Urokinase-mediated fibrinolysis in the synovial fluid of rheumatoid arthritis patients may be affected by the inactivation of single chain urokinase type plasminogen activator by thrombin

E A M Braat, A F H Jie, H K Ronday, B Beekman, D C Rijken

Abstract

Background—Excessive fibrin deposition within the inflamed joints of rheumatoid arthritis (RA) patients suggests that local fibrinolysis is inefficient, which seems to be in contrast with the observed increased levels of urokinase type plasminogen activator (u-PA). Thrombin-mediated inactivation of single chain u-PA (scu-PA) into an inactive form called thrombin-cleaved two chain u-PA (tcu-PA/T) may provide a possible explanation for this contradiction.

Aim—To assess the occurrence of tcu-PA/T in the synovial fluid of patients with RA and with osteoarthritis (OA), and in the synovial fluid of controls to find support for thrombin-mediated inactivation of scu-PA in RA.

Methods—Levels of scu-PA and tcu-PA/T were measured in the synovial fluid of 20 RA patients, nine OA patients and 14 controls using sensitive bioimmunoassays. Total urokinase antigen was quantified by a urokinase ELISA.

Results—tcu-PA/T was found in the synovial fluid of all RA and OA patients. Only in seven of 14 control samples, levels of tcu-PA/T could be measured above the detection limit of the assay (0.2 ng/ml). The concentrations of tcu-PA/T, scu-PA and u-PA:Ag were significantly higher in the synovial fluid of the RA and OA patients as compared with the controls, while the RA patients had significantly higher levels of tcu-PA/T and u-PA:Ag than the OA patients. In RA, tcu-PA/T seemed to account for more than 40% of total urokinase antigen, while the contribution of tcu-PA/T to total urokinase antigen was only minor in OA and the controls (9.0% and 6.6%, respectively).

Conclusion—A significant part of the high total urokinase antigen in the synovial fluid of RA patients can be attributed to tcu-PA/T, implying that a large amount of scu-PA is not available for fibrinolysis because of its inactivation by thrombin. Thus, thrombin may promote the inflammation process in RA by inhibiting the fibrinolytic system and preventing the removal of fibrin.

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterised by cartilage and bone destruction. Intra-articular fibrin deposition is a prominent finding in the disease and may be of major importance for the perpetuation of RA because of proinflammatory effects of fibrin.¹ Intra-articular fibrin formation is induced by thrombin generated extravascularly by coagulation proteins that are either leaked from the circulation or produced by synovial macrophages.¹ Several studies have shown that the excessive fibrin formation is counteracted by increased levels of fibrinolytic components, predominantly that of urokinase type plasminogen activator (u-PA).^{2–5} Recently, Busso *et al* demonstrated exacerbation of arthritis in u-PA-deficient mice, showing increased amounts of fibrin within the inflamed joints.⁶ Depletion of fibrinogen decreased the sustained joint inflammation in u-PA deficient mice, and all observations were comparable to what was found in plasminogen deficient mice. These findings strongly suggested that u-PA may play a beneficial part in arthritis by mediating the removal of fibrin via the activation of plasminogen. However, excessive deposition of fibrin within the inflamed joints of RA patients suggests that local fibrinolysis is inefficient, which seems to be in contrast with the previously observed increased levels of u-PA in synovial fluid (SF) and synovial tissue of RA patients.^{2–5} This contradiction may be explained by the presence of large amounts of thrombin in SF of RA patients. Thrombin is able to cleave single chain u-PA (scu-PA) into an inactive form called thrombin-cleaved two chain u-PA (tcu-PA/T).⁷ As thrombin is abundantly generated in SF of RA patients, we hypothesised that besides activation of scu-PA into two chain u-PA (tcu-PA), inactivation of scu-PA by thrombin into tcu-PA/T could take place. This may provide a possible explanation for the inefficient intra-articular fibrinolysis in RA patients. To find support for thrombin mediated inactivation of scu-PA, we investigated the occurrence of tcu-PA/T in SF of patients with RA and with osteoarthritis (OA), and in SF of controls.

Methods

Specimens of SF were obtained from 20 patients with RA and from nine patients with advanced OA, who required joint surgery for severe disease, and after death from 14 people without any sign of joint disease, who were regarded as controls. All RA patients fulfilled

TNO Prevention and Health, Gaubius Laboratory, Leiden, the Netherlands

E A M Braat
A F H Jie
H K Ronday
B Beekman
D C Rijken

Department of Rheumatology, University Hospital Leiden, the Netherlands
H K Ronday

Correspondence to:
Dr Rijken, TNO Prevention and Health, Gaubius Laboratory, PO Box 2215, 2301 CE Leiden, the Netherlands

Accepted for publication
22 November 1999

(Ann Rheum Dis 2000;59:315–318)

Figure 1 Measurements of tcu-PA/T (A), scu-PA (B) and u-PA:Ag (C) in SF of RA and OA patients and of controls. The horizontal lines indicate median values. Comparisons are made between RA patients and controls ($*p < 0.01$; $**p < 0.001$), between OA patients and controls ($*p < 0.01$; $**p < 0.001$) and between RA patients and OA patients ($\dagger p < 0.05$; $\ddagger p < 0.005$). Active tcu-PA was not detectable in SF of the controls nor the OA patients, and only in very low amounts (< 0.4 ng/ml) in some of the RA patients.

the established criteria for RA and were operated on at the Orthopaedic Department of the Leiden University Medical Centre, the Netherlands.⁸ The OA patients corresponded to grade 3–4 in the Kellgren classification system and were operated on at the Department of Orthopaedics of Rijnland Hospital, Leiderdorp, the Netherlands.⁹ Some of the RA patients were part of a previous study.¹⁰

Levels of tcu-PA, scu-PA and tcu-PA/T were measured using sensitive bioimmunoassays.^{10–12}

Table 1 Ratios (%) between tcu-PA/T and u-PA:Ag and between scu-PA and u-PA:Ag (median and interquartile ranges) in the synovial fluid of RA and OA patients and of controls

Parameter	RA (n=20)	OA (n=9)	Controls (n=14)
tcu-PA/T / u-PA:Ag (%)	42.4** $\ddagger$ (13.6–51.4)	9.0 (6.3–17.1)	6.6 (4.6–8.9)
scu-PA / u-PA:Ag (%)	14.1 (8.4–29.5)	18.8 (12.2–24.5)	22.9 (17.2–33.4)

Comparisons are made between RA patients and controls ($**p < 0.001$) and between RA patients and OA patients ($\ddagger p < 0.005$). No significant differences were found between the OA patients and the controls.

Briefly, samples in duplicate were incubated in 96 well plates coated with a rabbit polyclonal anti-u-PA antibody. After washing, the immobilised material was activated in parallel wells with buffer (BIA for tcu-PA), 1 nM plasmin (BIA for scu-PA) or 20 nM cathepsin C (BIA for tcu-PA/T). Urokinase activity was determined using Glu-plasminogen and the plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide. The concentrations of tcu-PA and scu-PA were calculated from a calibration curve obtained by serial dilutions of purified scu-PA activated in the BIA by plasmin, and the concentration of tcu-PA/T was calculated from a calibration curve obtained by serial dilutions of purified tcu-PA/T activated in the BIA by cathepsin C. Total urokinase antigen (u-PA:Ag) was quantitated by the urokinase ELISA as described before.¹³ In addition, scu-PA and tcu-PA/T were expressed as a percentage of total urokinase antigen.

Median and interquartile ranges were calculated for all values. Differences between groups were tested for significance with the Mann-Whitney test. Correlations between levels of tcu-PA/T and levels of total urokinase antigen and scu-PA were tested using Spearman rank correlation method.

Results

The values of tcu-PA/T, scu-PA and u-PA:Ag measured in SF of the controls, OA patients and RA patients are shown in figure 1. Active tcu-PA was not detectable in SF of the controls nor the OA patients, and only in very low amounts (< 0.4 ng/ml) in some of the RA patients.

tcu-PA/T was found in SF of all RA and OA patients (fig 1A). However, only in seven of 14 control SF samples, levels of tcu-PA/T could be measured above the detection limit of the assay (0.2 ng/ml). The concentrations of tcu-PA/T, scu-PA and u-PA:Ag were significantly higher in SF of the RA patients (median values of 5.4, 2.9 and 19.9 ng/ml, respectively) and OA patients (1.1, 1.9 and 12.6 ng/ml, respectively) as compared with the controls (0.2, 0.8, and 3.8 ng/ml, respectively), while the RA patients had significantly higher levels of tcu-PA/T and u-PA:Ag than the OA patients (fig 1).

The contribution of tcu-PA/T to total urokinase antigen was significantly higher in SF of the RA patients compared with the OA patients and the controls (table 1). In RA, tcu-PA/T seemed to account for more than 40% of total urokinase antigen, while the contribution of tcu-PA/T to total urokinase antigen was only minor in OA and the controls (9.0% and 6.6%, respectively). In addition, in the SF of the RA patients significant correlations were found between levels of tcu-PA/T and total urokinase antigen ($p < 0.05$, fig 2A) and between tcu-PA/T and scu-PA ($p < 0.01$, fig 2B). No significant correlations between these parameters were found in SF of the OA patients or the controls.

Discussion

The results of this study show that a significant part of the increased total urokinase antigen in

Figure 2 Relation between the levels of total urokinase antigen (u-PA:Ag) and tcu-PA/T (A) and between the levels of scu-PA and tcu-PA/T (B) in SF of RA patients. Significant correlations were found between levels of total urokinase antigen and tcu-PA/T (correlation coefficient $r = 0.520$, $p < 0.05$) and between levels of scu-PA and tcu-PA/T (correlation coefficient $r = 0.612$, $p < 0.01$), as tested by Spearman rank correlation method.

SF of RA patients can be attributed to tcu-PA/T. This implies that a large amount of scu-PA is not available for fibrinolysis because of its inactivation by thrombin. Thrombin may therefore promote the inflammation process in RA by inhibiting the fibrinolytic system and preventing the removal of fibrin. The finding of positive correlations between the levels of tcu-PA/T and the levels of both total urokinase antigen and scu-PA in the SF of the RA patients indicates that the inactivation of scu-PA by thrombin is a general phenomenon in RA. This can be explained by the fact that scu-PA is a substrate for thrombin. The levels of tcu-PA/T in RA may therefore not only depend on the amount of thrombin generated in the joint but also on the amount of urokinase available.

In the OA patients, the contribution of tcu-PA/T to total urokinase antigen was far lower than in the RA patients. This discrepancy between OA and RA can be explained by a higher expression of coagulation proteins in RA and thus a higher extent of thrombin formation.¹ Furthermore, increased SF levels of soluble thrombomodulin in RA patients, which is known to accelerate the inactivation of scu-PA by thrombin, may contribute to the high levels of tcu-PA/T.¹⁴⁻¹⁶ Finally, it has been suggested that a significant part of anti-thrombin, the main inhibitor of thrombin, is inactivated in SF of RA patients.¹⁷ Altogether these data suggest that the conditions for the inactivation of scu-PA by thrombin are highly

favourable in RA. Although tcu-PA/T accounts for about 42% of total urokinase antigen, a significant part of total urokinase antigen in SF of RA patients remains unexplained. No significant levels of active tcu-PA could be detected in SF of these patients, indicating that the unexplained part of total urokinase antigen should be ascribed to inactivated or inactive u-PA. Besides by thrombin, neutralisation of u-PA activity may also occur via other pathways. It is known that levels of PAI-1 are increased in the synovial fluid of RA.^{2,3,5} This inhibitor will rapidly neutralise u-PA activity by forming complexes with active tcu-PA. In addition, PAI-1 may form complexes with scu-PA, which might hamper the activation of scu-PA into tcu-PA.¹⁸ Besides by thrombin, degradation of scu-PA by other enzymes such as elastase may occur.¹⁹ These processes can also contribute to impaired u-PA-mediated intra-articular fibrinolysis. Furthermore, it has been described that levels of tissue type plasminogen activator (t-PA) were depressed in SF of RA patients compared with levels in plasma.^{2,3,5} Removal of intra-articular fibrin may therefore also be hampered by decreased t-PA mediated plasminogen activation. In SF of OA patients, the contribution of tcu-PA/T to urokinase antigen was only minor, implying that fibrinolysis may be more efficient than in RA patients. In SF of healthy people tcu-PA/T is negligible, as has been observed earlier in normal plasma.¹⁰

Inactivation of scu-PA by thrombin provides an explanation for the excessive amounts of fibrin being present in the inflamed joints of RA patients despite increased expression of u-PA in the synovial tissue and fluid of these patients. Thrombin might decrease u-PA mediated fibrinolysis and could therefore be of major importance for sustaining inflammation in RA.⁶

- Weinberg JB, Phippen AMM, Greenberg CS. Extravascular fibrin formation and dissolution in synovial tissue of patients with osteoarthritis and rheumatoid arthritis. *Arthritis Rheum* 1991;34:996-1005.
- Brommer EJP, Dooijewaard G, Dijkman BAC, Breedveld FC. Plasminogen activators in synovial fluid and plasma from patients with arthritis. *Ann Rheum Dis* 1992;51:965-8.
- Brommer EJP, Dooijewaard G, Dijkman BAC, Breedveld FC. Depression of tissue-type plasminogen activator and enhancement of urokinase-type plasminogen activator as an expression of local inflammation. *Thromb Haemost* 1992;68:180-4.
- Ronday HK, Smits HH, Van Muijen GNP, Pruszczynski MSM, Dolhain RJEM, Van Langelaan EJ, *et al.* Difference in expression of the plasminogen activation system in synovial tissue of patients with rheumatoid arthritis and osteoarthritis. *Br J Rheumatol* 1996;35:416-23.
- Busso N, Péclat V, So A, Sappino A-P. Plasminogen activation in synovial tissues: differences between normal, osteoarthritis, and rheumatoid arthritis joints. *Ann Rheum Dis* 1997;56:550-7.
- Busso N, Péclat V, Van Ness K, Kolodziejczyk E, Degen J, Bugge T, *et al.* Exacerbation of antigen-induced arthritis in urokinase-deficient mice. *J Clin Invest* 1998;102:41-50.
- Ichinose A, Fujikawa K, Suyama T. The activation of pro-urokinase by plasma kallikrein and its inactivation by thrombin. *J Biol Chem* 1986;261:3486-9.
- Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315-24.
- Kellgren JH, Lawrence JS. Radiologic assessment of osteoarthritis. *Ann Rheum Dis* 1957;19:494-502.
- Braat EAM, Nauland U, Dooijewaard G, Rijken DC. A sensitive bioimmunoassay for thrombin-cleaved two-chain urokinase-type plasminogen activator in human body fluids. *Thromb Haemost* 1996;75:908-14.
- Dooijewaard G, Van Iersel JLL, Brommer EJP. Quantitation of pro-UK, UK and UK-inhibitor levels in plasma of

- patients and healthy men. [Abstract 142]. In: Binder BR. *Fibrinolysis (8th International Congress on Fibrinolysis)*. Edinburgh: Churchill Livingstone, 1986.
- 12 Binnema DJ, Dooijewaard G, Turion PNC. An analysis of the activators of single-chain urokinase-type plasminogen activator (scu-PA) in the dextran sulphate euglobulin fraction of normal plasma and of plasmas deficient in factor XII and prekallikrein. *Thromb Haemost* 1991;65:144-8.
 - 13 Binnema DJ, Iersel van JJL, Dooijewaard G. Quantitation of urokinase antigen in plasma and culture media by use of an ELISA. *Thromb Res* 1986;43:569-77.
 - 14 Conway EM, Nowakowski B. Biologically active thrombomodulin is synthesized by adherent synovial fluid cells and is elevated in synovial fluid of patients with rheumatoid arthritis. *Blood* 1993;81:726-33.
 - 15 De Munk GAW, Groeneveld E, Rijken DC. Acceleration of the thrombin inactivation of single-chain urokinase-type plasminogen activator (pro-urokinase) by thrombomodulin. *J Clin Invest* 1991;88:1680-4.
 - 16 Braat EAM, Los P, Rijken DC. The inactivation of single-chain urokinase-type plasminogen activator by thrombin in a plasma milieu: effect of thrombomodulin. *Blood Coag Fibrinol* 1998;9:419-27.
 - 17 Jones HW, Bailey R, Zhang Z, Dunne KA, Blake DR, Cox NL, et al. Inactivation of antithrombin III in synovial fluid from patients with rheumatoid arthritis. *Ann Rheum Dis* 1998;57:162-5.
 - 18 Manchanda N, Schwartz BS. Interaction of single-chain urokinase and plasminogen activator inhibitor type 1. *J Biol Chem* 1995;270:20032-5.
 - 19 Schmitt M, Kanayama N, Henschen A, Hollrieder A, Hafter R, Gulba D, et al. Elastase released from human granulocytes stimulated with N-formyl-chemotactic peptide prevents activation of tumor cell prourokinase (pro-uPA). *FEBS Lett* 1989;255:83-8.