Identification and functional importance of plasma kallikrein in the synovial fluids of patients with rheumatoid, psoriatic, and osteoarthritis

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Abstract

Objectives—To determine and identify, unequivocally, if plasma kallikrein (PK) is present in the synovial fluid of patients with rheumatoid (RA), psoriatic (PA) and osteo (OA) arthritis, and to consider its functional importance in the inflamed joint.

Methods—Therapeutically aspirated synovial fluids (pooled and individual samples, $n = 66$) were obtained from patients with arthritis. In addition, serum ($n = 14$) was collected from RA patients, and saliva ($n = 10$) and urine ($n = 10$) from normal individuals. Enzymic (amidase) and immunoreactive activities of PK and its precursor, prokallikrein (PPK), were determined. The presence of PK was assessed by incubation with soya bean trypsin inhibitor (SBTI), and by adsorption with anti-PK antibody linked to Sepharose. An enzyme-linked immunosorbant assay (ELISA) for PK was developed for quantitative measurement of total PK in biological fluids. Enhancement of the PK dose-response by RA synovial fluid made it necessary to remove RF from synovial fluids before determination of PK by ELISA.

Results—Amidase activity was demonstrated in synovial fluid pools and shown to be inhibited completely by SBTI, and removed by prior treatment with anti-PK Sepharose. Total PK activity (PK + PPK) from individual synovial fluid specimens did not differ significantly between patients with RA (median activity 76 mU/g protein), PA (80 mU/g protein) or OA (60 mU/g protein). Similar results were obtained when active PK alone was measured. No correlation was found between active PK or total PK values and the severity score for individual joints. Most of the measured immunoreactivity was removed by adsorption with anti-PK antibody linked to Sepharose.

Conclusions—The results support the hypothesis that plasma kallikrein is present in synovial fluid. The enzyme may be important in the pathogenesis of inflamed joints.

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Kinins are vasoactive peptides that cause constriction of venules, dilatation of arterioles,

and increased permeability of capillary membranes, and interact with sensory nerve terminal transmitters to evoke pain. They are formed by the cleavage of H- and L- kininogens by serine proteases known as kallikreins (plasma and tissue), which occur in various biological fluids, tissues, and cells.¹ It has been proposed that the pain and swelling in inflammatory joint disease may be mediated by kinins, and the evidence in support of this view has been reviewed elsewhere.²

We have previously identified tissue kallikrein in synovial fluid of patients with inflamed joints, particularly patients with rheumatoid arthritis (RA), and thus established the presence of one of the two major kinin forming enzymes (kininogenases) in arthritic joints.³ Plasma kallikrein (PK) was first implicated in gout by Kellermeyer and Breckenridge.⁴ Although PK has been demonstrated to occur in the synovial fluid (SF) of patients with RA,^{5,6} the amidase activity present has not been shown to be attributable exclusively to this enzyme. The purpose of the present study was to examine critically and identify levels of enzymic and immunoreactive PK, to enable their determination in the SF of patients with inflamed joints.

Patients, samples and methods

PATIENTS

Knee joint SF was collected from randomly selected patients with diagnoses of RA (pooled $n = 14$; individual $n = 15$), osteoarthritis (OA) (pooled $n = 13$; individual $n = 8$) and psoriatic arthritis (PA) (pooled $n = 11$; individual $n = 8$), attending the Rheumatology Unit of Bristol Royal Infirmary (BRI) (table 1). The severity of joint inflammation was assessed clinically at the time of the SF aspiration, and was based on the degree of swelling, joint tenderness, local temperature, and erythema.

SAMPLES

Synovial fluid—Therapeutically aspirated SF from the knee joint was centrifuged at 3000 *g* for 10 minutes at room temperature. The volume of SF aspirated varied with each patient (table 1). After centrifugation the supernatants were thoroughly mixed, and 1 ml aliquots stored at -20°C . To reduce viscosity, before assay the samples were treated with 22.5 IU/ml of hyaluronidase (Sigma Chemicals) for 30 minutes at 37°C , and subsequently

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Table 1 Clinical details of pooled and individual synovial fluid samples from patients with RA, PA, or OA

	Pooled samples			Individual samples		
	RA	PA	OA	RA	PA	OA
No of patients	14	11	13	15	8	5
Male	4	10	2	2	7	1
Female	10	11	11	13	1	4
Age (yr)	60 (27–80)	43 (24–73)	70 (54–83)	67 (44–88)	41 (24–73)	70 (54–83)
Duration of disease (yr)						
< 2	1	3	2	0	2	0
2–10	3	4	5	5	3	2
> 10	10	4	6	10	3	3
Symptoms						
Severe	6	3	2	10	1	1
Mild/moderate	8	6	11	5	5	4
None	0	2	0	0	2	0
Aspiration volume (ml)	31 (5–90)	168 (10–550)	17 (2–55)	25 (10–73)	213 (10–550)	25 (15–50)

Values are numbers, or mean (range).

centrifuged at 3000 *g* for 10 minutes at room temperature.

Serum—Blood samples obtained from 14 RA patients attending the Rheumatology Unit at BRI were centrifuged at 180 *g* for 10 minutes. The supernatants were pooled and aliquots stored at –20°C. Before assay the samples were re-centrifuged at 3000 *g* for 10 minutes at room temperature.

Urine—Urine samples, collected from 10 healthy subjects attending the Nephrology Unit of Southmead Hospital Bristol (SHB) were pooled and aliquots stored at –70°C. Before measurement, each sample was centrifuged at 3000 *g* for 10 minutes.

Saliva—Saliva, obtained from healthy subjects attending the Nephrology Unit of SHB, was pooled and aliquots immediately frozen and stored at –70°C. Before measurement, each sample was centrifuged at 3000 *g* for 10 minutes.

AMIDASE ACTIVITY OF PK

Because the enzymic activity of PK in biological fluids is believed to be regulated mainly by C1 inhibitor and α_2 -macroglobulin,^{7,8} samples were denatured with an equal volume of 0.167 mol/l HCl (pH 1.0).⁹

To measure the total amidase activity of PK and its precursor, prokallikrein (PPK), we used the selective synthetic chromogen, H-D-Pro-Phe-Arg-pNA (S2302; Kabivitrum, Sweden).¹⁰ The assay method was adapted from that described by De La Cadena *et al.*,¹¹ using wells on microtitre plates as reaction sites for measurement of PK + PPK. Samples (50 μ l) were incubated with 50 μ l of 0.167 mol/l HCl (pH 1.0) for 25 minutes at room temperature, then 50 μ l of buffer (0.1 mmol/l sodium phosphate buffer in 0.15 mmol/l NaCl and 1 mmol/l EDTA, pH 7.6) was added to the mixture. Finally, the pH of the reaction mixture was adjusted to 7.4 with 50 μ l of 0.167 mol/l NaOH.

Standard curves were generated with purified PPK (Protogen, Switzerland). PPK (66.67 μ g) was dissolved in 1 ml of assay buffer (0.1% polyethylene glycol in 0.05 mol/l Tris-HCl buffer, pH 7.9). Dilutions of PPK (50 μ l per well) were added to the wells of the microtitre plate; similarly, blanks prepared with assay

buffer were added to designated wells. Next, 50 μ l of the PPK activation mixture (25 μ g/ml mixture of H-kininogen and L-kininogen, gift from Professor Werner Müller-Esterl, University of Mainz; 5 μ g/ml Factor XIIa, Nova Biochem; 0.1 mol/l ellagic acid, Sigma Chemicals and 1 μ g/ml 1- α -phosphatidyl-ethanolamine, Sigma Chemicals) was added, and the plate incubated for 15 minutes at 37°C. Finally, for the measurement of the activated PPK and the PK in the SF samples, 50 μ l of substrate (S2302) was added to each well, the plate incubated for a further 30 minutes at 37°C, and the absorbance read in a BioRad microtitre plate reader at 405 nm. Hydrolysis of 1 μ mol of substrate (S2302) per minute equalled 1 mU.

Specificity of the amidase activity of PK—The specificity of the synovial fluid PK amidase activity was examined with the selective enzyme inhibitor, soya bean trypsin inhibitor (SBTI).¹² SF samples were incubated for 30 minutes at 37°C with SBTI (300 μ g of a solution of 1 mg/ml) before the addition of substrate (S2302). As a further test of specificity, SF samples were also adsorbed with anti-PK Sepharose to remove PK + PPK before incubation with the substrate.

Units of PK amidase activity—Non-specific absorbance attributable to the samples was obtained by incubating them in the absence of substrate; assay values were corrected for the non-specific absorbance. PK activity in the test samples was determined by reference to a simultaneously measured standard curve using purified PK, and amidase activity expressed as mU/g protein. The protein concentration of samples was measured as described by Bradford.¹³

MEASUREMENT OF PK + PPK

Removal of rheumatoid factor from synovial fluid—Once the concentration dependency of purified PK was determined and optimised, the influence of RA synovial fluid (diluted 1:200) on PK immunoreactivity (examined by spiking the PK standard curve) showed marked enhancement of the PK dose-response curve when a constant amount of diluted RA synovial fluid was added to each PK standard. It was considered that this effect was probably

Figure 1 Schematic representation of the stages involved in the PK ELISA method using an avidin-biotin system. E = alkaline phosphatase. Steps 1, 3, 4, 5, and 6: Microtitre plate incubated for one hour at 37°C. Step 2: Microtitre plate incubated for 30 minutes at room temperature. Step 6: Microtitre plate incubated for one hour at 24°C. After each step the plate was washed with PBS-Tween 20 (three times for 10 seconds).

the result of a combination of PK and rheumatoid factor (RF) present in SF. Therefore, as part of routine sample preparation RF was removed from SF samples before the measurement of PK immunoreactivity in SF. Samples of SF were incubated with 0.3 volumes of heat aggregated rabbit IgG coupled to cyanogen bromide Sepharose and 0.2 volumes of 0.1 mol/l Tris/0.5 mol/l NaCl (pH 8.5) for 30 minutes at room temperature. Enzyme linked immunosorbent assay (ELISA)—Figure 1 illustrates the method for PK immunoassay. To coat microtitre plates with human PK, a stock solution, prepared at a concentration of 66.67 µg/ml in bicarbonate buffer (15 mmol/l Na₂CO₃, 35 mmol/l NaHCO₃, pH 9.6), was diluted and applied to the wells at a concentration of 1.91 µg/ml; bicarbonate buffer alone was added to the

blank wells. The plates were incubated for one hour at 37°C. Unoccupied sites were blocked by incubation for 30 minutes at room temperature with 100 µl per well of 5% fat free bovine milk (Marvel) in 0.02 mol/l Tris, 0.14 mol/l NaCl and 0.05% Tween 20, pH 7.2. Next, each SF sample of PK dose (in 5% Marvel) was incubated with 15 µg/ml of purified anti-PK rabbit IgG (Nova Biochem, Switzerland) at 37°C for one hour. The mixture (100 µl) was added to the wells and the plate incubated for one hour at 37°C. Thereafter, the plate was incubated sequentially with 1:1000 anti-rabbit IgG biotin conjugate (Sigma Chemicals) and 1:250 extravidin alkaline phosphatase (Sigma Chemicals) for one hour each at 37°C. The wells were washed with phosphate buffered saline containing 0.1% Tween 20, three times between each step. The final incubation was performed with disodium p-nitrophenyl phosphate (1 mg/ml, Sigma Chemicals) for one hour at 24°C, and absorbancies read at 405 nm in a BioRad (UK) Colorimetric plate reader. Absorbance values for the samples and standard curves were corrected by subtracting the non-specific absorbance (blanks). The immunoassay measured both PK and PPK because the antibody does not discriminate between the two molecular species.

STATISTICAL ANALYSIS
Significance was determined by analysis of variance.

Terminology
Throughout this paper, PK = active plasma kallikrein, PPK = pre-plasma kallikrein (the plasma kallikrein precursor), and PK + PPK = total PK after activation of PPK.

Results
AMIDASE ACTIVITY
To determine the amounts of PK and PPK in synovial fluids, as a first step the relationship between amidase activity and PK concentration was examined. As the amidase activity increased linearly up to a PK concentration of 4.4 µg/ml, all samples were diluted to give an activity below this value.

The amidase activity of the pooled SF samples was measured before and after activation of PPK in order to investigate the amount of active PK relative to the total PK (PPK + active PK). The proportion of the total PK present as active PK was 43%, 59% and 4.2% for RA, PA and OA pools, respectively (table 2). Median values for active PK were: RA 27 mU/g of protein, PA 20 mU/g of protein, and OA 20 mU/g of protein (fig 2); those for PK + PPK were: RA 76 mU/g of protein, PA 80 mU/g of protein, and OA 60 mU/g of protein (fig 3). These differences between RA, PA and OA SF samples did not reach statistical significance (p > 0.05), and comparisons of clinical scoring showed no clear correlation with PK activity.

Table 2 Amidase activity of PK in pooled samples of RA (n = 14), PA (n = 11), and OA (n = 13) synovial fluid and RA serum (n = 14)

Biological fluid	Activity (mU/g protein)	
	Active PK	PPK
RA synovial fluid	28 (1.4)	65 (1.6)
PA synovial fluid	13 (0.4)	22 (0.5)
OA synovial fluid	3 (0.5)	71 (1.1)
RA serum	43 (1.3)	70 (1.7)

Values are mean (SEM).

Figure 2 PK amidase activity of individual synovial fluid samples of patients with RA, PA, and OA. Each measurement was performed in duplicate.

Comparison of PK and PPK values for the RA SF and serum pool samples showed greater values for both enzyme forms in serum than in SF (table 2). No PK + PPK activity was detectable in saliva or urine using the selective substrate S2303 (< 0.05 mU/g protein).

As shown in table 3, the enzymic activity of PK + PPK in the RA, PA, and OA pooled SF samples was almost completely inhibited by the addition of SBTI. In the experiments in which PK + PPK were removed from the pooled SF samples by absorption with anti-PK Sepharose before incubation with substrate, absorption of both PK and PPK by the antibody from each SF pool was even greater than the inhibition by SBTI (table 3).

IMMUNOREACTIVE PLASMA KALLIKREIN

Figure 4 illustrates the marked enhancement of the PK dose-response curve when a constant amount of diluted RA synovial fluid was added to each PK standard—an effect believed to be due to a combination of PK and RF present in SF. Routine preparation of SF samples therefore included removal of RF before measurement of PK immunoreactivity in SF. Most of the immunoreactive PK in RF depleted

Figure 3 PK + PPK amidase activity of individual synovial fluid samples of patients with RA, PA, and OA. Each measurement was performed in duplicate.

Table 3 Percentage inhibition by SBTI and removal by anti-PK Sepharose (anti-PKS) of both PK and PPK amidase activity from RA, PA, and OA synovial fluids

Synovial fluid	Active PK		PK + PPK	
	Inhibition by SBTI (%)	Removal by anti-PKS (%)	Inhibition by SBTI (%)	Removal by anti-PKS (%)
RA	90.3	98	96.5	99
PA	95.6	98	96.5	98.9
OA	97.6	95	98	99

samples was removed by anti-PK Sepharose (table 3), but there remained a residual immunoreactivity (32%, 28%, and 48% for RA, PA and OA, respectively) which could be attributable to residual RF in the SF samples. When immunoreactive PK was determined in the pooled SF samples, similar levels were observed in RA (4.7 (SEM 0.5) μ g/mg protein), PA (6.5 (0.7) μ g/mg protein) and OA (5.0 (0.7) μ g/mg protein) synovial fluids (fig 5). As with amidase activity, no immunoreactive PK + PPK was measured in saliva or urine.

Discussion

This study has identified PK + PPK in the synovial fluids taken from the knee of patients with arthritis. Because of the relatively small numbers of individual samples we cannot draw any definite conclusions about the contribution of plasma kallikrein to the clinical features of these arthropathies, but, while earlier reports had shown the occurrence of PK in the SF of RA patients, we have achieved the first precise measurements of the amidase activity attributable to PK in the inflamed joints. We chose two primary methods to determine the concentration of PK in the synovial fluids: measurement of PK functional activity by microassay on the selective peptide substrate, H-D-Pro-Phe-Arg-pNA, and PK immunoreactivity by ELISA. The identification and verification of PK + PPK amidase activity was performed on SF pools, but in order to determine the range of activity, individual SF samples were examined for PK + PPK amidase activity.

Various evidence suggests that the amidase activity measured in SF reflected true plasma kallikrein values. First, the PK + PPK amidase activity was inhibited by SBTI (PK is completely inhibited by SBTI).¹² Second, the amidase activity was removed with anti-PK Sepharose. Finally, saliva and urine (which contain considerable amounts of tissue kallikrein^{14,15}) failed to hydrolyse the selective PK + PPK substrate (H-D-Pro-Phe-Arg-pNA). Therefore, the amidase activity of the RA, PA, and OA synovial fluids on this selective substrate was attributable to PK (intrinsic activity) and PPK (increase in activity after activation).

This study is the first to report immunoreactive PK in synovial fluids using an ELISA. RA synovial fluid enhanced the PK dose-response curve; removal of RF reduced this enhancement, indicating that RF interfered with the measurement of PK + PPK by the ELISA. It is therefore necessary to remove RF for the estimation of immunoreactive

Figure 4 Concentration-dependent curve of immunoreactive PK + PPK (●), and effect of RA synovial fluid on it (▲). Each experiment was performed three times in duplicate ($n = 6$).

PK + PPK. It may be argued that other factors were present in the SF which interfered with the immunoassay and which were not removed by pretreatment of SF with IgG Sepharose. Because PK was the primary coating molecule in the ELISA we expected minimal, if any, interference by inhibitors; none was observed. The results were further verified by demonstration that the PK + PPK immunoreactivity was removed by adsorption with anti-PK Sepharose.

Evidence for a proinflammatory role for kallikreins and kinins in arthritic joints has now accumulated, and a sequence of molecular events can be outlined. At the onset of inflammation, serum molecules and cells of the immune and phagocytic systems migrate into the inflamed joint tissue. The route of entry into the inflamed joint, of both the kallikreins and kininogens, may be by transudation or with the migrating neutrophil.^{2,16} The discovery of tissue kallikrein in neutrophil

granules,¹⁷ and the attachment of kininogens and PPK to the external surface of the neutrophil membrane^{16,18} provide a novel mechanism for the delivery of the kinin forming enzymes and their endogenous substrates to inflamed joints. During episodes of acute inflammation within RA joints, neutrophils degranulate in response to immune complexes,¹⁹⁻²¹ resulting in the formation of kinin by the released tissue kallikrein. In contrast, conversion of PPK to its active form in the inflamed joint may be triggered through activation of Hageman factor by tissue matrix components, such as proteoglycans, urate crystals or pyrophosphates.²² The resulting PK is believed to cause significant conversion of latent collagenase to its active form *in vitro*,⁵ and this could be an important property of PK within the joint space.

Kinins formed by plasma and tissue kallikreins are considered to modulate pain and swelling in the inflamed joint, and these effects are probably effected through the bradykinin-1 (BK-1) receptor.²³ In addition, kinins are known to release transmitters from nerve terminals (substance P), stimulate the synthesis of cytokines (interleukin-1, tumour necrosis factor), induce the formation of prostaglandins and leukotrienes by activating phospholipase α_2 , and release endothelium derived relaxing factor (nitric oxide) from endothelial cells.¹ Effective BK-1 antagonists may play a pivotal role in inhibiting the cascade of proinflammatory molecules formed in the inflamed joint, and therefore should be of considerable therapeutic value.

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Figure 5 ELISA measurement of immunoreactive PK + PPK in RA, PA, and OA synovial fluids, before (1) and after (2) adsorption of the samples with anti-PK antibody linked to Sepharose. Each experiment was performed three times in duplicate ($n = 6$).

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