Basic calcium phosphate crystals induce synthesis and secretion of 92 kDa gelatinase (gelatinase B/matrix metalloprotease 9) in human fibroblasts

Geraldine M McCarthy, Allison M Macius, Pamela A Christopherson, Lawrence M Ryan, Tayebah Pourmotabbed

Abstract

Objective—Synovial fluid basic calcium phosphate (BCP) crystals are associated with severe joint degeneration accompanied by synovial hypertrophy. The metalloprotease 92 kDa gelatinase (MMP-9) has been implicated in the degradation of extracellular matrix in osteoarthritis, but the ability of BCP crystals to induce gelatinase in human fibroblasts or in adult porcine chondrocytes has not previously been studied. The hypothesis that the mitogenic response to BCP crystals is accompanied by induction and secretion of MMP-9 was studied.

Methods—MMP-9 messenger RNA (mRNA) was detected by northern blot and reverse transcription-polymerase chain reaction (RT-PCR). Gelatinase secretion was identified by western blot and zymography of conditioned media.

Results—BCP crystals caused a concentration dependent induction of MMP-9 mRNA accumulation and protein secretion in human fibroblasts but not in adult porcine chondrocytes.

Conclusion—BCP crystals induce MMP-9 production by HF but not adult porcine chondrocytes. Fibroblast MMP-9 may be an important mediator of the joint destruction associated with synovial fluid BCP crystals.

(Ann Rheum Dis 1998;57:56-60)

cuff. Large, non-inflammatory joint effusions and pronounced synovial proliferation with frequent formation of pedunculated loose bodies are typical.

The biological effects of BCP crystals that may promote articular damage have been investigated in vitro. BCP crystals induce mitogenesis in a number of cell types, including fibroblasts and chondrocytes.² This property presumably accounts for the synovial proliferation characteristic of both Milwaukee Shoulder Syndrome and BCP crystal associated OA. BCP crystals also induce coordinate synthesis of the metalloproteases collagenase and stromelysin in human fibroblasts.³ Secretion of these enzymes permits degradation of many matrix components including type II collagen, fibronectin, laminin, and proteoglycan. These two enzymes have been postulated to mediate, at least in part, the joint destruction associated with the presence of BCP crystals in SF.³

The 92 kDa gelatinase (gelatinase B/matrix metalloprotease 9) (MMP-9), a 92 kDa metalloprotease, has also recently been implicated in the degradation of extracellular matrix in joint degeneration as it is expressed in osteoarthritic, but not in normal, human articular cartilage.⁴ It is also inducible in normal human cartilage and in normal human fibroblasts treated with interleukin1 (IL1).⁴ Gelatinases may influence the breakdown of cartilage in OA by accelerating degradation of matrix components or by digesting matrix molecules such as types IV, V, and XI collagens as well as denatured collagens (gelatins).⁴ Although MMP-9 may contribute to the pathogenic effects of BCP crystals in joint degradation, the ability of BCP crystals to induce gelatinase in human fibroblasts or cartilage has not previously been studied. We hypothesised that the mitogenic response to BCP crystals is accompanied by the induction and secretion of MMP-9 in both human fibroblasts and adult porcine chondrocytes.

Methods

CRYSTAL SYNTHESIS

BCP crystals were synthesised by a modification of published methods.⁵ 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

Department of
Medicine, Division of
Rheumatology,
Medical College of
Wisconsin, Milwaukee,
Wisconsin

G M McCarthy
A M Macius
P A Christopherson
L M Ryan

Department of
Biochemistry, The
University of
Tennessee, Memphis,
Tennessee

T Pourmotabbed

Correspondence to:
Dr G M McCarthy, Division
of Rheumatology, Froedtert
Hospital, East 650, 9200 W
Wisconsin Ave, Milwaukee,
WI 53226, USA.

Accepted for publication
1 October 1997

Concurrence of deposits of calcium containing crystals such as basic calcium phosphate (BCP) (hydroxyapatite, tricalcium phosphate, and octacalcium phosphate) and degenerative joint disease is well established. The prevalence of BCP crystals in synovial fluid (SF) from patients with knee osteoarthritis (OA) is between 30 and 60%.¹ Ample data support the role of these crystals in cartilage degeneration as their presence correlates strongly with radiographic evidence of cartilage degeneration and larger joint effusions are seen in affected knee joints when compared with joint fluid from OA knees without crystals.¹ The pathogenicity of these crystals is further supported by their association with a distinctive type of non-inflammatory destructive arthropathy affecting the shoulders and knees called Milwaukee Shoulder Syndrome.¹ Milwaukee Shoulder Syndrome is characterised by severe glenohumeral degeneration with loss of articular cartilage and dissolution of the rotator

sieved to yield 10–20 micron aggregates, which were sterilised and rendered pyrogen free by heating at 200°C for 90 minutes. Crystals were weighed and suspended by sonication in Dulbecco's modified Eagle's media (DMEM). The amounts of crystals used were based on our previous studies of dose response relations between BCP crystals and the mitogenic response.² In those studies, maximal mitogenic responses were achieved with BCP concentrations of 50–100 µg/ml. These values are equivalent to concentrations of BCP crystals found in pathologic joint fluids.

PROBES AND REAGENTS

The MMP-9 complementary DNA (cDNA) probe was a 2.3 Kb EcoRI fragment from the pETNG clone.⁶ The pHcGAP plasmid containing glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA was from the American Type Culture Collection.⁷ The MMP-9 antibody was a polyclonal antibody raised in rabbits against a synthetic peptide of MMP-9.⁸ Enhanced chemiluminescence was performed with a kit from Amersham (Arlington Heights, IL). Type I gelatin from porcine skin was from Sigma Chemical Co, St Louis, MO. Recombinant tumour necrosis α (TNF α) and interleukin 1 α (IL1) were obtained from Genzyme Corporation (Boston, MA). Cycloheximide and 12-*o*-tetradecanoylphorbol 13-acetate (TPA) were from Sigma (St Louis, MO). Fetal bovine serum (FBS), Hanks's balanced salt solution, serumless media (Neuman and Tytell), and DMEM were from Gibco (Grand Island, NY).

CELL CULTURE

A model system of human foreskin fibroblast cultures was used for these experiments as their responses to BCP crystals have been shown to be similar to those of synovial fibroblasts.² Human fibroblast cultures were established from explants, transferred, grown and maintained as previously described.⁹ 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 or fourth passage cells.

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 or epidermal growth factor.^{10,11} Chondrocytes were prepared by digestion of cartilage from adult porcine knees as described previously.¹⁰

PREPARATION OF RNA AND NORTHERN BLOT ANALYSIS

One 100 mm plate was harvested for each human fibroblast analysis and one 60 mm plate for each chondrocyte analysis. Total RNA was recovered, 10 µg fractionated, transferred to nitrocellulose filters, and northern blot analysis performed as previously described.³ Probes were labelled to a specific activity of $>5 \times 10^8$

counts per min/µg. Autoradiography was performed with Kodak XAR-5 film (Eastman Kodak, Rochester, NY), and signal intensity was quantified by densitometry, using a scanning laser densitometer (LKB Instruments, Stockholm, Sweden).

IMMUNOBLOTTING

Gelatinase protein secretion into conditioned media was confirmed by western blot.³ Samples of conditioned media (20 µl) were electrophoresed through 10% polyacrylamide gels under reducing conditions, transferred onto nitrocellulose membranes, and western blot was performed using a 1:1000 dilution of anti-MMP-9 antibody as described previously.³ Immunoreactive bands were detected using enhanced chemiluminescence reagents (Amersham Corp) and autoradiography.

ENZYME ACTIVITY

Gelatinase enzyme activity in conditioned media was determined using gelatin substrate gels.¹² Aliquots (115 µl) of conditioned media were electrophoresed on 10% polyacrylamide gels containing 1 mg/ml type I gelatin under non-reducing conditions. After electrophoresis, gels were washed in 2.5% Triton X-100 for one hour to allow proteins to renature. Gels were then incubated overnight in substrate buffer (50 mM TRIS, pH 8.0, 150 mM NaCl, 10 mM CaCl₂, 1 µM ZnCl₂, 0.02% Na azide) at 37°C, rinsed in water, and stained in 0.5% w/v Coomassie brilliant blue in 10% acetic acid and 30% isopropyl alcohol. Destained gels showed a clear zone of lysis where gelatin degradation, an index of gelatinase activity, had occurred.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA (2 µg) was converted to cDNA by incubation with random hexamer primers as described previously.¹² The MMP-9 cDNA was detected by amplifying a 600 bp 92 kDa gelatinase fragment cDNA from the library (2.5 µl) by PCR using two specific 92 kDa gelatinase primers (5'-CCTCCTGGCCCC GGCATTC AGGGA CACCCCAT and 5'-ATGATCTAAGCCCCAGCGCGTGGCCG AACCCATGCGCCGCCAC, the 5' and 3' primers respectively), as described previously.¹² The total RNA isolated from human chronic granulocytic leukaemia cells was used as a positive control.⁶ The PCR product was analysed by electrophoresis on a 1.0% agarose gel.

Results

BCP CRYSTAL INDUCTION OF MMP-9 MRNA

While BCP concentrations as low as 9 µg/cm² resulted in significant accumulation of MMP-9 mRNA, a positive dose responsiveness was demonstrated with maximal accumulation seen after stimulation with 18 or 36 µg/cm² (fig 1). BCP crystal induced accumulation of MMP-9 mRNA in HF was detectable within 16 hours after stimulation and continued for at least 24 hours (data not shown). TPA, a known inducer of MMP-9,¹³ served as a positive control. As it has been demonstrated that new protein synthesis is required for accumulation of

Figure 1 BCP crystal induced accumulation of 92 kDa gelatinase (MMP-9) messenger RNA (mRNA). Confluent cultures of human fibroblasts in 100 mm plates were incubated in DMEM and 0.5% FBS for 24 hours and were then stimulated with BCP crystals in the quantities shown. Control cultures were stimulated with TPA (200 nm) or left unstimulated. BCP crystals were also added to some cultures treated with the protein synthesis inhibitor, cycloheximide (CHX, 10 μ g/ml). After 24 hours, all plates were harvested and total RNA isolated. Northern blot analysis was performed using a specific cDNA probe for MMP-9 (GL) followed by autoradiography. The MMP-9 probe was then removed by washing, and the blot was re-probed with glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA, to ensure analysis of equivalent quantities of total mRNA under each condition. The positions of the 18S and 28S ribosomal bands are shown.

collagenase and stromelysin mRNAs in response to BCP crystals and to other stimuli,³ we studied the effect of protein synthesis inhibition by cycloheximide. When cycloheximide (10 μ g/ml) and BCP crystals (36 μ g/cm²) were added to human fibroblasts concurrently, there was complete inhibition of BCP crystal induced MMP-9 mRNA accumulation 24 hours after stimulation (fig 1).

In contrast, no accumulation of MMP-9 mRNA was seen in either untreated adult porcine chondrocytes or in adult porcine chondrocytes 24 hours after treatment with BCP crystals (at various concentrations), TNF or TPA (data not shown). Using PCR, constitutive expression of MMP-9 mRNA in chondrocytes was identified. Treatment with BCP crystals, IL1, TNF or TPA did not affect the level of MMP-9 mRNA expression (data not shown).

IDENTIFICATION OF MMP-9 PROTEIN IN CONDITIONED MEDIA FROM CULTURES TREATED WITH BCP CRYSTALS

Figure 2 shows the induction by BCP crystals, TPA or TNF of the secretion of a protein of molecular weight 92 kDa, immunoreactive

Figure 2 BCP crystal induced stimulation of MMP-9 synthesis. Confluent, quiescent human fibroblast cultures incubated in DMEM containing 0.5% FBS were stimulated with either BCP crystals (B) at varying concentrations, TPA (200 nm) or TNF (10 ng/ml); fibroblasts in 0.5% FBS alone (C) were used as a control. The culture medium was harvested after 48 hours and analysed by western blotting using a specific polyclonal antibody to MMP-9. Molecular weight markers are shown on the left.

with a polyclonal antibody to MMP-9, into conditioned media. The secretion of this protein was maximum 48 hours after stimulation, the longest incubation tested. This protein was not evident in control cultures incubated in 0.5% FBS alone. In contrast, in adult porcine chondrocytes there was no evidence of secretion of a 92 kDa protein, immunoreactive with a polyclonal antibody to MMP-9, into conditioned media when cultures were either unstimulated or treated with BCP crystals, TPA, TNF or 10% FBS (data not shown).

IDENTIFICATION OF MMP-9 ENZYME ACTIVITY IN CONDITIONED MEDIA FROM CULTURES TREATED WITH BCP CRYSTALS

To assess gelatinase activity in the conditioned media from human fibroblast cultures stimulated with BCP crystals, aliquots of conditioned media were analysed by SDS-PAGE on polyacrylamide gels containing gelatin substrate (fig 3). Conditioned media from cultures treated with TPA were used for comparison. Bands of lytic activity at 92 kDa consistent with MMP-9 were seen in conditioned media from human fibroblast cultures treated with BCP crystals or TPA. No lytic activity at 92 kDa was found in conditioned media taken from unstimulated control cultures. Lytic activity at 72 kDa resulted under all conditions consistent with 72 kDa gelatinase but seemed to be increased in the conditioned media of cultures treated with BCP crystals, TNF or TPA.

In contrast, in adult porcine chondrocytes there was no evidence of lytic activity at 92 kDa in conditioned media from cultures treated with TPA, IL1 or TNF although lytic activity at 72 kDa was apparent under all conditions (fig 3).

Discussion

This is the first demonstration of the induction of MMP-9 mRNA, followed by production and secretion of active MMP-9 enzyme, in human fibroblasts treated with BCP crystals. The time course of induction indicated that it occurred in coordination with collagenase and stromelysin.³ Connective tissue gelatinases are

Figure 3 Gelatinase activity in human fibroblast and adult porcine chondrocyte conditioned media. Confluent, quiescent human fibroblast cultures were stimulated with BCP crystals at varying concentrations for 24 hours. Control cultures were in DMEM containing 0.5% FBS (C) or TPA (200 nm). Confluent, quiescent cultures of chondrocytes in 60 mm plates were stimulated with BCP crystals (20 $\mu\text{g}/\text{cm}^2$), TNF (10 ng/ml), TPA (200 nm) or IL1 (100 u/ml) for 48 hours. Untreated control cultures remained in serum free media. The conditioned media was then collected and analysed by electrophoresis on a 10% polyacrylamide gel containing 1 mg/ml gelatin. After overnight incubation at 37°C, the gels were stained with Coomassie brilliant blue. Digestion of the gelatin substrate is seen as clear areas of lysis. Molecular weight markers are shown at the left. The positions of the 92 and 72 kDa gelatinases are shown to the right.

thought to be important in both normal matrix turnover and in pathological remodelling of cartilage because of their ability to act synergistically with collagenase and stromelysin in the degradation of the macromolecules of extracellular matrices.⁴ Both collagenase and neutral protease activity in synovial fluid from patients with Milwaukee Shoulder Syndrome was reported in the original and subsequent descriptions of this arthropathy.¹ The neutral protease activity was probably caused by stromelysin but unfortunately, the presence of gelatinase was not evaluated at that time.

We were unable to demonstrate accumulation of MMP-9 mRNA in normal adult porcine chondrocytes treated with BCP, TPA, IL1 or TNF; nor was there evidence of production or secretion of MMP-9, or both, in conditioned media as assessed by western blot and zymogram. Using PCR, constitutive expression of MMP-9 mRNA was identified in adult porcine chondrocytes but it was not increased after treatment with either BCP crystals, TNF, TPA or IL1. The chondrocyte data reported here contrast with studies of articular chondrocytes from human osteoarthritic cartilage in which MMP-9 mRNA was found with associated secretion of active enzyme. Expression of MMP-9 was increased in fibrillated cartilage. Active MMP-9 was also identified in the concentrated conditioned media from normal human articular chondrocytes treated with IL1.⁴

Such variation of expression of MMP-9 between APC and human osteoarthritic cartilage and human articular chondrocytes may reflect interspecies differences. None the less, adult porcine chondrocytes are capable of producing other MMPs as we have shown the induction of collagenase in adult porcine chondrocytes treated with BCP crystals.¹⁰ TNF

and epidermal growth factor both induce collagenase and stromelysin in adult porcine chondrocytes.¹¹ However, our data suggest that, in vivo, synovial fibroblasts are probably the most important source of intra-articular MMPs resulting in matrix degradation associated with BCP crystals and that chondrocytes are unlikely to produce biologically significant quantities of MMP-9. Although neutrophils secrete MMP-9,⁸ BCP crystal associated joint effusions contain few of these cells. Therefore, neutrophils are a possible but improbable source of MMP-9 in BCP crystal associated arthropathies.

In this study, there was constitutive production of 72 kDa gelatinase in human fibroblast conditioned media as assessed by western blot and in human fibroblasts and adult porcine chondrocytes as assessed by zymography. Enzyme activity in human fibroblasts, but not adult porcine chondrocytes conditioned media seemed to be slightly increased after treatment with BCP crystals, TNF or TPA. Although recent studies have shown that both 92 kDa and 72 kDa gelatinases have similar substrate specificity for type IV and V collagens as well as gelatin, the full complement of substrates of either gelatinase has yet to be explored.¹⁴ Because of this, the relative intensities of the zones of lysis created by the 72 and 92 kDa gelatinases on the gelatin substrate gel may be misleading in terms of their physiological significance.

Although MMP-9 mRNA accumulation and protein secretion in response to BCP crystals was less than that noted in response to TPA, enzyme activity in conditioned media from both TPA and BCP crystal treated cultures was comparable. The level of induction of MMP-9 in fibroblasts in response to BCP crystals is probably biologically significant. The data presented in this paper support our hypothesis that the mitogenic response to BCP crystals is accompanied by 92 kDa gelatinase induction and secretion. The coordinate induction and subsequent secretion of 92 kDa gelatinase, collagenase and stromelysin, probably from synovial fibroblasts, may underlie the pronounced, non-inflammatory degradation of cartilage and other matrix rich joint structures found in association with synovial fluid BCP crystals.

This study was supported by grants from the American Federation for Aging Research and the National Institutes of Health AR-01953 (GMC), AR-38656 (LMR) and AR-41843 (TP).

- 1 Halverson PB, McCarty DJ. Basic calcium phosphate (apatite, octacalcium phosphate, tricalcium phosphate) crystal deposition diseases; calcinosis. In: Koopman WJ, ed. *Arthritis and allied conditions. A textbook of rheumatology*. Baltimore, MD: Williams and Wilkins, 1997:2127-46.
- 2 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.
- 3 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.
- 4 Mohtai M, Lane Smith R, Schurman DJ, Tsuji T, Torti FM, Hutchinson NI, et al. Expression of 92-kD type IV collagenase/gelatinase (gelatinase B) in osteoarthritic cartilage and its induction in normal human articular cartilage by interleukin 1. *J Clin Invest* 1993;92:179-85.
- 5 Evans RW, Cheung HS, McCarty DJ. Cultured human monocytes and fibroblasts solubilize calcium phosphate crystals. *Calcif Tissue Int* 1984;36:645-50.

- 6 Pourmotabbed T, Solomon TL, Hasty KA, Mainardi C. Characteristics of 92 kDa type IV collagenase/gelatinase produced by granulocyte leukemia cells: structure, expression of cDNA in *E. Coli* and enzymic properties. *Biochem Biophys Acta* 1994;1204:97-107.
- 7 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.
- 8 Hibbs MS, Hasty KA, Seyer MJ, Kang AH, Mainardi CL. Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. *J Biol Chem* 1985;260:2493-500.
- 9 McCarthy GM, Mitchell PG, Cheung HS. The mitogenic response to stimulation with basic calcium phosphate crystals is accompanied by induction and secretion of collagenase in human fibroblasts. *Arthritis Rheum* 1991;34:1021-30.
- 10 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.
- 11 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.
- 12 Bu CH, Pourmotabbed T. Mechanism of Ca^{2+} -dependent activity of human neutrophil gelatinase B. *J Biol Chem* 1996;271:14308-15.
- 13 Huhtala P, Tuuttila A, Chow LT, Lohi J, Keski-Oja J, Trygvalson K. Complete structure of the human gene for 92-kDa type IV collagenase. Divergent regulation of expression for the 92- and 72-kilodalton enzyme genes in HT-1080 cells. *J Biol Chem* 1991;266:16485-90.
- 14 Unemori EN, Hibbs MS, Amento EP. Constitutive expression of a 92-kD gelatinase (type V collagenase) by rheumatoid synovial fibroblasts and its induction in normal human fibroblasts by inflammatory cytokines. *J Clin Invest* 1991;88:1656-62.