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Identification of differential pattern of protein expression in canine osteoarthritis serum after anterior cruciate ligament transection: A proteomic analysis

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ABSTRACT

Osteoarthritis (OA) management remains a great challenge and there is considerable effort to understand its pathophysiology and to identify new therapeutic targets and biomarkers. Canine OA surgically induced by the transection of the anterior cruciate ligament (ACLT) is a widely used and relevant model. This study reports a proteome mapping of dog serum and an analysis of the differentially expressed proteins between before and after ACLT. In the first part of the study, 261 picked protein spots were identified from preparative 2D gels and 71 different proteins were identified among the 261 spots present on the reference map. Canine serum proteome mapping reveals the presence of proteins of interest, such as fetuin B, complement C3 and C1s and pregnancy zone protein. The comparison between serum from dogs before and after ACLT reveals the differential expression of several proteins that could play a key role in the pathogenesis of OA.

A number of proteins, such as fetuin B and complement C3, were increased in dog OA serum whereas others, such as hyaluronan binding protein 2, inter-alpha-trypsin inhibitor H4 (ITIH4), complement C1s and C4 and haptoglobin were decreased. Some of these proteins could be candidate biomarkers for diagnosis, prognosis and treatment evaluation. The results of the study also reinforced the similarities between dog experimental OA and human cases of OA.

Introduction

Osteoarthritis (OA) is a highly prevalent chronic health condition that represents a real social and economic burden, even in the young population (Dibonaventura et al., 2011). The pathology can evolve over decades leading to loss of joint function. It is complex and multifactorial involving mechanical processes (i.e. joint instability, loading abnormalities) and inflammatory reactions. An understanding of the intimate pathophysiological pathways and the discovery of therapeutic targets are paramount as no cure exists to date.

Animal models are valuable tools not only to elucidate the pathophysiology (Little and Smith, 2008) but also for the pre-clinical evaluation of new therapies (Pelletier et al., 2010). The anterior cruciate ligament transection (ACLT) OA model in dogs corresponds to an instability model associated with inflammation processes that mimic the molecular pathology and the histopathology of the human disease (Little and Smith, 2008). It is one of the most widely used experimental models of OA (Cook et al., 2010).

The ACLT model has been used for the evaluation of therapeutic interventions (Pelletier et al., 2010) and has provided important information regarding the role of the different joint tissues, i.e. cartilage, subchondral bone and synovial membrane. It was recently shown that biomarkers of collagen degradation (i.e. Coll2-1 and Coll2-1NO_2) measured in OA dog sera could be correlated with the severity of OA lesions evaluated macroscopically and histologically (Henrotin et al., 2012b). Many efforts have been carried out to standardize animal studies and sample evaluation (Cook et al., 2010).

The identification of a biomarker or a panel of biomarkers related to the OA process in the ACLT model could be of great interest for the early characterization of the disease process and to monitor a treatment effect. However, to our knowledge nothing has been reported to date using a standardized approach to find biomarkers of OA progression in this model. Proteomics applied to the dog could represent an interesting technology and in recent years

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proteomic analysis has widened the horizon of research and knowledge in human OA (De Ceuninck and Berenbaum, 2009; Fernandez-Puente et al., 2011; Gharbi et al., 2011; Henrotin et al., 2012a).

The aim of the present study was to generate a map of the normal crossbred dog serum proteome and to further compare the proteomic profiles of dog sera before and after ACLT. The aim was to reveal the differential expression of relevant proteins in the sera from OA dogs that could be further studied to improve knowledge of OA pathogenesis, diagnosis or the identification of new biomarkers or therapeutic targets.

Materials and methods

Samples

OA was experimentally induced by the surgical transection of the cranial cruciate ligament of the knee joint in eight crossbred dogs as previously described (Boileau et al., 2009, 2010; Moreau et al., 2011). Sera from these dogs were obtained before the induction of OA and 12 weeks after surgery. The dogs corresponded to the placebo-treated group included in a study evaluating an experimental therapeutic intervention.

The study protocol was approved by the institutional ethics committee (protocol number A062-INT07D) and conducted in accordance with the Canadian Council on Animal Care guidelines.

Canine serum proteome mapping

Sera from three dogs with experimentally-induced OA were pooled with three sera from the same three dogs taken before induction of experimental OA (CTRL). This process was repeated three times to obtain different reference populations. Sera were depleted using the IgY-D11–dog specific column (GENWAY Biotech) that removes the top 11 highly abundant proteins from dog serum (Albumin, IgG, Fibrinogen, IgA, IgM, Haptoglobin, α2-Macroglobulin, α1-antitrypsin, α1-Acid glycoprotein, Apo AI HDL, Apo AII HDL). Sera (20 μL) were subjected to two passages on the column to ensure optimal depletion. This technique has showed a good specificity in man (Huang et al., 2005).

Two-dimensional difference gel electrophoresis (2D-DIGE) comparison of protein distribution between OA and control dogs

Sera from eight dogs with experimentally-induced OA (ACLT) were pooled to obtain the OA population. Sera from the same eight dogs taken before induction of experimental OA (CTRL) were pooled to obtain the control population. Sera were depleted using the IgY-D11–dog specific column (GENWAY Biotech) as above. Sera (20 μL) were again subjected to two passages on the column to ensure optimal depletion.

Protein labelling

Protein samples for canine serum proteome mapping were labelled on lysine residues with Cy2 CyDye DIGE Fluor using the minimal labelling technique. Three gels were made. Protein samples for 2D-DIGE comparison of protein distribution between OA and CTRL were labelled on lysine residues with Cy3 or Cy5 CyDye DIGE Fluors. Three gels were made. Proteins from CTRL samples were labelled with Cy5 CyDye DIGE Fluor and proteins from OA samples were labelled with Cy3 CyDye DIGE Fluor on the third gel. An internal standard (MIX) comprising equal amounts of CTRL and OA samples was labelled with Cy2 CyDye DIGE Fluor and loaded on each gel.

Two-dimensional electrophoresis

For canine serum proteome mapping, protein samples (250–300 μg) labelled with Cy2 DIGE Fluor were separated by 2D electrophoresis using an IEF (isoelectric focusing) buffer which was loaded into an Immobiline DryStrip (pH 3–7 NL, 24 cm) (GE Healthcare). The isoelectric focusing was performed for minimum 60,000 Vh using an Ettan IPGphor (GE Healthcare) at 20 °C. Next, the gels were equilibrated for 12 min in equilibration buffer I and II.

A 12.5% SDS–polyacrylamide slab gel (24 cm) was used for the second-dimension gel electrophoresis. The IPG strips were placed on the top of the second-dimension gel. The gels were then placed in SDS electrophoresis buffer and run overnight at 1 W per gel. Gels were scanned while still between two low-fluorescence glass plates using a Typhoon Trio + fluorescent scanner and saved in gel format using ImageQuant software (GE Healthcare).

DeCyder 2D v7 software (GE Healthcare) was used for the matching of three preparative gels made from different reference populations and simultaneous comparison of spots distribution. This enabled the evaluation of replicates between the reference population and the determination of protein pick list on each gel. Automatic detection of spots was completed by a manual refining procedure. This procedure corresponds to the removing of spots present on the edge of the map and spots corresponding to artefacts or portion of abundant spots. Some mismatching of missed spots were added on the map, whereas other spots were merged. Indeed, spot detection on gel maps were performed automatically using DeCyder software. However, according to the quality of migration or to the protein load on the gel, some abundant spots were detected as two distinct spots or, on the contrary, distinct spots were detected as single spot. Moreover, low abundant spots could be missed by automatic detection. Manual refining was needed to improve the automatic detection of spots.

2D-DIGE comparison of protein distribution between OA and control dogs

The experimental procedure was the same as described above except that the protein samples (37.5 μg) were labelled with Cy3, Cy5 or Cy2 DIGE Fluor. Decyder 2D v7 software (GE Healthcare) was used for simultaneous comparison of abundance changes across sample groups. The Decyder differential in-gel analysis (DIA) module generated ratios for each protein spot by comparing Cy3 and Cy5 signals to the Cy2 control signal. The Decyder biological variation analysis (BVA) module matched all protein spot maps from the gels and normalized the DIA-generated Cy3:Cy2 and Cy5:Cy2 ratios relative to the Cy2 signals for each resolved feature separately. This enabled the calculation of average abundance changes across all three samples within each test group and the application of univariate statistical analyses (Student's *t* test).

Protein identification

Protein spots were automatically cut out of the polyacrylamide gel using an Ettan Spot Picker and automatically digested using an Ettan Digester Robot. The identity of proteins was determined by MALDI-TOF mass spectrometry (MS) (Matrix-assisted laser desorption ionization-time of flight, Ultraflex II; Bruker Daltonics). Some complex protein mixtures were further analyzed by tandem MS (Amazon ion trap; Bruker Daltonics): fragments of peptides were separated on C18 hydrophobic column (Ultimate 3000 HPLC, C18 pepmap100, 3 μm, 15 cm column; Dionex) before ionization and analysis using tandem MS.

Fragmentation spectra of peptides were compared to theoretical spectra present in NCBI database. Mascot software (Matrix Sciences) was used in order to establish the degree of similarity between analyzed peptides and known sequenced proteins. This similarity was expressed as Mowse score: $-10\log(P)$ where P is the probability that the observed match was a random event. Significant identification was obtained when at least two unique peptides are identified per protein hits with at least one peptide showing an identity Mascot Score. NCBI database was chosen instead of SWISS-Prot because it contains more entries for *Canis lupus familiaris* and gives higher Mowse scores. Further technical details are provided as Supplemental material (Appendix A).

Results

Canine serum proteome mapping (reference map)

The reference map corresponds to the protein mapping of dog before and after ACLT induction. Results of image analysis and spot detection on preparative gels can be seen in Appendix A. It is important to note that preparative gel 3 was manually analyzed on Decyder with a slightly different area of interest. Nevertheless, this gel was considered in the matching process because it was less loaded in proteins than the two others gels (250 μg of proteins instead of 300 μg) then allowing for a better separation of the most abundant spots. However, it was of too low intensity to allow MS identification. Overall, 261 picked spots of proteins were identified from preparative gels. In total, 71 different proteins were identified among the 261 spots presents on the reference map. A gel view of this map and the list of the identification of protein spots are provided as Supplemental material (Appendix A).

The diversity of the identified proteins seems to be limited. Indeed, the majority of spots were gathered in clusters containing different isoforms of a single protein. A new set of abundant proteins like hemopexin or serotransferrin appears after depletion. A great number of identified proteins are factors of complement (C1s, C1r, C2, C3, C4, C5, C6, C7, C9, factor B, factor H, factor I, ficolin), protease (carboxypeptidase N) and protease inhibitors (pregnancy zone protein, alpha-2 macroglobulin, inter-alpha-inhibitor