Plasminogen activators and their inhibitors in synovial fluids from normal, osteoarthritis, and rheumatoid arthritis knees

Carolyn Belcher, Fiona Fawthrop, Rowena Bunning, Michael Doherty

Rheumatology Unit,
City Hospital,
Hucknall Road,
Nottingham NG5 1PB,
United Kingdom
C Belcher
F Fawthrop
M Doherty

Division of Biomedical
Sciences and Health
Research Institute,
Sheffield Hallam
University,
Sheffield S1 1WB,
United Kingdom
R Bunning

Correspondence to:
Ms C Belcher.

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Abstract

Objectives—To establish baseline concentrations of plasminogen activators and their inhibitors in normal knee synovial fluids, and to compare them with well characterised osteoarthritis (OA) and rheumatoid arthritis (RA) knee fluids.
Methods—A total of 26 normal subjects, 71 patients with OA, and 17 patients with RA underwent knee aspiration. Patients with OA were subclassified according to presence of nodal generalised OA (NGOA) and synovial fluid calcium pyrophosphate crystals. Clinical assessment of inflammation (graded 0–6) was undertaken in OA and RA patients. Plasminogen activator (PA), plasminogen activator inhibitor (PAI), and urokinase-type PA receptor (uPAR) antigen concentrations were determined by enzyme linked immunosorbent assay. The species of PAs present were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
Results—Concentrations of all antigens (uPA, tissue-type PA (tPA), uPAR, and PAI-1), were significantly greater in RA than OA; those in OA were significantly greater than normal. The concentrations showed no direct association with clinically assessed inflammation of the knee. In normal fluids, no associations with age were observed. Antigen concentrations (uPA, tPA, and uPAR) in NGOA differed from those in other subclasses of OA, but the species of PA present did not appear to vary between disease groups. The predominant PA appeared to have identity with uPA.
Conclusion—Because of the greater concentrations of these antigens in OA compared with normal fluids, OA cannot be used as a surrogate normal control in studies of the PA/PAI system. Alteration of the PA/PAI system was confirmed in RA and OA knee fluids, with greater changes evident in RA. The finding of different concentrations of PA antigens in NGOA compared with other OA fluids further supports a different pathogenic mechanism in this subset.

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Plasminogen activators (PAs) are serine proteases that convert the zymogen, plasminogen,
to plasmin. There are two forms of PA: tissue-type (tPA) and urokinase-type (uPA); they are products of different genes, having different molecular weights and immunological reactivity, and differing in their affinity for fibrin.[1] [2]

The PA system can have both direct and indirect effects on extracellular matrix degradation. Plasminogen activators have a direct role in the degradation of extracellular matrix glycoproteins,[3] and plasmin, formed by activation of plasminogen, can degrade connective tissue components, including proteoglycans.[4] [5] The metalloproteases collagenase and stromelysin are also implicated in cartilage degradation,[6] and plasmin can transform their latent forms, procollagenase and prostromelysin, into the active forms,[7] [8] which can then specifically attack the extracellular cartilage matrix.

The activity of uPA and tPA is regulated by specific plasminogen activator inhibitors (PAIs). Both PAI-1 and PAI-2 form stable complexes with tPA and uPA. PAI-1 is the main PAI in plasma[9] and is also bound to extracellular matrix, where it may regulate matrix breakdown.[10] It is produced by endothelial cells, platelets, and other cells resident in the joint, such as synovial cells and chondrocytes.[2] [11] [12] PAI-2 is produced by inflammatory cells such as monocytes and polymorphonuclear leucocytes, and also by chondrocytes and synovial cells.[2] [12] [13] In common with PAI-1, it may regulate extracellular tissue remodelling. It is important to study PAIs, as the balance between PA and PAI concentrations is an important determinant of overall PA activity.

The presence of a cellular receptor for uPA was first demonstrated by Vassalli *et al*.[14] The uPA receptor (uPAR) binds the proform and enzymatically active uPA with high affinity,[15] focusing uPA activity on the cell surface and enhancing the rate of activation of pro-uPA and plasminogen activation.[15] [16] uPARs have been described on a variety of cell types, including monocytes, chondrocytes, and synovial cells.[17] [18] In addition, the number of uPARs appears to be increased on monocytes in RA, and on synovial cells and chondrocytes in OA.[18] [19]

Few studies have examined the PA/PAI system in human joint fluids. Plasminogen activators have been detected in synovial fluid from patients with rheumatoid arthritis (RA) and osteoarthritis (OA).[20–22] Kikuchi *et al*[21] found that PAs could be detected in both types of arthritis, but concentrations were greatest in RA. Mochan *et al*[20] also found a significantly

greater PA activity in RA compared with OA, which they believed to be the result of an increase in PA antigen concentrations, rather than a decrease in PAI activity. Saxne *et al*[22] found increases in uPA, PAI-1, and PAI-2 concentrations in RA compared with OA.

No data are currently available concerning normal synovial fluid concentrations of PAs or PAIs, or any possible associations with the age of the subject. Furthermore, no study has examined possible differences between subsets of OA or the relationship between PA/PAI concentrations and clinically assessed inflammation of the aspirated joint. The objectives of this study were therefore to establish baseline concentrations in normal knees, to examine possible age related changes, and to determine PA/PAI concentrations in well characterised OA and RA knees.

Subjects and methods

This study was approved by the local Research Ethics Committee.

SUBJECTS

Normal subjects had never experienced knee symptoms, had normal knees on clinical examination, and had no clinical evidence of peripheral joint OA or other joint disease. Knee radiographs were obtained in those older than 50 years, to exclude unsuspected mild OA or chondrocalcinosis.

Patients with symptomatic knee OA all had radiographic evidence of joint space reduction and bone response (osteophyte with or without sclerosis) in at least one compartment (medial or lateral tibiofemoral, patellofemoral). Full clinical examination, radiographic screening, and synovial fluid crystal analysis were used to assign these patients to OA subsets: *pauci-articular, large joint OA* (OA)—patients with isolated knee OA or with additional involvement of other large joints (hips, shoulders), but not hand joints; *chronic pyrophosphate arthropathy* (CPA)—knee OA with calcium pyrophosphate crystals identified in knee synovial fluid by compensated polarised light microscopy (with or without radiographic chondrocalcinosis of the knee); *nodal generalised OA* (NGOA)—knee OA and coexistent Heberden's (with or without Bouchard's) nodes and radiographic evidence of interphalangeal OA affecting at least three rays of each hand.

Standing anteroposterior and lateral 30° plain radiographs taken at the time of, or within six months of, aspiration were obtained in all OA patients. Joint space narrowing and osteophyte formation were each scored 0–3 in each of the three compartments, using an atlas.[23] A summated score (0–18) was calculated for each knee.

Patients with rheumatoid arthritis (RA) fulfilling American College of Rheumatology criteria[24] and with symptomatic knee involvement were also studied. Synovial fluid was also obtained from two patients during episodes of acute pseudogout (self limiting florid acute

synovitis with calcium pyrophosphate crystals demonstrated in synovial fluid, resolving completely within three weeks).

In all patients (OA subsets, RA, pseudogout) clinical inflammation of the knee at the time of aspiration was designated active (score 4–6) or inactive (score 0–2) using a summated score of six clinical parameters.[25]

SAMPLE COLLECTION

Knees were aspirated to apparent dryness via a medial approach, and the aspirated volume recorded. A small sample was examined for the presence of crystals and the remainder collected into sterile plastic containers on ice, centrifuged at 2500 *g* for 15 minutes at 4°C to remove cells and crystals, and the resulting supernatant stored frozen at –80°C.

MEASUREMENT OF PAI-1 ANTIGEN, PAI-2 ANTIGEN, uPA ANTIGEN, AND uPAR ANTIGEN BY ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Imubind ELISA kits (American Diagnostica Inc) were used. Absorbances were read on a Dynatech MR5000 plate reader, using BioLinx software. The kits detected active, inactive, and complexed forms of each antigen. The lower limits of detection were 1 ng/ml PAI-1, 50 pg/ml PAI-2, 10 pg/ml uPA, and 0·1 ng/ml uPAR.

MEASUREMENT OF tPA ANTIGEN BY ELISA

A Chromogenix Coaliza kit (supplied by Quadratech) was used. Absorbances were read on a Dynatech MR5000 plate reader, using BioLinx software. The lower limit of detection of the assay was 0·5 ng/ml tPA.

MOLECULAR WEIGHT CHARACTERISATION OF PLASMINOGEN ACTIVATORS BY SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was carried out according to the method of Roche *et al.*[26] A 4% (w/v) acrylamide stacking gel and 9% (w/v) resolving gel were used. Two gels were run in parallel, one of the resolving gels containing casein 1 mg/ml and human plasminogen 5 µg/ml and the other containing casein only, to show that the activity seen was plasminogen dependent. Samples for electrophoresis were diluted in 0·5 mol/l Tris/HCl (pH 6·8) containing 10% (w/v) SDS, 32% (v/v) glycerol, and 0·5% (v/v) bromophenol blue, and were applied to gels without boiling. Synovial fluid samples were diluted 1 in 2 and had 5 µg cyanogen bromide digested fibrinogen fragments added as tPA stimulator. Prestained standards covered the molecular weight range 36 000–198 000. Gels were run overnight and then washed in 2·5% (v/v) aqueous Triton X-100 for one hour to remove the SDS. For development of plasminogen activator bands, the gels were incubated at 37°C for 24 hours in 0·1 mol/l Tris-HCl (pH 8·1). Staining was with Coomassie

brilliant blue R250. Plasminogen activator activity appeared as clear bands against a dark background where plasminogen activators had converted plasminogen to plasmin and the plasmin had degraded the casein in the gel.

STATISTICAL ANALYSIS
Comparison between disease groups was by the Mann-Whitney U test with a Bonferroni correction. Correlations were calculated using Spearman's correlation coefficient.

Results

PATIENT CHARACTERISTICS
The table shows details of the patients studied. Although a good age range was obtained in normal subjects, their mean age was lower than that of patients in all the disease groups ($p < 0.05$). Minor differences in radiographic scores were noted in the OA subsets: osteophyte formation and joint space narrowing were significantly lower in OA than in CPA ($p < 0.01$, $p < 0.05$, respectively).

uPA ANTIGEN CONCENTRATIONS IN SYNOVIAL FLUID
uPA concentration was significantly greater in the RA group (median 3.11 ng/ml) than in the OA (median 1.34 ng/ml) and normal (median 0.73 ng/ml) groups ($p < 0.001$). Patients with OA had significantly greater concentrations of uPA than normal subjects ($p < 0.001$) (fig 1A). The two samples from patients with pseudogout had uPA concentrations similar to those in patients with RA (1.48 ng/ml and 3.69 ng/ml). The CPA and NGOA groups had significantly smaller uPA concentrations than the OA group ($p < 0.001$) (fig 1B).

tPA ANTIGEN CONCENTRATIONS IN SYNOVIAL FLUID
Patients with RA had significantly greater tPA concentrations (median 3.94 ng/ml) than those with OA (median 1.63 ng/ml) and normal subjects (median 1.28 ng/ml) ($p < 0.001$), and the concentration in those with OA was significantly greater than that in normal subjects ($p < 0.001$) (fig 2A). The samples from patients with pseudogout had concentrations of tPA similar to those in patients with RA (2.35 ng/ml and 4.76 ng/ml). Concentrations of tPA in patients with NGOA were significantly smaller than those in patients with OA ($p < 0.001$) (fig 2B).
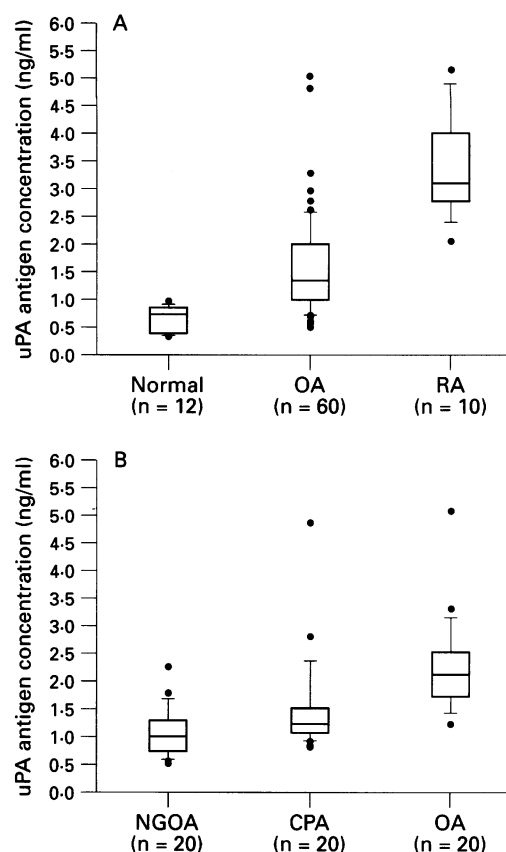

Figure 1   Comparison of urokinase-type plasminogen activator (uPA) antigen concentration between disease groups (A) in normal, osteoarthritis (OA), and rheumatoid arthritis (RA) subjects, and (B) in subclasses of OA. Central solid line = median; box = middle 50% of the data; error bar cap lines = 10th and 90th centiles. Dots represent individual data points outside the 10th and 90th centiles. NGOA = Nodal generalised OA; CPA = chronic pyrophosphate arthropathy; OA = pauciarticular, large joint OA.

uPAR ANTIGEN CONCENTRATIONS IN SYNOVIAL FLUID
The concentration of uPAR was significantly greater in patients with RA (median 14.2 ng/ml) than in those with OA (median 4.33 ng/ml) and normal subjects (median 0.95 ng/ml) ($p < 0.001$), and the concentration in patients with OA was significantly greater than that in normal subjects ($p < 0.001$) (fig 3A). The samples from patients with pseudogout had uPAR concentrations similar to those in RA patients (11.23 ng/ml and 19.2 ng/ml). Those in the NGOA subgroup had significantly smaller uPAR concentrations than those in the CPA subgroup ($p < 0.01$) (fig 3B).

PAI-1 ANTIGEN CONCENTRATIONS IN SYNOVIAL FLUID
Patients with RA had significantly greater PAI-1 concentrations (median 117.53 ng/ml) than

Patient characteristics

Patient group	Number of patients	Number of knees	Sex (M:F)	Age (yr)	Osteophyte formation	Joint space narrowing
RA	13	17	4:9	60 (30–85)	—	—
Normal	24	26	9:15	39 (21–81)	—	—
OA	22	31	13:9	71 (41–88)	4·0 (0·38)	3·59 (0·22)
CPA	15	20	11:4	72 (59–88)	5·8 (0·44)	4·7 (0·36)
NGOA	16	20	2:14	75 (63–87)	5·33 (0·46)	4·39 (0·35)

Values are mean (range) or mean (SEM).
RA = Rheumatoid arthritis; OA = osteoarthritis; CPA = chronic pyrophosphate arthropathy; NGOA = nodal generalised OA.
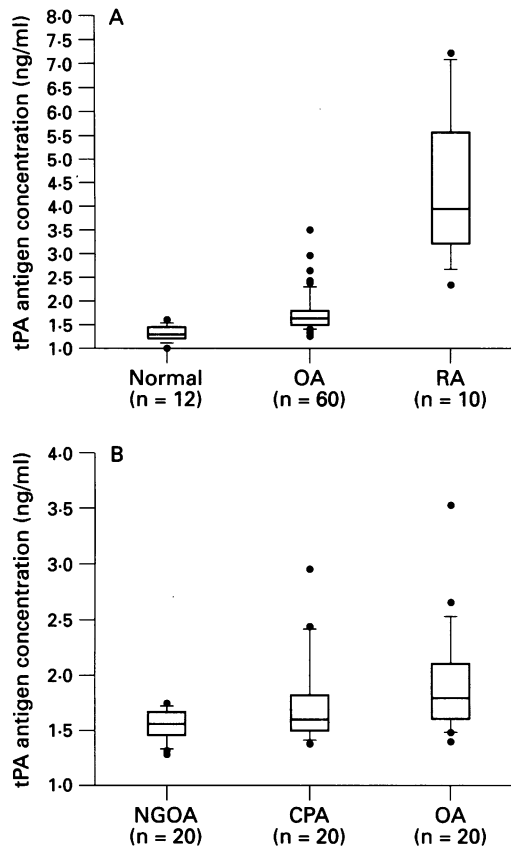
Figure 2 Comparison of tissue-type plasminogen activator (tPA) antigen concentration between disease groups (A) in normal, OA, and RA subjects, and (B) in subclasses of OA. Central solid line = median; box = middle 50% of the data; error bar cap lines = 10th and 90th centiles. Dots represent individual data points outside the 10th and 90th centiles. Abbreviations as in figure 1.
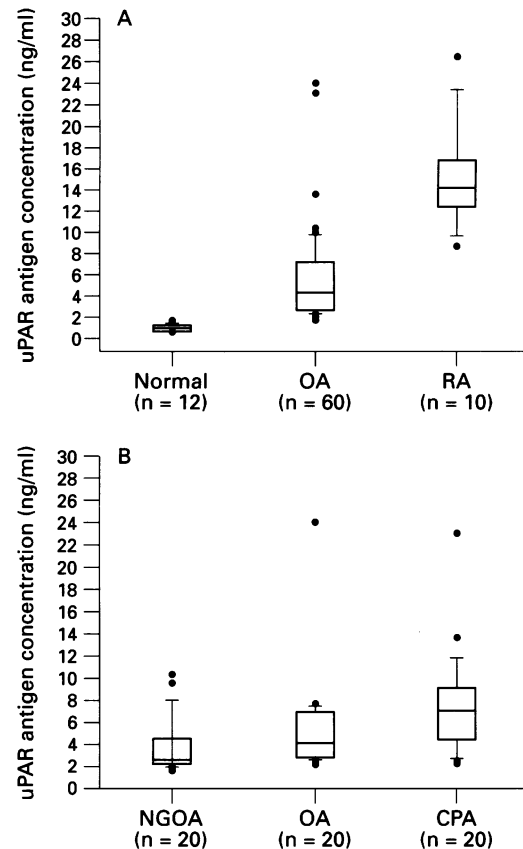
Figure 3 Comparison of urokinase-type plasminogen activator receptor (uPAR) antigen concentration between disease groups (A) in normal, OA, and RA subjects, and (B) in subclasses of OA. Central solid line = median; box = middle 50% of the data; error bar cap lines = 10th and 90th centiles. Dots represent individual data points outside the 10th and 90th centiles. Abbreviations as in figure 1.

those with OA (median 65·84 ng/ml) and normal subjects (median 27·8 ng/ml) (p < 0·01), and those with OA had significantly greater PAI-1 concentrations than normal subjects (p < 0·001) (fig 4). Samples from patients with pseudogout had PAI-1 concentrations similar to those with RA (68·59 ng/ml and 186·52 ng/ml).

PAI-2 ANTIGEN CONCENTRATIONS IN SYNOVIAL FLUID
PAI-2 was undetectable in 12 samples from normal individuals, was detectable in only seven of 20 OA samples (median 1·05 ng/ml), and was detected in all RA samples (median 7·98 ng/ml).

EFFECT OF CLINICALLY ASSESSED INFLAMMATION ON PA/PAI PARAMETERS
There were no differences between active (inflamed) and inactive (uninflamed) samples for all parameters measured.

EFFECT OF AGE ON PA/PAI PARAMETERS
None of the parameters measured showed a significant correlation with age in normal subjects.

CORRELATIONS BETWEEN PA AND PAI PARAMETERS
All parameters measured (uPA, tPA, PAI-1, PAI-2, and uPAR) were increased in parallel, with Spearman's correlation coefficient ranging from 0·63 to 0·82 (p values 0·001–0·05), demonstrating that the antigen concentrations do not vary in isolation.
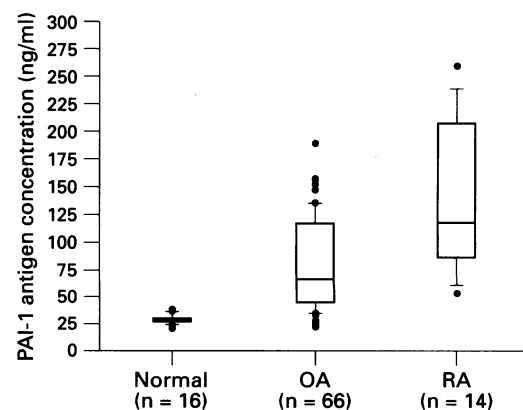

Figure 4 Comparison of plasminogen activator inhibitor-1 (PAI-1) antigen concentration between disease groups. Central solid line = median; box = middle 50% of the data; error bar cap lines = 10th and 90th centiles. Dots represent individual data points outside the 10th and 90th centiles. Abbreviations as in figure 1.

CHARACTERISATION OF PLASMINOGEN
ACTIVATORS BY SDS-PAGE
SDS-PAGE performed on synovial fluid from
different disease groups revealed similar
patterns of enzymes in all groups. Intensities of
bands varied between disease groups, but PA
species did not (fig 5). A band produced at
apparent $M_r \approx 42\,000$ did not appear in the
absence of plasminogen, identifying it as a PA.
This species comigrated with a standard uPA
sample, suggesting identity with uPA. However,
a doublet produced at apparent $M_r \approx 91\,000$
and $98\,000$ appeared on both plasminogen
containing and control gels. These enzymes
would appear not to be PAs, and their identity
remains uncertain; however, they are probably
not metalloproteases, because development of
the bands could not be inhibited by 1 mmol/l
EDTA.

Discussion

This is the first study to examine PA and PAI
parameters, including uPA receptor concen-
tration, in normal human synovial fluid, and to
determine the effect of age on these par-
ameters. It is also the first study to investi-
gate possible differences in concentrations of
PA/PAI antigens in OA subsets. As a group, the
normal subjects were younger than the OA and
RA patients. However, no correlation between
PA/PAI parameters and age was seen within the
broad range of the normal group, so age alone
is unlikely to account for the differences
observed between the disease groups.

Differences in antigen concentrations between
RA patients, OA patients (including sub-
classes), and normal subjects were consistent.
Concentrations in synovial fluid from RA
patients were significantly greater than those in
OA, and those in synovial fluid from OA
patients were significantly greater than those in
normal synovial fluid. tPA antigen concen-
trations in all groups were within the normal

plasma range of 1–12 ng/ml. This supports
previous reports of tPA in very small concen-
trations in synovial fluid, mainly from patients
with RA.[20] [22] [27] Cytokine stimulated chondro-
cytes and endothelial cells are potential sources
of tPA in these joints.[28] [29]

uPA has been identified as the predominant
synovial fluid PA in joint disease.[20] [22] [30] In our
study, concentrations of uPA found in OA and
RA joints were greater than the normal plasma
values of <1 ng/ml, agreeing with previous
findings.[22] [27] [30] This suggests that cells within
the joint, both resident and inflammatory,
may be a source of uPA. uPA is a product of
chondrocytes, synovial cells, monocytes, and
polymorphonuclear leucocytes;[28] [31-33] greater
concentrations of uPA in RA may thus
reflect an increase in inflammatory cells in the
joint and increased cellular activation by
cytokines.[22] [32]

In plasma, uPAR concentrations are less
than 0·1 ng/ml.[34] Concentrations in synovial
fluid are greater than this, in both normal and
diseased joints. Shedding of uPARs may
represent a mechanism of control of uPA
activity, the soluble receptors competing with
cell surface receptors for uPA binding, and thus
limiting cell surface plasminogen activation.
Increased concentrations of cytokines such as
tumour necrosis factor in inflamed joints may
increase expression and, perhaps, shedding of
receptors.[17]

PAI-1 concentrations remained in the
normal plasma range of 4–43 ng/ml in normal
synovial fluid, but were increased in diseased
joints. Brommer et al[30] found that PAI-1
concentrations in synovial fluid from patients
with RA were greater than in those in normal
plasma, but Saxne et al[22] found concentrations
in synovial fluid from patients with RA and OA
to be within the normal plasma range. PAI-1
can be produced by a number of cell types in
the joint.[2] [11] [12] Increased concentrations in RA
may be the result of increased cytokine con-
centrations.[32] However, cytokines may have
differential effects on PAI-1 production in
different cell types: for example, IL-1 increases
PAI-1 in endothelial cells, but decreases it in
chondrocytes and synovial cells.[11] [12] [35] Such
effects may give rise to variability in PAI-1
concentrations in different patients.

PAI-2 was undetectable in normal synovial
fluid and concentrations in OA were close to
the limit of detection of the assay; however, it
was detected in all synovial fluid samples from
RA patients. This is in agreement with the
findings of other workers.[22] Increased PAI-2
concentration may be an index of infiltration of
the joint by inflammatory cells, as these are a
major source.[2] [22] In addition, IL-1 stimulates
PAI-2 production in synovial fibroblasts.[12] An
increase in PAI concentration may indicate an
attempt to maintain the PA/PAI balance in
joint disease.

When our OA group was subdivided into
three subsets, results varied. The NGOA sub-
set appeared to differ for uPA antigen, tPA
antigen, and uPAR antigen, while the CPA
subset showed a difference only for uPA antigen
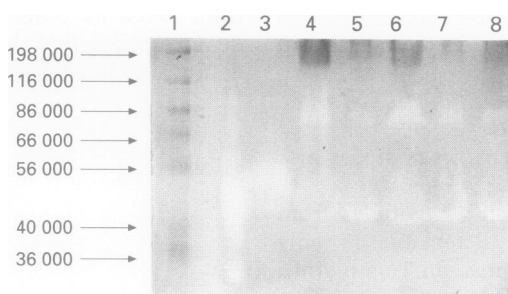and uPAR antigen. Such differences support

Figure 5   Electrophoretic separation of plasminogen activator
species present in synovial fluid from patients in different
disease groups, on sodium dodecyl sulphate-polyacrylamide
gel containing plasminogen and casein. LANE 1: prestained
molecular weight markers; LANE 2: urokinase-type
plasminogen activator (uPA) standard (high molecular
weight) at a concentration of 6·25 U/ml (approximately
78 ng/ml); LANE 3: tissue-type plasminogen activator
(tPA) standard (Bowes melanoma, two chain) at a
concentration of 6·25 U/ml (approximately 9·5 ng/ml);
LANE 4: pseudogout synovial fluid (estimated
uPA = 1·48 ng/ml; tPA = 2·35 ng/ml); LANE 5: normal
synovial fluid (estimated uPA not measured;
tPA = 1·61 ng/ml); LANE 6: RA synovial fluid (estimated
uPA = 3·12 ng/ml; tPA 3·73 ng/ml); LANE 7: NGOA
synovial fluid (estimated uPA = 1·01 ng/ml;
tPA = 1·68 ng/ml); LANE 8: OA synovial fluid
(PA estimations not done).

differentiation between NGOA and other forms of OA, with possible differences in pathogenesis. Measurement of other biochemical markers in subsets of OA would therefore be of interest.

For all antigen concentrations, samples from patients with pseudogout had values similar to those from patients with RA. This may indicate some link between PA/PAI parameters and florid inflammation. However, none of the antigen concentrations showed a difference between active and inactive disease samples. This could reflect insensitivity of the clinical assessment of inflammation, though clear correlation between complement activation and this clinical score[25] would argue against this. It may be, therefore, that the greater concentrations observed in RA and pseudogout relate to qualitative rather than quantitative differences in inflammation.

All antigen concentrations were increased in parallel, that of no one antigen varying in isolation. This would suggest an upregulation of the entire PA/PAI system. As PA concentrations increased, PAI concentrations also increased, perhaps reflecting a mechanism to neutralise any increased enzyme activity. However, there may still be an increase in the direct and indirect effects that the PA system has on extracellular matrix degradation. This, in turn, could affect the pathophysiology of RA and OA.

The presence of tPA was difficult to demonstrate in our study. tPA activity on the gels may have been masked by the doublet banding that occurred at $M_r \approx 91$–98 000 in both control and plasminogen containing gels. Alternatively, this doublet may represent tPA, as other workers have shown that tPA from synovial fluid runs at $M_r \approx 90$ 000—possibly the result of association with other proteins in synovial fluid.[21 36] The occurrence of a similar doublet in the control gel could be the result of activation of plasminogen, derived·from synovial fluid, which runs close to tPA on the gel.[36] However, spiking of synovial fluid with tPA did not produce additional bands of lysis, perhaps suggesting degradation of tPA by other proteolytic enzymes in synovial fluid. Combination of tPA with PAIs would have produced additional bands of lysis, $M_r \approx 100$ 000 corresponding to enzyme-inhibitor complexes, as described by Levin.[29] The identity of lysis bands could be confirmed by immunoprecipitation of samples before electrophoresis, using antibodies to tPA or uPA.

Our findings comparing concentrations of PAs and PAIs in synovial fluid from patients with RA and OA are broadly in agreement with those of other workers.[22 27 30] However, we were also able to compare these values with those in normal synovial fluid, and to measure uPAR concentrations. The increased concentrations of PA/PAI parameters in OA compared with normal indicate that it is not valid to use OA as a surrogate control in this type of study. Our data also confirm that alterations in the PA/PAI system occur in both OA and RA, and these may contribute to the pathophysiology of these diseases.

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