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Cellular Responses to Whitlockite

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Abstract. Whitlockite crystals have been observed in both degenerating and normal articular cartilages. To determine their potential for inducing cartilage degeneration, we studied their ability to induce mitogenesis and synthesis and secretion of metalloproteases *in vitro.* Whitlockite crystals were found to stimulate cell proliferation and to stimulate synthesis and secretion of stromelysin and collagenase. However, they were less stimulatory than crystals that contained calcium (Ca) and phosphate without magnesium substitution for Ca. Whitlockite crystals elicit biologic cellular responses that suggest potential pathogenicity in arthritis, but are less potent than Ca phosphate crystals without magnesium.

Key words: Whitlockite — Mitogenesis — Metalloprotease.

Cuboid crystals have been described in both normal and pathologic articular cartilages [1–4]. Their crystalline composition is magnesium-substituted tricalcium phosphate [β-TCMP, $(Ca,Mg)_3(PO_4)_2$, whitlockite] as suggested morphologically [1, 5] and as determined by electron and X-ray diffraction [6]. The frequent occurrence of these crystals in normal cartilages raises questions about their pathogenicity in osteoarthritic cartilages.

In sharp contrast to β-TCMP, the presence of other calcium-containing crystals in cartilage is strongly linked to degenerative joint disease. For instance, articular deposition of basic Ca phosphate [(BCP), a term restricted to various admixtures of carbonate substituted apatite, octacalcium phosphate, and tricalcium phosphate (TCP)] is associated with an exaggerated form of osteoarthritis [7, 8]. In addition, Ca pyrophosphate dihydrate (CPPD) crystal deposition disease affects articular cartilage and is a frequent concomitant of severe osteoarthritis [9, 10]. These latter two calcium-containing (BCP and CPPD) crystals are believed to either initiate the degenerative process or amplify ongoing degeneration [11]. The mechanisms by which BCP and CPPD crystals promote cartilage degeneration have been reviewed [12]. These crystals stimulate synthesis and secretion of cytokines and proteases by phagocytic cells. They also induce mitogenesis in synovial lining cells, thereby increasing the number of cells able to secrete cytokines and proteases in response to crystals shed into the joint fluid.

The Ca moiety in BCP and CPPD crystals appears to play an important role in eliciting biologic responses by phagocytic cells. Particulates that do not contain Ca do not stimulate mitogenesis after phagocytosis [13]. The mitogenic response to Ca-containing crystals requires crystal endocytosis and dissolution in the acidic environs of the phagolysosome [14–16]. However, inhibition of intracellular Ca crystal dissolution with the vacuolar-type H+-ATPase attenuates but does not abolish the mitogenic response to these crystals, implying pathways for mitogenic stimulation unrelated to intracellular Ca release by the crystals [17]. We sought to determine whether the substitution of Mg for Ca in TCP, resulting in β-TCMP, could diminish the biologic responses induced by these crystals and explain the lack of degenerative change in some cartilages containing β-TCMP crystals.

Materials and Methods

Cell Cultures

A model system of human foreskin fibroblast (HFF) cultures was used, since the responses of these cells to Ca containing crystals are identical to those of synovial fibroblasts [13]. HF cultures were established from explants, transferred, and grown and maintained in Dulbecco's modified Eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin, streptomycin, and Fungizone, as previously described [18]. All experiments were performed on confluent monolayers that had been rendered quiescent by removing media, washing with DMEM containing 0.5% FBS, and subsequently incubated in this media for 24 hours. All cultures were third or fourth passage.

Reagents

Antibody to matrix metalloprotease 1 (MMP1, collagenase) was a polyclonal rabbit antibody against purified MMP1 from human gingival fibroblasts (kind gift of Dr. Peter Mitchell, Pfizer, Groton, CT). Polyclonal rabbit anti-MMP9 (gelatinase B, 92 kD gelatinase) was raised against a synthetic peptide of MMP9 (kind gift of Dr. Tayebeh Pourmotabbed, University of Tennessee, Memphis, TN) and results were confirmed by using a second commercial antibody from Chemicon International (Temecula, CA). Rabbit polyclonal MMP3 (stromelysin) antibody was raised against a synthetic peptide based on the human sequence of the hinge region of MMP3 (Triple Point Biologics, Forest Grove, OR). Enhanced che-

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miluminescence was performed with a kit from Amersham Life Sciences. Horseradish-peroxidase (HRP)-labeled goat anti-rabbit Ig was from Amersham Life Sciences. FBS, Hanks' balanced salt solution, and DMEM were from Gibco (Grand Island, NY).

Crystal synthesis: BCP crystals were synthesized by a modification of a published method [15]. Mineral prepared by this method had a calcium/phosphorus ratio of 1.59 and contained partially carbonate substituted hydroxyapatite with admixed octacalcium phosphate by Fourier transform infrared spectroscopy. The amounts of crystals used were based on previous studies of dose-response curves for mitogenesis [17]. BCP crystals were a positive control, since they have consistently enhanced mitogenesis and stimulated protease secretion in the HF model system.

Mg-substituted tricalcium phosphate [$(Ca,Mg)_3(PO_4)_2$], crystals were prepared by the dropwise addition of solutions containing Ca and Mg to a stirring phosphate solution maintained at 60 or 95°C. The initial pH was either 6.0 or 7.5. Previous studies [19, 20] demonstrated that pure β-TCMP could be obtained at pH 6.0, when the Mg/Ca ratio varied between 0.2 and 0.3. At higher pH (7.5) the solution Mg/Ca molar ratio has to be increased. Mg-free tricalcium phosphate, β-TCP, $Ca_3(PO_4)_2$, cannot be obtained from aqueous systems and is therefore never observed in biologic systems. β-TCP (Ig) was obtained by sintering Ca-deficient apatite at 950°C [20]. A precipitated β-TCMP was ignited at the same temperature so that the cellular responses to Mg-free and Mg-containing TCP could be compared [β-TCP (Ig) versus β-TCMP (Ig)]. All preparations were characterized using X-ray diffraction which indicates the purity of the β-TCMP phase (i.e., not mixed with apatite) and the extent of Mg-for-Ca substitution which is determined from the decrease in *a*-axis with increasing Mg substitution [21].

Elemental dispersive X-ray analysis was performed in conjunction with scanning electron microscopy at Marquette University in order to determine the atomic percents of Ca, Mg, and P in the TCP and TCMP samples. β-TCP (Ig) contained less than 1% Mg. The Ca:Mg molar ratios in β-TCMP (Ig) and β-TCMP (97-38) were 9.9:1. The Ca:Mg molar ratio of β-TCMP (97-35) was 12.6:1.

All crystal species were of approximately the same size, as estimated by light microscopy, and all were rendered pyrogen free by heating to 200°C for 90 minutes prior to experiments.

Mitogenesis

Cells grown to confluence in 24-well plates and rendered quiescent by incubation in 0.5% FBS for 24 hours were assessed for ^{3}H-thymidine incorporation, as described elsewhere [17].

Immunoblotting

MMP1, 3, and 9 secretion were confirmed by Western blot [22]. Samples of conditioned media from cultures treated with BCP crystals, β-TCP (Ig), β-TCMP, β-TCMP (Ig), or trimagnesium phosphate crystals and untreated control cultures were subjected to immunoblotting, as described elsewhere [17].

Results

All Ca-containing crystals consistently produced a mitogenic response (Fig. 1). Doses of 5 μg/ml were as stimulatory as 20 μg/ml. Thymidine incorporation induced by β-TCMP (Ig), which contains Mg, was less than that induced by β-TCP (Ig), which does not contain Mg. Unignited crystals were also compared. Thymidine incorporation induced by β-TCMP (97-38), which contains more Mg, was less than that induced by β-TCMP (97-35). Identical trends were identified on studies of fibroblasts from three donors in addition to the results presented in Figure 1. Tri-Mg phosphate crystals were not mitogenic (data not shown).

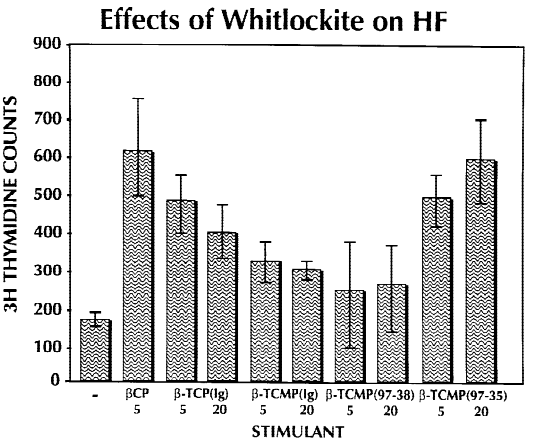

Fig. 1. Counts per minute of ^{3}H thymidine incorporated by human foreskin fibroblasts (HF) exposed to 5 or 20 μg/ml of sintered tricalcium phosphate crystals (β-TCP (Ig)) and sintered whitlockite (β-TCMP (Ig)); unsintered whitlockite with greater [β-TCMP (97-38) and lesser Mg substitution for Ca (β-TCMP (97-35)]; basic Ca phosphate (BCP) as a positive control; and negative control incubations without crystals (−). Bars represent standard deviation of triplicate determinations.

Protease release is a mechanism by which Ca-containing crystals may engender cartilage degeneration. HF incubated with each Ca-containing crystal species elaborated MMP1 (collagenase) and MMP3 (stromelysin) into the ambient media (Fig. 2). Levels of MMP1 induced by BCP and β-TCP (Ig) were comparable. Lesser amounts of MMP1 and 3 appeared when cells were incubated with the partially Mg-substituted β-TCMP (Ig) than with β-TCP (Ig). When the unignited crystalline β-TCMP species were compared, β-TCMP (97-38), containing the larger portion of Mg, induced less MMP1 and 3 release than did β-TCMP (97-35). Calcium-free trimagnesium phosphate elicited the least MMP1 and MMP3 response of all of the unignited crystals. Approximately equivalent amounts of MMP9 appeared in conditioned media after incubation of HF with every crystal type tested, levels being comparable to those in media of unstimulated control HFF. The differences in MMP1 and MMP3 production could not be accounted for by variation of gel loading. Each gel was loaded with identical volumes of conditioned media containing 16–18 μg of protein. Differences also could not be accounted for by variation in cellularity of the monolayer. Although the monolayers exhibit different rates of mitogenesis, only small differences in cell layer protein, DNA, or cell number are observed at 24 hours. This is due to the relatively small proportion of cells incorporating thymidine and dividing during the 24-hour incubation.

Discussion

The putative mechanisms by which BCP and CPPD crystals contribute to joint destruction include induction of mitogenesis, enhancement of the synthesis and secretion of proteases, induction of prostaglandin synthesis, phospholipase activation, and stimulation of cytokines [12, 23]. Whitlockite appears to share some of the same biologic properties. We have demonstrated enhanced mitogenesis and synthesis and secretion of two proteases in response to these crystals. These observations support a potential role for

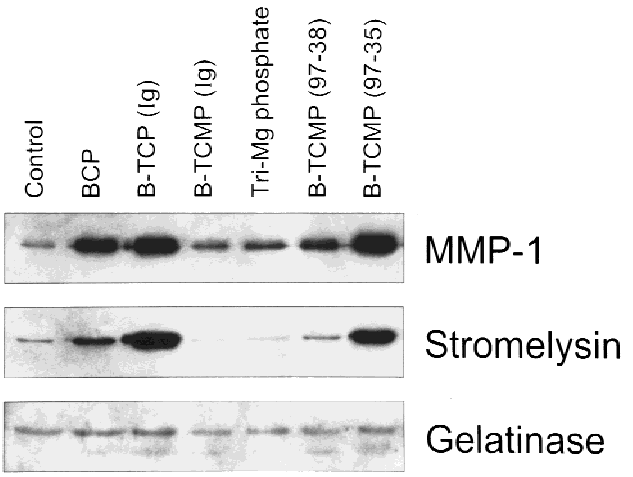

Fig. 2. Western blots of conditioned media from human foreskin fibroblasts exposed to control media without crystals; media with basic calcium phosphate (BCP); sintered tricalcium phosphate [B-TCP (Ig)]; sintered whitlockite [B-TCMP (Ig)]; unsintered Ca-free trimagnesium phosphate (Tri-Mg phosphate); and two unsintered whitlockite species [B-TCMP (97-38) with more Mg and less Ca content than B-TCMP (97-35)]. Gels show results for collagenase (MMP1), stromelysin (MMP3), and gelatinase (92 kD gelatinase; gelatinase B; MMP9). Crystal concentrations were 20 μg/ml.

whitlockite in fostering joint degeneration, just as postulated for other Ca-containing crystals, BCP and CPPD.

However, some of the biologic responses to whitlockite are attenuated compared with Ca-containing crystals that are devoid of Mg. Mitogenesis induced by β-TCMP (Ig), which has approximately 9.2% atomic substitution of Mg for Ca, was less than that induced by the Mg-free β-TCP (Ig). A similar trend was observed when the two unignited species of TCMP were compared. β-TCMP (97-35), which by EDAX had 7.4% atomic substitution of Mg for Ca, was more mitogenic than β-TCMP (97-38), which had 9.2% atomic substitution of Mg for Ca. (Fig. 1)

The events leading to mitogenesis induced by Ca-containing crystals are complex. Ingestion of crystals is followed by dissolution in the acidic pH of the phagolysosome. Interfering with either phagocytosis or lysosomal acidity partially interferes with mitogenesis [16, 17]. It is understandable that substitution of Mg for Ca would diminish this signaling mechanism. Ca-containing crystals also stimulate other cellular pathways. Phospholipase C activity is increased, leading to diacylglycerol accumulation, a response similar to that observed when cells are stimulated with the competence growth factor, platelet-derived growth factor [24]. A potential downstream effector of diacylglycerol-induced mitogenesis is protein kinase C (PKC). Downregulation of PKC inhibits the mitogenic response to BCP crystals [25]. Another and earlier event in mitogenesis induced by Ca-containing crystals is a rapid influx of Ca from extracellular fluids that is unrelated to crystal dissolution. This influx occurs within seconds of exposure of fibroblasts to Ca-containing crystals and lasts approximately 8 minutes [26]. Lastly, mitogen-activated protein (MAP) kinase activation is probably an important signaling pathway involved in Ca-containing crystal induction of mitosis [27].

Potential explanations for the reduced mitogenic response to the whitlockite crystals are multiple. Foremost, less Ca should be released upon intracellular dissolution of β-TCMP compared with crystals containing Ca without Mg substitution; or Mg released intracellularly may oppose the effects of Ca. Other effects of Mg substitution have not been studied and cannot be predicted but might include reduced phagocytosis, altered crystal solubility, interference of the Mg with the early and rapid influx of Ca by lessening crystal-membrane interactions, or interference with MAP kinase induction.

Induction of synthesis and secretion of proteases by fibroblasts fed Ca-containing crystals has not been as well studied as has mitogenesis. Therefore, comments about the role of Mg substitution for Ca are even more conjectural, but phosphocitrate (PC) is a potent and specific inhibitor of metalloprotease secretion in response to Ca-containing crystals [28]. PC likely coats Ca-containing crystals, interfering with crystal-membrane interactions and with the MAP kinase cascade [27]. Possibly Mg substitution similarly interferes with Ca-membrane interactions important for cell activation. This might explain the attenuation of collagenase (MMP1) and stromelysin (MMP3) secretion induced by the more highly Mg substituted crystals [β-TCMP (97-38)] compared with those of lesser or no Mg. One might expect parallel decreases in gelatinase B (MMP9). The failure to observe decreased MMP9 secretion serves to emphasize the contrasting pathways involved in induction of different MMPs.

The *in vivo* effects of Mg substitution are not entirely speculative. It is of interest that Mg treatment in CPPD crystal deposition disease has reportedly decreased symptoms without diminishing crystal deposits, as estimated radiographically [29]. In one case, treatment of a patient with Mg and colchicine remarkably improved previously refractory shoulder hydrops associated with BCP and CPPD deposits [30]. Altering crystal Mg content may be one approach to altering the natural course of Ca-containing crystal deposition diseases, which are not currently treatable. Perhaps biomaterials with increased Mg content may also be less biologically active.

Whitlockite crystals demonstrate *in vitro* properties that would support a role in the pathogenesis of joint degeneration. Nonetheless, some of the effects of whitlockite are attenuated compared with crystals with Ca as the sole cation.

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