Thrombin-cleaved Osteopontin Is Increased in Urine of Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To measure concentrations of the thrombin-cleaved isoform of osteopontin (OPN) in urine and plasma of patients with rheumatoid arthritis (RA), and to assess whether levels of thrombin-cleaved OPN are associated with measures of RA.

Methods. Subjects comprised 70 patients with RA, 20 patients with osteoarthritis (OA), and 46 healthy controls. RA disease activity was evaluated by tender joint count, swollen joint count, patient's global assessment of disease activity, erythrocyte sedimentation rate (ESR), and levels of C-reactive protein (CRP), matrix metalloproteinase-3 (MMP-3), and rheumatoid factor (RF), as well as 28-joint count Disease Activity Score (DAS28). OPN levels in plasma and urine were measured by ELISA.

Results. Median levels of thrombin-cleaved OPN in urine (U-half) were significantly higher in RA patients (143.5 pmol/mmol Cr) than in healthy controls (67.9 pmol/mmol Cr) or OA patients (69.8 pmol/mmol Cr). Thrombin-cleaved OPN was not detected in plasma. U-half levels correlated significantly with levels of CRP ($r = 0.26$, $p = 0.03$), ESR ($r = 0.26$, $p = 0.03$), and RF ($r = 0.28$, $p = 0.03$). Median U-half levels were significantly higher in patients with stage III (249.9 pmol/mmol Cr) and IV (251.6 pmol/mmol Cr) disease than in patients with stage I (98.6 pmol/mmol Cr) disease.

Conclusion. Our results suggest that urine levels of the thrombin-cleaved isoform of OPN may reflect the severity of active inflammatory arthritis in patients with RA. (J Rheumatol First Release Feb 15 2010; doi:10.3899/jrheum.090582)

Key Indexing Terms:
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Biologic disease-modifying antirheumatic drugs (DMARD) have revolutionized the management of rheumatoid arthritis (RA). However, investigations of new markers to assess disease activity and longterm prognosis are still required to enable physicians to make rational decisions about early intervention. Further studies are also required to develop new therapies that are more specific for synovitis or bone destruction, as current available biologic DMARD systemically suppress inflammatory cytokines, thereby increasing the risk of infectious disease. Osteopontin (OPN) is one of the key molecules in the pathogenesis of RA, due to its unique function bridging bone and the immune system.

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OPN is a secreted phosphoglycoprotein with a molecular weight of 44,000–75,000. First isolated from bone extracellular matrix[1], OPN is expressed by various cells, including osteoclasts, macrophages, and activated T cells. OPN interacts with a variety of cell-surface receptors, including the αvß3, αvß5, α4ß1, and α9ß1 integrins, as well as CD44. Binding of OPN to these cell-surface receptors stimulates cell adhesion, cell migration, and other specific cell-signaling functions. OPN has also been recognized as an inflammatory cytokine that promotes efficient type-1 immune responses by regulating macrophage differentiation via expression of interleukin 12 (IL-12) and IL-10[2].

The major integrin-binding site in OPN is the arginine-glysine-aspartate (RGD) integrin-binding motif, which is required for the adherence of various types of cells to OPN. Other sequences within OPN have also been shown to mediate cell adherence. For example, cleavage of human OPN by thrombin exposes the SVVYGLR sequence (SLAYGLR in mice), promoting adherence of cells that express α9 and α4 integrins[3].

OPN is abundant in bone, facilitating the attachment of osteoclasts to bone matrix via interaction with cell-surface αvß3 integrin and CD44. Overexpression of OPN has been detected in various cell types, including synovial fibroblasts, synovial lining cells, peripheral blood mononuclear cells, and synovial CD4+ T cells, in patients with RA[4-7].

Overexpression of OPN has also been detected at sites of bone erosion in a murine model of collagen-induced arthritis[8]. Sennels, *et al* reported that concentrations of the full-length isoform of OPN were higher in plasma from RA patients with high disease activity than in healthy controls, and levels correlated with levels of C-reactive protein (CRP)[9]. Ohshima, *et al* have shown that levels of full-length OPN were significantly higher in synovial fluid from RA patients than those from patients with osteoarthritis (OA) and correlated with serum CRP levels[5]. In contrast, Hasegawa, *et al* reported no significant differences in levels of full-length OPN in synovial fluid of RA and OA patients, whereas levels of the thrombin-cleaved isoform of OPN were significantly higher in synovial fluid from RA patients than those from OA patients[10]. Interestingly, Ohba, *et al* revealed high levels of thrombin activity in synovial fluid of RA patients and strong mitogenic activity of thrombin toward synovial fibroblast-like cells[11].

OPN is thus strongly suggested to be associated with both synovitis and bone destruction in RA. However, the role and distribution of the thrombin-cleaved OPN isoform and relationships between full-length and thrombin-cleaved isoforms have yet to be fully elucidated. To evaluate the clinical significance of thrombin-cleaved OPN in RA, we measured levels of the 2 isoforms of OPN in plasma and urine of patients with RA, compared the levels of each isoform to those in healthy individuals and patients with OA, and assessed relationships between each of the isoforms of OPN and biomarkers of RA.

MATERIALS AND METHODS

Patients and samples. Subjects included 70 patients with RA, 20 patients with OA, and 46 healthy controls (Table 1). Patients with acute or chronic infectious disease, liver disease, renal disease, or malignant disease were excluded. Patients with RA fulfilled the American Rheumatism Association 1987 revised criteria for the classification of RA[12]. Patients with RA did not fulfill the criteria for any other rheumatic diseases, with the exception of one patient with Sjögren's syndrome. RA patients underwent routine clinical and laboratory assessments (Table 2). OA was diagnosed based on clinical and radiological findings. Individuals without rheumatic disease or musculoskeletal disease served as healthy controls. The study was conducted according to the Helsinki Declaration and was approved by the institutional ethics committee. All subjects provided informed consent.

Venous blood was collected into tubes containing ethylenediaminetetraacetic acid and was centrifuged immediately at 4˚C. Urine samples were centrifuged at 4˚C to remove cell debris. Plasma and urine samples were frozen at –80˚C until use.

Clinical assessment of RA. Clinical activity was evaluated by tender joint count, swollen joint count, patient's global assessment of disease activity, ESR, and levels of CRP, matrix metalloproteinase (MMP-3), and rheumatoid factor (RF). Disease activity score using ESR (DAS28-4ESR) was calculated as follows: DAS28 = 0.56 √(T28) + 0.28 √(SW28) + 0.70Ln(ESR) + 0.014VAS[13]. Classification of functional status (from class I to IV) and radiological progression (from Steinbrocker stage I to IV) was also determined according to established criteria[14,15].

Table 1. Characteristics of the study population.

	RA, n = 70	OA, n = 20	Controls, n = 46
Male/female, no.	16/54	2/18	15/31
Age, yrs, median (range)	55.0 (24–75)	53.5 (29–90)	46.0 (27–60)

Table 2. Clinical characteristics of patients with RA.

Characteristic	Median (range)
Disease duration, yrs	5 (0.2–40)
Tender joint count	5 (0–27)
Swollen joint count	5 (0–26)
Patient's global assessment of disease activity (100-mm VAS)	36.5 (0–100)
DAS28-4ESR	4.8 (1.5–8.5)
CRP, mg/dl	1.3 (0.1–16.5)
ESR, mm/h	39 (5–130)
MMP-3, mg/dl	155 (31.3–703)
RF, mg/dl	72 (1–1715)
Steinbrocker stage, no.	I:25, II:14, III:13, IV:18
Steinbrocker class, no.	1:23, 2:41, 3:6, 4:0

VAS: visual analog scale; DAS28: 28-joint count disease activity score; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; MMP-3: matrix metalloproteinase-3; RF: rheumatoid factor.

Determination of OPN levels in plasma and urine. Concentrations of full-length and thrombin-cleaved OPN were measured by ELISA according to the protocol provided by the manufacturer (IBL, Gunma, Japan)[10,16]. In brief, 96-well microtiter plates were coated with capture antibody, then blocked with 1% bovine serum albumin in phosphate buffered saline. Plasma was diluted 1:10, and urine was used at 1:200 dilution for full-length OPN and 1:10 dilution for thrombin-cleaved OPN with dilution buffer. Samples were then added to the plates (100 μl/well, in duplicate) and incubated 1 h at 37˚C. After plates were washed with wash buffer, 100 μl of horseradish peroxidase-labeled detection antibodies was added to each well, and plates were incubated 30 min at 4˚C. After extensive washes with wash buffer, 100 μl of tetramethyl benzidine buffer (used as a substrate) was added to each well, and plates were incubated 30 min at room temperature in the dark. Color development was stopped by adding 100 μl stop solution. A plate reader (Bio-Rad, Hercules, CA, USA) was used to quantify the signal at 450 nm. Figure 1 shows the binding sites of each antibody used in this ELISA system.

Since urinary OPN concentration has been shown to vary inversely with urinary volume[17], each OPN value obtained by ELISA was divided by the urinary creatinine level to adjust OPN concentration in urine samples.

Statistical analysis. Values are expressed as median (range). The Steel-Dwass test was used to assess the significance of differences between study groups. Spearman's correlation coefficient by rank test was used to assess correlations between OPN values and clinical variables in RA patients. Values of $p < 0.05$ were considered significant.

RESULTS

Patient characteristics. Median ages of RA patients, OA patients, and healthy controls were 55.0 (24–75), 53.5 (29–90), and 46.0 (27–60) years, respectively. No significant differences in age were observed between groups.

The following values were obtained for RA patient clinical characteristics (Table 2): disease duration, 5 (0.2–40) years; tender joint count, 5 (0–27); swollen joint count, 5 (0–26); patient's global assessment of disease activity, 36.5 (0–100) mm; DAS28-4ESR, 4.8 (1.5–8.5); CRP, 1.3

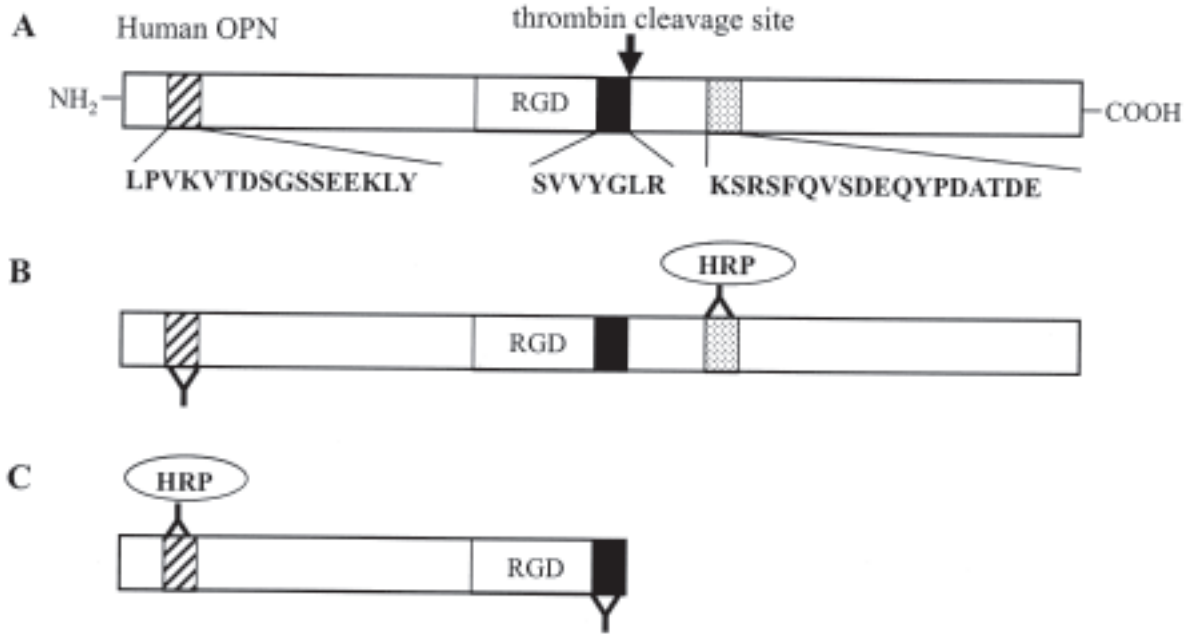

Figure 1. Antibodies used for osteopontin (OPN) ELISA. Cleavage of human OPN by thrombin exposes the SVVYGLR sequence (A). The capture antibody for full-length OPN binds to the N-terminal region of OPN, and the detection antibody binds to a non-thrombin-cleaved site in OPN (B). The capture antibody for thrombin-cleaved OPN binds to the SVVYGLR sequence, and the detection antibody binds to the N-terminal region (C). RGD: arginine-glysine-aspartate.

(0.1–16.5) mg/dl; ESR, 39 (5–130) mm/h; MMP-3, 155 (31.3–703) mg/dl; and RF, 72 (1–1715) mg/dl. Radiological progression varied from early (stage I) to terminal (stage IV) bone destruction. No patients were classified as class IV with respect to functional status. Among the 70 RA patients, 62 had been treated with the following drugs: nonsteroidal antiinflammatory drugs (n = 30), prednisolone (n = 27, 2–10 mg/day), nonbiologic DMARD (n = 33: methotrexate, n = 15; salazosulfapyridine, n = 13; gold sodium thiomalate, n = 3; bucillamine, n = 5; actarit, n = 1), and biologic DMARD (infliximab, n = 1).

In patients with OA, distal interphalangeal joints of the fingers were mostly affected in 7 patients, and knees were mostly affected in 13 patients. Levels of CRP were below the limit of detection in all OA patients.

OPN levels in plasma and urine. Levels of thrombin-cleaved OPN in urine (U-half) were significantly higher in RA patients (143.5 pmol/mmol Cr) than in healthy controls (67.9 pmol/mmol Cr) and OA patients (69.8 pmol/mmol Cr) (Table 3). No significant differences in urine levels of full-length OPN (U-full) were observed between groups. Levels of full-length OPN in plasma (P-full) were significantly higher in RA patients (499.4 ng/ml) and OA patients (413.9 ng/ml) than in healthy controls (277.9 ng/ml). However, no significant differences in P-full levels were observed between patients with RA and those with OA. Thrombin-cleaved OPN was not detected in plasma. No significant differences in any OPN levels were observed between large-joint destruction and small-joint destruction.

Correlation between OPN levels and RA clinical measures. U-half levels correlated significantly with CRP ($r = 0.26$, $p = 0.03$), ESR ($r = 0.26$, $p = 0.03$), and RF ($r = 0.28$, $p = 0.03$) values (Figure 2). In contrast, no significant correlations were found between U-full levels and CRP, ESR, MMP-3, or RF values (Figure 3). P-full levels correlated significantly with levels of CRP ($r = 0.30$, $p = 0.015$) and MMP-3 ($r = 0.35$, $p = 0.007$), but not with ESR or RF levels (Figure 4). No correlations were observed between levels of

Table 3. OPN levels in plasma and urine.

Levels of OPN, Median (range)	Controls	OA	RA
U-half, pmol/mmol Cr	67.9 (1.3–148.4)	69.8 (5.8–430.7)	143.5 (3.2–2431.8)*†
U-full, ng/mmol Cr	1549.2 (22.7–3949.4)	945.7 (47.5–2358.9)	2044.6 (54.8–8055.5)
P-full, ng/ml	277.9 (114.0–655.1)	413.9 (195.6–1047.8)*	499.4 (58.8–1492.8)*

* $p < 0.05$ compared to healthy controls. † $p < 0.05$ compared to OA. OPN: osteopontin; U-half: thrombin-cleaved OPN in urine; U-full: full-length form of OPN in urine; P-full: full-length form of OPN in plasma.

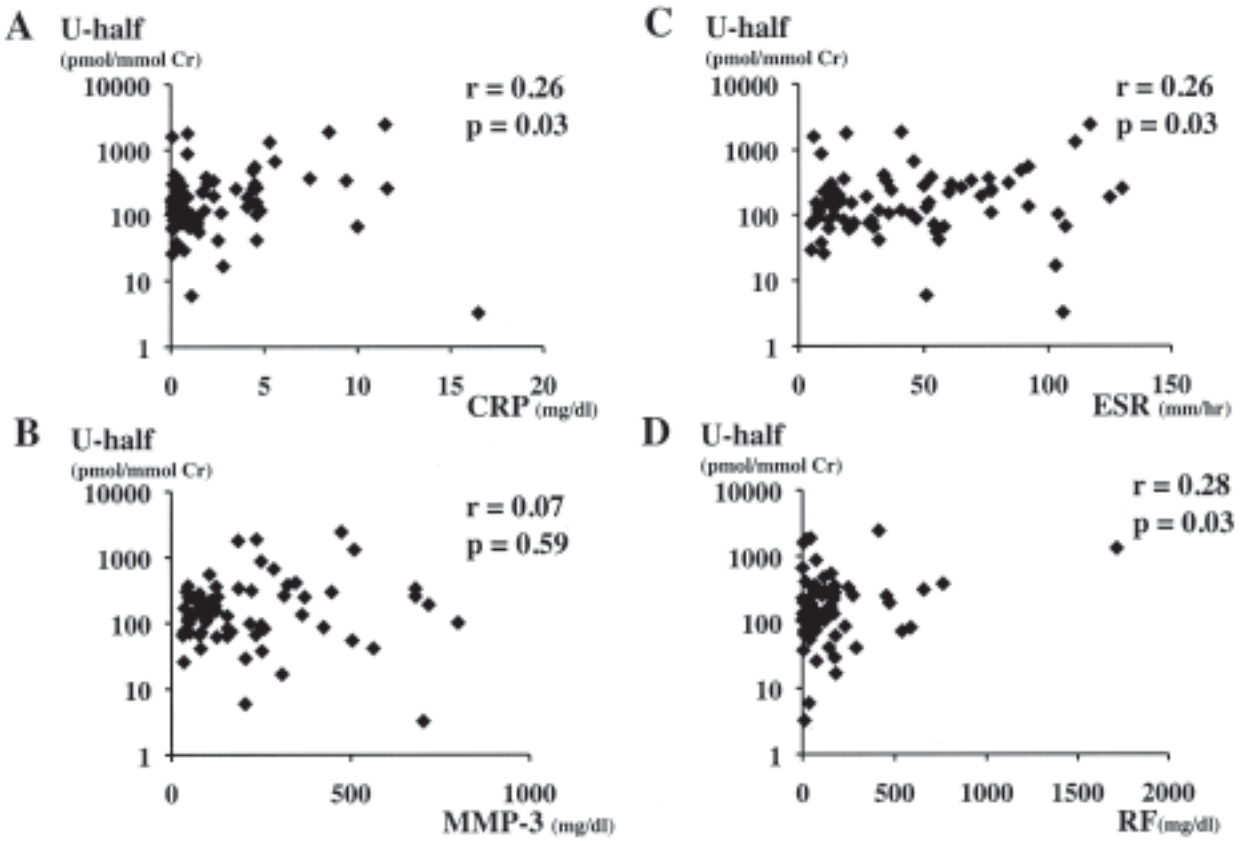

Figure 2. Correlation coefficients between levels of thrombin-cleaved OPN in urine (U-half) and indicators for RA. U-half levels correlated significantly with values of CRP (r = 0.26, p = 0.03; panel A), ESR (r = 0.26, p = 0.03; C), and RF (r = 0.28, p = 0.03; D), but not with MMP-3 values (r = 0.07, p = 0.59; B).

each OPN isoform and disease duration, tender joint count, swollen joint count, patient's global assessment of disease activity, or DAS28-4ESR (data not shown).

U-half levels were significantly higher in patients with stage III (249.9 pmol/mmol Cr) and IV (251.6 pmol/mmol Cr) disease than in patients with stage I disease (98.6 pmol/mmol) (Table 4). No significant differences in U-full or P-full levels were identified between stages. Further, no significant differences were observed in any OPN levels between functional status classes.

DISCUSSION

Our study demonstrated that the thrombin-cleaved isoform of OPN is significantly increased in urine from patients with RA. No previous study has shown the presence of thrombin-cleaved OPN in urine of patients with RA. Further, levels of U-half correlated with CRP, ESR, and RF levels, and were significantly higher in patients with progressive-stage bone destruction compared to those with early-stage bone destruction. In contrast, levels of U-full were not significantly increased in RA patients. Although P-full levels correlated with CRP and MMP-3 values, no significant differences in P-full levels were observed between RA and OA patients. Together, these results indicate that U-half appears to be more useful than U-full or P-full for the assessment of articular inflammation in patients with RA.

Clinical assessment of RA includes 2 key aspects: degree of synovitis (inflammation) during the acute phase; and severity of bone damage for longterm prognosis. Our results suggest that U-half is associated with inflammatory activity in RA patients, as U-half levels correlated with CRP and ESR values. U-half levels also appear to be associated with bone damage in RA patients, and they were higher in patients with progressive-stage disease and correlated with RF levels. High-titer RF is known to be an important variable in predicting the severity of radiographic bone damage[18,19].

Although U-half levels were not associated with MMP-3 levels, P-full levels were. MMP-3 has been identified as a marker for predicting bone damage during early-stage RA[20,21]. To assess cartilage destruction, P-full may be more useful than U-half, as MMP-3 is considered to play a pivotal role in cartilage degradation. One notable finding in our study was an increased P-full level not only in patients with RA, but also in those with OA. OPN is also overexpressed in OA cartilage[22], and has been detected in synovial tissues

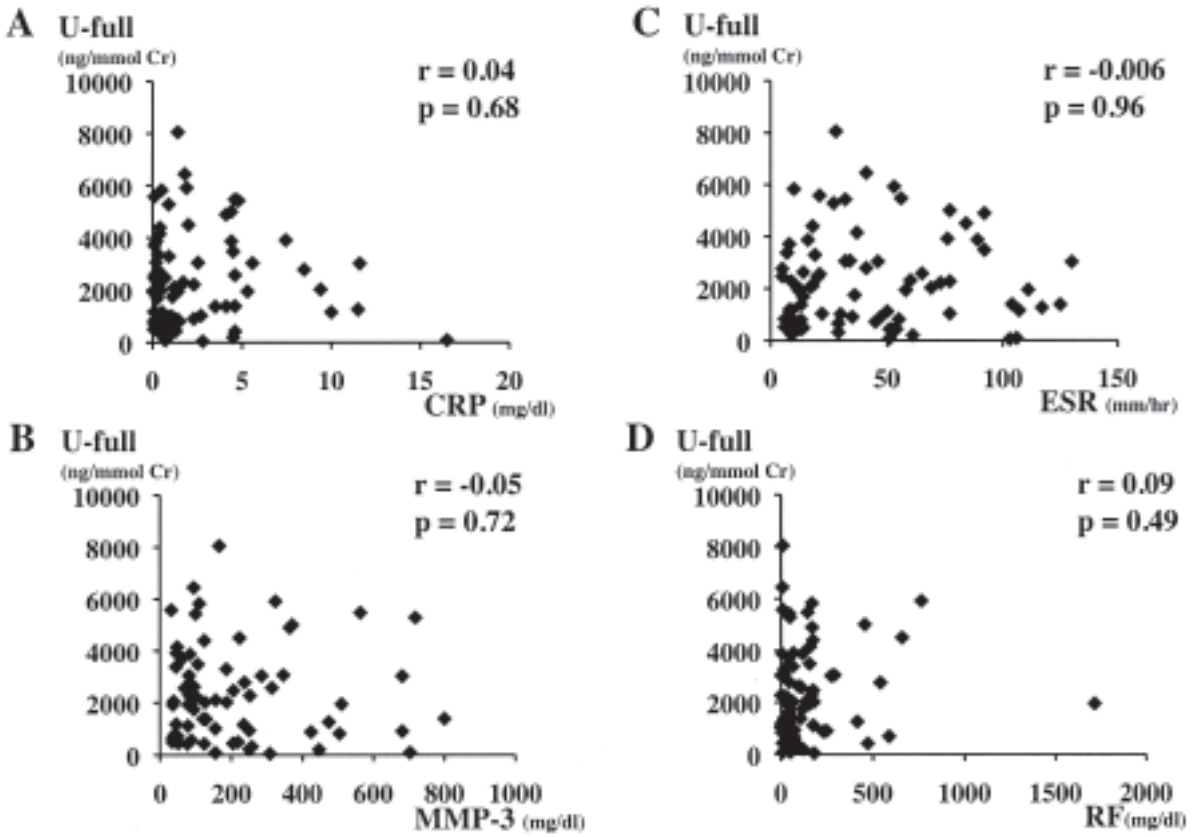

Figure 3. Correlation coefficients between urine levels of full-length OPN (U-full) and indicators for RA. No significant correlations were identified between urine levels of U-full and CRP (panel A), MMP-3 (B), ESR (C), or RF (D) values.

and fluid of OA patients[5]. U-half thus presumably reflects activity of arthritis in RA, whereas P-full reflects cartilage destruction.

Of note, OPN has received attention as a new target molecule in the development of therapies for RA. In a murine model of arthritis, antibodies recognizing thrombin-cleaved OPN have been shown to inhibit proliferation of the synovium, bone erosion, and inflammatory cell infiltration in arthritic joints, whereas antibodies recognizing full-length OPN also inhibit development of arthritis[23-25]. No studies have compared the efficacy of these antibodies. However, the results of our study and a study by Hasegawa, *et al*[10] indicate that thrombin-cleaved OPN could potentially be a new target molecule for DMARD, as both studies show that thrombin-cleaved OPN is more highly associated with arthritis in patients with RA than in patients with OA.

This study shows some limitations in the small number of subjects and the fact that we could not exclude the effects of medication, as most RA patients were taking medication. No previous study has clearly shown whether full-length or thrombin-cleaved OPN is associated with clinical response in patients with RA. Changes to OPN levels in plasma and urine in RA patients before and after standard treatment should be determined. We are conducting a prospective study with a population of patients with untreated RA to evaluate whether U-half is associated with disease activity and bone destruction in RA. We previously could not determine why thrombin-cleaved OPN is detected in synovial fluid[10] and urine, but not plasma. We speculate that our ELISA system did not detect P-half, as P-half levels may be too low to be detected by our system; U-half may be excreted by both synovium and tubules, and/or P-full may be cleaved in the kidneys.

Despite these limitations, this is the first report to describe that levels of the thrombin-cleaved isoform of OPN are significantly increased in the urine of patients with RA, particularly during progressive-stage disease. Clarifying the role of thrombin-cleaved OPN in the pathogenesis of RA may contribute to development of specific markers of disease progression and new treatments for RA.

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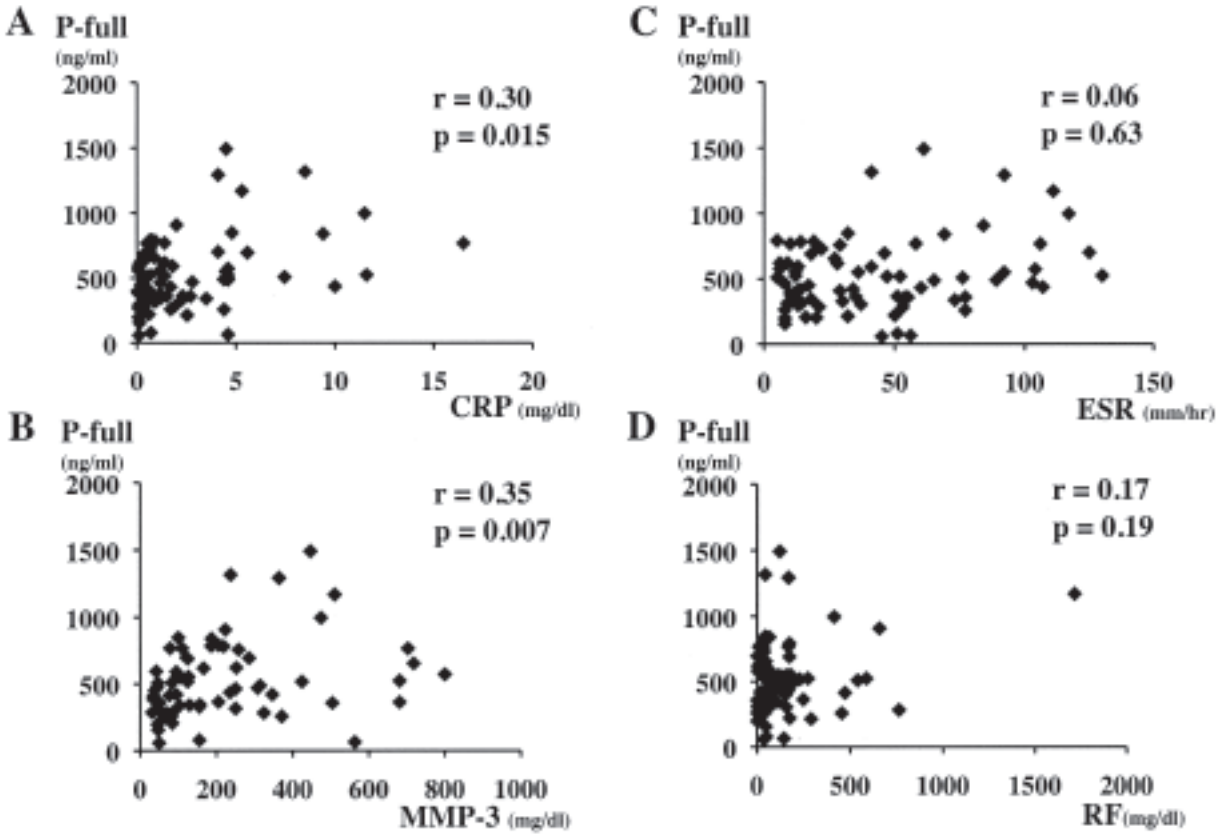

Figure 4. Correlation coefficients between levels of full-length OPN in plasma (P-full) and indicators for RA. P-full levels correlated significantly with values of CRP (r = 0.30, p = 0.015; panel A) and MMP-3 (r = 0.35, p = 0.007; B), but not with values of ESR (C) or RF (D).

Table 4. OPN levels by Steinbrocker stage in patients with RA.

Levels of OPN, median (range)	Stage I	Stage II	Stage III	Stage IV
U-half, pmol/mmol Cr	98.6 (5.9–380.8)	116.5 (16.7–1311.1)	249.9 (83.1–548.2)*	251.6 (29.1–2431.8)*
U-full, ng/mmol Cr	1375.1 (95.0–6445.4)	2248.1 (54.8–8055.5)	2574.0 (430.4–5827.3)	1785.9 (90.1–4498.0)
P-full, ng/ml	436.8 (58.8–1492.8)	475.2 (286.2–1294.3)	479.5 (259.6–1317.6)	521.1 (220.4–769.3)

* $p < 0.05$ compared to Stage I. OPN: osteopontin; U-half: thrombin-cleaved OPN in urine; U-full: full-length form of OPN in urine; P-full: full-length form of OPN in plasma.

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