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Original Research

Gene expression and histological studies of articular chondrocytes in cam-type femoroacetabular impingement demonstrates chronic and sustained inflammation and age related abnormal extracellular matrix ☆,☆☆

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ABSTRACT

Introduction: Femoroacetabular impingement (FAI) is a frequent cause of hip pain associated with the degeneration of cartilage in hip joint. However, the molecular events linking the bone and cartilage deformation with joint degeneration are unclear.

Objective: Using gene expression and histological analyses of cam-type FAI tissues to discover abnormal biological changes of chondrocytes that contribute to the molecular pathophysiology of FAI.

Methods: Full-thickness cartilage specimens obtained from donors who underwent hip arthroscopy to address symptomatic cam-type FAI were analyzed. Quantitative real-time polymerase chain reaction (RT-PCR) was performed to assess gene expressions of markers for inflammation, extracellular matrix (ECM) synthesis, and cellular senescence. Histological specimens were prepared with safranin O/fast green staining as well as immunohistochemistry for evaluation.

Results: Compared to normal cartilage, cam-type FAI tissues demonstrated decreased expression of ACAN, COL2, and Sox9. Additionally, chondrocytes in these tissues showed increased expressions of MMP13, ADAMTS4, and IL-1β, as well as p21, Bcl-2, and FasL. Histological analyses of the FAI tissues revealed two distinct phenotypes: safranin O positive (SO+) and negative (SO-) that

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☆☆ All specimens used in this study were obtained with tissue donation programs approved by Northwell Health Tissue Donation Program (TDP) Committee for Participant Protection (COPP) (TAP1711, TAP1902) of Northwell Health.

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demonstrated different stages of FAI related to patient age. Immunohistochemical studies of COL2, ACAN, MMP3, and PCNA showed differences between SO+ and SO- groups.

Conclusions: Gene expression and histological analyses indicated that chronic and sustained inflammation and age related degradation of extracellular matrix associated with cell senescence were major characteristics of FAI tissue.

Introduction

Femoroacetabular impingement (FAI) is a common source of hip pain and dysfunction. The etiology of FAI is not fully discovered, however, the pathogenesis may relate to the irregular development of the musculoskeletal system or possible preceding injury of the hip.[1] The abnormal contact between the acetabulum and proximal femur is often divided into three types: cam, pincer, and combined.[2] Cam lesions are characterized by an aspherical head-neck junction while pincer lesions are marked by regions of the acetabulum that extend beyond the normal socket rim. Both deformities impede normal movement of the femoral head within the acetabulum thereby stimulating the mechanical destruction of articular cartilage.[3]

Epidemiological studies have shown that the presence of FAI can be associated with up to a ten-fold increased risk for osteoarthritis (OA) within five years in patients more than 45 years old.[4] However, while the clinical association between FAI and the development of OA is well known, many patients remain asymptomatic for years.[5] Unlike traditional OA with consistent joint pain and swelling, FAI patients often suffer from intermittent pain. Current evidence suggests that initial cartilage damage stimulates chondrocytes to release a combination of extracellular matrix (ECM) proteins including catabolic enzymes such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs).[6,7] We hypothesized that the intrinsic molecular profile of chondrocytes in FAI tissue pose a chronic and sustained negative influence on the joint cartilage beyond the mechanical damage secondary to physical impingement.

Methods

Acquisition of cartilage samples

Experimental specimens from 29 donors were obtained from symptomatic FAI patients who underwent hip arthroscopic cam resection from the Institutional-Review-Board-approved tissue donation program. The diagnosis of FAI was based on a history of activity related hip pain, the limitation of the hip motion, positive impingement test, standing anteroposterior (AP) pelvis, 45° Dunn lateral view and false profile radiographs and three dimensional (3D) computed tomography (CT) scans confirming cam type impingement and magnetic resonance (MR) imaging confirming labral tear. Patients were included between the ages over 18 and under 65, joint preservation with less than Tönnis Grade 2 on radiographs,[8] documented alpha angle greater than 55° measured utilizing radiographs[9] and 3D CT imaging. Exclusion criteria included prior hip surgery, dysplasia, Perthes disease, avascular necrosis and synovial chondromatosis. All cartilage samples harvested by the arthroscopic cam resection were biopsied from the apex of the cam deformity based on preoperative 3D CT scan assessment and intraoperative correlation (Fig. 1A, B). Control specimens were obtained from 4 cadavers with no evidence of FAI or other joint disease. Specimens from both groups were then subdivided for molecular and histological analyses with the methods similar as previously published studies.[7,10-12]

Gene expression analysis

Total ribonucleic acid (RNA) was extracted from the specimens by using MACS M Tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) with RNeasy MinElute Cleanup kit (QIAGEN, Venlo, Netherlands). The cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, California) and T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, California). Gene expression analysis was performed using the Applied Biosystems QuantStudio 3 (ThermoFisher Scientific, Grand Island, NY) with SYBR green master mix (ThermoFisher Scientific, Grand Island, NY) following standard protocols. Quantitative real-time polymerase chain reaction (RT-PCR) was then performed, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control. The gene expression analyses included aggrecan (ACAN), collagen type II (COL2), sex-determining region Y-box-9 (Sox9), matrix metalloproteinase-3 (MMP3), matrix metalloproteinase-13 (MMP13), a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS4), interleukin-1β (IL-1β), B-cell lymphoma 2 (Bcl-2), cyclin-dependent kinase inhibitor 1A (p21), cyclin-dependent kinase inhibitor 2A (p16), tumor protein p53, and Fas ligand (FasL). The primers were produced with custom designed sequences (Table 1) (Integrated DNA technologies, Coralville, Iowa). The data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

Histological analyses

Cartilage specimens were fixed in 10% formalin, dehydrated, and then embedded in paraffin using standard technique. Sections were cut at 7 μm thickness and stained with safranin O/fast green (American MasterTech Scientific, Inc., Lodi, California). Immuno-

Table 1
Primer sequences.

Primers	Sequences	Product length (bp)
GAPDH	5′-ACCCAGAAGACTGTGGATGG-3′ 5′-GAGGCAGGGATGATGTTCTG-3′	80
ACAN	5′-GGCACTAGTCAACCCTTTGG-3′ 5′-CTGAACCCTGGTAACCCTGA-3′	95
COL2A1	5′-CGCACCTGCAGAGACCTGAA-3′ 5′-TCTTCTTGGGAACGTTTGCTGG-3′	163
SOX9	5′-ACCACCCGGATTACAAGTACCA-3′ 5′-TTGAAGATGGCGTTGGGGGAG-3′	112
MMP3	5′-TGAAGAGTCTTCCAATCCTACTGTTG-3′ 5′-CTAGATATTTCTGAACAAGGTTCATGCA-3′	114
MMP13	5′-GGACAAGTAGTTCCAAAGGCTACAA-3′ 5′-CTTTTGCCGGTGTAGGTGTAGATAG-3′	130
ADAMTS4	5′-GGCTAAAGCGCTACCTGCTA-3′ 5′-GAGTCACCACCAAGCTGACA-3′	93
IL-1β	5′-TCCAGGAGAATGACCTGAGC-3′ 5′-GTGATCGTACAGGTGCATCG-3′	111
CCL3L1	5′-GTCCTCTCTGCACCACTTGC-3′ 5′-GGAAGATGACACTGGGCTTG-3′	136
BCL2	5′- CTTTGAGTTCGGTGGGGTCA -3′ 5′- GGGCCGTACAGTTCCACAAA -3′	162
ASF1A	5′-GTGCATCGAGGACCTGTCTG-3′ 5′-CGGGAACAGGACCCACTAAA-3′	113
P21	5′-AGGATGACAAGCAGAGAGCCC-3′ 5′-AAGGGGAGGATTTGACGAGTG-3′	186
P16	5′-CTGAGGCGCCCTTTGGTTA-3′ 5′-AAACTACGAAAGCGGGGTGG-3′	238
P53	5′-GTTCCGAGAGCTGAATGAGG-3′ 5′-TTATGGCGGGAGGTAGACTG-3′	123
FasL	5′-TCAATGAAACTGGGCTGTACTTT-3′ 5′-AGAGTTCCTCATGTAGACCTTGT-3′	101

histochemical staining of the specimens was performed with primary antibodies of COL2 (1:200 dilution, Abcam, Cambridge, MA), ACAN (1:100 dilution, Abcam, Cambridge, MA), MMP3 (1:400 dilution, Aviva Systems Biology, San Diego, CA) and proliferating cell nuclear antigen (PCNA) (1:1000 dilution, Cell Signaling Technology, Danvers, MA). Chromogen DAB (3,3′-Diaminobenzidine) (Cell Signaling Technology, Danvers, MA) was used for the staining.

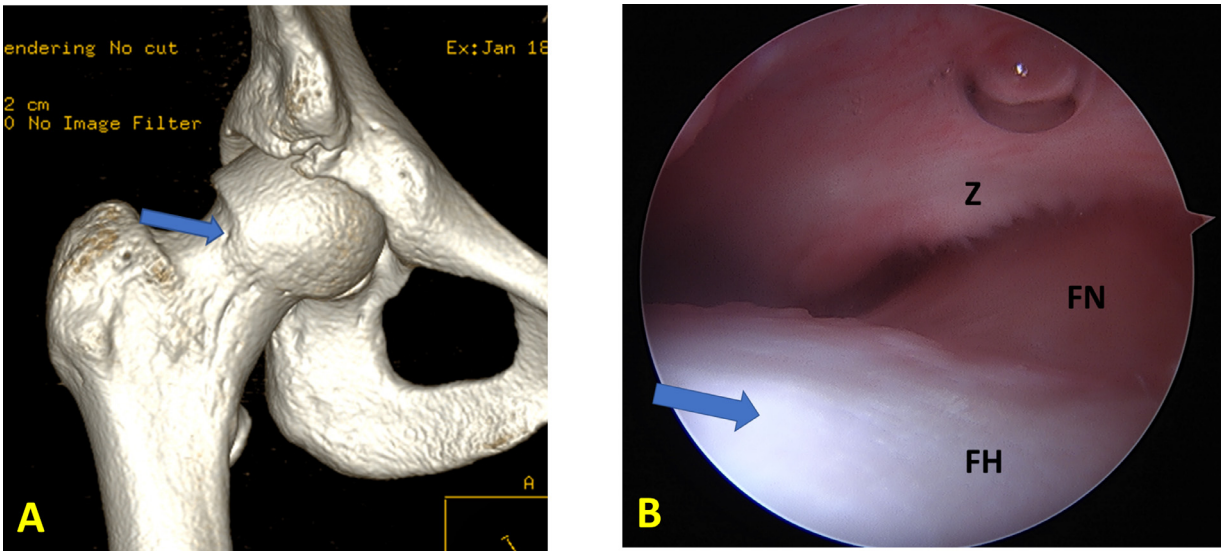

Fig. 1. The location of the sample collection (arrow). (A) 3d-CT reconstruction image. (B) Arthroscope view. Z: Zona orbicularis; FN: Femoral neck; FH: Femoral head.

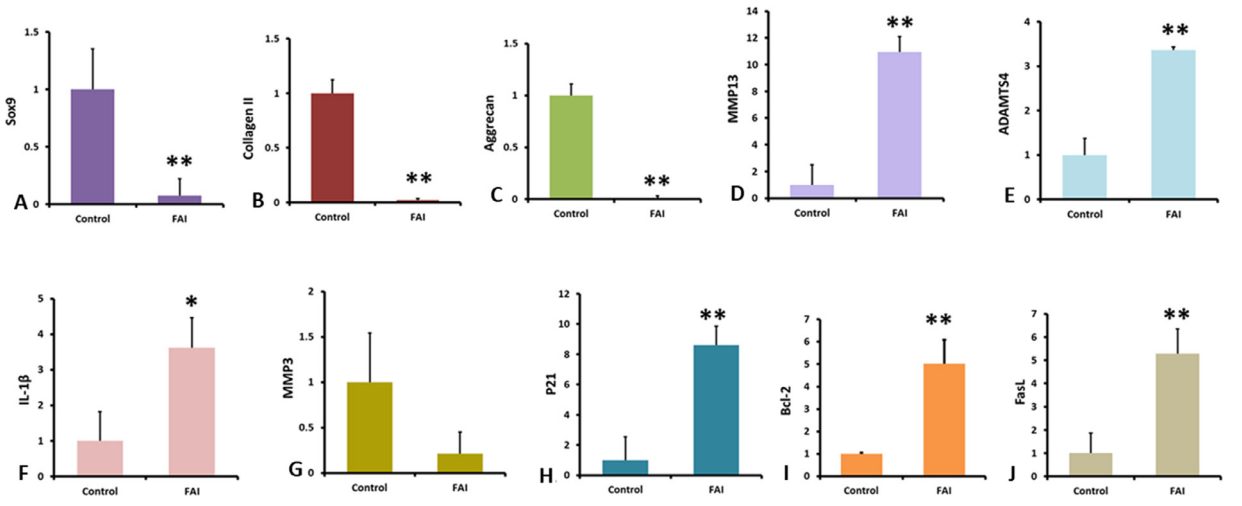

Fig. 2. Comparison of relative gene expression between FAI and normal cartilage. The results are shown as relative fold change. $^{*}P < .05$; $^{**}P < .01$. (A) Sox9; (B) type II collagen; (C) aggrecan; (D) MMP13; (E) ADAMTS4; (F) IL-1β; (G) MMP3; (H) p21; (I) Bcl-2; (J) FasL.

Statistical analysis

The statistical analyses were performed with open source bio-statistical software on the website (https://www.statskingdom.com/index.html). The results of safranin O staining were compared with age and alpha angle by Mann-Whitney U test. The compare with gender was calculated by using chi-square test. Gene expression study results were compared by *t*-test. Significance was determined if $P < .05$.

Results

Gene expression analysis

The expressions of Sox9 ($P < .01$), COL2 ($P < .01$), and ACAN ($P < .01$)—as markers of cartilage health—in FAI specimens were all significantly lower compared to the control group (Fig. 2A, B, C). The degradation of ECM and inflammation-related factors, MMP13 ($P < .01$), ADAMTS4 ($P < .01$), and IL-1β ($P < .05$), were all significantly higher in the FAI group (Fig. 2D, E,F). However, the difference of the expression of MMP3 did not reach a statistical significance (Fig. 2G). The expressions of p21 ($P < .01$), Bcl-2 ($P < .01$) and FasL ($P < .01$) were significantly higher compared to the control (Figure2H, I, J). The expression of p16 was increased in the FAI group compared to the control; however, the difference between the two groups did not reach statistical significance (data not shown). The expression of p53 in the FAI group was too low to be detected (data not shown).

Safranin o staining analysis

The cartilage samples obtained from the controls displayed a surface devoid of fibrillation or edema. Positive safranin O staining was distributed uniformly throughout most part of the matrix. Cell density was similar among different zones (Fig. 3A, B). In contrast, The ECM in the center region of the FAI tissue demonstrated sparse safranin O positive staining. Most of the positive safranin O staining was observed in the pericellular area (Fig. 3C, D). After reviewing all of the specimens, the FAI samples could be grouped phenotypically as safranin O positive (SO+) or negative (SO-) based on whether positive stained safranin O could be seen in the entire tissue. In the SO+ group, the positive staining was seen primarily in the pericellular area in the tissue (Mankin score 2 or 3) .[13] Some of the cells exhibited a large size and formed clusters (Fig. 3E,F). However, in the specimens with SO-, the safranin O positive staining was hardly seen in the entire tissue (Mankin score 4) .[13] The cell size in this group was small with low density (Fig. 3G,H).

Based on these results of safranin O staining and the resulting partition into the SO+ and SO- designations, the age, alpha angle and gender of the donors were then compared between groups. Donors with SO- tissue (32 ± 7 years old) tended to be younger than donors with SO+ tissue (42 ± 10 years old) ($P < .05$) (Fig. 3I). The alpha angle (Fig. 3J) and gender proportion (Table 2) between two groups showed no significant difference.

Immunostaining analyses

In the control specimens, COL2 stained densely positive in the ECM of the superficial zone but less in the deeper zones. All of the cells in the control group demonstrated positive staining for COL2 throughout (Fig. 4A). In SO- specimens, most of the tissue

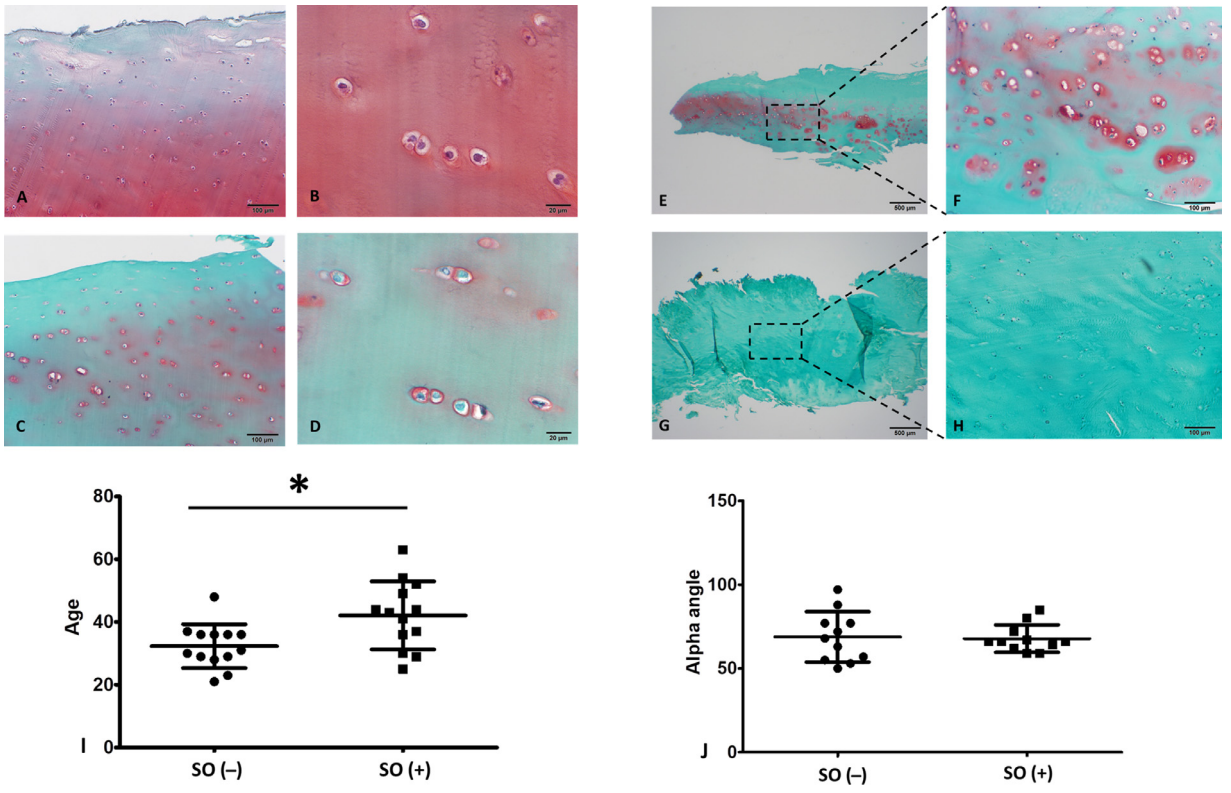

Fig. 3. Safranin O/fast green staining of normal cartilage (control) and FAI tissues. (A) Control cartilage at low magnification; (B) Control cartilage at high magnification; (C) FAI tissue at low magnification; (D) FAI tissue at high magnification; (E) Safranin O positive (SO+) specimen at low magnification; (F) SO+ specimen at high magnification; (G) Safranin O negative (SO-) specimen at low magnification; (H) SO- specimen at high magnification; (I) Compare the ages between SO- and SO+ groups; (J) Compare the alpha angle between SO- and SO+ groups. $^{*}P < .05$.

Table 2
Compare safranin O staining results (SO+/SO-) with genders.

		SO+	SO-	*P* value
Gender	Male	50.0% (n = 6)	50.0% (n = 6)	0.999
	Female	53.8% (n = 7)	46.2% (n = 6)	

stained positively for COL2 both inside the cell and the ECM (Fig. 4B). In contrast, in the SO+ specimens, the ECM and the cells close to the surface displayed faintly positive COL2 staining. However, the cells in the deep zones showed little-to-no COL2 staining (Fig. 4C).

ACAN staining of the control group showed a broadly positive, uniform pattern in the ECM around the chondrocytes (Fig. 4G). In the SO- specimens, positive staining of ACAN in the ECM was seen in the deep zone close to bone. Near the cartilage surface, there was little-to-no staining for ACAN in the ECM; however, some cells in the superficial zone possessed positive staining (Fig. 4H). In contrast, the SO+ group demonstrated more positive staining of ACAN in the ECM than SO- group but less than the control group especially in the pericellular area (Fig. 4I).

MMP3 was analyzed by immunohistochemical staining in order to evaluate levels of ECM turnover. In the control samples, the superficial areas stained positively within the ECM; however, in the deep region, the positive staining was only seen intracellularly (Fig. 4M). In the SO- specimens, the ECM stained positively for MMP3 but with a markedly lighter color than the ECM in control group. The cells in the SO- group possessed little-to-no positive staining (Fig. 4N). In the SO+ specimens, there was positive staining in the ECM, but only in the superficial layer with a much lighter color than the other two groups. Some lightly positively stained cells were also present (Fig. 4O).

The immunohistochemical staining of PCNA was used to detect cell proliferation. In the control group, cells throughout the entire sample stained positive with a higher density in the superficial regions (Fig. 4S). Cells positive for PCNA in the SO- group were also distributed throughout the whole tissue (Fig. 4T). However, in the SO+ group, almost no cells stained positively for PCNA (Fig. 4U).

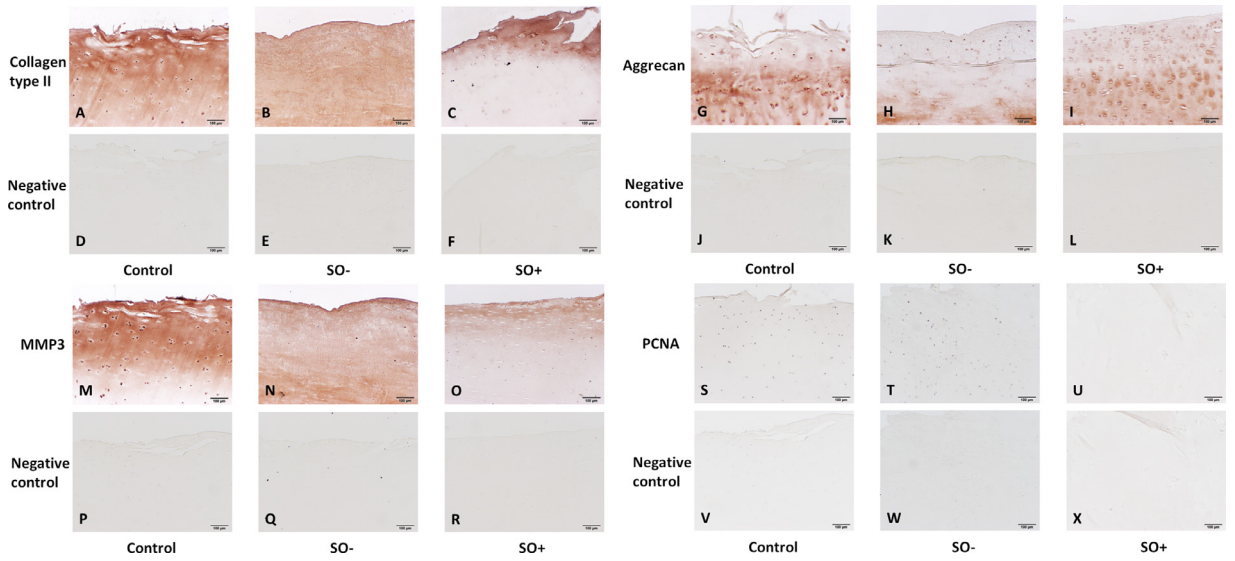

Fig. 4. Immunohistological staining. (A-F) Type II collagen staining and negative controls; (G-L) Aggrecan staining and negative controls; (M-R) MMP3 staining and negative controls; (S-X) PCNA staining and negative controls.

Discussion

This investigation compared the cartilage harvested from cam-type FAI patients with non-pathological cartilage using both gene expression and histological analyses. The production of ECM in FAI cartilage was found to be decreased in both gene expression and histological studies relative to normal cartilage. The integrity of the ECM in FAI, however, was not significantly damaged. Cells from FAI tissue were found to have both elevated inflammatory factors as well as cell senescence-related factors.

FAI has in the past been considered an inflammatory state that eventually leads to the development of OA.[2,3] The expression of catabolism-related genes, such as IL-1β, ADAMTS4, MMP13, were found in much higher levels in the FAI tissue than in normal cartilage, which is consistent with other studies.[10,11] The expression of MMP3, however, a stromelysin with broad substrates, was found to be no higher in FAI tissue than normal cartilage.[14,15] MMP3 is a marker of ECM turnover and is utilized as an indicator for OA.[15,16] This discrepancy of the expression of MMP3 in FAI and OA may suggest that FAI and OA have different pathophysiologic mechanisms. Chinzei et al. reported a decrease in MMP3 expression in FAI tissue compared to OA, but their study used synovium and labrum tissues from FAI donors.[11] In the histological analyses of this present study, FAI tissue was shown to have ECM that maintained its structural integrity. The stability of the FAI tissue reflected that even with higher level of inflammatory factors than normal tissue, the tissue integrity was not decreased during the long developing period of the disease. Considering the gene expression of SOX9, Collagen 2 and aggrecan in FAI tissue was lower than normal tissue, the metabolism of the cells in FAI tissue was considered even lower than normal cartilage tissue. This conclusion is in contrast with the report from Haneda et al.[17] In their study, they also found increased inflammatory factors in FAI tissue. They considered it was due to a metabolic hyperactivity of the cells. Higher levels of the expression of inflammatory factors but lower level of the metabolism is seen in situations of cell Senescence Associated Secretory Phonotype (SASP).[18,19] SASP is a cellular process by which cells in a senescent state produce pro-inflammatory and matrix-degrading enzymes.[20] Elevated p21, MMP13, IL-1β, as well as other pro-inflammatory factors are known constituents of SASP.[21-23]

Cell senescence and apoptosis were evaluated in this study via expression of p21, p16, p53, Bcl-2 and FasL. P21, p16 and p53 proteins are all involved in cell senescence and apoptosis with inhibition of cell proliferation.[24] In the present study, the expression of p21 was significantly higher in the FAI tissue compared to normal cartilage. The FAI group also had an increased level of p16, although this difference did not reach statistical significance. The inhibition of cell proliferation by p21 and p16 occur through the pathways of p21-CDK2 and p16-CDK4,6 respectively.[25] Therefore, it is possible that the predominant mechanism of growth inhibition in FAI tissue occurs through the p21-CDK2 pathway. On a related note, the expression of p53 in FAI tissue fell below the detection threshold. P53, whose activation relies on stress-related damage to DNA,[24] is widely considered a major upstream inducer of apoptosis and senescence.[24,26] Undetectable levels of p53 suggest that there is either low environmental stress in the FAI tissue or a blunted reaction of the chondrocytes to environmental stimuli. Aside from p53, p21 influences cell proliferation through other p53-independent regulatory control methods.[27] High level of p21 have been shown to negatively feedback on p53.[28]

Another regulatory mechanism of senescence is the anti-apoptotic effects of the Bcl-2 signal pathway. The activation of Bcl-2 provides pro-survival effects that help the cell avoid damage caused by inflammation and apoptosis.[29-31] In the present study, the expression of Bcl-2 was significantly higher in FAI tissue than in control cartilage. The chondrocytes in FAI tissue demonstrated higher levels of p21 and Bcl-2 as well as a lower level of p53 relative to controls. It is possible that in this situation, cell survival was maintained with reduced proliferation while avoiding apoptosis. Haneda et al. reported the viability of the cells in FAI tissue was similar among early, late stages FAI and normal cartilage.[7,17] Their results suggested no extensive cell apoptosis in FAI tissues.

Chondrocyte proliferation was further assessed in this study by PCNA immunohistochemical staining. Both the SO- and SO+ FAI phenotypes exhibited fewer stained cells than normal cartilage. The positive staining of PCNA was extremely weak in SO+ specimens. These results reinforce the hypothesis that chondrocytes from FAI tissue demonstrate significant senescence. Transmembrane protein FasL has been shown to interact with infiltrating pro-inflammatory cells.[32] Specifically, FasL from chondrocytes induces apoptosis of macrophages which renders the cartilage immune-privileged.[33] In the present investigation, the expression of FasL was higher in the FAI tissue than in the control samples. The combination of reduced cellular activity and protection against infiltrating leukocytes may be a way for FAI tissue to maintain environmental stability and senescence.

One of the significant influences on the histological results of FAI tissue was the age of the donors. SO- samples (donors with younger age) were considered as an early stage of FAI. SO- samples demonstrated livelier cell biological activity than SO+ samples. SO+ samples were considered representative of a later stage of FAI, with cell proliferation almost stopped and ECM turnover significantly decreased. This reduced ECM productivity of FAI tissue was confirmed by gene expressions with decreased Sox9, COL2 and ACAN, which differed from the report by Hashimoto et al.[10] This may be explained by the difference in patient age across the two studies. The mean age of the donors from Hashimoto et al. study was 24.1 years, which is considerably younger than the patients from the present study (mean = 37.2 years). Handeda et al. also distinguished FAI patients to two groups, early and late stages.[7,17] In their report, late stage FAI represented OA that had already developed in the hip joint. Age was also one of the significant differences between two groups in their study. In a review by Morris et al., abnormalities in the ossification of the epiphysis during adolescence was found to strongly correlate to cam-type FAI.[34] This brings up the possibility that cam-type FAI tissue may start to be formed during adolescence and may then be maintained by cell senescence over time. Since positive safranin O staining was seen more in the late stage FAI, we consider that safranin O staining can be used as an important measurement of the progression of FAI and in prediction of its development.

While the results of this study show promise, it is important to note that the small sample size precludes the capacity to establish definitive conclusions without experimental replication. Future investigations should examine whether molecular differences exist between the different phenotypes of FAI and compare them to chondrocytes derived from age-matched patients with advanced OA. Additional genes involved in ECM synthesis, inflammatory, and cellular senescence pathways also ought to be tested in order to localize where the pathological changes in cartilage degradation occur.

Conclusion

Gene expression and histological analyses suggest that cam-type FAI is a disease with chronic and sustained inflammation associated with cell senescence. This chronic pathological situation led to an age related degradation of extracellular matrix and sustained release of inflammatory factors. Eventually, FAI formed at the early age of the patient developed OA in the hip joint at a late age. If this is the case, the removal of FAI tissue as early as possible may help in preventing the development of OA in the future.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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