Osteoarthritis and Cartilage (2009) **17,** 91–99

doi:10.1016/j.joca.2008.05.010

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Inhibition of cyclooxygenase 2 expression by diallyl sulfide on joint inflammation induced by urate crystal and IL-1β

H.-S. Lee M.D., Ph.D.†*, C.-H. Lee M.D.‡, H.-C. Tsai M.S.† and D. M. Salter M.D.§

† *Department of Pathology, Tri-Service General Hospital and National Defense Medical Center, Taipei, Taiwan, ROC*

‡ *Department of Orthopaedic Surgery, Tri-Service General Hospital and National Defense Medical Center, Taipei, Taiwan, ROC*

§ *Osteoarticular Group, Centre for Inflammation Research, Edinburgh University, Little France, Edinburgh, UK*

Summary

Objective: Investigation of the effects of diallyl sulfide (DAS), a garlic sulfur compound, on joint tissue inflammatory responses induced by monosodium urate (MSU) crystals and interleukin-1β (IL-1β).

Design: The HIG-82 synovial cell line was used to establish the experimental model and DAS regime. Primary cultures of articular chondrocytes and synovial fibroblasts obtained from patients undergoing joint replacement for osteoarthritis were used in experimental studies. Cyclooxygenase (COX) expression following MSU crystal and IL-1β stimulation with/without DAS co-incubation was assessed by reverse transcription-polymerase chain reaction (RT-PCR), western blotting, and immunocytochemistry and nuclear factor-kappa B (NF-κB) activation determined by electrophoretic mobility shift assay. Prostaglandin E2 (PGE_2) production was measured by enzyme-linked immunosorbent assay (ELISA). DAS effects on *COX* gene expression in an MSU crystal-induced acute arthritis in rats were assessed by RT-PCR.

Results: MSU crystals upregulated COX-2 expression in HIG-82 cells and this was inhibited by co-incubation with DAS. DAS inhibited MSU crystal and IL-1β induced elevation of COX-2 expression in primary synovial cells and chondrocytes. Production of PGE_2 induced by crystals was suppressed by DAS and celecoxib. MSU crystals had no effect on expression of COX-1 in synovial cells. NF-κB was activated by MSU crystals and this was blocked by DAS. Increased expression of COX-2 in synovium following intraarticular injection of MSU crystals in a rat model was inhibited by co-administration of DAS.

Conclusions: DAS prevents IL-1β and MSU crystal induced COX-2 upregulation in synovial cells and chondrocytes and ameliorates crystal induced synovitis potentially through a mechanism involving NF-κB. Anti-inflammatory actions of DAS may be of value in treatment of joint inflammation.

Key words: Cyclooxygenase 2, Diallyl sulfide, Chondrocyte, Synovium, Urate crystal, IL-1β.

Introduction

The cyclooxygenase (COX) pathway, through the constitutively expressed COX-1 and the inducible enzyme COX-2, leads to the generation of prostaglandins. COX-2 is absent in most tissues under normal resting conditions but is upregulated in inflamed tissues and is responsible for elevated prostaglandin E2 (PGE_2) production. Agents that suppress COX-2 activity have been promoted as potential drug targets to suppress the inflammatory conditions involving most tissues[1]. COX-2 over expression in articular tissues is a characteristic feature of both inflammatory joint disease, including crystal-induced arthritis, and osteoarthritis (OA)[2]. COX-2 is induced in human joint tissues including chondrocytes and synoviocytes by a variety of inflammatory stimuli including interleukin-1β (IL-1β), IL-17, and tumor necrosis factor (TNF)[3–11]. These cytokines appear to play a major role in regulation of COX-2 expression in joint disease and subsequent production of PGE_2 resulting in cartilage degradation, inflammation, and angiogenesis[2,7,12–15].

COX-2 selective inhibitors are of benefit in the management of arthritis but evidence of associated increased cardiovascular risk has limited their general use. Recently there has been increased interest in possible roles for natural derived compounds that may have anti-inflammatory effects in treatment of diverse conditions, although the specific mechanisms of action of many of these compounds remain unclear. Garlic has been shown to have beneficial effects against several diseases. It exhibits antithrombotic[16–18], anti-oxidative[19–21] and anti-carcinogenic activities[22,23]. Its bioactive components are being identified and tested for the specific treatment of diseases[24]. Diallyl sulfide (DAS), a flavor compound derived from garlic oil, is one such agent. Following oxidation at the sulfur atom, the allylic carbon, and the terminal double bonds of DAS are converted to diallyl sulfoxide (DASO) and diallyl sulfone ($DASO_2$) by cytochrome enzymes[25]. These compounds have been demonstrated to inhibit chemically induced hepatotoxicity and carcinogenesis in animal models[26].

*Address for correspondence and reprint requests to: Dr H. S. Lee, Department of Pathology, Tri-Service General Hospital and National Defense Medical Center, 325, Section 2, Cheng-Kung Road, Neihu 114, Taipei, Taiwan, ROC. Tel: 886-2-66069319; Fax: 886-2-66000309; E-mail: herngsheng131419@gmail.com
Received 26 November 2007; revision accepted 12 May 2008.

DAS may also have anti-infectious properties[27]. Although garlic has been suggested as having potential benefit in age-related conditions such as OA[28], a role for the pure garlic extract DAS on joint inflammation has not been extensively explored.

This work was carried out to investigate the potential anti-inflammatory effect of DAS on joint inflammation induced by monosodium urate (MSU) crystals and IL-1β. Both *in vitro* and *in vivo* models were tested. We identified that COX-2 upregulation, induced by urate crystals and IL-1β in chondrocytes and synoviocytes was inhibited by DAS. Urate crystal induced production of PGE_2 by chondrocytes and synoviocytes was also inhibited by DAS and the positive control celecoxib. The mechanism appears to involve inhibition of nuclear factor-kappa B (NF-κB) activation. Our data suggest that DAS may have potential for a drug target in the control of inflammation in arthritic disease.

Materials and methods

MATERIALS

DAS, a liquid product, was purchased from Fluka Chemie (Switzerland), corn oil from Sigma-Aldrich, Inc. (St. Louis, MO, USA). DAS stock solution at a concentration of 1 M was prepared in corn oil (64 μl of DAS in 436 μl of corn oil). Preparation of MSU crystals has been described previously[29]. Briefly, 0.4 g sodium hydroxide pellets (Sigma) were dissolved in 400 ml distilled water; 1.68 g uric acid (Sigma) was then added and the preparation left overnight at room temperature. The following day crystals were harvested by decanting of the supernatant solution and washing three times in cold sterile phosphate buffered saline (PBS). Crystals were left to dry at room temperature for 3 days before re-suspension in PBS at a concentration of 24 mg/ml and sterilizing in an autoclave.

CELL CULTURE

Synovial cell line

The rabbit synovial cell line HIG-82 (ATCC CRL-1832) was maintained in Nutrient Mixture F-12 HAM medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 I.U./ml penicillin (Gibco, Carlsbad, CA, USA) and 100 μg/ml streptomycin (Gibco).

Human chondrocytes and synovial cells

Human cartilage and synovial tissue samples were obtained from knee joints at total knee replacement of OA patients with consent ($n = 35$, mean age, 68.7 years; range 55–83 year). Residual OA cartilage with predominantly grade II and III lesions (Collins/McElligot system) from each joint was removed and pooled[30,31]. Chondrocytes were extracted by sequential enzymatic digestion at 37°C in 95% air/5% CO_2 with 0.25% trypsin (Gibco) for 30 min and 3 mg/ml blend collagenase type H (Sigma). Extracted cells were re-suspended in 10 ml Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 HAM medium (Sigma) supplemented with 10% FBS (Sigma), 100 I.U./ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) and seeded in complete medium at a density of 5×10^5 cell/ml in 55 mm petri dishes, and cultured in a humidified 5% CO_2 atmosphere at 37°C. First-passage of OA chondrocytes was used in all experiments. A minimum of three donors was used for each experiment.

For primary human synovial fibroblasts culture, OA synovial tissues were minced, incubated in anti-microbial solution for 1 h at room temperature, and stirred with 3 mg/ml of blend collagenase type H (Sigma) in serum-free Nutrient Mixture F-12 HAM medium (Sigma) for 6 h, filtered through nylon mesh, and washed extensively. Synovial fibroblasts were maintained in Nutrient Mixture F-12 HAM medium supplemented with 10% FBS (Sigma), 100 I.U./ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). Cells between passages 2 and 4 were used.

TETRAZOLIUM ASSAY

DAS cytotoxicity was assessed by a tetrazolium methylthiotetrazole (MTT) assay. 1×10^4 HIG-82 synovial cells were seeded on 96-well microtiter plates in 0.2 ml growth medium described above. DAS (10, 20, 25, 30, 40, 50 mM) was added to cultured wells and incubated for 0, 2, 4, 6,12, 24 and 48 h with triplication of each condition. Fifty microliters of tetrazolium was added to each well and incubated for 3 h at 37°C. After aspiration of medium and tetrazolium, 25 μl of Sorenson's glycine buffer (0.1 mol/l glycine, 0.1 mol/l NaCl, pH 10.5) and 200 μl of dimethyl sulfoxide (DMSO) were added to each well. The plate was shaken for 5 min. The optical density at 540 nm was determined on a microplate reader (μQuant, BIO-TEK Instruments, Inc.) with analyzing software of KC junior, version 1.5 (BIO-TEK Instruments, Inc.)

EXPERIMENTAL PROTOCOL

Petri dishes of OA chondrocytes and synovial cells were placed in serum-free media overnight, then incubated with either 2 mg/ml of MSU crystals, 10 ng/ml of IL-1β (R&D Systems Inc., Minneapolis, MN, USA), or 20 mM of DAS for treatment at 37°C for 6 h.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using TriZOL Reagent (Invitrogen Cor., Carlsbad, CA, USA). For first strand cDNA synthesis, 3 μg of total RNA was used in a single-round RT reaction, containing 0.75 μg $Oligo(dT)_{14}$ primer, 1 mM deoxyribonucleotide triphosphate (dNTP), 1× first strand buffer, 4 mM dithiothreitol (DTT), 40 units RNaseOut recombinant ribonuclease inhibitor, and 200 units of superscript II reverse transcriptase (Invitrogen). Polymerase chain reaction (PCR) was run using 0.9 μl of the RT reaction mixture as template, 0.4 mM of gene specific primers, 1× PCR buffer, 0.25 mM dNTPs, and 1.5 units of KlenTaq DNA polymerase (Ab Peptides Inc., St. Louis, MO, USA). The amplification was carried out at 94°C for 2 min, then for 30 cycles at 94°C for 45 s, 56°C for 1 min, and 72°C for 45 s followed by a final extension at 72°C for 10 min. All PCR products were size-fractionated by a 1.5% agarose gel electrophoresis, and DNA bands were visualized by staining the gel with 0.1 μg/ml ethidium bromide. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) simultaneously served as an internal positive control and allowed normalization of the various mRNA levels against the total mRNA content in the samples. The bands were then scanned (Vilber Lourmat, France) and analyzed using gel documentation system (Bio-Profil, Bio-1D version 99, Viogene, USA), according to the manufacturer's instructions. The values were expressed as ratio of the *COX-2* gene to the control GAPDH. The primers used are summarized in Table I.

Protein extraction and western blotting

Following stimulation, cells were immediately washed with ice-cold PBS containing 100 μM Na_3VO_4 (Sigma) and lysed *in situ* with ice-cold lysis buffer at 4°C for 15 min. Lysis buffer contained 1% Igepal (Sigma), 100 μM Na_3VO_4, and protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Whole cell lysates were collected after centrifugation at 13,000 rpm for 15 min. Protein concentration was determined by the Lowry method. Equal amounts of protein (20 μg) were loaded onto 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Immobilon-P, Sigma). Membranes were blocked overnight at 4°C with 2% bovine serum albumin (BSA) in tris-buffered saline tween-20 (TBST) (12.5 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20). After washing three times with TBST, blots were incubated for 1 h at room temperature with anti-COX-2 antibody (NeoMarkers, Fremont, CA, USA) diluted in TBST. After washing six times with TBST, the blots were then incubated with horseradish peroxidase (HRP) labeled secondary antibody for 1 h at room temperature. Membranes were rewashed extensively and the binding was detected using Enhanced Chemiluminescense plus western blotting detection system (Amersham Biosciences, UK), according to the manufacturer's instructions. The bands were also scanned and analyzed by densitometry (Bio-Profil, Bio-1D version 99, Viogene, USA), according to the manufacturer's instructions and values expressed as ratio of COX-2 protein to the control tubulin.

Immunocytochemistry

Cells were cultured at a concentration of 5×10^4/ml as a monolayer for 1–2 weeks in 55 mm tissue culture petri dishes (Nunc). The cells were washed with 10 ml tris-buffered saline (TBS) and then fixed using 2 ml of a 1:1 methanol/acetone mixture per dish for 5 min at −20°C. Immunodetection for COX-2 was performed with a standard avidin–biotin–peroxidase complex detection kit (DakoCytomation, Glostrup, Denmark). Fixed cells were rinsed with PBS for three times. Endogenous peroxidase activity and non-specific background were blocked by incubation with 3% hydrogen peroxide and non-immune goat serum, respectively. The cells were then incubated sequentially with primary antibody (1:200) for 60 min, biotinylated secondary antibody for 30 min, and peroxidase-conjugated streptavidin for 30 min before washing with 5 ml TBS three times for 5 min. In the negative controls, the primary antibody was omitted. The chromogen 3-amino-9-ethylcarbazole (AEC) was used to localize positive staining by light microscopy.

Table I
Oligonucleotides primers for semi-quantitative RT-PCR analysis

Gene		Size (bp)	Accession no.
Human			
COX-1	5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ 5′-GTGCATCAACACAGGCGCCTCTTC-3′	303	NM_080591
COX-2	5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ 5′-AGATCATCTCTGCCTGAGTATCTT-3′	304	NM_000963
GAPDH	5′-GAAGGTGAAGGTCGGAGTCA-3′ 5′-GAAGATGGTGATGGGATTTC-3′	225	NM_002046
Rabbit			
COX-2	5′-TCAGCCACGCAGCAAATCCT-3′ 5′-GTCATCTGGATGTCAGCACG-3′	278	NM_001082388
GAPDH	5′-TCACCATCTTCCAGGAGCGA-3′ 5′-CACAATGCCGAAGTGGTCGT-3′	293	DQ403051
Rat			
COX-2	5′-GTCTCTCATCTGCAATAATGTG-3′ 5′-ATCTGTGTGGGTACAAATTTG-3′	801	S67722
GAPDH	5′-CCC ATC ACC ATC TTC CAG GAG-3′ 5′-GTTGTCATGGATGACCTTGGCC-3′	284	X02231

For each primer pair, the top sequence is forward and the bottom is reverse.

Measurement of PGE_2 production in culture medium

Cells were seeded at a density of 5×10^4 cells/dish in culture medium containing 10% FBS. Cells were then cultured with 2 mg/ml of MSU crystals in the presence or absence of DAS and celecoxib for 6 h. The culture supernatant was harvested, and PGE_2 concentration was measured by enzyme-linked immunosorbent assay (ELISA) (R&D) according to the manufacturer's instructions. Celecoxib (Celebrex 200 mg) was obtained from Pfizer Co. (New York, USA) and dissolved in DMSO (Sigma).

Activation of NF-κB by electrophoretic mobility shift assay (EMSA)

Cells were incubated with 2 mg/ml of MSU crystals only or combined treatment with DAS at 37°C for 0, 30 min, and 2 h. Nuclear and cytoplasmic extracts were prepared using nuclear extract-protein extraction reagent (NE-PER) nuclear and cytoplasmic extraction reagents according to the manufacturer's protocols (Pierce, Rockford, IL, USA). A non-radioactive EMSA was performed using an EMSA kit according to the manufacturer's instructions

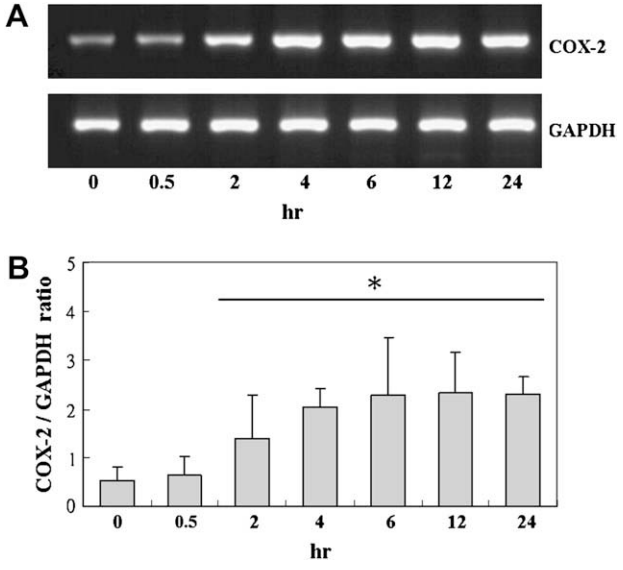

Fig. 1. Upregulation of COX-2 induced by MSU crystal stimulation in HIG-82 cells. HIG-82 cells were incubated with 2 mg/ml MSU crystals for 0, 0.5, 2, 4, 6, 12, and 24 h following which total RNA was isolated and RT-PCR undertaken. *COX-2* gene expression was significantly upregulated following MSU crystal stimulation from 2 h, reaching a maximum at 6 h, with continued expression at high levels to 24 h. GAPDH served as an internal control. (A) *COX-2* and *GAPDH* gene expression. (B) Results presented as semi-quantitative data. COX-2/GAPDH ratio is significantly elevated after 2 h stimulation ($^*P < 0.05$). The results shown in (A) are from a single experiment and are representative of a total of six separate experiments. B shows the data from six experiments with the ratios given as a mean ± SD.

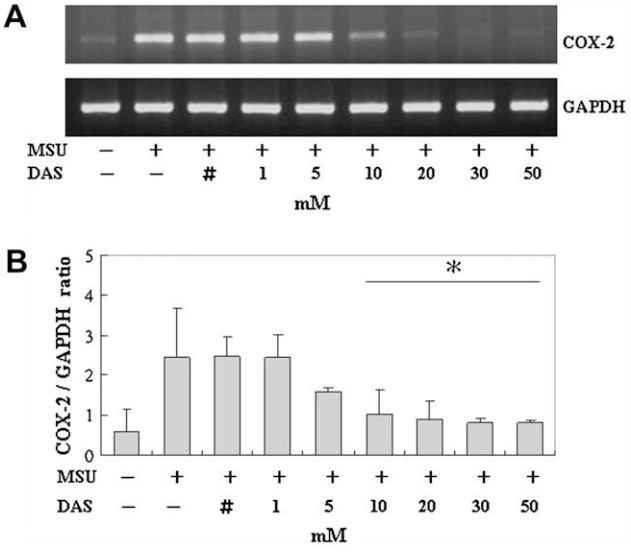

Fig. 2. DAS inhibits MSU crystal induced upregulation of *COX-2* gene expression in HIG-82 cells. HIG-82 cells were incubated with 2 mg/ml MSU crystals in the presence of DAS over a concentration range of 0, 1, 5, 10, 20, 30, and 50 mM for 6 h following which total RNA was isolated and RT-PCR undertaken. (A) *COX-2* and *GAPDH* gene expression. (B) Results presented as semi-quantitative data. COX-2 upregulation following MSU crystal stimulation was significantly inhibited by DAS at a concentration of greater than 10 mM ($^*P < 0.05$). The solvent corn oil alone (#) did not alter the effect of MSU crystals. The results shown in (A) are from a single experiment and are representative of a total of six separate experiments. B shows the data from six experiments with the ratios given as a mean ± SD.

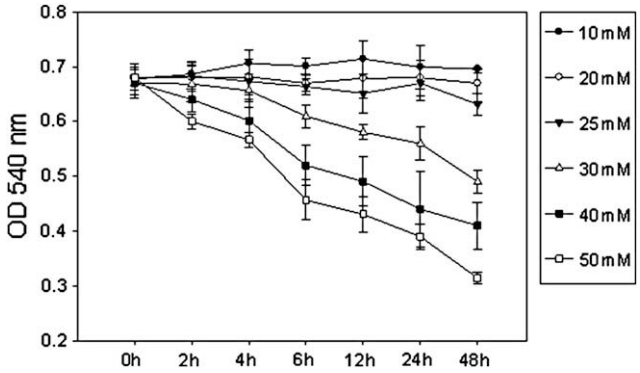

Fig. 3. Effects of DAS on cell viability. The effects of DAS on HIG-82 cell viability were assessed using an MTT assay. DAS at a concentration of 10, 20, and 25 mM, unlike concentrations of 30 mM and over showed no significant effect on cell viability with incubation up to 48 h.

(Panomics, Inc., Redwood City, CA, USA). Six micrograms of nuclear protein were used to bind biotinylated oligonucleotides containing the NF-κB-binding site for 30 min at room temperature. The samples were separated in a non-denaturing polyacrylamide gel (6%, with 2.5% glycerol) and blotted on a Biodyne B (0.45 μm) positively charged nylon membrane (Pall Schweiz AG, Basel, Switzerland). The biotin was labeled with alkaline phosphatase-conjugated streptavidin and alkaline phosphatase was detected with Enhanced Chemiluminescense detection system (Santa Cruz, CA, USA).

MSU-induced inflammation in rat knee joints

Male Sprague–Dawley rats weighing 300–400 g were obtained from the National Applied Research Laboratories and National Laboratory Animal Center (Taiwan). Experiments were approved by the local Institutional Review Board and were performed in adherence to the National Institutes of Health Guidelines for the treatment of experimental animals. Direct knee joint cavity injection with 2 mg/ml of MSU crystals (volume 50 μl) was performed according to previous reports[32] with the contralateral knee joint, injected with normal saline being used as a control. The treatment group was injected with DAS (48.9 mg/kg) and MSU crystals into one knee joint and MSU crystals only into the contralateral knee joint[33]. Sixteen hours after injection animals were sacrificed and fresh synovial tissues were collected for RT-PCR studies. Knee joint tissues ($n = 3$ for each group) were also fixed in formalin, processed into paraffin wax, sections cut and subjected to routine hematoxylin and eosin (H&E) staining.

Statistical analysis

All values were expressed as mean ± standard deviation (SD). Statistical evaluation of the semi-quantification data of COX-2 mRNA expression levels was performed by Student's *t* tests. For the animal study ($n = 4$ for each group), Wilcoxon signed-rank test was used. The results were considered significant at a value of $P < 0.05$.

Results

MSU CRYSTALS UPREGULATE COX-2 EXPRESSION IN THE HIG-82 SYNOVIAL CELL LINE

The rabbit synovial cell line HIG-82 cells were co-cultured with MSU crystals at a concentration of 2 mg/ml for up to 24 h. *COX-2* gene expression, as assessed by RT-PCR,

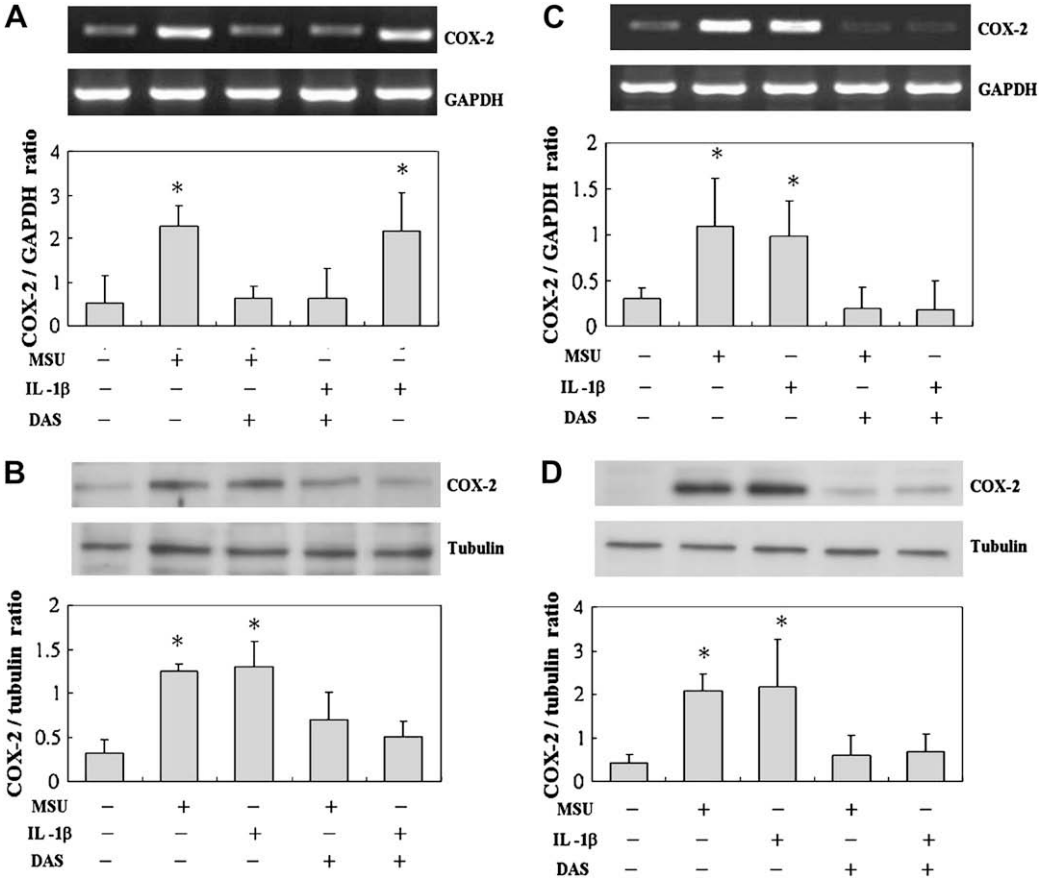

Fig. 4. DAS effects on 2 mg/ml MSU crystal and 10 ng/ml IL-1β induced upregulation of *COX-2* gene ($n = 6$) and protein ($n = 3$) expression in human OA synovial cells (A and B) and articular chondrocytes (C and D). *COX-2* gene expression was significantly increased in both synovial cells and chondrocytes by 2 mg/ml MSU crystals and 10 ng/ml IL-1β (*$P < 0.05$). This effect of MSU crystals was inhibited by 20 mM DAS.

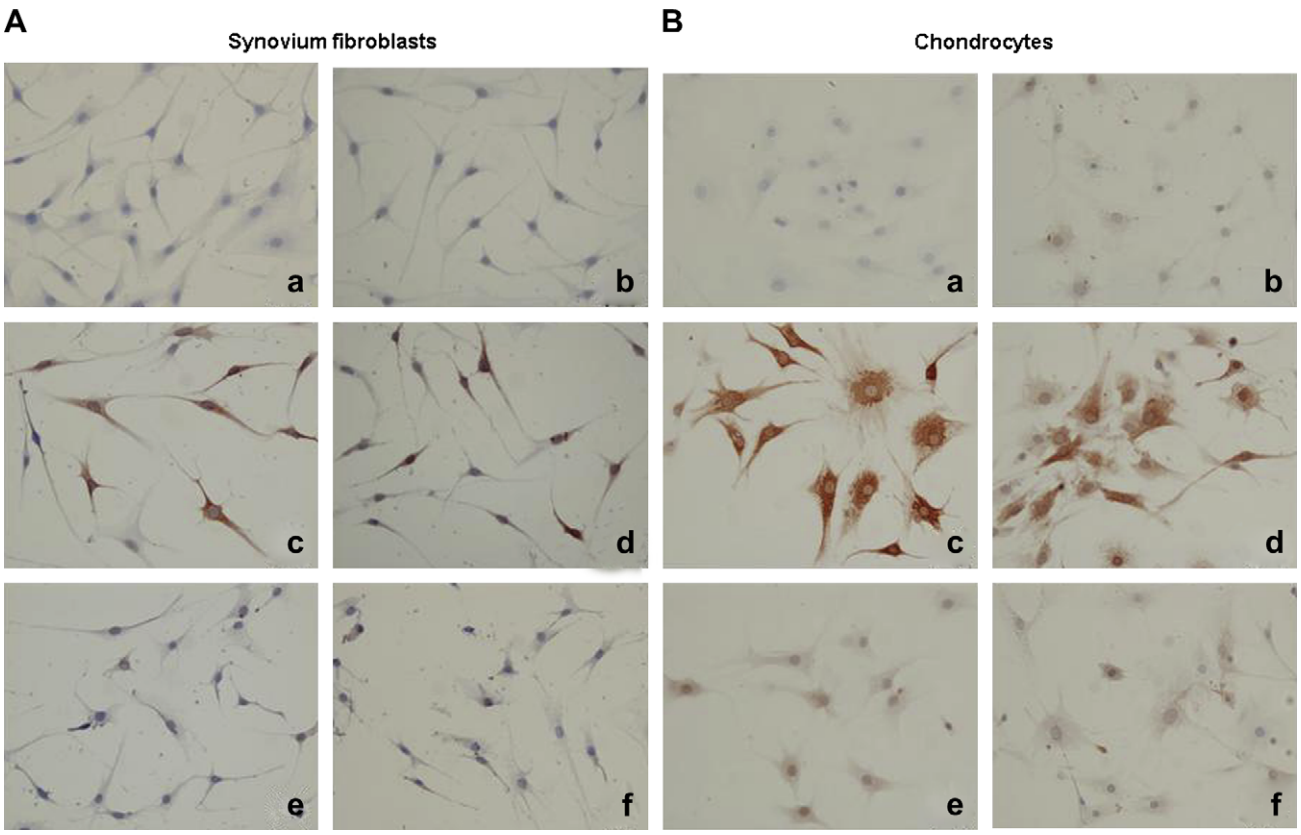

Fig. 5. DAS effects on 2 mg/ml MSU crystal and 10 ng/ml IL-1β induced upregulation of COX-2 expression in human OA synovial cells (A) and articular chondrocytes (B) ($n = 3$). (a) Negative control: secondary antibody only. (b) No stimulation: anti-COX-2 antibody. (c) 10 ng/ml IL-1β: anti-COX-2 antibody. (d) 2 mg/ml MSU crystals stimulation: anti-COX-2 antibody. (e) 10 ng/ml IL-1β plus DAS: anti-COX-2 antibody. (f) 2 mg/ml MSU crystals plus DAS: anti-COX-2 antibody. COX-2 induction by MSU crystal and IL-1β is suppressed by DAS co-incubation.

was increased significantly after 2 h exposure to MSU crystal stimulation, reaching a plateau by 6 h, with elevated levels being seen up to 24 h. Semi-quantitative analysis of *COX-2* gene expression after 6 h stimulation showed a 4.19 ± 1.21-fold increase (six repeats, $P < 0.05$) (Fig. 1).

DAS BLOCKS MSU CRYSTAL-INDUCED UPREGULATION OF COX-2 IN THE HIG-82 SYNOVIAL CELL LINE

HIG-82 cells were co-incubated both MSU crystals (2 mg/ml) and DAS (0, 1, 5, 10, 20, 30, and 50 mM) or the solvent corn oil. By RT-PCR analysis, COX-2 upregulation-induced by MSU crystals was significantly inhibited by DAS in a dose dependent manner from 10 mM, approaching basal levels at 20 mM ($P < 0.05$) (Fig. 2). The solvent corn oil alone did not influence the effects of MSU crystals on *COX-2* gene expression. DAS alone (20 mM) did not affect *COX-2* gene expression (data not shown). MTT assays showed no effect on cell viability of DAS at a concentration of 10, 20, or 25 mM (Fig. 3), however, at higher concentrations the MTT assay suggested an adverse influence of DAS on cell activity/viability and a 20 mM concentration was therefore chosen for further experiments.

COX-1 AND COX-2 REGULATION BY MSU CRYSTALS IN HUMAN OA SYNOVIAL CELLS

Cultures of human OA synovial cells were incubated with MSU crystals, 2 mg/ml, for up to 24 h to characterize the effects on *COX-1* and *COX-2* gene regulation. RT-PCR showed COX-2 upregulation, whereas COX-1 showed no discernible change over the time course of the experiment. COX-2 induction in human OA synovial cells was very similar to rabbit HIG-82 synovial cells (data not shown). By semi-quantitative analysis a 5.24 ± 1.16-fold increase in *COX-2* gene expression was identifiable at 6 h compared to basal levels ($n = 6$) ($P < 0.05$).

DAS BLOCKS MSU CRYSTAL AND IL-1β-INDUCED UPREGULATION OF *COX-2* GENE AND PROTEIN IN OA SYNOVIAL CELLS AND CHONDROCYTES

We next studied whether DAS influenced both MSU crystal and IL-1β effects on COX-2 expression in OA chondrocytes and synovial cells. Treatment of OA synovial cells with 2 mg/ml of MSU crystals or 10 ng/ml of IL-1β for 6 h resulted, to a similar degree, in a significant increase in *COX-2* gene and protein expression. DAS alone (20 mM) did not significantly affect *COX-2* gene expression at basal levels (data not shown). The upregulation of *COX-2* gene and protein expression was suppressed by DAS treatment [Fig. 4(A) and (B)]. Chondrocytes from OA cartilage were similarly treated; analysis showing that MSU crystals increased *COX-2* gene expression by 3.65 ± 0.95-fold and IL-1β had similar activity increasing *COX-2* gene expression by 3.31 ± 1.04-fold. The increase in gene and protein expression of COX-2 was inhibited by DAS treatment [Fig. 4(C) and (D)]. Immunocytochemical analysis of COX-2 expression in OA synovial cells and chondrocytes

supported the findings of western blotting, showing increased COX-2 immunoreactivity following stimulation of cells by MSU crystals that was blocked by the presence of DAS [Fig. 5(A) and (B)].

To assess whether increased expression of COX-2 was associated with changes in prostaglandin production levels of PGE_2 released into the media of MSU crystal treated OA synovial cells and chondrocytes were measured. The results demonstrated that treatment of both OA synovial cells and chondrocytes by MSU crystals significantly increased PGE_2 release into the media. This effect was suppressed by the addition of DAS and celecoxib [Fig. 6(A) & (B)].

NF-κB ACTIVATION BY MSU CRYSTALS IN OA SYNOVIAL CELLS AND CHONDROCYTES

As IL-1β is known to upregulate COX-2 expression by an NF-κB dependent pathway in both synoviocytes and chondrocytes, experiments were undertaken to establish whether NF-κB was similarly involved in MSU crystal upregulation of COX expression. Following incubation with 2 mg/ml MSU crystals for 30 min and 2 h NF-κB activation, in both synovial cells and chondrocytes, was identified using a gel mobility-shift assay. This activation of NF-κB in both OA synovial cells and chondrocytes following stimulation by MSU crystals was significantly inhibited by the presence of DAS [Fig. 7(A) and (B)].

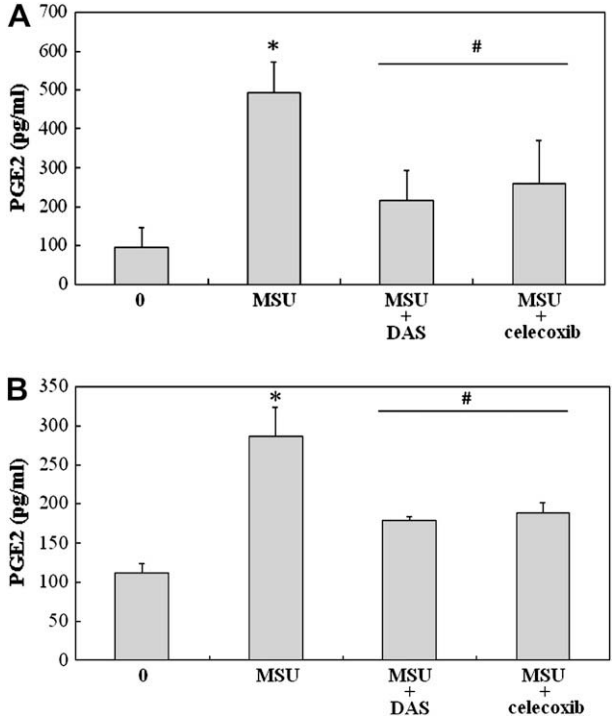

Fig. 6. DAS effects on MSU crystal-induced PGE_2 production. The supernatants from OA human synovial cells (A) and articular chondrocytes (B) following stimulation with MSU crystals, crystals plus DAS, and crystals plus celecoxib for 6 h were collected. PGE_2 levels in the supernatants were assessed by ELISA ($n = 3$). MSU crystals significantly induced PGE_2 secretion ($^*P < 0.05$). PGE_2 production-induced by MSU crystal was blocked by DAS and celecoxib ($\#P < 0.05$).

DAS INHIBITS MSU-INDUCED COX-2 UPREGULATION *IN VIVO* AND AMELIORATE SYNOVIAL INFLAMMATION

Sixteen hours following injection of MSU crystals into rat knee joints ($n = 4$) there was a significant increase in *COX-2* gene expression in synovial tissue when compared to contralateral joints injected with saline (Fig. 8, group 1). In a further group of rats, MSU crystals were injected into knee joints with or without DAS and at 16 h *COX-2* gene expression in synovial tissue measured. The results (Fig. 8, group 2), demonstrate that DAS significantly inhibited the increase of *COX-2* gene expression induced by MSU crystals. Histologically, the acute synovitis induced by MSU crystals was characterized by massive influx of polymorphoneclear leukocytes and tissue necrosis. These changes were markedly suppressed by DAS injection [Fig. 8(C)].

Discussion

The present study has demonstrated that *COX-2* gene and protein expression and PGE_2 secretion are increased in primary human OA chondrocytes and synovial cells following stimulation by MSU crystals and IL-1β. *COX-2* gene expression is elevated in synovial tissue in a rat model of MSU crystal induced arthritis. The increased expression of COX-2 is significantly inhibited by DAS both *in vitro* and *in vivo*, possibly through the prevention of NF-κB activation.

Extracts of garlic have for sometime been known to have anti-carcinogenic properties. Recently DAS a flavor compound derived from garlic that is sequentially converted to DASO and $DASO_2$ by cytochrome P_{450} 2E1 has been shown to have anti-carcinogenetic effects[26,34,35], believed to be secondary to modulation of COX-2 expression. Inhibition of inducible COX-2 expression in lipopolysaccharide (LPS)-stimulated macrophages by DAS[36] indicated possible roles for this compound in treatment of conditions such as arthritis where COX-2 over expression is believed to have important roles in the inflammatory response[2]. Urate crystals increase COX-2 expression in monocytes[37]

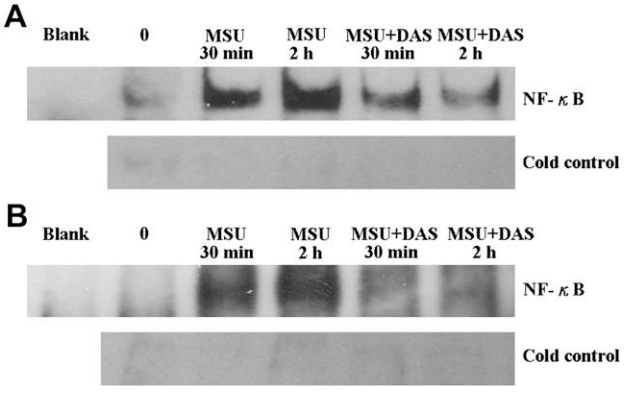

Fig. 7. Electrophoresis mobility-shift assay with NF-κB-binding site. Gel mobility-shift assay was performed using a digoxigenin (DIG)-labeled synthetic oligonucleotides and nuclear extract from cultures of OA human synovial cells (A) and articular chondrocytes (B) following stimulation with 2 mg/ml MSU crystals for 0, 30 min, 2 h. The competition assay used the same unlabeled oligonucleotide sequence with 10× concentration. NF-κB translocation was identified in both OA synovial cells (A) and articular chondrocytes (B) following with MSU crystals at 30 min and 2 h. The effect of MSU crystals was inhibited by DAS treatment. Blank lane was free of oligonucleotides ($n = 3$).

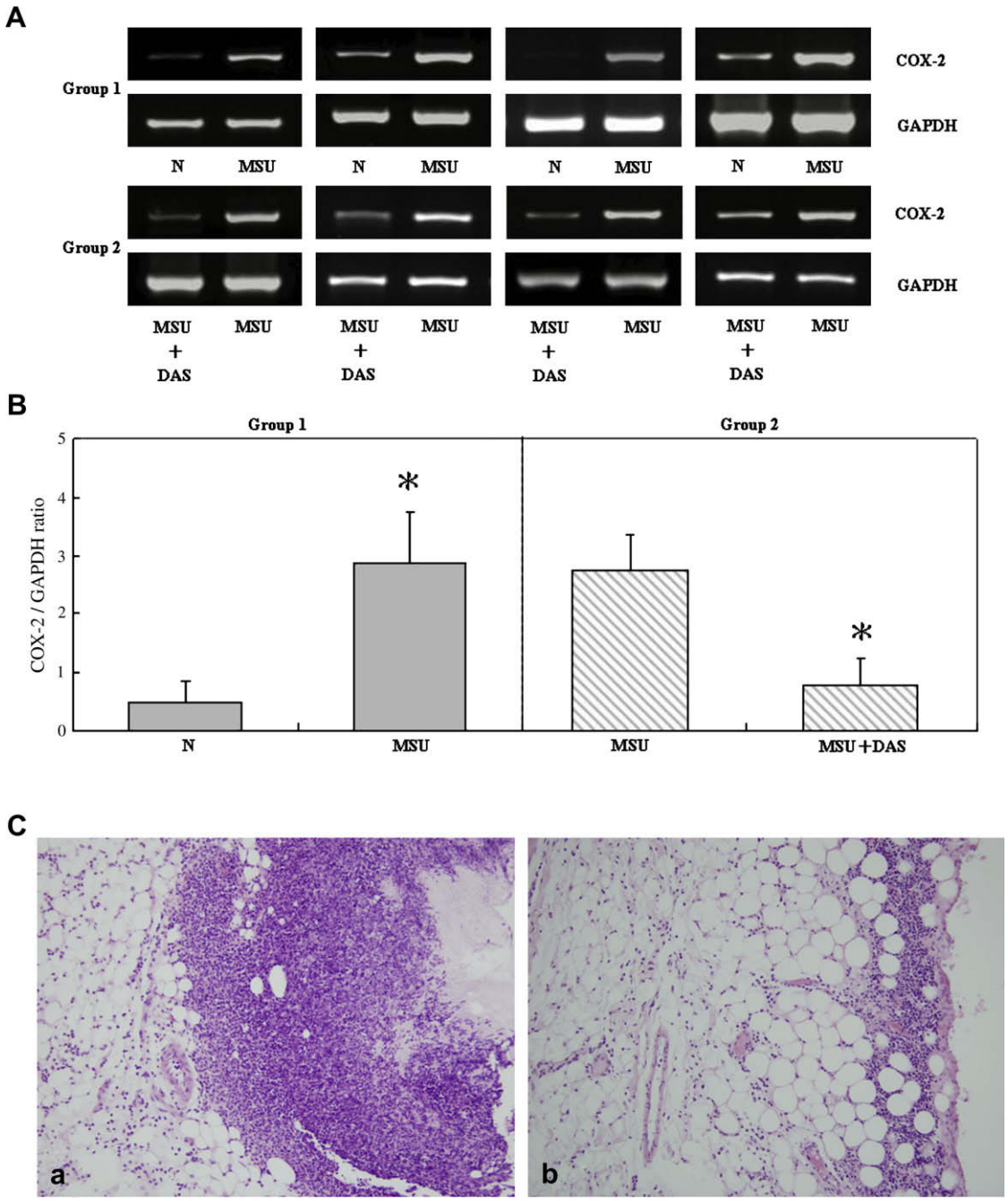

Fig. 8. DAS effects on synovial *COX-2* gene expression and synovial inflammation in an MSU crystal-induced arthritis model. *COX-2* gene expression was significantly induced 16 h following injection of MSU crystals into rat knee joints (group 1 and group 2 − MSU) in comparison to saline injected joints (group 1 − N) but this increase was inhibited by the presence of DAS (group 2 − MSU + DAS). (A) Gene expression. (B) Results presented as semi-quantitative data. $^*P < 0.05$. Group 1 = 4 animals, N: left knee joint with normal saline injection; MSU: right knee joint with MSU crystal injection. Group 2 = 4 animals, MSU: right knee joint with MSU crystal injection; MSU + DAS: left knee joint. (C) Severe acute synovitis induced by MSU crystal injection (a). DAS ameliorated the degree of acute synovitis (b); H&E, 200×.

and in synovial fluid inflammatory cells[38] in acute gout. In the current work we now demonstrate that MSU crystals also increase COX-2 expression in a rabbit synovial cell line, human articular chondrocytes and synovial cells whereas levels of constitutive COX-1 showed no significant change following exposure to MSU crystals. This modulation of COX-2 by MSU crystals and the increased expression of COX-2 induced by IL-1β was blocked by DAS suggesting that this compound may have potential anti-inflammatory roles in treatment of crystal-induced arthritis.

Several studies have reported that NF-κB activation is essential in the transcriptional activation of COX-2 in response to IL-1β in chondrocytes and synovial fibroblasts[39–42] and we have previously shown that NF-κB translocation is seen following treatment of chondrocytes with IL-1β and fibronectin fragments[43]. Similar to IL-1β activation shown

by Yan *et al.*[44], here we show that not only is NF-κB translocation seen in both chondrocytes and synovial cells following MSU crystal stimulation but also that NF-κB translocation is inhibited by DAS co-incubation. Interestingly DAS appears to have differential effects on NF-κB activation in different systems. DAS does not inhibit tumor necrosis factor-alpha (TNF-α) induced NF-κB DNA binding nor NF-κB transactivation in endothelial cells[45] and appears to have no effect on LPS stimulated NF-κB activation in RAW 264.7 macrophages[46] although the other major organosulfur compounds, diallyl trisulfide (DATS) and diallyl disulfide (DADS), in garlic oil show inhibitory activity in the latter model system.

DAS also reduced the COX-2 expression in synovial tissues in the MSU crystal-induced arthritis model system used in the current study. Treatment of acute gout normally involves medication with non-steroidal anti-inflammatory drugs (NSAIDs) with allopurinol most often used in long-term treatment. COX-2 selective inhibitors have been suggested as being useful in both prophylactic management of gout as these have been shown to suppress acute MSU crystal induced inflammation in a rat subcutaneous air pouch model[11] and in urate crystal induced acute synovitis in dogs[47]. A randomised double blind trial comparing a COX-2 inhibitor with an NSAID in acute gout has also suggested that they may be equally efficacious[48]. There is increasing interest in the use of naturally derived products in treatment of a variety of conditions although knowledge of mechanisms by which these may function remains limited. Use of colchicine, a derivative of the autumn crocus used to treat gout for thousands of years, can provide relief in acute attacks and can help prevent future attacks but is less now used because of side effects. Unlike non-steroidal anti-inflammatory agents colchicine does not exert its anti-inhibitory effect through inhibition of COXs[49].

The current study demonstrates potential for DAS, a flavor compound derived from garlic oil, in the treatment of inflammatory arthritis and provides evidence to support the idea that anti-inflammatory effects are through suppression of NF-κB activation and COX-2 expression. DAS was used at a concentration of 20 mM in the current study. At concentrations of 30 mM and above the MTT assay showed increasing toxicity. DAS has been shown to induce apoptosis in several cancer cells including colon cancer cells[50], glioblastoma[51], and neuroblastoma[52] tumor cells acting, at least in part by activation of caspases. Indeed in the current study we cannot exclude that effect on neutrophil apoptosis may contribute to the inhibition of the inflammatory response. It is unlikely that effects seen *in vitro* are a result of induction of apoptosis but clearly potential toxicity may influence use in a clinical setting. Both DAS and DADS have been tested for cytotoxic and genotoxic effects in a Chinese hamster ovary cell line. DAS exhibits less cytotoxicity and genotoxicity including chromosome aberrations and sister chromatid exchanges than DADS. The use of such compounds therefore should be cautioned to avoid any potential deleterious effects[53]. The administration of this agent by mouth or intraarticular use, however, needs to be further examined. Combined use with other intraarticular reagents such as colcemid or hyaluronic acid might be a way to explore.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

This study was supported by a grant from the National Science Council and National Defense Medical Center, Tri-Service General Hospital, Taiwan (NSC94-2320-B-016-018, DOD95-10-04, and TSGH-C94-48).

References

1. Flower RJ. The development of COX2 inhibitors. Nat Rev Drug Discov 2003;2:179–91.
2. Martel-Pelletier J, Pelletier JP, Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. Semin Arthritis Rheum 2003;33:155–67.
3. Farahat MN, Yanni G, Poston R, Panayi GS. Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. Ann Rheum Dis 1993;52:870–5.
4. Fernandes JC, Pelletier JM, Pelletier JP. The role of cytokines in osteoarthritis pathophysiology. Biorheology 2002;39:237–46.
5. Faour WH, He Y, He QW, de Ladurantaye M, Quintero M, Mancini A, *et al.* Prostaglandin E(2) regulates the level and stability of cyclooxygenase-2 mRNA through activation of p38 mitogen-activated protein kinase in interleukin-1 beta-treated human synovial fibroblasts. J Biol Chem 2001;276:31720–31.
6. Dinarello CA. The IL-1 family and inflammatory diseases. Clin Exp Rheumatol 2002;20(5 Suppl 27):S1–13.
7. Woods JM, Mogollon A, Amin MA, Martinez RJ, Koch AE. The role of COX-2 in angiogenesis and rheumatoid arthritis. Exp Mol Pathol 2003;74:282–90.
8. LeGrand A, Fermor B, Fink C, Pisetsky DS, Weinberg JB, Vail TP, *et al.* Interleukin-1, tumor necrosis factor alpha, and interleukin-17 synergistically up-regulate nitric oxide and prostaglandin E2 production in explants of human osteoarthritic knee menisci. Arthritis Rheum 2001;44:2078–83.
9. Kojima F, Naraba H, Miyamoto S, Beppu M, Aoki H, Kawai S. Membrane-associated prostaglandin E synthase-1 is upregulated by proinflammatory cytokines in chondrocytes from patients with osteoarthritis. Arthritis Res Ther 2004;6:R355–65.
10. van Doornum S, Ryan PF. Clinical manifestations of gout and their management. Med J Aust 2000;172:493–7.
11. Nalbant S, Chen LX, Sieck MS, Clayburne G, Schumacher HR. Prophylactic effect of highly selective COX-2 inhibition in acute monosodium urate crystal induced inflammation in the rat subcutaneous air pouch. J Rheumatol 2005;32:1762–4.
12. Hardy MM, Seibert K, Manning PT, Currie MG, Woerner BM, Edwards D, *et al.* Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. Arthritis Rheum 2002;46:1789–803.
13. McCoy JM, Wicks JR, Audoly LP. The role of prostaglandin E2 receptors in the pathogenesis of rheumatoid arthritis. J Clin Invest 2002;110:651–8.
14. Honda T, Segi-Nishida E, Miyachi Y, Narumiya S. Prostacyclin-IP signaling and prostaglandin E2-EP2/EP4 signaling both mediate joint inflammation in mouse collagen-induced arthritis. J Exp Med 2006;203:325–35.
15. Lainer DT, Brahn E. New antiangiogenic strategies for the treatment of proliferative synovitis. Expert Opin Investig Drugs 2005;14:1–17.
16. Makhej AN, Vanderhoek YJ, Bailey JM. Inhibition of platelet aggregation and thromboxane synthesis by onion and garlic. Lancet 1979;1:781.
17. Rahman K, Billington D. Dietary supplementation with aged garlic extract inhibits ADP-induced platelet aggregation in humans. J Nutr 2000;130:2662–5.
18. Steiner M, Li W. Aged garlic extract, a modulator of cardiovascular risk factors: a dose-finding study on the effect of AGE on platelet functions. J Nutr 2001;131:980–4.
19. Yang GC, Yasaei PM, Page SW. Garlic as anti-oxidants and free radical scavengers. J Food Drug Anal 1993;4:357–64.
20. Lau BH. Suppression of LDL oxidation by garlic. J Nutr 2001;131:985–8.
21. Durak I, Ozturk HS, Olcay E, Can B, Kavutcu M. Effects of garlic extract on oxidant/antioxidant status and atherosclerotic plaque formation in rabbit aorta. Nutr Metab Cardiovasc Dis 2002;12:141–7.
22. Milner JA. Garlic: its anticarcinogenic and antitumorigenic properties. Nutr Rev 1996;54:S82–6.
23. Milner JA. Preclinical perspectives on garlic and cancer. J Nutr 2006;136(3 Suppl):827S–831.
24. Amagase H. Clarifying the real bioactive constituents of garlic. J Nutr 2006;136(3 Suppl):716S–725.

25. Fanelli SL, Castro GD, de Toranzo EG, Castro JA. Mechanisms of the preventive properties of some garlic components in the carbon tetrachloride-promoted oxidative stress. Diallyl sulfide; diallyl disulfide; allyl mercaptan and allyl methyl sulfide. Res Commun Mol Pathol Pharmacol 1998;102:163–74.
26. Yang CS, Chhabra SK, Hong JY, Smith TJ. Mechanisms of inhibition of chemical toxicity and carcinogenesis by diallyl sulfide (DAS) and related compounds from garlic. J Nutr 2001;131:1041S–1045.
27. Chung JG, Chen GW, Wu LT, Chang HL, Lin JG, Yeh CC, *et al.* Effects of garlic compounds diallyl sulfide and diallyl disulfide on arylamine N-acetyltransferase activity in strains of *Helicobacter pylori* from peptic ulcer patients. Am J Chin Med 1998;26:353–64.
28. Rahman K. Garlic and aging: new insights into an old remedy. Ageing Res Rev 2003;2:39–56.
29. McCarty DJ Jr, Faires JS. A comparison of the duration of local anti-inflammatory effect of several adrenocorticosteroid esters – a bioassay technique. Curr Ther Res Clin Exp 1963;5:284–90.
30. Lee HS, Millward-Sadler SJ, Wright MO, Nuki G, Salter DM. Integrin and mechanosensitive ion channel-dependent tyrosine phosphorylation of focal adhesion proteins and beta-catenin in human articular chondrocytes after mechanical stimulation. J Bone Miner Res 2000; 15:1501–9.
31. Lee HS, Millward-Sadler SJ, Wright MO, Nuki G, Jamal AI, Salter DM. Activation of Integrin – RACK1/PKC signalling in human articular chondrocyte mechanotransduction. Osteoarthritis Cartilage 2002;10: 890–7.
32. Getting SJ, Christian HC, Flower RJ, Perretti M. Activation of melanocortin type 3 receptor as a molecular mechanism for adrenocorticotropic hormone efficacy in gouty arthritis. Arthritis Rheum 2002;46: 2765–75.
33. Szutowski MM, Zalewska K, Jadczak M, Marek M. *In vivo* effect of diallyl sulfide and cimetidine on phenacetin metabolism and bioavailability in rat. Acta Biochim Pol 2002;49:249–56.
34. Elango EM, Asita H, Nidhi G, Seema P, Banerji A, Kuriakose MA. Inhibition of cyclooxygenase-2 by diallyl sulfides (DAS) in HEK 293T cells. J Appl Genet 2004;45:469–71.
35. Sengupta A, Ghosh S, Das S. Modulatory influence of garlic and tomato on cyclooxygenase-2 activity, cell proliferation and apoptosis during azoxymethane induced colon carcinogenesis in rat. Cancer Lett 2004;208:127–36.
36. Chang HP, Chen YH. Differential effects of organosulfur compounds from garlic oil on nitric oxide and prostaglandin E2 in stimulated macrophages. Nutrition 2005;21:530–6.
37. Pouliot M, James MJ, McColl SR, Naccache PH, Cleland LG. Monosodium urate microcrystals induce cyclooxygenase-2 in human monocytes. Blood 1998;91:1769–76.
38. Iñiguez MA, Pablos JL, Carreira PE, Cabré F, Gomez-Reino JJ. Detection of COX-1 and COX-2 isoforms in synovial fluid cells from inflammatory joint diseases. Br J Rheumatol 1998;37:773–8.
39. Lianxu C, Hongti J, Changlong Y. NF-kappaBp65-specific siRNA inhibits expression of genes of COX-2, NOS-2 and MMP-9 in rat IL-1beta-induced and TNF-alpha-induced chondrocytes. Osteoarthritis Cartilage 2006;14:367–76.
40. Shakibaei M, John T, Schulze-Tanzil G, Lehmann I, Mobasheri A. Suppression of NF-kappaB activation by curcumin leads to inhibition of expression of cyclo-oxygenase-2 and matrix metalloproteinase-9 in human articular chondrocytes: implications for the treatment of osteoarthritis. Biochem Pharmacol 2007;73:1434–45.
41. Mbvundula EC, Bunning RA, Rainsford KD. Arthritis and cannabinoids: HU-210 and Win-55,212-2 prevent IL-1alpha-induced matrix degradation in bovine articular chondrocytes *in-vitro*. J Pharm Pharmacol 2006;58:351–8.
42. Stuhlmeier KM. The anti-rheumatic gold salt aurothiomalate suppresses interleukin-1beta-induced hyaluronan accumulation by blocking HAS1 transcription and by acting as a COX-2 transcriptional repressor. J Biol Chem 2007;282:2250–8.
43. Su SL, Tsai CD, Lee CH, Salter DM, Lee HS. Expression and regulation of Toll-like receptor 2 by IL-1beta and fibronectin fragments in human articular chondrocytes. Osteoarthritis Cartilage 2005;13:879–86.
44. Yan X, Wu Xiao C, Sun M, Tsang BK, Gibb W. Nuclear factor kappa B activation and regulation of cyclooxygenase type-2 expression in human amnion mesenchymal cells by interleukin-1beta. Biol Reprod 2002;66:1667–71.
45. Dirsch VM, Keiss HP, Vollmar AM. Garlic metabolites fail to inhibit the activation of the transcription factor NF-kappaB and subsequent expression of the adhesion molecule E-selectin in human endothelial cells. Eur J Nutr 2004;43:55–9.
46. Liu KL, Chen HW, Wang RY, Lei YP, Sheen LY, Lii CK. DATS reduces LPS-induced iNOS expression, NO production, oxidative stress, and NF-kappaB activation in RAW 264.7 macrophages. J Agric Food Chem 2006;54:3472–8.
47. Hazewinkel HA, van den Brom WE, Theyse LF, Pollmeier M, Hanson PD. Comparison of the effects of firocoxib, carprofen and vedaprofen in a sodium urate crystal induced synovitis model of arthritis in dogs. Res Vet Sci 2008;84:74–9.
48. Schumacher HR Jr, Boice JA, Daikh DI, Mukhopadhyay S, Malmstrom K, Ng J, *et al.* Randomised double blind trial of etoricoxib and indometacin in treatment of acute gouty arthritis. BMJ 2002;324: 1488–92.
49. Ben-Chetrit E, Fischel R, Hinz B, Levy M. The effects of colchicine and hydroxychloroquine on the cyclo-oxygenases COX-1 and COX-2. Rheumatol Int 2005;25:332–5.
50. Sriram N, Kalayarasan S, Ashokkumar P, Sureshkumar A, Sudhandiran G. Diallyl sulfide induces apoptosis in Colo 320 DM human colon cancer cells: involvement of caspase-3, NF-kappaB, and ERK-2. Mol Cell Biochem 2008;311:157–65.
51. Das A, Banik NL, Ray SK. Garlic compounds generate reactive oxygen species leading to activation of stress kinases and cysteine proteases for apoptosis in human glioblastoma T98G and U87MG cells. Cancer 2007;110:1083–95.
52. Karmakar S, Banik NL, Patel SJ, Ray SK. Garlic compounds induced calpain and intrinsic caspase cascade for apoptosis in human malignant neuroblastoma SH-SY5Y cells. Apoptosis 2007;12:671–84.
53. Musk SR, Clapham P, Johnson IT. Cytotoxicity and genotoxicity of diallyl sulfide and diallyl disulfide towards Chinese hamster ovary cells. Food Chem Toxicol 1997;35:379–85.