ORIGINAL ARTICLE

Subchondral cyst development and MMP-1 expression during progression of osteoarthritis: An immunohistochemical study

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KEYWORDS
Osteoarthritis; Subchondral bone cysts; MMP-1; Immunohistochemistry

Summary

Background: Subchondral bone cyst (SBC) formation is often identified in patients with osteoarthritis. Furthermore, several studies have shown that expression of matrix metalloproteinases (MMPs) is elevated in patients with OA.

Objectives: The aim of our study is to correlate the presence of SBCs and MMP-1 expression with the osteochondral alterations during OA progression.

Methods: We studied the cartilage and subchondral bone of 15 patients who had undergone total knee or hip replacement due to primary OA. As controls, we used the femoral heads of three patients without macroscopic OA changes. We evaluated three specimens per patient.

Results: Specimens were divided in four groups based on the Mankin histological severity score. Using immunohistochemistry, we noted SBCs at the site of greatest disease severity. Specifically, these were present more frequently in group III (Mankin score: 6–7) and IV (Mankin: ≥8), compared with group I (Mankin: 1–3) and II (Mankin: 4–5). Mild OA stages (Mankin: 1–6) were characterized by degeneration and thinning of the cartilage, followed by increased osteoblast and osteoclast activity of the subjacent bone and the subsequent appearance of SBCs. Simultaneously, we observed expression of MMP-1 in groups I and II in the cartilage and III and IV in

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Introduction

Osteoarthritis (OA) is the major cause of disability in the adult population. Although in the past it was considered as a primary disorder of articular cartilage, it is now generally considered as a disease of the whole joint, including the calcified cartilage, subchondral cortical and trabecular bone, joint capsular tissues and the synovium [1]. Not only the structural support, but also the biological cross-talk between bone and cartilage, make subchondral bone and cartilage become a closely functional unit that cannot be separated [2]. A characteristic of bone adaptation associated with OA is the presence of subchondral bone cysts (SBC) [3] that are present in about 50% of subjects with knee OA [4,5].

Subchondral bone cysts (SBCs), "pseudocysts" or "geodes" [6–8], were first identified by Ondrouch [9] and Landells [10] in the load-bearing regions of the femur, patella, and shoulder of arthritic patients, although the exact cause is not well known [8]. Currently, there are two main theories: the "Bone Contusion Theory" where bony microcontusions below the joint surface, associated with increased intra-articular pressure lead to extension of synovial fluid into the subchondral bone through tiny gaps in the articular surface, and the "Synovial Breach Theory" where a breach in the subchondral plate caused by a diminished local cartilage leads to a rapid inflammation response and the proliferation of myxomatous tissue within the bone marrow [6,8,9,11]. Subchondral cystic lesions appear as well-delimited areas of fluid signal on magnetic resonance imaging (MRI), corresponding to radiolucencies encompassed with sclerotic margins on standard radiographs [12,13]. They are currently gaining increasing attention for their potential etiologic role in the development and progression of OA. Moreover, their presence is associated with increased cartilage loss and risk of knee joint replacement [14].

The matrix metalloproteinases (MMPs) are a family of 23 proteolytic enzymes that share several structural and functional characteristics with different substrate specificities. MMPs degrade proteins of the extracellular matrix and they have been considered the main enzymes responsible for degradation of collagens in OA cartilage [15]. MMP-1 is an interstitial collagenase that is capable of degrading interstitial collagens (types I, II and III) and is thought to be a multifunctional molecule with important roles in diverse physiologic processes maintaining osteochondral integrity [16,17].

In this study, we correlated SBCs presence and MMP-1 expression in different OA stages by using histological and immunohistological measurements, in order to shed light on SBCs role during the progression of OA and to explore their correlation with MMP-1 expression at the whole cartilage-subchondral bone functional unit. Our hypothesis was that SBCs might be associated with increased MMP-1 expression.

Patients and methods

Overall, 15 patients aged 60–89 years diagnosed with idiopathic OA of the hip or of the knee in accordance with the criteria of the American College of Rheumatology [18,19] were included and compared to three controls aged 68–83 years (Table 1). Exclusion criteria were as follows: secondary arthritis, haemochromatosis, hip fractures, inflammarory or autoimmune diseases, malignancies, intra-articular administration of steroids. Eight patients suffered of knee OA and seven of hip OA. OA severity was estimated based on pain, functionality, and clinical data using the Harris score for hip OA [20] and the index of severity (ISK) for knee OA [21]. Radiographically, assessment of OA was based on the Ahlbach classification for the knee OA [22] and the Kellgren & Lawrence for hip OA [23]. The same orthopaedic surgeon specialized in OA evaluated all X-rays. Controls consisted of three femoral heads [24–27], which were harvested in patients undergoing hip hemiarthroplasty following fracture of the femoral neck. All control patients had no known history of bone or joint disease and their cartilage was free of OA lesions (grade 0), according to the Collins scale modified by Muehleman et al. [28].

Ethical approval and informed consent were received, according to the 1964 Helsinki Declaration.

Histology and immunohistochemistry

We evaluated three specimens per patient, and per control.

Histology

The specimens were fixed in 10% buffered formalin for 24–36 h, decalcified in neutral ethylene-diamine-tetra-acetic acid (EDTA) for 6–8 weeks at room temperature, and embedded in paraffin blocks. Three-micrometer thick histological sections were obtained and stained with Haematoxylin/Eosin. The quantitative histological evaluation of the specimens was based on a modified form of the Mankin scale [29,30]. Normal cartilage was graded as 0, while the scale went up proportionally to the severity of the OA changes. In addition, the status of chondrocytes and the subchondral area were similarly graded. The total score of all parameters characterized the severity of the OA changes in

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every histological section. Four groups of OA were formed: Group I was constituted of sections with Mankin score 1–3, group II with Mankin score 4–5 and group III and IV with Mankin score 6–7 and greater or equal to 8, respectively. The sums of scores of all sections in each group were statistically analysed and compared with immunohistochemical staining for MMP-1 in the same sections.

**Immunohistochemistry**

The slices were deparaffinised in xylene and degraded alcohols, immersed in distilled water. The endogenous peroxidase was blocked with 3% H$_2$O$_2$ for 30 min in a dark chamber at room temperature. Sections were then washed in distilled water and three times with TBS, incubated for 1 hour at room temperature with anti-MMP 1/8 (H-300) (Santa Cruz sc-30069) diluted 1:50 in antibody diluents (DAKO REAL S2022), incubated for 45 min at room temperature with peroxidase-labelled anti-mouse/rabbit IgG (En-vision Kit, DAKO Detection System, Peroxidase/DAB+, Rabbit/Mouse K5007), washed three times with TBS, and stained for 10 minutes in a dark chamber at room temperature with 3-amino-9-ethylcarbazole/H$_2$O$_2$, washed in distilled and counterstained with haematoxylin.

**Statistical analysis**

Kruskal-Wallis test was used for comparing the histological and immunohistochemical evaluation results of the sections between the groups of patients with OA results. Differences were considered significant when $P$-values were inferior to 0.05.

**Results**

**Subchondral bone cysts and Mankin score in OA**

Histological findings were identical for all the non-communicating cysts, which contained necrotic bone fragments with dead denuclearized cells. The cavities were surrounded by a layer of fibrous connective tissue containing adipocytes and osteoblasts. Overall, the frequency of SBCs was significantly greater in groups III and IV (Fig. 1A). Specifically, their frequency in patients with knee OA was higher in the group III (Fig. 1B). Their frequency in patients with hip OA (Fig. 1C) was remarkably increased in group IV ($P = 0.028$).

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**Table 1** Characteristics of the study population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>OA Patients ($n = 15$)</th>
<th>Controls ($n = 03$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex$^a$</td>
<td>14 females, 1 male</td>
<td>2 females, 1 male</td>
</tr>
<tr>
<td>Mean age (years)$^b$</td>
<td>72.4 $\pm$ 8.29 (Min: 63, Max: 89)</td>
<td>72.3 $\pm$ 6.66 (Min: 65, Max: 78)</td>
</tr>
<tr>
<td>Mean height (cm)$^b$</td>
<td>164.4 $\pm$ 5.355 (Min: 156, Max: 173)</td>
<td>172.67 $\pm$ 2.52 (Min: 170, Max: 175)</td>
</tr>
<tr>
<td>Mean weight (kg)$^b$</td>
<td>85.9 $\pm$ 15.16 (Min: 65, Max: 110)</td>
<td>77.10 $\pm$ 7.94 (Min: 68, Max: 83)</td>
</tr>
</tbody>
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$^a$ Expressed as number of individuals.

$^b$ Expressed as mean $\pm$ standard deviation.

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**Figure 1** A. Overall results from the immunohistochemical analysis processed statistically. B. Results from the immunohistochemical analysis of the knee OA patients. C. Immunohistochemical findings in patients with Hip OA.

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and fourth group and its staining rose significantly with the severity of OA changes. Additionally, the frequency of MMP-1 expression in the cartilage and in the subchondral bone was increased in groups III and IV compared with the groups I and II in both patients with knee (P = 0.03 and P = 0.002, respectively) and hip OA (P = 0.0019 and P = 0.0014, respectively).

MMP-1 was detected in the matrix during the aggregation of chondrocytes (Fig. 3A) and in the fibrocartilage (Figs. 3B and C), pannus (Fig. 3D) or fissures (Fig. 4A). In the subchondral bone, MMP-1 was detected around the osteoblast-like cells (Fig. 4B) and in the bone-lining cells (Fig. 4B). The presence of osteoblast-like cells was accompanied by the simultaneous presence of osteoclasts, mainly in group III. However, expression of MMP-1 in the osteoclasts around the cysts was not observed (Fig. 4C). In group IV there was an increased number of apoptotic osteocytes positive for MMP-1 (Fig. 4D).

The wall osteoblasts of SBCs in groups III and IV also expressed MMP-1 (Figs. 5 and 6A and B), whereas MMP-1 expression was not detectable in groups I and II (Fig. 6C).

Discussion
To the best of our knowledge, this is the first study that examines the relationship between the presence of SBC and

Expression of MMP-1 in OA tissues
Cartilage and subchondral bone matrix and cells, as well as the cystic lesions of OA patients were stained positively for MMP-1, while it was undetectable in controls (Fig. 2). MMP-1 expression was observed in the sections of the second, third

Figure 2  Cartilage and subchondral bone without OA changes in a control negative for MMP-1.

Figure 3  A. Cartilage matrix (black arrows) with positive expression for MMP-1 in a patient with a Mankin score of 5 and chondrocytes in aggregations (red arrows). B. Fibrocartilage (black arrows) and cartilage matrix (red arrows) positive for MMP-1 in a patient with a Mankin score of 7. C. A large number of subchondral cysts (black arrows) with overlying fibrocartilage tissue (red arrows) in a patient with a Mankin score of 6. D. MMP-1 expression in cartilage with pannus (black arrows) and chondrocytes in aggregation (red arrows), from a slice with a Mankin score of 7.

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MMP-1 expression. Despite the small number of patients in the control group the results reached significance. It is important to note that our observations mostly derived from female patients, reflecting an epidemiological proportion of OA in our region.

The frequency of SBC was related to the severity of the degenerative changes and they were found to develop at the site of greatest disease severity, which is consistent with other studies in humans [31] and in animal models, such as the rat anterior cruciate ligament transection model [3]. It also looks in agreement with the theory of Ondrouch, according to which SBCs appearance is preceded by joint narrowing that would induce subchondral bone micro-fracture in response to repeated overloading.

SBCs were found to coexist with increased cell number of osteoblasts and osteoclasts. Additionally, defects filled by fibrocartilage were observed around the cysts (Fig. 4A), which were also surrounded by large numbers of apoptotic osteocytes. This suggests incomplete healing following repeated microtrauma, leading to further deterioration and finally to focal osteonecrosis [3].

Another important finding is the concurrent secretion of MMP-1 in the subchondral bone-lining cells in Mankin stages 6 or more (Fig. 6D). Although the heterogeneity of their osteoblastic phenotype has been well established, bone-lining cells constitute a subpopulation of the osteoblastic family, which responds to abnormal mechanical loading. Before osteoclastic attachment and resorption, bone-lining

Figure 4  A. Presence of subchondral cysts presenting osteoblastic expression of MMP-1 in overlying cartilage with crevices (black arrows). We note fibrous tissue (red arrows) in the cyst wall. From a slice with a Mankin score of 7. B. Intense MMP-1 expression in the walls of subchondral cysts and the subchondral bone by osteoblast-like cells (black arrows) and lining cells (red arrows) in a slice with a Mankin score of 8. C. Subchondral bone cyst showing mild expression of MMP-1, from mural osteoblast-like cells (black arrows). Adjacent, we observed MMP-1 expression by subchondral osteoblast-like cells and an aggregation of multinuclear cells (osteoclasts, red arrows) from a slice with a Mankin score of 5. D. Expression of MMP-1 in fibrocartilage tissue, in osteoblast-like cells (black arrows) and in apoptotic osteocytes (red arrows) around a SBC (Mankin score 8).

Figure 5  We note MMP-1 expression in osteocytes (black arrows), in cartilage tissue with pannus (red arrows) and mild expression in areas of cystic processes in the cartilage and subchondral bone (Mankin 6).
cells digest non-mineralized collagen protruding from the bone surface, action that would be activated by MMPs [32].

The SBC osteoblast-like cells showed an increased expression of MMP-1 in Mankin stages greater than 6, which would account for degradation of non-mineralized collagen type I, exposing binding sites on the surface of the bone. This has been shown to be a prerequisite for osteoclast stimulation and attachment to the bone [33–36]. Our data are in agreement with the study of Sasaki et al. showing that stromal lining cells and osteoblasts would express MMP-1, upon mechanical stimulation, thus preparing recruitment sites for osteoclasts [37]. Furthermore, the presence study showed the coexistence of SBCs with apoptotic osteocytes expressing MMP-1, in late stages of OA. The relationship of MMP-1 with cell apoptosis has been suggested by recent reports providing evidence for the role of MMP-1 in targeting non-matrix substrates, such as cell bound cytokines, enzymes and cell surface receptors [38].

These findings have clinical significance and show that SBCs, as well as MMP-1, may be directly involved in the progress of the disease. It has been reported that the presence of SBCs increases the risk of joint replacement [14] and revision arthroplasty [39]. In fact, increased expression of MMP-1 is implicated in the aseptic loosening of joint replacement implants [40].

Conclusion

In conclusion, MMP-1 expression by osteoblasts, the lining cells of the subchondral bone and SBCs in advanced OA stages, may contribute to the pathological tissue remodelling and the osteochondral changes in OA. Hence, targeting MMP-1 may provide an important therapeutic alternative for OA that affect not only the cartilage but the subchondral bone, as well.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

References


