Basic calcium phosphate crystals activate human osteoarthritic synovial fibroblasts and induce matrix metalloproteinase-13 (collagenase-3) in adult porcine articular chondrocytes

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Abstract

Objective—To determine the ability of basic calcium phosphate (BCP) crystals to induce (a) mitogenesis, matrix metalloproteinase (MMP)-1, and MMP-13 in human osteoarthritic synovial fibroblasts (HOAS) and (b) MMP-13 in cultured porcine articular chondrocytes.

Methods—Mitogenesis of HOAS was measured by [³H]thymidine incorporation assay and counts of cells in monolayer culture. MMP messenger RNA (mRNA) accumulation was determined either by northern blot analysis or reverse transcriptase-polymerase chain reaction (RT-PCR) of RNA from chondrocytes or HOAS treated with BCP crystals. MMP-13 secretion was identified by immunoprecipitation and MMP-1 secretion by western blot of conditioned media.

Results—BCP crystals caused a 4.5-fold increase in [³H]thymidine incorporation by HOAS within 20 hours compared with untreated control cultures ($p \leq 0.05$). BCP crystals induced MMP-13 mRNA accumulation and MMP-13 protein secretion by articular chondrocytes. In contrast, in HOAS, MMP-13 mRNA induced by BCP crystals was detectable only by RT-PCR, and MMP-13 protein was undetectable. BCP crystals induced MMP-1 mRNA accumulation and MMP-1 protein secretion by HOAS. MMP-1 expression was further augmented when HOAS were co-incubated with either BCP and tumour necrosis factor α (TNF α ; threefold) or BCP and interleukin 1 α (IL1 α ; twofold).

Conclusion—These data confirm the ability of BCP crystals to activate HOAS, leading to the induction of mitogenesis and MMP-1 production. MMP-13 production in response to BCP crystals is substantially more detectable in porcine articular chondrocytes than in HOAS. These data support the active role of BCP crystals in osteoarthritis and suggest that BCP crystals act synergistically with IL1 α and TNF α to promote MMP production and subsequent joint degeneration.

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Intra-articular basic calcium phosphate (BCP) (hydroxyapatite, octacalcium phosphate, and tricalcium phosphate) crystal deposition is found in the joint fluid of up to 70% of

osteoarthritic (OA) knees and is associated with severe degenerative arthritis.^{1 2} Perichondrocyte BCP crystal deposition is found in the articular cartilages of more than 50% of patients with more advanced hip or knee degeneration.³ OA knees containing BCP crystals exhibit synovial lining hyperplasia and have more severe radiographic degeneration and larger joint effusions than OA knees without crystals.^{4 5} BCP crystals are also associated with severe destructive arthropathies, such as Milwaukee shoulder syndrome. This condition is characterised by marked synovial proliferation and non-inflammatory degradation of articular structures, such as cartilage, tendons, ligaments, and adjacent bone.⁶

Matrix metalloproteinases (MMP) play a major part in cartilage degradation and BCP crystals cause production of MMP (collagenase, stromelysin, and 92 kDa gelatinase) from human fibroblasts in vitro.^{7 8} They also induce collagenase (MMP-1) production from adult porcine articular chondrocytes.⁹ BCP crystal induction of these MMP from intra-articular cells probably promotes the severe joint degeneration associated with their presence. In addition, BCP crystals themselves are at least partly responsible for the associated synovial lining proliferation, and increased cell numbers in the synovial lining enhance the capacity for secretion of proteolytic enzymes.^{10 11}

Tumour necrosis factor α (TNF α) and interleukin 1 (IL1) have both been implicated in the pathogenesis of OA. TNF α and IL1 promote the release of MMP and suppress the synthesis of proteoglycans and collagen by human chondrocytes and synovial cells.¹² Also, TNF α induces MMP-1 and MMP-3 production from adult porcine chondrocytes.¹³ A more recently isolated MMP, MMP-13, has a 10-fold greater catalytic activity towards type II collagen than MMP-1. It has been identified in human OA cartilage and is inducible in human and porcine articular chondrocytes.¹⁴ Reports of the expression of MMP-13 in human osteoarthritic synoviocytes (HOAS) have varied.^{15 16} Furthermore, although BCP crystals induce MMP-1 in chondrocytes, the ability of BCP crystals to induce MMP-13 in chondrocytes has not been reported.

BCP crystals have been shown to induce mitogenesis and MMP-1 production in a number of cell lines, including human foreskin fibroblasts, porcine articular chondrocytes, and canine synovial fibroblasts, but their effect on

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human synovial cells has not previously been shown. Furthermore, in OA joints BCP crystals probably exert their effects in association with cytokines such as IL1 and TNF α , allowing an opportunity for synergism.

To elucidate further the biological effects of BCP crystals we suggested that BCP crystals induce MMP-13 in adult porcine articular chondrocytes and HOAS. We also suggested that BCP crystals activate HOAS leading to mitogenesis and MMP-1 production, thereby enhancing TNF α and IL1 induced MMP-1 production.

Materials and methods

PROBES AND REAGENTS

The MMP-13 probe was a full length human cDNA.¹⁷ The MMP-1 probe was a 2.05 kb *Hind-III/SmaI* insert from the pC11ase 1 clone,¹⁸ obtained from the repository of human DNA probes of the American Type Culture Collection (Rockville, MD). The pHcGAP plasmid containing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA¹⁹ was also from the American Type Culture Collection. TRIzol reagent (a monophasic solution of phenol and guanidine isothiocyanate) was from Gibco BRL Life Technologies (Grand Island, NY). FORMAzol (formamide) and BCP phase separation reagent (1-bromo-3-chloropropane) were from Molecular Research, Inc. ⁴⁵Ca was from DuPont NEN Research Products (Boston, MA). Two MMP-13 antibodies were used: a polyclonal antibody which was raised in rabbit against full length recombinant human MMP-13 and a monoclonal antibody (No F17 IB-3-C5) (Pfizer Central Research). The MMP-1 antibody was a polyclonal antibody raised in rabbit against purified human MMP-1 from gingival fibroblasts. Biotin labelled, goat anti-rabbit IgG and peroxidase labelled streptavidin were from Kirkegaard and Perry Laboratories, Inc (Gaithersburg, MD). Enhanced chemiluminescence was performed using a kit from Amersham Life Sciences (Buckinghamshire, UK). Tritiated thymidine (50 Ci/mmol) was from Amersham Life Sciences and α -[³²P]dATP and ³⁵S labelled L-methionine (1051 Ci/mmol) was from ICN Pharmaceuticals, Inc (Irvine, CA). SUPERScript II, oligo(dT)15 primer and Taq DNA polymerase were from Gibco BRL Life Technologies. Fetal bovine serum (FBS), Hanks's buffered saline solution, HEPES buffer, and Dulbecco's modified Eagle's medium (DMEM) were from Gibco.¹⁴C-Methylated protein standards (31–48 μ Ci/mg protein) were from Amersham (Arlington Heights, IL). Protein A-Sepharose was from Pharmacia Biotech, Inc (Piscataway, NJ). Cycloheximide and 12-*o*-tetradecanoylphorbol-13-acetate were from Sigma Chemical Co (St Louis, MO). Recombinant TNF α and IL1 α were obtained from Genzyme Corporation.

CRYSTAL SYNTHESIS AND PREPARATION

BCP crystals were synthesised by a modification of published methods.²⁰ Mineral prepared by this method had a calcium/phosphorus

molar ratio of 1.59 and contained partially carbonate substituted hydroxyapatite with admixed octacalcium phosphate by Fourier transform infrared spectroscopy. Retention of the crystal character was confirmed by x ray diffraction and Fourier transform infrared spectroscopy. The crystals were crushed and sieved to yield 10–20 μ m aggregates, which were sterilised and rendered pyrogen free by heating at 200°C for 90 minutes. Crystals were weighed and suspended by sonication in DMEM. The amounts of crystals used were determined based on our previous studies of dose-response relations between BCP crystals and the mitogenic response.¹¹ In those studies, maximal mitogenic responses were achieved with BCP levels of 50–100 μ g/ml. These levels are equivalent to concentrations of BCP crystals found in pathological joint fluids.

CELL CULTURE

Adult porcine chondrocytes were used as a model for human chondrocytes because they have been shown to have numerous biological responses to treatment with BCP crystals and to produce collagenase and stromelysin when treated with TNF α or epidermal growth factor.^{9,13} Chondrocytes were prepared by digestion of cartilage from adult porcine knees as described previously.⁹ The cells were plated at 4×10^3 /cm² in DMEM supplemented with 10% FBS, allowed to attach, and incubated at 37°C in a humidified atmosphere containing 10% CO₂. Cultures were routinely re-fed with 10% FBS in DMEM on days 2 and 4 after plating. These were replaced with serum-free (Neumann and Tytell) medium on day 6. Plates were used for experiments 24 hours later. Chondrocytes plated under these conditions continued to secrete type II collagen, as described previously.²¹

HOAS were derived from synovial tissue obtained from three patients with OA at the time of arthroplasty. The tissue was minced and enzymatically dissociated with 1 mg/ml collagenase and 0.1% DNase 1 in DMEM (50 ml) and 1% phenylmethylsulphonyl fluoride (PMSF) for 90 minutes at 37°C. After 90 minutes, 0.25% trypsin/DMEM (50 ml) and 1% PMSF was added for an additional 30 minutes' incubation. Liberated cells were spun at 400 *g* for 10 minutes and washed twice with 50% phosphate buffered saline (PBS)-50% DMEM. Cells were resuspended, filtered through nylon, and then grown and maintained in DMEM supplemented with 10% FBS containing 1% PMSF and 0.2% gentamycin. They were incubated at 37°C in a humidified atmosphere containing 10% CO₂. All experiments were performed on confluent cell monolayers that had been rendered quiescent by removing the medium, washing with DMEM containing 0.5% FBS, and subsequently incubating in this medium for 24 hours. All cultures used were third to fourth passage cells. All experiments were repeated at least three times.

NORTHERN BLOT ANALYSIS

Northern blot analysis of total cellular RNA was used to study the expression of MMP-13

Figure 1 Basic calcium phosphate (BCP) crystal induced accumulation of matrix metalloproteinase-13 (MMP-13) messenger RNA (mRNA) in adult articular porcine chondrocytes. Time course. Confluent cultures of chondrocytes, in 60 mm plates, were incubated in serum-free media for 24 hours before being stimulated with BCP crystals ($24 \mu\text{g}/\text{cm}^2$). At various times after treatment, cultures were harvested and total RNA was isolated. Northern blot analysis was performed using a specific probe for MMP-13, followed by autoradiography. The probe was then removed by washing and the blot was re-probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as a control. The positions of the 18S and 28S ribosomes are shown. B = BCP crystals; C = unstimulated control cultures.

mRNA in chondrocytes or HOAS after stimulation with BCP crystals or TNF α . Control cultures were treated with 12-O-tetradecanoylphorbol 13-acetate or IL1 or left untreated. Confluent, quiescent monolayer cultures of chondrocytes in 60 mm plates, or HOAS in 100 mm plates, were washed twice with cold PBS 24 hours after treatment. Total RNA was recovered with TRIzol reagent based on the methods of Chomczynski and Sacchi²² but using 1-bromo-3-chloropropane reagent instead of chloroform. RNA was solubilised in FORMAZOL. Ten micrograms of RNA were fractionated on a 1.2% agarose-formaldehyde gel, ribosomal RNA was visualised with ethidium bromide, and the fractionated RNA was transferred to supported nitrocellulose membrane (Micron Separations Inc, Westborough, MA) by the capillary method.²³ The RNA was cross linked to the membrane after transfer by UV cross linking in a Fisher brand UV cross linker (Fisher Pittsburgh, PA). The blot was pre-hybridised for four hours at 42°C and hybridisation of the filters with DNA probes was performed overnight at 42°C. Probes were labelled using the random primer method to a specific activity of $>5 \times 10^8$ cpm/ μg .²⁴ The filters were washed at a maximal stringency of $0.25 \times$ standard saline citrate (SSC; $1 \times \text{SSC} = 0.15 \text{ M}$ sodium chloride, 0.015 M sodium citrate, pH 7.0) at 60°C for 30 minutes.

Figure 2 Basic calcium phosphate (BCP) crystal induced stimulation of matrix metalloproteinase-13 (MMP-13) protein synthesis and secretion in adult articular porcine chondrocytes. One millilitre samples of media, conditioned for 24 or 48 hours in the presence of $50 \mu\text{Ci}/\text{ml}$ of [^{35}S]methionine, from confluent cultures of chondrocytes untreated or treated with various quantities of BCP crystals, were incubated with protein A-Sepharose and pre-immune rabbit serum at 4°C for one hour. Samples were processed as in "Materials and methods" and incubated overnight with $10 \mu\text{l}$ of MMP-13 antiserum. Samples were washed with immunoprecipitation buffer and examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by autoradiography. B = BCP crystals; C = unstimulated control cultures. Numbers to the left refer to the molecular weight in kDa.

Autoradiography was performed with Kodak XAR-5 film (Eastman Kodak, Rochester, NY) and signal intensity was quantified by scanning laser densitometry (LKB Instruments, Stockholm, Sweden).

DETERMINATION OF MITOGENESIS BY UPTAKE OF [^3H]THYMIDINE

HOAS were grown to confluence in 24 well plates and rendered quiescent by incubation in 0.5% FBS for 24 hours. [^3H]Thymidine ($1 \mu\text{Ci}/\text{ml}$) was added to the wells 23 hours after the addition of 10% FBS or BCP crystals ($18 \mu\text{g}/\text{cm}^2$) and pulse labelled for one hour. Control cultures were in 0.5% FBS. The cells were then washed three times with PBS, and macromolecules were precipitated with 5% trichloroacetic acid (TCA) solution. The precipitate was washed again with PBS and dissolved in 1 ml 0.1 N NaOH/1% sodium dodecyl sulphate (SDS). Levels of TCA precipitable ^3H were determined in quadruplicate, using a liquid scintillation counter (Packard Instruments, Downers Grove, IL). In separate experiments, mitogenic stimulation of monolayers was followed by trypsinisation after 48 hours and cell counts were determined with a Coulter counter (Coulter Electronics, Hi-aleah, FL).

IMMUNOBLOTTING

MMP-1 secretion by HOAS was confirmed by western blot.²⁵ Briefly, samples of conditioned media from cultures treated with BCP crystals, TNF α , IL1 α , or unstimulated control cultures were electrophoresed through 10% polyacrylamide gels under reducing conditions and then transferred onto nitrocellulose membranes. After transfer, the membranes were incubated in 2.5% non-fat dry milk in Tris buffered saline

Figure 3 Mitogenic effects of basic calcium phosphate (BCP) crystals in human osteoarthritic synovial fibroblasts. Confluent, quiescent cultures of synovial fibroblasts grown in 24 well plates were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% fetal bovine serum (FBS). Twenty four hours later, fresh DMEM containing either 0.5% control (C) or 10% FBS (FBS) or BCP crystals (BCP) (25 µg/cm²) was added. At the times indicated, cultures were pulse labelled with [³H]thymidine (1 µCi/ml) for one hour. The plates were then processed, and thymidine incorporation was determined as described in "Materials and methods". All values (SEM), n=4. Significant mitogenic response to BCP at 20, 22.5, and 25 hours compared with C ($p \leq 0.05$).

Figure 4 Basic calcium phosphate (BCP) crystal induced accumulation of matrix metalloproteinase-1 (MMP-1) messenger RNA (mRNA) in human osteoarthritic synovial fibroblasts. Time course. Confluent cultures of synovial fibroblasts, in 100 mm plates, were incubated in 0.5% fetal bovine serum-Dulbecco's modified Eagle's medium for 24 hours before being stimulated with BCP crystals (18 µg/cm²). Control cultures were either untreated or treated with tumour necrosis factor α (10 ng/ml) or interleukin 1α (100 U/ml). At various times after treatment, cultures were harvested and total RNA was isolated. Northern blot analysis was performed using a specific probe for MMP-1, followed by autoradiography. The probe was then removed by washing and the blot was re-probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as a control. The positions of the 18S and 28S ribosomes are shown. C = unstimulated control cultures; B = BCP crystals; IL1 = interleukin 1α; TNF = tumour necrosis factor α.

(TBS) (20 mM Tris, 500 mM NaCl, pH 7.5) to eliminate non-specific binding of the antibodies to the membranes. The membranes were washed twice with Tween-20 wash solution (20 mM Tris, 500 mM NaCl, 0.05% Tween-20) (TTBS). The membranes were then incubated with antibody buffer containing a 1:1000 dilution of MMP-1 antibody for two hours at room temperature. The blots were then washed with TTBS and incubated with a

1:1000 dilution of biotin conjugated, goat anti-rabbit IgG for one hour. This was followed by washing with TTBS and incubation with a 1:1000 dilution of peroxidase labelled streptavidin for one hour. Membranes were washed twice with TTBS and once with TBS. Immunoreactive bands were detected using enhanced chemiluminescence reagents and autoradiography. The bands were then normalised and scanned for optical density using Scanalytics (Ambis, San Diego, CA).

IMMUNOPRECIPITATION

MMP-13 secretion was confirmed by immunoprecipitation of the conditioned media by a polyclonal antibody to MMP-13. Chondrocyte cultures were incubated overnight in DMEM with 20 mM HEPES buffer and 1% PMSF. Cultures were then incubated in methionine-free media. One millilitre samples of methionine-free DMEM, conditioned in the presence of 50 µCi/ml of [³⁵S]methionine, from cultures stimulated with BCP crystals or TNFα were collected after 24 or 48 hours. Control cultures were in 0.5% FBS with or without IL1α. Fifty microlitres of 10 × immunoprecipitation buffer (100 mM Tris, pH 7.5, 5% triton X-100, 1% SDS, 5% deoxycholate), 40 µl of a 50% suspension of protein A-Sepharose CL-4B, and 10 µl of normal rabbit serum were added and samples mixed at 4°C for one hour. The supernatant was removed after brief centrifugation, and 40 µl of fresh protein A-Sepharose added. The sample was mixed at 4°C for 30 minutes. The supernatant was again removed after brief centrifugation. Ten microlitres of MMP-13 antiserum, 10 µl immunoprecipitation buffer, and 40 µl 50% protein A-Sepharose were added to each sample. The samples were mixed overnight at 4°C and the immunoprecipitates washed three times in 1 ml × immunoprecipitation buffer (10 mM Tris, pH 7.4, 0.5% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) and once in PBS. After the final spin the supernatant was discarded. Samples were placed in SDS reducing sample buffer and separated on a 10% polyacrylamide gel according to Laemmli. The gel was then dried and autoradiography performed.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

The reverse transcriptase reaction was performed with 1 µg total RNA using the SUPERScript II (moloney-murine leukaemia virus reverse transcriptase) and an oligo(dT)₁₅ primer. Aliquots of cDNA were amplified by PCR using Taq DNA polymerase up to a cycle number of 40. The PCR products were electrophoresed in 2.0% agarose gels containing ethidium bromide. The cycle number was determined as the amplification in the linear range. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control. The following primers were used in the amplification reactions: GAPDH, 5'-AACTCCCTCAAGATTGTCAGCA-3', 5'-TCCACCACCCTGTTGCTGTA-3', resulting in a 553 bp product;

Figure 5 Basic calcium phosphate (BCP) crystal induced stimulation of matrix metalloproteinase-1 (MMP-1) protein synthesis and secretion in human osteoarthritic synovial fibroblasts. Confluent, quiescent human osteoarthritic synovial fibroblasts incubated in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum (FBS) were stimulated with either BCP crystals ($18 \mu\text{g}/\text{cm}^2$), interleukin 1a ($100 \text{ U}/\text{ml}$) or tumour necrosis factor α ($10 \text{ ng}/\text{ml}$). Untreated cultures were in 0.5% FBS. The culture medium was analysed by western blotting using a specific monoclonal antibody to MMP-1. Numbers refer to hours after treatment. Molecular weight markers are shown to the left. C = unstimulated control cultures; B = BCP crystals; IL1 = interleukin 1a; TNF = tumour necrosis factor α .

Figure 6 Basic calcium phosphate (BCP) crystal induced stimulation of matrix metalloproteinase-1 (MMP-1) protein synthesis and secretion in human osteoarthritic synovial fibroblasts (HOAS). Effect of co-incubation with tumour necrosis factor α (TNF α) or interleukin 1a (IL1a). Confluent, quiescent HOAS incubated in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum (FBS) were stimulated with either BCP crystals ($18 \mu\text{g}/\text{cm}^2$) and IL1a ($100 \text{ U}/\text{ml}$) or TNF α ($10 \text{ ng}/\text{ml}$). For comparison, some cultures were treated with both IL1a and TNF α . Untreated cultures were in 0.5% FBS. The culture medium was analysed by western blotting using a specific monoclonal antibody to MMP-1. Numbers refer to hours after treatment. Molecular weight markers are shown to the left. C = unstimulated control cultures; B = BCP crystals.

MMP-13, 5'-GTGCCCTTCTTCACACAGAC-3', 5'-CAGAATTCAAAGGCCACATC-3', resulting in a 100 bp product. Sequences for MMP-13 to design primer sets were found from Genbank accession number X75308.²⁶

STATISTICS

Statistical analysis was performed using the Wilcoxon rank sum test.²⁷

Results

BCP CRYSTAL INDUCTION OF MMP-13 IN CHONDROCYTES

Although low levels of MMP-13 mRNA were apparent in samples from unstimulated control cultures, BCP crystal induction of MMP-13 mRNA accumulation in chondrocytes was

detectable within eight hours after treatment and continued for at least 24 hours (fig 1). As it has been shown that new protein synthesis is required for accumulation of MMP-1, MMP-3, and MMP-9 mRNAs in response to BCP crystals and other stimuli, we studied the effect of protein synthesis inhibition by cycloheximide. When BCP crystals and cycloheximide ($10 \mu\text{g}/\text{ml}$) and BCP crystals ($48 \mu\text{g}/\text{cm}^2$) were added concurrently, BCP crystal induced MMP-13 mRNA was markedly inhibited. This inhibition persisted when cycloheximide was added up to four hours after treatment with BCP crystals (data not shown). When chondrocytes were incubated for 24 or 48 hours with various concentrations of BCP crystals the secretion of a protein of approximately 63 kDa, immunoprecipitable with a polyclonal antibody to MMP-13, was induced at all concentrations tested (fig 2). This protein was not evident in unstimulated control cultures.

BCP CRYSTAL INDUCTION OF MITOGENESIS IN HOAS

The addition of BCP crystals ($25 \mu\text{g}/\text{cm}^2$) in 0.5% FBS-DMEM stimulated an increase in [^3H]thymidine incorporation, an index of mitogenesis, at all time points tested (fig 3). The increase in thymidine incorporation reached statistical significance at 20, 22.5, and 25 hours compared with control ($p \leq 0.05$). Maximal [^3H]thymidine incorporation was noted in cultures harvested 20 hours after treatment, when there was a fourfold increase in [^3H]thymidine incorporation compared with unstimulated control cultures. We also performed cell counts four days after treatment to confirm that the increased [^3H]thymidine incorporation was accompanied by an increase in cell number. The mean (SD) number of cells ($\times 10^4/\text{cm}^2$) in control cultures incubated with 0.5% FBS was 1.65 (0.3). The number of cells ($\times 10^4/\text{cm}^2$) in cultures incubated with BCP crystals was 2.92 (0.51). The number of cells in cultures incubated with 10% FBS was 4.09 (0.31). The increase in cell number in response to both BCP crystals and 10% FBS was significant ($p \leq 0.05$).

BCP CRYSTAL INDUCTION OF MMP-1 MRNA AND PROTEIN SECRETION IN HOAS

BCP crystals induced significant accumulation of MMP-1 mRNA in HOAS, first evident at eight hours and continuing to at least 48 hours, the longest time point tested (fig 4). At 24 and 48 hours, MMP-1 mRNA accumulation induced by BCP crystals was comparable with that induced by IL1 and TNF α (fig 4). MMP-1 mRNA accumulation was followed by significant MMP-1 protein secretion in conditioned media which was maximal when the conditioned media was harvested at 48 hours (fig 5). Similarly, when HOAS were treated with IL1a or TNF α , there was significant MMP-1 protein secretion, which was maximal at 48 hours. MMP-1 protein expression was further increased when HOAS were co-incubated with

Figure 7 Reverse transcriptase-polymerase chain reaction (RT-PCR) determination of matrix metalloproteinase-13 (MMP-13) mRNA in human osteoarthritic synovial fibroblasts. Total RNA was isolated from synovial fibroblasts and subjected to RT-PCR analysis for MMP-13 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR amplification was performed for 40 cycles as described in "Materials and methods". Numbers refer to time in hours after treatment with BCP crystals ($18 \mu\text{g}/\text{cm}^2$), tumour necrosis factor α (TNF α ; 10 ng/ml), or interleukin 1 α (IL1 α ; 100 U/ml).

either BCP and TNF α (approximately three-fold) or BCP and IL1 α (approximately two-fold), as assessed by normalisation and scanning for optical density, compared with treatment with either TNF α or IL1 α alone (fig 6).

BCP CRYSTAL INDUCTION OF MMP-13 MRNA IN HOAS

BCP crystal induction of MMP-13 mRNA accumulation in HOAS was not detectable by northern blot (data not shown). However by RT-PCR, MMP-13 mRNA induction in response to TNF α was identified after 24 hours and in response to BCP crystals and IL1 α after 48 hours (fig 7). The increased MMP-13 mRNA detected by RT-PCR was not followed by detectable MMP-13 protein expression.

Discussion

In this work we show that BCP crystals cause the accumulation of MMP-13 mRNA, followed by the production and secretion of MMP-13 protein in adult porcine articular chondrocytes. We also show here that BCP crystals induce cell replication and MMP-1 production by HOAS. The mitogenic effects of BCP crystals and their ability to induce a number of MMP has been shown in a variety of human and animal cell types but not previously in human articular cells.

The time course of induction of MMP-13 in response to BCP crystals was similar to that of crystal induction of MMP-1, MMP-3, and MMP-9, suggesting the coordinate production of all four MMP in response to BCP crystals.^{7,8} In HOAS we found that MMP-13 mRNA accumulation was detectable only by RT-PCR in response to BCP crystals, TNF α , or IL1 α . No MMP-13 protein secretion was identified, presumably because quantities secreted were below the level of detection of our methods. BCP crystal induction of MMP-13 mRNA was inhibited by the protein synthesis inhibitor cycloheximide, suggesting that new protein synthesis is required for the transcriptase activation of MMP-13 by BCP. Structural analysis of the 5' flanking region of the MMP-13 gene has shown the presence of an activator protein 1 (AP1) motif.²⁸ AP1 is a heterodimer composed of the protein products of Fos and Jun. BCP crystals induce *c-fos* and

c-jun and AP1 in human fibroblasts.²⁹ It is therefore likely that the inhibition of BCP crystal induction of MMP-13 mRNA by cycloheximide, at least in part, reflects inhibition of the synthesis of an AP1 complex, normally induced in response to BCP crystals.

Although this is the first report of MMP-13 production in chondrocytes of any species treated with BCP crystals, MMP-13 expression has been consistently described in human OA chondrocytes and in chondrocytes treated with various inflammatory cytokines, including TNF α , IL1 α , and IL1 β .^{14,17} Reported synovio-cyte production and expression of MMP-13 suggests that it is less abundant and less consistent than that seen in chondrocytes. MMP-13 mRNA, identified by northern blot, was reported in the synovial membranes of one patient with rheumatoid arthritis (RA) and one patient with OA.¹⁵ MMP-13 expression, assessed by immunohistochemistry, was identified in the synovial membranes of six patients with RA and seven patients with OA. The overall expression of MMP-13 in the synovial stroma was substantially higher in RA than in OA.³⁰ MMP-13 was identified by RT-PCR and by immunohistochemistry in the synovial lining of patients with aseptic loosening of a total hip replacement and with OA. MMP-13 was found in the synovial fluid of these patients by western blotting.³¹ MMP-13 mRNA expression was detected in the synovial membranes of 21 of 36 patients with RA, and increased synovio-cyte MMP-13 expression correlated with increases in the clinical measurements of erythrocyte sedimentation rate and C reactive protein.³² Four of 10 primary RA synovial fibroblast cell cultures manifested basal expression of MMP-13 mRNA, which was stimulated two- to fourfold in response to IL1 β or TNF α .³² Finally, Vincenti *et al* have shown that MMP-13 mRNA and protein is inducible in rabbit synovial fibroblasts by IL1 β , TNF α , or phorbol esters.³³ In contrast, however, Reboul *et al* showed MMP-13 expression by RT-PCR in OA chondrocytes but not HOAS.¹⁶ Borden *et al* studied patients with RA and found MMP-13 gene expression in RA chondrocytes but not RA synovial fibroblasts.³⁴

We compared healthy adult porcine chondrocytes with OA fibroblasts, thus introducing the variables of species differences and healthy versus diseased states. None the less, current data, including ours, suggest that MMP-13 is more consistently inducible and expressed in greater quantities in chondrocytes than in synovio-cytes. Therefore, MMP-13 derived from chondrocytes probably has a more important role in OA, RA, and in BCP crystal associated joint degeneration than MMP-13 derived from synovio-cytes.

Furthermore, enhanced and coordinated expression of IL1 β , TNF α , and inducible nitric oxide synthase by chondrocytes compared with HOAS has previously been seen in OA.³⁵ We have shown here that the production of MMP-13 in response to BCP crystals is also enhanced in chondrocytes compared with synovial cells, thus following a similar pattern.

Taken together, these data support the hypothesis that chondrocytes are a major site of production of mediators of inflammation in human OA.³⁵

Previous studies of BCP crystal induced cell activation have been performed using model systems of human skin and foreskin fibroblasts, canine synovial and murine 3T3 fibroblasts, and porcine chondrocytes. Data presented here confirm that BCP crystals also activate human (OA) synovial fibroblasts, leading to mitogenesis and MMP-1 production. These observations confirm the relevance of previous work using other model systems, including human, non-articular fibroblasts to study BCP crystal mediated events.

BCP crystals occur commonly in OA joints. None the less, whether BCP crystals are simply an epiphenomenon of cartilage damage or whether BCP crystals cause cartilage damage has long been contested.³⁶ If the crystals were present simply as a consequence of cartilage damage and joint destruction, we would expect them to be present in other arthropathies characterised by cartilage dissolution and synovial lining proliferation, such as RA. However, BCP crystals are rarely found in RA joint fluids.¹ Thus current data support the active role of BCP crystals in the exacerbation of OA.

We have shown that BCP crystals induce MMP-1 in HOAS in vitro. Furthermore, BCP crystals, IL1 α , and TNF α appear to act in synergy to increase MMP-1 production by HOAS. In vivo, because BCP crystals and cytokines such as TNF α and IL1 α co-exist, they probably act in concert to augment joint degeneration. Historically, the role of cytokines in the pathogenesis of OA was also considered to be speculative.¹² Furthermore, as with BCP crystals, levels of cytokines such as IL1 or TNF α are not routinely measured in joint fluid from patients with arthritis. After considerable further investigation, however, the roles of IL1 and TNF α in mediating joint degeneration in OA are now well accepted.³⁵ As a consequence of such recognition, Pelletier and coworkers have prevented the development of OA in an experimental model by transfer of the IL1 receptor antagonist gene.³⁷ Efforts continue to discover methods to inhibit the pathogenic effects of IL1 and TNF α . Because BCP crystals act in synergy with these cytokines in vitro, BCP crystals in vivo are a likely therapeutic target in OA. To this end, more studies of the molecular mechanism of BCP crystal mediated cell activation are required.

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1 Swan A, Chapman B, Heap P, Seward H, Dieppe P. Submicroscopic crystals in osteoarthritic synovial fluids. *Ann Rheum Dis* 1994;53:467-70.
2 McCarthy GM. Crystal-related tissue damage. In: Smyth CJ, Holers VM, eds. *Gout, hyperuricemia, and other crystal-associated arthropathies*. New York, NY: Marcel Dekker, 1999:39-57.
3 Doyle D. Tissue calcification and inflammation in osteoarthritis. *J Pathol* 1982;136:199-216.
4 Halverson PB, McCarty DJ. Patterns of radiographic abnormalities associated with basic calcium phosphate and calcium pyrophosphate crystal deposition in the knee. *Ann Rheum Dis* 1986;45:603-5.

5 Carroll GJ, Stuart RA, Armstrong JA, Breidahl PD, Laing BA. Hydroxyapatite crystals are a frequent finding in osteoarthritic synovial fluid, but are not related to increased concentrations of keratan sulfate or interleukin 1 β . *J Rheumatol* 1991;18:861-6.
6 McCarty DJ, Halverson PB, Carrera GF, Brewer BJ, Kozin FK. Milwaukee shoulder: association of microspheroids containing hydroxyapatite crystals, active collagenase, and neutral protease with rotator cuff defects. i. Clinical aspects. *Arthritis Rheum* 1981;24:464-73.
7 McCarthy GM, Mitchell PG, Struve JS, Cheung HS. Basic calcium phosphate crystals cause co-ordinate induction and secretion of collagenase and stromelysin. *J Cell Physiol* 1992;153:140-6.
8 McCarthy G, Macius A, Christopherson P, Ryan L, Pourmotabbed T. Basic calcium phosphate crystals induce synthesis and secretion of 92-kD gelatinase (matrix metalloproteinase 9/gelatinase B) in human fibroblasts. *Ann Rheum Dis* 1998;57:56-60.
9 Mitchell PG, Struve JA, McCarthy GM, Cheung HS. Basic calcium phosphate crystals stimulate cell proliferation and collagenase message accumulation in cultured adult articular chondrocytes. *Arthritis Rheum* 1992;35:343-50.
10 Garancis JC, Cheung HS, Halverson PB, McCarty DJ. Milwaukee shoulder: association of microspheroids containing hydroxyapatite crystals, active collagenase and neutral protease with rotator cuff defects. iii. Morphologic and biochemical studies of an excised synovium showing chondromatosis. *Arthritis Rheum* 1981;24:484-91.
11 Cheung HS, Story MT, McCarty DJ. Mitogenic effects of hydroxyapatite and calcium pyrophosphate dihydrate crystals on cultured mammalian cells. *Arthritis Rheum* 1984;27:668-74.
12 Pelletier J-P, Roughley P, DiBattista J, McCollum R, Martel-Pelletier J. Are cytokines involved in osteoarthritic pathophysiology? *Semin Arthritis Rheum* 1991;20(suppl 2):12-25.
13 Mitchell PG, Cheung HS. Tumor necrosis factor α and epidermal growth factor regulation of collagenase and stromelysin in adult porcine articular chondrocytes. *J Cell Physiol* 1991;149:132-40.
14 Shlopov B, Lie W-R, Mainardi C, Cole A, Chubinskaya S, Hasty K. Osteoarthritic lesions: involvement of three different collagenases. *Arthritis Rheum* 1997;40:2065-74.
15 Wernicke D, Seyfert C, Hinzmann B, Gromnica-Ihle E. Cloning of collagenase 3 from the synovial membrane and its expression in rheumatoid arthritis and osteoarthritis. *J Rheumatol* 1996;23:590-5.
16 Reboul P, Pelletier J-P, Tardif G, Cloutier J-M, Martel-Pelletier J. The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis. *J Clin Invest* 1996;97:2011-19.
17 Mitchell P, Magna H, Reeves L, Lopresti-Morrow L, Yocum S, Rosner P, et al. Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. *J Clin Invest* 1996;97:761-8.
18 Whitham SE, Murphy G, Angel P, Rhamdsdorf HJ, Smith BJ, Lyons A, et al. Comparison of human stromelysin and collagenase by cloning and sequence analysis. *Biochem J* 1986;240:913-16.
19 Tso JY, Sun XH, Kao TH, Reece KS, Wu R. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenases cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res* 1985;13:2485-502.
20 Evans RW, Cheung HS, McCarty DJ. Cultured human monocytes and fibroblasts solubilize calcium phosphate crystals. *Calcif Tissue Int* 1984;36:645-50.
21 Watt F. Effect of seeding density on stability of the differentiated phenotype of pig articular chondrocytes in culture. *J Cell Sci* 1988;89:373-8.
22 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
23 Davis LG, Dibner MD, Battey JF. Formaldehyde gel for electrophoretic separation of RNA and northern blot. In: *Basic methods in molecular biology*. New York: Elsevier, 1986:143-6.
24 Feinberg AP, Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1983;132:6-9.
25 MacNaul KL, Chartrain N, Lark M, Tocci MJ, Hutchinson NI. Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinases-1 in rheumatoid human synovial fibroblasts. Synergistic effects of interleukin-1 and tumour necrosis- α on stromelysin expression. *J Biol Chem* 1990;265:17238-45.
26 Freije JMP, Diez-Itza I, Balbin M, Sanchez LM, Blasco R, Tolivia J, et al. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. *J Biol Chem* 1994;269:16766-73.
27 Rimm AA, Hartz AJ, Kalbfleisch JH, Anderson AJ, Hoffmann RG. Nonparametric techniques. In: *Basic biostatistics in medicine and epidemiology*. New York, NY: Appleton-Century-Crofts, 1980:267-81.
28 Pendas A, Balbix M, Llano E, Jimenez M, Lopez-Otin C. Structural analysis and promoter characterization of the human collagenase-3 gene (MMP13). *Genomics* 1997;40:222-33.

- 29 McCarthy GM, Augustine JA, Baldwin AS, Christopherson PA, Cheung HS, Westfall PR, *et al*. Molecular mechanism of basic calcium phosphate crystal-induced activation of human fibroblasts. Role of nuclear factor κ B, activator protein 1 and protein kinase C. *J Biol Chem* 1998;273:35161–9.
- 30 Lindy O, Konttinen Y, Sorsa T, Ding Y, Santavirta S, Ceponis A, *et al*. Matrix metalloproteinase 13 (collagenase 3) in human rheumatoid synovium. *Arthritis Rheum* 1997;40:1391–9.
- 31 Imai S, Konttinen Y, Jumppanen M, Lindy O, Ceponis A, Kempainen P, *et al*. High levels of expression of collagenase-3 (MMP-13) in pathological conditions associated with a foreign-body reaction. *J Bone Joint Surg Br* 1998;80:701–10.
- 32 Westhoff C, Freudiger D, Petrow P, Seyfert C, Zacher J, Kriegsmann J, *et al*. Characterization of collagenase 3 (matrix metalloproteinase 13) messenger RNA expression in the synovial membrane and synovial fibroblasts of patients with rheumatoid arthritis. *Arthritis Rheum* 1999;42:1517–27.
- 33 Vincenti M, Coon C, Mengshol J, Yocum S, Mitchell P, Brinckerhoff C. Cloning of the gene for interstitial collagenase-3 (matrix metalloproteinase-13) from rabbit synovial fibroblasts: differential expression with collagenase-1 (matrix metalloproteinase-1). *Biochem J* 1998;331:341–6.
- 34 Borden P, Solymar D, Sucharczuk A, Lindman B, Cannon P, Heller R. Cytokine control of interstitial collagenase and collagenase-3 gene expression in human chondrocytes. *J Biol Chem* 1996;271:23577–81.
- 35 Melchiorri C, Meliconi R, Frizziero L, Silvestri T, Pulsatelli L, Mazzetti I, *et al*. Enhanced and coordinated in vivo expression of inflammatory cytokines and nitric oxide synthase by chondrocytes from patients with osteoarthritis. *Arthritis Rheum* 1998;41:2165–74.
- 36 Schumacher HR. Synovial inflammation, crystals, and osteoarthritis. *J Rheumatol* 1995;22(suppl 43):101–3.
- 37 Pelletier J-P, Caron JP, Evans C, Robbins PD, Georgescu HI, Jovanovic DEA. In vivo suppression of early experimental osteoarthritis by interleukin-1 receptor antagonist using gene therapy. *Arthritis Rheum* 1997;40:1012–19.