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Original Articles

Activation of stress-activated protein kinase in osteoarthritic cartilage: evidence for nitric oxide dependence

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Summary

Objective: We have demonstrated in bovine chondrocytes that nitric oxide (NO) mediates IL1 dependent apoptosis under conditions of oxidant stress. This process is accompanied by activation of c-Jun NH2-terminal kinase (JNK; also called stress-activated protein kinase). In these studies we examined activation of JNK in explant cultures of human osteoarthritic cartilage obtained at joint replacement surgery and we characterized the role of peroxynitrite to act as an upstream trigger.

Design: A novel technique to isolate chondrocyte proteins (<10% of total cartilage protein) from cartilage specimens was developed. It was used to analyse JNK activation by a western blot technique. To examine the hypothesis that chondrocyte JNK activation is a result of increased peroxynitrite, *in vitro* experiments were performed in which cultured chondrocytes were incubated with this oxidant.

Results: Activated JNK was detected in the cytoplasm of osteoarthritis (OA) affected chondrocytes but not in that of controls. *In vitro*, chondrocytes produce NO and superoxide anion. IL-1 (48 h), which induces nitric oxide synthase, resulted in an activation of JNK; this effect was reversed by N-monomethylarginine (NMA). TNFα treated chondrocytes at 48 h produce superoxide anion (EPR method). Exposure of cells to peroxynitrite led to an accumulation of intracellular oxidants, in association with JNK activation and cell death by apoptosis.

Conclusion: We suggest that JNK activation is among the IL-1 elicited responses that injure articular chondrocytes and this activation of JNK is dependent on intracellular oxidant formation (including NO peroxynitrite). In addition, the extraction technique here described is a novel method that permits the quantitation and study of proteins such as JNK involved in the signaling pathways of chondrocytes within osteoarthritic cartilage. © 2001 OsteoArthritis Research Society International

Key words: Stress-activated protein kinase, Nitric oxide, Osteoarthritic cartilage.

Introduction

Chondrocytes are capable of adaptation and remodeling in response to mechanical force that, in healthy tissue, stimulates anabolic pathways[1]. However, this response is highly sensitive to local environmental changes, often modified by altered biomechanical forces, which can result in the release of autocrine catabolic cytokines leading to a loss of proteoglycan[2]. We and others have implicated inducible nitric oxide synthase (iNOS) as an inflammatory mediator of osteoarthritis (OA)[3–5]. NO can react with other free radicals to generate molecules such as peroxynitrite leading to the accumulation of injurious intracellular oxidants and DNA damage[6–8]. It is possible that NO and peroxynitrite account for the local environmental changes which interfere with chondrocyte anabolic pathways.

We have previously reported that the MAPK extracellular signal regulated kinase 1 and 2 (ERK) acts to promote constitutive and growth factor elicited (e.g. IGF-1, FGF) proteoglycan synthesis and that iNOS expression is associated with the inhibition of (ERK)[9]. Two other MAPK pathways, p38 and the stress-activated protein kinases [SAPKs, also known as C-Jun terminal kinase (JNK)], appear to serve roles in cellular responses to stress and/or apoptosis. p38 is uniquely sensitive to activation by osmotic stress or bacterial endotoxin (LPS)[10]. Although the role of NO was not evaluated, activation of JNK led to overexpression of metalloproteinases and in some cases it promoted apoptosis[11,12]. Taken together, the regulation and balanced activation of MAPK signaling pathways in chondrocytes regulates physiological and pathophysiological cell responses.

A systematic examination of the relationship between MAPK pathway activation, iNOS expression and chondrocyte injury in OA has not been undertaken. However, because of a central role of MAPK in cell function it is tempting to speculate that chondrocyte injury in OA reflects a dysregulation of physiologic MAPK activation pathways. Accordingly, the present study was initiated to examine two MAPK pathways, ERK and JNK, in chondrocytes of patients with OA. To define a molecular basis for the MAPK

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activities, cultures of bovine chondrocytes were established to examine the role of nitric oxide and resultant peroxynitrite to alter chondrocyte MAPK activation.

Methods

CARTILAGE ORGAN CULTURE

Articular cartilage was collected from patients undergoing total knee joint replacement. Total tissue was pooled without discrimination to normal or focal disease areas. The diagnosis of OA was confirmed by pathology examination. Each patient sample was analysed separately, the patient population included three male and nine female patients, and we found no relation between measurements (JNK activation) and the sex or age of the patients or the use of particular medications. Normal human cartilage was obtained from autopsy specimens (NDRI, Philadelphia). Tissue protocols included (1) fixation in 4% paraformaldehyde, (2) immediate freezing of tissue at −80°C or preparation as uniform cartilage slices measuring 10×10 mm which were placed in Falcon six-well flat-bottomed tissue culture plates. The cartilage organ cultures were maintained in complete Ham's F-12 plus P/S, as previously described[13]. Cartilage explants were incubated in the absence and presence of NMA (1 mM) and IL-1β (5 ng/ml) After 48 h, fluid was analysed for NO release by measuring NO_2- by Griess assay and tissue was immediately frozen at −80°C.

CARTILAGE EXTRACTION TECHNIQUES

Slices from articular cartilage affected by OA were frozen at −80°C, milled to a fine particulate in liquid nitrogen using a mortar and pedestal and separated into two fractions. Extract was obtained as described by Amin *et al*.[13]. Briefly, the guanidine SDS treatment involved a treatment of cartilage with extraction buffer A (10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA); then a sequential treatment with 10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA plus 4 M guanidine HCl and 10% SDS (extraction buffer B).

Alternatively, we developed an adsorption chromatography method, which we describe for the first time in this text. Milled cartilage (0.5 g) is homogenized for 30 s at 4°C in 1.4 ml extraction buffer C (10 mM Tris, 1 mM EDTA, 5 mM vanadate, 1 mM EGTA, 25 mM NaF, 5 μg/ml aprotinin, 1 mM PMSF). An additional 0.6 ml of extraction buffer C was added, followed by a second homogenization (30 s, 4°C). The addition of 1 ml of 20 mM Tris, 0.1 M NaCl, 0.4 M LiBr, 0.1% NP40 and 1 mM EDTA leads to a new mixture (extraction buffer D). A slurry (0.2 ml) of controlled pore glass beads (Sigma, dissolved 1:1 in 50 mM acetate pH 5.5) per ml of sample in extraction buffer D and 3 ml of 50 mM acetate pH 5.5, 1 mM EGTA, 2 mM Na vanadate, 25 mM NaF, 0.1 M LiBr. This mixture is incubated at 4°C on a rotating platform. After 3 h, the beads were harvested, washed in 10 mM Tris, 1 mM EDTA (4°C). The sample was either placed in SDS sample buffer and boiled or analysed for catalase activity (see below). Aliquots of beads containing 500 units of catalase were stored at −80°C.

WESTERN BLOT ANALYSIS

Proteins were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose. Blots were incubated with anti-actin (Sigma), anti-phosphoERK (New England Biolabs), anti-total JNK, or activated JNK (both New England Biolabs) and rabbit anti-Nitrotyrosine (UBI). Blots were incubated with goat anti-rabbit antibody with peroxidase and then with detection by chemiluminescence. Protein bands were quantitated by densitometric gel scans (UMAX scanner, model UC630).

HISTOLOGY

Cartilage sections were fixed in paraformaldehyde and cryostat sections were prepared for staining. The staining protocol involved reaction with a rabbit polyclonal antibody to activated JNK (New England Biolabs). Sections were then incubated with a secondary antibody which was conjugated to alkaline phosphatase and then with a detection dye. The preparations were counterstained with eosin and phase contrast was used to detect cells positive for anti-JNK staining, which were recorded by photography.

ANALYSIS OF CARTILAGE EXTRACTS FOR CATALASE AND HYALURONIC ACID CONTENT

Catalase (samples in extraction buffer A–D) was measured by quantitating oxygen generated from the decomposition of H_2O_2 by catalase using a Clark electrode. Hyaluronic acid was analysed using a RIA by following the instructions of the manufacturer (Biomedical Technologies Inc.).

EPR MEASUREMENTS

Spin trapping measurements of oxygen radicals were performed on 107 cells per ml in phosphate-buffered saline (PBS) with 50 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO; Sigma). EPR spectra were recorded in a flat cell at room temperature with a Bruker ER 300 spectrometer as previously described[14].

DATA VARIABILITY

Data were analysed as the mean and the standard error of the mean. The levels of significance were calculated using the Student's *t*-test.

Results

Activated JNK is detected in OA cartilage but not in normal cartilage. MAPK activation was examined in 12 OA patients and two normal controls as described below. Cartilage sections were prepared from osteoarthritic human knee obtained at the time of joint replacement (Fig. 1). MAPK activation was measured by probing the tissue section of patient CD with either an antibody to either the activated form of JNK or an antibody that recognizes total (both inactive and active) JNK. As shown in panel C, activation of stress-activated protein kinase was detected in the majority of cells in osteoarthritic cartilage. All cells in the cartilage section reacted positive with an antibody that recognized total JNK; the cells failed to react with an isotype control antibody (panel A). Although it has been previously reported that exposure of chondrocytes to catabolic cytokines such as IL-1 results in an acute activation of

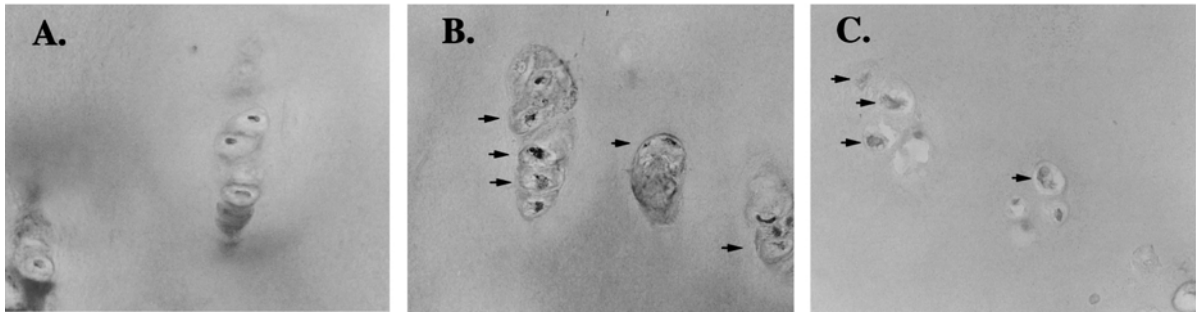

Fig. 1. Activation of stress activated protein kinase in osteoarthritic cartilage. Cartilage sections were isolated from osteoarthritic human knee obtained at the time of joint replacement (age 80, female). Cartilage chips from OA affected cartilage were fixed in paraformaldehyde and cryostat sections were prepared for staining with primary antibody (anti-activated JNK, anti-total JNK or isotype control antibody) and detection by a secondary antibody which was conjugated to alkaline phosphatase. After employing a detection dye the preparation were counterstained with eosin prior to recording by photography. Stains of OA cartilage specimen correspond to polyclonal rabbit IgG control (A), total (active and inactive) JNK (B) and activated form of JNK (C).

JNK[11], this is the first report of activation of JNK in diseased cartilage.

In order to further characterize this finding and compare osteoarthritic with normal cartilage more quantitatively, we developed a novel extraction technique employing adsorption chromatography to isolate chondrocyte proteins. A common property of many proteins from a wide spectrum of sources is a capacity to reversibly adsorb to glass surfaces[15]. We adapted this technique to cartilage as we sought: (1) to retrieve cellular proteins in a functionally active state and (2) to exclude matrix components (collagen, proteoglycan, hyaluronic acid). Osteoarthritic cartilage was obtained and divided into two equal fractions. The first was extracted using a SDS-guanidine extraction and the second was treated with the adsorption technique. In both isolates we evaluated the recovery of catalase (chondrocyte protein) and hyaluronic acid (matrix component). The adsorption technique completely eliminated the contamination of the isolate by hyaluronic acid or type II collagen (Fig. 2). In addition, the isolate was enriched in intracellular proteins, JNK (described below), rhoA, and catalase. The latter was recovered in a functionally active form and we obtained a protein fraction that was enriched three-fold in the specific activity of chondrocyte catalase. In contrast, the protein isolate using the SDS-guanidine extraction failed to increase the specific activity of catalase or to eliminate hyaluronic acid.

When the adsorption technique was used to extract cellular proteins from cartilage chondrocytes, we observed that activated JNK was detected in OA patients, but not in normal human cartilage (Fig. 3). The blot was sequentially probed with an antibody to activated JNK and then total JNK. In addition, the blot was probed for actin as a protein loading control. Consistent with the immunostaining, the OA extract contained activated JNK; two isoforms were detected (JNK-2 and JNK-1, Mr 55 kDa and 46 kDa, respectively; lane 3). In a different patient activated JNK was also detected (lane 4). Moreover, activated JNK was detected using the immunoblot technique in all OA patients (N=12, not shown). A common feature is that the intensity of activated JNK Mr 55 kDa (JNK 2) band was three-fold stronger than the 46 kDa (JNK 1) band. The recovery of actin and total JNK (phosphorylated+non-phosphorylated JNK, not shown) was similar between normal and OA cartilage specimens.

Endogenous NO is linked to the expression of the activated form of JNK in human osteoarthritic cartilage. Osteoarthritic cartilage explants were pre-incubated for 48 h in the presence or absence of IL-1β (5 ng/ml) and L-N-monomethylarginine (NMA) (1.0 mM). Spontaneous NO release was 1.5 μM/g wet weight, consistent with the previous report that human OA-affected cartilage spontaneously produces NO[13]. IL-1 increased NO production to 8.3 μM, which was reduced to 2.9 μM in the presence of L-NMA. Chondrocyte protein was isolated and

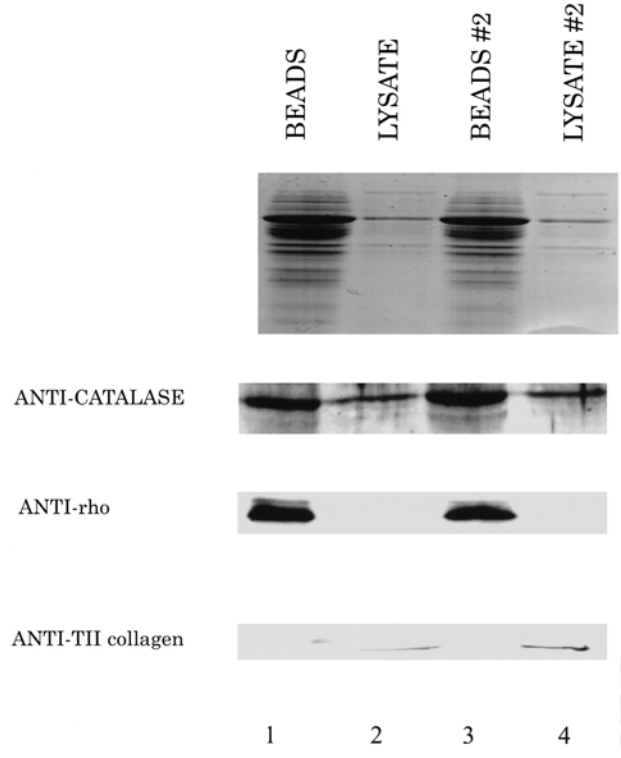

Fig. 2. Characterization of OA cartilage extract (from two separate donors) after use of the adsorption extraction technique to isolate chondrocyte protein. Extract of chondrocyte proteins was analysed by Western blot for catalase, rho A andTII collagen. Human OA cartilage chips from two separate donors were homogenized (lysate) and purified by adsorption chromatography (beads). After separation by SDS gels, the gel was stained by coomaasie (upper panel). A duplicate set was transferred to nitrocellulose and probed for catalase (upper western blot), the ras related G protein, rho A (middle panel) or Type II collagen (lower panel). Note that the protein extract (beads) are enriched in chondrocyte proteins (catalase, rho A) but not matrix (type II collagen).

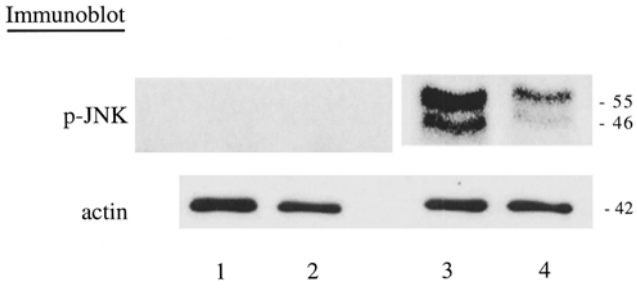

Fig. 3. Detection of activated JNK in OA cartilage but not normal cartilage. Chondrocyte protein was extracted using controlled pore glass beads of two OA patients and two healthy controls. The blot was probed with an antibody to actin or activated JNK. Note that activated JNK was detected in both patients (lane 3,4). In contrast, active JNK was absent in healthy control. The intensity of activated JNK Mr 55 kDa band was three-fold stronger than the 46 kDa band.

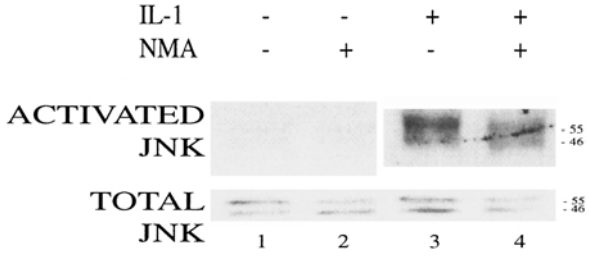

Fig. 4. Endogenous NO is linked to the expression of the activated form of JNK in human OA cartilage. Human OA cartilage chips were exposed to IL-1 in the absence and presence of NMA (48 h, 37°C). Protein fraction obtained using adsorption chromatography were separated by SDS gels and transferred to nitrocellulose. Blots were sequentially probed with anti-activated JNK, anti-total JNK (active and inactive) (N=3).

analysed for JNK activation in osteoarthritic cartilage specimens. IL-1 increased JNK activation above baseline by 5.3 fold (Fig. 4, assessed by phosphoimager; control=1 phosphoimager unit); IL-1 augmentation was reduced by L-NMA treatment (5.3 vs 2 phosphoimager units).

Exposure of chondrocytes to TNFα leads to release of peroxynitrite and superoxide anion. A focus on 'upstream' events that act to trigger JNK activation was next evaluated in studies that measured the capacity of chondrocytes to produce peroxynitrite and superoxide anion. In these studies, we examined TNFα, which, like IL-1, is a known activator of JNK[8,11]. Cells were pre-incubated in the presence or absence of TNFα (10 ng/ml, 48 h) and cells were stained with antinitrotyrosine, a 'footprint' of peroxynitrite, or were analysed for superoxide anion release by an EPR method. The expression of nitrotyrosine was present in TNFα treated chondrocytes but not controls (not shown). Because nitrated protein forms when cells undergo oxidant stress, a focus was placed on oxidant release, which was measured for 5 min in the presence of DMPO by an EPR method. We observed that TNFα treated chondrocytes, but not control cells, released oxidants as indicated by an EPR signal with a typical intensity ratio of 1:2:2:1 and hyperfine coupling of 15G (DMPO-OH, Fig. 5).

Exposure of chondrocytes to peroxynitrite results in activation of JNK (and not ERK) in association with apoptosis (Fig. 6). To test the hypothesis that JNK activation is secondary to peroxynitrite generated by increased expression of iNOS, JNK activation was measured in the

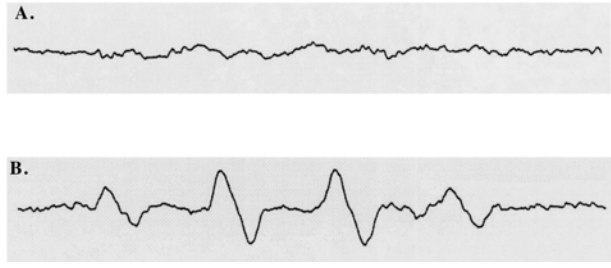

Fig. 5. Exposure of chondrocytes to TNFα led to release of superoxide anion. Chondrocytes were cultured as passage one cells with or without TNFα (10 ng/ml; 48 h). Cells were then removed from culture and the release of superoxide anion was determined by the EPR method described in Methods. Note the characteristic spectrum for DMPOH, which is present in TNFα treated cells (lower spectrum) and not control (upper spectrum). (Experiment was performed twice in duplicate.)

presence or absence of peroxynitrite. We found that exposure of chondrocytes to peroxynitrite (10 μM; 30 min) resulted in the activation of JNK and not ERK. We characterized the nuclear morphology of control and peroxynitrite-challenged cells. Cells adhered to glass coverslips were pre-incubated as control and peroxynitrite treated cells (10 μM peroxynitrite, 30 min). After treatment cells were stained with Hoescht 33258. We observed that peroxynitrite treatment induced apoptosis (nuclear fragmentation); in addition the nuclei in this group were markedly condensed. The apoptosis response was increased by co-addition of the polyADP ribose inhibitor, 3 aminobenzimide (58% vs 30%).

Discussion

Our results indicate that activated C-Jun terminal kinase (JNK) was detected in the cytoplasm of OA chondrocytes, but not in healthy controls. Several findings indicated a role of NO derived from iNOS to act as a signal to trigger JNK. (1) Treatment with cytokines, which induce nitric oxide synthase, results in further activation of JNK; this effect is reversed by N-monomethylarginine (NMA). (2) Exposure of chondrocytes to cytokines led to the accumulation of intracellular oxidants including peroxynitrite and superoxide anion. (3) Peroxynitrite treatment resulted in the activation of JNK but not ERK (Fig. 7). This redirection of MAPK signaling pathways is associated with an apoptosis-inducing activity.

Intracellular reactive oxygen species (ROS) have been recognized to act as signaling intermediates for cytokines, including IL-1 and TNFα and to activate JNK/SAPK[16,17]. A possible link among nitric oxide, MAPK and chondrocyte dysfunction was indicated by the finding that experimental rodent adjuvant-induced arthritis (AIA) is attenuated by co-infusion of either oxygen radical scavengers[18] or the NOS inhibitor, L-NMA[19]. Previous *in vitro* studies have been limited to monolayer chondrocyte studies because of the difficulty involved in isolation and characterization of chondrocyte proteins in cartilage specimens. In this study, we report a novel technique to isolate chondrocyte proteins from osteoarthritic cartilage obtained at surgery from patients undergoing joint replacement. MAPK signaling was assessed by Western blot of extracted chondrocyte proteins using specific antibodies that report activated and total SAPK/JNK. For example, SAPK/JNK (Thr183/Tyr185)

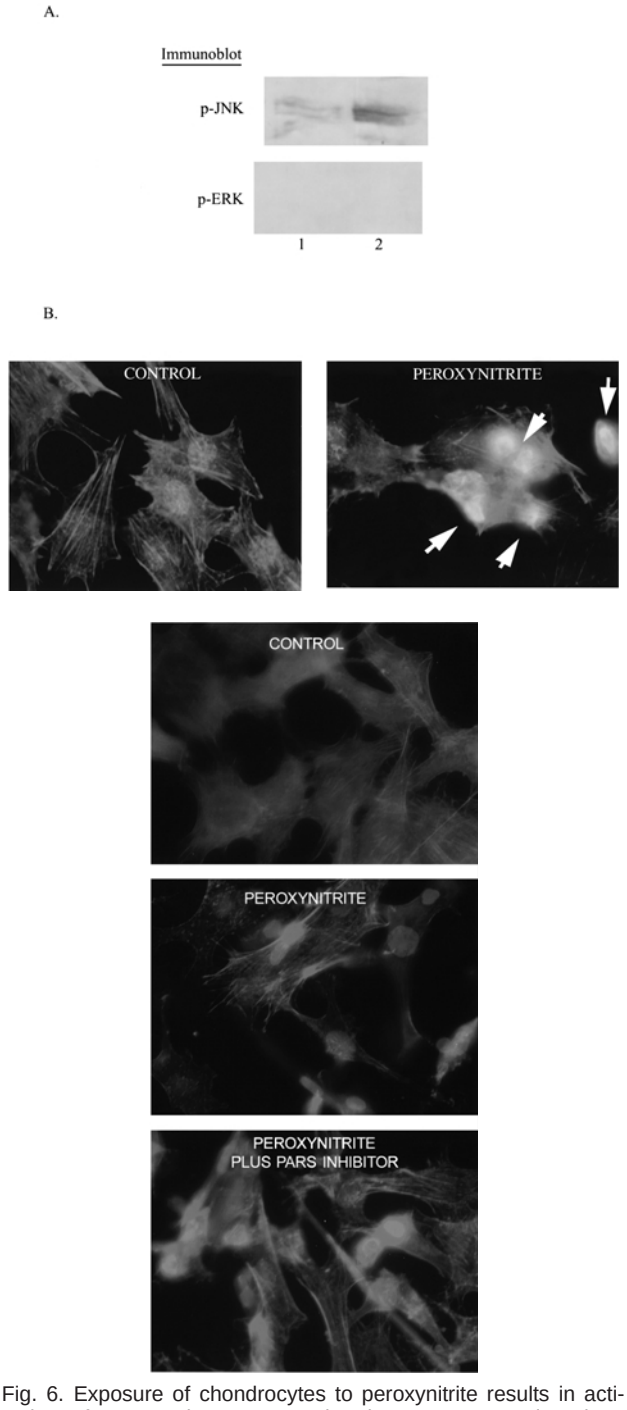

Fig. 6. Exposure of chondrocytes to peroxynitrite results in activation of JNK and not ERK. Chondrocytes were cultured as passage one cells with or without peroxynitrite (10 μM, 30 min). Panel A. Cells were lysed. The sequential stains using the Western blot technique determined the levels of phosphoJNK and phosphoERK (Representative data from *N*=3). Panel B. Cells were stained with Hoescht 33258 and rhodamine phalloidin. The peroxynitrite treated chondrocytes were apoptotic as evidenced by nuclear fragmentation. In contrast, control exhibit symmetrically round nuclei. Note in panel B (middle and lower pictures) that condensed nuclei in the peroxynitrite group and in the peroxynitrite+PolyADP ribose polymerase inhibitor were 30% and 58% of the total cells respectively (average determined at three separate fields).

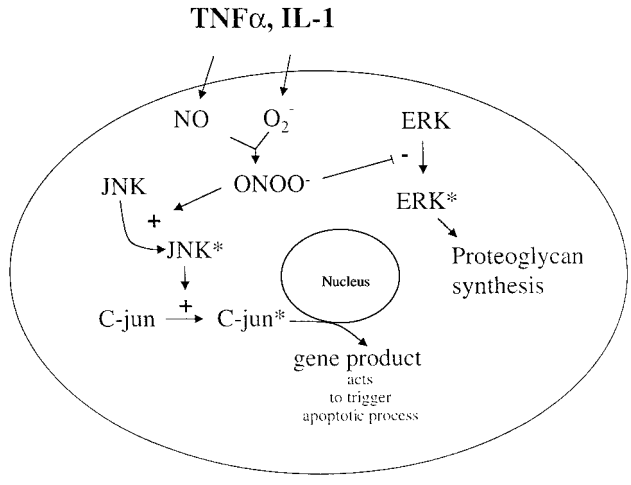

Fig. 7. A model hypothesis explains the role of peroxynitrite to contribute to the catabolic phenotype of OA chondrocytes. Exposure of chondrocytes to TNFα or IL-1 leads to an inhibition of ERK and an activation of JNK secondary to peroxynitrite formation.

antibody detects SAPK/JNK only when dually phosphorylated at Thr183/Tyr185[20]. As this dual phosphorylation is essential for kinase activity, phosphorylation at this site is a marker of SAPK/JNK activation. Using this extraction technique, we were able to demonstrate activated MAPK signaling of chondrocytes within osteoarthritic cartilage.

These data suggest a cascade of events that is initiated by the expression of inducible nitric oxide synthase (iNOS). The induction of iNOS in OA chondrocytes results in the inhibition MAPK extracellular signal-regulated kinases 1 and 2 (ERK), which regulate constitutive and growth factor elicited (e.g. IGF-1, FGF) proteoglycan synthesis[9]. In contrast, increased NO due to iNOS up-regulation in concert with increased oxidant production results in peroxynitrite generation which we show activates the JNK pathway. Since Frisch and Ruoslahti suggested that in normal cells the apoptosis-inducing activity of JNK is counteracted by extracellular signal-regulated kinases (ERK)[21], it is possible that the altered JNK/ERK balance secondary to peroxynitrite underlie the death pathway via apoptosis.

In summary, increased NO and oxidant production in chondrocytes, as is observed in osteoarthritic cartilage, results in the potential activation of the JNK over the ERK MAP kinase pathway. We suggest that this imbalance of normal MAP kinase pathway activation contributes to chondrocyte injury in OA.

Acknowledgments

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