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Brief Report

Usefulness of specific OA biomarkers, Coll2-1 and Coll2-1NO$_2$, in the anterior cruciate ligament OA canine model

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Introduction

Osteoarthritis (OA) is a frequent and disabling rheumatic condition. Its diagnosis relies mainly on clinical examination and imaging. Most of the time, there is a discrepancy between patient symptoms and the appearance of the joint structural changes on either X-ray or magnetic resonance imaging (MRI). There is an obvious need for specific biomarkers that could be useful for diagnostic purposes, detectable at an early disease stage, and that could predict and follow disease progression[1]. In addition, specific biomarkers could also be useful tools to evaluate treatment efficacy of disease modifying OA drugs (DMOAD).

Collagen degradation is one of the main features of cartilage breakdown during OA. Type II collagen degradation product is a specific OA biomarker that can assess both disease progression and activity[1,2]. Specific immunoassays have been developed to measure a specific peptide located in the triple helix, Coll2-1 and its nitrated form Coll2-1NO$_2$, a marker associated with local oxidative stress[3,4]. These biomarkers have been studied *in vivo* in mouse, guinea pig and horse[5–8] and also in healthy human[3] and OA patients[4,9]. The serum concentrations of these biomarkers were compared to the serum concentration of myeloperoxidase (MPO), a marker of neutrophil activation.

The aim of this study was to measure the changes over time of both Coll2-1 and Coll2-1NO$_2$ during the development of OA induced by the transection of the anterior cruciate ligament (ACL) in dog. The concentrations of biomarkers 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

Specimens used in this study were obtained from dog experimental groups included in previous studies[10] performed in Arthrolab, Inc facilities in Canada.

Sixteen adult crossbred dogs (24–48 months old), weighing 25 ± 3 kg, were used. Surgical transection of the ACL of the right knee was performed under general anesthesia as previously reported[10]. Following surgery, the dogs were housed and they had free access to exercise in a large enclosure. All dogs exercised in exterior runs for a 2-h period during the morning, 5 days a week (Monday to Friday). The dogs were sacrificed by an intravenous injection of barbiturates 8 weeks after the surgical procedure. The study protocol was approved by the ArthroLab Animal Protection Committee, fully accredited by the Canadian Council on Animal Care (CCAC).

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Serum sampling

Serum samples were obtained for each dog at baseline, 2, 4, 6 and 8 weeks after surgery. Samples were kept frozen at −80°C until the biomarker analysis was performed.

Macroscopic grading

Each knee was examined for gross morphologic changes. The degree of osteophyte formation was graded by measuring the maximal width (in mm) of the spur on both the medial and the lateral femoral condyles, using a digital caliper (Digimatic Caliper; Mitutoyo, Kawasaki, Japan). Cartilage changes on the medial and the lateral femoral condyles and tibial plateaus were graded separately as previously described[11]. Briefly, changes to the articular surface area were measured, with results expressed in mm^2. In addition, the depth of erosions was graded on a scale of 0–4, where 0 = normal appearance of the surface, 1 = minimal fibrillation or a slight yellowish discoloration of the surface, 2 = erosion extending into the superficial or middle layers, 3 = erosion extending into the deep layers, and 4 = erosion extending to the subchondral bone. The cartilage macroscopic score was then calculated using the formula, surface × depth. The final macroscopic score consisted of the sum of the scores (surface × depth) of each of the lesions present on the medial and lateral femoral condyles and tibial plateaus. The total joint score consisted of the sum of the final scores obtained for both condyles and the tibial plateaus.

Histologic grading

Cartilage was removed from the areas of the lesions identified by the macroscopic grading and histologic evaluation was then performed on sagittal sections of cartilage from the lesional areas of the femoral condyles and tibial plateaus[10]. Specimens were dissected, fixed in TissuFix #2 (Laboratoires Gilles Chaput, Montreal, Quebec, Canada), and embedded in paraffin. Serial sections (5 μm) of paraffin-embedded specimens were prepared and stained with safranin-O. The severity of OA lesions was graded on a histologic scale of 0–29, adapted from Sakakibara *et al*[12], by two independent observers. This scale evaluates the severity of OA lesions based on structural changes (scale 0–10), cellular changes (scale 0–12), stainability with safranin-O (scale 0–4), and pannus formation (0–3). The final score corresponds to the score of the most severe lesions. The total score corresponds to the compilation of the score from the histologic parameters obtained from the different compartments of the knee.

Coll2-1 and Coll2-1NO$_2$ immunoassays

The serum concentrations of Coll2-1 and Coll2-1NO$_2$ were measured by two specific competitive immunoassays[3]. Briefly, Coll2-1 and Coll2-1NO$_2$ conjugated to bovine serum albumin (BSA) by BS[3] were coated by adding either 100 μl of Coll2-1 conjugate at 50 ng/ml or 100 μl of Coll2-1NO$_2$ conjugate at 8 ng/ml for 24 h at 4°C. After washing, 50 μl of calibrators (synthetic peptide) or 50 μl unknown serum samples diluted 5-fold, were applied to the wells, followed by 100 μl either of D3 or of D37 antibody, diluted 1/40,000 and 1/250,000 respectively, and incubated 1 h at room temperature. During the procedure, a competition for binding the antibody takes place between the immobilized peptide and the peptide contained in the samples. After washing, 100 μl of peroxidase-conjugated goat antibodies to rabbit IgG (Invitrogen, Belgium) diluted 1/10,000, were incubated 1 h at room temperature. After washing, 100 μl of freshly prepared enzyme substrate (TMB, Invitrogen, Belgium) were added into each well. After 15 min, the reaction was stopped with 100 μl of 4 M H_3PO_4. The coloration was read with a microplate reader (Labsystem) at 450 nm, corrected for absorbance at 650 nm. The coating buffer was a 10 mM phosphate buffer saline (PBS), 138 mM NaCl pH 7.4. The washing buffer was a solution of 25 mM Tris, 50 mM NaCl pH 7.3. The preparation of the standard curve, the dilutions of serum samples, of antisera and of the secondary antibody were done in 10 mM PBS, 138 mM NaCl, 0.2% (w/v) BSA, 0.1% (v/v) Tween 20 pH 7.0 for the Coll2-1 immunoassay and in 50 mM Tris, 138 mM NaCl, 0.2% (w/v) BSA, 0.1% (v/v) Tween 20 pH 8.0 for the Coll2-1NO$_2$ immunoassay.

MPO immunoassay

The serum concentration of MPO was measured by a commercially available solid phase two-site enzyme linked immunosorbent assay according to the manufacturer's recommendations (Elizen MPO, Zentech SA, Liège, Belgium).

Statistical analysis

The Coll2-1, Coll2-1NO$_2$ and MPO concentrations were expressed as median (minimum and maximum). Statistical analyses were carried out with GraphPad InStat3. Friedman test followed by the Dunn's multiple comparison post-test were used to analyze the variation of the biomarker concentration. Correlations between biomarkers and the other evaluated parameters were established using the non-parametric Spearman's rank correlation coefficient. Data were considered statistically significant when P value was below 0.05.

Results

Macroscopical and histological analyses

The macroscopic appearance of cartilage lesions in the dog knees 8 weeks after ACL transection corresponded to mild to moderate OA as previously reported[10] (Table I). The size of the osteophytes was measured and reported Table I. The histological evaluation illustrated the OA lesion observed with the macroscopic observation. It revealed cartilage surface fibrillation, changes in cellularity and cartilage matrix discoloration (Table I). The presence of a pannus was reported by the histological score.

Serum concentration of the studied biomarkers

Following ACL transection a significant increase in the serum concentrations of the three studied biomarkers was found (Friedman test; Coll2-1: $P = 0.003$, Coll2-1NO$_2$: $P = 0.0007$ and MPO: $P < 0.0001$) (Fig. 1). The Coll2-1NO$_2$/Coll2-1 ratio was also increased (Friedman test; Coll2-1NO$_2$/Coll2-1; $P = 0.008$). The concentrations of Coll2-1 [Fig. 1(A)] and MPO [Fig. 1(C)] were significantly increased as early as 2 weeks after transection compared to

Table I
Cartilage macroscopic and histological evaluation of the total joint 8 weeks after ACL transection

Macroscopic evaluation	Osteophytes (mm)	6.82 ± 0.60
	Size of lesions (mm^2)	63.57 ± 15.89
	Global macroscopic score (size × grade)	**139.8 ± 34.95**
Histological evaluation	Structure	6.2 ± 0.4
	Cellularity	
	Tangential	1.6 ± 0.1
	Transitional	5.4 ± 0.3
	Safranin-O staining	1.7 ± 0.1
	Pannus	0.7 ± 0.2
	Total score	**15.7 ± 0.9**

Values are mean ± S.E.M.

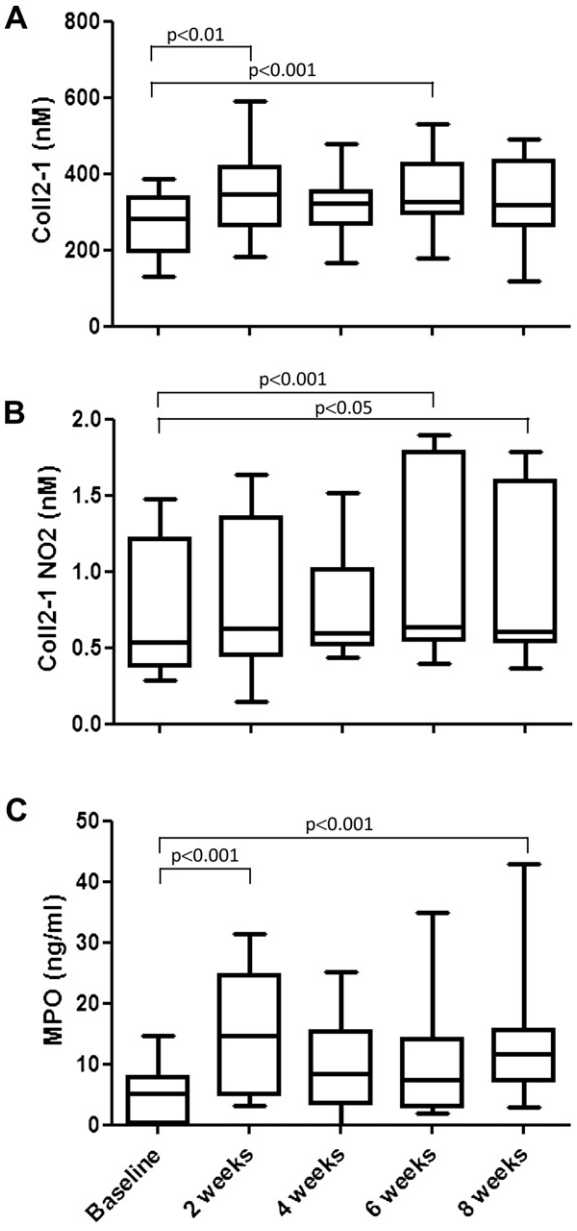

Fig. 1. Coll2-1 (A), Coll2-1NO_2 (B) and MPO (C) serum concentrations at baseline and 2, 4, 6 and 8 weeks after ACL transection. Data are presented as bow and whiskers plot. Data were analyzed using Friedman test followed by Dunn's multiple comparison post-test. $P < 0.05$ is considered significant. $N = 16$ at each time point for Coll2-1 and MPO and $N = 15$ at each time point for Coll2-1NO_2.

baseline (Coll2-1: $P < 0.01$ and MPO: $P < 0.001$) and remained stable until sacrifice at week 8. The Coll2-1NO_2 [Fig. 1(B)] concentration was found to be significantly increased starting at 6 ($P < 0.001$) and 8 weeks ($P < 0.05$) after transection compared to baseline. Notably, although Coll2-1 and Coll2-1NO_2 were evidently correlated ($r = 0.67$, $P < 0.0001$), Coll2-1NO_2 expectedly showed stronger association with MPO than Coll2-1 ($r = 0.39$, $P = 0.0005$ vs $r = 0.22$, $P = 0.055$, for Coll2-1NO_2 and Coll2-1, respectively).

Correlation of biomarkers with macroscopic lesions and cartilage histological scores

A positive and highly significant correlation was found between Coll2-1 concentrations (from 4 weeks until 8 weeks) and the global macroscopic score (4 weeks: $r = 0.54$, $P = 0.029$; 6 weeks: $r = 0.50$, $P = 0.045$; 8 weeks: $r = 0.79$, $P = 0.0002$). Interestingly, the same correlation was found between Coll2-1 concentration (from 2 weeks until 8 weeks) and the size of the cartilage lesions (2 weeks: $r = 0.62$, $P = 0.009$; 4 weeks: $r = 0.60$, $P = 0.013$; 6 weeks: $r = 0.50$, $P = 0.044$; 8 weeks: $r = 0.80$, $P = 0.0002$). Coll2-1 concentration at 8 weeks was also found to be significantly correlated with the changes in cartilage structure as evaluated by the histological score ($r = 0.54$, $P = 0.028$). On the other hand, Coll2-1NO_2 concentrations were found to be significantly correlated with the size of the osteophytes (baseline: $r = 0.70$, $P = 0.003$; 2 weeks: $r = 0.57$, $P = 0.02$; 4 weeks: $r = 0.60$, $P = 0.017$; 6 weeks: $r = 0.72$, $P = 0.002$; 8 weeks: $r = 0.67$, $P = 0.006$). At 8 weeks, the ratio Coll2-1NO_2/Coll2-1 was correlated with the global macroscopic score ($r = -0.6929$, $P = 0.0042$), the size of the macroscopic lesions ($r = -0.5643$, $P = 0.0284$) and the total histological score ($r = -0.6206$, $P = 0.0136$), but not with the osteophyte size.

Discussion

There is a need for reliable OA biomarkers that could detect the early changes that take place during the disease process and that could also monitor the disease progression. Coll2-1 and Coll2-1NO_2 represent a double interest since they reflect not only cartilage degradation but also inflammation and oxidative stress, key features of OA and are considered biomarkers of cartilage catabolism[13]. These were reported to be useful to assess the diagnosis, the burden of disease, the prognosis by the burden of disease, investigative, prognosis, efficacy of treatment, diagnosis (BIPED) classification[2] and were recently proven reliable to assess the efficacy of intervention[14].

This study showed that both biomarkers were increased after the transection of ACL in dogs. The increase in the level of Coll2-1 was seen as early as 2 weeks after surgery. The importance of collagen degradation through the activation of specific proteolytic enzymes, e.g., matrix metalloproteases, cathepsin K or aggrecanases, has already been demonstrated in the ACL transection dog model of OA[15,16]. In addition, this finding is in accordance with the concept that damage to the collagen network would occur early in the OA process. In addition, this hypothesis is supported by the previous data obtained in the Hartley guinea pig OA model[7] in which serum Coll2-1 level increase is related to early detectable type II collagen molecule degradation. The precocity of Coll2-1 variation accounted for the validation of this biomarker. Furthermore correlations showed that Coll2-1 reflected both macroscopic and histological changes as its concentrations were significantly correlated with both parameters. These results confirmed the previous observations made in a well-validated guinea pig model[7] in which Coll2-1 increase was shown to be associated with the appearance of early disruption of collagen fibrils and correlated with histological severity of cartilage lesions.

The level of Coll2-1NO_2 was found to be increased throughout the study period to reach its maximum levels 6 and 8 weeks after surgery. Coll2-1NO_2 reflects the oxidative stress that occurs in the joint during OA. Based on previous studies in animals and human, the ratio Coll2-1NO_2/Coll2-1 has been considered to reflect the oxidative stress related to inflammation. One may therefore expect a strong correlation of the ratio with oxidative stress parameters. This statement is supported by the correlation found between Coll2-1NO_2 and MPO, another marker of oxidative stress, even if it requires further investigations. In contrast, the ratio Coll2-1NO_2/Coll2-1 changes over time was negatively correlated with histological and macroscopic parameters. This is explained by the fact that the increase of Coll2-1 concentration over time was more important than the increase of Coll2-1NO_2. The present results showed that Coll2-1NO_2 is also associated with osteophytes formation, a process involving nitric oxide. One may also suggest that the biomarker

Coll2-1NO_2 could reflect the process of endochondral ossification that takes place for the development of osteophytes. However, this observation needs to be further studied. Finally, this is important to note that MPO was note correlated with either of the OA evaluated parameters, meaning that it is not a specific marker of OA.

Whereas interesting, this is important to pursue these investigations in order to test the variations of both biomarkers in longer range studies and in comparison with a control group. In addition, it would be valuable to measure their variations in a DMOAD study.

In conclusion, this study added information regarding the validity of these two OA biomarkers, i.e., Coll2-1 and Coll2-1NO_2. These results showed that they clearly reflect key events that happened in the knee joint during the development of OA in animal model. They should be further studied in human in order to test their ability to detect early structural cartilage changes and to reflect the natural course of OA and to complete our previous work in human[9].

Contribution

Study design: YH, JMP, MD.
Data acquisition: MD, JMP.
Data analysis: YH, JMP, MD.
Data interpretation: YH, JMP, PM, GG, MD.
Manuscript preparation: YH, MD.
Manuscript revision: YH, JMP, PM, GG, MD.

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Conflict of interest

P Msika and GB Guillou are employees of Laboratoires Expanscience. Y Henrotin is the founder of the university spin-off Artialis sa. M Deberg is the R&D director of Artialis. Other authors declare no conflict of interest.

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Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.joca.2012.03.016.

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