Regulation of p38 MAPK phosphorylation inhibits chondrocyte apoptosis in response to heat stress or mechanical stress

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Abstract. Activation of p38 MAPK has been associated with a stress response and with apoptotic processes. However, the function of p38 MAPK in chondrocytes is not clearly understood. In this study, we analyzed the expression of p38 and phosphorylated p38 in cartilage of patients with osteoarthritis (OA) was compared to those in normal cartilage by immunohistochemistry and Western blotting. Human knee chondrocytes were exposed to heat stress or mechanical stress. Normal knee chondrocytes were pre-treated with SB203580 or p38 small interfering RNA (siRNA) before induction of heat stress or mechanical stress. Chondrocyte apoptosis was detected by TUNEL staining and Western blotting of cleaved caspases. OA and normal chondrocytes expressed p38; however, OA chondrocytes showed much higher phosphorylated p38 compared to normal chondrocytes. Heat stress or mechanical stress induced apoptosis and increased phosphorylated p38 in normal chondrocytes. The TUNEL positive cells and expression levels of phosphorylated p38 in response to stress decreased when chondrocytes were incubated with SB203580 or transfected with siRNA against p38. In conclusion, we have demonstrated that heat stress or mechanical stress increased chondrocyte death induced by heat stress or mechanical stress.

Introduction

Osteoarthritis (OA) is the most common degenerative disease of the human articular cartilage, especially in the population aged ≥65 years (1). In the US, approximately 37% of the population has OA demonstrable by X-ray examination (2). OA is characterized by extracellular matrix damage and an important loss in tissue cellularity (3). Chondrocyte apoptosis, a type of programmed cell death, is an important component of the pathogenesis of OA cartilage degradation (4).

The mitogen-activated protein kinase (MAPK) pathways are major signal pathways from the cell surface to the nucleus. These signaling cascades control complex programs, such as embryogenesis, differentiation, proliferation and cell death (5,6). There are three major classes of MAPKs in mammals, the extracellular signal-regulated kinases (ERKs), the c-jun N-terminal kinase (JNK) and p38 (7). ERKs are activated by mitogens and growth factors, while JNK and p38 MAPK are activated in response to the inflammatory cytokines, TNFα and IL-1, as well as by cellular stress, such as heat stress, osmotic stress, reactive oxygen metabolites, and ultraviolet irradiation (8-13).

Activation of p38 MAPK results in cancer cell apoptosis initiated by retinoids, cisplatin and other chemotherapeutic agents (14). Mechanical stretch in the human saphenous vein induces activation of p38 MAPK, which is associated with apoptosis (15). Phosphorylation of p38 MAPK induced by oxidative stress has been linked to the activation of caspase 9-mediated apoptotic pathway in dopaminergic neurons (16). A p38 MAPK inhibitor was shown to decrease the number of apoptotic cells and to prevent delayed progressive degeneration in the injured area of oligodendrocytes (17). Moreover, Pelletier et al reported that chondrocyte death in experimental osteoarthritis is mediated by the p38 MAPK pathway (18). The p38 MAPK signal transduction pathway has also been implicated as a critical factor in NO-induced rabbit articular chondrocyte apoptosis (19).

Therefore, we speculated that p38 MAPK might play an important role in stress-induced apoptosis of articular cartilage, and that down-regulation of p38 MAPK might contribute to a decrease in chondrocyte apoptosis. In this study, we investigated the function of p38 MAPK in chondrocytes after heat stress or mechanical stress.
Materials and methods

Isolation and culture of chondrocytes. Cartilage tissue samples were obtained from five patients with OA undergoing total knee replacement surgery. Diagnosis of OA was based on clinical, laboratory, and radiographic evaluations. Normal cartilage tissue samples were obtained from five age-matched patients undergoing surgery for femoral neck fracture. These five patients had no history of joint disease and had macroscopically normal cartilage. All samples were obtained in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. The femoral condyles and the femoral head were fixed in 4% paraformaldehyde for 24 h, dehydrated in graded alcohol solutions, and embedded in paraffin wax. Chondrocytes were isolated from cartilage tissue and cultured. Tissue samples were minced and digested in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) containing 0.2% collagenase (Sigma, St. Louis, MO) at 37°C for 2 h. Dissociated cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD) and 100 U/ml of penicillin-streptomycin. After overnight culture, non-adherent cells were removed, and adherent cells were further incubated in fresh medium. All experiments were conducted using first-passage cells. We confirmed that all samples synthesized type II collagen, and that OA chondrocytes synthesized type X collagen analyzed by reverse transcription polymerase chain reaction (RT-PCR) (data not shown).

RT-PCR analysis of chondrocytes. Chondrocytes were cultured in 6-well plates with various stimulants, and RNA was extracted with the QIA-shredder and the RNaseasy mini kit, according to the recommendations of the manufacturer (Qiagen, Hilden, Germany). Total RNA (1 µg) was reverse-transcribed to first-strand complementary DNA with a 1.25 µM oligo (dT) primer in 40 µl of PCR buffer II containing 2.5 mM MgCl₂, 0.5 mM dNTP mixture, 0.5 units RNase inhibitor, and 1.25 units murine leukemia virus RT (Perkin-Elmer, Foster City, CA) at 42°C for 60 min. The PCR buffer contained 1.5 mM MgCl₂, 0.2 mM dNTP mixture, 0.5 µM sense and antisense primers, 1.5 units of AmpliTaq Gold DNA polymerase, and 1 µl of RT reaction mixture in 20 µl of PCR buffer II (Perkin-Elmer). Thermal cycling conditions for p38 consisted of 40 cycles of denaturation at 95°C for 45 sec, annealing at 65°C for 45 sec, and extension at 72°C for 2 min. The primers used were p38 sense, 5'-CGAAATGACCAGCCTACGTGG-3', and antisense, 5'-CATTTCACTGTAGGTCAAGGC-3'.

Cell fractionation. Chondrocytes were washed three times with phosphate buffered saline and lyzed in MOPS buffer (25 mM Tris, 1% Nonidet P-40, 150 mM NaCl, 1.5 mM EGTA) supplemented with a protease and phosphatase inhibitor mixture (Roche Diagnostics, Basel, Switzerland) on ice for 20 min. The lysates were centrifuged at 15,000 revolutions per minute for 20 min to remove cellular debris, and the supernatants were collected, followed by the addition of 3X electrophoresis sample buffer (Bio-Rad, Hercules, CA) was added (20). Western blot analysis. The concentration of proteins was quantified by the Bradford method with the protein assay reagent (Bio-Rad). The proteins were diluted to an equal concentration with MOPS buffer. Each sample was mixed with 3X electrophoresis sample buffer and electrophoresed on a 7.5-15% polyacrylamide gradient gel (Biocraft, Tokyo, Japan) and transblotted electrically onto a blotting membrane (GE Healthcare, Buckinghamshire, UK). Phosphorylation of p38 was detected using an anti-human p38 monoclonal antibody (Cell Signaling Technology) and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (GE Healthcare), and was visualized with ECL Plus reagent (GE Healthcare) with the Chemilumino analyzer LAS-3000 mini (Fujiﬁlm, Tokyo, Japan).

Apoptosis is known to be associated downstream with the sequential activation of caspases. Apoptosis via the p53 pathway is related to the mitochondrial pathway. Apoptotic signals were conﬁrmed by detection of cleaved caspase 9. Cytochrome c released from mitochondria associates with procaspase 9/Apaf-1, and this complex processes procaspase 9 into large and small fragments by self-cleavage under apoptotic stimulation (21). The expression of cleaved caspase 9 indicates the activation of the mitochondrial apoptotic pathway. The expression of cleaved caspase 9 was detected using mouse anti-human caspase 9 mAb (Upstate Biotechnology, Temecula, CA) and conjugated sheep anti-mouse IgG antibody. Protein expression was determined by semi-quantification of digitally captured image using the public domain NIH Image program (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/nih-image/). Values were normalized to α-tubulin expression.

Culture of normal human knee chondrocytes and exposure to heat stress or mechanical stress. Normal human knee chondrocytes (Cambrex, Charles City, IA) were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C in a Bullet kit. Before performing the experiments, we confirmed that normal human knee chondrocytes expressed type II collagen and sulfated proteoglycans but not type X collagen. Cells were grown to a subconfluent state and were then plated onto 6-well plates at a density of 3x10⁵ cells per well in DMEM supplemented with 10% FBS and 100 U/ml of penicillin-streptomycin. The cultured cells underwent heat stress in an incubator (humidified atmosphere of 5% CO₂ and 95% air at 39°C for 1 h, 3 h, and 5 h). Cells were grown to a subconfluent state and were then plated onto type I collagen-coated BioFlex.
plates with a deformable silicone rubber bottom surface at a density of 3x10^5 cells per well in DMEM/F-12 supplemented with 10% FBS and 100 U/ml of penicillin-streptomycin. Cyclic stretch experiments were performed using an FX-2000 Flexercell system (Flexcell International, McKeesport, PA). Shear strain was enforced at 10% elongation for 12 h (0.25 Hz).

Pretreatment of chondrocytes with SB203580 prior to heat stress or mechanical stress induction. SB203580 (Sigma, St. Louis, MO) is a specific inhibitor of p38 MAPK (22). Chondrocytes were pre-treated with 10 µM SB203580 for 1 h in DMEM medium supplemented with 10% FBS before the induction of heat stress or mechanical stress.

Small interfering RNA (siRNA) transfection. Lipofectamine 2000 was used to transfected p38 siRNA and non-specific siRNA control into normal human knee chondrocyte monolayers, according to the recommendations of the manufacturer (Invitrogen, San Diego, CA). Briefly, 1 day before transfection, cells were plated on a 6-well plate in growth medium without antibiotics so that they would be 30-50% confluent at the time of transfection. Then, 100 pmoles of siRNA and Lipofectamine 2000 complexes were prepared and added to each well. After 6 h of transfection, the complexes were removed and fresh medium containing 10% FBS was added. After 48 h of transfection, the cells were placed onto a 6-well plate at a density of 3x10^5 cells per well, and heat stress or mechanical stress was enforced. Transfection efficiency was determined by RT-PCR and Western blotting.

TUNEL staining. Before induction of heat stress or mechanical stress, 3x10^5 chondrocytes were cultured in 6-well plates. After exposure to heat stress or mechanical stress, the chondrocytes cultured on the plates were fixed with 4% neutral buffered formalin for 10 min, and apoptotic cells were determined using a TUNEL assay kit, according to the recommendations of the manufacturer (Wako, Osaka, Japan).

Statistical analysis. Data are expressed as the mean ± SD. For normally distributed data, the Student's two-tailed t-test was used for comparisons between the 2 groups. P<0.05 were considered significant.

Results

Phosphorylation of p38 MAPK is elevated in OA cartilage. Immunohistochemistry showed that phosphorylated p38 was expressed at the superficial zone in OA cartilage. However, phosphorylated p38 MAPK was not expressed at the superficial zone in normal cartilage (Fig. 1A). Phosphorylated p38 MAPK was expressed at the hypertrophic zone in both OA and normal cartilage (Fig. 1A).

Phosphorylation of p38 MAPK is elevated in OA chondrocytes. Western blotting showed that p38 protein was expressed in OA and normal chondrocytes. However, the expression of phosphorylated p38 was significantly higher in OA chondrocytes compared to normal chondrocytes (Fig. 1B).

Heat stress or mechanical stress induced chondrocyte apoptosis. The percentage of TUNEL-positive apoptotic cells significantly increased in a time-dependent manner after exposure to a 39°C heat stress (Fig. 2A). Western blotting showed that phosphorylation of p38 increased after exposure to a 39°C heat stress for 1, 3, or 5 h (Fig. 2B). Expression of cleaved caspase 9 increased in a time-dependent manner

Figure 1. (A) Expression of phosphorylated p38 in osteoarthritis (OA) and normal cartilage analyzed by immunohistochemistry. The specimens were stained with anti-phosphorylated p38 antibody. (a,b) Sections from normal cartilage. (c,d) Sections from OA cartilage. The arrows indicate stained chondrocytes. (B) Expression of p38 and phosphorylated p38 in OA and normal chondrocytes, detected by Western blotting. Ratios were obtained using the NIH Image software. Values were normalized to α-tubulin. The bar graph shows the mean and SD of five individual samples.
after exposure to 39°C heat stress (Fig. 2B). The percentage of TUNEL-positive apoptotic cells significantly increased after exposure to 10% shear stress (Fig. 2C). Western blotting showed that phosphorylation of p38 increased after exposure to 10% shear stress for 12 h (Fig. 2D). Expression of cleaved caspase 9 also increased after exposure to 10% shear stress (Fig. 2D).

**Figure 2.** Effects of exposure of normal human knee chondrocytes to a 39°C heat stress for 1, 3, or 5 h or to 10% mechanical stress for 12 h. (A) The white bar shows the percentage of TUNEL-positive apoptotic cells after exposure to 37°C (regular culture conditions). The black bars show the percentage of TUNEL-positive apoptotic cells after exposure to a 39°C heat stress. At least 300 cells were counted by an observer blinded to the duration of heat stress. Bars show the mean and SD of four individual samples. (B) Western blotting of the expression of p38, phosphorylated p38, and cleaved caspase 9 after exposure to a 39°C heat stress. (C) Percentage of TUNEL-positive apoptotic cells after exposure to 10% mechanical stress. At least 300 cells were counted by an observer blinded to the duration of mechanical stress. Bars show the mean and SD of four individual samples. (D) Western blotting of the expression of p38, phosphorylated p38, and of cleaved caspase 9 after exposure to 10% mechanical stress.

**Figure 3.** Efficiency of p38 small interfering RNA (siRNA) transfection in normal human knee chondrocytes (NHAC-kn). (A) Significant decrease in the expression of p38 mRNA after siRNA transfection, determined by reverse transcriptase-polymerase chain reaction. Bars show the mean and SD of four individual samples. (B) Western blot showing decreased p38 protein levels. Bars show the mean and SD.

Down-regulation of endogenous p38 MAPK reduced heat stress or mechanical stress-induced chondrocyte apoptosis. RT-PCR showed that expression of p38 mRNA was significantly inhibited after 48 h of transfection with specific p38 siRNA (Fig. 3A). The expression of p38 mRNA decreased to 1.9% of the non-specific control siRNA (Fig. 3A) and that of p38 protein decreased to 16.2% of the control (Fig. 3B).
The percentage of TUNEL-positive apoptotic cells was increased by heat stress. However, the number of apoptotic cells induced by heat stress after p38 siRNA transfection (Fig. 4A) was significantly lower in comparison with that of apoptotic cells when p38 siRNA was not transfected. Western blotting confirmed that expressions of p38 and phosphorylated p38...
MAPK were inhibited with p38 siRNA transfection (Fig. 4B). Cleaved caspase 9 expression was not detected after p38 siRNA transfection (Fig. 4B). Similarly, the percentage of TUNEL-positive apoptotic cells increased by 10% shear stress. However, the number of apoptotic cells induced by shear stress was significantly inhibited when p38 siRNA was transfected in comparison with that of apoptotic cells when p38 siRNA was not transfected (Fig. 4C). Western blotting confirmed that phosphorylated p38 expression was inhibited with p38 siRNA transfection (Fig. 4D). Cleaved caspase 9 expression was also decreased with p38 siRNA transfection (Fig. 4D).

Inhibition of p38 activity reduced heat stress or mechanical stress-induced chondrocyte apoptosis. The percentage of TUNEL-positive apoptotic cells was decreased when chondrocytes were pre-treated with SB203580 for 1 h before exposure to 39°C heat stress (Fig. 5A). Western blotting showed that the expressions of phosphorylated p38 and of cleaved caspase 9 were decreased when chondrocytes were pre-treated with 10 µM SB203580 (Fig. 5B). The percentage of TUNEL-positive apoptotic cells was decreased when chondrocytes were pre-treated with SB203580 for 1 h before exposure to 10% shear stress for 12 h (Fig. 5C). Western blotting showed that phosphorylation of p38 and expression of cleaved caspase 9 decreased compared to that in untreated samples when chondrocytes were pre-treated with 10 µM SB203580 (Fig. 5D).

Discussion

Cartilage cells are mainly responsible for the anabolic-catabolic balance required for matrix maintenance and tissue function (23). Chondrocytes in OA receive physical stresses such as unphysiological high-weight loading and high temperature (24). Various biological and chemical stress factors are believed to be involved in the onset and progression of the pathogenesis of OA (25).

In patients with OA, the intra-articular temperature is possibly elevated to a higher degree due to local inflammation and aberrant frictional force induced by non-physiological mechanical loading (26). Mitrovic et al reported that hyperthermia induced damage to articular cartilage (27). Ye et al showed that heat stress induced articular chondrocyte apoptosis in a rat model (28). It was reported that physiological movement of a joint elevates the temperature in the joint cavity by 2.5°C (26). Hojo et al reported that appropriate heat stimulation positively affects cell viability and the proteoglycan metabolism of articular cartilage, whereas too much heat stimulation produces negative effects (29). However, the mechanism of signal transduction of heat stress remains unclear in articular chondrocytes.

Previous studies have shown that intermittent hydrostatic pressure induces apoptosis in vitro in a load- and a time-dependent manner and is characterized by a loss of chondrocyte viability, internucleosomal DNA fragmentation, and activation of caspases (30). Furthermore, optimal mechanical stress stimulation was shown to maintain the structure and function of articular cartilage, whereas excessive mechanical force was shown to lead to a loss of cartilage and the onset of OA (31-33). In addition, we have previously demonstrated that excessive shear stress induces chondrocyte apoptosis via the p53 pathway (34).

In this study, we have demonstrated that heat stress or mechanical stress increases the phosphorylation of p38 MAPK, and induces chondrocyte apoptosis via the caspase 9 pathway. Furthermore, we have shown that down-regulation of p38 MAPK expression by specific p38 MAPK siRNA transfection decreased chondrocyte apoptosis in response to both heat stress and mechanical stress. Our findings indicate that p38 MAPK plays a critical function in chondrocyte apoptosis induced by heat stress or mechanical stress.

Under physiological conditions, OA cartilage has a higher number of apoptotic chondrocytes compared to that of normal cartilage (4). Hashimoto et al reported that chondrocyte apoptotic cells are found in the superficial and middle zone of OA cartilage (35). In this study, we have demonstrated that phosphorylated p38 MAPK was expressed in the superficial and middle zone in OA cartilage, but not in normal cartilage. Furthermore, OA chondrocytes expressed phosphorylated p38 MAPK protein more than normal chondrocytes. These results suggest that chondrocyte apoptosis in response to stress is closely linked to activation of p38 MAPK.

We also found that phosphorylated p38 MAPK was expressed in the deep zone (hypertrophic zone) of both OA and normal cartilage. This phenomenon is explained by previous studies showing that hypertrophic chondrocytes direct the formation of the mineralized matrix, attract blood vessels, and undergo apoptosis (36-38).

Finally, we demonstrated that inhibition of p38 MAPK activity by SB203580 reduced chondrocyte apoptosis in response to heat stress. This finding supports the notion that SB203580 could be used as a drug for OA therapy.

In conclusion, we have demonstrated that heat stress or mechanical stress increases chondrocyte apoptosis via phosphorylation of p38. Stress-induced chondrocyte apoptosis was decreased due to inhibition of p38 MAPK activation when chondrocytes were pre-treated with SB203580 or transfected with p38 specific siRNA. The phosphorylation of p38 MAPK was increased in OA chondrocytes. Based on our results, down-regulation of p38 MAPK activation inhibits chondrocyte death induced by heat stress. Therefore, evaluation of the interaction between phosphorylation of p38 and chondrocyte apoptosis could be one of the keys to elucidate OA etiology.

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References