Enhanced Local Production of Osteopontin in Rheumatoid Joints

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ABSTRACT. ***Objective.*** To investigate the involvement of osteopontin (OPN) in the pathogenesis of rheumatoid arthritis (RA), localization and production of OPN were examined in patients with RA.

Methods. Localization of OPN in the rheumatoid synovium was examined by immunohistochemistry. *In vitro* OPN production by cultured synovial cells from patients with RA (n = 5) and with osteoarthritis (OA) (n = 5) was assessed by ELISA. OPN concentrations in plasma and synovium were quantified in patients with RA (n = 23) by 2 distinct ELISA systems to measure both thrombin cleaved and non-cleaved OPN. The same experiments were done in patients with OA (n = 15) and healthy volunteers (n = 10) as a control.

Results. OPN was highly detected by immunohistochemistry predominantly in the RA synovial lining cells, while less and scattered OPN was detected in OA synovial tissues. ELISA revealed that cultured RA synovial cells secreted significantly more OPN than OA cells. ELISA also showed a marked increase of OPN levels in synovial fluid (SF) of patients with RA and with OA compared to the control plasma OPN levels, although OPN levels were not increased in RA and OA plasma compared to healthy controls. SF OPN levels of patients with RA were significantly higher than those of patients with OA, and correlated with serum C-reactive protein levels. The ratios of thrombin cleaved versus non-cleaved OPN were significantly increased in RA plasma and SF compared with OA plasma and SF and plasma from healthy controls.

Conclusion. Our results revealed enhanced local production of OPN in rheumatoid joints, suggesting involvement of OPN in the pathogenesis of RA. (J Rheumatol 2002;29:2061–7)

Key Indexing Terms:

OSTEOPONTIN	RHEUMATOID ARTHRITIS	ELISA
IMMUNOHISTOCHEMISTRY	SYNOVIAL FLUID	PLASMA

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Osteopontin (OPN) is a secreted phosphoglycoprotein, originally isolated from bone extracellular matrix[1,2]. OPN is expressed by various cell types, including osteoclasts, macrophages, activated T cells, smooth muscle cells, and epithelial cells, and is present in several tissues including bone, kidney, placenta, smooth muscle, and secretory epithelia[3]. OPN contains an Arg-Gly-Asp (RGD) sequence that interacts with αvß1, ß3, and ß5 integrins and is capable of promoting cell attachment, chemotaxis, and signal transduction in several different cell types[4-6]. It is now thought to be involved in normal tissue remodeling processes such as in bone resorption, angiogenesis, wound healing, and tissue injury as well as diseases such as restenosis, atherosclerosis, renal diseases, and tumorigenesis[7-15].

Rheumatoid arthritis (RA) is a major chronic inflammatory disease characterized by bone destructive polyarthritis[16]. Although the mechanism of bone destruction in RA has not been fully elucidated, activated synovial cells and infiltrating inflammatory cells contribute to the bone destruction directly as well as indirectly through various soluble chemical mediators they produce. Recent evidence

has revealed that osteoclasts play a critical role for bone resorption not only in physiological bone remodeling but also in pathological states such as osteoporosis[17]. At the sites of bone erosion of RA, we can observe histopathologically the invasion of active osteoclasts from the activated synovial tissue (so-called pannus), suggesting that synovial osteoclasts also play a critical role for bone resorption in RA. Bone resorption by osteoclasts is known to be initiated by the attachment of osteoclasts to the surface of bone. This attachment is known to be mediated through the ligation between OPN and one of its receptors, αvß3 integrin[18-20]. In addition, we recently described the enhanced local expression of OPN in the proliferating lining layer of synovium and αvß3 integrin on the surface of cartilage in inflamed joints of murine arthritis models such as collagen induced arthritis and antigen induced arthritis, using immunohistochemistry and *in situ* hybridization[21]. We also observed the elevation of plasma OPN concentrations during the progression of arthritis by ELISA[21]. These results suggest that OPN plays a critical role in pathogenesis and especially bone resorption of RA. There have been other reports regarding OPN in RA[22].

In this study to investigate the involvement of OPN in the pathogenesis of RA, localization of OPN was examined by immunohistochemistry in rheumatoid synovial tissues. In addition, *in vitro* OPN production by synovial cells and plasma/synovial OPN concentrations were quantified using 2 distinct ELISA systems in patients with RA.

MATERIALS AND METHODS

Patients and tissue preparation. Synovial tissue samples were obtained with informed consent at the time of surgery from 3 patients with persistent RA. All patients fulfilled the American College of Rheumatology (ACR, formerly, the American Rheumatism Association) 1987 revised criteria for RA[23]. Synovial tissue samples were also obtained at the time of arthroplasty from 3 patients with osteoarthritis (OA). The diagnosis of OA was based on both clinical and radiographic findings. The tissue specimens were fixed in 4% paraformaldehyde for 2 h at room temperature, dehydrated in alcohol, embedded in paraffin, and then used for further experiments. This human experimentation was approved by the Human Ethics Review Committee of Osaka University Medical School.

Immunohistochemistry for OPN. Expression of OPN in synovial tissues was determined by immunohistochemistry using the avidin-biotin complex method. A mouse monoclonal IgG anti-human OPN antibody, 4C1, was used as a primary antibody. Equal concentration of irrelevant mouse IgG (Pharmingen, San Diego, CA, USA) was used as a negative control for the primary antibody. Biotinylated goat anti-mouse IgG antibody (Dako, Glostrup, Denmark) was used as a secondary antibody. Paraffin sections (5 μm slices) were pretreated in 10 mM citrate buffer, pH 6.0, in a microwave oven for 5 min for antigen retrieval, and then treated with 0.3% H_2O_2, blocked with 2% bovine serum albumin (BSA) and incubated with either the primary or the control antibodies overnight at 4°C. Sections were washed with phosphate buffered saline (PBS) and reacted with the secondary antibody for 1 h at room temperature. After washing, they were incubated with avidin-peroxidase complex for 30 min. Sections were then developed with diaminobenzidine tetrahydrochloride substrate solution, counterstained with hematoxylin, and mounted in glycerol gelatin.

Preparation and culture of synovial cells. Synovial cells were isolated by enzymatic digestion from tissues obtained from the patients with RA (n = 5) and OA (n = 5) undergoing surgical treatment (joint replacement or synovectomy) as described[24]. After digestion, synovial cells were expanded in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. At confluency, they were trypsinized and passaged. After 3 passages, cultured cells were fibroblast-like in morphology and more than 90% of them were CD14 negative. The cultured cells were used for the experiment after 4–8 passages. They were cultured at 1×10^5 cells/well in 24 well plates for 7 days and then culture supernatants were collected for measurement of OPN by ELISA (System 1) as described below.

Enzyme linked immunosorbent assay for OPN. All blood and synovial fluid (SF) samples (RA, n = 23; OA, n = 17; healthy controls, n = 10) were collected under heparin to avoid being fragmented *in vitro* by thrombin, centrifuged to remove cells and debris, and stored at –80°C until used. All patients fulfilled the ACR 1987 revised criteria for RA[23]. The mean age of patients was 62.4 years (range 40–78) and the mean disease duration was 16.3 years (range 2.2–42). Two distinct sandwich ELISA systems, denoted System 1 and System 2, originally established by our laboratory[25] were used to quantify OPN from each sample. For System 1, a rabbit polyclonal anti-human OPN antibody, OPN1, was used for a coating antibody and a mouse monoclonal anti-human OPN antibody, OPN3, was used for a detecting antibody. For System 2, another rabbit polyclonal anti-human OPN antibody, OPN5, was used for a coating antibody and OPN1 was used for a detecting antibody. These antibodies were generated by our laboratory as reported[18].

Briefly, to generate each antibody the following synthetic peptides were used for immunization. OPN1, IPVKQADSGSSEEKQ; OPN3, KSKKFRRPDIQYPDATDE; OPN5, VDTYDGRGDSVVYGLRS, which correspond to the I_1–Q_{15}, K_{154}–E_{171}, and V_{137}–S_{153} internal amino acid sequences of human OPN, respectively. The individual possible recognition sites are shown in Figure 1, and the specificity of each antibody was checked by Western blot analysis[25]. According to the Western blot data, OPN1 and OPN5 can bind both non-thrombin cleaved (full length) OPN and thrombin cleaved (N-terminal half) OPN, while OPN3 can bind non-thrombin cleaved but not the N-half of cleaved OPN. Thus, although the binding capacity of each antibody may be different comparing Western blot and ELISA results, in practice, System 1 can detect only non-thrombin cleaved OPN but not cleaved OPN. On the other hand, System 2 can detect both non-thrombin cleaved and cleaved OPN.

ELISA was performed as described[25]. Briefly, 96 well microtiter plates were coated with either OPN1 or OPN5 (20 μg/ml, 100 μl in 0.1 M carbonate buffer, pH 9.5) overnight at 4°C, then blocked with 1% BSA in PBS containing 0.05% NaN_3. Samples and purified recombinant OPN derived from CHO cells as a standard OPN were diluted with dilution buffer (1% BSA in PBS containing 0.05% Tween 20), added to the plates (100 μl/well), and incubated 1 h at 37°C. After extensive washing with buffer (PBS containing 0.05% Tween 20), 100 μl of 2 ng/ml horseradish peroxidase labeled OPN3 (for System 1) or OPN1 (for System 2) were added to each well and incubated 30 min at 37°C. After extensive washes with washing buffer, 100 μl tetramethyl benzidine buffer as substrate were added to each well and incubated 30 min at room temperature in the dark. Color development was stopped by addition of 100 μl of stop solution (1 N H_2SO_4). A BioRad plate reader (Bio-Rad, Hercules, CA, USA) was used to quantify the signal at 450 nm. Reproducibility of the data was confirmed by testing each sample at least twice.

Statistical analysis. The Mann-Whitney U test was used to assess the significance of the difference between RA patients and either OA patients or controls. Pearson's correlation coefficient was used to assess correlation between variables. Data were expressed as a mean ± SEM. P values < 0.05 were considered significant.

RESULTS

Localization of OPN in rheumatoid synovial tissues. Localization of OPN was examined by immunohistochemistry in synovial tissues from 3 patients with RA and 3 with

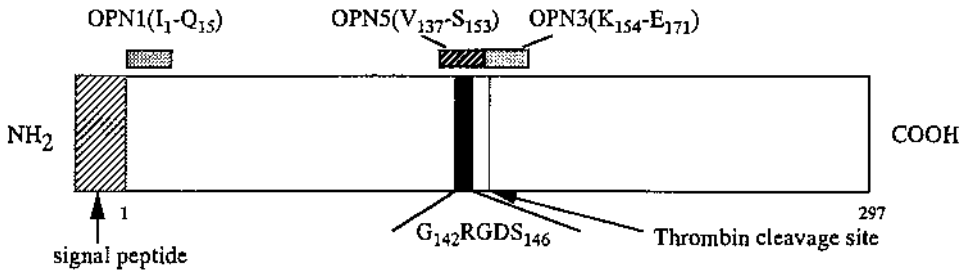

Figure 1. Structure of osteopontin (OPN) and possible recognition sites of the individual anti-human OPN antibodies[25].

OA. A representative example is shown in Figure 2: OPN was detected as brown colored stains, predominantly in the synovial lining layer rather than in perivascular and sublining areas. It was absent in lymphoid aggregates. In contrast, although the OA synovium also showed some OPN staining, the expression was less notable and was scattered. Thus, OPN was localized predominantly in the lining layer of rheumatoid synovium.

Spontaneous production of OPN by RA synovial cells. Synovial cells were isolated by enzymatic digestion from tissues obtained from patients with RA (n = 5) and OA (n = 5) undergoing surgical treatment (joint replacement or synovectomy) as described[24]. After 3 passages, the cultured cells were fibroblast-like in morphology and more than 90% of them were CD14 negative. As shown in Table 1, cultured RA synovial cells secreted significantly more OPN than OA cells: mean OPN concentration in RA 1695 ± 1258 ng/ml versus OA, 415 ± 155 ng/ml ($p < 0.05$).

OPN in blood and SF samples of RA patients. OPN levels were measured in both plasma and SF from 23 patients with RA by a sandwich ELISA system (System 1), using 2 distinct anti-human OPN antibodies, OPN1 and OPN3. Samples from OA patients or healthy volunteers were used as a control. The results are summarized in Figure 3: there was no significant difference among the blood samples. In contrast, SF OPN levels were markedly elevated in both RA and OA patients compared to OPN levels of the blood samples (SF OPN levels vs plasma OPN levels: RA, $p < 0.05$; OA, $p < 0.05$). In addition, SF OPN levels of RA patients were significantly higher than those of OA patients

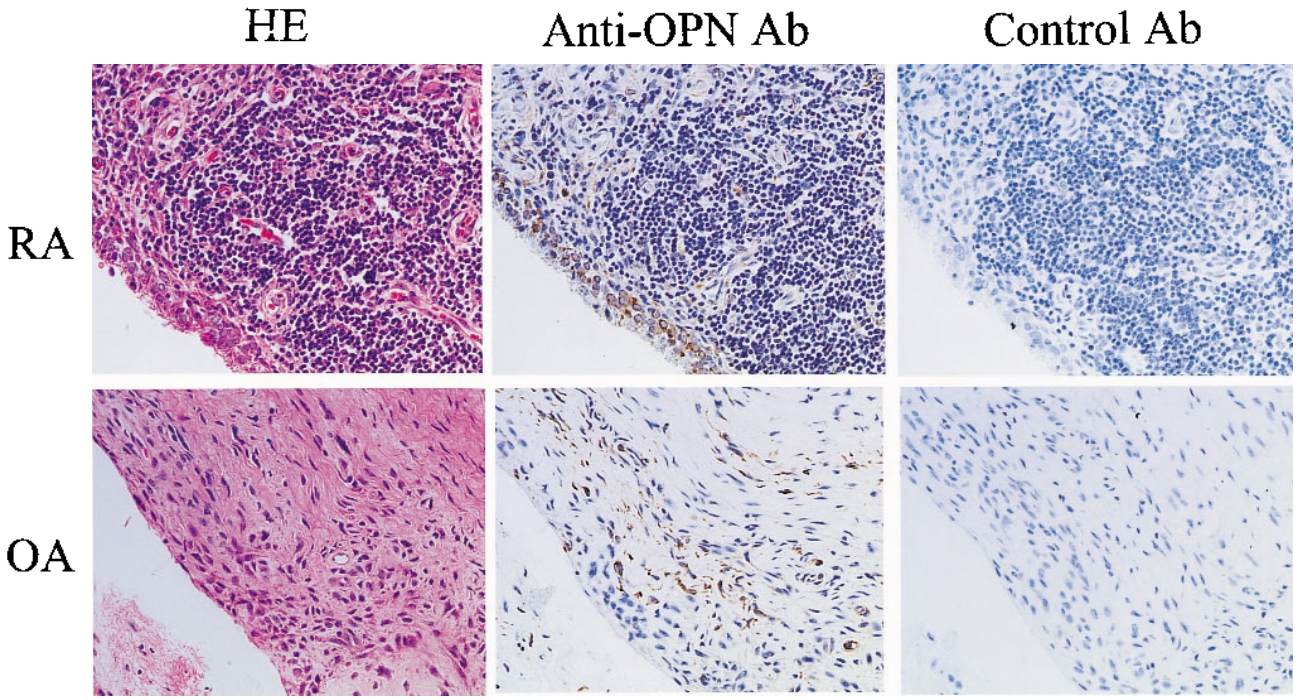

Figure 2. Localization of OPN in rheumatoid synovium was examined by immunohistochemistry in synovial tissues from 3 RA patients and 3 OA patients. In RA synovium, OPN was detected as brown colored stains, predominantly in synovial lining cells rather than in perivascular and sublining areas. It was absent in lymphoid aggregates. In contrast, in OA synovium OPN expression was less remarkable and scattered (original magnification ×200). HE: hematoxylin-eosin stain; Ab: antibody.

Table 1. Spontaneous OPN production in cultured synovial cells. Synovial cells were isolated enzymatically from RA (n = 5) and OA (n = 5) synovial tissues. Concentrations of OPN were measured by ELISA System 1.

	OPN, ng/ml
RA, n = 5	1695 ± 1258
OA, n = 5	415 ± 155

* $p < 0.05$.

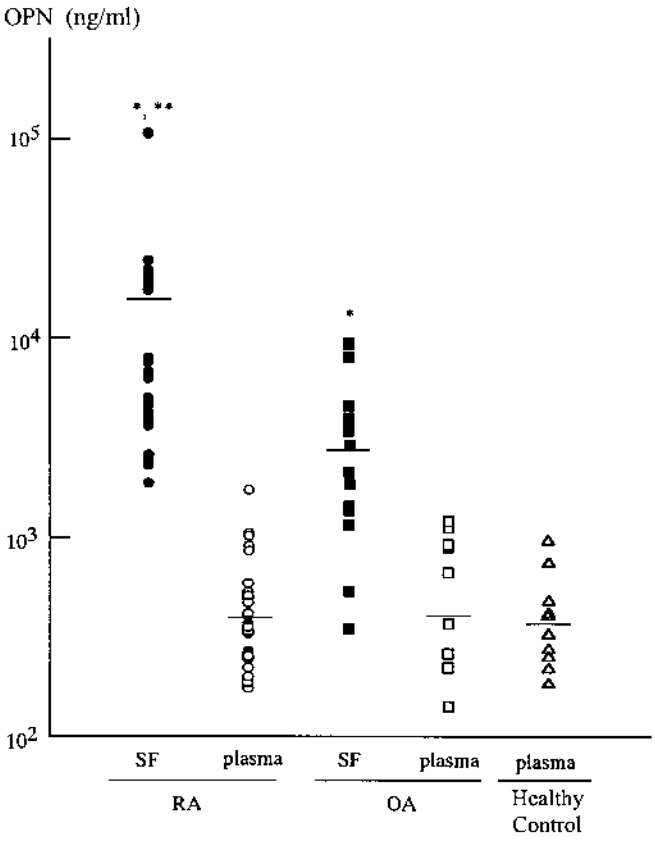

Figure 3. OPN levels were measured in plasma and SF samples from 23 RA patients by sandwich ELISA (System 1), using 2 distinct anti-human OPN antibodies, OPN1 and OPN 3. Samples from patients with OA and healthy volunteers were used as a control. *SF OPN levels vs plasma, $p < 0.05$. **SF OPN levels, RA vs OA, $p < 0.05$.

(RA vs OA, $p < 0.05$). These results suggest enhanced local OPN production in the rheumatoid joints.

Thrombin cleaved OPN was increased in RA plasma and SF. A similar result was obtained by the other ELISA system, System 2, as shown in Figure 4. The result of System 2 was correlated with that of System 1 ($r = 0.639$, $p = 0.0168$) (Figure 5). As described in Material and Methods, considering the possible recognition sites of the individual anti-human OPN antibodies (OPN1, OPN3, and OPN5), in practice, System 1 can detect only non-thrombin cleaved OPN, but not cleaved OPN. On the other hand, System 2 can detect both non-thrombin cleaved and cleaved OPN. Thus,

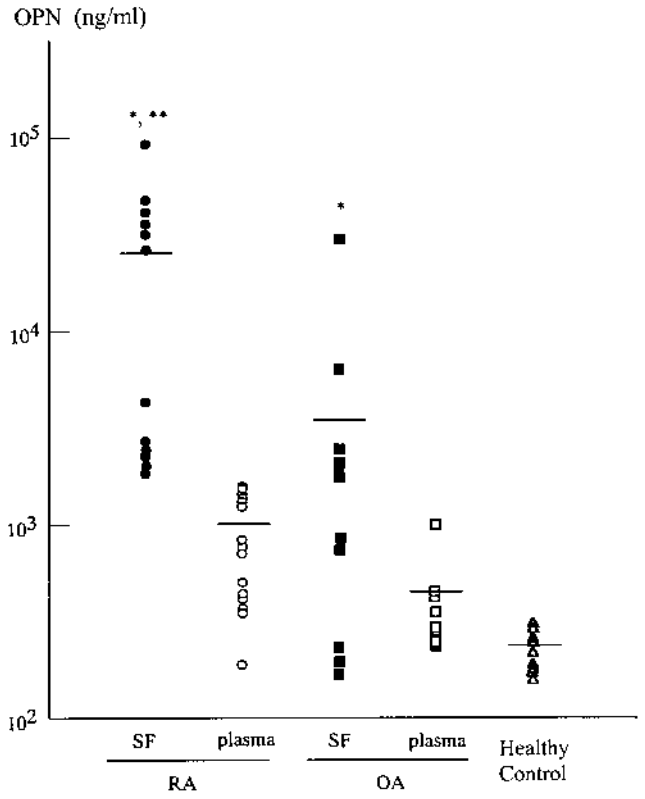

Figure 4. OPN levels were measured in plasma and SF from 23 RA patients by a second sandwich ELISA system (System 2), using 2 distinct anti-human OPN antibodies, OPN1 and OPN 5. Samples from patients with OA and healthy volunteers were used as a control. *SF OPN levels vs plasma, $p < 0.05$. **SF OPN levels, RA vs OA, $p < 0.05$.

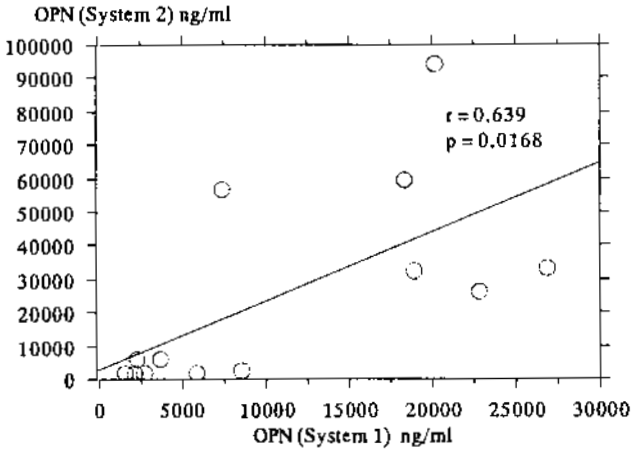

Figure 5. Correlation in OPN measurement by System 2 compared to System 1 ($r = 0.639$, $p = 0.0168$).

the ratio of the value of System 1 versus that of System 2 may reflect the ratio of thrombin cleaved versus non-cleaved OPN. If the ratio is small, the proportion of thrombin cleaved OPN may be increased. As shown in Figure 6, the ratios of the value of System 1 versus that of

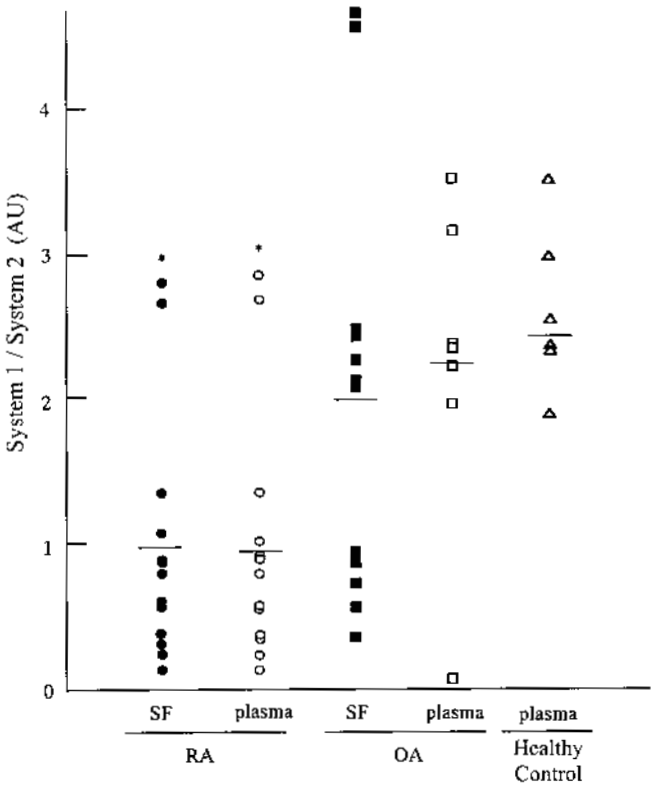

Figure 6. Thrombin cleaved OPN was increased in plasma and SF of patients with RA. The ratio of the value of System 1 versus that of System 2 may reflect the ratio of thrombin cleaved versus non-cleaved OPN. The ratios of the value of System 1 versus that of System 2 were significantly smaller in both plasma and SF of the RA patients compared with those in plasma and SF of OA patients or plasma of healthy controls. RA plasma: 0.938 ± 0.312, SF: 0.969 ± 0.244. OA plasma: 2.266 ± 0.412, SF: 1.975 ± 0.435. Healthy control plasma: 2.576 ± 0.281 (RA versus OA or healthy controls, *p < 0.05). AU: arbitrary unit.

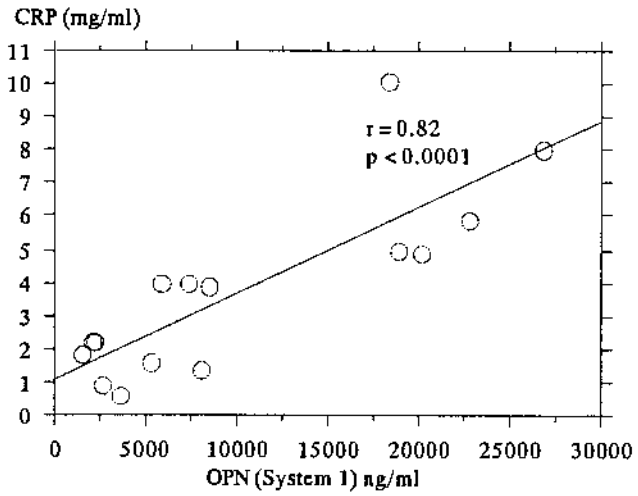

Figure 7. SF OPN levels were strongly correlated with serum concentration of CRP in patients with RA (r = 0.82, p < 0.0001, Pearson correlation coefficient), but not with any other clinical variable.

System 2 were significantly smaller in both plasma and SF from RA patients compared with those in plasma and SF of the OA patients or in plasma from healthy controls (RA plasma: 0.938 ± 0.312, SF: 0.969 ± 0.244; OA plasma: 2.266 ± 0.412, SF: 1.975 ± 0.435; healthy control plasma: 2.576 ± 0.281; RA vs OA or healthy controls, $p < 0.05$). This suggests that the proportion of thrombin cleaved OPN was increased in both blood and SF of RA patients.

SF OPN levels were correlated with serum concentration of C-reactive protein (CRP) in RA patients. Clinical data for patients with RA are summarized in Table 2. We examined whether SF OPN levels were correlated with any clinical measures of patients with RA. As shown in Figure 7, the SF OPN levels were strongly correlated with serum concentration of CRP ($r = 0.82$, $p < 0.0001$), but not with any other clinical variable. This suggests that SF OPN levels may reflect RA disease activity.

DISCUSSION

Our findings indicate enhanced local production of OPN in rheumatoid joints.

Immunohistochemistry studies revealed that OPN was highly expressed in the rheumatoid synovium, predominantly in the lining cells. Petrow, *et al* reported a similar observation[22]. We also observed that cultured synovial cells from patients with RA secreted significantly more OPN than those from patients with OA. Moreover, we recently reported high expression of OPN in inflamed synovium in collagen induced arthritis[21]. In murine arthritis, we also observed αvß3 integrin expression on the surface of cartilage attached to the proliferating synovium, of which the lining layer expressed OPN predominantly. It is well known that αvß3 is one of receptors for OPN[18-20]. These data suggest the predominant OPN expression of the lining layer of rheumatoid synovium may have pathological implications, such as the role for attachment of activated rheumatoid synovium to cartilage.

ELISA studies revealed a marked increase of OPN levels in SF of both patients with RA and with OA compared to the control plasma OPN levels, although OPN levels were not increased in the RA and OA plasma compared to the healthy controls. In addition, SF OPN levels of patients with RA were significantly higher than those of patients with OA. In contrast, Petrow, *et al* reported rather wide variations in concentration and no significant differences of SF OPN levels between patients with RA and those with OA[22]. One possible reason for the difference in these results may be the molecular fragility of OPN. OPN can easily be cleaved into 2 fragments by thrombin, since the thrombin cleavage site is present in the OPN molecule, as shown in Figure 1. This characteristic of OPN has prevented the development of reliable or stable ELISA. Thus, we collected all samples in the presence of heparin to avoid *in vitro* fragmentation by thrombin. In addition, we employed 2 distinct sandwich

Table 2. ClinIcal data of patients with RA.

Patient	Age, yrs/ Sex	Disease Duration, yrs	Stage	CRP, mg/dl	Treatments PSL, mg/day	Treatments MTX, mg/week
1	58F	14	4	3.9	5.0	0.0
2	58F	3	4	5.9	0.0	4.0
3	66F	11	3	0.9	0.0	0.0
4	65F	42	4	4.9	7.5	5.0
5	40F	2.2	2	1.8	5.0	5.0
6	62M	20	2	4.0	7.5	7.5
7	53F	19	4	4.0	7.5	0.0
8	69F	27	4	2.2	5.0	0.0
9	63F	6	4	10.1	0.0	0.0
10	74M	8	4	2.2	10.0	4.0
11	58F	22	4	8.0	10.0	0.0
12	58F	11	3	5.0	0.0	5.0
13	58F	33	4	0.6	10.0	5.0
14	70F	12	4	1.4	2.5	8.0
15	66F	6	3	1.6	5.0	5.0
16	78F	11	4	4.5	5.0	5.0
17	65F	30	4	2.0	5.0	0.0

PSL: prednisolone, MTX: methotrexate.

ELISA systems we had developed[25]. In these ELISA systems, we use different pairs of the anti-human OPN antibodies, each of which recognizes different epitopes on the OPN molecule. These ELISA systems enable us to detect thrombin cleaved OPN as well as non-cleaved OPN. System 1 can detect only non-thrombin cleaved OPN, but System 2 can detect both non-thrombin and thrombin cleaved OPN. Both ELISA systems showed a similar tendency regarding OPN levels. Interestingly, the proportion of thrombin cleaved versus non-cleaved OPN levels was increased in both plasma and SF of RA patients compared with OA patients. This seems to be logical, because thrombin activity is enhanced in conditions of inflammation, such as RA[26]. Although the implication of this remains unknown, the thrombin cleaved OPN fragments may also be involved in the pathogenesis of RA: recent reports indicate that OPN is bound to another integrin, α5ß1, after the thrombin cleavage[27].

The OPN levels of patients with RA were correlated with serum CRP. The results suggest that SF OPN levels reflect disease activity. Matsumoto, *et al* have demonstrated the induced expression of NF-IL6, a CCAAT/enhancer binding protein family of transcription factors, led to increased OPN gene expression by the subtraction cloning method[28]. In addition, we have shown that NF-IL6 is expressed and activated in the rheumatoid synovium using immunohistochemistry and the electrophoretic mobility shift assay[29]. Interestingly, the distribution of NF-IL6 is similar to that of OPN in the rheumatoid synovium. These results suggest that the production of OPN may be regulated in part by NF-IL6 and its upstream molecules, especially proinflammatory cytokines such as interleukin 1 (IL-1) and IL-6.

In addition, Petrow, *et al* report that OPN induced collagenase 1 (matrix metalloproteinase 1, MMP-1) production *in vitro* in articular chondrocytes[22], suggesting some role of OPN in matrix degradation.

These results revealed enhanced local production of OPN in rheumatoid joints, suggesting the involvement of OPN in the pathogenesis and bone destruction of RA.

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