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Development and Clinical Application in Arthritis of a New Immunoassay for Serum Type IIA Procollagen NH2 Propeptide

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Summary

Type II collagen, the most abundant protein of cartilage matrix, is synthesized as a procollagen molecule including the N-(PIINP) and C-(PIICP) propeptides at each end. Type II procollagen is produced in two forms as the result of alternative RNA splicing. One form (IIA) includes and the other form (IIB) excludes a 69-amino acid cysteine-rich globular domain encoded by exon 2 in PIINP. During the process of synthesis, these N-propeptides are removed by specific proteases and released in the circulation, and their levels are believed to reflect type II collagen synthesis. In this chapter we describe the development of a specific enzyme-linked immunosorbent assay (ELISA) for the measurement of the IIA form of PIINP (PIIANP) in serum based on a polyclonal antibody raised against recombinant human exon 2 fusion protein of type II procollagen. We show that this ELISA is highly specific for circulating PIIANP and has adequate technical precision. In patients with knee osteoarthritis and rheumatoid arthritis, serum PIIANP was decreased by 53% ($p < 0.0001$) and 35% ($p < 0.001$), respectively, suggesting that type IIA collagen synthesis is altered in these arthritic diseases. The measurement of serum PIIANP may be useful for the clinical investigation of patients with joint diseases.

Key Words: Type II collagen; N-propeptide; joint disease; osteoarthritis; rheumatoid arthritis; cartilage; biological marker; ELISA.

1. Introduction

The hallmark of osteoarthritis (OA), the most common joint disease, is cartilage loss leading to joint destruction. Molecular markers are molecules or fragments thereof of connective tissue matrices that are released into biological fluids during the process of tissue biosynthesis and turnover and that can be measured by immunoassays. Several molecular markers of bone, cartilage, and synovium have been described, and their changes have been investigated in

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patients with OA, mainly in cross-sectional studies (for a review, *see* **ref. *1***). Because the loss of cartilage is believed to result from the combination of a decreased reparative process coupled with an increased degradative phenomenon, thereby limiting the capacity of cartilage repair, and because type II collagen is the most abundant protein of cartilage matrix, the assessment of type II collagen synthesis and degradation is an attractive approach for the investigation of OA. After many years of unsuccessful research, the recent development of assays specific for type II collagen breakdown is probably a breakthrough in the field of biological markers for OA, given that degradation of collagen fibers is associated with irreversible cartilage destruction ***(2–5)***. Conversely, there is still a lack of a specific and sensitive biological marker reflecting the rate of type II collagen synthesis.

Type II collagen is synthesized as a procollagen molecule including the N-(PIINP) and C-(PIICP) propeptides at each end. Type II procollagen is produced in two forms as the result of alternative RNA splicing. One form (IIA) includes and the other form (IIB) excludes a 69-amino acid cysteine-rich globular domain encoded by exon 2 in the PIINP (**Fig. 1**). During the process of synthesis, these N-propeptides are removed by specific proteases before the mature molecules are incorporated into fibrils ***(6)***. Theoretically, serum levels of these propeptides can be used as specific markers of collagen synthesis. This IIA form is known to be expressed in skeletal progenitor cells and noncartilaginous tissues in embryos but to be absent from mature and normal cartilage, in which IIB is known to be expressed ***(7–10)***. Aigner et al. ***(11)*** showed the re-expression of type IIA collagen by adult articular chondrocytes in OA disease. This re-expression may contribute to the reported increase of mRNA and protein expression of type II collagen during OA disease ***(12–16)*** but also implies a significant modulation of chondrocyte phenotype. Together, these results suggest that the N-propeptide IIA could be a specific marker of OA disease, reflecting the synthesis of IIA collagen. Currently, no immunoassays have been developed allowing the measurement of N-propeptide IIA. This chapter describes the development of an immunoassay for serum N-propeptide IIA, the characterization of circulating immunoreactive forms detected by this assay, and preliminary clinical evaluation in arthritis.

2. Materials

2.1. Materials for Immunoassay

1. Maxisorp microtiter plates (Nunc, Roskilde, Denmark).
2. Bovine serum albumin (BSA), radioimmunoassay (RIA) grade (Sigma, St Louis, MO).
3. Tween-20 (Sigma).

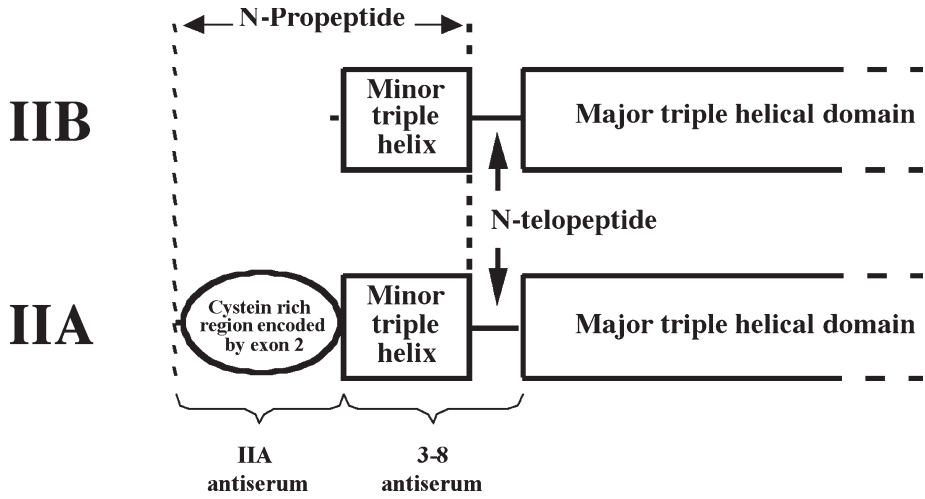

Fig. 1. Schematic reprensentation of type IIA/IIB procollagen. Type II collagen is expressed in two forms of IIA and IIB, as the result of alternative splicing of exon 2 in the NH2-propeptide region. IIA polyclonal antibodies specifically recognize IIA N-propeptide, and exon 3–8 polyclonal antibodies recognize the two forms (IIA and IIB) of the N-propeptide.

4. Phosphate-buffered saline (PBS), pH 7.4.
5. Anti-rabbit IgG peroxidase conjugate (Sigma).
6. H_2O_2 and tetramethylbenzidine substrate indicator solution (Sigma).
7. H_2SO_4 (VWR, Gibbstown, NJ).
8. Dynatech microtiter plate reader MR 7000 (Labsystems, Vantaa, Finland).

2.2. Materials for Specificity Characterization

1. 5% Tris/tricine gel electrophoresis and Western blot equipment.
2. Nitrocellulose membrane (Hybond ECL, Amersham, Buckinghamshire, England).
3. Polyclonal anti-von Willebrand factor (Dako, Carpinteria, CA).
4. Polyclonal antithrombospondin (NeoMarkers, Fremont, CA).
5. Polyclonal anti-GST–exon 2 recombinant protein (IIA antiserum).
6. Polyclonal anti-GST–exon 3–8 recombinant protein (3–8 antiserum, developed in chicken).
7. Rabbit nonimmune serum.
8. Goat antirabbit horseradish peroxidase (HRP) conjugate (Jackson Immuno-Research, West Grove, PA).
9. Antichicken IgG (whole molecule) peroxidase conjugate (Sigma).
10. Western Lightning Chemiluminescence reagent (Perkin Elmer, Boston, MA).
11. BioMax MS-1, Kodak film (Sigma).

2.3. Recombinant Protein and Antibodies

These have been previously described in **refs. *11*** and ***17***.

1. Recombinant fusion protein GST-exon 2 obtained from Dr. L. Sandell's laboratory (St. Louis, MO).
2. Polyclonal antibodies anti-IIA and anti-exon 3–8 obtained from Dr. L. Sandell's laboratory.

3. Methods

3.1. ELISA Technique for Serum PIIANP

1. Incubate each microtiter plate well overnight at 4°C with 100 µL of recombinant fusion GST-exon 2 protein (10 ng/mL) in PBS. Do not shake.
2. Empty well contents and tap the plate firmly two to three times on absorbent paper.
3. Saturate for 2 h at room temperature with PBS, BSA 1% on a microwell plate rotation apparatus (gentle agitation).
4. Wash microwells with 200 µL of PBS, 1% BSA, 0.05% Tween-20.
5. Repeat three times and check that there is no residual washing solution in wells after each washing cycle. Repeat washes after each step of the protocol.
6. Dispense 100 µL/well of standards (recombinant fusion protein GST-exon 2; 0 [PBS/BSA/Tween], 2.5, 5, 7.5, 10, 15, 20, 30, 40 ng/mL) or serum (samples and controls). Serum must be centrifuged for 15 min at 4°C (14,000*g*) before use.
7. Add 100 µL/well of IIA antiserum (1:1650 final) and incubate for 4 h at room temperature on a microwell plate rotation apparatus (40 rpm).
8. Add the peroxidase-conjugated antirabbit antibodies (diluted 1:8000; 100 µL/well) and incubate at room temperature for 1 h on a microwell plate rotation apparatus (40 rpm).
9. Incubate at room temperature with 100 µL/well H_2O_2/tetramethylbenzidine substrate indicator solution (one tablet in 10 mL of 0.05 *M* phosphate citrate buffer, pH 5.0 with 2 µL H_2O_2) on a microwell plate rotation apparatus (40 rpm).
10. After 30 min, stop the color reaction by addition of 100 µL 2 *M* H_2SO_4/well.
11. Read the optical density at 450 nm with a Dynatech Reader.

3.2. Characterization of Antigens Recognized by Antiserum IIA

1. Select a pool of sera from healthy donors and dilute this pool 10 times in PBS buffer.
2. Boil the samples for 5 min and resolve them (15 µL/well) in a 5% Tris/tricine gel under reducing conditions.
3. Transfer overnight (50 V) in 100 m*M* CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), 5% methanol on a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech).
4. Saturate the membranes for 1 h with 5% nonfat milk in PBS with shaking.
5. Wash the membranes 4 × 10 min in PBS, 0.05% Tween-20.

6. Incubate with primary antibodies overnight at 4°C with shaking (IIA antiserum, 1:400; 3–8 antiserum, 1:500; nonimmune serum, 1:400; anti-von Willebrand factor, 1:400; antithrombospondin, 1:400).
7. Wash the membranes 4 × 10 min in PBS, 0.05% Tween-20.
8. Incubate with the specific secondary antibodies (anti-rabbit 1:3000 or anti-chicken 1:20,000) for 1 h at room temperature with shaking.
9. Wash the membranes 4 × 10 min in PBS, 0.05% Tween-20.
10. Use the Perkin Elmer kit for the revelation of the membranes.

3.3. Populations Used in the Study

1. Healthy subjects. Reference values were obtained from 67 women ranging in age from 31 yr to 75 yr (mean age: 53 ± 13 yr) and 21 men ranging in age from 53 yr to 77 yr (mean age: 63.4 ± 6.9 yr). All belonged to large population-based cohorts involved in prospective studies on the determinants of bone loss in women (OFELY [Os des Femmes de Lyon] study) ***(18)*** and men (MINOS study) ***(19)***. The cohort of the OFELY study comprises 1039 healthy white female volunteers (31–89 yr of age), and the cohort of the MINOS study comprises 842 healthy white male volunteers (50–85 yr of age). All healthy subjects came from the same region of France as the patients with knee OA and rheumatoid arthritis (RA). None of the control subjects had evidence of OA as assessed by clinical examination, questionnaire, and X-ray films of the thoracic and lumbar spine, and all subjects were without disease or treatment that could interfere with bone or joint metabolism including hormone replacement therapy in postmenopausal women.
2. Osteoarthritis patients. The study group included 43 outpatients suffering from knee OA ranging in age from 41 yr to 83 yr (23 women and 20 men; median age: 62.6 ± 9.6 yr). They consulted at the Department of Rheumatology of the Centre Hospitalier Lyon Sud (Lyon, France). All patients fulfilled the American College of Rheumatology criteria for primary knee OA ***(20)***. All patients had had chronic daily pain of the knee for at least 3 mo (median: 10 yr) and also had radiographical evidence of OA with joint space narrowing when using the posteroanterior view of the knees flexed at 30° (Schuss view). Patients presenting with an advanced stage of OA with a minimum joint space width of less than 1 mm were excluded. All women were postmenopausal and all patients were without treatment that could interfere with bone metabolism including estrogen replacement therapy, dilantin, thyroid replacement therapy, and diuretics. Pain and physical function were assessed by the Western Ontario and McMasters Universities multifunctional (WOMAC) index ***(21)***, using a visual analog scale as a grading system. A subset of 53 healthy age and sex-matched subjects (32 women and 21 men, mean age: 62.4 ± 7.7 yr) was used as a control for patients with knee OA.
3. Rheumatoid arthritis patients. We investigated 66 women who met the American College of Rheumatology criteria for RA disease revised in 1987 ***(22)*** ranging in age from 15 to 84 yr (median age: 54 ± 16 yr; median disease duration: 6 yr). Twenty-four were on low-dose corticosteroids (<10 mg/d prednisone). Patients

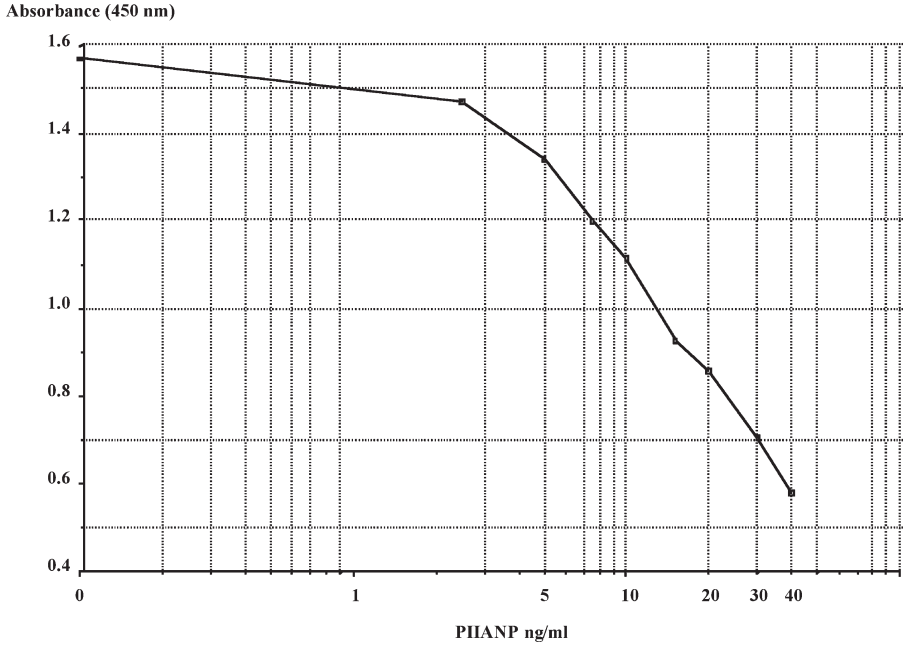

Fig. 2. Standard curve produced by duplicate measurements of the mean optical density at 450 nm of nine PIIANP standards.

with renal insufficiency were excluded. A subset of 67 healthy age-matched women (mean age: 53 ± 13 yr) was used as controls for patients with RA.

3.4. Statistical Analysis

Comparison of serum PIIANP levels between healthy controls and patients with OA or RA was assessed by the nonparametric Mann-Whitney U test.

3.5. Analytical Performances of PIIANP ELISA

A typical standard curve using the recombinant fusion protein GST-exon 2 as a standard is shown on **Fig. 2**. The competitive polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) demonstrated adequate intra- and interassay precisions (coefficient of variation [CV] <10%) and dilution recovery (**Tables 1–3**). The detection limit, defined as the concentration 2 SDs above that of the lowest calibrator, was 1.13 ng/mL.

3.6. Characterization of Serum Antigens Recognized by Antiserum IIA

1. The major part of PIIANP is encoded by exon 2 (**Fig. 1**), which has the highest sequence homology with the interstitial collagen chain α1(I). It also has signifi-

Table 1
Intraassay Precision of the PIIANP Assay[a]

Serum PIIANP (ng/mL)	CV (%)
9.6	7.4
12.8	8.5
25.4	9.0

[a]Intraassay variability was assessed by measuring three serum samples 10 times in the same run.

Table 2
Interassay Precision on the PIIANP Assay[a]

Serum PIIANP (ng/mL)	CV (%)
9.0	8.6
27.0	6.0
36.3	1.5

[a]Interassay variability was assessed by measuring three serum samples in duplicate in five runs.

Table 3
Dilution Recovery

Sample	Dilution (%)	Serum PIIANP (ng/mL) Expected	Serum PIIANP (ng/mL) Measured	Recovery (%)
A	Net	—	259	—
	80	20.7	21.1	102
	60	15.5	13.3	86
	40	10.3	9.5	92
B	Net	—	24.2	—
	80	19.3	14.0	72
	60	14.5	13.9	96
	40	9.7	12.4	128
C	Net	—	30.4	—
	80	24.3	23.2	95
	60	18.2	17.8	98
	40	12.2	12.1	99

cant sequence homology with thrombospondins 1 and 2 and the von Willebrand factor C domain ***(10)***. Consequently we analyzed the specificity of IIA antiserum with these circulating proteins.

2. Western blot analysis showed that antiserum IIA recognizes two immunoreactive forms with molecular weights of 90 kDa (band a) and 120 kDa (band b) respectively (**Fig. 3A**, lane 1 and **Fig. 3B**, lane 1). These two bands were also recognized by an antiserum raised against the protein part encoded by exon 3–8 in the collagen II N-propeptide, which is present in both PIIANP and PIIBNP (**Fig. 3B,** lane 2). Thus circulating PIIANP appears to be present in at least two different immunoreactive forms (*see* **Note 1**). The antibody raised against exon

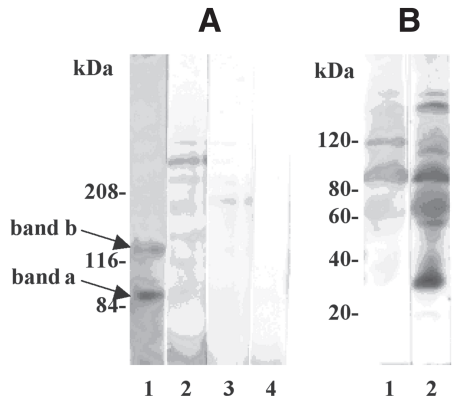

Fig. 3. Western blot analysis of the serum pool from healthy patients. The membranes were probed with **(A)** anti-IIA (lane 1), anti-von Willebrand factor (lane 2), antithrombospondin (lane 3), or nonimmune serum (lane 4) and **(B)** with anti-IIA (lane 1) or anti-exon 3–8 (lane 2).

3–8 recognized additional immunoreactive forms that are not detected by anti-IIA antibody and that probably correspond to circulating PIIBNP forms (*see* **Note 2**).

3. We then verified that bands a and b were not recognized by antithrombospondin and anti-von Willebrand factor antibodies (**Fig. 3A**, lanes 2 and 3) and additionally that antiserum IIA did not recognize bands corresponding to thrombospondin and von Willebrand factor (**Fig. 3A**, lanes 1 and 2). As negative control, a nonimmune serum showed no crossreactivity with bands a and b (**Fig. 3A**, lane 4).
4. The specificity of ELISA for PIIANP was also confirmed by the lack of significant crossreactivity of the PIIANP antiserum with purified intact human N-propeptide of type I collagen (PINP) composed of two α1(I) chains and one α2(I) chain, in agreement with previously reported findings ***(10)*** (data not shown).
5. The mol wt of bands a and b were higher than the estimated weight of monomeric N-propeptide IIA, approx 20–30 kDa. It is unlikely that these bands correspond to aggregates with binding serum protein because Tris/tricine gels were performed under reducing conditions after samples were boiled. We can hypothesize that bands a and b are composed of multiple propeptides with triple-helical regions bound to each other by the crosslinking molecules such as pyridinoline in the N-telopeptide region, located upstream to the cleavage site of matrix metalloproteinases (MMPs)-3, -7, -9, -13, and -14 ***(17)***.

3.7. Concentration of Serum PIIANP in Patients With Knee OA and Rheumatoid Arthritis

The mean serum concentration of PIIANP was significantly decreased ($p <$ 0.0001) in patients with knee OA (12.0 ± 3.2 ng/mL vs 25.8 ± 7.5 ng/mL; **Fig. 4A**)

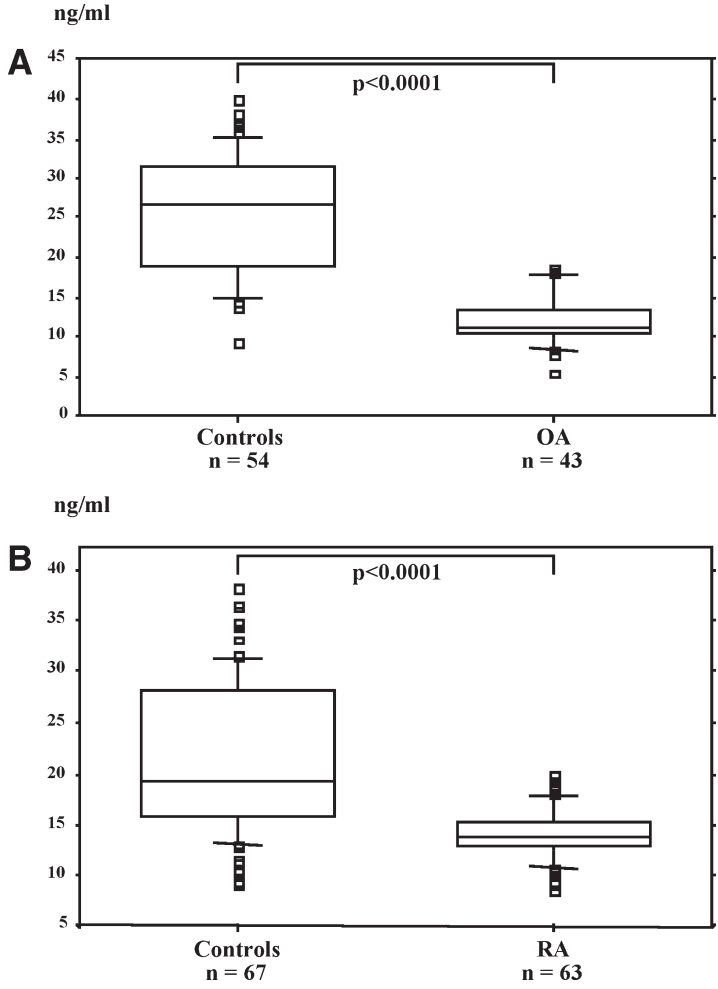

Fig. 4. Box plot distribution of serum PIIANP levels in healthy controls and in patients with **(A)** osteoarthritis (OA) or **(B)** rheumatoid arthritis (RA). From the bottom up, the box indicate the 25th, 50th (median), and 75th percentiles, and the bars indicate the 10th and 90th percentiles.

and RA (14.1 ± 2.5 ng/mL vs 21.7 ± 7.6 ng/mL; **Fig. 4B**) compared with sex- and age-matched healthy controls (*see* **Notes 3–5**). When using the mean –1 SD of the controls as the lower limit of the normal range, 97 and 57% of patients with knee OA and RA, respectively, had serum levels below that limit. The distribution of PIIANP serum levels was narrower in patients with OA or RA than in healthy controls **(Figs. 4A** and **4B)**.

3.8. Concluding Remarks

We have developed a specific immunoassay for serum PIIANP. This assay has adequate technical performance and should be useful to predict the progression of OA—probably in combination with markers of type II collagen degradation, as recently reported *(33)*—and also to monitor the efficacy of disease-modifying OA therapy, especially anabolic treatments.

4. Notes

1. We showed that IIA antiserum recognizes two forms of circulating PIIANP without significant crossreactivity with other proteins in the serum of healthy people. Additionally, the sequence encoded by exon 2 is highly conserved between human and mouse (85%) or human and rabbit (92%), suggesting that this immunoassay could be useful in the evaluation of preclinical studies using these animal models.
2. The development of an assay specific for the PIIBNP form would be an alternative complementary approach to assay for PIIANP. Indeed, this would allow the investigation of potential alterations in the ratio between type IIA and type IIB collagen synthesis in arthritic diseases.
3. The tissue origin of N-propeptide IIA in healthy controls is still a matter of discussion. Although immunohistochemistry studies were unable to detect type IIA procollagen in mature cartilage of healthy controls even after extensive protease treatments *(11)*, animal studies showed mRNA expression of type IIA procollagen in culture of adult bovine chondrocytes *(23)* and in articular cartilage of healthy dogs *(24)* and mice *(25)*. In addition, detectable concentrations of type IIA procollagen protein were shown in the pellets of adult bovine chondrocytes *(26)*. Thus, it may be possible that part of the circulating PIIANP arises from adult cartilage. Besides articular cartilage, type IIA collagen may also arise in part from other tissues such as adult vitreous humor or intervertebral disc tissues *(27–29)*. Nevertheless, it remains unclear whether or not these tissues synthesize type IIA in adults, and their contributions to the serum concentration are likely to be low compared with articular cartilage. Additional studies are needed to elucidate the relative contribution of articular cartilage and other tissues to the global serum levels of PIIANP.
4. In patients with knee OA, we found decreased serum levels of PIIANP compared with controls. The clinical significance of decreased serum PIIANP levels in patients with knee OA should, however, be interpreted with caution. Nelson et al. *(30)* showed that the content of PIICP—another index of type II collagen synthesis—in cartilage tissue extract was increased in patients with knee OA. Measurements of PIICP in synovial fluid and serum have yielded conflicting results. Increased levels of PIICP in the synovial fluid of the signal joint were found in patients with knee OA compared with healthy controls *(31)*. These increased levels are, however, mainly found in early and mid-stage OA, whereas levels decrease in end-stage disease, possibly because of decreased cartilage mass and

chondrocytes end-stage failure. Contrasting with increased PIICP levels in synovial fluid, Nelson et al. ***(30)*** reported in another population depressed levels of that marker in the serum of patients with knee OA compared with healthy controls. Thus, it could be speculated that decreased serum PIICP levels in OA may reflect systemic alterations of type II collagen metabolism, which may not be related to the signal joint ***(30)***. In our study, we only analyzed serum, and thus it remains to be determined whether the conflicting data between synovial fluid and serum PIICP levels will also apply to PIIANP. Clearly further studies comparing serum and synovial fluid levels of both PIICP and PIINP in well-characterized populations of patients with OA and controls are required to delineate the clinical significance of the serum levels of these markers. The PIIANP decrease was larger (58% vs 19%) than that of PIICP, suggesting that serum PIIANP may be more sensitive than serum PIICP in detecting alteration of type II collagen metabolism in OA. However, because no direct comparison of these two markers was performed in the same subjects, an increased sensitivity of PIIANP over PIICP needs to be confirmed in other studies.

5. Our study showed that serum PIIANP levels are markedly decreased in RA patients compared with controls. Conversely, levels of serum PIICP were found to be increased in serum ***(30)*** without significant differences between aggressive and nonaggressive disease ***(32)***. The reasons for this discrepancy between serum levels of PIIANP and PIICP in patients with RA is unclear, but it may result from differences in population characteristics, our patients being characterized by long-standing disease. It seems unlikely that this decrease was the result of increased proteinase degradation of PIIANP at the tissue level. Indeed, Fukui et al. ***(17)*** showed recently that the antibody raised against IIA that was used in the ELISA still recognizes recombinant N-propeptide IIA even after cleavage by the differents MMPs known to be involved in the destruction of articular cartilage.

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