Urokinase-type Plasminogen Activator, Receptor, and Inhibitor Correlating with Gelatinase-B (MMP-9) Contribute to Inflammation in Gouty Arthritis of the Knee

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ABSTRACT. ***Objective.*** To determine the possible role of the plasminogen activator (PA)/plasmin system in gouty arthritis.

Methods. We compared the antigenic values of urokinase-type PA (uPA), soluble uPA receptor (suPAR), and PA inhibitor type-1 (PAI-1) and the levels of matrix metalloproteinase-2 (MMP-2) and MMP-9 in 63 synovial fluid (SF) samples from knee joints of 38 patients with gouty arthritis and 20 SF samples from knee joints of 20 patients with osteoarthritis (OA).

Results. The increases of uPA, suPAR, and PAI-1 antigenic values in SF were associated with increased levels of latent MMP-9 (pro-MMP-9) ($p < 0.001$, $p < 0.001$, $p < 0.001$, respectively) in gouty arthritis, whereas a correlation was not observed between uPA, suPAR, and PAI-1 antigenic values and MMP-2 levels. Increased uPA, suPAR, and PAI-1 antigenic values in gouty arthritis SF were also correlated to each other ($p < 0.001$, $p < 0.001$, $p < 0.001$). In gouty arthritis SF, no significant relations were observed between uPA and suPAR antigenic values and leukocyte, neutrophil, or monocyte counts, while increased values of PAI-1 corresponded closely with increased leukocyte and neutrophil counts ($p = 0.005$, $p = 0.004$). Significantly higher values of the uPA, suPAR, and PAI-1 ($p < 0.001$, $p < 0.001$, $p = 0.012$) appeared in SF of gouty arthritis than in SF from patients with OA.

Conclusion. These results show a correlation of the PA/plasmin system and MMP-9 in SF from patients with gouty arthritis, resulting in increased PA catalytic activity and contributing to inflammation in gouty arthritis. (J Rheumatol 2006;33:311–7)

Key Indexing Terms:
PLASMINOGEN ACTIVATOR PLASMINOGEN RECEPTOR GOUTY ARTHRITIS
PLASMINOGEN INHIBITOR MATRIX METALLOPROTEINASE-9

Cartilage destruction is the terminal event of inflammatory joint diseases, such as gouty arthritis, clinically associated with edema, erythema of the joints, and severe pain. Urate crystals are also typically present in lavage fluids aspirated from gouty arthritis[1]. There is intense infiltration of blood-borne neutrophils into the joint space[1,2], mostly likely due to the release of an array of cytokines and chemokines, including interleukin 1 (IL-1) and IL-8[3-5], where inflammation of the synovium contributes to the breakdown of cartilage. Such a mechanism is not fully valid for degenerative joint diseases such as osteoarthritis (OA), where a relatively small number of neutrophils are detected in the synovial fluid (SF)[6-8]. An acute gout attack may be treated with the use of nonsteroidal antiinflammatory drugs (NSAID), colchicines, or corticosteroids. In addition to partial relief of the symptoms and an analgesic action, NSAID may down-regulate the matrix metalloproteinase-9 (MMP-9) in SF of gouty arthritis[9].

The MMP belong to the family of Zn^{2+} metalloproteinases that together can degrade all extracellular matrix (ECM) components. These MMP are secreted as latent precursor enzymes and can be activated by limited proteolysis, which results in a loss of molecular weight of about 10 kDa. Type IV collagenases (gelatinases) are members of the family of MMP and can be divided into gelatinase-A (MMP-2) and gelatinase-B (MMP-9). In gouty arthritis of the knee, concentrations of the latent MMP-9 (pro-MMP-9) in SF are the result of local production by either the inflammatory cells invading the affected tissue, or by synovial cells that have been stimulated as a result of inflammatory cell influx, and therefore pro-MMP-9 levels work locally for the inflammatory process[9]. MMP-2 and MMP-9 in SF contribute to the activity of gouty arthritis during physiologic turnover and pathologic destruction.

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Plasminogen activators (PA), urokinase-type (uPA) and tissue-type PA (tPA), are serine proteases that catalyze the conversion of the circulating zymogen, plasminogen, to generate the less specific serine protease, plasmin[10]. Plasmin, which is the final product of the fibrinolytic activation, can degrade basement membrane and cartilage macromolecules and activate both latent collagenases and certain enzyme cascades (complement, clotting, and the kinin system) that are important in the development of inflammatory responses[11,12]. PA inhibitor-1 (PAI-1) is the major circulating PAI, and controls the rate of plasmin generation by forming irreversible inhibitory complexes with uPA and tPA[13]. A specific cellular receptor for uPA[14], evident in rheumatoid arthritis (RA) and OA, binds uPA and localizes it at specific sites on the cell surface, e.g., cell-to-cell and cell-to-matrix contact sites[15]. uPA receptor (uPAR) may become cleaved at its cell surface anchor, thus forming a free soluble uPAR (suPAR)[16].

A pathophysiological role of the PA/plasmin system in destructive joint disease has been suggested before[17-19]. A positive correlation between both uPA and PAI concentrations in rheumatoid SF and markers of disease activity[20,21], as well as high expression of uPA, suPAR, and PAI in synovial tissue of patients with RA has been demonstrated[22,23]. In OA, uPA, uPAR, and PAI are barely detectable. Moreover, uPA was the predominant PA form in OA, while a decreased amount of PAI-1 has been found in OA cartilage, which contributes to the increased PA activity[24]. These observations suggest involvement of the PA/plasmin system in destructive arthropathy; however, detailed knowledge of the mechanisms and components involved in gouty arthritis is lacking. We hypothesized that more PA/plasmin system activities correspond with higher gelatinase levels in SF in gouty arthritis. We compared the levels of MMP-2 and MMP-9 with uPA, suPAR, and PAI-1 in SF samples from knee joints of patients with gouty arthritis and OA for investigation of the possible role of the PA/plasmin system in gouty arthritis.

MATERIALS AND METHODS

Patients. Table 1 shows the clinical and laboratory data of 63 SF samples aspirated from knee joints (30 right, 33 left) of 38 patients with gouty arthritis (diagnosed by detection of crystals of monosodium urate in the SF) and 20 SF samples from knee joints (11 right, 9 left) of 20 patients with OA (fulfilled the American College of Rheumatology criteria for OA[25]). These patients comprised 36 men and 2 women (68 and 76 yrs) ranging from 18 to 88 years of age (mean 44.89 ± 16.44) with gouty arthritis, and 10 men and 10 women ranging from 48 to 82 years of age (mean 62.10 ± 9.93) with OA. This study was conducted in accord with the principles in the Declaration of Helsinki and was approved by the Institutional Review Board of Chung Shan Medical University Hospital, Taichung, Taiwan. All patients gave informed consent for the use of their SF samples. None of the patients had received intraarticular steroid injections or colchicine treatments. In gouty arthritis, 35 SF samples were repeatedly obtained from one knee of the 14 patients before and during treatment with NSAID, while 8 SF samples were obtained from both knees of 4 other patients before NSAID treatment. The other 20 patients contributed only one sample to the study before NSAID treatment and completely recovered use of their knees after treatment.

Synovial fluids. We used arthrocentesis to obtain fluid from the suprapatellar pouch from a superolateral approach. The procedure was carried out with a 19-gauge needle. Heparinized SF samples were examined for inflammation using laboratory indicators for the condition, including leukocyte counts and differential counts. The remaining samples were centrifuged for 10 min at 3000 RPM, and aliquots were prepared for casein zymography, the measurement of uPA, suPAR and PAI-1 antigens, and gelatin zymography. Protein concentration of SF was determined according to the method described by Bradford using bovine serum albumin as standards[26].

Casein zymography. The level of uPA was determined by casein zymogram protease assays[27]. From each SF sample, 16 μl of the sample containing 20 μg total protein was briefly loaded onto a precast 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 2% casein and 20 μg/ml plasminogen. Electrophoresis was performed, then gels were washed twice with 100 ml distilled water containing 2.5% Triton X-100 on a gyratory shaker for 30 min at room temperature to remove SDS. The gel was incubated in 50 ml reaction buffer (40 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.01% NaN_3) for 12 h at 37°C, stained with Coomassie brilliant blue R-250, and destained with methanol-acetic acid-water (50/75/875, v/v/v). The values of uPA antigen were then measured by ELISA.

Measurement of uPA, suPAR, and PAI-1 antigens. uPA, suPAR, and PAI-1 antigens in SF samples were measured with appropriate ELISA kits (Biopool, Umea, Sweden). From each SF sample, 200 μl of the sample containing 20 μg protein were directly transferred to the microtest strip wells of the ELISA plate. All further procedures were performed following the manufacturer's instructions. The absorbance at 495 nm was measured in a microtest plate spectrophotometer and uPA, suPAR, and PAI-1 antigenic values were quantitated with a calibration curve using human uPA, suPAR, and PAI-1 as a standard.

Gelatin zymography. MMP-2 and MMP-9 levels were assayed by gelatin zymography[28]. Of each SF sample, 20 μl SF containing 10 μg total protein was loaded onto a precast SDS-PAGE containing 0.1% gelatin. After electrophoresis, gels were washed twice with 100 ml distilled water containing 2.5% Triton X-100 on a gyratory shaker for 30 min at room temperature to remove SDS. The gel was incubated in 50 ml reaction buffer (40 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.01% NaN_3) for 12 h at 37°C, stained with Coomassie brilliant blue R-250, and destained with methanol-acetic acid-water (50/75/875, v/v/v). Nonstaining bands representing the levels of latent and activated forms of MMP-2 and MMP-9 were measured by spot density measurement using a digital imaging analysis system (Alpha Innotech, Mt. Prospect, IL, USA). Results were expressed as integrated density value (% of 100%; IDV). IDV is the sum of all the pixel values after background correction, i.e., IDV = ∑(each pixel value – background value)[29,30].

Table 1. Characteristics of 63 SF samples from knee joints of 38 patients with gouty arthritis (GA) and 20 SF samples from knee joints of 20 patients with OA. Results are shown as mean ± SD.

	Effusion, ml	Leukocytes/μl	Neutrophils/μl	Lymphocytes/μl	Monocytes/μl
GA	28.4 ± 15.4	8.209 ± 9.750	6.765 ± 9.051	0.556 ± 0.672	0.498 ± 0.504
OA	22.9 ± 17.0	0.359 ± 0.196	0.049 ± 0.123	0.172 ± 0.133	0.069 ± 0.071

Statistical analysis. All assays were repeated 3 times to ensure reproducibility. Linear regression analysis was employed for the correlation between PA/plasmin activity, MMP-2 and MP-9, and cell counts. Student's t test was used for the analysis of data concerning uPA, suPAR and PAI-1 between gouty arthritis and OA. Statistical significance was set at $p < 0.05$.

RESULTS

There were 63 SF samples obtained from 38 patients with gouty arthritis and 20 SF samples from 20 patients with OA. Figure 1A shows the casein zymograms of the SF. uPA appeared in all tested SF samples, and then the values of uPA, suPAR, and PAI-1 antigens in SF were measured by ELISA (Table 2). Figure 1B shows gelatin zymograms of the SF samples. Pro-MMP-2 migrated at 72 kDa in all SF. Pro-MMP-9 appeared at 92 kDa regions in some SF of patients with gouty arthritis. The activated form of MMP-2, which showed a loss of the propeptide of about 10 kDa, appeared in a few gouty arthritis SF. The nonstaining bands of MMP-2 and MMP-9 were quantitatively measured.

uPA, suPAR, and PAI-1 in relation to MMP-9. As shown in Table 2, uPA, suPA, and PAI-1 antigen values varied among individual SF samples. The increases of uPA, suPAR, and PAI-1 antigenic values showed a correlation to each other in SF samples from gouty arthritis (Figure 2), while no relationship appeared in the OA SF. Considering the gouty arthritis group samples, using linear regression analysis, increased uPA, suPAR, and PAI-1 antigenic values were found to correlate significantly with increased pro-MMP-9 levels in SF (Figure 3). The same type of influential relationship was not observed between uPA, suPAR, and PAI-1 antigenic values and MMP-2 levels. Analogously, in the 14 gouty arthritis patients who gave 35 SF samples before and during treatment with NSAID, a similar relation was found between uPA ($Y = 0.817 + 8.529X$, $r = 0.662$, $p < 0.001$), suPAR ($Y = 4.212 + 2.760X$, $r = 0.441$, $p = 0.009$), and PAI-1 values ($Y = -7.245 + 5.201X$, $r = 0.727$, $p < 0.001$) and pro-MMP-9 levels, while a negative correlation was observed between uPA, suPAR, and PAI-1 values and MMP-2 levels. We found no significant correlations between uPA, suPAR, and PAI-1 values and MMP-2 levels in OA SF samples.

uPA, suPAR, and PAI-1 in relation to cell counts. In gouty arthritis SF, uPA and suPAR antigens appeared irrespective of the leukocyte, neutrophil, and monocyte counts, while increased PAI-1 antigen values were associated with increased leukocyte and neutrophil counts (Figure 4). Nevertheless, in the 14 patients with gouty arthritis who gave 35 SF samples before and during treatment with NSAID, increased uPA values correlated significantly with increased leukocyte ($Y = 3570.7 + 1753.6X$, $r = 0.436$, $p = 0.01$) and neutrophil counts ($Y = 2556.1 + 1646.6X$, $r = 0.434$, $p = 0.01$). There were also significant associations between PAI-1 values and leukocyte ($Y = 766.1 + 1280.0X$, $r = 0.574$, $p < 0.001$) and neutrophil counts ($Y = -77.9 + 1201.9X$, $r = 0.571$, $p < 0.001$). In contrast, comparison of uPA, suPAR, and PAI-1 values and cell counts in OA SF samples revealed no significant relations.

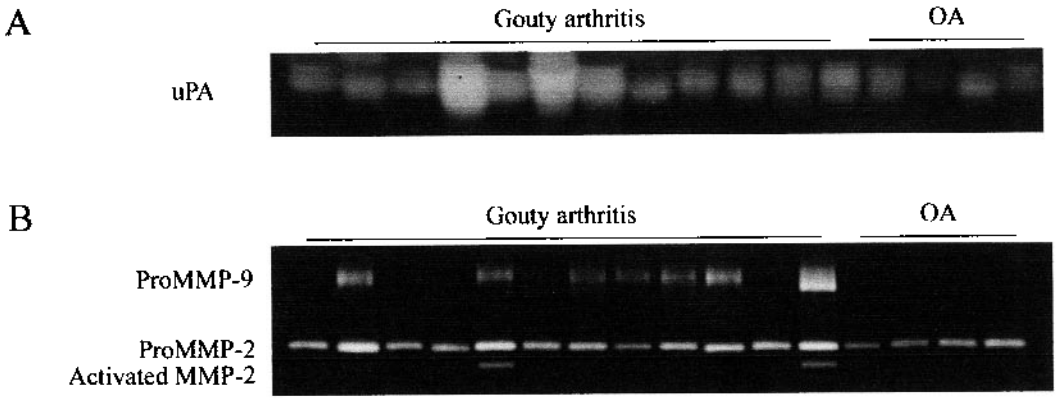
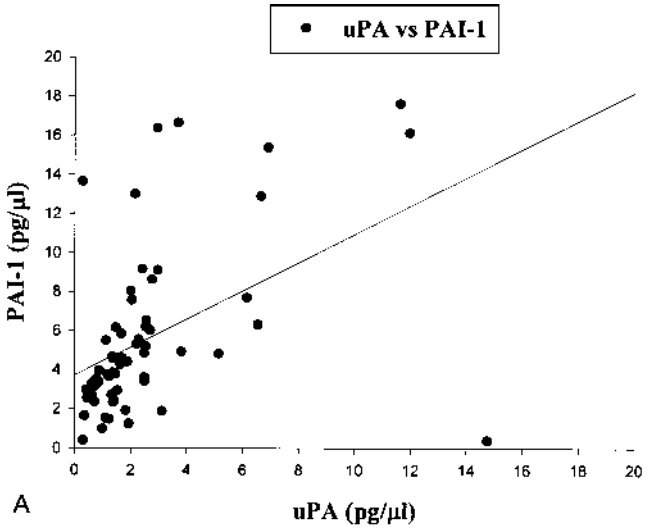


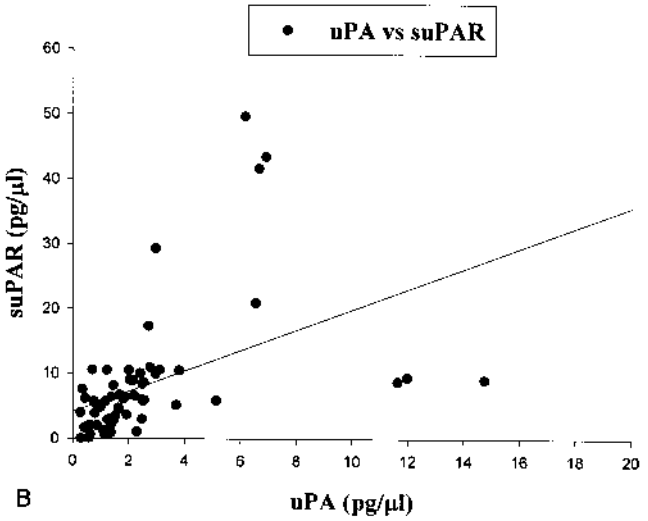
Figure 1. A. uPA levels in knee joint SF of patients with gouty arthritis and OA were determined by casein zymography. B. Gelatinase levels in knee joint SF of patients with gouty arthritis and OA were quantified by gelatin zymography. Pro-MMP-2 was produced in all SF in equivalent amounts. The active forms of MMP-2 and pro-MMP-9 appeared in some gouty arthritis SF in various amounts.

Table 2. Levels of MMP-2 and MMP-9 and values of uPA, suPAR, and PAI-1 in 63 gouty arthritis (GA) SF samples and 20 OA SF samples. Results are shown as mean ± SD.

	Pro-MMP-2, %	Active MMP-2, %	Pro-MMP-9, %	uPA, pg/μl	suPAR, pg/μl	PAI-1, pg/μl	PAI-1/uPA
GA	56.36 ± 14.91	2.01 ± 4.43	24.13 ± 32.94	2.52 ± 2.79*	8.07 ± 9.69*	5.53 ± 4.27**	3.60 ± 5.78
OA	60.16 ± 15.87	ND	ND	0.92 ± 0.37	2.74 ± 1.73	4.01 ± 1.09	4.75 ± 1.34

* $p < 0.001$, ** $p = 0.012$ versus corresponding values in OA (Student t test). ND: not detectable (see Results).





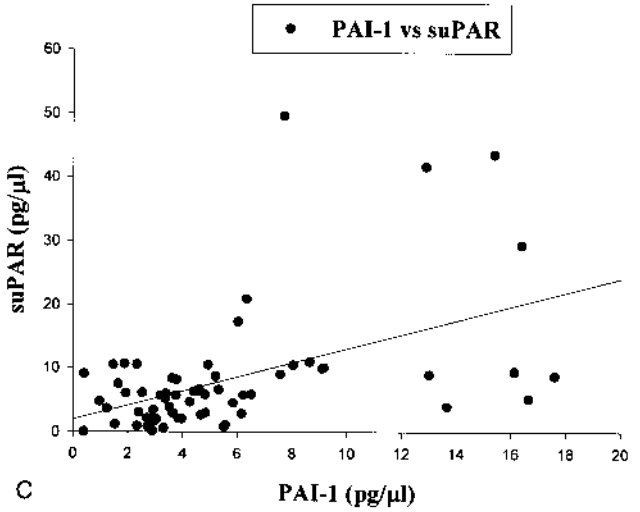


Figure 2. Associations between uPA, suPAR, and PAI-1 values in gouty arthritis SF. Linear regression analysis indicated a direct association between uPA and PAI-1 values ($Y = 3.705 + 0.723X$, $r = 0.472$, $p < 0.001$) (panel A); uPA and suPAR values ($Y = 4.099 + 1.574X$, $r = 0.453$, $p < 0.001$) (panel B); and PAI-1 and suPAR values ($Y = 1.946 + 1.107X$, $r = 0.488$, $p < 0.001$) (panel C) in SF.

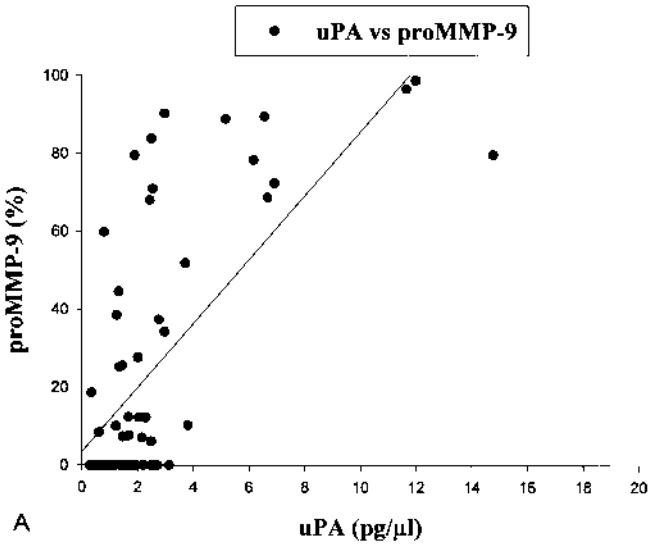
uPA, suPAR, and PAI-1 in gouty arthritis and OA. The values for uPA were significantly higher in SF from the gouty arthritis patients than in the OA patients ($p < 0.001$), and the gouty arthritis patients also had significantly higher values of suPAR and PAI-1 ($p < 0.001$ and $p = 0.012$, respectively) than the OA patients. However, the ratio of PAI-1 to uPA did not show any differences between gouty arthritis and OA ($p = 0.383$).

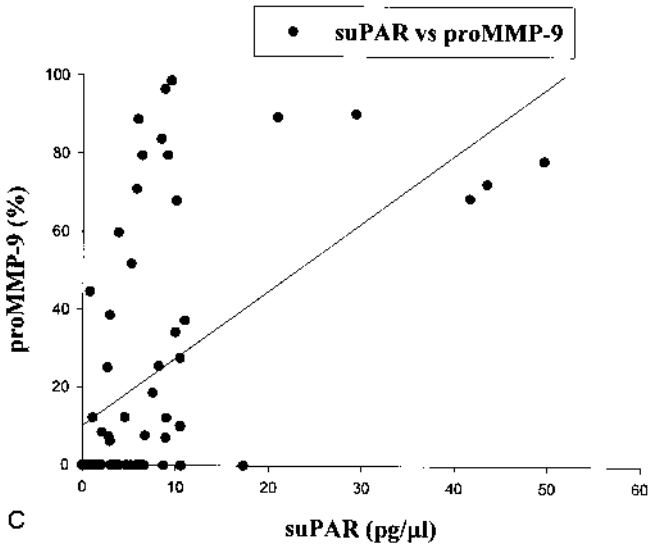
DISCUSSION

Proteolytic joint destruction in inflammatory arthropathy is mediated, at least in part, by the PA/plasmin system. The central enzyme of the PA/plasmin system, plasmin, is a broad-spectrum serine protease involved in fibrinolysis and thrombolysis, as well as in extracellular matrix degradation, required for normal and pathological forms of cell migration and tissue remodeling[31]. It is able to degrade extracellular matrix directly as well as by activating latent MMP[32]. Fibrin deposition, cell migration, and tissue remodeling are key components in the lesions of inflammatory joint diseases, such as gouty arthritis. Referring to our previous report[9], the findings of positive correlations between values for uPA, suPAR, and PAI-1 within individual patients with acute gout attack indicated that the PA/plasmin system is implicated in these aspects of an inflammatory response.

Both uPA and plasmin produce active forms of MMP that promote degradation of joint cartilage by single proteolytic cleavage[32], such as gelatinases[33] and stromelysins[34]. Recently, we have shown that MMP-9 works in the inflammatory conditions of the knee, such as gouty arthritis[9] and septic arthritis[35]. In a short-term *ex vivo* model using aprotinin, a plasmin/serine proteinase antagonist, we have also shown[36] that serine proteinase activity is required for the regulation of MMP-2 and MMP-9 in OA. In addition, the major effect of uPA in RA is deleterious, whereas that of tPA is protective[37]. Considering our current observations of significant relations between uPA, suPAR, and PAI-1 antigenic values and pro-MMP-9 levels in individual patients with acute gout before and during treatment with NSAID, we suggest the regulatory role of PA/plasmin system activation on production of gelatinases in an arthritic joint of gout.

It has been shown that PAI-1 is identified in polymorphs from normal individuals, and concentrations rise significantly in polymorphs from septic patients, but in contrast mononuclear cells from normal and septic patients contain no detectable quantities of PAI-1[38]. Interestingly, in the current study, PAI-1 antigenic values in total SF from gouty arthritis, and in repeated SF acute gout samples before and





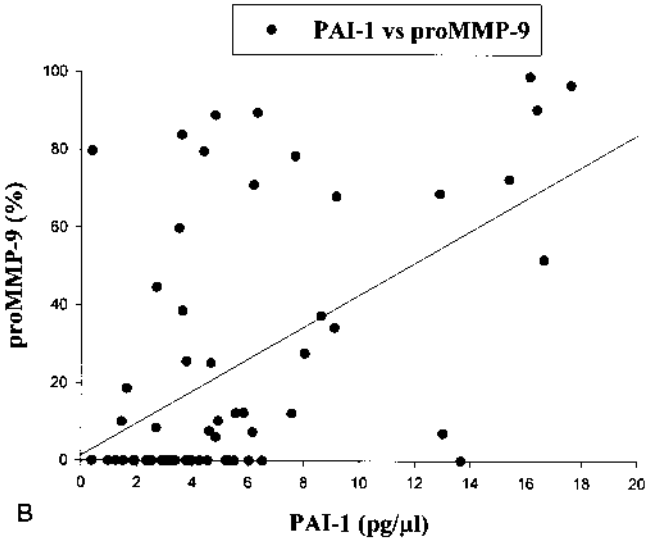


Figure 3. Correlation between uPA, suPAR, and PAI-1 values and increased pro-MMP-9 levels in gouty arthritis SF. Based on linear regression analysis, increased uPA (Y = 3.436 + 8.209X, r = 0.482, p < 0.001) (panel A); suPAR (Y = 10.125 + 1.736X, r = 0.511, p < 0.001) (panel B); and PAI-1 antigenic values (Y = 1.355 + 4.120X, r = 0.534, p < 0.001) (panel C) correlated significantly with increased pro-MMP-9 levels in SF.

during treatment with NSAID, showed associations with the leukocyte and neutrophil counts. It is known that monocytes/macrophages are a driving influence in the pathogenesis of inflammatory arthritis[39,40], and monocytes and lymphocyte cell populations play an arthritogenic role in uPA induced arthritis[41]. Somewhat unexpectedly, we observed positive correlations between uPA values and leukocyte and neutrophil counts in the repeat SF samples taken before and during treatment with NSAID, while no significant relations between uPA and suPAR antigenic values and cell counts in total SF of gouty arthritis were observed. Serially aspirating the same knees may decrease the statistical power of the analysis substantially, so the 35 SF repeat samples may skew the statistical analysis. Perhaps further studies that include more individual samples could answer this question. Intriguingly, we found instead that significantly higher values for uPA, suPAR, and PAI-1 appeared in SF of gouty arthritis than in the OA SF. These results indicate that the PA/plasmin system activity plays a pathogenic part in acute gout attack. The mechanism and regulation of PA production requires further investigation.

In OA synovium, levels of components from the PA/plasmin system are generally lower than in RA synovium[24]; however, there is a hypercoagulable and prothrombotic state in OA, with hypofibrinolysis and indirect evidence of increased fibrin generation[42]. Also, expression of u-PAR is evident in the breakdown of osteoarthritic cartilage, but not in healthy cartilage[43]. Therefore, the condition of OA increasing the PA/plasmin system activity cannot be used as a surrogate normal control[44]. However, according to our findings, the PA/plasmin system activity in gouty arthritis is actually higher than that in OA. In an acute attack of gouty arthritis, local synovial production of more PA, which subsequently accumulate in the SF, could promote more degradation of joint cartilage and bone through the PA/plasmin system-mediated proteolytic activity than in OA.

After intraarticular injection of urinary trypsin inhibitor, which has been shown to inhibit uPA, clinical improvement of patients with OA or RA was observed[45,46]. In our study, the correlations between components of the PA/plasmin system in total arthritic SF of gout and in the repeat SF samples before and during treatment with NSAID, and particularly between the increasing uPA, suPAR, and PAI-1 antigens and increasing pro-MMP-9 levels, indicate that fibrinolytic enzymes play an important role in the inflammatory joint and joint destruction. These findings, in combination with

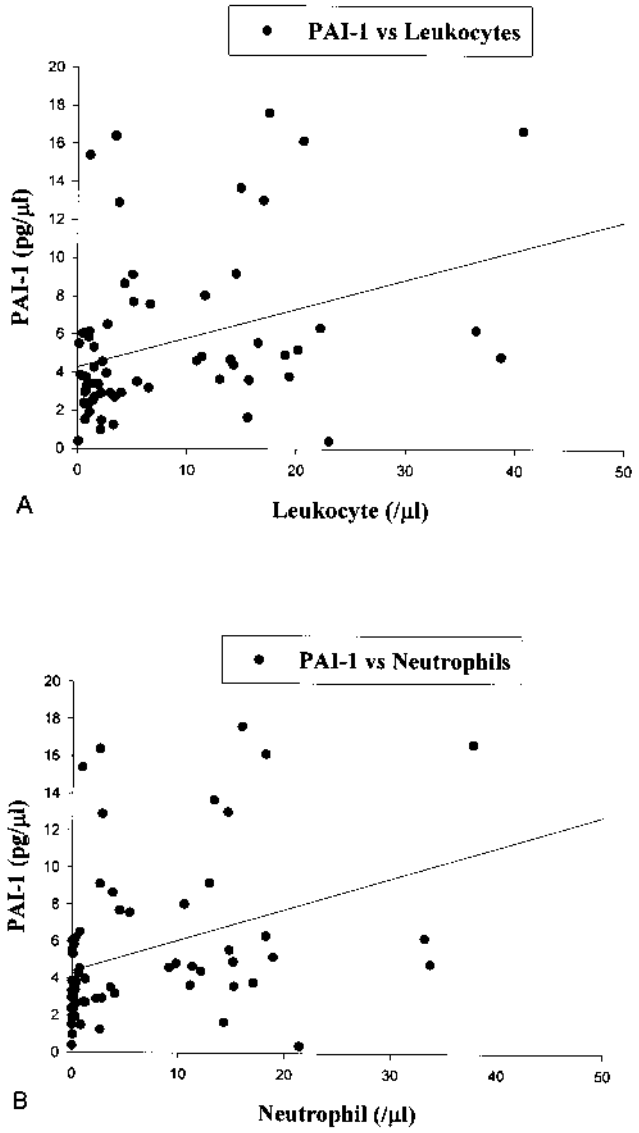


Figure 4. Relations between increased PAI-1 values and increased leukocyte and neutrophil counts in gouty arthritis SF. Increased PAI-1 antigenic values corresponded closely with increased leukocyte (Y = 3835.63 + 719.22X, r = 0.346, p = 0.005) (panel A) and neutrophil counts (Y = 2604.9 + 752.63X, r = 0.355, p = 0.004) (panel B).

our results with gelatinase B in gout[9], provide strong evidence in support of the hypothesis that increased PA/plasmin system activity correlates with higher gelatinase B concentrations that reveal the inflammatory condition of the knee joints with gouty arthritis. In addition to inhibiting uPA, suppression of uPAR signaling in gouty arthritis using antagonists of the PAI/uPAR system may be a novel therapeutic approach to downregulate degradative proteinases.

Taken together, these results support a pathophysiological role of the PA/plasmin system in the inflammation of gouty arthritis. In addition to reducing inflammation and pain, arthrocentesis for acute gouty arthritis of the knee may also decrease the MMP and PA/plasmin system activity available in SF to attack the extracellular matrix of the joint, whether it is normal or not. Although NSAID offer analgesic action, they may also downregulate the MMP and PA/plasmin system activity in SF in gouty arthritis. Further *in vivo* studies and studies of pharmacological agents that target MMP and the PA/plasmin system are required for the design of more efficacious therapies.

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