Osteoarthritis and Cartilage 20 (2012) 783–786

Osteoarthritis and Cartilage

Brief Report

Measurement of matrilin-3 levels in human serum and synovial fluid using a competitive enzyme-linked immunosorbent assay

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ARTICLE INFO

Article history:
Received 6 October 2011
Accepted 22 March 2012

Keywords:
Extracellular matrix
Marker
Metabolism

Introduction

Matrilin-3 (MATN3) is a skeletal-specific, tetrameric pericellular protein[1–3], localizing to the pericellular matrix of chondrocytes. It accumulates in higher amounts in osteoarthritic cartilage in human[4]. Indeed, *in vitro* characterization of MATN3 identified anti-anabolic[5] and pro-catabolic functions[6], although it was also found to favour chondrogenesis of synovial fibroblasts[7]. As MATN3 is virtually specifically expressed in cartilage, over-expressed upon osteoarthritis and as its functions may participate in the mechanisms of the disease, it may constitute a useful marker to diagnose osteoarthritis or to evaluate its progression, not only from synovial fluids (SF), but also from circulating fluids. However, methods to quantify MATN3 from complex solutions are lacking. Here, we report the establishment of a direct, competitive Enzyme-Linked Immunosorbent Assay (ELISA) method prepared from commercially available antibodies and reagents, which allows reproducible determination of MATN3 levels in the nanogramm to microgramm per millilitre range. The method will be useful to conclude whether MATN3 will be a useful marker to diagnose osteoarthritis and/or to follow its progression using a non-invasive and financially affordable technique.

Materials results and discussion

Patients

SF and sera were obtained in the context of total knee replacement due to end-stage osteoarthritis. Patients gave written informed consent in accordance with the usual ethical regulations in collaboration with our local bone bank. This study was approved by our local Research Institution review board (registration number UF 9757 - CPRC 2004 - Cellules souches et chondrogénèse). The average age of patients was 70 years (ranging from 47 to 88) and 68% of them were females. Their Kellgren & Lawrence grades ranged from 2 to 4. No correlations were evidenced between these and MATN3 levels. 20 sera and 15 SFs were collected from 25 patients. From 10 patients, both serum and SF were used to compareMATN3 levels in both fluids.

Sample collection, conservation and processing

SF samples were collected under general anaesthesia during total knee replacements. Sera were obtained during pre-operative procedures. Aliquots were centrifuged at 10,000 g for 5 min and clarified supernatants were transferred and frozen at −80°C. For measurement, aliquots were thawed on ice and clarified again using the same centrifugation procedure. Aliquots were not reused after more than one freeze-thaw cycle.

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1063-4584/$ – see front matter
doi:10.1016/j.joca.2012.03.017

Statistical analyses

Measurements were performed in triplicates unless stated otherwise. For assay validation, optical density (OD) was fitted against analyte concentration applying a logarithmic regression to the calibration curve. Average, standard deviations (SD) and differences from theoretical values were calculated for all standards and samples.

Direct, competitive ELISA procedure

In preliminary experiments, only one commercial antibody (MAB3017 from R&D) achieved sufficient specificity and sensitivity to MATN3 in direct ELISA assays (data not shown). Therefore, a competitive assay was required. The antibody was coated onto plastic surfaces and then exposed to mixed analyte and labelled competitor, the binding of the latter being then measured. A fraction of commercial recombinant MATN3 was biotinylated using Sulfo-NHS biotinylation kit (Pierce) and used as competitor after dialysis to PBS (Gibco) containing 0.1% azide. Another fraction was suspended in PBS and serial diluted in FCS for use as standards. 96-well Maxisorp (Nunc) plates were coated with 1 μg/ml antibody overnight at 4°C. Non-specific binding was blocked with 3% BSA (type IV BSA, from Euromedex) in PBS for 1 hour. Meanwhile, an identical concentration of 50 ng/ml competitor MATN3 was added to each standard or analyte fraction and homogenized. Standards (500 pg/ml–1 μg/ml) were then added to wells and incubated for 3 hours under constant shaking. Wells were washed five times in PBS containing 0.2% Tween-20 (PBST), streptavidin-HRP (from R&D) diluted in 3% BSA was added for 45 min in 3% BSA and washed five times again in PBST. Detection was performed as specified by the manufacturer (R&D) and measurements were performed on a Multiscan EX (Fisher Thermoscientific).

Standard curves

Standard curves were prepared using recombinant MATN3 diluted into FCS (FCS reacted insignificantly to the assay as determined using BSA as a negative control). Typical results using standard concentrations ranging from 500 pg/ml to 1 μg/ml exhibited classical saturation curves with SDs of all values falling within 10% of the average measure. The standard curves fitted to logarithmic regressions with sufficient correlation coefficients throughout this concentration range [Fig. 1(A)], allowing back-calculation of each concentration from the measured absorbance with errors lower than 15% compared to theoretical values.

Validation on sera and SFs

The assay was applied to 20 sera and 15 SFs obtained from osteoarthritic patients. Sera (100 μl) were directly used as diluents for competitors as described for standards. SFs were diluted 10-fold in FCS containing 55 μg/ml competitor. The range of concentration measurement using this assay fitted to concentrations found in all 35 samples, which ranged from 80 to 2700 ng/ml. When determining the recovery by dilution to patient samples [Supplemental Fig. 1 (A)], all recoveries were within ±20% as long as dilutions fell into a range between 2 ng/ml and 1 μg/ml. This was not a limit for calculations of MATN3 levels in either fluid, which all fell into this interval (taking into account that SFs were 10-fold diluted).

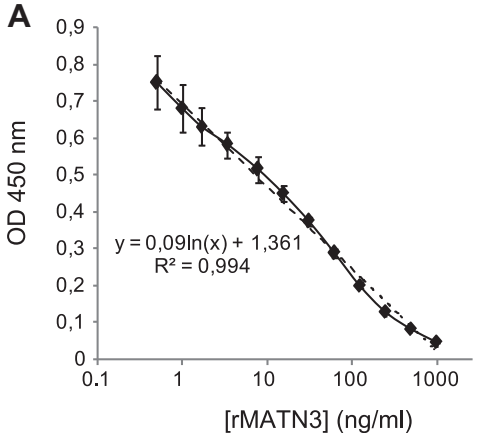
In order to further validate the assay, dilutions of standards into human fluids of previously characterized MATN3 concentrations were performed and their total concentrations were measured [Supplemental Fig. 1(B)]. In all tested fluids, measured MATN3 concentrations fitted to theoretical values with errors lower than 15%.

The average intra-assay variability was 6.1% and the average inter-assay variability was 9.1%.

Specifications of the assay

The numerical values of the specifications were compiled in [Fig. 1 (B)]. Recovery rates were calculated after measurements of up to 64-fold serial dilutions of two samples of each fluid into FCS. Initial concentrations were back-calculable as long as diluted concentrations remained higher or equal to 2 ng/ml. Competitions between samples and standards were performed from two samples of each fluid in which standards were diluted. The intra-assay and inter-assay precisions were measured from eight independent runs of one SF and two serum samples (all samples being prepared together). The inter-assay precision was determined by eight independent measurements at eight different days over a 3 weeks period.

The lower limit of detection [Fig. 1 (B)] was considered as the lowest concentration that could be measured with a an SD lower than 10% and that allowed back-calculation using the regression curve with an error lower than 15%, from three independent assays.



B

intra-assay SD	6.1% (n=8)
inter-assay SD	9.4% (n=8)
LDL for standards	500 pg/ml
LDL from diluted samples	2 ng/ml

Fig. 1. Saturation curve and specifications of the assay. (A) Classically observed dose-response of the assay represented on a logarithmic concentration scale. The equation of the regression curve together with the correlation coefficient (r^2) are annotated. The plot shows average measures and bars indicate SD. (B) summary of the specifications of the assay.

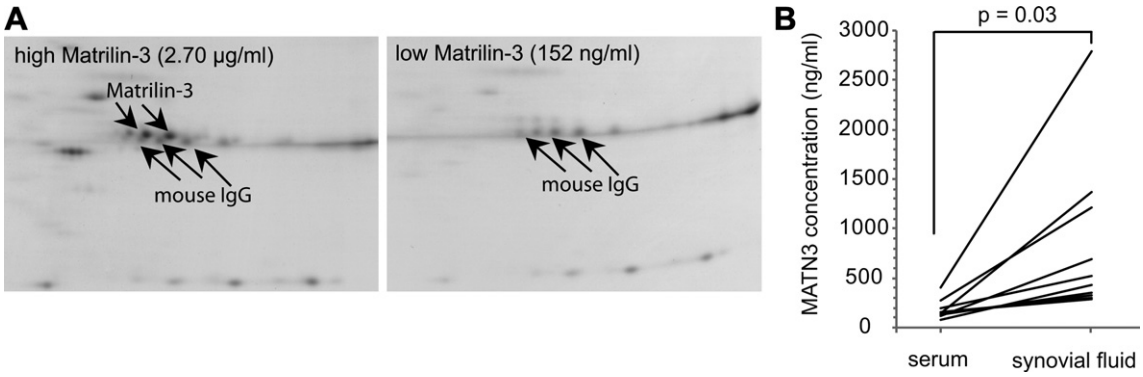


Fig. 2. Specificity of the antibody and adaptation of the assay to SFs and sera. (A) Proteomic investigation of the specificity of the antibody used in the assay. SFs found by ELISA to contain high versus low MATN3 amounts were subjected to immunoprecpitation using the same antibody and the immunoprecipitates were analyzed by 2D-electrophoresis coupled mass spectrometry. Spots of interest are indicated by arrows. (B) Comparison of SF and serum contents in Matrilin-3 from 10 patients. Segments link measures of both compartments for each individual patient. SD were not indicated to allow visualization of details. The indicated p value is as calculated by the student *t* test.

Stability of reagents

In order to address the stability of the reagents after preparation, test measurements from standards were performed twice at a 3 months interval all reagents being kept at 4°C in triplicates and exhibited an inter-assay variation of 12%. It is worth noticing that all reagents can be prepared from stable commercial products within 24 hours.

Specificity of the antibody

We investigated the specificity of the single antibody used in this method by immunoprecipitation coupled to 2D-gel electrophoresis and mass spectrometry [Fig. 2 (A)]. 1 ml aliquots of two SFs of respectively high and low MATN3 concentrations as determined by ELISA were used in this experiment. 10 µg biotinylated antibody was added to each sample and incubated overnight. On the next day, 10 µl neutravidin beads (PIERCE) were added, incubated for 1 hour, rinsed three times in PBST and eluted in PBST containing 100 mM DTT. The eluates were subjected to 2D-electrophoresis and revealed by Coomassie staining. Spots were identified by mass spectrometry of tryptic peptides with high confidence scores. Several spots were observed on both gels. A few corresponded to proteins such as serum albumin and alpha-antitrypsin (not shown), which most likely bound non-specifically to the beads. Mouse immunoglobulin chains were also found, as expected [Fig. 2 (A)]. Finally, two spots were observed only from the sample with high MATN3 content and these two spots were identified as human MATN3, verifying that the antibody bound to MATN3 and not at comparable levels to other SF constituents.

Comparison in individual patients of the MATN3 content of SFs and sera

Because MATN3 is virtually specific to cartilage, its content would be presumed to be much higher in SF than serum. However, markers of cartilage metabolism have previously been found measurable in serum[8]. We compared concentrations of MATN3 measured from 10 SFs to those observed in 10 sera originating from the same 10 patients, respectively [Fig. 2 (B)]. In all cases, SF levels were higher than serum levels, as intuitively thought. The fold difference ranged approximately from 2 to 10. The patients who exhibited highest MATN3 levels in SFs also exhibited the highest levels in their sera, suggesting that elevated MATN3 in a joint may be evidenced from its serum levels.

All biological fluids used here were obtained from patients suffering from end-point knee osteoarthritis. As described by others[4], MATN3 levels in osteoarthritic articular cartilage continuously increase throughout progression of the disease. Therefore, the population used here for assay validation may exhibit particularly high MATN3 levels. However, a conclusion of our initial investigations of MATN3 levels in SFs from those patients is that the release of MATN3 in SF may not be a homogenous hallmark of osteoarthritis: among 15 patients, the fold difference between most distant concentrations was almost 50 (data not shown). Future investigations from much larger and better controlled populations[8] will determine whether the measurement of MATN3 levels in serum can be used to predict or follow the evolution of osteoarthritis. If so, the assay will be useful as it is achievable from serum, affordable and technically feasible.

Author contribution

JBV, PG, DM, FG, ACR, PN, JM designed experiments. PG, DM, FG, ACR provided biological samples. JBV, PG, DM performed experiments. JBV, PG, FG, ACR, PN, JM analyzed data. JBV, PG, DM, FG, ACR, PN, JM wrote the manuscript.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgements

This work was supported by Association pour la Recherche contre le Cancer, Ligue Régionale contre le Cancer, Fondation pour la Recherche Médicale, Région Lorraine, Institut National pour la Santé et la Recherche Médicale and the KHOALA osteoarthritis study group. The study was performed under the framework of Fédération de Recherche 3209 and of Contrat Hospitalier de Recherche Translationnelle (CHRT).

Supplementary material

Supplementary data related to this article can be found online at 10.1016/j.joca.2012.03.017

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