

# Osteoarthritis and Cartilage



## Usefulness of specific OA biomarkers, thrombin-cleaved osteopontin, in the posterior cruciate ligament OA rabbit model

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### ARTICLE INFO

#### Article history:

Received 29 May 2012

Accepted 11 September 2012

#### Keywords:

Thrombin-cleaved osteopontin

Osteoarthritis

Posterior cruciate ligament

Articular cartilage

### SUMMARY

**Objective:** We undertook this study to determine whether thrombin-cleaved osteopontin (OPN) in synovial fluid (SF) represents a useful marker of osteoarthritis (OA) progression in the posterior cruciate ligament transection (PCLT) OA rabbit model.

**Method:** PCLT was performed on the right knee joints of 48 rabbits. The rabbits were then sacrificed separately at 4, 8, 16, and 24 weeks post-surgery, when the joint was harvested and macroscopic and histological assessments of articular cartilage were performed. Thrombin-cleaved OPN product in SF was determined using Western blotting and the levels were measured using an enzyme-linked immunoassay.

**Results:** The macroscopic and histological scores for PCLT knees were already elevated 4 weeks after surgery and increased with time. Western blotting showed the presence of thrombin-cleaved OPN in SF from PCLT knees. Thrombin-cleaved OPN levels in SF were elevated at 4 weeks ( $P < 0.001$ ) and were elevated peaking at 24 weeks ( $P < 0.00001$ ) after PCLT compared to baseline. A positive significant correlation was found between thrombin-cleaved OPN levels and the macroscopic scores (8 weeks:  $\rho = 0.695$ ,  $P = 0.012$ ; 16 weeks:  $\rho = 0.751$ ,  $P = 0.005$ ; 24 weeks:  $\rho = 0.660$ ,  $P = 0.020$ ). Furthermore, the same correlation was noted between thrombin-cleaved OPN levels and the histological scores (4 weeks:  $\rho = 0.609$ ,  $P = 0.036$ ; 8 weeks:  $\rho = 0.662$ ,  $P = 0.019$ ; 16 weeks:  $\rho = 0.827$ ,  $P = 0.001$ ; 24 weeks:  $\rho = 0.813$ ,  $P = 0.001$ ).

**Conclusion:** In this rabbit model of PCLT, thrombin-cleaved OPN levels in SF appear to provide a useful marker of OA disease severity and progression.

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### Introduction

Osteoarthritis (OA) is thought to be the most prevalent chronic joint disease. 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. Diagnosis of OA relies mainly on clinical examination and radiographic

measures. Radiographic measures, however, are less than adequate for diagnosing and assessing the actual progress of this disease. Despite efforts over the past decades to develop markers of disease, biochemical marker still need to be improved and possibly extended with more specific and sensitive methods to diagnose the disease at an early stage, reliably follow the course of disease and evaluate efficacy of disease-modifying agents<sup>1,2</sup>.

Osteopontin (OPN) is an extracellular matrix glycoprotein that has been recognized as a potential inflammatory cytokine. OPN is abundant in bone and can be secreted by many cell types such as osteoclasts, macrophages, lymphocytes, epithelial cells and vascular smooth muscle cells (SMCs)<sup>3,4</sup>. The function of OPN is modulated by protease digestion, and a thrombin-cleaved form of OPN is involved in the pathogenesis of various inflammatory disorders. The biomarker has been studied *in vivo* in rat<sup>5</sup>, healthy human and OA patients<sup>6,7</sup> and also rheumatoid arthritis (RA) patients<sup>8</sup>. The thrombin-cleaved form of OPN may be involved in

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the molecular pathogenesis of OA, contributing to progressive degeneration of articular cartilage<sup>8,9</sup>.

Surgically induced destabilization of joints is the most widely used induction method, where the underlying initiating mechanism is altered mechanical loading, one of the most common causes of secondary OA in humans. Many induction methods actually copy known injuries in humans, such as anterior cruciate ligament transection (ACLT) and meniscectomy. As a long-term complication, many patients with posterior cruciate ligament (PCL) injuries eventually develop OA in the injured knee joint<sup>10,11</sup>. Posterior cruciate ligament transection (PCLT) can also replicate the naturally occurring sequence of the disease process of human OA and has been used as a model in previous studies<sup>12–15</sup>.

The purpose of this study was to investigate the changes over time of thrombin-cleaved OPN in synovial fluid (SF) during the development of OA induced by PCLT in rabbits. We hypothesized that the levels of thrombin-cleaved OPN were correlated with the macroscopic and histological changes that occurred in this model. This study aimed at adding important details for the validation of these specific OA biomarkers.

## Materials and methods

### Experimental group

The experiment was absolutely approved by the Institutional Animal Care and Use Committee of Xiangya Hospital, Central South University (Changsha, China) and the animals were cared for in the Experimental Animal Center of Xiangya Hospital. 48 mature white rabbits (male,  $2.5 \pm 0.4$  kg, 6 months old) were used. The rabbits were acclimated to conditions for 1 week before the experiment and were housed individually at 25°C with a 12:12-h light–dark cycle and had free access to tap water and commercial rabbit diet. Surgical transection of the PCL of the right knee was performed under general anesthesia as previously reported<sup>14</sup>. Postoperatively, the rabbits were housed and they had free access to exercise in a large enclosure. For each rabbit, sodium aminobenzyl penicillin (500 mg) was administered intramuscularly daily for 7 days postoperatively. The rabbits were sacrificed by an intravenous injection of barbiturates 4, 8, 16 and 24 weeks after the surgical procedure.

### Macroscopic grading

After the rabbits were sacrificed, each knee was harvested and evaluated macroscopically by two independent, blinded investigators using the grading system of Tibesku *et al.*<sup>16</sup>, consisting of four different criteria: fibrillations and ulcerations of the hyaline cartilage, osteophyte formation, and joint effusion. Scores for fibrillations ranged from 0 for intact hyaline cartilage to 3 for marked fibrillations, scores for ulcerations ranged from 0 for normal to 2 for a large area of ulceration, scores for osteophyte formation ranged from 0 for no osteophytes to 3 for marked osteophyte formation, and scores for joint effusion ranged from 0 for no effusion to 3 for marked effusion. The total score ranged from 0 to 11, with 0 being a macroscopically intact knee joint and 11 being significant OA.

### Histologic grading

Cartilage was removed from the areas of the lesions identified by the macroscopic grading and then histologic evaluation was performed on sagittal sections of cartilage from the lesions of the femoral condyles and tibial plateaus. The specimens were dissected, fixed in 4% paraformaldehyde for 2 days. After decalcification with buffered ethylene diamine tetraacetic acid (EDTA), the samples then

sequentially dehydrated in alcohol and embedded into paraffin blocks. Sections were cut at a thickness of 5  $\mu$ m, mounted to the center of the glass slide, deparaffinized in xylene, and washed three times with distilled water and then with Tris-buffered saline for 2 min. Sections were stained with hematoxylin and eosin (H&E) or with safranin O–fast green to evaluate histologic changes of the cartilage and bone tissue according to the osteoarthritis research society international (OARSI) histopathology initiative (Table 1)<sup>17</sup>. This scale evaluates the severity of OA lesions based on safranin O–fast green staining (scale 0–6), structure (scale 0–11), chondrocyte density (scale 0–4), and cluster formation (scale 0–3). The final score corresponds to the score of the most severe lesions. The examination was performed by two independent researchers in a blind manner.

### SF sampling, Western blot analysis and enzyme-linked immunosorbent assay (ELISA)

SF samples were obtained for each rabbit at baseline, 4, 8, 16 and 24 weeks after surgery. 2 ml of sterile Ringer's solution was injected

**Table 1**  
Histochemical/histological assessment of articular cartilage changes

Parameter
<b>Safranin O–fast green staining</b>
0 Uniform staining throughout articular cartilage
1 Loss of staining in superficial zone of hyaline cartilage <50% the length of the condyle or plateau
2 Loss of staining in superficial zone of hyaline cartilage $\geq$ 50% the length of the condyle or plateau
3 Loss of staining in the upper 2/3's of hyaline cartilage <50% the length of the condyle or plateau
4 Loss of staining in the in the upper 2/3's hyaline cartilage $\geq$ 50% the length of the condyle or plateau
5 Loss of staining in all the hyaline cartilage <50% the length of the condyle or plateau
6 Loss of staining in all the hyaline cartilage $\geq$ 50% the length of the condyle or plateau
<b>Structure</b>
0 Normal
1 Surface irregularities
2 Fissures in <50% surface
3 Fissures in $\geq$ 50% surface
4 Erosion 1/3 hyaline cartilage <50% surface
5 Erosion 1/3 hyaline cartilage $\geq$ 50% surface
6 Erosion 2/3 hyaline cartilage <50% surface
7 Erosion 2/3 hyaline cartilage $\geq$ 50% surface
8 Full depth erosion hyaline cartilage <50% surface
9 Full depth erosion hyaline cartilage $\geq$ 50% surface
10 Full depth erosion hyaline and calcified cartilage to the subchondral bone <50% surface
11 Full depth erosion hyaline and calcified cartilage to the subchondral bone $\geq$ 50% surface
<b>Chondrocyte density</b>
0 No decrease in cells
1 Focal decrease in cells
2 Multifocal decrease in cells
3 Multifocal confluent decrease in cells
4 Diffuse decrease in cells
<b>Cluster formation</b>
0 Normal
1 <4 clusters
2 $\geq$ 4 but <8 clusters
3 $\geq$ 8 clusters

**Definitions for the recommended grading system.** See glossary in chapter X for terminology and synonyms. For the purposes of this assessment articular cartilage is divided into four zones: Zone 1: Superficial zone: upper 1/3 hyaline cartilage; Zone 2: Middle zone: middle 1/3 hyaline cartilage; Zone 3: Deep zone: deep 1/3 hyaline cartilage; Zone 4: The calcified cartilage. Artifact definition: fissure but no hypochondrocyte or loss of safranin O adjacent to the cleft; Focal: observed at one site on section; Multifocal: observed at more than one site; Multifocal confluent: observed at multiple sites that are in contact.

and the knees moved five times through a full range of motions. SF samples were centrifuged at 3000 rpm in a micro-centrifuge prior to freezing and were kept frozen at  $-70^{\circ}\text{C}$  until the biomarker analysis was performed.

Western blot analyses of SF were performed to examine the presence of thrombin-cleaved OPN (OPN N-half). 0.1 ml each sample was diluted twice with phosphate-buffered saline (PBS) and loaded onto gels, incubated in blocking buffer (PBS, 5% non-fat dry milk, 0.05% Tween 20) for 1 h at room temperature, washed five times with PBS, and then eluted with 1.0 ml of 0.7 M NaCl in PBS. The elutes were diluted twice with  $2\times$  sodium dodecyl sulfate (SDS) gel-loading buffer (100 mM Tris HCl, pH 6.8, 4% SDS, 20% glycerol), boiled, and applied on Western blotting with horseradish peroxidase (HRP)-labeled rabbit anti-mouse OPN polyclonal antibody (O-17) raised against LPVKVTDSGSSEELY peptide (Uscnlife Science & Technology Company, China).

Double-blinded quantitative detection of thrombin-cleaved OPN (OPN N-half) in SF was performed using commercial ELISA (Uscnlife Science & Technology Company, China) according to the manufacturers' instructions. Briefly, 96-well microtiter plates were coated with anti-OPN N-half mouse monoclonal antibody (in 0.1 M carbonate buffer, pH 9.5) at  $4^{\circ}\text{C}$  overnight, then blocked with 1% bovine serum albumin in PBS at  $4^{\circ}\text{C}$  overnight. The mouse monoclonal antibody (34E3) specifically reacts to SLAYGLR and SVVYGLR, exposed by thrombin cleavage of mouse and human OPN, respectively. The wells were then washed seven times with washing buffer and incubated for 30 min at  $4^{\circ}\text{C}$  with a HRP-labeled rabbit anti-mouse OPN polyclonal antibody (O-17). 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, color development was stopped by addition of stop solution. The optical density of each sample was measured at 450 nm. Each data was evaluated by three independent experiments. The assays had intra-assay coefficients of variation  $<5\%$  and inter-assay coefficients of variation  $<8\%$ <sup>18</sup>.

#### Statistical analysis

Macroscopic and histological scores were expressed as mean  $\pm$  standard deviation (SD). The levels of thrombin-cleaved

OPN were expressed as median and 95% confidence interval (CI). Statistical analysis was performed using GraphPad InStat 3. Friedman test followed by the Dunn's multiple comparison post-test was used to analyze the variation of the biomarker concentration. Correlations between thrombin-cleaved OPN levels in SF and the other evaluated parameters were established using the nonparametric Spearman's rank correlation coefficient. *P*-values less than 0.05 were taken as statistically significant.

## Results

### Macroscopic and histologic analyses

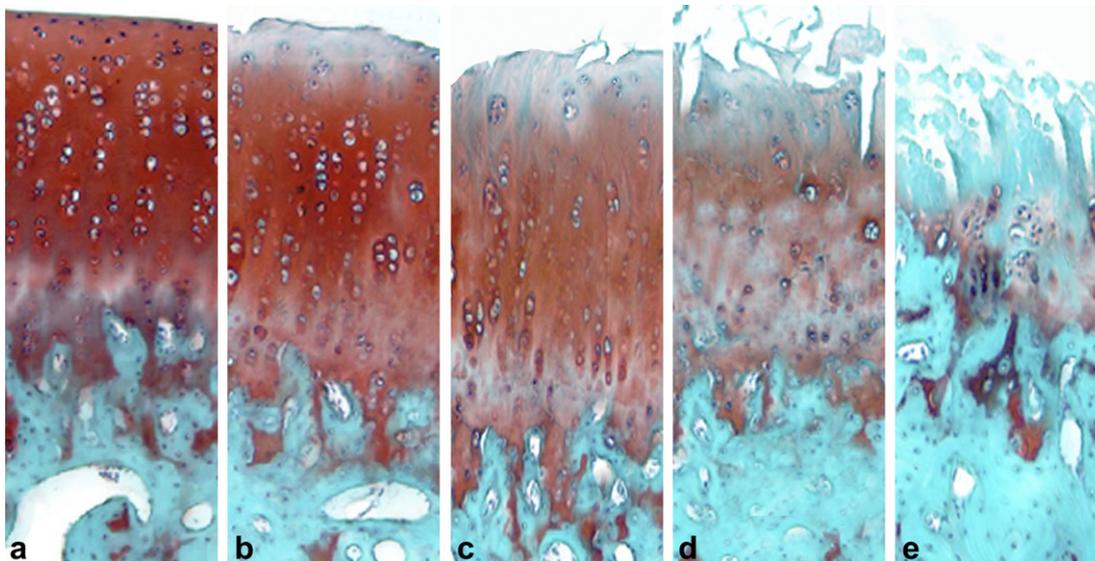
Macroscopically visible surface fibrillation and focal erosions were observed as early as 4 weeks after PCLT in the femorotibial joint. The histological evaluation illustrated the OA lesion observed with the macroscopic observation (Fig. 1). These characteristics of OA progressed gradually at later time points, predominantly on the medial tibial plateau and medial femur. The macroscopic and histological scores of cartilage from PCLT knees were already elevated 4 weeks after surgery and increased with time (Table II).

### The protein expression of thrombin-cleaved OPN

The protein expressions of thrombin-cleaved OPN are shown in Fig. 2. In SF, the antibody O-17 reacted with N-half OPN with molecular weights of 30 kDa. The results showed that samples of SF contained N-half OPN at baseline showed no band. A faint band was rarely detected at 4 weeks postoperatively. The faint band was visible at 8 weeks postoperatively. The band was more pronounced at 16 weeks postoperatively. The strongest band was noted at 24 weeks postoperatively. The protein expression of thrombin-cleaved OPN was considerably elevated in rabbits with advanced OA.

### The levels of thrombin-cleaved OPN

SF levels of thrombin-cleaved OPN are demonstrated in Fig. 3. The levels of thrombin-cleaved OPN in SF from baseline were 3.20 (95% CI: 1.94–6.71) pmol/L, those from 4 weeks postoperatively were 9.90 (95% CI: 7.92–13.20) pmol/L, those from 8 weeks



**Fig. 1.** Histology of cartilage after PCLT in the rabbit knee joint (safranin O/fast green). (a) Articular cartilage with smooth surface, and with regular chondrocytes arrangement was observed at baseline. (b) Slight irregularity of the superficial zone was found at 4 weeks post-surgery. (c) Superficial loss of chondrocytes with surface irregularity and sporadic cellular proliferation were seen at 8 weeks post-surgery. (d) Diffuse loss of chondrocytes in the whole zone with clone formation was noted at 16 weeks post-surgery. (e) Severe loss of safranin O staining, the loss of articular cartilage and multifocal decrease in cells were identified at 24 weeks post-surgery.

**Table II**  
Macroscopic and histological evaluation

Macroscopic evaluation	Mean $\pm$ SD
4 w	1.42 $\pm$ 0.90
8 w	3.25 $\pm$ 1.54
16 w	5.42 $\pm$ 2.19
24 w	7.83 $\pm$ 1.75
Histological evaluation	Mean $\pm$ SD
4 w	4.33 $\pm$ 2.23
8 w	9.08 $\pm$ 2.81
16 w	15.50 $\pm$ 2.15
24 w	18.00 $\pm$ 3.86

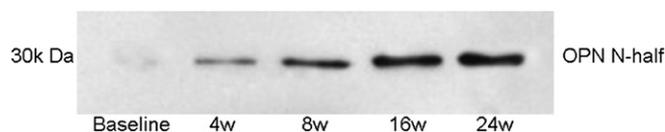
postoperatively were 14.45 (95% CI: 18.53–30.46) pmol/L those from 16 weeks postoperatively were 33.40 (95% CI: 20.27–46.87) pmol/L, and those from 24 weeks postoperatively were 48.55 (95% CI: 41.62–64.29) pmol/L. The data revealed that thrombin-cleaved OPN levels from 4, 8, 16, 24 weeks postoperatively were significantly elevated compared with those from baseline ( $P < 0.001$ ). Thrombin-cleaved OPN levels in SF were elevated peaking at 24 weeks after PCLT. There was no statistically significant difference in thrombin-cleaved OPN levels between 8 weeks postoperatively and 16 weeks postoperatively ( $P = 0.13$ ).

#### Correlation of SF levels of thrombin-cleaved OPN with macroscopic and histological scores

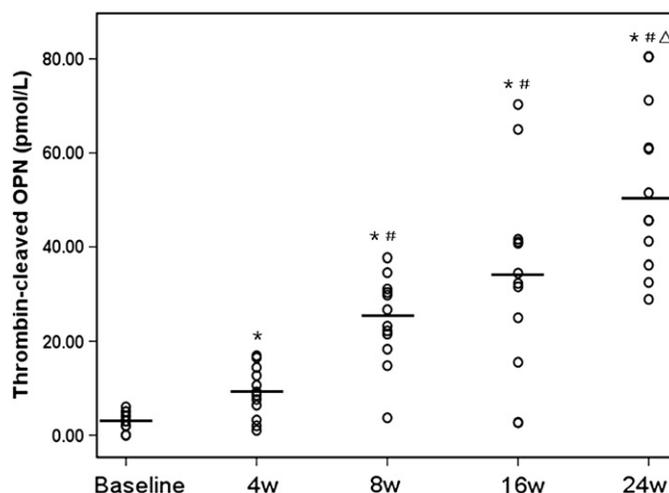
At 4 weeks, thrombin-cleaved OPN levels were not correlated with the macroscopic score ( $\rho = 0.482$ ,  $P = 0.113$ ). Thrombin-cleaved OPN levels showed a positive correlation with the macroscopic scores (8 weeks:  $\rho = 0.695$ ,  $P = 0.012$ ; 16 weeks:  $\rho = 0.751$ ,  $P = 0.005$ ; 24 weeks:  $\rho = 0.660$ ,  $P = 0.020$ ). Interestingly, the same correlation was found between thrombin-cleaved OPN levels and the histological scores (4 weeks:  $\rho = 0.609$ ,  $P = 0.036$ ; 8 weeks:  $\rho = 0.662$ ,  $P = 0.019$ ; 16 weeks:  $\rho = 0.827$ ,  $P = 0.001$ ; 24 weeks:  $\rho = 0.813$ ,  $P = 0.001$ ) (Fig. 4).

#### Discussion

This study revealed a marked increase of thrombin-cleaved OPN levels in SF after PCLT compared with the baseline. Elevated levels of OPN in the SF were possibly caused by either the release of OPN residing in the local tissues, including the synovium, bone, and articular cartilage, or the increase in its production, or both<sup>7,10</sup>. Previous studies have demonstrated the immunohistochemical expression of OPN in the synovial lining cells<sup>19</sup>, fibroblasts in the synovial tissues<sup>20</sup> and articular chondrocytes<sup>20</sup>. It is suggested that cell adhesion, migration or inflammation could be involved in the release of OPN<sup>21</sup>. Proinflammatory cytokines have been demonstrated to play a pivotal role in the development of the disease process. Xu *et al.*<sup>22</sup> reported 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. Sharif *et al.*<sup>8</sup> showed that increased levels of thrombin-cleaved OPN correlated with increased levels of multiple proinflammatory cytokines including TNF- $\alpha$  and IL-6. Thus, elevated levels of



**Fig. 2.** The protein expression of thrombin-cleaved OPN (OPN N-half) in SF at baseline, 4, 8, 16 and 24 weeks after PCLT detected by Western blot.

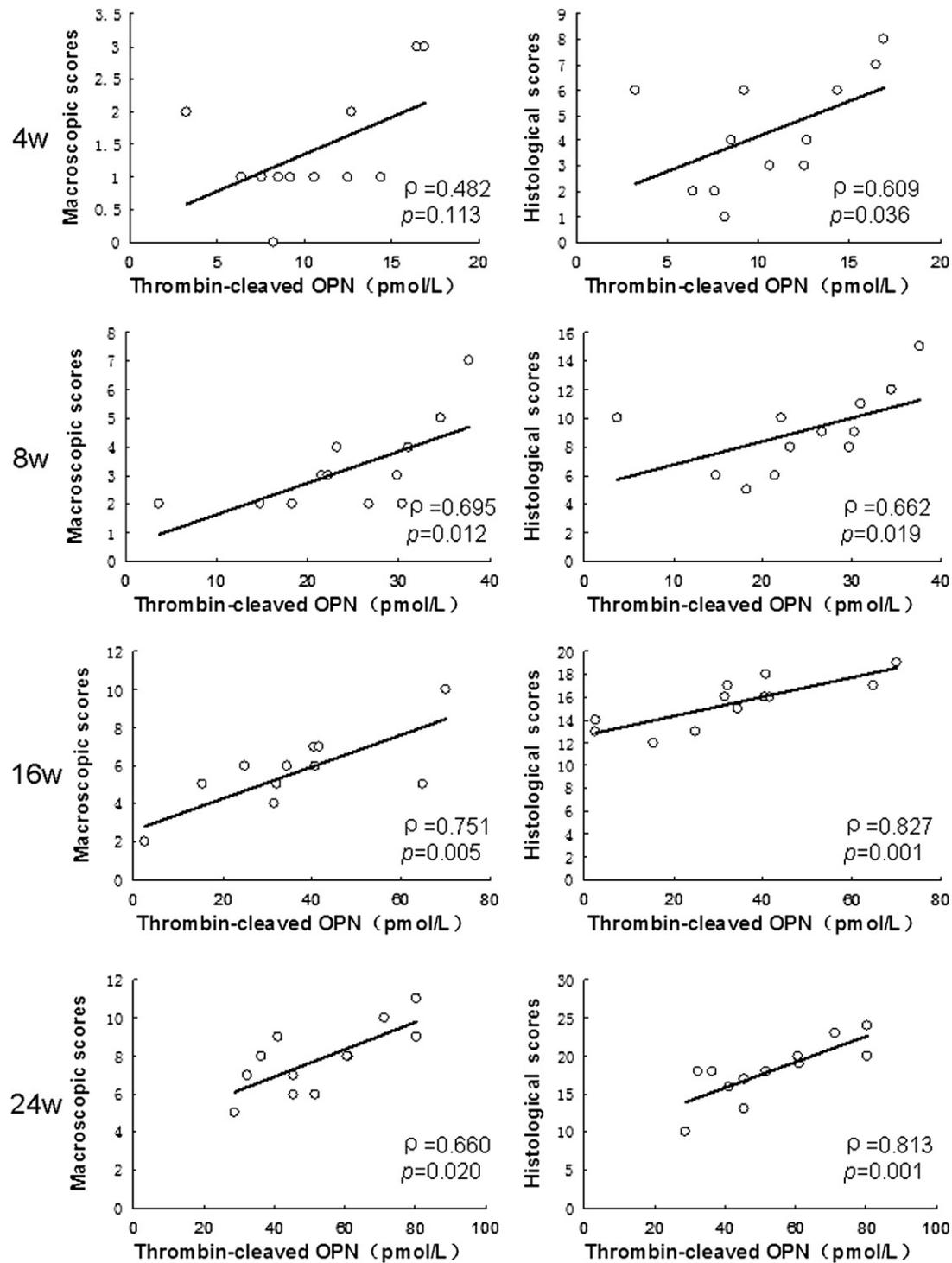


**Fig. 3.** Thrombin-cleaved OPN levels in SF at baseline, 4, 8, 16 and 24 weeks after PCLT. \* $P < 0.001$  vs baseline; # $P < 0.01$  vs 4 w;  $\Delta P < 0.01$  vs 8 w and 16 w.

thrombin-cleaved OPN may play a significant role in the pathogenesis of OA.

Previous study<sup>23</sup> also showed administration of an antibody directed against the SLAYGLR sequence, exposed by thrombin cleavage of murine OPN, inhibited synovitis, bone erosion, inflammatory cell infiltration in arthritic joints of animal models of RA and reduced the expression of inflammatory cytokine (IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and IL-10). Another very recent study<sup>24</sup> showed that the SVVYGLR sequence induces pro-MMP9 expression in isolated vascular SMCs and in diabetic mouse aortas. In addition, the SVVYGLR peptide has been shown to induce angiogenesis of *in vitro* and *in vivo*<sup>25</sup>. These studies suggested that a SLAYGLR epitope within OPN is involved in inflammatory cell migration, differentiation and activation of osteoclast, inflammatory cytokine production and synovial proliferation. However, antibodies recognizing full-length OPN also inhibit development of arthritis<sup>26</sup>. Thrombin has various proinflammatory activities, including cytokine release through activation of the receptors on cells. Tissue factor expression and fibrin deposition are common features of inflamed synovium in RA and OA, which indicates generation of thrombin in the lesions and suggests an involvement of thrombin in the joint inflammation. Sharif *et al.*<sup>8</sup> considered thrombin was generated and cleaves OPN to OPN-R (SLAYGLR) following initial insult, which enhanced tissue inflammation. Alternatively, thrombin can also bind to thrombomodulin on the synovial cell surface, which then activated thrombin-activatable procarboxypeptidase B (pCPB) locally to thrombin-activatable carboxypeptidase B (CPB) and converted OPN-R (SLAYGLR) to OPN-L (SVVYGL), thereby dampening inflammation. Myles *et al.*<sup>27</sup> found OPN cleavage by thrombin was dependent on both of the anion-binding exosites in thrombin that determined rates and specificity of thrombin proteolysis. Their results suggested OPN was a bona fide substrate for thrombin, and generation of thrombin-cleaved OPN with enhanced proinflammatory properties provided another molecular link between coagulation and inflammation.

The intact and thrombin-cleaved forms of OPN (SVVYGLR sequence) are recognized by  $\alpha 4\beta 1$  integrins, while only the thrombin-cleaved form of OPN (but not intact OPN) binds to  $\alpha 9\beta 1$  integrins<sup>28</sup>. Since synovial fibroblasts express  $\alpha 9\beta 1$  integrin and produce its ligands, OPN and tenascin-C, it is possible that there is an autocrine and paracrine interaction of  $\alpha 9\beta 1$  integrin and its ligands *in vivo*<sup>29</sup>. When a blocking antibody against  $\alpha 9\beta 1$  integrin was given at day 3, the augmented expression of the cytokine (IL-6, TNF- $\alpha$ , TGF- $\beta$ , IL-1 $\beta$ ) and chemokines (CCL3, CCL4, CXCL5, CXCL12,



**Fig. 4.** Correlation of SF levels of thrombin-cleaved OPN with macroscopic and histological scores. At 4 weeks, thrombin-cleaved OPN levels were found to be significantly correlated with the histological scores. At 8, 16, 24 weeks, thrombin-cleaved OPN levels were correlated with the macroscopic and histological scores.

CXCL14) was significantly reduced<sup>29</sup>, indicating that  $\alpha 9\beta 1$  integrin-mediated signaling *in vivo* leads to the production of inflammatory molecules. In addition, bone absorption, a critical outcome of arthritis, was inhibited by  $\alpha 9\beta 1$  integrin antibody treatment<sup>28</sup>. These studies indicated the interaction of the cleaved form of OPN and  $\alpha 9\beta 1$  integrin leads to the secretion of cytokine and chemokine, contributing the development of inflammatory tissue destruction. This mechanism, mediated by thrombin-cleaved OPN, is also likely to play a key role in OA. Thus, abrogation of the interaction between

cleaved form of OPN or tenascin-C and  $\alpha 9\beta 1$  integrin by specific antibodies or knock-down of protein or gene expression of OPN, tenascin-C, or  $\alpha 9\beta 1$  integrin by siRNA may be a novel therapeutic means for the treatment of OA in the future.

We have demonstrated that elevated levels of thrombin-cleaved OPN in SF correlated with macroscopic and histological scores of OA. Therefore, only by detecting the levels of thrombin-cleaved OPN in SF, it may be predictive of the degree of cartilaginous damage. In accordance with our observations, Hasegawa *et al.*<sup>9</sup> have

showed that statistically significant correlation was found between thrombin-cleaved OPN and disease severity by Kellgren–Lawrence grade. Honsawek *et al.*<sup>30</sup> also showed that OPN in plasma and SF was related to progressive joint damage in knee OA. These findings indicate that measurements of synovial levels of thrombin-cleaved 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.

Matsui *et al.*<sup>5</sup> showed that both structural changes and an increased loss of proteoglycan from cartilage tissue were augmented in the absence of OPN. OPN deficiency also led to the induction of matrix metalloproteinase 13 (MMP-13), which degraded a major component of the cartilage matrix protein type II collagen. They demonstrated that OPN could be a critical intrinsic regulator of cartilage degradation and that OPN deficiency resulted in accelerated development of OA-like tenascin-C deficiency. Okamura *et al.*<sup>31</sup> revealed that cartilage repair in tenascin-C knockout mice was significantly slower than that in WT mice and that the deficiency of tenascin-C progressed during cartilage degeneration. Nakoshi *et al.*<sup>32</sup> indicated that the distribution of tenascin-C was related to chondroitin sulfate production and chondrocyte proliferation in osteoarthritic cartilage and that tenascin-C had effects on DNA synthesis, proteoglycan content, and aggrecan mRNA expression *in vitro*. They considered tenascin-C may be responsible for repair in human osteoarthritic cartilage. These findings and our results suggested remodeling of cartilage could play some role in the elevated levels of thrombin-cleaved OPN.

There was considerable individual variation in the levels of thrombin-cleaved OPN detected at 16 and 24 weeks post-PCL transaction (with some at 8 weeks). Some animals were potentially so different from others for the following reasons. First, animals were not equally disabled by PCLT, nor they all developed severe OA at 16 and 24 weeks post-PCL transaction. Identical PCLT may result in inter-animal variations in instability and OA. Secondly, the degree of OA development following PCLT may also vary directly with the degree of biomechanical abnormality between individuals. Thirdly, a transected PCL can individually heal spontaneously and did not develop severe OA. Fourthly, the transection of PCL in some animals may be not completed successfully and result in inter-animal variations in the degree of OA. Therefore, some animals had lower histological scores at 16 and 24 weeks post-PCL transaction (with some at 8 weeks) in this study. Moreover, there was correlation between OPN-cleavage levels and histological scores at these points. Consequently, lower levels of thrombin-cleaved OPN in these animals were also noted.

Whereas interesting, this is important to pursue these investigations in order to test variations of the biomarker in longer range studies and in comparison with a control group. Furthermore, it would be valuable to measure levels of thrombin-cleaved OPN in serum and urine. 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. In addition, thrombin has various proinflammatory activities and the thrombin level in SF is probably a feasible marker to monitor ongoing joint inflammation<sup>33</sup>. Based on the above major point discussed regarding thrombin levels, measuring thrombin levels in SF from these animals to address those issues is necessary in the future.

In summary, our results demonstrated that thrombin-cleaved OPN levels in SF appeared to provide a useful marker of OA disease severity and progression in this rabbit model of PCLT. This study added information regarding the validity of thrombin-cleaved OPN. It should be further studied in human in order to test their ability to reflect the natural course of OA and to complete our previous study in human<sup>7</sup>.

## Contribution

Study design: SGG, LC, GHL; Data acquisition: SGG, WL; Data analysis: SGG, CZ; Data interpretation: SGG, CZ, FJZ, JT, MT; Manuscript preparation: SGG, GHL; Manuscript revision: SGG, LC, WL, GHL.

## Conflict of interest

No author has any conflict of interest related to this work.

## Acknowledgments

This work was supported by the National 863 Project of China (2011AA030101), National Natural Science Foundation of China (NO. 81201420, 81272034), the Provincial Science Foundation of Hunan (NO. 09JJ3048), the Young Teacher's Boosting Project of the Fundamental Research Funds for the Central Universities in Central South University, the freedom explore Program of Central South University (2012QNZT103) and National Clinical Key Department Construction Projects of China.

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