EXTENDED REPORTS

Magnesium whitlockite deposition in articular cartilage: a study of 80 specimens from 70 patients

Colin A Scotchford, S Yousuf Ali

Abstract

Objective—To examine articular cartilage from a number of joint sites, using a large sample group, for the presence of magnesium whitlockite crystal deposition.

Methods—Articular cartilage specimens were taken from a total of 70 patients. The majority of specimens were taken from femoral heads, with smaller numbers from femoral condyle, tibial plateau, radius, ulna, and several small peripheral joints. Normal and osteoarthritic articular cartilage specimens were obtained from patients undergoing prosthesis replacement or amputation. Specimens were resin embedded and examined using transmission electron microscopy and x ray microanalysis.

Results—Magnesium whitlockite crystals were identified, on the basis of morphology, size and elemental composition, in articular cartilage from all sites sampled. The distribution of crystals was similar in all samples (restricted to the superficial zone), although the density of deposition was extremely variable, with the greatest density observed in femoral head specimens. No magnesium whitlockite crystals were observed in osteophytic or epiphyseal cartilage.

Conclusions—This study demonstrated the widespread extent of magnesium whitlockite deposition in human articular cartilage, albeit at much lower density than previously reported in femoral head articular cartilage. In consideration of possible roles for these crystals in articular cartilage, it is concluded that an opportunistic mode of formation, possibly influenced by mechanical stresses, would be most plausible.

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Arthropathies associated with monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD) deposition are well established.^{1 2} More recently, basic calcium phosphate (BCP) crystals, including hydroxyapatite,³ carbonated apatites⁴ and octocalcium phosphate,^{4–6} have been assigned a role in crystal arthropathy.^{3 7}

BCP crystal deposition takes place at two main articular sites, periarticular tendon⁸ and intra-articular cartilage.^{3 9 10} In articular cartilage, apatite crystals tend to be deposited in smaller amounts than in tendon,¹⁰ and demon-

strate a pericellular distribution associated with an extension of the calcified zone.^{10 11} BCP crystals have been reported in the synovial fluid of approximately 30% of patients in a number of studies.^{3 12–14} Several patterns of arthritis have been associated with BCP crystals, including primary arthritis, polyarticular arthritis and Milwaukee shoulder syndrome. The deposition of BCP crystals in articular cartilage may be a secondary event resulting from other changes in cartilage metabolism or structure. Their presence, however, may induce further damage to the tissue by feedback mechanisms resulting in an amplification loop.¹⁵ Support for such a relationship may be drawn from a number of studies. The severity of knee joint degeneration has been correlated with hydroxyapatite concentration in synovial fluid^{12 16} and a rapidly progressive destructive disease of the shoulder associated with hydroxyapatite deposition has been described.^{17 18}

Anomalous calcium phosphate ‘cuboid’ crystals were first described in osteoarthritic and elderly human articular cartilage.^{9 10 19 20} On the basis of their calcium to phosphorus ratio and morphology, they were postulated to be magnesium whitlockite.^{10 21} More recently, crystals having the same morphology, size range and calcium to phosphorus ratio were reported in osteoarthritic (OA) articular cartilage from nine of 19 patients²² and consistently, in a study of normal human femoral head articular cartilage, in all of 12 patients across a broad age range.²³ Crystal density was significantly greater in superior region samples than inferior region samples; this difference was smaller in older specimens. A band of crystals 10–20 μm below the articular surface was observed in superior region samples. Using electron and x ray diffraction techniques, these crystals have since been identified as magnesium whitlockite; the possibility of artefactual formation was discounted by using a variety of tissue preparation methods.²⁴ Magnesium whitlockite is a basic calcium phosphate, similar to β -tricalcium phosphate, in which Mg^{2+} , H_2O and HPO_4^{2-} play a structural role.²⁵

In this study, normal articular cartilage from an extensive range of patients and joint sites has been examined for magnesium whitlockite crystal deposition.

Materials and methods**SPECIMENS**

Articular cartilage specimens were taken from a total of 70 patients. The majority of

Institute of Orthopaedics, University College London Medical School, Royal National Orthopaedic Hospital, Stanmore, United Kingdom
C A Scotchford
S Y Ali

Correspondence to: Professor S Y Ali, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Brockley Hill, Stanmore, Middlesex HA7 4LP, United Kingdom.

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specimens were normal femoral head articular cartilage, resected because of tumour located distant from the sampled cartilage, or fracture of femoral neck, or osteoarthritic cartilage from the same site. Cartilage was taken from other joint sites, when available, from patients having amputation or resection for bone tumour.

In all cases full depth blocks of articular cartilage, plus subchondral bone, were taken from sample sites. Specimens were generally obtained within 20 minutes of resection. Normal articular cartilage samples were taken from weight bearing regions of joints where possible. All tissues remained undecalcified.

TRANSMISSION ELECTRON MICROSCOPY

Tissue samples were subdivided to full depth blocks approximately 1 mm in the remaining two dimensions. A small amount of subchondral bone was left attached to minimise tissue distortion during processing and facilitate easy tissue orientation. The tissue blocks were fixed for two to four hours in 1.5% glutaraldehyde (Agar Scientific, Stansted, UK) in 0.085 mol/l sodium cacodylate buffer (pH 7.4) (Merck, Poole, UK). Specimens were then washed in three changes of 0.085 mol/l sodium caco-

dylate buffer containing 0.2 mol/l sucrose (Merck) (pH 7.4) (wash buffer). The tissue blocks were then divided and half the blocks underwent secondary fixation with 1% osmium tetroxide (Agar Scientific) in 0.085 mol/l sodium cacodylate for 90 minutes at room temperature. Buffer washing was repeated and all tissue blocks were dehydrated through a graded alcohol series. Specimens were transferred to propylene oxide (Agar Scientific) for 30 minutes before infiltration with a 1:1 propylene oxide:araldite CY212 resin (Agar Scientific) mixture for one hour, followed by infiltration in neat CY212 resin under vacuum (150 mbar) overnight, and embedding in fresh resin at 60°C for 48 hours. CY212 resin was mixed according to the manufacturer's instructions with the omission of methyl phthalate (plasticiser).

Ultrathin sections (70–100 nm) were cut with diatome diamond knives (Leica, Milton Keynes, UK) using a Reichert Ultracut E ultramicrotome (Leica) and floated onto 0.085 mol/l sodium cacodylate buffer before collection on to G200 HS copper grids (TAAB Laboratories, Aldermaston, UK). Where necessary the grids were precoated with 0.45% piolform (Agar Scientific) in chloroform (Merck). If required, ultrathin sections were stained on drops of aqueous saturated uranyl acetate (Agar Scientific) for 10 minutes at room temperature followed by lead citrate for five minutes at room temperature in the presence of sodium hydroxide pellets (Merck).

Table 1 Range of sample sites from which full depth articular cartilage specimens were taken, number of specimens obtained from each site listed, and age range and male to female ratio of patients. The extent of magnesium whitlockite crystal deposition is indicated on a presence/not observed basis, with a qualitative grading score for the density of deposition, as determined by transmission electron microscopy

Sample site	No of samples	Age range (yr)	M/F	No of samples	
				Crystals present (grade)	Crystals not observed
Femoral head (normal)	34	10–93	14–20	34 (3–4)	—
Femoral head (OA)	26	46–83	8/18	26 (1–2)	—
Peripheral osteophyte	5	53–73	3/2	— (0)	5
Femoral condyle	3	5–20	2/1	3 (2)	—
Tibial plateau	2	19–20	2/0	2 (1)	—
Radial head	1	19	1/0	1 (3)	—
Proximal ulna	1	19	1/0	1 (3)	—
1st metatarsal	2	17–61	2/0	2 (1–2)	—
Carpal	1	19	1/0	1 (1)	—
2nd metacarpal	2	19–62	2/0	2 (1–2)	—
Lunate	2	19–62	2/0	2 (1–2)	—
Phalanges	1	17	0/1	1 (1–2)	—
Epiphyseal growth plate	1	9	1/0	— (0)	1

Figure 1 Characteristic x ray microanalysis spectrum from magnesium whitlockite crystals in human articular cartilage. Peaks for calcium, phosphorus and magnesium (arrow) are clearly present.

X RAY MICROANALYSIS

Unstained sections were examined using a Philips CM12 transmission electron microscope with an EDAX PV9800 x ray microanalysis (XRMA) system. Spectra were recorded at 100 kV (operation at maximum available kV has been recommended to obtain maximum peak to background ratios²⁶), for 200 live seconds with a tilt angle of 20°, giving a total 'take off' angle of 40°. The electron probe size was selected to encompass individual crystals and was never greater than 200 nm diameter; a 70 nm 'top hat' condenser aperture was used to minimise the background effect of x rays generated higher in the column reaching the specimen area. Calcium to phosphorus ratios were calculated using the quantitative analysis of thin sections software package (EDAX PV9800) based on the ratio model.²⁷ The synthetic hydroxyapatite sample was used as the calcium phosphate standard for these analyses.²⁸

Results

Crystals were observed in articular cartilage from all sites sampled (table 1). Identification was based on the distinctive 'cuboid' morphology, electron density, size range (50–500 nm) and XRMA spectrum, and the calcium to phosphorus ratio generated from this. Crystals from areas of deposition all generated spectra with characteristic calcium, phosphorus and magnesium peaks (fig 1). Calcium to phosphorus ratios ranged from 1.30 to 1.47.

The distribution in all cases was similar to that described for normal femoral head articular cartilage.²³ Crystals were present either within a band running parallel to the articular surface from 5 μm to a maximum of 50 μm depth below the surface, or in close proximity to chondrocytes, and occasionally in dense clusters. A band of crystals was identified in many specimens with variable density and depth, frequently with a degree of patchiness. The depth of the band in some specimens was limited to extreme superficial articular cartilage extracellular matrix between the articular surface and the most superficial chondrocytes, a region frequently observed to

contain characteristic granular intramatrix lipidic debris, including membranous structures (fig 2A). Other specimens demonstrated a deepening of the band, encompassing initial superficial zone chondrocytes (fig 2B). Such differences between specimens appeared to be related to the age of the patient, specimens from older patients having deeper bands of crystal deposition. Crystals were commonly observed deposited pericellularly to chondrocytes, apparently associated with intramatrix lipidic debris (fig 2C). In many cases the crystal distribution was biased, with greater numbers adjacent to the articular surface aspect of the cells when viewed in sagittal

Figure 2 Transmission electron micrographs of magnesium whitlockite crystal deposition in human articular cartilage. A: Crystals (C) are present, forming a band immediately below the articular surface, together with intramatrix lipidic debris (L). (Patient 20 years; femoral condyle; section stained with uranyl acetate and lead citrate.) B: Denser crystal deposition in femoral head articular cartilage shows a broader band. (Patient 43 years; femoral head; unstained section.) C: Sparse deposition in specimens of metacarpal articular cartilage. (Patient 19 years; unstained section.) D: Pericellular distribution of crystals (C) and intramatrix lipidic debris (L) in the superficial zone of metatarsal articular cartilage. (Patient 61 years; unstained section.)

section. Crystals were frequently observed within chondrocyte territorial matrix, often in close proximity to the plasma membrane; however, crystals were not observed in living chondrocytes. In deeper zones, magnesium whitlockite crystals were rarely observed. Crystal deposition when encountered was, in the main, perilacunar, amongst areas of lipidic debris.

The density of deposition showed much variability between joint sites, with no site reaching the greatest densities observed in normal femoral head articular cartilage. A five point qualitative grading scale (table 2) was devised to compare crystal distribution in tissues from different sites. In the knee, whilst articular cartilage from both the femoral condyle (grade 2) and the tibial plateau had crystals present, deposition in the tibial plateau tissue was very sparse (grade 1). A small number of specimens from several smaller joints were examined. Crystal deposition density in cartilage from the proximal ulna and radial head was similar to that in the femoral condyle samples (grade 2–3). In samples from the remaining sites, deposition was more sparse than this, with the least density observed in samples of carpal and phalangeal cartilage (grade 1) (fig 2D). No crystals were observed in peripheral osteophytes from OA femoral heads or epiphyseal growth cartilage.

Despite occasional focal densities and dense banding of crystals in the superficial zone matrix, there was no evidence of structural disruption of collagen fibril orientation by crystals in normal articular cartilage, nor was there any evidence of chondrocyte degeneration induced by crystal deposition. Crystals appeared to be present in the interfibrillar space without disrupting the overall fibrillar arrangement.

Discussion

Previous studies using electron diffraction and x ray diffraction techniques have shown crystals with the same morphology, elemental composition, calcium to phosphorus ratio, and size range to be magnesium whitlockite.^{24–29} The positive identification of crystals in this study using the above criteria is considered sound. The results of this study highlight the extent of magnesium whitlockite crystal deposition in human articular cartilage.

Table 2 Criteria for qualitative grading of crystal deposition density

Grade	Definition
0	Crystals not observed.
1	At least one crystal observed, searching necessary.
2	Crystals present in a diffuse distribution, no apparent pattern, regular encounters, extensive searching not necessary.
3	Crystals present in localised groupings, generally pericellular to chondrocytes plus a diffuse intermittent band.
4	Crystals effectively form a continuous band, running parallel with, and immediately below the articular surface; local patchiness may be observed in the band at greater magnifications.

This work supports and broadens the original limited studies of Ali *et al*^{9–10–21} and Stockwell *et al*.^{19–20} With the exception of these authors, such crystal deposition in articular cartilage has not been previously reported, although the reports of the deposition of calcified bodies in human meniscus by Ghadially and Lalonde³⁰ and of tricalcium phosphate in articular cartilage by Bardin *et al*²² are most closely related. A number of points may be raised in an attempt to explain this apparent oversight.

The ultrastructural study of normal human femoral head articular cartilage—a site where deposition has been observed at its most dense—is limited. Ghadially³¹ commented that knowledge of the fine structure of normal articular cartilage is derived largely from a study of animal material, particularly rabbit, with more limited studies of human cartilage from fibrillated and normal looking OA specimens, juvenile specimens taken at autopsy and young adult specimens surgically obtained. Many of these studies were based on specimens from the femoral condyle—a site which, in this study, exhibited a lower maximum crystal deposition density than superior region femoral head specimens.²³ The commonly used thin section stain uranyl acetate, and distilled water, routinely used to float thin sections away from the ultratome knife edge, both showed a tendency towards crystal loss.^{32–33} Uranyl acetate and lead citrate staining imparts greater electron density to matrix components in thin section, possibly concealing the presence of crystals in cases of sparse deposition. With this in mind, re-examination of early studies of human articular cartilage³⁴ reveals electron densities amongst stained lipid debris bearing an arguable resemblance to ‘cuboid’ crystals.

Distribution of crystal deposition in this study conformed to that described for normal femoral head articular cartilage,²³ the main difference being the density of deposition at different locations. The earlier study demonstrated an association between load bearing and crystal density around the femoral head.²³ Evidence for the influence of load bearing stresses may be extrapolated from this study. Mechanical loading exerts a major influence on articular cartilage.^{35–36} It has been suggested that mechanical stress is involved in the regulation of chondrocyte metabolism.³⁷ Whilst crystals were present in cartilage from all joint sites examined, the density of such deposition was always less than in the superior region femoral head as determined by the qualitative grading system. This was most noticeable in specimens from the smaller, more peripheral joint sites which are normally subject to lower load bearing stresses.³⁸

Magnesium whitlockite deposition has been reported in a number of biological calcifications, both as calculi and in tissues. The most common of these are orally associated, with sites including dental calculi,³⁹ arrested carious dentine,⁴⁰ and sialoliths.⁴¹ Whitlockite has also been reported in urinary calculi,⁴² though this is considered a minor component

in such situations. Deposition of whitlockite within tissues has been reported at various sites including aortic valvular topoi,⁴³ pulmonary calcifications,⁴⁴ and in cartilage from the nasal septum, trachea, epiglottis, and intervertebral disc.⁴⁵ Marked differences in magnesium content have been reported between whitlockites found in calculi and those from cartilages.⁴⁵ The magnesium content of the whitlockite crystals in articular cartilage corresponds with those of tracheal and intervertebral disc cartilage.²⁴ The pathological nature of these depositions appears at odds with the current report of magnesium whitlockite deposition in apparently normal tissue.

The presence of these crystals requires consideration of their role in articular cartilage, physiological or pathological. The demonstration of crystal deposition in articular cartilage from normal adolescent to elderly cartilage in this study, suggests that they do not, per se, pose a pathological risk to the tissue.

This may indicate that they are the result of opportunistic deposition, with no defined function. Their circumstantial association with intramatrix lipidic debris may relate crystal deposition to the removal of material generated from chondrocyte necrosis from the extracellular matrix to the synovial fluid, a function considered by Ghadially.³¹ This would provide an opportunity for further speculation on crystal deposition: first, providing possible means of damping ion fluxes created by cell necrosis, minimising the effects on the pericellular environment and second, raising the question, do crystals stay at the site of formation, or is there a migration to the articular surface as suggested for necrotic debris?³¹ The existence of a crystal band in the superficial zone may support the latter.

The possibility of the involvement of subarticular matrix lipid in boundary lubrication—the formation of an adsorbed protective layer on articulating surfaces—has been raised.^{46–47} If lipid does act as a boundary lubricant, an involvement in crystal formation may reduce such activity. In the presence of synovial fluid, with the potential for boundary lubrication from hyaluronan,⁴⁸ it would be difficult to envisage a significant decrease in the coefficient of friction resulting from a loss of lipid as suggested. An increase in the coefficient of friction of cartilage samples has been reported in conjunction with increased incidence of crystal deposition, nominally CPPD, and severity of tissue fibrillation, although the relative contribution of either factor was not determined.⁴⁹

The presence of crystals in articular cartilage may alter tissue compliance. It would seem unlikely that the crystal deposition observed in this study, being primarily at the articular surface, would significantly alter the load spreading ability of the tissue. More pertinent may be the local differences in physical properties between cartilage matrix and crystals, providing potential for disruption of articular surface integrity. A correlation between CPPD crystal deposition and severity

of fibrillation has been reported by Clift *et al*;⁴⁹ however, no evidence for the correlation of fibrillation with magnesium whitlockite crystal deposition was observed in this study.

In conclusion, magnesium whitlockite crystal deposition has been demonstrated in articular cartilage across a broad age range at several joint sites. If an apparently opportunistic mode of crystal deposition is accepted, a role as an aggravator of pathological degeneration via an amplification loop, similar to that described by Dieppe and Calvert,¹⁵ would seem plausible. The potential for such a role may be clarified by further study.

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