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Osteoarthritis and Cartilage

Elevated osteopontin level of synovial fluid and articular cartilage is associated with disease severity in knee osteoarthritis patients

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Summary

Objective: To investigate osteopontin (OPN) levels in both synovial fluid and articular cartilage of patients with primary knee osteoarthritis (OA) and their relationship with severity of the disease.

Method: Fifty patients aged 48–81 years with knee OA and 10 healthy controls were enrolled in this study. Anteroposterior knee radiographs or/and Mankin score were taken to determine the disease severity of the affected knee. The radiographic grading of OA in the knee was performed by using the Kellgren-Lawrence criteria. OPN levels in synovial fluid were measured using enzyme-linked immunosorbent assay. OPN levels in articular cartilage were assessed by immunohistochemical methods.

Results: Compared to healthy controls, OA patients had higher OPN concentration in synovial fluid (4519.60 ± 1830.37, 95%CI 3999.42–5039.79 vs 1179.70 ± 303.39, 95%CI 1035.53–1438.74 pg/ml, $P < 0.001$)and articular cartilage(0.6 ± 0.06, 95%CI 0.59–0.62 vs 0.43 ± 0.07, 95%CI 0.38–0.48, $P < 0.01$). In addition, synovial fluid OPN levels showed a positive correlation with articular cartilage OPN levels ($r = 0.411$, 95%CI 0.150–0.619, $P = 0.003$). Subsequent analysis showed that synovial fluid OPN levels significantly correlated with severity of disease (Spearman's $\rho = 0.581$, 95%CI 0.335–0.726, $P < 0.001$). Furthermore, the articular cartilage levels of OPN also correlated with disease severity (Spearman's $\rho = 0.675$, 95%CI 0.500–0.808, $P < 0.001$).

Conclusions: OPN in synovial fluid and articular cartilage is associated with progressive joint damage and is likely to be a useful biomarker for determining disease severity and progression in knee OA.
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Key words: Osteopontin, Osteoarthritis, Synovial fluid, Articular cartilage.

Introduction

Osteoarthritis (OA), the most prevalent joint disease, is characterized by the progressive loss of articular cartilage that leads to chronic pain and functional restrictions in affected joints. Worldwide estimates are that about 10% of men and 18% of women and about 60–65% aged over 60 years have symptomatic OA and 80% of those have limitations in movement. The clinical features of OA include pain, stiffness, reduced motion, swelling, crepitus and deformity. Although clinical research on OA has been extensively investigated, the etiology of this disease remains poorly elucidated. Several biochemical and biomechanical factors are considered for the pathogenesis.

OPN (osteopontin) is a 44∼75 KD multifunctional phosphoprotein secreted by many cell types such as osteoclasts, macrophages, lymphocytes, epithelial cells and vascular smooth muscle cells (SMC)[1,2] and is present in the extracellular matrix of mineralized tissues and in extracellular fluids, at sites of inflammation[3,4]. Gene structure and chromosomal location identify OPN as a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family. This protein also known as early T cell activation gene-1 (Eta-1) is abundant in bone, where it mediates important cell-matrix and cell-cell interactions[5].OPN expression during chondrocyte maturation is one of the important events involved in cartilage-to-bone transitions in fracture repair[6,7]. OPN facilitates the attachment of osteoclasts to the bone matrix *via* an interaction with cell surface $\alpha v\beta 3$ integrin and CD44, the hyaluronic acid receptor[6,8–10]. OPN may be involved in the molecular pathogenesis of OA, contributing to progressive degeneration of articular cartilage[11]. Expression of OPN mRNA isolated from human OA cartilage was enhanced as compared with normal cartilage. OPN was shown to be upregulated in human OA chondrocytes[12]. Moreover, OPN was found to be expressed in bone forming cells and hypertrophic chondrocytes of the embryonic epiphyseal growth plates[13].

It has been reported that OPN was present in plasma and synovial fluid of patients with rheumatoid arthritis and with OA[6], There have been no detailed studies on synovial fluid

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and articular cartilage levels of OPN in various clinical stages of primary knee OA[14–19]. The relationship between OPN levels in the synovial fluid and OPN expression in articular cartilage and disease severity has never been previously reported in the literature. We hypothesized that OPN in synovial fluid and articular cartilage may be associated with the severity of clinical outcomes in knee OA patients. To prove this hypothesis we examine the synovial fluid and articular cartilage levels of OPN in knee OA patients and healthy controls.

The purpose of the present study was to investigate the concentrations of OPN in both synovial fluid and articular cartilage of patients with primary knee OA, and evaluate the possible correlations with the radiographic grading and Mankin score of knee OA, which may serve as a useful tool to mark the osteoarthritic disease process and to further elucidate the pathways involved in the progression of the disease.

Methods

PATIENTS AND PREPARATION OF SAMPLES

Fifty patients aged 48–81 years with primary knee OA according to the criteria of the American College of Rheumatology and 10 normal healthy individuals were enrolled in the study. Clinical data were carefully reviewed to exclude any forms of secondary OA and inflammatory joint diseases like rheumatoid arthritis. The severity of the disease was determined using weight-bearing anteroposterior radiographs of the affected knee. Knee radiographs were evaluated according to the Kellgren and Lawrence (KL) classification[20]. Grade 1, doubtful narrowing of joint space and possible osteophytic lipping. Grade 2, definite osteophytes and possible narrowing of joint space. Grade 3, moderate multiple osteophytes, definite narrowing of joints space, some sclerosis and possible deformity of bone contour. Grade 4, large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone contour. According to the KL grading scale, 13 patients were KL grade 2, whereas 20 patients were KL grade 3 and 17 patients were KL grade 4 OA. The grading scale used for analysis was the higher of the two knees.

Fifty osteoarthritic cartilage samples were obtained from 50 patients with primary OA undergoing a total knee replacement. Normal cartilage was collected from 10 human knees at the time of autopsy, within 18 h after death (37-58-year-old donors). Osteoarthritic changes were classified histomorphologically, using the grading system of Mankin[21]. Mankin score 0: Normal cartilage with a smooth surface and a regular zonal distribution of the chondrocytes. Mankin score 1–4: Cartilage surface shows fibrillations and a superficial loss of proteoglycans(safranin-O staining), the zonal structure is intact. Mankin score 5–8: Cartilage samples have clefts reaching down to the middle cartilage zone, clusters of proliferating chondrocytes are present. Mankin score $\geq$9: Severely affected cartilage samples, clefts are reaching down to the deep zone, the tangential zone is lost and chondrocyte clusters are present. Ten samples (Mankin score 0), 13 samples (Mankin score 1–4), 19 samples (Mankin score 5–8) and 18 samples (Mankin score $\geq$9) were included in this study.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Synovial fluid was aspirated from the affected knee during surgery, when a total knee arthroplasty was performed, centrifuged to remove cells and joint debris and stored immediately at -70°C until the day of measurement. Double-blined quantitative detection of OPN in synovial fluid was performed using commercial ELISA (Uscnlife Science & Technology Company, China) according to the manufacturers' instructions. Briefly, standards of recombinant human OPN and synovial fluid samples were added to 96-well microtiter plates precoated with rabbit polyclonal antibody against OPN and incubated for 1 h at room temperature. The wells were then washed seven times with washing buffer and incubated for 30 min at 4°C with a horseradish peroxidase-labeled mouse monoclonal antibody to human OPN. After nine washes, substrate solution was added to each well and the plate was incubated for 30 min at room temperature in the dark. Finally, the reaction was stopped with the stop solution and then absorbance was measured at 450 nm using automated microtiter plate reader. The OPN concentration was calculated by the standard curve. Twofold serial dilutions of recombinant human OPN with a concentration of 156–10,000 pg/ml were used as standards. Each data was evaluated by three independent experiments. The assays had intra-assay coefficients of variation $<$5% and inter-assay coefficients of variation $<$6%.

IMMUNOHISTOCHEMISTRY

Biopsies (cartilager/bone samples) were cut from both lateral and medial sides of tibia plateau including the loading zone, as well as the margin zone whenever possible. Cartilager/bone samples (1.0 cm thick) with a cartilage surface of approximately 2.0×0.5 cm were incubated in freshly prepared paraformaldehyde, then dehydrated in a grading concentration of ethanol and xylene and finally embedded in paraffin. Representative formalin-fixed, paraffin-embedded tissue blocks were retrieved and sectioned for the immunohistochemical study. The sections were deparaffinized, treated with 3% hydrogen peroxide for 10 min and then microwaved in 10 mM citrate buffer (pH 6.0) to unmask the epitopes. The sections were then incubated with diluted OPN antibody (AKm2A1, 1:100) for 1 h. After wash, horseradish peroxidase/Fab polymer conjugate (PicTureTM-Plus kit, Zymed, South San Francisco, CA) was applied to the sections for 30 min. Finally, the sections were incubated with diaminobenzidine for 5 min to develop the signals. A negative control was simultaneously performed by omitting the primary antibody. All the sections were evaluated by a pathologist who was unaware of the clinical data. To evaluate the expression of OPN, the sections were examined under a microscope at $100\times$ magnification. Positive OPN immunostaining was defined as detectable immunoreactivity in the perinuclear and/or other cytoplasmic regions in chondrocytes. The relative OPN distribution of cartilage tissue can be visualized and quantified as optical density (OD). Semiquantitative assessment of mean OD of OPN expression was performed on scanned auto-radiograms using MIAS-4400 Image J. Grayscale images were captured and converted to absorbance units and a region from the cartilage surface to the cartilage-bone junction was analyzed. All densities were normalized to phosphate-buffered saline (PBS). The experiment was repeated for three times. To reduce the error arising from the slightly varying section thickness, a total of three sections per sample were measured and averaged. The final data, which were applied in all analysis, consisted therefore of a mean of three independent measurements representing the average levels of OPN in articular cartilage. The coefficient of variation (CV) of OPN expression in articular cartilage was $<$2%.

Statistical analysis

SPSS for Windows (version 13.0) was used for data management and statistical analysis. Tests of normality and test of homogeneity of variances were performed to analyze the subject's age, body mass index, concentration of OPN in the synovial fluid and the articular cartilage OPN expression. The analysis of co-variance (ANCOVA) indicated that age, sex and body mass index were not potential confounding factors in this study. When the populations from which the samples were normally or approximate normal distribution and the variances of the populations were equal, Student's *t*-test was employed to compare the means of two independent groups and one way analysis of variance (ANOVA) was employed to compare the means of more than two independent groups. Comparisons between groups were made using Mann–Whitney *U* test (for two groups) or Kruskal–Wallis test (for more than two groups) when the variances were not equal among the groups. Spearman's correlation was employed to determine the correlation between synovial fluid levels of OPN and severity of OA. Spearman's correlation and linear regression were used to determine the correlation between OD of OPN in articular cartilage and Mankin score of OA. Pearson's correlation and linear regression were applied to determine the correlation between synovial fluid OPN levels and OD of OPN in articular cartilage. Spearman's correlation coefficients and Pearson's correlation coefficients were calculated using Prism 5.0 software (GraphPad, Inc., San Diego, CA). Data were expressed as mean $\pm$ standard deviation (SD). A *P*-value less than 0.05 was taken as statistically significant.

Results

A total of 60 knees were analyzed from 50 patients (35 female) and 10 controls (6 female). Characteristics of the

Table I
Characteristics of patients (n = 50) and controls (n = 10)

	Osteoarthritis patients	Controls	*P*
Age (years)	61.8 ± 7.4, 95%CI: 59.6–63.9	63.2 ± 6.0, 95%CI: 58.9–67.5	0.57
Gender (%female)	70%	60%	0.54
BMI (kg/m^2)	27.08 ± 0.44, 95%CI: 26.96–27.20	26.82 ± 0.44, 95%CI: 26.50–27.14	0.09

*BMI = body mass index.

study population are shown in Table I. There was no clinical meaningful difference in age between OA patients (61.8 ± 7.4, 95%CI 59.6–63.9) and controls (63.2 ± 6.0, 95%CI 58.9–67.5, $P = 0.57$). There was no difference between OA patients and controls for sex (70% vs 60% females, Chi-square $P = 0.54$) and body mass index (27.08 ± 0.44, 95%CI 26.96–27.20 vs 26.82 ± 0.44, 95%CI 26.50–27.14 kg/m^2, $P = 0.09$).

Synovial fluid OPN levels of knee OA patients and controls are demonstrated in Fig. 1. OA patients had higher synovial fluid OPN concentrations compared to healthy controls (4519.60 ± 1830.37, 95%CI 3999.42–5039.79 vs 1179.70 ± 303.39, 95%CI 1035.53–1438.74 pg/ml, $P < 0.001$). The synovial fluid levels of OPN were analyzed and compared in relation to radiological KL grading of OA. The synovial fluid levels of OPN from KL grade 2 were 3543.82 ± 811.19 (95%CI 2817.71–4269.93) pg/ml, those from KL grade 3 were 4013.63 ± 676.81 (95%CI 3228.86–4798.91) pg/ml and those from KL grade 4 were 6170.01 ± 773.50 (95%CI 3999.42–5039.79) pg/ml. The data revealed that synovial fluid OPN levels in KL grade 4 were significantly elevated compared with those of KL grade 2 and 3 ($P < 0.01$). The synovial fluid levels of OPN also correlated with disease severity (Spearman's $\rho = 0.581$, 95%CI 0.335–0.726, $P < 0.001$) (Fig. 2). In order to compare synovial fluid levels of OPN as a function of age among OA patients, they were categorized into three age groups as follows: less than 60 years of age, 60–75 years of age and more than 75 years of age. There was no statistically significant difference in synovial fluid OPN levels among patients with OA ($P = 0.15$).

In the normal cartilagerbone section (Mankin score 0), OPN expression wasn't found in all nucleated cells (Fig. 3). In the cartilagerbone section (Mankin score 1–4), a faint staining was visible in the territorial matrix surrounding deep zone chondrocytes (Fig. 3). In cartilage samples with a Mankin score of 5–8, the staining was more pronounced in deep zone chondrocytes and their surrounding matrix (Fig. 3). Sections with severe osteoarthritic (Mankin score ≥9), the strongest OPN expression was detected in these samples. Clusters of chondrocytes and chondrocytes from the deep cartilage zone showed cellular and extracellular OPN deposition. Osteocytes and bone trabeculae lining cells stained positive for OPN (Fig. 3). Average OD of OPN in articular cartilage of OA patients and healthy controls was 0.6 ± 0.06 (95%CI 0.59–0.62) and 0.43 ± 0.07 (95%CI 0.38–0.48) respectively. OA patients had higher articular cartilage OPN expression compared to healthy controls ($P < 0.01$). The articular cartilage OPN expression correlated with Mankin score (Spearman's $\rho = 0.675$, 95%CI 0.500–0.808, $P < 0.001$, regression equation: $\hat{y} = -15.442 + 37.772x$, $F = 42.055$, $P < 0.001$, β 95%CI 26.061–49.483) (Fig. 4). Interestingly, synovial fluid OPN levels showed a positive correlation with articular cartilage OPN expression (Pearson's $r = 0.411$, 95%CI 0.150–0.619, $P = 0.003$, regression equation: $\hat{y} = 0.541 + 1.33 * 10^{-5}x$, $F = 9.775$, $P < 0.001$, β 95%CI $0.47*10^{-5}$ $-2.18*10^{-5}$) (Fig. 5).

Discussion

This study revealed a marked increase of OPN levels in both synovial fluid and articular cartilage of patients with

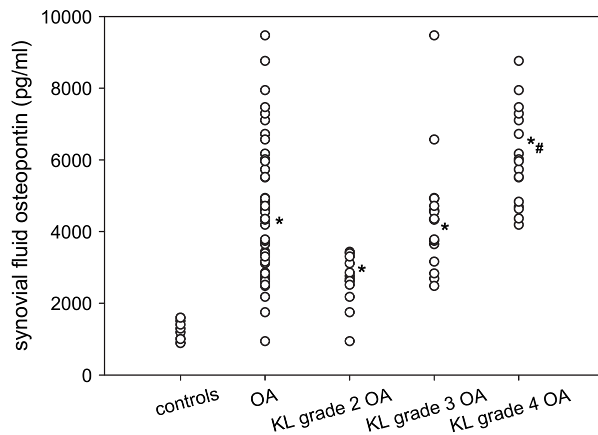

Fig. 1. Synovial fluid levels of OPN in healthy controls ($n = 10$), osteoarthritis patients ($n = 50$) and osteoarthritis patients from KL grade 2 ($n = 13$), KL grade 3 ($n = 20$) and KL grade 4 ($n = 17$). * $P < 0.001$ vs controls; # $P < 0.01$ vs KL grade 2 OA and KL grade 3 OA.

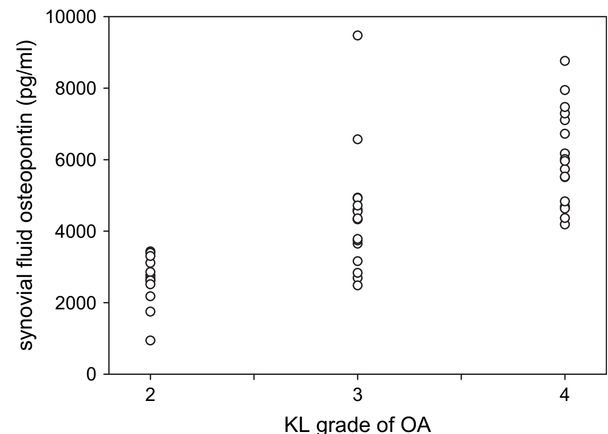

Fig. 2. Synovial fluid levels of OPN correlated with KL grade of osteoarthritis (Spearman's $\rho = 0.581$, 95%CI 0.335–0.726, $P < 0.001$). The severity of the disease was determined using weight-bearing anteroposterior radiographs of the affected knee. Knee radiographs were evaluated according to the KL classification. Spearman's correlation was employed to determine the correlation between synovial fluid levels of OPN and severity of osteoarthritis.

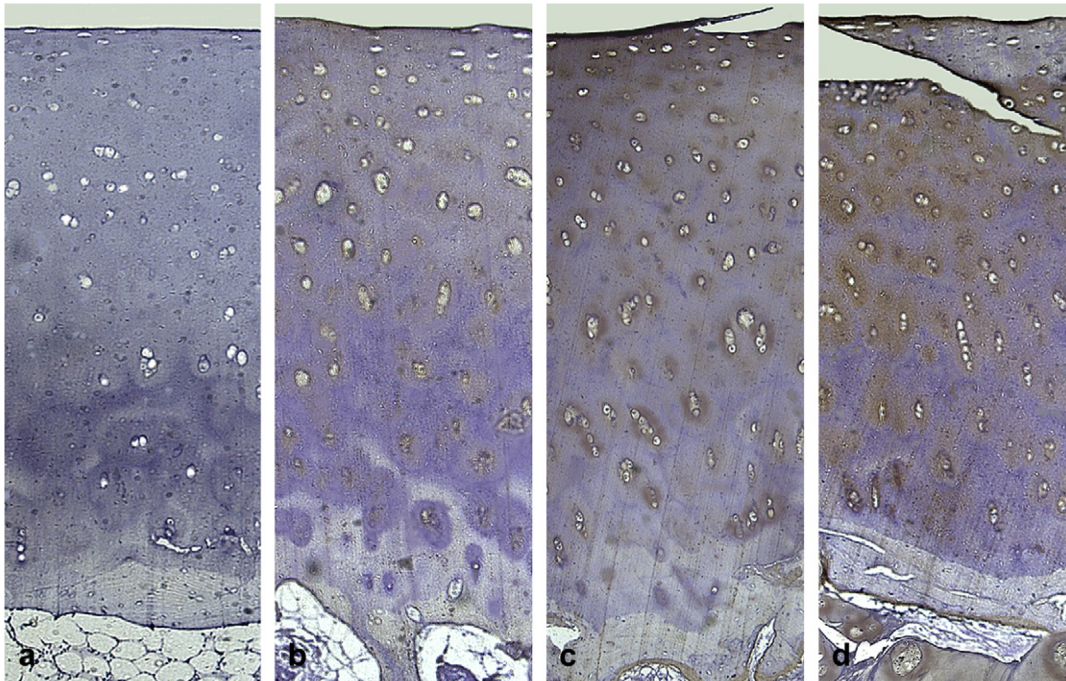

Fig. 3. OPN immunohistochemical staining in articular cartilage of knee osteoarthritis patients and controls. (a) OPN expression wasn't found in the perinuclear and/or other cytoplasmic regions in chondrocytes of controls. (b) The faint staining was visible in the territorial matrix surrounding deep zone chondrocytes of minor osteoarthritis patients with a Mankin score of 1–4. (c) The staining was more pronounced in deep zone chondrocytes and their surrounding matrix of moderate osteoarthritis patients with a Mankin score of 5–8. (d) The strongest OPN stained in chondrocytes, chondrocytes from the deep cartilage zone, chondrocytes surrounding matrix and bone trabeculae lining cells of severe osteoarthritis patients (Mankin score ≥ 9).

knee OA compared with the controls. Elevated levels of OPN in synovial fluid are possibly caused by either the release of OPN residing in extracellular matrix, or the increase in its production, or both processes. The source of the OPN in the synovial fluid is presumably to be the local tissues, such as the synovial membrane and articular cartilage. Many studies had demonstrated the expression of OPN in bone, thus, bone may also be a source of synovial fluid OPN. When moderate and severe lesions of the articular cartilage occur, it can begin to expose subchondral bone in the synovial fluid,

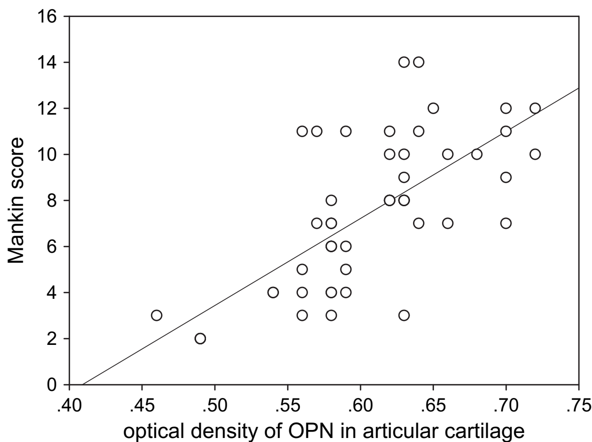

Fig. 4. OD of OPN in articular cartilage correlated with Mankin score of osteoarthritis (Spearman's $\rho = 0.675$, 95%CI 0.500–0.808, $P < 0.001$, regression equation: $\hat{y} = -15.442 + 37.772x$, $F = 42.055$, $P < 0.001$, β 95%CI 26.061–49.483). Osteoarthritic changes were classified histomorphologically, using the grading system of Mankin. Spearman's correlation and linear regression were used to determine the correlation between OD of OPN in articular cartilage correlated and Mankin score of osteoarthritis.

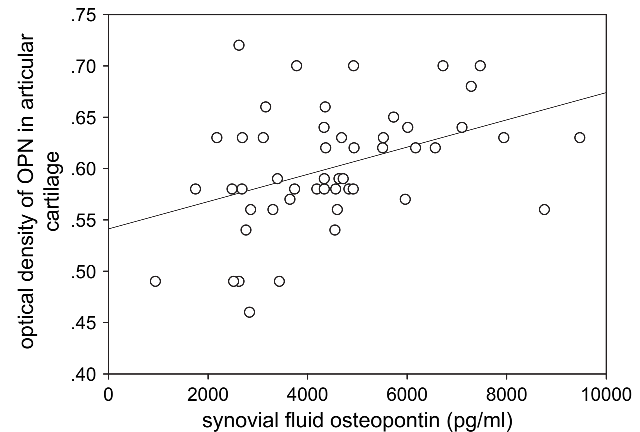

Fig. 5. Synovial fluid OPN levels correlated with OD of OPN in articular cartilage (Pearson's $r = 0.411$, 95%CI 0.150–0.619, $P = 0.003$, regression equation: $\hat{y} = 0.541 + 1.33 * 10^{-5}x$, $F = 9.775$, $P < 0.001$, β 95%CI $0.47*10^{-5}$–$2.18*10^{-5}$). Pearson's correlation and linear regression were applied to determine the correlation between synovial fluid OPN levels and OD of OPN in articular cartilage.

bone may be a source of synovial fluid OPN, but there is not reported evidence so far to support this hypothesis. Previous studies have demonstrated the immunohistochemical expression of OPN in the synovial lining cells[17], fibroblasts in the synovial tissues[18] and articular chondrocytes[12,18]. It is suggested that cell adhesion, migration or inflammation could be involved in the release of OPN[7].

Cartilage damage is one of the main pathological changes in OA. Synovitis and degenerative changes of articular cartilage is likely to be facilitating factors in the release of OPN into the synovial fluid. Interestingly, synovial fluid OPN levels showed a positive correlation with articular cartilage OPN expression. Therefore, only by detecting the levels of OPN in synovial fluid, it may be predictive of the degree of cartilaginous damage and disease severity. In accordance with our observations, recent studies have showed that OPN protein deposition and mRNA expression increased with morphological signs and the severity of matrix degradation in human osteoarthritic cartilage[12]. These findings indicate that OPN expression in osteoarthritic cartilage strongly correlates with the severity of OA disease. In addition, Attur *et al.* have revealed the increased expression of OPN in human OA cartilage, the expression of OPN mRNA was highly upregulated as compared with normal cartilage[11]. They also found that addition of recombinant OPN to human OA-affected cartilage under *ex vivo* conditions suppressed spontaneous production of nitric oxide and prostaglandin E2. These findings suggest that OPN is overexpressed in OA cartilage and function as an endogenous inhibitor of production of inflammatory mediators in cartilage. Furthermore, Yaki *et al.* showed that the advanced OA cartilage had significantly higher OPN mRNA expression than the minimal OA cartilage[22]. The patient matched comparison of minimal and advanced OA cartilage displayed alterations in gene expression that may be involved in OA progression.

These results indicate that synovial fluid levels and cartilage expression of OPN may play a significant role in the pathogenesis of OA. Previous studies have demonstrated overexpression of OPN induces proinflammatory chemokines and cytokines (e.g., IL-1, IL-8, CXCL1, CCL2, and so on) and activates nuclear factor-kappa B pathway[23]. OPN deficiency is consistently shown to prevent the destruction of joint cartilage and joint swelling through suppression of chondrocyte apoptosis and angiogenesis in the rheumatoid arthritis model[23]. This mechanism, mediated by OPN, is also likely to play a key role in OA. Further investigations are under way in our laboratory to define the signaling events induced by OPN and potential experimental strategies for the inhibition of OPN-mediated OA process. Measurements of synovial levels of OPN could possibly serve as a biochemical parameter for determining disease severity and may be predictive of prognosis with respect to the progression of osteoarthritic disease process. Longitudinal studies may provide further information regarding the value of OPN as a potential marker to monitor the course of OA. Additional investigation will be needed to evaluate the knee radiographs among the control group because they presumably has no knee pain, but might have radiographic evidence of OA.

This study has had several limitations. First, the sample size was not large enough to arrive at definitive conclusions. Secondly, we investigated only those patients of knee OA who attended Xiangya Hospital, Central South University. Thirdly, the cross-sectional design of our study precluded addressing whether the analyzed level of OPN predicted alteration in severity in patients with knee OA.

The documented normal circulating OPN levels in previous reports are highly variable, with a range from 31 ng/ml to 200 ng/ml[24–26]. In this study, the commercial ELISA with a concentration of 156–10,000 pg/ml were used for detecting OPN in human synovial fluid. The exact reason is unclear but it could be attributed to the different assay systems and conditions of sample. A critical issue that limits the applicability of plasma OPN as a specific marker for OA is that the OPN level is also increased in a variety of cancers. Elevation of circulating OPN levels has been recently reported in pancreatic cancer, colon cancer, breast cancer, lung cancer, ovarian cancer, hepatocellular carcinoma and melanoma[27–31]. Further investigations with standardized assay system in a large population are warranted to gain insight into the potential utility of OPN in OA patients, especially in synovial fluid.

In summary, patients with primary knee OA had higher levels of synovial fluid OPN compared with healthy controls. OPN concentrations in synovial fluid and articular cartilage significantly correlated with the severity of disease. Further studies are in progress to elucidate the contribution of OPN to the pathogenesis of the degenerative process of OA.

Conflict of interest

The authors have no conflict of interest to report.

Acknowledgements

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