Phase 2 enzyme inducer sulforaphane blocks matrix metalloproteinase production in articular chondrocytes

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Objectives. In addition to its chemopreventive activity, phase 2 enzyme inducers have been recently found to have anti-inflammatory activity. In this study, we examined the influence of sulphoraphane (SPN), one of the most potent inducers of the phase II enzymes on the production of MMPs by pro-inflammatory cytokines in human articular chondrocytes.

Methods. Articular cartilages were obtained from knee OA patients and were cultured in monolayers and explants. Induction of a phase II enzyme, NAD(P)H:quinone oxidoreductase 1 (NQO1), in chondrocytes was assayed after incubation with various concentrations of SPN. Chondrocytes were stimulated with IL-1 or TNF-α with or without pre-incubation with SPN. The expression and activation of MMP-1, -3 and -13 was evaluated by an ELISA, gel zymography and RT–PCR. MAP kinases [p38, extracellular signal-regulated protein kinase (ERK) and C-Jun N terminal kinase (JNK)] and NF-κB activation were evaluated by western blotting and by an electrophoretic mobility shift assay, respectively.

Results. SPN significantly induced NQO1 activity in chondrocytes and the induction was maximal at 24 h. SPN inhibited the production of MMP-1, -3 and -13 protein and mRNA induced by either IL-1 or TNF-α in a dose-dependent manner. This inhibition of MMP by SPN was accompanied by the inhibition of NF-κB and JNK activation.

Conclusions. SPN was found to inhibit MMP production in pro-inflammatory cytokine-stimulated chondrocytes. Delineation of the biochemical mechanism regulating cartilage catabolism by SPN may identify safe and effective therapeutic targets for the inhibition of cartilage degradation.

Key words: Chondrocyte, Phase 2 enzymes, Sulphoraphane, MMP, IL-1, TNF-α.

Introduction

Degradation of the extracellular matrix (ECM) in articular cartilage is a central event leading to joint destruction in many arthritic conditions, including OA and RA. Among the important mediators of matrix degradation are the MMPs, a family of enzymes that are active at physiological pH and temperature. In humans, MMPs can be subdivided into six classes, consisting of the collagensases (MMP-1, -8 and -13), the gelatinases (MMP-2 and -9), the stromelysins (MMP-3, -10 and -11), the matrilysins (MMP-7 and -26), the membrane-type MMPs (MMP-14, -15, -16, -17, -24 and -25), and a diverse subgroup [1]. MMP-1 and -13 specifically degrade native type II collagen, and are synthesized in increased amounts by OA chondrocytes; thus, they are postulated to have an important role in the destruction of cartilage [2]. On the other hand, proteoglycan loss involving MMP-3 results in a reduction of cartilage stiffness [3]. Chondrocytes respond to a variety of stimuli such as pro-inflammatory cytokines, mechanical loading, fibronectin fragments and toll-like receptor ligands by producing MMPs [4–6]. As current treatments for OA only act on symptoms and do not prevent or cure OA, investigators have tried to find effective agents that inhibit the degeneration of articular cartilage. Modulation of MMP synthesis or activity has been one of the major targets in this endeavour. The highly conserved structure of the MMP catalytic domain has made it difficult to maintain target specificity, however, and direct inhibition of MMPs usually results in considerable side-effects [1]. As OA is a slowly progressing, indolent disease, minimal toxicity is a prerequisite for any disease-modifying anti-OA drug (DMOAD) that is proposed.

Phase II enzymes including NAD(P)H:quinone oxidoreductase 1 (NQO1), haem oxygenase (HO)-1 and γ-glutamylcysteine ligase (GCLCR) have been traditionally recognized as those enzymes converting carcinogens to inactive metabolites, thus conferring protection from carcinogenesis [7]. Epidemiological data linking the dietary intake of cruciferous vegetables and reduction of the risk of different types of malignancies are attributed to the phase 2 enzyme-inducing constituents, the isothiocyanates (ITCs) [8]. Sulphoraphane [SPN; 1-isothiocyanato-4-(methylsulphinyl)-butane], a naturally occurring ITC obtained through the consumption of broccoli, is one of the most potent inducers of the phase 2 enzymes implicated in carcinogen detoxification [9]. In addition to its chemopreventive activity, phase 2 enzyme inducers have been recently found to have anti-inflammatory activity, such as the ability to inhibit the inducible nitric oxide synthetase (iNOS) and cyclo-oxygenase (COX)-2 in IFN-γ-stimulated mouse macrophages [10]. The purpose of this study was to determine whether SPN influences the production of MMPs by human OA chondrocytes stimulated with pro-inflammatory cytokines. In addition, we sought to determine the effect of SPN on the activation of MAP kinases and NF-κB in chondrocytes.

Materials and methods

Reagents

SPN was purchased from LKT Laboratories (St Paul, MN, USA). DNA fragmentation ELISA kit was purchased from Roche Diagnostics (Mannheim, Germany). Anti-phospho-extracellular signal-regulated protein kinase (ERK) 1/2, anti-phospho-p38, anti-phospho-SAPK/JNK N terminal kinase (JNK), anti-ERK 1/2 and anti-p38 were purchased from Cell Signaling Technology (Beverly, MA, USA); anti-JNK1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human recombinant IL-1, TNF-α and ELISA assay kits for MMP-1, -3 and -13 were obtained from R & D Systems (Minneapolis, MN, USA). All other reagents were obtained from Sigma (St Louis, MO, USA) unless otherwise specified.
Chondrocyte monolayer culture

Cartilage samples were obtained from the femoral condyle and tibial plateau of the knee from OA patients at the time of joint replacement surgery. Informed consent for cartilage collection was obtained from each patient and the whole study was approved by the Institutional Review Board of Hallym University Sacred Heart Hospital (Anyang, Korea). Full-thickness cartilage slices were obtained from above the subchondral bone from a relatively lesion-free area. For monolayer cultures, slices were minced and incubated sequentially with pronase and collagenase in Dulbecco’s modified Eagle’s medium (DMEM) until the fragments were digested. Released cells were seeded at a density of 5x10^4 cells/plate in 10 cm culture plates in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 1% Fungizone (Gibco, Grand Island, NY, USA) and penicillin/streptomycin (150 U/ml and 50 mg/ml each). After about 7 days, confluent chondrocytes were split once and seeded at high density and these first passage chondrocytes were used within 2 days in subsequent experiments. For the explant culture, full-thickness slices were obtained from the femoral condyle. Each slice was cut further and a piece of ~2 mm width × 5 mm length × full thickness was weighed and cultured in 200 μl/well in a 48-well culture plate in the same medium as described above for the monolayer culture. Chondrocytes were incubated with DMEM containing 0.5% fetal calf serum prior to treatment with SPN and pro-inflammatory cytokines. For the blocking experiments, SPN was dissolved in distilled water just before the initiation of an experiment and were added to the chondrocytes at various concentrations 1 and 24 h before stimulation with the pro-inflammatory cytokines for monolayer and explant culture, respectively.

Quantification and verification of cell death

The amount of cell death was quantitated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay, as described [11]. Briefly, chondrocytes were seeded at 5 x 10^3 cells/well in 96-well microtiter plates, and incubated with various concentrations of SPN for 24 h. MTT was added to each well to a final concentration of 0.125 mg/ml, and the plate incubated at 37 °C for 3 h, after which, the formazan product was solubilized with 100 μl of dimethylsulfoxide (DMSO) and the optical density was read at a wavelength of 595 nm. The percentage of cell survival was calculated by taking the optical density of cells given a particular treatment, dividing this by the percentage of cell survival was calculated by taking the optical density was read at a wavelength of 595 nm. The expression of cell death was calculated by comparing the absorbance at 570 nm (background absorbance) and absorbance at 630 nm (formazan product). The formazan product of chondrocytes treated with 1 mM sodium nitroprusside (SNP) was set at 100.

NQO1 activity assay

The enzyme activity of NQO1 in cell lysates was determined using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. RNA was treated with RNase-free DNase I (Ambion, Austin, TX, USA) to remove contaminating DNA. Six hundred nanograms of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (In Vitrogen, Burlington, ON, Canada) as detailed in the manufacturer’s guidelines. Real-time RT-PCR analysis of NQO1, -3 and -13 was performed in a total volume of 20 μl containing one-tenth of the reverse transcriptase reaction, 100 nM of sense and anti-sense primers, and 2 μl of Lightcycler FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany). The following sense and anti-sense primers for each molecule were used: for MMP-1, 5'-GGC-CTT-GGC-AAA-TCT-GGC-GTG-TAA-3' (sense) and 5'-GCT-CTT-GGC-AAA-TCT-GGC-GTG-TTA-3' (anti-sense); for MMP-3, 5'-GGC-TGG-TAT-GGC-CAT-TAT-AGG-TTG-3' (sense) and 5'-AAA-CCT-AGG-GGC-CAT-TAT-AGG-TTG-3' (anti-sense); for MMP-13, 5'-AAAG-GAC-CTT-GGC-CAT-TAT-AGG-TTG-3' (sense) and 5'-AAA-CCT-AGG-GGC-CAT-TAT-AGG-TTG-3' (anti-sense); and for GAPDH, 5'-GGC-CTT-GGC-AAA-TCT-GGC-GTG-TTA-3' (sense) and 5'-AAA-CCT-AGG-GGC-CAT-TAT-AGG-TTG-3' (anti-sense). After incubation at 95 °C for 10 min, the mixtures were subjected to 45 amplification cycles (10 s at 95 °C for denaturation, 5 s at 62 °C for annealing, 10 s at 72 °C for extension). Incorporation of SYBR Green dye into PCR products was monitored in real-time using a LightCycler 2.0 instrument (Roche) allowing determination of the threshold cycle (CT) at which exponential amplification of PCR products begins. Relative mRNA amount was calculated by 1/2^(-ΔΔCT) of a specific gene/1^2-ΔCT of GAPDH. Specificity of PCR products was determined by melting curve analysis, agarose gel electrophoresis and sequencing of the PCR products. Each PCR reaction was performed only the expected specific amplicon as shown by the melting temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed.
in duplicate on two separate occasions for three independent cartilage donors.

Western blot

First passage chondrocytes were seeded (500 000/well) in 6-well culture plates and serum starved before treatment with SPN, IL-1 or TNF-α. Cellular protein was extracted in lysis buffer containing 50 mM sodium acetate, pH 5.8, 10% v/v SDS, 1 mM Na2EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin, 1 mM sodium fluoride and 1 mM sodium orthovanadate at 4°C and protein concentrations were measured using the BCA method with BSA as a standard. Proteins were separated on a 12% SDS–PAGE, and were transferred to a polyvinylidene fluoride membrane. Blots were blocked with Tris buffered saline (TBS) containing 5% non-fat milk at room temperature for 1 h and were incubated with the respective antibodies overnight at 4°C. The blots were then incubated with 1:5000 peroxidase-conjugated secondary antibody for 1 h. Bound immunoglobulin was detected by using an enhanced chemiluminescence kit (Amersham, Amersham, UK).

Electrophoretic mobility shift assay

Nuclear extracts from chondrocytes were prepared from 2×10⁶ cells as described previously with minor modifications [13]. Protein content was measured and 5 μg aliquots of extracts were used for the binding reaction. A consensus double-stranded NF-κB probe was obtained from Promega (Madison, WI, USA) and was end-labelled by using γ-[³²P]-adenosine-5-triphosphate. Nuclear extracts were incubated in gel binding buffer (Promega) in a volume of 9 μl. Later, the end-labelled probe was added (100 000 cpm/sample). Samples were then incubated for 20 min and were loaded onto a 4% non-denaturing polyacrylamide gel. Electrophoresis was performed for 3 h in a cold room. The protein complexes were identified by autoradiography.

Statistical analysis

Data are expressed as the mean ± s.d. Differences between treatment groups were tested by using the Mann–Whitney U-test (GraphPad Prism, version 3, GraphPad Software, San Diego, CA, USA). Significance was established at the 95% CI (P < 0.05).

Results

Induction of chondrocyte death by SPN

As the anti-cancer effect of SPN is largely explained by its pro-apoptotic effect [8], we first treated chondrocytes with various concentrations of SPN to define its cytotoxicity in chondrocytes. As shown in Fig. 1A, SPN was not cytotoxic at up to 20 μM, while at 50 μM, it significantly induced chondrocyte death. To further confirm that SPN does not induce apoptosis which is not detectable by MTT assay, we performed DNA fragmentation ELISA. SPN did not significantly induce chondrocyte apoptosis at up to 10 μM, while at 20 μM, a small amount of apoptosis was detected (Fig. 1B). At 50 μM, SPN significantly increased apoptosis in chondrocytes. Therefore, we used SPN concentrations between 1 and 20 μM in subsequent experiments.

Induction of NQO1 activity by SPN

Next, we tested whether treatment of human articular chondrocytes with SPN leads to the up-regulation of the phase 2 enzyme, NQO1. activity. As shown in Fig. 2, treatment of chondrocytes with SPN led to significant increase of NQO1 activity and the increase of NQO1 activity was concentration dependent.

Inhibition of MMPs induced by pro-inflammatory cytokines with SPN

We determined whether SPN inhibits MMP proteins induced by pro-inflammatory cytokines in articular chondrocytes. One hour pre-treatment of monolayer chondrocytes with SPN led to significant inhibition of all three MMPs measured (MMP-1, -3 and -13) induced by either IL-1 or TNF-α (Fig. 3A, B). The inhibition was
concentration dependent and invariably significant at 10\(\mu\)M. We then performed a time-course analysis to test whether the suppression of MMPs by SPN was effective even after the addition of pro-inflammatory cytokines. As shown in Fig. 4, SPN effectively inhibited MMP expression even when it was added 4 h after the addition of each cytokine. Induction of MMPs in cartilage explants after 3-day incubation with IL-1 or TNF-\(\alpha\) was also suppressed by 24 h pre-treatment with SPN (Fig. 3C, D).

The inhibition of MMP activation induced by IL-1 or TNF-\(\alpha\) was examined by gelatin and casein gel zymography (Fig. 5). The 72 kDa band in gelatin zymography which represents pro-MMP-2 was suppressed from 5\(\mu\)M SPN in both IL-1- and TNF-\(\alpha\)-treated chondrocytes, while the 55 kDa band that represents either activated MMP-2 or pro-MMP 13 was suppressed at 10\(\mu\)M. The 45 kDa band which represents activated MMP-13 was significantly inhibited at 10\(\mu\)M. Casein zymography revealed 2 bands at 55 kDa which represents pro-MMP-1, -3, -10 or -12, and 45 kDa which represents activated MMP-1, -3 or -10. Both bands were suppressed invariably by 10\(\mu\)M SPN in both IL-1- and TNF-\(\alpha\)-treated chondrocytes.

In order to determine whether SPN suppresses MMP mRNA transcription, real-time RT-PCR was performed (Fig. 6). Although the effective concentration for inhibition differed among each MMP analysed and the stimuli used, SPN mostly inhibited the up-regulation of MMP-1, -3 and -13 mRNA by IL-1 or TNF-\(\alpha\) from 5\(\mu\)M.

**Suppression of MAP kinases and NF-\(\kappa\)B by SPN**

As the activation of MAP kinases has been reported in OA cartilage, and signalling downstream leading to NF-\(\kappa\)B activation could contribute to cartilage degradation through the up-regulation of MMP expression [14], we wished to define the regulation of MAP kinases and NF-\(\kappa\)B in SPN-treated articular chondrocytes. As was reported previously, chondrocytes treated with IL-1 or TNF-\(\alpha\) led to phosphorylation of all three MAP kinases, p38, ERK and JNK and the translocation of NF-\(\kappa\)B (Fig. 7). Pre-treatment with SPN reduced activation of JNK while it did not have any effect on the activation of p38 or ERK induced by either IL-1 or TNF-\(\alpha\). NF-\(\kappa\)B activation induced by IL-1 or TNF-\(\alpha\) was inhibited by SPN pre-treatment.

**Discussion**

There is a continuing challenge to find the inhibitory mechanism of the catabolic signalling of articular chondrocytes in pursuit of the disease modification in various arthritides including OA and RA. Findings from this study suggest that the phase 2 enzyme inducer SPN potently inhibits MMP production and activation induced by two prototypical inflammatory cytokines, IL-1 and TNF-\(\alpha\).

SPN has been extensively investigated for its effect on protection against carcinogenesis. Although significant overlap exists in the mechanisms underlying malignancy and inflammation, a paucity of data exists on the anti-inflammatory effect of SPN. In a previous report studying the effects of SPN on human breast cancer cells, 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cancer cell invasion was significantly inhibited along with MMP-9 activity [15]. In a subsequent report, however, SPN was found to inhibit angiogenesis in human umbilical vein endothelial cells without affecting the production of the active or proenzyme form of MMP-2 or -9 [16]. In our study, SPN inhibited MMP-1, -3 and -13 induced by either IL-1 or TNF-\(\alpha\) in human OA chondrocytes.
to a similar extent. In addition, gelatin zymography revealed that SPN inhibits MMP-2 activation induced by pro-inflammatory cytokines. As we did not assay the inhibitory effect of SPN on other MMPs, such as MMP-9 and -14, we cannot rule out whether this reflects a non-specific general anti-inflammatory effect of SPN. However, the MMPs tested are the ones most relevant in the degradation of articular cartilage [17]; thus, we believe that our findings do have clinical implications.

Regarding the pro-inflammatory signalling pathway affected by SPN, effects on NF-κB have been the most extensively reported. SPN was shown to down-regulate the lipopolysaccharide-mediated induction of iNOS and cyclo-oxygenase (COX)-2 expression, two important mediators of inflammation in arthritis, in raw 264.7 macrophages via impairment of NF-κB activation [18]. In another report using a human malignant glioblastoma cell line, SPN significantly decreased NF-κB 65-kDa protein expression along with an increase of IkBα [19]. As NF-κB is a key mediator in the induction of MMPs in chondrocytes, we observed the influence of SPN on NF-κB activation. As expected, SPN down-regulated IL-1 or TNF-α-mediated NF-κB activation in chondrocytes. Contrary to the pro-survival role of NF-κB, however, this inhibition of NF-κB at a SPN concentration of 10 μM in the presence of cytokines did not lead to decreased survival in chondrocytes (data not shown). Whereas suppression of MMPs was observed with a concentration from 2 μM, cytotoxicity was apparent from a concentration as high as 50 μM. This concentration is relatively high as compared with the cytotoxic concentration reported in cancer cells (10–40 μM). Whether SPN induces apoptosis in synovial fibroblasts at a lower concentration than that required in chondrocytes is an interesting subject, and is currently being explored by our laboratory. SPN can interfere with multiple cell signalling pathways and some SPN molecular targets are involved in overlapping pathways forming molecular cross-talk [20]. One of the most important signaling pathway components induced by SPN is nuclear factor-like 2 (Nrf2). Nrf2 is sequestered in the cytoplasm by the Kelch-like ECH-associated protein 1 (Keap1), and SPN potently induces
FIG. 6. Inhibition of MMP mRNA up-regulation induced with IL-1 or TNF-α by SPN. (A) SPN was added to the chondrocytes 1 h before stimulation with IL-1 or TNF-α. Total RNA was isolated 6 h after cytokine stimulation and real-time RT–PCR was performed using MMP-1, -3 and -13, and GAPDH primer sets. Incorporation of SYBR Green dye into PCR products was monitored in real-time using a LightCycler 2.0 instrument allowing determination of the CT at which exponential amplification of PCR products begins. Relative mRNA amount was calculated by $1/2^{\text{CT}}$ of a specific gene/$1/2^{\text{CT}}$ of GAPDH. Messenger RNA expression level of IL-1 or TNF-α alone was set at 1. *$P<0.05$ compared with IL-1 or TNF-α treatment alone. (B) PCR products were separated on an agarose gel and stained with ethidium bromide. MMP-1, -3 and -13 bands are shown in the upper panel and GAPDH in the lower panel. The data represent duplicate samples from four different donors.

FIG. 7. The regulation of MAP kinases (A) and NF-κB (B) by SPN in chondrocytes. Monolayer-cultured human articular chondrocytes were treated with 10 μM SPN 1 h before the addition of IL-1 or TNF-α. (A) After 15 min, proteins were extracted from the chondrocytes and 20 μg of each protein sample was separated by 12% SDS–PAGE. Phosphorylation of ERK1/2, p38 and SAPK/JNK was analysed by a western blot. Blots were stripped and were re-probed with non-phospho-specific antibodies. The data represent five samples from different donors. (B) Nuclear proteins were extracted 30 min after treatment with IL-1 or TNF-α. Activation of NF-κB was analysed from nuclear proteins by an electrophoretic mobility shift assay. The arrow denotes activated NF-κB bands. The data represent three samples from different donors.
were postulated to represent a safe alternative to COX-2 shear-activated cyclo-oxygenase 2 protein, prostaglandin E2. The phase 2 enzyme inducer D3T was shown to down-regulate reports support the possibility of a benefit. In chondrocytes, rheumatic disease have not yet been fully elucidated, but recent short-term activation of p38 or ERK induced by pro-inflammatory cytokines, while it suppresses JNK activation. Therapeutic implications of a phase 2 enzyme inducer on rheumatic disease have not yet been fully elucidated, but recent reports support the possibility of a benefit. In chondrocytes, the phase 2 enzyme inducer D3T was shown to down-regulate shear-activated cyclo-oxygenase 2 protein, prostaglandin E2 release and apoptosis. Based on this result, phase 2 inducers were postulated to represent a safe alternative to COX-2 inhibitors. In a study of MRL/lpr mice, a murine model of SLE, conjugated linoleic acid was found to down-regulate oxidative stress and anti-DS DNA production via phase 2 enzyme induction. Phase 2 enzymes include haem oxygenase-1, an inducible haem-degrading enzyme with anti-inflammatory properties that suppress pro-inflammatory cytokine production in rheumatoid synovial fibroblasts. All these reports including our results suggest the novel therapeutic role of a phase 2 enzyme inducer on the pathogenesis of arthritis and autoimmune diseases.

The concentration of SPN used in our experiment was 10 μM, but this is higher than the reported steady-state plasma level for chemoprevention after oral dosing in mice, which is around 200 nM. Animal experiments using systemic and intra-articular administration are currently under way in our laboratory to test the feasibility of SPN treatment for cartilage protection.

In conclusion, SPN was found to inhibit MMP production and activation in pro-inflammatory cytokine-stimulated chondrocytes. Delineation of the biochemical mechanisms of cartilage catabolism regulated by SPN may identify potentially safe and effective therapeutic targets for inhibition of cartilage degradation in arthritic diseases.

Rheumatology key messages
- SPN suppresses MMP production from articular chondrocytes stimulated with either IL-1 or TNF-α.
- SPN inhibits NF-κB and JNK activation in pro-inflammatory cytokine-stimulated articular chondrocytes.

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References