentration, the amount of cell growth was found to be time dependent. Based on these results, we set the mechanical stress exposure conditions to be an HA concentration of 5 μg/ml and a shaking time of 48 hr, which gave a cell survival rate of approximately 50%.

Mechanical stress-induced NF-κB phosphorylation activity

Figure 2 shows the NF-κB phosphorylation activity (total NF-κB/phospho NF-κB) after exposure of the SW982 cells to mechanical stress. Compared to the shake (−) HA (−) group (1.14 ± 0.049; n = 9), the shake (+) HA (−) group (0.887 ± 0.064; n = 8) showed significant activation ($P < 0.01$), and a more marked activation was verified in the shake (+) HA (+) group (0.673 ± 0.059; n = 9, $P < 0.05$).

Mechanical stress-induced TNF-α levels

Figure 3 shows the generation of the inflammatory cytokine TNF-α in SW982 cells after exposure to mechanical stress. Compared to the shake (−) HA (−) group (5.98 ± 0.06; n＝10), there was a significant increase in the shake (−) HA (+) (26.00 ± 5.61; n＝5; $P < 0.01$), shake (+) HA (+) (20.4 ± 2.0; n＝6; $P < 0.01$) and shake (+) HA (−) groups (17.41 ± 3.77; n＝8; $P < 0.05$). Thus, the production of TNF-α significantly increased with exposure to either type of stress.
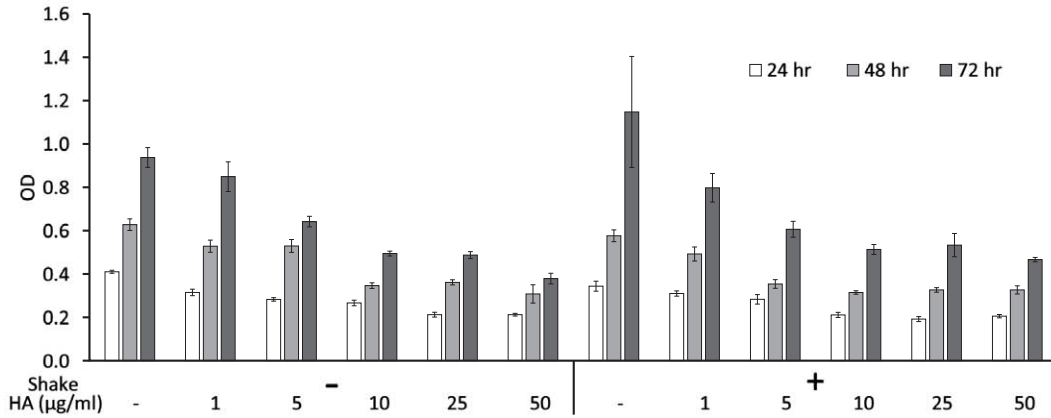

Fig. 1. Effects of exposure to hydroxyapatite (HA) and/or shaking stress on the survival rate of SW982 cells. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method to evaluate the effects of the addition of HA and shaking stress on SW982 cells. The vertical axis shows the cell survival rate. The horizontal axis shows the added HA concentration and the existence of shaking. Values are mean ± standard error, n＝6.
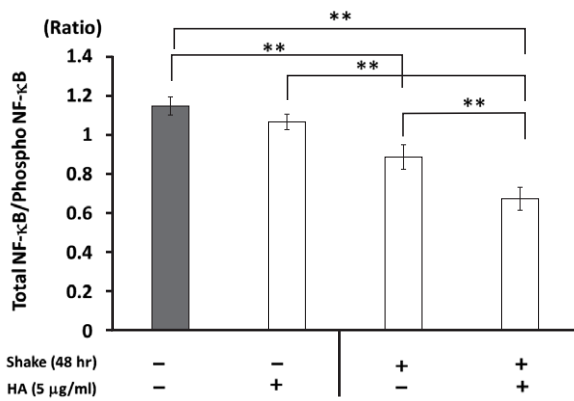

Fig. 2. Mechanical stress-induced nuclear factor (NF)-κB phosphorylation activity of SW982 cells. We measured NF-κB activity after applying mechanical stress loads of 5 μg/ml HA and shaking for 48 hr to SW982 cells. The vertical axis shows NF-κB activity (expressed as total NF-κB/phosphorylated NF-κB), and the horizontal axis shows the presence or absence of HA and shaking. Values are mean ± standard error; n＝3 to 9; **$P < 0.01$.
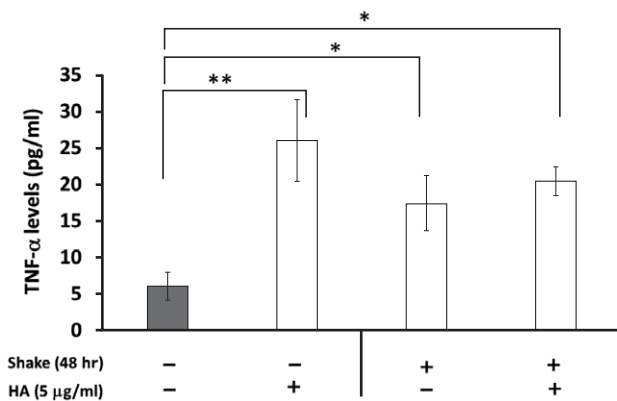
Fig. 3. Mechanical stress-induced tumor necrosis factor (TNF)-α levels of SW982 cells. We measured the TNF-α level within the medium after applying mechanical stress loads of 5 μg/ml HA and shaking for 48 hr to SW982 cells. The vertical axis shows the TNF-α level (expressed in pg/ml), and the horizontal axis shows the presence or absence of HA and shaking. Values are mean ± standard error; n＝5 to 10; *$P < 0.05$, **$P < 0.01$.

Mechanical stress-induced IL-6 levels

Figure 4 shows the generation of the inflammatory cytokine IL-6 in SW982 cells after exposure to mechanical stress.　There was a significant increase in the shake（+）HA（+）group（25.5 ± 1.4；n＝5）, compared to the three other groups［shake（−）HA（−）, 12.33 ± 1.16, n＝6；shake（−）HA（+）, 11.75 ± 0.81, n＝6；shake（+）HA（−）13.59 ± 1.26, n＝6；all $P < 0.01$］.　Thus, a marked increase in IL-6 was found when the cells were exposed to both stresses at the same time.

Mechanical stress-induced MMP-3 levels

Figure 5 shows the generation of the cartilage destruction marker MMP-3 in SW982 cells after exposure to mechanical stress.　There was a significant increase in the shake（+）HA（+）group（12.4 ± 2.56；n＝6）, compared to the three other groups［shake（−）HA（−）, 3.53 ± 0.13；n＝6；$P < 0.05$；shake（−）HA（+）.　4.59 ± 0.14；n＝6；$P < 0.01$；shake（+）HA（−）, 4.96 ± 0.29；n＝6；$P < 0.05$）.　Thus, a large increase in MMP-3 was found when the cells were exposed to both stresses at the same time.

Discussion

Recently, the most important treatment method for OA has been "pharmacotherapy（pain management)," followed by "exercise therapy（rehabilitation)" and "artificial joints and femoral head prostheses".　There are also great expectations for new drugs in the future from fundamental research in molecular biology.

In this study, we used SW982 cells, provided motion and DAMPs, which are considered to be
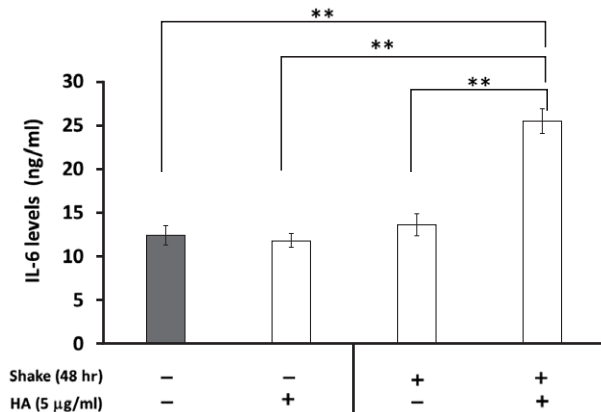

Fig. 4.　Mechanical stress-induced interleukin（IL)-6 levels of SW982 cells. We measured the IL-6 level within the medium after applying mechanical stress loads of 5 μg/ml HA and shaking for 48 hr to SW982 cells. The vertical axis shows the IL-6 level (expressed in pg/ml), and the horizontal axis shows the presence or absence of HA and shaking. Values are mean ± standard error；n＝5 to 9；**$P < 0.01$.
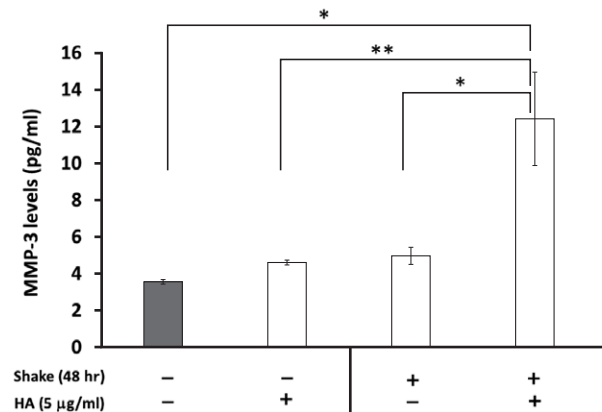
Fig. 5.　Mechanical stress-induced matrix metalloproteinase（MMP)-3 levels of SW982 cells. We measured the MMP-3 level within the medium after applying mechanical stress loads of 5 μg/ml HA and shaking for 48 hr to SW982 cells. The vertical axis shows the MMP-3 level (expressed in pg/ml), and the horizontal axis shows the presence or absence of HA and shaking. Values are mean ± standard error；n＝3 to 6；*$P < 0.05$, **$P < 0.01$.

mechanical stress exposures （shaking and HA）, and measured the induced transcription factor NF-κB, as well as the inflammatory cytokines, TNF-α and IL-6, and the cartilage destruction marker, MMP-3, to investigate their appropriateness as a new OA model. NF-κB is activated by various stresses and plays an important role in many types of physiological reactions and disease conditions, such as immune responses, RA, allergies, cancer, and inflammatory reactions. We verified that the phosphorylation of NF-κB was significantly activated by a shaking stress, but it was most activated by shake （+） HA （+）. Moreover, the level of TNF-α was significantly increased by shaking stress alone. Whereas, the levels of IL-6, TNF-α and MMP-3 were markedly increased by shake （+） HA （+）.

The synovium contains various types of cells, such as osteoclasts, fibroblasts, macrophages, T-cells, and B-cells. The SW982 cells used in this study are derived from fibroblasts, and have been reported to be useful for analysis of inflammatory cytokines and MMP[12]. The cytokines （IL-1, TNF-α, etc.） that are produced from RA synovium increase inflammation[13], and among them, IL-6 exists in high concentrations in the synovial fluid of RA patients. Indeed, a correlation has been reported between the IL-6 concentration and the activity of RA lesions, such as increased C-reactive protein, pannus formation and joint destruction[14]. Moreover, these inflammatory cytokines are known to contribute to the induction of prostaglandin E_2, vascular endothelial growth factor, MMP, and receptor activator of nuclear factor kappa-B ligand within the synovial membrane, and therefore, they have become a target of treatment[15].

Inflammation-related substances, such as IL-1β, TNF-α, cyclooxygenase 1 and 2, and prostaglandin E_2, and transcription modifying factors, such as NF-κB, are expressed within the synovial fluid tissue in OA from the early stages of inflammation[16]. Thus, although RA and OA are different with regard to the extent and pattern of joint deformation, many similar aspects have been recognized at the cellular level, and in both cases, inflammatory cytokines contribute to the disease condition[4].

Many points are still unclear about the cause of OA. Broken cartilage fragments induce inflammation in the synovial membrane which spreads to the cartilage tissue again. Thus, a negative chain reaction of inflammation is known to exacerbate cartilage and bone destruction[6,17].

Joint cartilage receives nutrients from the synovial fluid that is exuded by joint movement. Therefore, a moderate mechanical load is important for cartilage metabolism[18]. However, an abnormal or excessive mechanical stress （aging, weight gain and external injury） might cause changes in the extracellular matrix, cytoskeleton, or cell membrane, such that signals are transmitted into the cells[19] that transform the biochemical reactions. It is unclear how cartilage tissue receives mechanical stress; one report suggests the contribution of the cell surface receptor, integrin[20]. This receptor is a heterodimer consisting of two subunits （α chain and β chain） and a signal is transmitted into the cell when some fluid factor is secreted as the result of the hyperpolarization of integrin by mechanical stress[20]. In our current study, we have verified that, with just shaking stress load alone, the phosphorylation activity of NF-κB and the TNF-α level were significantly increased, but no changes were found in IL-6 and MMP-3 levels.

Moreover, as a mechanism of onset of chronic inflammation, an immune response via the

toll-like receptor, which exists on the cell surface, has been proposed. During an immune or inflammatory reaction, the toll-like receptor recognizes two types of microscopic particles as ligands. One is the pathogen-associated molecular pattern type of microscopic particle that is emitted from pathogenic microorganisms during an infection, and the other is the DAMP type of endogenous molecule that is emitted from cells that have sustained some kind of injury[21,22]. The innate immune system of the synovial membrane recognizes microscopic cartilage cell fragments as DAMPs[23]. In our current study, as a result of experiments using HA as a DAMP, we have found that only the TNF-α level showed a significant increase after HA stress load alone.

With simultaneous loads of HA and shaking stress, various factors significantly increased, namely, NF-κB activity, and levels of TNF-α, IL-6 and MMP-3. TNF-α acts on fibroblasts, promotes the production of the inflammatory cytokine IL-6, and also plays a central role in the pathogenesis of RA[24,25]. If a TNF-α receptor blocking agent is administered, then IL-6 concentration is reduced, and thus an anti-inflammatory effect is achieved by suppressing the production of IL-6 from macrophages and fibroblasts[26]. TNF-α also plays an important role in inducing MMPs in arthritis[27,28], and in the cartilage surface of OA[29,30]. MMPs are upregulated by the inflammatory cytokines, IL-1β and TNF-α, whose expression has been verified in the cartilage surface[30]. Most of the MMP genes have an activator protein 1-binding region within the transcription modifying region. TNF-α activates activator protein 1 structural factors such as Fos and Jun, as well as NF-κB, and stimulates MMP transcription via p38 mitogen-activated protein kinase and c-jun-N-terminal kinase signals[31,32], to accelerate the decomposition of the cartilage cell matrix[30,33].

In summary, in this study, transcription factor NF-κB was activated by two types of mechanical stress in SW982, and promoted the production of TNF-α and IL-6 which were target genes of NF-κB. Our results suggest that these cytokines act on the fibroblasts of the synovium by two mechanisms. The first mechanism is that TNF-α and IL-6 bind to each receptor on the cell membrane and then exacerbate inflammation. The other mechanism is that TNF-α induces MMP-3 which leads to cartilage destruction （joint destruction）. In conclusion, because markers related to inflammation and cartilage destruction （IL-6, TNF-α, and MMP-3） were significantly increased in our study, the mechanical stress load conditions used in this study might be useful as an OA model.

Conflict of interest disclosure

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