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

Objectives—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 24 hours compared with untreated control cultures (p < 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 undetected 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 (IL1α; twofold).

Conclusion—These data confirm the ability of BCP 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 IL1α and TNF-α to promote MMP production and subsequent joint degeneration.

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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 TNFα, 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 TNFα and IL1 induced MMP-1 production.

**Materials and methods**

**PROBES AND REAGENTS**

The MMP-13 probe was a full length human cDNA. The MMP-1 probe was a 2.05 kb HindIII-EcoR1 insert from the pCI1ase 1 clone, obtained from the repository of human DNA probes of the American Type Culture Collection (Rockville, MD). The pHcGAP DNA probes of the American Type Culture Collection (Rockville, MD). Recombinant TNFα acetate were from Sigma Chemical Co (St Louis, MO). Cloheximide and o-tetradecanoylphorbol-13-acetate were from Sigma Chemical Co (St Louis, MO). Cy- tochrome b5 reductase was from Goerz, Germany. The MMP-1 antibody was a polyclonal antibody raised in rabbit against purified human MMP-1 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 PIY IB-8-C5) (Pierce Central Research). The MMP-1 antibody was a polyclonal antibody raised in rabbit against purified human MMP-1 from gingival fibroblasts. Biotin labelled, goat antirabbit IgG and peroxidase labelled streptavidin were from Kerkogard and Perry Laboratories, Inc (Gaithersburg, MD). Enhanced chemiluminescence was performed using a kit from Amersham Life Sciences (Buckinghamshire, UK). Tritiated thymidine (50 Ci/mmole) was from Amersham Life Sciences (Buckinghamshire, UK). Formaxol (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 PIY IB-8-C5) (Pierce Central Research). The MMP-1 antibody was a polyclonal antibody raised in rabbit against purified human MMP-1 from gingival fibroblasts. Biotin labelled, goat antirabbit IgG and peroxidase labelled streptavidin were from Kerkogard and Perry Laboratories, Inc (Gaithersburg, MD). Enhanced chemiluminescence was performed using a kit from Amersham Life Sciences (Buckinghamshire, UK). Tritiated thymidine (50 Ci/mmole) was from Amersham Life Sciences (Buckinghamshire, UK).

**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 TNFα or epidermal growth factor. Chondrocytes were prepared by digestion of cartilage from adult porcine knees as described previously. The cells were plated at 4 x 10^5/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.

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 in DMEM, 0.1% 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.
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 TriReagent reagent based on the methods of Chomczynski and Sacchi. 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 $^3$H were determined in quadruplicate. Control cultures were in 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0 at 60°C for 30 minutes.

Immuno blotting by HOAS was confirmed by western blot. Briefly, samples of conditioned media from cultures treated with BCP crystals, TNF-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.
before being stimulated with BCP crystals (18 µg/cm2 incubated in 0.5% fetal bovine serum–Dulbecco’s modified Eagle’s medium for 24 hours.

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 stimulated with BCP crystals; IL1 = interleukin 1 (10 ng/ml) or interleukin 1 (1 µCi/ml) for one hour. The plates were then 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 containing 0.5% fetal bovine serum (FBS). T wenty four hours later, fresh DMEM containing 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]methionine (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<0.05).

Figure 3 Mitogenic effects of basic calcium phosphate (BCP) crystals in human osteoarthritic synovial fibroblasts. Confluent, monolayer 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<0.05).

Figure 2 Northern blot analysis of MMP-1 mRNA levels. (a) GAPDH and MMP-1 mRNA positions of the 18S and 28S ribosomes are shown. C = unstimulated control cultures; B = untreated or treated with tumour necrosis factor (1 µg/ml) or interleukin 1 (10 ng/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<0.05).

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)20 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'-AATCCTCCCTCACAAGAT GTCAGCA-3’, 5’-TCCACACCCCTGTGCT CTTGTA-3’, resulting in a 553 bp product;
Induction of matrix metalloproteinase in porcine articular chondrocytes

Although low levels of MMP-13 mRNA were apparent in samples from unstimulated 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 µg/ml) were added concurrently, BCP crystal induced MMP-1 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, immunoprecipitatable with a polyclonal antibody to MMP-13, was induced at all concentrations tested (fig 2). No protein was not evident in unstimulated control cultures.

BCP CRYSTAL INDUCTION OF MITOGENESIS IN HOAS

The addition of BCP crystals (25 µg/cm²) 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<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 (>10⁵/cm²) in control cultures incubated with 0.5% FBS was 1.65 (0.3). The number of cells (>10⁵/cm²) 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<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 TNFα (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 IL1α or TNFα, there was significant MMP-1 protein secretion, which was maximal at 48 hours, and increased when HOAS were co-incubated with BCP crystals.
Table 7

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<th>Condition</th>
<th>MMP-13</th>
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<tr>
<td>TNF-α</td>
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<td>IL-1β</td>
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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 μg/cm² analysis for MMP-13 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR fibroblasts. Total RNA was isolated from synovial fibroblasts and subjected to RT-PCR matrix metalloproteinase-13 (MMP-13) mRNA in human osteoarthritic synovial fibroblasts. 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 demonstrated in human articular cells. We also show here that BCP crystals induce cell replication and MMP-1 protein secretion 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.  In HOAS we found that MMP-13 mRNA accumulation was detectable only by RT-PCR in response to BCP crystals, TNF-α, or IL-1β. No MMP-13 protein secretion was identified, presumably because quantitative comparisons 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 transcriptional activation of MMP-13 by BCP. Structural analysis of the 5’ flanking region of the MMP-13 gene has shown the presence of an AP1 motif.  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. 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, IL-1β, and IL-1α.  Reported synoviocyte 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.  The overall expression of MMP-13 in the synovial stroma was substantially higher in RA than in OA.  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. MMP-13 mRNA expression was detected in the synovial membranes of 21 of 36 patients with RA, and increased synoviocyte MMP-13 expression correlated with increases in the clinical measurements of erythrocyte sedimentation rate and C reactive protein.  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 IL1β or TNF-α.  Finally, Vincenti et al have shown that MMP-13 mRNA and protein is inducible in rabbit synovial fibroblasts by IL1β, TNF-α, or phorbol esters.  In contrast, however, Reboul et al showed MMP-13 expression by RT-PCR in OA chondrocytes but not HOAS.  Borden et al studied patients with RA and found MMP-13 gene expression in RA chondrocytes but not RA synovial fibroblasts.  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 derived from synoviocytes. 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 synoviocytes.

Furthermore, enhanced and coordinated expression of IL1β, TNF-α, and inducible nitric oxide synthase by chondrocytes compared with HOAS has previously been seen in OA.  We have shown here that the production of MMP-13 in response to BCP crystals is 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. 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. 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. 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 and TNF appear to act in synergy to increase MMP-1 production by HOAS in vivo, because BCP crystals and cytokines such as TNF and IL1 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. Furthermore, as with BCP crystals, levels of cytokines such as IL1 or TNF are not routinely measured in joint fluid from patients with arthritis. After considerable further investigation, however, the roles of IL1 and TNF in mediating joint degeneration in OA are now well accepted. As a consequence of such recognition, Pelle- tier and coworkers have prevented the development of OA in an experimental model by transfer of the IL1 receptor antagonist gene. Efforts continue to discover methods to inhibit the pathogenic effects of IL1 and TNF. 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, in more studies of the molecular mechanism of BCP crystal mediated cell activation are required.

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