





Basic science

A new ex vivo human model of osteoarthritis cartilage calcification

Elodie Faure¹, Julien Wegrzyn², Ilaria Bernabei¹, Guillaume Falgayrac³, Nicolas Bertheaume³, Tristan Pascart^{3,4}, Thomas Hugle¹, Nathalie Busso^{1,‡}, Sonia Nasi ® ^{1,*,‡}

- ¹Service of Rheumatology, Department of Musculoskeletal Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland
- ²Service of Orthopedics, Department of Musculoskeletal Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland
- ³ULR 4490-MABLab- Adiposité Médullaire et Os, Univ. Lille, CHU Lille, Univ. Littoral Côte d'Opale, Lille, France
- ⁴Department of Rheumatology, Lille Catholic Hospitals and Lille Catholic University, Lille, France
- *Correspondence to: Sonia Nasi, Service of Rheumatology, Department of Musculoskeletal Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Chemin des Boveresses 155, 1066 Epalinges, Lausanne, Switzerland. E-mail: sonia.nasi@chuv.ch

Abstract

Objective: Cartilage pathologic calcification is a hallmark of osteoarthritis (OA). Here, we aimed to describe a new *ex vivo* human model to study the progression of cartilage calcification.

Method: Cartilage explants (n=11), as well as primary chondrocytes (n=3), were obtained from OA patients undergoing knee replacement. Explants and chondrocytes were cultured in control (NT) or calcification (CM) medium (supplemented with ascorbic acid and β-glycerophosphate). Calcification was evaluated by micro-CT scan at day 0 and 21 in explants, and by Alizarin red staining in chondrocyte monolayers. Raman spectrometry allowed characterization of the crystal type. Interleukin-6 (IL-6) secretion in explant and cell supernatants was measured by ELISA. Finally, matrix degradation was evaluated by Safranin-O staining of explant sections and by glycosaminoglycans (GAG) released in supernatants.

Results: Micro-CT scan showed calcifications in all explants at baseline (day 0), which in the CM group increased significantly in number and size after 21 days compared with the NT group. Raman spectrometry revealed that crystals were exclusively basic calcium phosphate crystals (carbonated hydroxyapatite) both in NT and CM. IL-6 secretion was significantly increased in calcifying conditions. Finally, CM significantly increased cartilage catabolism as assessed by decreased Safranin-O staining of tissue explants and increased GAG release in supernatants. CM effects (enhanced calcification, IL-6 secretion and proteoglycans turn-over) were recapitulated *in vitro* in OA chondrocytes.

Conclusions: We have described a new *ex vivo* human model of cartilage calcification that can summarize the triad of events seen during osteoarthritis progression, i.e. calcification, inflammation and cartilage degradation. This model will allow the identification of new anticalcification compounds.

Keywords: human cartilage, ex vivo model, osteoarthritis, calcification, inflammation, cartilage degradation.

Rheumatology key messages

- Pathologic cartilage calcification is a key feature and an etiologic factor for osteoarthritis development.
- Human experimental models to study pathologic cartilage calcification are missing.
- Our model can be useful for understanding cartilage calcification and for identifying new anti-calcifying compounds.

Introduction

Factors involved in osteoarthritis (OA) include calcification, inflammation and cartilage degradation [1]. Pathologic calcification in OA consists of the deposition of calcium-containing crystals in cartilage [2], encompassing basic calcium phosphate (BCP) and calcium pyrophosphate dihydrate (CPP) crystals. Crystals are found in 100% of patients after knee replacement surgery [3] and are considered of

etiologic importance in OA. Indeed, crystal presence in cartilage and synovial fluid positively correlates with histological and radiological knee OA [4].

Accumulated evidence indicates a contribution of the proinflammatory cytokine IL-6 to OA progression. IL-6 is increased in cartilage, synovial fluid and serum of OA patients [5]. In experimental OA, *Il-6* ablation resulted in decreased cartilage degeneration, whereas intra-articular injections of

[‡]N.B. and S.N. contributed equally.

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IL-6 induced cartilage destruction [5]. Accordingly, inhibition of IL-6/Stat3 signalling revealed protective in OA mice [5].

Both crystals and inflammation ultimately contribute to cartilage degradation via catabolic enzymes MMPs and ADAMTs [6]. However, the impact of newly formed calcification within cartilage is not known, and human experimental models are lacking. In this study, we set up a novel human model of *de novo* cartilage calcification, useful for the study of cartilage calcification in OA.

Methods

Human cartilage explants

Lateral and medial, tibial and femoral cartilage from 11 OA patients (Kellgren-Lawrence score 3.6 ± 0.5 , age $70.4 \pm$ 5.7 years, 6 females and 5 males) was obtained from the Orthopedics Department (CHUV, Lausanne) at time of joint replacement. Cartilage punches of 6 mm diameter were dissected using a dermal punch (six punches/patient) from macroscopically undamaged cartilage. To match for location across treatments, each punch was divided into two halves. Each half was cultured for 21 days in control medium [not treated (NT)]: DMEM high glucose containing 0.9 mM phosphate, 1.8 mM calcium + 1%Penicillin Streptomycin + 10%FBS, or in calcification medium (CM condition): DMEM high glucose (same as NT)+50 µg/ml ascorbic acid + 20mM β-glycerophosphate. Media were changed twice a week and supernatants collected for ELISAs. At day 21, explants were analysed by micro-CT scan, fixed with 4% paraformaldehyde and embedded in paraffin for histological staining (Safranin-O and 0.5% Alizarin Red). We could not use healthy donors as these are difficult to obtain.

Cartilage histopathology

Sections 4 μ m-thick of cartilage explants were stained with Safranin-O (Sigma). Blind score for Safranin-O loss was performed: 0 = intact proteoglycans; 1 = proteoglycan loss up to 20% of cartilage deepness; 2 = proteoglycan loss up to 50%; 3 = proteoglycan loss up to 80%; 4 = proteoglycan loss up to 100%. For each patient, the average score from six different explants was plotted in graphs.

Micro-CT scan

Explant images were acquired at time of explant isolation (t0) and after 21 days (t21), using micro-CT scan (MILabs): ultrafocus, 50 kV, 0.20 mA, 0.375° rotation step over 360°, 75 ms. Three-dimensional reconstructions at 18 μm resolution were performed and analysed (Imalytics Preclinical, MILabs, Houten, Netherlands). A reference phantom with known HA concentrations was used for transformation from Hounsfield Units to mg/cm³ HA. Gauss filter 1.5, threshold for calcification 200 mg/cm³ were applied. Calcification volume (bone volume) (mm³) and bone mineral density (BMD) (mg/cm³) were calculated by the software, and crystal content (μg) obtained by multiplying the two parameters.

Raman microspectroscopy

Sections 10 µm-thick of cartilage explants were mounted on quartz microscope slides. Cell pellets from monolayers were spread on quartz microscope slides. Raman spectra of crystals were acquired with a Raman microspectrometer LabRAM HR800 (HORIBA JY, Lille, France), with a XYZ

motorized stage and a 785 nm laser diode (power 10 mW at the sample). Acquisitions were done with an objective Olympus $\times 100$ (N.A.=0.9) for 30 s averaged twice (total acquisition time of 60 s), with a spectral domain of 350–1700 cm⁻¹ and spectral resolution of $4 \, \text{cm}^{-1}$. The spectra were processed by Savitzky–Golay smoothing filter (width: 3; polynomial order: 2) with a polynomial baseline correction (degree 4).

Human chondrocyte isolation

Cartilage from OA patients undergoing knee replacement (Kellgren–Lawrence score 4 ± 0 , mean age 70 ± 8 years) was digested overnight with Liberase TM (Roche, Basel, Switzerland) as previously described [7]. Monolayer of chondrocytes $(1.2\times10^5/\text{cm}^2)$ were cultured at passage 3 in NT or CM medium for 21 days. Crystal formation was analysed by Alizarin red staining and quantified by spectrophotometry (570 nm–630 nm) and dissolution of crystals in cetylpyridinium chloride.

Apoptosis

Apoptotic chondrocytes were detected by ApopTag Kit (MilliporeSigma) on paraffin sections and quantified by NDP2.view2 (Hamamatsu).

Glycosaminoglycans (GAG)

A quantity of 1,9-dimethyl-methylene blue (DMMB) was used at 0.115 mM DMMB in deionized water containing 142 mM of glycine and 160 mM of NaCl, pH 3.0. Standard curve was performed using chondroitin 4-sulphf (0–50 μ g/ml) in DMEM + 10% FBS. For GAG measurement, 50 μ l of explant or chondrocyte supernatant was added to an equal volume of DMMB dye, and absorbance was read at 535 nm and 450 nm.

Cytotoxicity

Lactate dehydrogenase (LDH) in supernatants was measured using CytoTox-ONETM Assay (Promega).

IL-6 and MMPs quantification

Explant supernatants were assayed using human IL-6, MMP-3, -13, ADAMTS-5 ELISA kits (eBioscience).

Statistical analysis

Values are expressed as mean \pm SD. Variation between paired data sets was evaluated using parametric (paired t test) or non-parametric (Wilcoxon) Student's t test. For unpaired data sets, we used the Mann–Whitney test. Differences were considered statistically significant at *P <0.05, **P <0.01. Data was analysed with GraphPad Prism software Version 9 (GraphPad).

Ethics statement

Human cartilages have been obtained at time of joint replacement, upon approval by the ethical committee of the Lausanne University Hospital and patients' written informed consent.

Results

Calcification medium promotes BCP formation in an ex vivo human model of OA

We first established an *ex vivo* model to study *de novo* cartilage calcification. In 10 out of 11 patients, cartilage calcification was seen by X-rays (Supplementary Table S1, available at *Rheumatology* online). We demonstrated that CM had no toxic effect over 21 days on cartilage explants (LDH levels mean ± SD: 1867 ± 168 for NT, 2248 ± 289 for CM).

Micro-CT scans at t0 already showed some calcifications in all explants (mean crystal content \pm SD/explant: $11\pm16~\mu g$ in NT group, $10\pm15~\mu g$ in CM group) (Fig. 1A and Supplementary Table S2, available at *Rheumatology* online). However, in CM-treated explants the initial calcifications became bigger and new calcifications appeared at t21 (Fig. 1A and B, Supplementary Table S2). Crystals were localized in superficial cartilage (Supplementary Fig. S1A, available at *Rheumatology* online) in most explants while, in some explants, crystals were also found in deep layers. This was confirmed by Alizarin red staining at t21 (Supplementary Fig. S1A, available at *Rheumatology* online, right panel, black arrows).

Micro-CT scan at t21 confirmed that CM triggered significant increased calcification volume (Fig. 1B), while NT explants showed no increase (NT: 1 ± 1.21 at t0 vs 1.21 ± 1.27 at t21, P=0.36; CM: 1 ± 1.22 at t0 vs 1.39 ± 1.68 at t21, P=0.04). Similarly, crystal content was increased at t21 in CM conditions, although not significantly (Supplementary Fig. S1B, available at *Rheumatology* online) (NT: 1 ± 1.3 at t0 vs 1.11 ± 1.45 at t21, P=0.2; CM: 1 ± 1.31 at t0 vs 1.55 ± 2.13 at t21 P=0.06).

We hypothesized that chondrocytes cultured in CM would produce mainly BCP crystals, as previously demonstrated in OA cartilage [6, 8]. Raman microspectroscopy of cartilage

explant sections (Supplementary Fig. S1C, available at *Rheumatology* online) revealed bands characteristic of carbonated hydroxyapatite crystals (CA: 432, 586, 960 and $1070\,\mathrm{cm^{-1}}$) (Fig. 1C). Interestingly, CA was the only type of crystal found in superficial cartilage of all patients, while no crystals were detectable in deep cartilage (Supplementary Table S3, available at *Rheumatology* online). Similarly, primary chondrocytes cultured *in vitro* in CM produced more crystals compared with NT (Fig. 1D and E) (NT: $100 \pm 7~vs$ CM: 236 ± 21 , results expressed as % of NT), and again these crystals were characterized as CA by Raman analysis (Fig. 1F).

Although increased calcification can result from increased apoptosis [9], explants cultured in non-calcifying and calcifying conditions showed similar percent of apoptotic chondrocytes (Supplementary Fig. S2, available at *Rheumatology* online) (NT: $65 \pm 13\%$ vs CM: $72 \pm 9\%$), ruling out apoptosis as a calcification trigger in our experimental settings.

De novo calcification promotes IL-6 secretion and cartilage degradation

We previously demonstrated that BCP crystals induce IL-6 secretion in chondrocytes [6]. Here we found that, at both t10 and t21, IL-6 secretion was significantly increased by CM (NT: 6 ± 5 pg/mg tissue vs CM: 11 ± 8 pg/mg tissue at t10 and NT: 18 ± 14 pg/mg tissue vs CM: 24 ± 16 pg/mg tissue in CM group at t21) (Fig. 2A). The same was true in primary chondrocytes (Fig. 2G).

We next evaluated if calcification could impact cartilage matrix integrity. We examined cartilage explant sections and found that CM increased loss of Safranin-O staining (Fig. 2B and C). In the corresponding explant supernatants, we found higher release of GAG in the CM condition (Fig. 2D). We hypothesized that these effects could be accounted for by

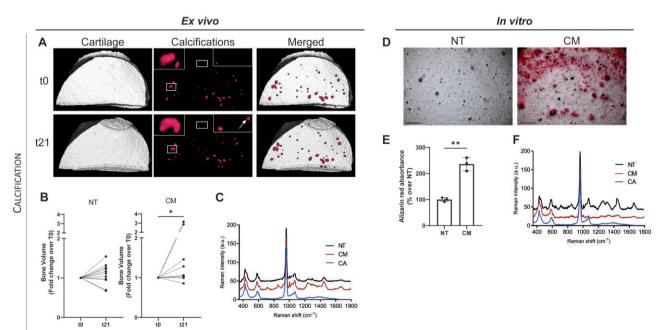


Figure 1. Induction of *de novo* calcification in *ex vivo* and *in vitro* models of OA. (**A**) Micro-CT scans of cartilage explants. Calcifications (red) increased in size (squares) and number (arrow) at t21. (**B**) Volume of calcification deposits in explants. Each dot represents the mean of six explants/patient. Wilcoxon test. n = 11. (**C**) Raman spectrum of crystals in NT (black) or CM-stimulated cartilage explants (red), compared to CA reference spectrum (blue). (**D**) Alizarin red staining of chondrocytes from one representative patient out of three patients. Scale bars: $500 \, \mu m$. (**E**) Crystal content quantified in one representative patient out of three patients. Triplicate from one representative patient is shown. Unpaired test with Welch's correction. (**F**) Raman spectrum of crystals in NT (upper line) or CM-stimulated chondrocytes (middle line), compared to CA reference spectrum (lower line)

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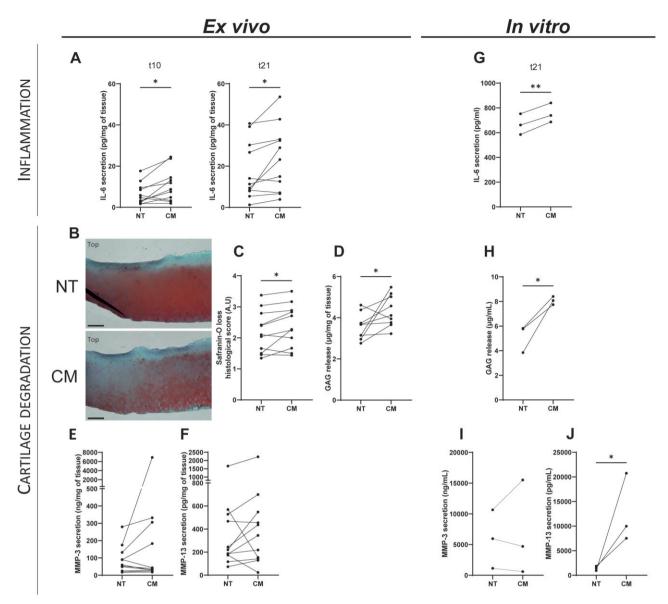


Figure 2. IL-6 secretion and cartilage degradation are increased by $ex\ vivo$ and $in\ vitro$ calcifications. (A) IL-6 in explant media. Each dot represents the mean of six explants/patient. Paired t test. n=11. (B) Safranin-O staining of explants at t21. Scale bars: $500\ \mu\text{m}$. (C) Safranin-O loss quantification. Paired t test. n=11. (D) GAG in explant supernatants at t21. Paired t test. n=9. (E) MMP-3 and (F) MMP-13 in explant supernatants. Wilcoxon test. n=11. Each dot represents the mean of six explants/patient. (G) IL-6 in OA chondrocytes. Each dot represents the mean of triplicates from one patient. Paired t test. n=3. (H) GAG release, (I) MMP-3 and (J) MMP-13 in chondrocytes supernatants. Each dot represents the mean of triplicates from one patient. Paired t test. n=3

increased matrix-degrading enzymes. While ADAMTS-5 was not detectable in our samples, MMP-3 and -13 were higher in supernatants of CM explants although not significantly (MMP-3 NT: 90 ± 80 ng/mg of tissue *vs* CM: 721 ± 2051 ng/mg of tissue; MMP-13 NT: 402.5 ± 451 pg/mg of tissue *vs* CM: 489 ± 612 pg/mg of tissue in CM group) (Fig. 2E and F). Finally, CM stimulation led to significant increased GAG release (Fig. 2H) and MMP-13 secretion (Fig. 2I) in primary chondrocytes, while no effect was observed on MMP-3 (Fig. 2J).

Discussion

Cartilage calcification is a crucial hallmark of OA, found in 100% of OA knee cartilage [3], and associates with inflammation (IL-6) and cartilage degradation [6]. While these

events have been extensively studied in OA chondrocytes *in vitro*, no human 3D experimental model, mimicking the complexity of cartilage structure, has been developed so far. In this study, we demonstrated that culturing human cartilage explants in calcification medium can promote *de novo* calcifications. These calcifications were identified as BCP crystals (CA) by Raman analysis, while no other BCP crystals nor CPP crystals were detected. Recently, Raman has been demonstrated to be a reliable method for the discrimination between BCP and CPP crystals [10].

Additionally, we found that *de novo* cartilage calcification induced both IL-6 secretion and cartilage degradation (MMP-13). These results are in agreement with our previous study, indicating that exogenously added hydroxyapatite crystals induced IL-6 in human cartilage explants [6]. As IL-6 is also a pro-mineralizing cytokine, it could be anticipated

that its production will sustain calcification. Thus, biologicals targeting IL-6 could be of therapeutic relevance to prevent the onset or reduce the progression of joint calcification. We found previously that tocilizumab decreased crystal deposition and matrix degradation in human OA cartilage explants [6]. However, no pain relief by tocilizumab was obtained in hand OA patients [11].

Proteolytic activity by MMPs is believed to be the main mediator of cartilage degradation in OA. Crystals can upregulate various matrix-degrading enzymes including MMP-3 and MMP-13 [2, 6], the latter involved in chondrocyte hypertrophy [12]. Moreover, activated MMPs cause cartilage degradation and apoptosis of chondrocytes, which might be preceded by matrix calcification [13]. Here we found increased MMP-3 and MMP-13 levels in explants cultured in CM, in 7/11 and 8/11 patients, respectively. Proteoglycan degradation in OA cartilage, with GAG-rich degradation products being released from the tissue into the culture medium has been previously observed [14]. We found here that de novo calcification exacerbated proteoglycan loss in explant sections (Safranin-O loss) and GAG release in explant supernatants. These changes in both proteoglycans and GAG can in turn impact ongoing calcification. For instance, proteoglycans from cartilage inhibited HA growth [15], while GAG accelerated collagen mineralization in a tissue-based in vitro model [16]. Clearly, the molecular effect of proteoglycans and GAG should be assessed in our model.

Overall, calcification, IL-6 production and MMPs secretion are interlinked processes. Calcification can trigger IL-6 production, and conversely, IL-6 can stimulate chondrocyte calcification [6]. IL-6 induces MMP as well [17]. In our model, the triggering factor is BCP crystal formation induced by CM. Whether BCP crystals induce MMPs directly or via IL-6 production remains to be established. However, this study has some limitations. First, we used cartilage from OA knees, known for its inherent calcifications. While we acknowledge the potential influence of pre-existing calcifications on the secretion of IL-6, MMPs and GAGs, our data unequivocally support the involvement of de novo calcification induced by calcification medium (CM) in amplifying these detrimental factors. Ideally, healthy cartilage without calcification should be used, but such cartilage has limited accessibility. Furthermore, we observed significant variability among cartilages from different patients, as well as within explants from the same patient. This variation may be attributed to the diverse origins of the explants, dissected from different areas of the knee cartilage. Nevertheless, this approach was necessary to ensure an adequate supply of undamaged explants for our experimental conditions.

To conclude, we described a novel *ex vivo* model of cartilage calcification, recapitulating some OA aspects. This model can be useful for studying cartilage calcification and for identifying new anti-calcifying compounds.

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

All data are available in the manuscript or upon request to the corresponding author.

Contribution statement

E.F. performed and evaluated most experiments, and edited the manuscript; I.B. and S.N contributed to experiments; N. Bu. and S.N. designed the project, evaluated results and wrote the manuscript; J.W. collected human cartilage; N.Be. and G.F. performed Raman analysis; T.H. and T.P. contributed writing, reviewing and editing the manuscript. All authors have read and agreed to the final version of the manuscript. S.N take responsibility for the integrity of the work (sonia.nasi@chuv.ch).

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