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ROLE OF CALCIUM-CONTAINING CRYSTALS IN OSTEOARTHRITIS

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1. ABSTRACT

The deposition of calcium-containing crystals in articular tissues is probably an underrecognized event. Clinical observations indicate that exaggerated and uniquely distributed cartilage degeneration is associated with these deposits. Perhaps the most compelling argument favoring a role for crystals in causing osteoarthritis stems from their in vitro effects on articular tissues. In this review, we will highlight some of the recent findings that further reinforce the thesis that basic calcium phosphate (**BCP**) and calcium pyrophosphate dihydrate (**CPPD**) crystals can cause the degeneration of articular tissues in 2 separate pathways. In the "Direct" pathway, crystals directly induce fibroblast-like synoviocytes to proliferate and produce metalloproteinases and prostaglandins. The other "Paracrine Pathway" involves the interaction between crystals and macrophages/monocytes which leads to synthesis and release of cytokines which can reinforce the action of crystals on synoviocytes and/or induce chondrocytes to secrete enzymes and which eventually cause the degeneration of articular tissues.

2. INTRODUCTION

Crystalline calcium pyrophosphate dihydrate (**CPPD**) and basic calcium phosphate (**BCP**) are the 2 most common forms of pathologic articular mineral. Each occurs frequently in OA joints, and each may be phlogistic, causing acute attacks of pseudogout in the case of CPPD crystals and acute calcific periarthritis in the case of BCP crystals (1;2;3).

Evidence for a causal role of crystals in cartilage degeneration is primarily inferential, based on correlative data. However, clinical observations and experimental evidence of their *in vitro* effects support the thesis that articular crystals promote cartilage degeneration. More definitive investigations of causality are impeded by the lack of a suitable animal model for studying non-inflammatory aspects of crystal deposition, and the slow pace of degeneration (3;4).

Correlative data indicate that CPPD and BCP crystals are more common in degenerative joints than in normal joints or joints affected with inflammatory forms of arthritis. Conversely, OA is both more common and more severe in patients with calcium-containing crystals. One or both crystals are present in up to 60% of fluid from knees of patients with advanced OA (5-7;7;8). The presence of CPPD crystals predicts a poor clinical and radiographic outcome (9).. Similarly a prospective study of radiographic chondrocalcinosis in Slovakian kindred with autosomal dominant CPPD deposition demonstrated that radiographic evidence of crystal deposition antedated radiographic evidence of degeneration (7). Although there is no satisfactory animal model of crystal deposition disease, there are useful models of OA. In the 2 studies of the anterior cruciate injury model, BCP crystals have been detected early, at a time when they might contribute to ongoing cartilage damage (6;10).

Studies of synovial fluid containing CPPD crystals suggest associated chondrolytic activity in the involved joints, usually of a greater magnitude than seen in fluids from patients with OA but no crystals. Patients with acute "pyrophosphate arthropathy" (CPPD in joint fluids and OA) had the highest synovial fluid proteoglycan fragment concentrations of 6 disease categories evaluated (6;11-13). Moreover, these CPPD-containing fluids also had the highest levels of matrix metalloproteinase (**MMP**)-1, MMP-3, and the highest ratio of MMP3: tissue inhibitor of metalloproteinase (**TIMP**), all factors, which would support increased matrix catabolism (13). Although these studies do not prove a causative role of the crystals in producing OA, they strongly support an association of accelerated matrix degradation with the presence of calcium-containing crystals.

Perhaps the most compelling argument favoring a role for crystals in causing osteoarthritis stems from their *in vitro* effects on articular tissues. Crystals can cause the

degeneration of articular tissues in two separate pathways. In the "**Direct**" pathway, crystals directly induce fibroblast-like synoviocytes to proliferate and produce metalloproteinases and prostaglandins (**PGE_2**). The other "**Paracrine or Indirect Pathway**" centers on the interaction between crystals and macrophages/monocytes which leads to synthesis and release of cytokines which can reinforce the action of crystals on synoviocytes and/or induce chondrocytes to secrete enzymes and which eventually cause the degeneration of articular tissues (3).

3. "DIRECT PATHWAY"

Much of the research was stimulated by recognition of the Milwaukee shoulder syndrome as a paradigm for non-inflammatory yet destructive arthritis associated with synovial fluid BCP crystals and active proteases(14;15).. Subsequent studies revealed that both BCP and CPPD crystals could elicit mitogenesis, synthesis and secretion of proteases, and synthesis and secretion of PGE_2 by articular tissues(3).

3.1. Crystal-induced mitogenesis

Synovial lining proliferation is observed in crystal-associated arthritis (14) and the crystals themselves induce mitogenesis in this tissue (16;17). The increased cellularity of the synovium in turn enhances the capacity for cytokine and protease secretion, both of which can promote chondrolysis.

A variety of calcium-containing crystals stimulate (^{3}H)-thymidine incorporation into phagocytic cells, whereas other crystals have weak or no mitogenic properties (16;18). Particularly well studied has been the mitogenic response to BCP crystals. At concentrations routinely observed in pathologic joint fluids, these crystals are endocytosed and then are solubilized in the acidic environment of the phagolysosome. Mitogenesis induced by these crystals can be inhibited by preventing endocytosis or by raising lysosomal pH. The latter effect can be produced by lysosomotropic agents NH_4Cl or chloroquine (19) or by the specific vacuolar ATPase inhibitor, bafilomycin (20).. Cells that are exposed to such inhibitors remain responsive to other mitogenic stimuli, e.g. serum.

Another way by which calcium crystals induce mitogenesis may involve diacylglycerol (DAG), a hydrolysis product of PLC action on phosphatidylinositol 4,5-bisphosphate. Elevated phospholipase C activity and DAG accumulation were observed in cells exposed to BCP crystals, a response similar to that observed when cells are stimulated with the platelet-derived growth factor (21;22). A downstream effector of DAG-induced mitogenesis may be PKC**,** since down-regulation of PKC inhibited the mitogenic response to crystals (22-25).

Proto-oncogenes are important in the control of cell proliferation. BCP crystals induce c-*fos* and c-*myc* expression in fibroblasts (22;25). Transcription of c-*fos* begins within minutes and peaks at 30 minutes after stimulation with BCP. Messenger RNA for c-*myc* accumulates within 1 h with maximal levels at 3 h. In PKC down-regulated cells, there is an attenuated proto-oncogene response to BCP, reinforcing the importance of PKC in regulating BCP-induced biologic responses.

In view of the above data, we proposed that the crystal-induced mitogenesis occurs through a 2 step-mechanism. The first is a rapid membrane-associated event resulting in activation of PLC and subsequent PKC stimulation with proto-oncogene induction. The second is a more gradual process associated with the intracellular dissolution of the crystals and consequent elevation of cytoplasmic calcium concentrations, leading to calcium-dependent events (3;7).

3.2. Crystal-induced MMP synthesis.

The original and subsequent reports of Milwaukee shoulder syndrome indicated the presence of active neutral protease and collagenase in synovial fluids from affected individuals (14;15).. Such proteases were felt to explain the profound articular tissue (rotator cuff and cartilage) destruction observed. Due to the consistent finding of BCP crystals and frequent finding of CPPD crystals (50% prevalence) in these joint fluids, it was postulated that these particulates might induce protease secretion by synovial lining cells. Natural and synthetic BCP and CPPD crystals were fed to human and canine synovial cells in culture, following which ambient media were analyzed for proteases. Neutral protease and collagenase activities were identified after 8 h with peak levels measured at 24 h (26). Levels in the presence of crystals were much higher than in the media of cells cultured without crystals.

Subsequent studies confirmed that both BCP and CPPD crystal-induced the synthesis of metalloproteinases (MMP–1, -3, -8, –9 and -13), (27-30) in cultured cells. More recently, Bai et al (29) reported that while BCP crystal upregulated expression and synthesis of MMP, the crystals also downregulated TIMP significantly. Since *in vivo* degradative effect of MMPs is dependent on the presence of TIMP, this would explain the earlier observation that the CPPD-containing fluids have the highest levels of MMP1, MMP-3, and the highest ratio of MMP3/TIMP (13) and support the thesis that crystals increase matrix catabolism.

3.3. Crystal-induced Early Growth Response (EGR) gene expression and synthesis

EGR genes were identified originally based on their rapid induction of gene expression in quiescent fibroblasts stimulated by serum (31).. While the amino acid sequences of 4 family members are distinct, they all interact with the Sp1-type of DNA target element. EGR protein alters gene transcription through mechanisms dependent on both co-activators and co-repressors. Transcriptional co-activators such as CREB-binding protein (**CEP**) and p300 can interact directly with the activation domain of EGR1 and increase its trans-activating activity (32). EGR proteins serve as sensors of extracellular signaling pathways that play key roles in regulating cell proliferation, differentiation and function. Recently, Zeng at al showed that BCP crystals could induce the message levels of EGR-1 and –3, which peaked at about 1 hr. In contrast, the message level of EGR-2 increased steadily and peaked at 24 hours after BCP crystals stimulation. This induction was crystal concentration-dependent, and could be abolished by either phosphocitrate, p44/42 MAPK inhibitor U0126, or

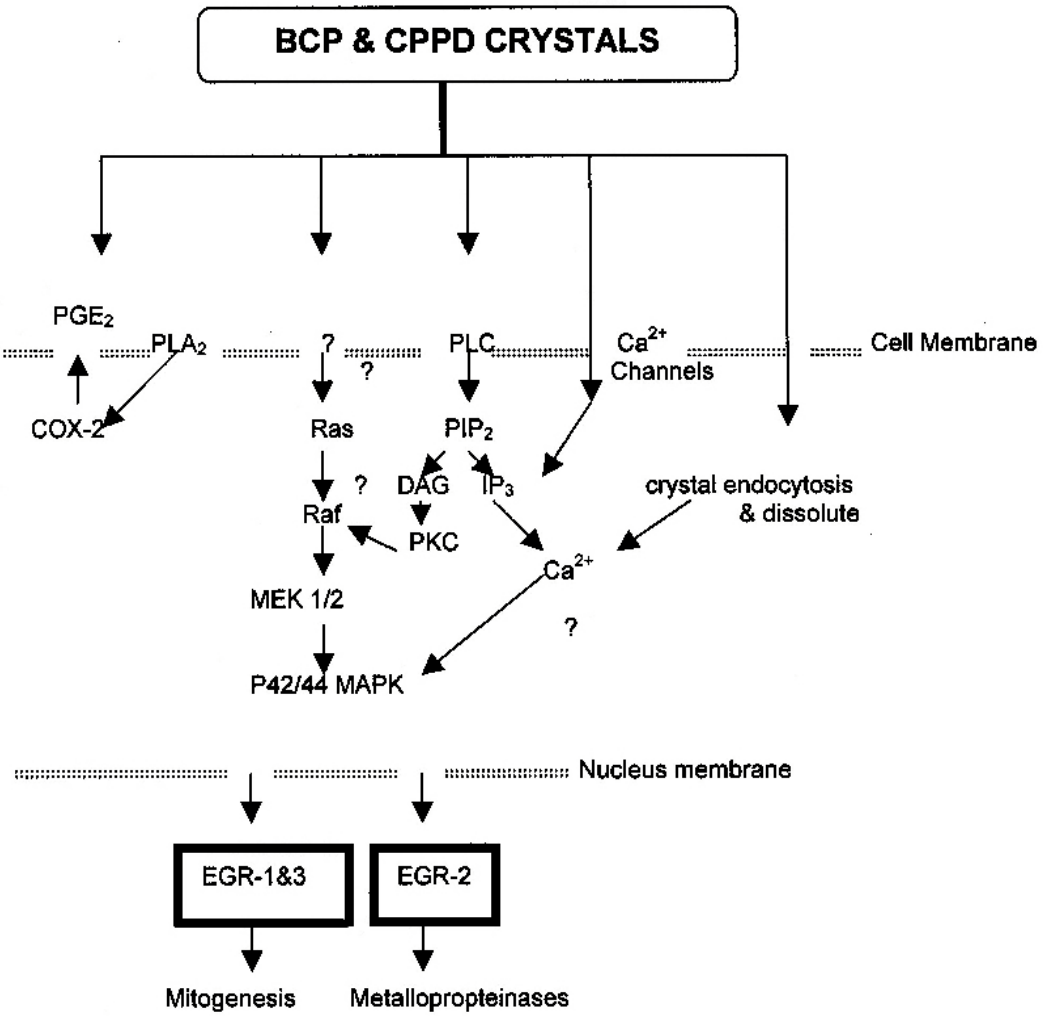

Figure 1. *In vitro* biological effect of .BCP and CPPD crystals. Both crystals activate: (a) phospholipase A_2 (PLA_2), which leads to Prostaglandins E_2 (PGE_2) synthesis and release: (b) Phospholipase C (PLC) and (c) calcium influx via the Calcium channel which leads to the Mitogen-activating Protein Kinase (p42/44 MAPK) activation and ultimately mitogenesis and metalloproteinase synthesis and release.

calcium chelators EGTA and TMB-8, but not by SAPK2/p38 and the PKC inhibitor Bis-I. The induction of Egr2 expression significantly enhanced the binding of transcription factors such as c-fos, SRF and *c-myc* to enhancer elements and activated the p44/42 MAPK signaling pathway. This study demonstrate that crystals induce Egr-2 transcription through a PKC-α-independent p44/42 MAPK pathway, and that induction of Egr2 may subsequently activate genes regulated by SRF, *c-myc* and *c-fos*, which may play key roles in regulating fibroblast proliferation (33).. (Figure 1).

4. "INDIRECT PATHWAY"

Both BCP and CPPD crystals exert a number of biologic responses that may injure articular structures. Crystals provoke prostaglandin (**PG**) generation; especially PGE_2 (34-36) Phosphatidylcholine and phosphatidylethanolamine are the major sources of arachidonic acid for PGE_2 synthesis, confirming that the phospholipase A_2/cyclooxygenase pathway is the predominant route for PGE_2 production (37).. Recently, Morgan *et al* confirmed that BCP crystals upregulated Cylco-oxygenase (**COX**) enzymes in particular COX 2, which in turn induced PGE2 production and IL-β expression in fibroblasts. This suggests that BCP crystal might be an important amplifier of the PGE2 production through the induction of the COX enzymes and the pro-inflammatory cytokine IL-1 β (36).

TGF-ßs are found in synovial fluids from patient with various arthritic conditions (38). Recently, Rosenthal at al (39) showed that synovial fluids from patients with CPPD deposits have significantly higher TGF-ß than fluid from OA and RA patients. Since TGF-ß is a stimulator of PPi (the obligatory anion for the formation of CPPD crystals) extrusion from chondrocytes (40), potentially can promote new crystal formation.

During acute episodes of pseudogout and likely during BCP-induced periarthritis, neutrophils play an important role. Ishikawa reported that in 3 cartilage specimens from patients with pseudogout, neutrophils were attached to superficial deposits of CPPD crystals (41). The adjacent matrix was eroded. Mechanisms of matrix damage during acute episodes of crystal-induced arthritis include release of leukotrienes, lysosomal proteases, chemotactic factors, fibronectin fragments, and active oxygen species. These mechanisms are probably not prominent in the more common instances of non-inflammatory arthritis associated with calcium-containing crystals. CPPD and BCP are known to induce the release of IL -6 and to a much lesser IL-1β by synovial cells (42)..

BCP crystals also stimulate the endocytotic activity of cells. Since calcium-containing crystals are associated with many different macromolecules including DNA fragments cytokines, these particulate may be endocytosed together with crystals disturbing the homeostasis of normal molecular signaling. This finding could be important for our understanding of the potential pathological role of crystals in crystal arthropathy (43)

5. CONCLUSION

In summary, evidence for a causal role of crystals in cartilage degeneration in osteoarthritis is primarily inferential and based on correlative data. Clinical observations indicate that exaggerated and uniquely distributed cartilage degeneration is associated with these deposits. Measurements of putative markers of cartilage breakdown suggest that these crystals magnify the degenerative process. Studies reveal 3 potential mechanisms by which crystals cause degeneration. These involve the stimulation of mitogenesis in synovial fibroblasts and the secretion of MMPs by cells that phagocytose these crystals. New information as how crystals form and how they exert their biologic effects will help us to design an effective therapeutic approach.

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