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Brief Report

Chondrocyte enlargement is a marker of osteoarthritis severity

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SUMMARY

Objective: We aimed to assess whether an increase in chondrocyte size might be a feature of the articular cartilage (AC) hypertrophic-like phenotype both in experimental and in human osteoarthritis (OA). The anatomical location of these enlarged cells in the cartilage layers was also evaluated.
Methods: Experimental OA was carried out in female rabbits alone or in combination with osteoporosis (OPOA). The rabbits were subjected to destabilization knee surgery to develop OA. Osteoporosis was induced with ovariectomy and methylprednisolone administration. Human OA samples obtained from knee replacement surgery were also studied. Cartilage lesions and chondrocyte size were assessed in AC sections. Immunostaining of type-X collagen and metalloproteinase-13 were used as markers of the AC hypertrophic transformation. Both the cell size and the gene expression of type-X collagen were further analyzed in primary murine chondrocyte cultures.
Results: Compared to healthy AC, chondrocyte size was increased both in experimental and in human OA, in correlation with the severity of cartilage damage. No differences in chondrocyte size were found between deeper or more superficial regions of AC. In cell cultures, accretion of hypertrophic markers and cell enlargement were found to occur synchronized.
Conclusions: We observed an enhancement in the mean size of chondrocytes at the OA cartilage, which showed correlation with cartilage damage, both in human and in experimental OA. The enlarged chondrocytes were homogeneously distributed throughout the AC. Our results suggest that chondrocyte size could be a reliable measure of disease progression, of potential use in the histopathological assessment of OA cartilage.

Introduction

Endochondral ossification primarily drives skeletal development at the growth plate. Chondrocytes undergo various stages of differentiation from a proliferating zone of flattened cells to a hypertrophic phenotype of column-shaped cells which mature into bone[1]. Hypertrophic chondrocytes are mainly characterized by a marked volume enlargement[1]. Cellular mechanisms responsible for this physiological transformation remain largely unknown. Recent data point to possible factors involved, including changes in osmolarity, degradation of pericellular matrix, or the accumulation of cell organelles[2]. Even though cell enlargement is sure to alter chondrocyte functions, it is unclear whether this phenomenon positively contributes to the expression of specific hypertrophic markers or, conversely, it is the expression of terminal differentiation markers which eventually leads to the volume growth[3]. Along with *Indian Hedgehog (IHH)* or *Runx-2* genes, type-X Collagen (ColX) stands as one of the major up-regulated molecules in hypertrophic chondrocyte[1,2,4,5].

Osteoarthritis (OA) is a complex disease involving all joint tissues, but primarily defined by a progressive degradation of articular cartilage (AC)[4]. During OA progression, AC chondrocytes seem to retrieve in some way the characteristic gene expression profile of the terminal differentiating chondrocytes at the growth plate, although some differences may exist between these two processes. Both in human and experimental OA, an enhancement of IHH, Runx-2, ColX, and alkaline phosphatase has been documented[4,5], this accounting for a reason why OA chondrocytes are frequently termed hypertrophic-like chondrocytes[5].

In spite of the up-regulation of hypertrophic markers, the morphological phenotype of OA hypertrophic-like cells is not yet

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completely characterized. We aimed to study whether an increase in cell size might be a hypertrophic-like feature of chondrocytes both in human and in experimental OA.

Methods

Experimental model of OA

32 New Zealand female 8 months old rabbits (4.5 ± 0.5 kg, Granja San Bernardo) were housed in individual cages exposed to 12-h light/dark cycles, with free access to water and standard chow (Panlab). Rabbits were randomly assigned to four equal groups: healthy controls, osteoporosis (OP), osteoarthritis (OA) and OA preceded by OP (OPOA). OP and OA models were induced as described elsewhere[6]. One animal did not survive ovariectomy. Rabbits were euthanized 6 weeks after the OA surgery. The right tibia of each animal was decalcified and embedded in paraffin, where Safranin-O fast green staining and immunodetection techniques were carried out. Animal handling was done in accordance with the Spanish Regulations and the Guidelines for the Care and Use of Laboratory Animals (NIH).

Human tissue collection

Human OA cartilage from nine patients (6w/3 m, 73 ± 4 y.o.) was obtained during knee joint replacement surgery (FJD Hospital), after gaining informed consent and approval from the local Committee. Medial tibial plateaus were decalcified and embedded in paraffin. All procedures were done in accordance with the ethical principles of the Declaration of Helsinki.

Histological damage assessment

Safranin O-fast green sections were scanned using an automated iScanCoreo slide scanner (Ventana Medical Systems). Cartilage damage was evaluated by two blinded observers using a modified Mankin score in rabbit samples[6] (Scale: 0–22). The OARSI histopathological score[7] (Scale: 0–6) was employed in human samples. They were divided into regions according to their different grade of damage. In order to avoid tissue processing artefacts, only clear sections, simultaneously stained were analyzed.

Chondrocyte measurement

Chondrocyte size was measured in Safranin-O Fast Green stained sections (Image Viewer software, Ventana Medical Systems) with a magnification of 20×. Cartilage height was measured at the midpoint of the medial plateau which was thereafter divided into two areas of similar length. On each of them, the height and width of 30 chondrocytes were measured, and cell areas were calculated with the ellipse formula. In human samples, measurements were done in the same fashion in each of the predetermined regions according to OARSI's scoring.

Immunohistochemistry of ColX and metalloproteinase (MMP)-13

Monoclonal antibodies to ColX (1/100; ab49945) and MMP13 (1/200; ab39012) (Abcam) were employed[8]. Positive-to-total cell ratios were calculated in the whole cartilage area from medial tibial plateaus. The antibody specificity was confirmed by Western blot (Sup. Fig. 2(A)). Negative controls of the immunostaining technique are shown in Supl. Fig. 2(B)–(E).

Murine primary cell culture

Articular and costal cartilage samples were isolated from 5-days old C57BL/6 mice[9]. First passaged chondrocytes were seeded in 20 μL droplets (10^6 cells/mL) and maintained for 3 h at 37 °C to let micromass formation. Cells were changed to DMEM/F-12 medium with 2% FBS and 1/100 ITS (1.72 μM insulin, 68.75 nM transferrin, 38.73 nM selenite, Gibco), 1 mM pyruvate (Lonza) and 0.25 mM L-ascorbic acid (Sigma–Aldrich) to differentiate (IAP). After 14 days, micromasses were processed for RNA isolation or embedded in paraffin[8]. Micromass sections (3 μm) were stained with Safranin-O fast green. Chondrocytes with a diameter higher than 20 μm were considered for the analysis.

Expression studies

1 μg RNA was reverse-transcribed (High Capacity cDNA kit, Applied Biosystems), and gene expression studies were performed using real-time RT–PCR (Step One Plus, Applied Biosystems) employing specific TaqMan ColX (Mm00487041_m1) and GAPDH assays (4352932E, endogenous control) (Applied Biosystems)[8]. Cells at day 0 of differentiation were chosen as control.

Statistical analysis

Based on previously published data, the sample size was determined on the expected difference to be detected regarding the chondrocyte size. By accepting a significance level (alpha) of 5% and a power of 80%, a pairwise *t*-test between conditions requires eight animals per group to demonstrate an approximate 1.5 fold-change (187 vs 131) assuming a standard deviation of 40. No adjustment for multiple comparisons between groups was made a priori, but to preserve type one error, individual tests were only to be undertaken following a significant omnibus ANOVA test.

On collection of the data, non-parametric methods were substituted due to an observed lack of normality based on a Kolmogorov–Smirnov test. An overall Kruskal–Wallis test was undertaken prior to individual Mann–Whitney tests adopting a Bonferroni correction. A *P*-value of less than 0.05 was considered significant. Associations were expressed using Spearman's rank coefficient (rho, ρ). The analysis was done with SPSS (v23.0, IBM SPSS Inc., Chicago, IL, USA) or GraphPad Prism (v.6, GraphPad, San Diego, CA, USA). Data are shown as median with interquartile range (IQR).

Results

Chondrocyte size and cartilage histopathology

Cartilage damage was evident in the groups of rabbits undergoing knee surgery as compared to healthy animals. The most severe cartilage lesions were found in the OPOA group[6] [Fig. 1(A) and (B)].

The mean cell size of both OA and OPOA groups was larger than that observed in the healthy cartilages. Chondrocytes from the OPOA group tended to be bigger than those from the OA group [Fig. 1(C) and (D)]. The number of enlarged chondrocytes was higher in the rabbits showing more severe histopathology scores, while there were conversely fewer small cells ($\rho = 0.72$, $p < 0.01$) [Supl. Fig. 1]. The distribution of large size chondrocytes was similar in the superficial and deep regions of the AC for each group of rabbits [Fig. 1(E)].

An association between mean chondrocyte size and Mankin scores was also found in human OA cartilages ($\rho = 0.92$, $p < 0.01$) [Fig. 1(F)].

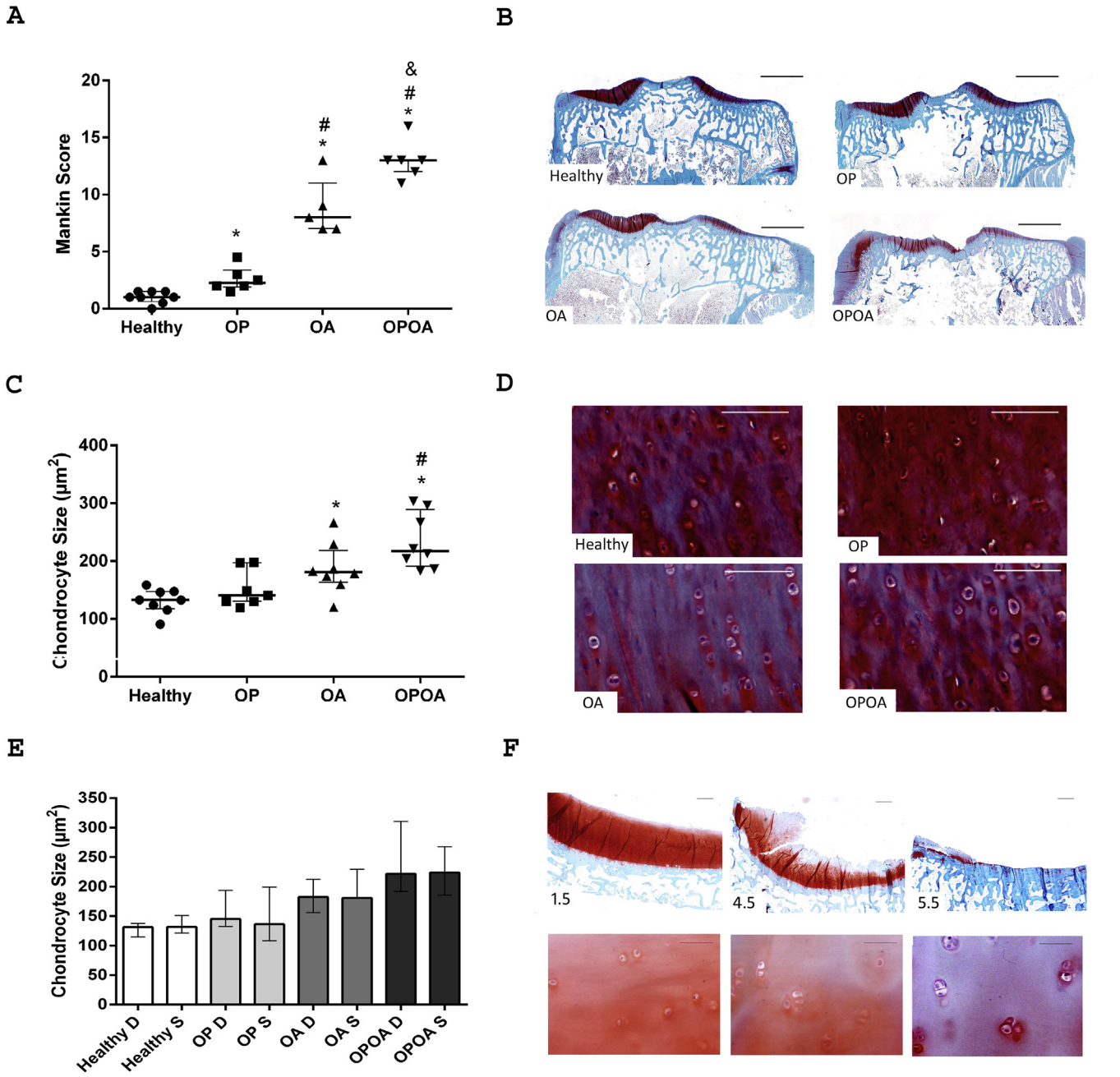

Fig. 1. Chondrocyte size in articular cartilage (AC) increases according to osteoarthritis severity (A) Assessment of cartilage damage in rabbit tibia samples (B) Representative Safranin-O fast green stained rabbit tibia sections, bars represent 3 mm (C) Measurement of the mean chondrocyte size in rabbit tibia samples (D) Representative Safranin-O stained sections of chondrocyte size of (C), bars = 100 µm (E) Different mean chondrocyte size between superficial (S) and deeper (D) area in rabbit tibial articular cartilage ($n = 5–8$) (F) Representative Safranin-O stained slides from human samples at 1× and 20× objectives, bars = 1 mm and 50 µm respectively ($n = 5–8$, Data are shown as Median ± interquartile range (IQR), $^*p < 0.05$ vs Healthy, $\#p < 0.05$ vs OP, $\&p < 0.05$ vs OA).

ColX immunoreactivity in OA cartilage and micromass culture

A higher deposition of ColX was observed in cartilages from OA and OPOA rabbits as compared to healthy, while it was further increased in OA with regard to OP cartilages [Fig. 2(A) and (B)].

An association between ColX deposition and cell enlargement was observed when all the experimental groups were integrated ($\rho = 0.44$, $p = 0.02$). However, the association was lost if only damaged cartilage samples were assessed (OA and OPOA) ($\rho = 0.11$, $p = 0.73$). In human samples, ColX staining appeared at the extracellular matrix, pericellularly and intracellularly [Fig. 2(C),(D),(E)]. We could not confirm an association between ColX and cell size in human sections ($\rho = 0.35$, $p = 0.28$).

In micromass cultured chondrocytes, ColX gene expression was strongly up-regulated by insulin, ascorbic acid and pyruvate (IAP) presence [Fig. 2(F)]. In addition, IAP induced a higher percentage of large chondrocytes (over 20 µm of diameter) [Fig. 2(G) and (H)].

MMP-13 immunoreactivity in OA cartilage

There was an association between MMP-13 staining and chondrocyte size in rabbit damaged cartilage sections (OA and OPOA,

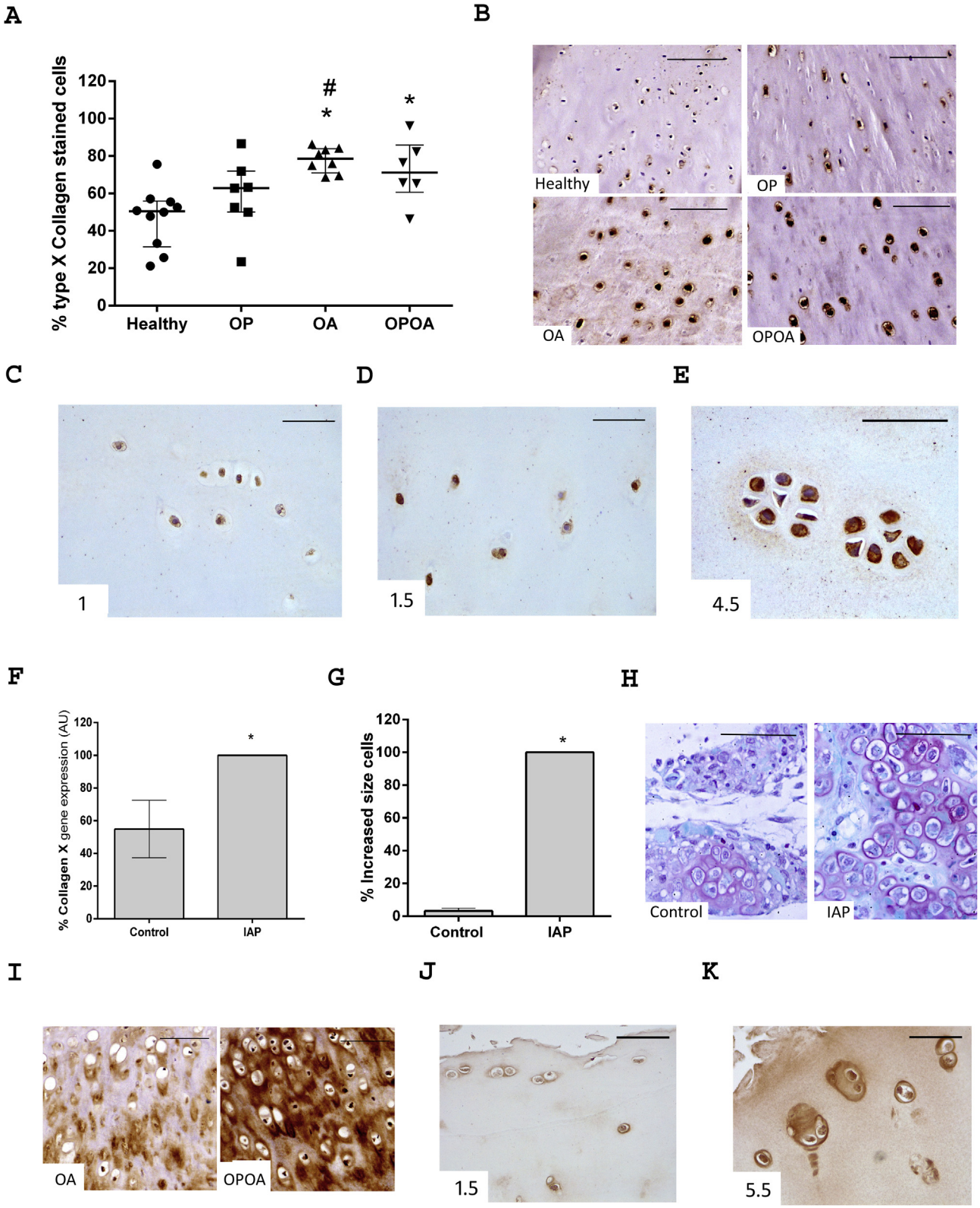

Fig. 2. Increased levels of type-X collagen (ColX) in osteoarthritis (A) Percentage of ColX staining in AC samples from rabbits ($n = 5–8$, Data are shown as Mean ± interquartile range, *p < 0.05 vs. Healthy; #p < 0.05 vs. OP). (B) Representative ColX immunochemistry sections of rabbit tibia samples, bars = 100 μm (C) Representative sections of immunochemistry to ColX in human samples at 40× magnification, bars = 50 μm (D) Representative sections of immunochemistry to ColX in human samples at 40× magnification, bars = 50 μm (E) Representative section of immunochemistry to ColX in human sample at 63× magnification, bars = 50 μm (F) Increased % of ColX expression in chondrocytes differentiated with insulin, ascorbic acid and pyruvate (IAP) as regards control group ($n = 10$, Mean ± SEM, *$p < 0.05$ vs. Control). (G) Increased frequency of enlarged chondrocytes (higher than 20 μm diameter) differentiated with IAP in comparison to unstimulated cells ($n = 5–7$, Mean ± SEM, *$p < 0.05$ vs. Control). (H) Representative sections of micromass primary mice culture stained with Safranin-O, bars = 100 μm (I) Representative MMP13 immunochemistry sections of rabbit tibia samples (bars = 100 μm) (J) Representative MMP13 immunochemistry sections of human tibia samples (bars = 100 μm) with their respective grade of severity (K) Representative MMP13 immunochemistry sections of human tibia samples (bars = 100 μm) with their respective grade of severity.

$\rho = 0.60$, $p = 0.048$) [Fig. 2I]. However, we could not observe an association between both features in human sections ($\rho = 0.53$, $p = 0.08$) [Fig. 2(J) and (K)].

Discussion

Our results show that a growth in cell size takes place in AC during experimental OA. In our model, this feature progressed in relationship with histopathological damage. Of note, the tight statistically significant association between cell enlargement and severity of cartilage damage could also be confirmed in human OA. As a whole, these data support that the variation in chondrocyte size evolves in parallel to disease severity, both in human OA and in an experimental model.

During development of OA, there is an enhancement in subchondral bone remodeling activity, which results in a net bone gain in established disease[10,11]. Accordingly, there is an increase in the thickness of calcified cartilage, which has been described as a duplication or an advance of the tidemark[12]. At the same time, the deepest layer of calcified cartilage is apparently replaced by new bone arising from endochondral ossification. These modifications along with the recapitulation of a hypertrophy-related protein profile by OA chondrocytes (hypertrophic-like phenotype) possibly indicate an attempt of the AC to activate secondary ossification centers[13].

Therefore, we expected hypertrophic chondrocytes to be placed at the deepest layers of AC, immediately above calcified cartilage, and arranged in resemblance to the growth plate's organization. However, we could not find differences in cell size between layers in rabbit AC. Although this comparison was not feasible in the human samples, because of the advanced destruction of superficial layers in some areas, previous published data indicate that these oversized chondrocytes appear at different cartilage layers in human OA as well[14].

We studied the appearance of ColX and MMP-13, as well-established markers of hypertrophy, aiming at mapping the hypertrophic-like phenotype throughout the tissues. As compared to healthy AC, both ColX and MMP-13 presence was increased in cartilages from diseased rabbits, in whom it had a uniform distribution pattern. Intriguingly, these widespread hypertrophic-like changes across the OA cartilage were matched by the non-selective localization displayed by enlarged chondrocytes. Therefore, whilst according to our data the protein expression profile of OA resembles that of physiological endochondral ossification, it nevertheless acquires a diffuse pattern of distribution in all cartilage layers during OA. This remarkable difference probably reflects functional differences as well. Thus, the fact that hypertrophic-like OA chondrocytes might drive the OA-associated increase in cartilage calcification cannot be drawn from our data.

No association between ColX and chondrocyte size increase was found either in experimental or in human AC. A lack of relationship between OA severity and ColX presence had already been noticed, in relationship with the abrupt decline of the latter in advanced OA[15]. This could derive from a loss of its expression by apoptotic chondrocytes. It could also occur that surviving chondrocytes ceased to express high levels of hypertrophy-related molecules in late disease. Altogether, these observations imply that in spite of being a good indicator of hypertrophy, ColX cannot be taken as a similarly reliable marker of OA severity[15].

Mechanisms accounting for chondrocyte morphologic alterations during OA are only partially comprehended[13]. Even though the same growth factors and additional mediators which trigger differentiation of growth plate's chondrocytes could be involved[13], extrinsic molecules becoming locally increased during OA might also play a role. Inflammatory cytokines can evoke hypertrophy-inducing mechanisms[8]. Alternative theories suggest that chondrocyte enlargement arises as a result of tissue swelling caused by the loss of collagen network integrity, although additional mechanisms could be involved[14].

A limitation of this study is the small sample size employed in the animal model. Therefore, additional research would be recommended in order to confirm the relationship between cartilage damage severity and the chondrocyte size.

In summary, our studies show that a progressive increment in chondrocyte size parallels progression of OA, and point to this feature as a reliable marker of cartilage damage. Measurement of cell enlargement in AC could be standardized in OA histopathology scoring systems, and be potentially useful in the assessment of response to experimental treatments. It could account for an objective measure of cartilage damage, but it would also allow the evaluation of severely damaged regions of cartilage.

Author contributions

PG, AM, GH-B, RL and were in charge with conception, design, analysis, and interpretation of data. PG, IP-P and AL performed the animal experiments. PG, IP-P, and AL contributed to the acquisition of data. PG, AM, OS-P, IP-P, AL, GH-B and RL were involved in drafting the article or revising it critically for important intellectual content and approved the definitive version to be published. GH-B and RL have full access to overall data and takes responsibility for the integrity and accuracy of the data analysis.

Competing interests

PG, AM, OS-P, IP-P, AL, GH-B and RL do not have any disclosures.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2019.04.013.

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