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Discovery and development of the N-terminal procollagen type II (NPII) biomarker: a tool for measuring collagen type II synthesis

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Summary

Objective: Progression of joint damage in osteoarthritis (OA) is likely to result from an imbalance between cartilage degradation and synthesis processes. Markers reflecting these two components appear to be promising in predicting the rate of OA progression. Both N- and C-terminal propeptides of type II collagen reflect the rates of collagen type II synthesis. The ability to quantify the procollagen peptides in biological fluids would enable a better understanding of OA disease pathology and provide means for assessing the proof of mechanism of anabolic disease modifying OA drugs (DMOADs).

Methods: A polyclonal antibody that recognizes the sequence GPKGQKGEPGDIKDI in the propeptide region of rat, dog, and human type II collagen was raised in chicken and peptide-affinity purified. The immunoaffinity liquid chromatography mass spectrometry (LC-MS/MS) was used to extensively characterize N-terminal procollagen type II (NPII) peptides found in biological fluids. The novel competition enzyme-linked immunosorbent assay (ELISA) assay was developed to quantitatively measure the NPII peptides.

Results: Several peptides ranging from 17 to 41 amino acids with various modifications including hydroxylations on proline and lysine residues, oxidation of lysines to allysines, and attachments of glucose and galactose moieties to hydroxylysines were identified in a simple system such as *ex vivo* cultures of human articular cartilage (HAC) explants as well as in more complex biological fluids such as human urine and plasma. A competitive ELISA assay has been developed and applied to urine, plasma, and synovial fluid matrices in human, rat and dog samples.

Conclusion: A novel NPII assay has been developed and applied to OA and normal human subjects to understand the changes in collagen type II synthesis related to the pathology of OA.

Key words: Biomarkers, Procollagen type II.

Articular cartilage has an important role in cushioning the joints in the skeleton. It is composed mainly of type II collagen and various proteoglycans including aggrecan. When type II collagen is synthesized by chondrocytes, the immature protein contains three extra domains: a signal peptide and N- and C-terminal propeptide domains. The signal sequence directs the protein to be secreted, whereas propeptide regions upon cleavage allow mature collagen molecules to be incorporated into the extracellular matrix. As these N- and C-terminal propeptides are released only during synthesis of the new molecules, their production is known to reflect the rate of type II collagen synthesis[1]. As these propeptides span from 87 to 245 amino acids for N- and C- terminal regions, respectively, there are several immunoassays to measure them depending on the epitope that was used to generate the antibody.

A homeostatic balance between matrix synthesis and degradation is maintained in healthy cartilage[1]. However, these metabolic processes are disrupted in degenerative joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA), resulting in a net increase in catabolism of matrix molecules, especially proteoglycans[2,3] and collagen type II[4,5], and eventually in loss of joint function.

Recently it has been discovered that in the early stages of OA, chondrocytes proliferate and synthesize more collagen molecules presumably to compensate for the loss of collagen type II due to the increased proteolytic activities. Synthesis of new type II collagen has been shown to be increased in OA patients as evaluated by the CPII assay that quantifies a C-terminal propeptide region within collagen type II molecule[6–10]. Another collagen synthesis biomarker, which is specific to the embryonic form of procollagen type II, was first identified by Aigner *et al.* and found to be synthesized by chondroprogenitor cells in developing tissues[11]. It is characterized by the presence of an extra cysteine-rich region in the N-terminus propeptide of type II collagen and termed PIIANP. Healthy adults do not appear to express PIIANP[11], but adults suffering from OA have been shown to re-express it. Rousseau *et al.*[12] developed an enzyme-linked immunosorbent assay (ELISA) assay using antibodies to type IIA procollagen amino terminal propeptide and showed that PIIANP is decreased in the plasma of knee OA and RA patients. Garnero *et al.*[13] proposed that disequilibrium between synthesis of collagen type II, as

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measured by the levels of PIIANP, and degradation of the articular cartilage, as measured by the levels of C-terminal telopeptides (CTX-II), represents a predictive marker of OA disease progression and severity. Patients who exhibited a greater degree of disease progression as determined by radiography had lower levels of PIIANP and higher levels of CTX-II. The combined analysis of these two biomarkers as the "uncoupling index" of cartilage turnover was more effective in predicting 1-year radiological progression in knee OA than the measurement of each single marker. However, the recent paper by Sharif *et al.*[14] shows that OA patients with serum levels of PIIANP in the highest quartile had a significantly higher risk of disease progression. As PIIANP data in OA progression studies is not always consistent, further understanding of the PIIANP data is needed before it can be fully utilized as a biomarker of anabolic activity. More recently, an assay for the adult type II collagen N-propeptide (PIINP) has been developed[15]. This PIINP biomarker, which recognizes the N-terminal region spanning from amino acid 30 to 43, demonstrated a decreased anabolic activity in RA subjects suggesting that not only PIIANP, but also PIINP might be regulated in OA disease[11–15].

Several markers that reflect collagen matrix degradation have been reported in the literature including the C-terminal telopeptides[16–18], collagen type II neoepitope peptides such as TIINE[19,20], C2C[4,5], and HELIX peptides[21] with some of them such as CTX-II being extensively characterized. However, markers that reflect the synthesis of collagen type II and its adult form, in particular including CPII and PIINP, are not characterized to the same extent. Specifically, the N-terminal collagen type II peptides are not well characterized in biological fluids. As several post-translational modifications (PTMs) of the propeptide region are known, it is unclear whether a published PIINP assay detects all PIINP peptides or only unmodified portion that binds to the antibody that was raised using unmodified sequence. Moreover, the N-terminal propeptide region is known to be processed by several enzymes including matrix metalloproteinases (MMPs)[22] resulting in potentially shorter fragments that may not be detected by the PIINP assay that utilizes an antibody generated by Olsen *et al.*[15] to the region starting only at position 29 from the N-terminus.

Using the adult form of procollagen type II as a biomarker of type II collagen synthesis will enable development of disease modifying OA drug (DMOAD) anabolic therapies. As there are numbers of regulatory factors and anabolic mediators, e.g., osteogenic protein 1 (OP-1), which may have therapeutic potential as anabolic DMOADs, there is a need for biomarkers capable of measuring matrix synthesis that would facilitate testing proof of mechanism of these drugs in clinic.

This paper presents a description of the methods used for the identification and characterization of the N-terminal procollagen type II (NPII) peptides from various biological fluids including urine and plasma as well as simple *ex vivo* system of human articular cartilage (HAC) explants. The sequences of specific peptides that result from processing of the NPII which are secreted into systemic biofluids as well as PTMs of these peptides are described. Moreover, this paper describes the development and application of the ELISA to measure both modified and unmodified peptides as well as peptides of varying length starting from the N-terminus in various biological fluids. An application of the NPII biomarker to cross-sectional human OA and non-OA (control subjects) samples provided a preliminary indication of decreased adult collagen type II synthesis in subjects with symptomatic OA.

Materials and methods

PEPTIDES FOR ANTIBODY GENERATION

The NPII standard peptide sequence CZ-GPKGQKGEPGDIKDI, where Z represents a proprietary to Aves Labs, Inc. (Tigard, OR, USA) linker was synthesized by Aves. Biotin-GPKGQKGEPGDIKDI was synthesized in house using solid-phase peptide synthesis technology. Mass spectroscopy and quantitative HPLC (high performance liquid chromatography) were performed on all peptides to confirm the purity and fidelity of these peptides.

ANTIBODY PRODUCTION AND PURIFICATION

Polyclonal antibody production was completed by Aves Labs, Inc. (Tigard, OR, USA). Two hens were immunized with keyhole limpet hemocyanin (KLH) conjugated to the standard peptide. After a total of four injections, immune eggs were collected and following a lipid extraction, the IgY fraction was peptide-affinity purified from the yolks. Egg yolks contained approximately 25 mg of IgY per ml.

EX *VIVO* INDUCTION OF CARTILAGE DEGRADATION

Articular cartilage was dissected from a knee obtained from a patient undergoing knee replacement (65-year-old female). Visually normal (i.e., not fibrillated) cartilage was cut into small pieces (~2 mm) and cultured in 96-well plates with 200 μl of dulbecco/vogt modified eagle's minimal essential medium (DMEM) media (Gibco BRL high glucose, 25 mM Hepes, containing 2 mM L-glutamine and 1 mM sodium pyruvate) freshly supplemented with 1× hybridoma lymphocytes-1 (HL-1) (Bio Whitaker) and 5 μg/ml ascorbic acid (Sigma), containing 0.1 ng/ml interleukin (IL)-1β (R&D) + 50 μg/ml oncostatin M (R&D) or vehicle as described in Ref. 4. Media was replaced every 3–5 days and cartilage was cultured for a total of 22 days. Supernatants at day 3 were frozen and stored at −70°C prior to LC-MS/MS analysis for procollagen type II peptides.

IMMUNOAFFINITY COLUMN PREPARATION

On-line immobilized antibody (immunoaffinity) columns were prepared as described in Ref. 17. Briefly, the antibody was immobilized onto a Poros™ Epoxide immunodetection cartridge (Applied Biosystems, Framingham, MA, USA). The cartridge was equilibrated with 0.5 M Na_2SO_4, 0.1 M sodium phosphate (pH 8.8) at 1 ml/min. The 1.5 M Na_2SO_4, 0.1 M sodium phosphate solution was added until the A_{280} was less than 10% of the maximum A_{280} and re-circulated for another 30 min. The column was then removed and incubated at room temperature for 24–72 h. After incubation, the remaining epoxide sites were blocked with 0.2 M Tris base (pH 10.5) at room temperature for 2 h.

PEPTIDE IDENTIFICATION BY LC-MS/MS

Samples were analyzed on a quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters, Beverly, MA, USA) coupled to a CapLC (Waters, Milford, MA, USA) system equipped with an autosampler, gradient and auxiliary pump. The peptides were separated on a Pepmap column (75 μm × 150 mm, 3 μm particle) (LC Packings, San Francisco, CA, USA) prior to introduction into the mass spectrometer. Separation was achieved using a 50 min linear gradient of 95% H_2O, 0.1% HCOOH to 95% MeCN, 0.1% HCOOH.

Data dependent MS/MS experiments were performed using a modified nanospray source designed to hold a distally coated picotip (360 μm OD × 20 μm ID × 10 μm tip diameter) (New Objective, Cambridge, MA, USA). The data was processed by ProteinLynx version 3.5 (Waters, Beverly, MA, USA) to generate searchable.pkl files. The data was searched against NCBI database with no enzyme specificity to identify endogenously produced peptides. Several variable modifications including methionine oxidation and proline or lysine hydroxylation were added to the search parameters. All identifications of peptides exceeded the significant homology threshold with some identifications exceeding identity threshold.

ELISA PROCEDURE

Nunc 96-well flat-bottom MaxiSorp microtiter ELISA plates (Nalge Nunc International, Roskilde, Denmark) were pre-coated with peptide-affinity purified IgY (2 μg/ml) in 0.1 N $NaHCO_3$ buffer pH 8.2 for 18 h at room temperature and 100% humidity. Unbound antibody was removed and the un-reactive sites on the plates were blocked with blocking buffer (3% protease-free bovine serum albumin (BSA), 0.05% Tween-20, 0.02% thimerosal, added to dulbecco's phosphate-buffered saline (DPBS) with pH adjusted to 7.4) for 1 h, 37°C and 100% humidity, followed by three washes with 0.05% Tween-20 in normal saline. NPII standard was prepared in triplicates by diluting a 1 mg/ml stock

solution in ELISA assay buffer (0.1% BSA (protease-free), 0.05% Tween-20, 0.02% thimerosal, added to DPBS, pH adjusted to 7.4) at six concentrations: 128 ng/ml, 32 ng/ml, 8 ng/ml, 2 ng/ml, 0.5 ng/ml and 0.125 ng/ml final concentration. This was accomplished by making a 2× upper standard at 256 ng/ml and performing 1:4 serial dilutions in the assay plates. Biotin-labeled standard was prepared at a 2× concentration of 1 ng/ml by diluting a 1 mg/ml stock of biotin–peptide in ELISA assay buffer. Total assay volume equaled 150 μl/well. For maximum binding (Bo) control, three wells received biotin label without standard. Biotinylated NPII peptide was allowed to compete with standard peptide or unknown samples for 2 h at room temperature. Unbound NPII is removed and biotinylated peptide is detected with horseradish peroxidase (HRP) conjugated anti-biotin and developed using tetramethylbenzidine (TMB) substrate. The absorbance of the resultant blue color was read at 650 nm and was inversely proportional to bound NPII concentration in the sample.

SAMPLES

Urine and plasma samples were collected from men and women over 55 years of age in polypropylene tubes, aliquoted, and stored at −70°C until sample analysis. Typically, 100 μl of urine was aliquoted separately for measurement of creatinine. All subjects were identified for the presence of joint pain and radiographic signs of OA (KL $\geq$ 1). Two groups of subjects were determined including symptomatic OA patients with radiographic OA of the hip(s) and/or knee(s) (n = 66) and asymptomatic subjects with either radiographic OA of the hand(s)/spine (n = 17) (i.e., cervical and lower lumbar) or no radiographic signs of OA (non-OA) in the knee(s)/hip(s)/hand(s)/spine (n = 12). The latter group is referred to as a reference control group.

Results

NPII ANTIBODY CHARACTERIZATION

The collagen type II molecule contains two propeptide regions: N- and C-terminal. The N-terminal region of collagen type II is 87 amino acids long and contains GXP or GXK motifs, whereas the C-terminal propeptide region spans 245 amino acids and does not contain GXP/GXK motifs (Table I). The 15-amino acid region GPKGQKGEPGDIKDI in the N-terminal propeptide region (Table I, underlined sequence) was selected for polyclonal antibody generation. This sequence is unique to collagen type II and conserved among human and most widely used preclinical species such as rat and dog. Polyclonal antibodies that recognize GPKGQKGEPGDIKDI were developed in chicken and affinity purified using a standard peptide. Online immobilization of the NPII antibody onto porous support using epoxide chemistry resulted in a high capacity immunoaffinity column that was used to pull-down NPII peptides from various fluids.

IDENTIFICATION AND CHARACTERIZATION OF NPII PEPTIDES

HAC explants after 3 days of stimulation with IL-1β and oncostatin M were used for the identification of the NPII peptides. In addition, urine and plasma from adult human subjects were used to identify NPII peptides. For this, 50 μl of HAC supernatant, 0.3 ml of human plasma, and 1 ml of human urine were passed through NPII antibody column and eluted with low pH solvent to disrupt antibody–antigen interactions. Several peptides of various lengths were detected in the HAC supernatants, human plasma and urine (Table II). An example of LC-MS spectra of QDVRQPGPKGQKGEPGDIKD peptide that was observed in all three samples is shown in the Fig. 1(A–C). Both triply and quadruply charged ions of the peptide with various modifications have been observed. The difference between major peaks was 40.5 and 54 m/z for quadruply and triply charged ions, respectively, corresponding to the series of 162 Da differences in molecular weight (MW) of the peptide. This difference was attributed to the sugar moiety attached to the peptide sequence and based on the unique mass of 162 was ascribed to be a hexose. Up to four hexose moieties within the peptide sequence were observed. Moreover, for many of the detected peptides, shifts in 4.0 and 5.3 m/z for quadruple and triple charge states, respectively, were detected. These shifts resulted in a 16 Da increase in the MW of the peptide which for the collagen molecule has been known to be due to hydroxylation of either proline and lysine residues.

As a result of the immunoaffinity pull-down experiments several peptides that varied in length, hydroxylation and sugar attachment patterns were identified (Table II). All identified peptides contained the N-terminal sequence corresponding to the beginning of the N-terminus, whereas their C-terminal ends varied depending on the processing site. The C-terminal ends of two major peptides had an aspartic acid at positions 17 and 20 from the N-terminus, respectively. These two cleavages after the aspartic acid residue are reported to be initiated by the members of the MMP enzyme family[20]. Additional C-terminal processing sites included the lysine residue at position 19, glycine residues at positions 34 and 40, and the proline residue at position 29. Each peptide was found to be present with one, two or up to three hydroxylations and up to four glycosylation states. Furthermore, upon detailed investigation of the LC-MS spectrum of the QDVRQPGPKGQKGEPGDIKD peptide it was found that its calculated MW was one mass unit less than theoretical. Utilizing LC-MS/MS capabilities, we found that a modification on the lysine residue arising from the oxidation of the side chain producing allysine was contributing to the decrease in the MW of the peptide by one. Manual interpretation of the MS/MS spectrum of QDVRQPGPKGQKGEPGDIKD provided evidence that the lysine residue at the ninth position from the N-terminus was modified to allysine [Fig. 1(D)]. The difference between singly charged product ions at m/z 1005.52 and 877.4 resulted in the loss of 128 from the QDVRQPGP (aK), where aK stands for allysine, ion that corresponded to the loss of allysine. Moreover, digestion of the immunoaffinity isolated peptide from the human urine with LysC enzyme resulted in cleavages only after lysine residues in positions 19 and 24 from the N-terminus, whereas no cleavages were observed after lysine residues in positions 9 and 12. This suggests that both lysines in 9 and 12 positions are modified and resistant to the enzymatic digestion (Table II). This finding is consistent with literature reports suggesting that hydroxylysines are typically further modified by O-linked glycosylation to give ((Glcα1–2)Galβ1-O-Lys)[23].

NPII ELISA ASSAY DEVELOPMENT

A novel competition ELISA for detection of human procollagen (NPII) was developed. The assay is based on competition

Table I
Sequence of human procollagen type II

QDVRQPGPKGQKGEPGDIKDI*VGPKGPPGPQGPAGEQGPRGDRGDKGEKGAPGPRGRDGEPGTLGNPGPPGPPGPPGPPGLGGNFAA

*Underlined sequence was used to raise an antibody.

Table II
Peptides and their PTMs identified in HAC, human urine, and plasma using NPII antibody

QDVRQPGPKGQKGEPGD	1−2OH/1−4 Hex*
QDVRQPGPKGQKGEPGDIK	1−2OH/1−4 Hex
QDVRQPGPKGQKGEPGDIKD	2−3OH/1−4 Hex*
QDVRQPGPKGQKGEPGDIKDIVGPKGPPGP	2OH/1−4 Hex
QDVRQPGPKGQKGEPGDIKDIVGPKGPPGPQGPAG	2OH/1−4 Hex
QDVRQPGPKGQKGEPGDIKDIVGPKGPPGPQGPAGEQGPRG	2OH/1−4 Hex

*The most abundant peptides.

of standard or sample NPII with biotin-labeled NPII peptide for IgY (chicken anti-NPII peptide) coated on a 96-well microtiter plate. Standard peptide CZ-GPKGQKGEPGDIKDI used in the ELISA assay contains an amino acid sequence that has been identified to be a part of the most abundant peptides identified-by LC-MS. The concentrations of standard, label and sample dilutions were predetermined by checkerboard ELISA and diluted in 0.1% BSA−PBST, pH 7.4. To establish a standard curve, NPII peptide was diluted fourfold in a concentration range between 0.125 and 128 ng/ml. Following an incubation period of 2 h at 37°C, plates were washed four times and incubated for 1 h with 150 μl/well HRP anti-biotin which was diluted 1:10,000 in PBST. The color reaction was developed by adding 150 μl/well peroxidase substrate

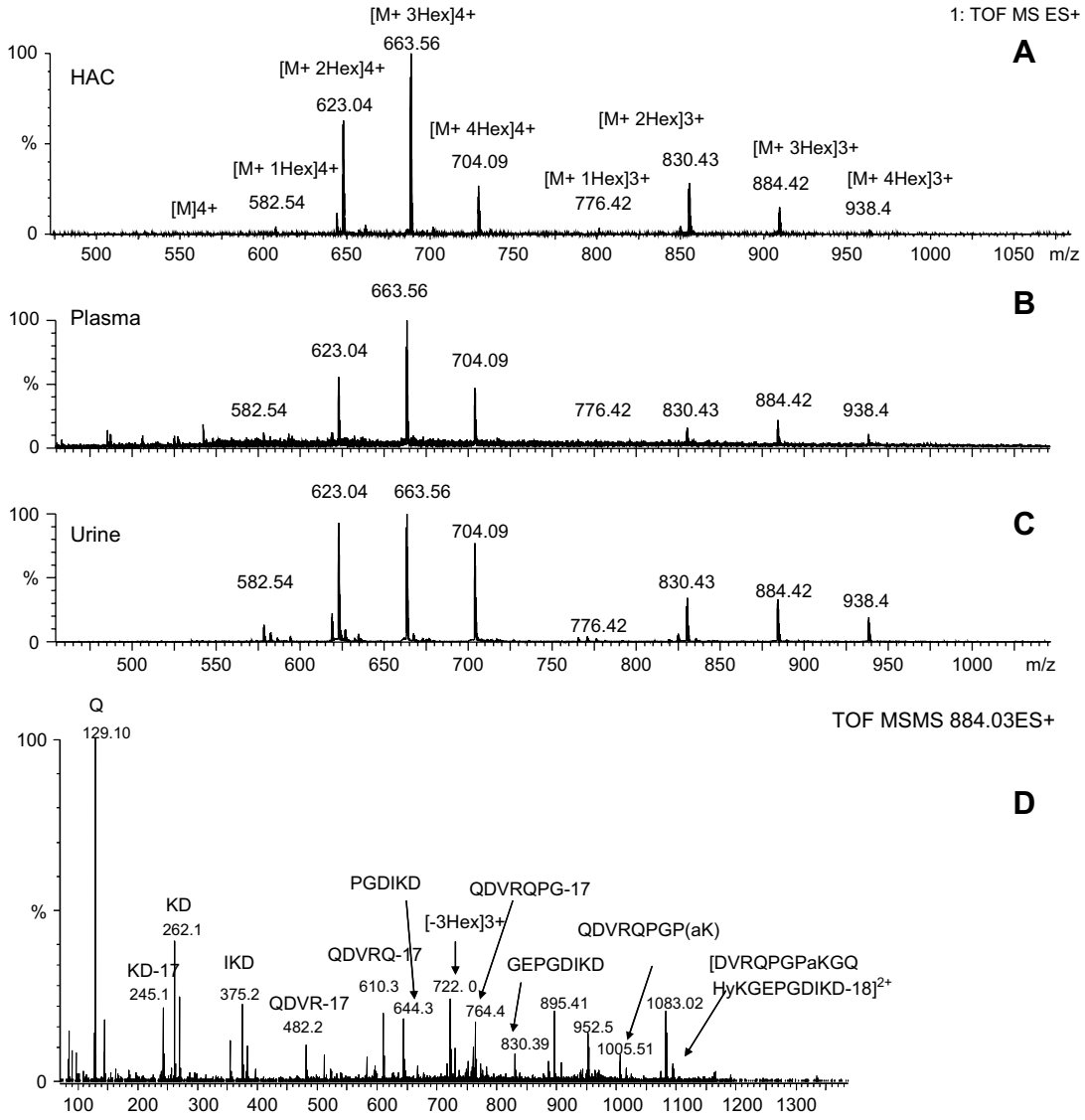

Fig. 1. LC-MS spectra of the NPII fragments. Each spectrum represents one peak in the reverse phase-high performance liquid chromatogram (RP-HPLC) obtained by immunoaffinity pull-down of the procollagen II peptides from (A) HAC, (B) plasma, and (C) urine samples. Different charge states for various forms of the QDVRQPGPKGQKGEPGDIKD peptide with up to four hexose moieties were detected. (D) An MS/MS spectrum of the QDVRQPGP(aK)GQ(HyK)GEPGDIKD with three Hex moieties, m/z 884.03 for the triple charged parent ion. Both N- and C-terminal fragment ions were observed allowing for manual sequence identification. In addition, a facile loss of sugar moieties was observed.

(2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)). Each run of the assay included data for a single standard curve across a range of concentrations, in duplicate.

Intra-assay precision was determined by testing three replicates of twofold dilutions (seven total dilutions) of a positive control (NPII peptide spiked into human plasma or urine) on the same day. The results for each assay are reported as percent coefficient of variations (CVs) of the endpoint titer and the range for all dilution points are also reported in the table below (Table III).

For the NPII ELISA, the limit of detection (LOD) was established to be 0.1 ng/ml. The lower limit of quantification (LLOQ) is the lowest amount of NPII peptide in a sample that can be quantitatively determined with suitable precision and accuracy. The LLOQ for the NPII ELISA was 0.5 ng/ml in either human plasma or urine. The upper limit of quantification (ULOQ) was 128 ng/ml.

APPLICATIONS OF THE NPII ASSAY

The NPII ELISA assay was utilized to measure the levels of the NPII peptides in various biofluids and to understand its variability in various species and feasibility for future applications. The basal levels for plasma and urine were measured in healthy human, dog, and rat subjects (Table III). In all three species, the levels in urine were higher than in plasma approximately two-fold to three-fold. NPII was also detected in synovial fluid lavage (about 10-fold diluted with PBS) at 257.7 ± 71.2 ng/ml.

Table III
*Summary of NPII assay validation results and levels of NPII peptides detected in the urine, plasma or synovial fluid of healthy human, dog, and rat subjects**

Analyte	NPII peptide	NPII peptide
Species	Human	Human
Matrix	Plasma	Urine
MRD*	1:16 final	1:16 final
Sample volume, μl	75	75
Method type	Competition ELISA	Competition ELISA
Linearity	Four orders of magnitude	Four orders of magnitude
Sensitivity (LOD)*, ng/ml	~0.1	~0.1
Sensitivity (LLOQ), ng/ml	0.5	0.5
Range (ULOQ), ng/ml	128	128
Intra-assay precision (%CV*), ($n = 3$)	0.5–9	3–11
Mean % recovery	95.30	108.80*

Species	*n*	Fluid	NPII (ng/ml) (mean ± SD)
Human	29	Plasma	17.45 ± 8.95
	6	Urine	36.8 ± 33.2
Dog	20	Plasma	4.14 ± 5.53
	4	Urine	9.43 ± 6.59
Rat	2	Plasma	40.2 + 4.3
	8	Urine	125.3 ± 24.5

Human reference control subjects were 55–70 years old males and females. Dogs were mixed-breed of gray labs with hounds and ranged in weight from 20 to 30 kg. Rats were 180–200 g Lewis male rats.

*MRD - minimum required dilution; LOD - lower limit of detection; CV - coefficient of variation.

The assay was subsequently used to evaluate the difference in levels of the NPII biomarker between age and sex matched reference non-OA subjects and patients with both confirmed radiographic OA in the hip(s) and/or knee(s) and symptomatic disease. The mean value for plasma NPII in the reference control group was 17.45 ng/ml ($n = 29$), whereas the mean value for the symptomatic OA group was 3.25 ng/ml ($n = 22$). An almost fivefold decrease in the NPII levels in the symptomatic OA group was detected. The mean NPII levels in the symptomatic OA group were statistically significant lower as compared to the mean NPII values in the reference controls ($P < 0.0001$) (Fig. 2).

Discussion

Imbalance between degradative and reparative processes characterized by loss of cartilage matrix molecules and their synthesis has been implicated in OA. Development of biomarkers that will allow the quantitative measurement of the net loss of cartilage by assessing both rate of cartilage degradation and synthesis will tremendously aid the understanding of OA disease pathology.

In this report, we describe the development of a polyclonal antibody that recognizes the N-terminal propeptides from amino acid in position 7 to 21. This antibody was used to immunoaffinity capture and enrich for peptides from HAC, urine and plasma, thus making the process of peptide identification feasible.

Several peptides from the N-terminal region of collagen type II were identified. None of the peptides contained a cysteine-rich region as described by Sandell to be characteristic of the embryonic type IIA form of type II collagen. All of the peptides matched sequences reported to be derived from the adult procollagen type II even though the sequence used to generate the antibody is also found in the embryonic procollagen type IIA form. These findings suggest that embryonic form was not abundant in healthy reference control subjects or was too long to be detected by the mass spectrometry (MS) technology. We did not attempt to

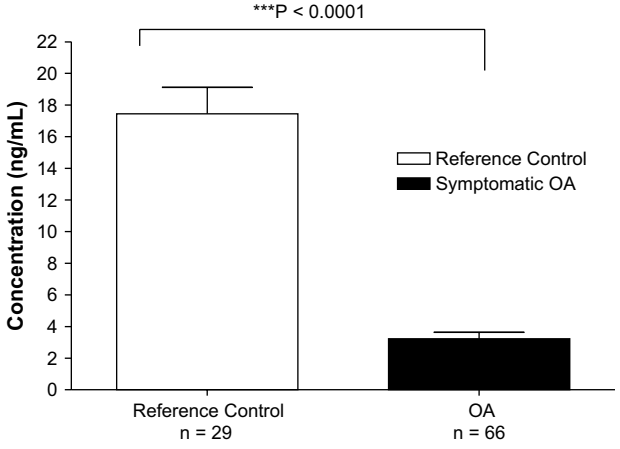

Fig. 2. Cross-sectional evaluation of the NPII levels in the plasma of reference control and OA patients. Statistically significant (***$P < 0.0001$) difference was observed between symptomatic OA patients with radiographic OA in the hip(s) and/or knee(s) and a reference control group consisting of healthy volunteers without symptoms and radiographic OA in the hip(s) and/or knee(s); a subset of the first group ($n = 17$) was presented with radiographic OA in the smaller joints such as hand(s) and/or spine.

isolate peptides from the OA subjects. Moreover, all identified peptides contained the N-terminus starting with residues QDVR… that corresponds to the beginning of the N-terminal region of procollagen type II. However, the C-termini of the peptides were different. Among several identified peptides only two peptides have at least partial epitope that would have been recognized by the PIINP antibody. This suggests that the pool of peptides that the NPII assay measures is different than that of PIINP. All identified peptides were heavily modified by hydroxylations at proline and lysine residues as well as glycosylated by glucose and galactose at hydroxylysine residues. The lysine residue at position 9 was also identified to be modified to allysine residue by the oxidation of the side chain amino group to aldehyde moiety. It is important to note that even though antibody was generated to the sequence of type II collagen propeptide only, it was found to bind both unmodified and post-translationally modified peptides. This suggests that our antibody recognizes all of the expected peptides regardless of the modification and not just a small subset of them.

For the development of the quantitative assay, two considerations were given: LC-MS/MS and ELISA assays. An LC-MS/MS assay typically is developed for a single most abundant peptide representing the region of the molecule, whereas an ELISA assay measures all peptides that have immunoreactivity to the peptide sequence allowing for the quantification of the total signal coming from the region of the molecule. Resulting from our experience of developing a quantitative immunoaffinity LC-MS/MS assay to measure collagen type II 45-mer peptide[17], we considered applying a similar approach for the measurement of the most abundant NPII peptide utilizing a unique combination of the immuno-affinity and LC-MS/MS technology. However, due to the complexity of identified peptides and difficulties making synthetic standards with hydroxylysine, allysine and complex sugar moieties, no attempts were made to develop an LC-MS/MS assay. For the quantification of the N-terminal collagen type II peptides, a competitive ELISA assay was chosen as it will provide a means of detecting all of the various forms of NPII peptides. This technique is based on immunological detection and quantitation of a single or multiple molecular species in biological samples. To achieve robust assay performance, key assay parameters such as reagent concentrations, volumes, dilution factors, assay incubation times, and temperatures were optimized. Among many key assay parameters, establishing the most optimum dilution factor at which no matrix interferences are observed while still measuring a robust signal was the most challenging step. We found that it was necessary to dilute both urine and plasma samples 16-fold to minimize matrix interference with analyte recovery ranging from 95 to 109%. In addition, intra-assay precision as measured by the %CV has been established to be less than 15% for both plasma and urinary assays.

Basal levels of NPII were measured in urine and plasma samples from human subjects and other preclinical species such as rat and dog. A common trend showing higher levels of the NPII in urine as compared to plasma was observed across all species (2–3 fold). This suggests that the small, polar NPII peptides concentrate in urine once cleared through the glomerulus. The NPII peptides were also detected in the synovial fluid lavage, at least in rat samples, as other species synovial fluid samples were not available at the time of testing. The ability to apply the same assay to measure NPII peptides across many species including human subjects allows for the translation of the NPII biomarker from preclinical to clinical studies.

Inter-subject variability for NPII was evaluated and shown to be greater than 50% CV for 29 reference control subjects. This indicates that clinical studies using NPII as biomarker will have to be appropriately powered with enough subjects to achieve statistical significance. Utilizing the NPII assay to analyze cross-sectional human OA and non-OA reference control samples demonstrated a significant difference in plasma NPII levels between reference control subjects and patients exhibiting both symptoms and structural changes of OA. A comparable difference between PIINP levels in healthy controls and RA subjects was reported by Olsen *et al.*[15] suggesting that decreased circulating levels of the NPII peptides are consistent with reduced anabolic activity of the articular cartilage in various joint diseases such as RA and symptomatic OA.

Conflict of interest

There are no real or potential conflicts of interest.

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