

Research article

Urokinase, a constitutive component of the inflamed synovial fluid, induces arthritisTao Jin¹, Andrej Tarkowski¹, Peter Carmeliet² and Maria Bokarewa¹¹Department of Rheumatology and Inflammation Research, Sahlgrenska University Hospital, Göteborg, Sweden²Center of Transgene Technology and Gene Therapy, Flanders Interuniversity Institute of Biotechnology, Campus Gasthuisberg, Leuven, BelgiumCorresponding author: Maria Bokarewa (e-mail: maria.bokarewa@rheuma.gu.se)

Received: 11 June 2002 Revisions received: 13 September 2002 Accepted: 25 September 2002 Published: 17 October 2002

Arthritis Res Ther 2003, **5**:R9-R17 (DOI 10.1186/ar606)© 2003 Jin *et al.*, licensee BioMed Central Ltd (Print ISSN 1478-6354; Online ISSN 1478-6362). This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any non-commercial purpose, provided this notice is preserved along with the article's original URL.**Abstract**

Urokinase plasminogen activator (uPA) is an important regulator of fibrinolysis in synovial fluid. An increase of uPA activity and expression of its receptor have been reported in joints of patients with rheumatoid arthritis (RA). The aim of the present study was to assess the arthritogenic capacity of uPA and the mechanisms by which this effect is mediated. uPA was injected into the knee joints of healthy mice, and morphological signs of arthritis were assessed 4 days after the injection. The prerequisite of different leukocyte populations for the development of uPA-triggered arthritis was assessed by selective cell depletion. The inflammatory capacity of uPA was assessed *in vitro*. Finally, levels of uPA were measured in 67 paired blood and synovial fluid samples from RA patients. The synovial fluid from RA patients displayed higher levels of uPA compared with blood samples.

Morphological signs of arthritis were found in 72% of uPA-injected joints compared with only 18% of joints injected with PBS ($P < 0.05$). Synovitis was characterised by infiltration of CD4-Mac-1⁺ mononuclear cells, by the formation of pannus and by occasional cartilage destruction. The absence of monocytes and lymphocytes diminished the frequency of synovitis ($P < 0.01$), indicating an arthritogenic role of both these leukocyte populations. Synthetic uPA inhibitor downregulated the incidence of uPA-triggered arthritis by 50%. uPA induced arthritis, stimulating the release of proinflammatory cytokines IL-6, IL-1 β and tumour necrosis factor alpha. Accumulation of uPA locally in the joint cavity is a typical finding in erosive RA. uPA exerts potent arthritogenic properties and thus may be viewed as one of the essential mediators of joint inflammation.

Keywords: arthritis, inflammation, urokinase plasminogen activator**Introduction**

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterised by the inflammatory cell infiltration and proliferation of synovial tissue, followed by cartilage and bone destruction. Accumulation of fibrin is a prominent morphologic finding in the cavity of inflamed joints implied in the aggravation of joint damage [1]. Plasmin-mediated degradation of the fibrin net within the joint might be viewed as an anti-inflammatory defence reaction. Urokinase plasminogen activator (uPA) is a serine protease indicated as the principal regulator of plasmin activity during arthritis [2–5] and may be produced within the

joint cavity. Indeed, cultured chondrocytes produce uPA spontaneously [6,7], while synoviocytes and mononuclear cells respond with a prominent uPA production upon stimulation with proinflammatory cytokines [8–10] or with growth factors [11].

When released into the joint cavity, uPA participates in various biological processes. uPA as a serine protease converts plasminogen to plasmin, a broad-spectrum enzyme able to degrade not only fibrin, but also proteins of the joint extracellular matrix and cartilage [12]. By single proteolytic cleavage, both uPA and plasmin produce

BSA = bovine serum albumin; ELISA = enzyme-linked immunosorbent assay; FACS = fluorescence-activated cell sorting; FCS = foetal calf serum; H & E = haematoxylin and eosin; HGF = hepatocyte growth factor; HMW = high molecular weight; IFN- γ = interferon gamma; IL = interleukin; LMW = low molecular weight; MMP = matrix metalloproteinase; PBS = phosphate-buffered saline; PPACK = *H*-D-Pro-Phe-Arg-chloromethylketone; RA = rheumatoid arthritis; RF = rheumatoid factor; TNF- α = tumour necrosis factor alpha; uPA = urokinase plasminogen activator; uPAR = urokinase plasminogen activator receptor.

active forms of matrix metalloproteinases (MMPs), a group of enzymes that promote degradation of joint cartilage [13,14]. uPA also exhibits an indirect stimulatory effect by activating latent growth factors [15]. On the contrary, uPA has been shown to be essential for the alleviation of antigen-induced arthritis [16] and in defence during endotoxin challenge [17]. Moreover, uPA null mice show diminished T-cell proliferation capacity and a shifted cytokine pattern to the T helper cell type 2 direction, displaying low IFN- γ and IL-12 production but high IL-10 production [18]. uPA binds through its amino-terminal fragment to the urokinase plasminogen activator receptor (uPAR), a glycosylphosphatidylinositol-anchored molecule on the cell wall that attracts proteolysis to the cell surface. Pericellular proteolytic processes triggered by the uPA/uPAR interaction evoke various cell events including adhesion and proliferation, chemotactic migration and differentiation that are of vital importance for the innate immune response (reviewed in [19]).

The complexity of the processes taking place in the inflamed joint does not permit a definitive conclusion about the role of uPA in the development of arthritis, at least not in a human setting. We demonstrate that intra-articular injection of highly purified uPA induces arthritis in naïve mice. The inflammation is characterised by abundant mononuclear cell infiltration, pannus formation and occasional development of erosions. Furthermore, we demonstrate that the serine proteinase activity of uPA rather than its interaction with the uPAR is essential for the development of joint inflammation.

Materials and methods

Synovial fluid and blood samples

Synovial fluid and blood samples were collected from 67 patients with RA (aged 29–87 years) who attended the Rheumatology Clinic, Sahlgrenska University Hospital, Göteborg for acute joint effusion. Forty-six patients displayed erosive joint disease, as detected by X-ray imaging, and 47 patients were positive for rheumatoid factor (RF). Synovial fluid was aseptically aspirated and transferred into tubes containing sodium citrate (0.129 mol/l; pH 7.4). Blood samples from all 67 RA patients were simultaneously obtained from the cubital vein and directly transferred into sodium citrate medium. Blood samples from 22 healthy individuals (aged 32–66 years) were used in the control group. The collected blood and synovial fluid samples were centrifuged at $800 \times g$ for 15 min, aliquoted and stored frozen at -20°C until use.

Mice and reagents

Female NMRI mice (6–8 weeks old, weighing 25–30 g) were purchased from ALAB (Stockholm, Sweden). SCID mice were obtained from Charles River Laboratories (Uppsala, Sweden). The breeding pairs of IL-1 receptor type I knockout mice and their transgenic counterpart

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were bred and housed in the animal facility of the Department of Rheumatology, University of Göteborg, under standard conditions of temperature and light, and were fed laboratory chow and water *ad libitum*. The uPAR knockout mice and their transgenic counterpart (75% BL6/25% 129) [20] were bred at the animal facility of the Center for Transgene Technology and Gene Therapy, University of Leuven, Belgium. Human low molecular weight (LMW)-uPA was purchased from American Diagnostica (Greenwich, CT, USA), and high molecular weight (HMW)-uPA was from Medac (Hamburg, Germany).

Injection protocol and cell depletion procedures

NMRI mice were used in all the experiments unless stated otherwise. Both HMW-uPA and LMW-uPA were injected intra-articularly into the right knee joint in a total volume of 20 μl . Control mice received an equivalent volume of PBS buffer.

For monocyte depletion, mice were injected subcutaneously with etoposide (Bristol-Myers Squibb, Bromma, Sweden; 12.5 mg/kg body weight in a volume of 100 μl) on three consecutive days before and after the uPA injection. FACS analysis showed that such a procedure depletes the monocyte population by more than 90% [21]. To assess the role of lymphocytes, uPA was injected into SCID mice lacking functional T lymphocytes and B lymphocytes.

Inactivation of uPA with synthetic inhibitor

Synthetic peptide *H*-D-Pro-Phe-Arg-chloromethylketone (PPACK; Bachem, Feinchemikalien AG, Switzerland) was used to inhibit the serine proteinase activity of uPA. To allow complex formation, uPA (3 μM) was incubated with 30 μM PPACK in Tris-HCl buffer (pH 7.4) in a 37°C water bath for 30 min. The inhibitory effect of PPACK was evaluated by hydrolysis of S-2251 (0.3 mM; Chromogenix, Mölndal, Sweden) in the presence of 300 nM Glu-plasminogen (Biopool, Umeå, Sweden). The preincubated uPA-PPACK complex was injected intra-articularly into the right knee joint of each mouse. Control mice received PPACK alone (40 nmol/joint). In the parallel experiment, 40 nmol/mouse PPACK was injected intraperitoneally, twice daily during three consecutive days after the intra-articular injection of uPA.

Histopathological and immunohistochemical examination of joints

Three days after the joint injection, the mice were sacrificed by cervical dislocation and the right knee was removed for histopathologic and immunohistochemical examination. Histological examination of joints was carried out after routine fixation, decalcification and paraffin embedding of the tissue. Tissue sections of the knee joints

were cut and stained with H & E. All the slides were coded and evaluated blindly by two investigators with respect to synovial hypertrophy, to the inflammatory cell infiltration of the synovia, to pannus formation, and to cartilage and subchondrial bone destruction. Synovial hypertrophy was defined as a synovial membrane thickness of more than two cell layers. The intensity of inflammatory cell infiltration of the synovia (arthritis index) was graded arbitrarily from 0 to 8.

For immunohistochemistry, the knee joints were demineralised in 10% EDTA–0.1 M Tris buffer (pH 6.95) by an enzymatic procedure previously described in detail [22]. The demineralised specimens were frozen in isopentane, and stored at -70°C . Serial cryosections (6 μm thick) were fixed in acetone and depleted of endogenous peroxidase activity by treatment with H_2O_2 . Sections were stained with rat monoclonal anti-CD11b (Mac-1, M1/70), CD4⁺ (GK 1.5), or CD8⁺ (all antibodies obtained from PharMingen, San Diego, CA, USA) in a humid chamber overnight, followed by incubation with biotinylated secondary antibodies (rabbit anti-rat IgG; DAKO, Glostrup, Denmark) and finally with avidin–biotin–peroxidase complexes. The enzymatic reaction was developed using H_2O_2 in 3-amino-9-ethyl-carbazole buffer (pH 5.5). Joint sections were also stained with goat antimouse uPAR monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and then incubated with horseradish peroxidase-labelled secondary antibodies (rabbit anti-goat; DAKO). Control staining was performed using irrelevant goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). All sections were counterstained with Mayer's haematoxylin.

***In vitro* spleen cell stimulation**

Mice spleens were aseptically removed, and passed through a nylon mesh. Erythrocytes were depleted by lysis in 0.83% ammonium chloride, and the remaining cells were carefully washed in PBS. The resulting single-cell suspension was adjusted to a cell density of $1 \times 10^6/\text{ml}$ in Iscove's complete medium (10% FCS, 2 mM L-glutamine, 5×10^{-5} M mercaptoethanol and 50 $\mu\text{g}/\text{ml}$ gentamicin). Splenocyte cultures were stimulated with LMW-uPA and HMW-uPA (final concentration between 60 and 0.6 nM). Control cultures were stimulated with lipopolysaccharide (10 $\mu\text{g}/\text{ml}$) and concavalin A (2.5 $\mu\text{g}/\text{ml}$). Supernatants were collected at defined time points for the determination of cytokine levels (see later).

To assess proliferative responses, a splenocyte suspension ($1 \times 10^6/\text{ml}$) in Iscove's complete medium was added to 96-well, flat-bottomed microtitre plates (Nunc, Copenhagen, Denmark) in triplicate and incubated with various preparations of uPA (final concentrations between 60 and 0.6 nM) for 72 hours. Splenocyte stimulated with lipopolysaccharide (10 $\mu\text{g}/\text{ml}$) served as a positive control.

During the last 12 hours, the cells were pulsed with 1 μCi [^3H]thymidine (Amersham, Bucks, UK). Cells were harvested into a glass fibre filter and thymidine incorporation was measured in a β -counter. The results were expressed as a stimulation index (mean \pm SEM) and were compared with nonstimulated cells.

Measurement of cytokine levels

IL-6 levels were measured by a bioassay employing the cell clone B13.29, subclone B9, which is dependent on IL-6 for its growth [23]. The cell suspension ($1 \times 10^6/\text{ml}$) was incubated with sample supernatants for 72 hours and pulsed with 1 μCi [^3H]thymidine. Incorporation of thymidine corresponded to the amount of IL-6 in the culture. The results were compared with recombinant IL-6 standard (Genzyme, Cambridge, MA, USA).

IFN- γ levels were quantified by an ELISA. The 96-well, flat-bottomed polyester plates (Nunc) were coated with rat antimouse IFN- γ antibodies (2 $\mu\text{g}/\text{ml}$; Pharmingen, San Diego, CA, USA) in carbonate buffer (pH 9.6) overnight. After blocking with 1% Tris–BSA (pH 7.4), samples were added and incubated for 2 hours at 37°C followed by incubation with biotinylated antimouse antibodies (2 $\mu\text{g}/\text{ml}$) overnight. Colour development was registered at 450 nm using extravidin alkaline phosphatase (0.5 mg/ml) and substrate. Sample concentrations were calculated using recombinant mouse IFN- γ (Genzyme).

Tumour necrosis factor alpha (TNF- α) and IL-1 β levels were determined using Quantikine™ ELISA kits (R&D systems Inc., Abingdon, UK) following the manufacturer's instructions.

Measurement of uPA levels

The uPA levels in the plasma and synovial fluid samples as well as in various preparations of uPA were determined as a total uPA antigen level, by a sandwich ELISA (Haemochrom Diagnostica GmbH, Mölndal, Sweden) following the manufacturer's recommendation. Primary antibodies consisted of a mixture of monoclonal antibodies reactive with single-chain and two-chain variants of uPA. Samples were tested in a 1:20 dilution. The obtained values were recalculated using the reference curve.

Statistical analysis

The frequency of arthritis in groups was analysed by Fisher's exact test. The severity of arthritis was compared using the Mann–Whitney U test. The level of uPA in the blood and synovial fluid samples as well as the level of cytokines in the supernatants were expressed as the mean \pm SEM. Differences in the matched blood and synovial fluid samples were analysed by the paired *t* test. Differences in the groups were calculated by the Mann–Whitney U test. $P < 0.05$ was considered significant.

Results

Accumulation of uPA in synovial fluid of RA patients

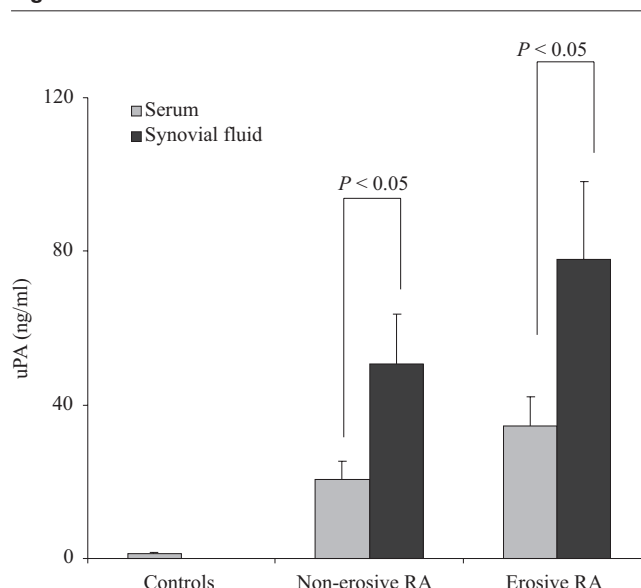
The plasma levels of uPA in RA patients ($n=67$) proved to be significantly higher compared with those in healthy controls ($n=22$) (29.9 ± 4.4 versus 1.3 ± 0.3 ng/ml, $P<0.0001$). The concentration of uPA in the joints of RA patients (71.5 ± 11.9 ng/ml) was even higher than in the matching blood samples ($P<0.0001$). The uPA levels in synovial fluid were strongly related to the corresponding blood level ($r=0.80$, $P<0.0001$) (Fig. 1) and to the duration of the disease ($P<0.05$). Levels of uPA correlated neither to the blood levels of acute phase reactants (C-reactive protein, sedimentation rate) nor to the white blood cell/neutrophil counts in synovial fluid. The RA patients positive for RF had higher levels of uPA compared with those patients negative for RF. This observation was true both in the case of blood samples (36.5 ± 6.0 versus 20.0 ± 5.5 ng/ml, not significant) and in the case of synovial fluid samples (79.2 ± 11.9 versus 38.9 ± 8.2 ng/ml, $P=0.032$).

The most striking difference with respect to the uPA levels in blood and in synovial fluid was observed when the patients were stratified with respect to erosive joint disease and RF. The uPA level was high in patients with erosive joint disease who were positive for RF ($n=38$), compared with those with erosive disease who were RF negative ($n=8$) (blood, 37.3 ± 7.1 versus 5.9 ± 1.9 ng/ml, $P<0.05$; synovial fluid, 83.8 ± 14.0 versus 22.5 ± 9.2 ng/ml, $P<0.05$). In contrast, patients with nonerosive joint disease revealed no differences of uPA levels with respect to the presence or absence of RF.

Intra-articularly deposited uPA is arthritogenic

The arthritogenic capacity of uPA was tested by a single intra-articular injection of both HMW-uPA and LMW-uPA into the knee joint of healthy mice. The concentrations tested spanned between 60 and 0.6 pmol/knee (from 2000 to 20 ng/knee). Morphological evaluation of the injected joints with respect to hypertrophy and inflammatory cell infiltration of the synovia, to pannus formation and to the development of bone erosions was performed on the fourth day after instillation of uPA. The reason for choosing this timeframe was to avoid potential immune reactivity to the heterologous protein. Both the frequency and severity of arthritis exhibited a dose-dependent pattern. The highest frequency of arthritis was observed using 60 pmol/knee LMW-uPA and reached 72% (22 out of 31 mice), being significantly higher than that of control joints injected with an equal volume of PBS buffer (four out of 20 mice, $P<0.05$). Synovitis (severity 1–8, mean 2.8 ± 1.9) was observed in all cases of arthritis. In addition, pannus formation ($n=5$) and bone erosions ($n=2$) were found (Fig. 2). The equivalent dose of HMW-uPA gave rise to arthritis in 40% of cases (four out of 10 mice), and the frequency remained low even

Figure 1



Accumulation of urokinase plasminogen activator (uPA) in the synovial fluid of patients with rheumatoid arthritis (RA). The level of uPA is presented as the mean \pm SEM (ng/ml). Significance regarding differences between the groups is indicated.

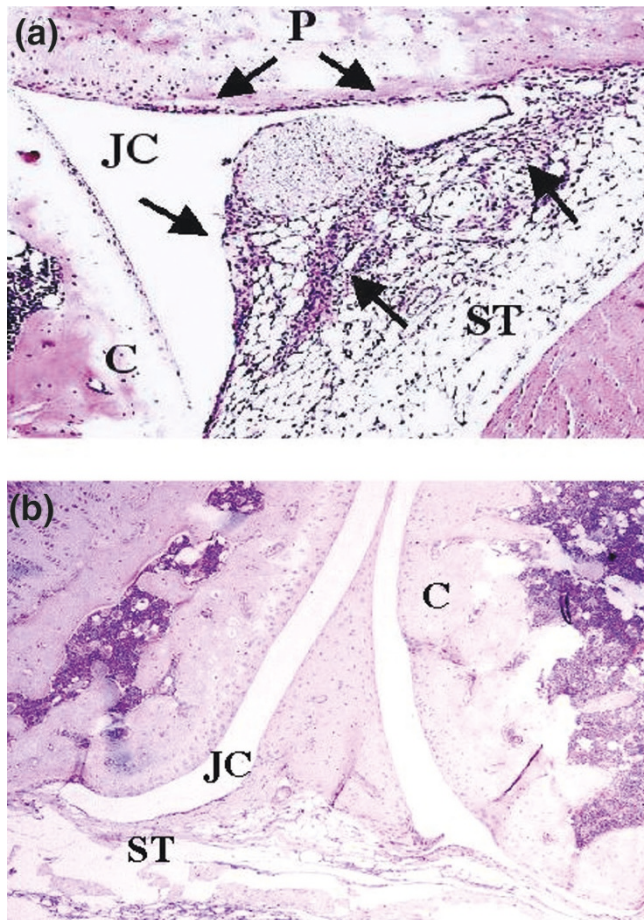
when a 50 times higher concentration was used (data not shown).

To assess the role of the uPAR in the mediating of inflammation after injection of uPA, uPAR^{-/-} mice and their genetic counterpart (75% BL6/25% 129, as indicated in Materials and methods) were injected with 60 pmol/knee LMW-uPA. Morphological features of arthritis were found in 83% of uPAR^{-/-} mice, demonstrating no significant difference from the control group possessing uPAR (5/6 versus 6/8, not significant).

The cellular composition of the synovial infiltrates as assessed by immunohistochemical staining of joint sections revealed the dominance of Mac-1⁺ mononuclear cells, while CD4⁺ and CD8⁺ cells were rarely observed in the synovial tissue (Fig. 3). uPAR expression was absent in the joints injected with LMW-uPA.

Monocytes and lymphocytes are essential for the uPA-induced inflammation

LMW-uPA (60 pmol/joint) was used to evaluate the role of different leukocyte populations in the development of uPA-induced arthritis. Depletion of peripheral monocytes with etoposide resulted in a remarkable reduction in the frequency and severity of synovial inflammation, since only one out of 12 mice (8%, severity mean 0.5 ± 0.2) developed arthritis. Analogously, injection of LMW-uPA into SCID mice lacking functional T lymphocytes and B lymphocytes resulted in arthritis only in two out of eight mice

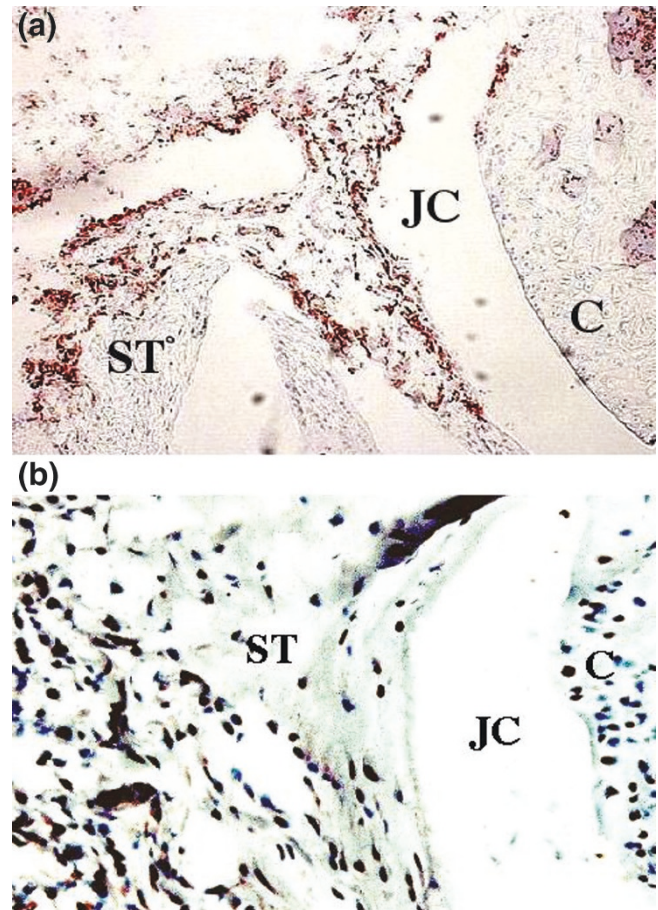
Figure 2

Histopathological appearance of arthritis after intra-articular injection of low molecular weight urokinase plasminogen activator (LMW-uPA). **(a)** Arthritic knee joint 4 days after the LMW-uPA injection (60 pmol/joint). Infiltration of mononuclear cells in the synovial tissue is apparent. Original magnification $\times 10$. **(b)** Knee joint injected with PBS revealed no signs of inflammation. Original magnification $\times 10$. JC, joint cavity; C, cartilage; ST, synovial tissue; P, pannus. Arrows indicate inflammatory cell infiltration in the synovium and pannus formation.

(25%, severity mean 0.75 ± 0.15). In both cases, the frequency and severity of arthritis were significantly lower than in the untreated animals exposed intra-articularly to uPA ($P < 0.05$) (Fig. 4). The results allow us to conclude that monocyte and lymphocyte cell populations are both important for the development of uPA-induced arthritis.

uPA induces cytokine production *in vitro*

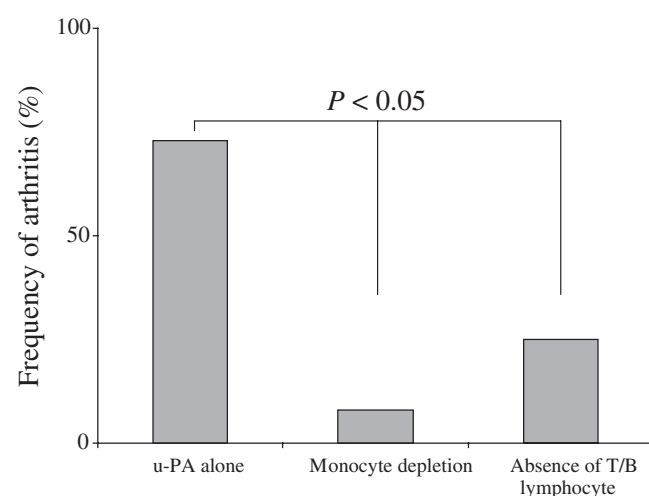
To evaluate cytokine production by mouse spleen cells exposed to uPA, single cell cultures were incubated with LMW-uPA in a final concentration ranging from 0.6 to 60 nM. Supernatants were collected after 6, 24 and 48 hours of stimulation. IL-6 was found already after 6 hours of stimulation, and its concentration remained high during 48 hours of culturing. The level of IL-6 was directly

Figure 3

Immunohistochemical staining of an arthritic knee joint injected with low molecular weight urokinase plasminogen activator. **(a)** Abundance of mononuclear cells expressing Mac-1 (red-brown colour) in the inflamed synovial tissue. Original magnification $\times 20$. **(b)** Arthritic knee joint stained with irrelevant goat IgG. JC, joint cavity; C, cartilage; ST, synovial tissue.

related to the concentration of LMW-uPA (Fig. 5). Indeed, treatment with 60 nM LMW-uPA resulted in 100-fold increased IL-6 production compared with nonstimulated cells (889 ± 134 versus 9 ± 6 pg/ml, $P < 0.001$). A different pattern of stimulation was registered for TNF- α and IL-1 β . Both TNF- α and IL-1 β appeared in supernatants first after 24 hours of stimulation, and required lower doses of LMW-uPA (6 nM) for the induction. Production of IFN- γ was not detected in uPA-stimulated spleen cell cultures.

Stimulation of spleen cells from uPAR $^{-/-}$ mice ($n = 3$) with LMW-uPA for 48 hours demonstrated a similar dose-dependent increase in IL-6 (3093 ± 564 pg/ml when stimulated with 60 nM) and no change in the TNF- α levels in the supernatants. The level of IL-6 did not differ from that detected in the uPAR $^{+/+}$ supernatants (2972 ± 437 pg/ml, $n = 3$).

Figure 4

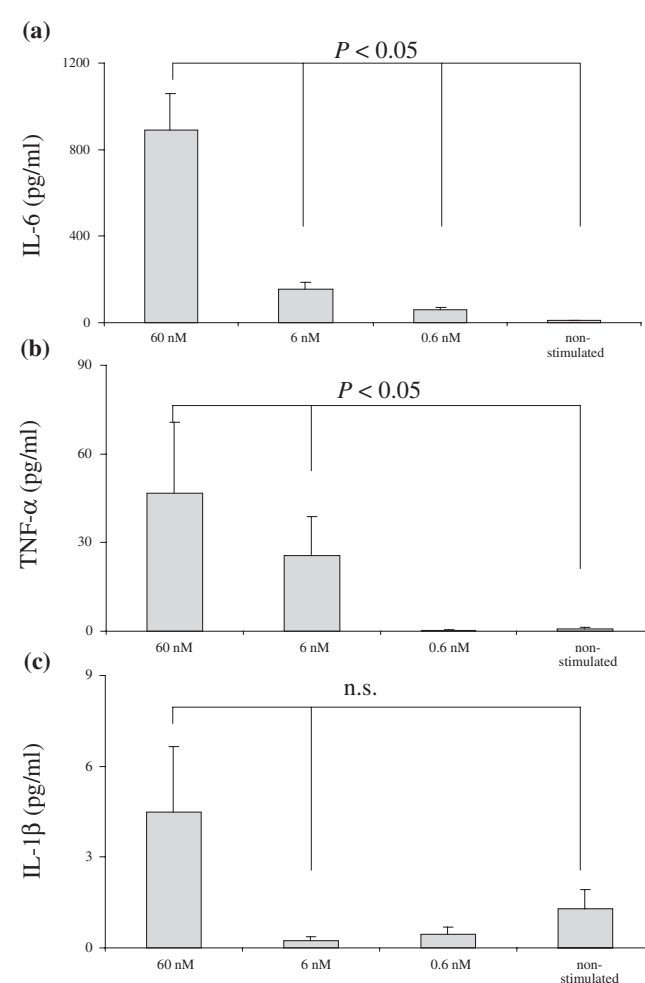
Essential role of monocytes and lymphocytes for the development of urokinase plasminogen activator (uPA)-induced arthritis. Frequency of histological signs of arthritis 4 days after a single injection of low molecular weight uPA (60 pmol/knee). Significance regarding the difference of incidence of arthritis between the groups is indicated.

To further assess the role of IL-1 in the development of uPA-induced arthritis, LMW-PA was injected intra-articularly (60 pmol/knee) into the IL-1 receptor type 1 null mice. A significant reduction in the frequency of arthritis was not observed compared with the control group of C57BL/6 mice (4/10 versus 6/10, not significant).

Several preparations of uPA were tested in the *in vitro* experiments. None of the uPA preparations (HMW-uPA or LMW-uPA) induced proliferative activity of mouse spleen cells. No change in the IL-6, TNF- α and IL-1 β levels were observed in the supernatants of NMRI, uPAR^{-/-} and uPAR^{+/+} spleen cells after stimulation with HMW-uPA.

Serine proteinase activity of uPA is important for the induction of arthritis

PPACK was used to inhibit the serine proteinase activity of uPA. Systemic (intraperitoneal) and local (intra-articular) methods of PPACK administration were tested. In both sets of experiments, PPACK was efficient in diminishing the frequency of the uPA-induced arthritis. When PPACK was administered intraperitoneally, uPA-triggered arthritis developed only in 36% of uPA-injected joints ($P < 0.05$). The severity of arthritis was also significantly reduced compared with that in untreated mice (1.2 ± 0.8 versus 2.8 ± 1.9 , $P < 0.05$). Direct intra-articular administration of PPACK was less efficient since, in 50% of joints injected with the uPA-PPACK complex, the arthritis was visible (not significant). These results indicate that the enzymatic function of uPA as a serine proteinase is of importance in mediating the inflammatory processes in the joint.

Figure 5

In vitro cytokine production following stimulation with low molecular weight urokinase plasminogen activator for 48 hours. (a) IL-6 ($n = 10$), (b) tumour necrosis factor alpha (TNF- α) ($n = 6$), and (c) IL-1 β ($n = 4$). Levels of cytokines are presented as the mean \pm SEM (pg/ml). Significant difference in the groups is indicated; n.s., not significant.

Discussion

The present results indicate that uPA is a constitutive component of the inflamed synovial fluid in RA joints. Increased levels of uPA detected locally in joints as compared with those in plasma of the same patients indicate either accumulation or, more probably, production of uPA by the cells invading the synovial tissue during the process of arthritis. The significant correlation observed between the uPA levels in the synovial fluid and the erosivity of arthritis suggests the regulatory role of uPA in joint destruction. However, direct proof of the arthritogenic properties of uPA is still lacking. The present study demonstrates that injection of uPA directly into the joints of healthy mice triggers arthritis, characterised by synovial inflammation and occasional cartilage destruction, and

provides the first direct evidence for the inflammatory/destructive role of this molecule *in vivo*.

Using a synthetic serine protease inhibitor (PPACK), we demonstrated that serine protease activity is essential for the proinflammatory effect of uPA. Substrates to be activated by uPA include plasminogen, MMPs and plasminogen-related growth factors (hepatocyte growth factor [HGF] and macrophage stimulating proteins), molecules participating in the degradation of the extracellular matrix and the regulation of angiogenesis [12,15]. The clinical relevance of MMPs and plasminogen in joint inflammation is based on the studies of the synovial fluid and tissues of RA patients that revealed an association between the level and localisation of these molecules with uPA [24,25]. Further support was obtained in animal models when downregulation of MMPs resulted in alleviation of antigen-induced arthritis [26–28]. In the present experiments, it has been clearly demonstrated that LMW-uPA gave rise, in a dose-dependent way, to the production of inflammatory and tissue-destructive cytokines (Fig. 4). IL-6, TNF- α and IL-1 β have all been previously demonstrated to participate in joint inflammation in human RA [29,30] and in experimental arthritis [31]. However, other effects of LMW-uPA once in the joint cavity cannot be excluded. Indeed, plasmin and HGF may be considered possible mediators of the uPA-induced inflammation, since both molecules act as chemoattractants for mononuclear cells [32–34], an important constituent of the synovial infiltrate in the uPA-induced arthritis. However, in our experimental model, intra-articular injection of plasmin/plasminogen resulted neither in a mononuclear cell influx in the synovial tissue nor in IL-6 production *in vitro* (results not shown). These observations allow one to suggest that plasminogen is not the major mediator of the uPA-induced inflammation.

Pannus formation was a remarkable finding in uPA-induced arthritis, taking into consideration the short time course of our experiments and the self-limiting character of inflammation. HGF is proposed to regulate the development of synovial hyperplasia [35,36]. In this context, uPA controls the formation of functional HGF on the surface of endothelial cells and monocytes [37,38]. The earlier findings together with the present data make HGF a possible candidate for the mediation of uPA-induced synovitis. This suggestion finds further support in the HGF-mediated release of inflammatory cytokines (IL-6 and granulocyte-macrophage colony-stimulating factor) by human monocytes [34].

The uPA-induced arthritis may be mediated, besides its proteolytic and cytokine triggering effects, by a direct interaction with the uPAR, a high affinity receptor that is constitutively expressed on peripheral blood leukocytes and may be found on the cultured cells of joint tissues,

such as synoviocytes and chondrocytes [11]. In the present study, however, we have demonstrated that interaction with the uPAR is not essential for the development of uPA-induced arthritis. First, the LMW-uPA used for the intra-articular injections lacks the N-terminal fragment necessary for binding to the receptor. Second, the injection of LMW-uPA to uPAR null mice resulted in arthritis. In addition, we were unable to detect the expression of uPAR in the murine inflamed joints injected with uPA.

Mononuclear cell infiltration of synovia is a typical sign of early RA, which is found in the majority of asymptomatic subjects preceding clinically overt arthritis [39,40]. The Mac-1⁺CD4⁺ mononuclear cells were by far the most predominant cell population in the inflamed synovial tissue triggered by delivery of uPA, thereby sharing this feature with early RA.

Intriguingly, the HMW-uPA, possessing both full serine protease activity and the amino-terminal fragment interacting with the uPAR, appeared to be less efficient both in giving rise to the cytokine production and in evoking joint inflammation. Analogous to our observations, HMW-uPA did not induce proinflammatory signalling in polymorphonuclear cells [41]. Both HMW-uPA and LMW-uPA are the two naturally existing forms of the uPA. Cleavage of the HMW-uPA is performed by several proteases including MMPs and plasmin, resulting in the release of the LMW-uPA molecule, and may serve as a feedback mechanism of the uPA-induced inflammation. Analysis for different forms of uPA in the synovial fluid indicated an excess of thrombin-cleaved uPA molecules that may be further activated by cathepsin C [42]. Little is known about the physiological role of LMW-uPA. It has been hypothesised that fragments of neutral and immunologically inert substances (e.g. hyaluronan, collagen-like polypeptides, fibrin degradation products, fibronectin) have biological activities different from their high molecular mass precursor molecules [43–46]. Similar reasoning could be applied with respect to uPA.

Conclusion

In conclusion, we have demonstrated that uPA, a constitutive component of inflamed synovial fluid in RA patients, is proinflammatory and may be responsible for an induction/perpetuation of intra-articular inflammation. In addition, the present results demonstrate that uPA mediates its effect through a uPAR-independent but serine protease-dependent mechanism.

Acknowledgements

The study was approved by the Ethic Committee of Sahlgrenska Hospital, and animal experimentation guidelines were followed. The work has been supported by Göteborg Medical Society, Swedish Association Against Rheumatism, King Gustaf V's Foundation, Swedish Medical Research Council, Nanna Svartz' Foundation, Börje Dahlin's Foundation, National Inflammation Network and the University of Göteborg.

References

- Weinberg JB, Wortham TS, Misukonis MA, Patton KL, Chitneni SR: **Synovial mononuclear phagocytes in rheumatoid arthritis and osteoarthritis: quantitative and functional aspects.** *Immunol Invest* 1993, **22**:365-374.
- Del Rosso M, Fibbi G, Matucci Cerinic M: **The urokinase-type plasminogen activator system and inflammatory joint diseases.** *Clin Exp Rheumatol* 1999, **17**:485-498.
- Brommer EJ, Dooijewaard G, Dijkmans BA, Breedveld FC: **Plasminogen activators in synovial fluid and plasma from patients with arthritis.** *Ann Rheum Dis* 1992, **51**:965-968.
- Kummer JA, Abbink JJ, de Boer JP, Roem D, Nieuwenhuys EJ, Kamp AM, Swaak TJ, Hack CE: **Analysis of intraarticular fibrinolytic pathways in patients with inflammatory and noninflammatory joint diseases.** *Arthritis Rheum* 1992, **35**:884-893.
- Busso N, Peclat V, So A, Sappino AP: **Plasminogen activation in synovial tissues: differences between normal, osteoarthritis, and rheumatoid arthritis joints.** *Ann Rheum Dis* 1997, **56**:550-557.
- Medcalf RL, Hamilton JA: **Human synovial fibroblasts produce urokinase-type plasminogen activator.** *Arthritis Rheum* 1986, **29**:1397-1401.
- Martel-Pelletier J, Faure MP, McCollum R, Mineau F, Cloutier JM, Pelletier JP: **Plasmin, plasminogen activators and inhibitor in human osteoarthritic cartilage.** *J Rheumatol* 1991, **18**:1863-1871.
- Ito A, Itoh Y, Sasaguri Y, Morimatsu M, Mori Y: **Effects of interleukin-6 on the metabolism of connective tissue components in rheumatoid synovial fibroblasts.** *Arthritis Rheum* 1992, **35**:1197-1201.
- Gyetko MR, Shollenberger SB, Sitrin RG: **Urokinase expression in mononuclear phagocytes: cytokine-specific modulation by interferon-gamma and tumor necrosis factor-alpha.** *J Leukoc Biol* 1992, **51**:256-263.
- Ronday HK, Smits HH, Van Muijen GN, Pruszczynski MS, Dolhain RJ, Van Langelaan EJ, Breedveld FC, Verheijen JH: **Difference in expression of the plasminogen activation system in synovial tissue of patients with rheumatoid arthritis and osteoarthritis.** *Br J Rheumatol* 1996, **35**:416-423.
- Hamilton JA, Campbell IK, Wojta J, Cheung D: **Plasminogen activators and their inhibitors in arthritic disease.** *Ann NY Acad Sci* 1992, **667**:87-100.
- Collen D: **The plasminogen (fibrinolytic) system.** *Thromb Haemost* 1999, **82**:259-270.
- Daci E, Udagawa N, Martin TJ, Bouillon R, Carmeliet G: **The role of the plasminogen system in bone resorption in vitro.** *J Bone Miner Res* 1999, **14**:946-952.
- Kikuchi H, Shimada W, Nonaka T, Ueshima S, Tanaka S: **Significance of serine proteinase and matrix metalloproteinase systems in the destruction of human articular cartilage.** *Clin Exp Pharmacol Physiol* 1996, **23**:885-889.
- Comoglio PM, Tamagnone L, Boccaccio C: **Plasminogen-related growth factor and semaphorin receptors: a gene superfamily controlling invasive growth.** *Exp Cell Res* 1999, **253**:88-99.
- Busso N, Peclat V, Van Ness K, Kolodzieczyk E, Degen J, Bugge T, So A: **Exacerbation of antigen-induced arthritis in urokinase-deficient mice.** *J Clin Invest* 1998, **102**:41-50.
- Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, Mulligan RC: **Physiological consequences of loss of plasminogen activator gene function in mice.** *Nature* 1994, **368**:419-424.
- Gyetko MR, Libre EA, Fuller JA, Chen GH, Toews G: **Urokinase is required for T lymphocyte proliferation and activation in vitro.** *J Lab Clin Med* 1999, **133**:274-288.
- Chapman HA, Wei Y, Simon DI, Waltz DA: **Role of urokinase receptor and caveolin in regulation of integrin signaling.** *Thromb Haemost* 1999, **82**:291-297.
- Dewerchin M, Nuffelen AV, Wallays G, Bouche A, Moons L, Carmeliet P, Mulligan RC, Collen D: **Generation and characterization of urokinase receptor-deficient mice.** *J Clin Invest* 1996, **97**:870-878.
- Verdrengh M, Tarkowski A: **Role of neutrophils in experimental septicemia and septic arthritis induced by *Staphylococcus aureus*.** *Infect Immun* 1997, **65**:2517-2521.
- Jonsson R, Tarkowski A, Klareskog L: **A demineralization procedure for immunohistopathological use. EDTA treatment pre-**
- serves lymphoid cell surface antigens.** *J Immunol Methods* 1986, **88**:109-114.
- Helle M, Boeije L, Aarden LA: **Functional discrimination between interleukin 6 and interleukin 1.** *Eur J Immunol* 1988, **18**:1535-1540.
- Koolwijk P, Miltenburg AM, van Erck MG, Oudshoorn M, Niedbala MJ, Breedveld FC, van Hinsbergh VW: **Activated gelatinase-B (MMP-9) and urokinase-type plasminogen activator in synovial fluids of patients with arthritis. Correlation with clinical and experimental variables of inflammation.** *J Rheumatol* 1995, **22**:385-393.
- Longstaff C, Merton RE, Fabregas P, Felez J: **Characterization of cell-associated plasminogen activation catalyzed by urokinase-type plasminogen activator, but independent of urokinase receptor (uPAR, CD87).** *Blood* 1999, **93**:3839-3846.
- Singer, Il, Kawka DW, Bayne EK, Donatelli SA, Weidner JR, Williams HR, Ayala JM, Mumford RA, Lark MW, Glant TT, Nabozny GH, David CS: **VDIPEN, a metalloproteinase-generated neopeptide, is induced and immunolocalized in articular cartilage during inflammatory arthritis.** *J Clin Invest* 1995, **95**:2178-2186.
- van der Laan WH, Pap T, Ronday HK, Grimbergen JM, Huisman LG, TeKoppele JM, Breedveld FC, Gay RE, Gay S, Huizinga TW, Verheijen JH, Quax PH: **Cartilage degradation and invasion by rheumatoid synovial fibroblasts is inhibited by gene transfer of a cell surface-targeted plasmin inhibitor.** *Arthritis Rheum* 2000, **43**:1710-1718.
- van Meurs JB, van Lent PL, Holthuysen AE, Singer, Il, Bayne EK, van den Berg WB: **Kinetics of aggrecanase- and metalloproteinase-induced neopeptides in various stages of cartilage destruction in murine arthritis.** *Arthritis Rheum* 1999, **42**:1128-1139.
- Taylor PC, Williams RO, Maini RN: **Immunotherapy for rheumatoid arthritis.** *Curr Opin Immunol* 2001, **13**:611-616.
- Feldmann M: **Pathogenesis of arthritis: recent research progress.** *Nat Immunol* 2001, **2**:771-773.
- Bao L, Zhu Y, Elhassan AM, Wu Q, Xiao B, Zhu J, Lindgren JU: **Adjuvant-induced arthritis: IL-1beta, IL-6 and TNF-alpha are up-regulated in the spinal cord.** *Neuroreport* 2001, **12**:3905-3908.
- Falcone DJ, Borth W, Khan KM, Hajjar KA: **Plasminogen-mediated matrix invasion and degradation by macrophages is dependent on surface expression of annexin II.** *Blood* 2001, **97**:777-784.
- Syrovets T, Tippler B, Rieks M, Simmet T: **Plasmin is a potent and specific chemoattractant for human peripheral monocytes acting via a cyclic guanosine monophosphate-dependent pathway.** *Blood* 1997, **89**:4574-4583.
- Galimi F, Cottone E, Vigna E, Arena N, Boccaccio C, Giordano S, Naldini L, Comoglio PM: **Hepatocyte growth factor is a regulator of monocyte-macrophage function.** *J Immunol* 2001, **166**:1241-1247.
- Yukioka K, Inaba M, Furumitsu Y, Yukioka M, Nishino T, Goto H, Nishizawa Y, Morii H: **Levels of hepatocyte growth factor in synovial fluid and serum of patients with rheumatoid arthritis and release of hepatocyte growth factor by rheumatoid synovial fluid cells.** *J Rheumatol* 1994, **21**:2184-2189.
- Koch AE, Halloran MM, Hosaka S, Shah MR, Haskell CJ, Baker SK, Panos RJ, Haines GK, Bennett GL, Pope RM, Ferrara N: **Hepatocyte growth factor. A cytokine mediating endothelial migration in inflammatory arthritis.** *Arthritis Rheum* 1996, **39**:1566-1575.
- Ried S, Jager C, Jeffers M, Vande Woude GF, Graeff H, Schmitt M, Lengyel E: **Activation mechanisms of the urokinase-type plasminogen activator promoter by hepatocyte growth factor/scatter factor.** *J Biol Chem* 1999, **274**:16377-16386.
- Mars WM, Kim TH, Stolz DB, Liu ML, Michalopoulos GK: **Presence of urokinase in serum-free primary rat hepatocyte cultures and its role in activating hepatocyte growth factor.** *Cancer Res* 1996, **56**:2837-2843.
- Koizumi F, Matsuno H, Wakaki K, Ishii Y, Kurashige Y, Nakamura H: **Synovitis in rheumatoid arthritis: scoring of characteristic histopathological features.** *Pathol Int* 1999, **49**:298-304.
- Kraan MC, Versendaal H, Jonker M, Bresnahan B, Post WJ, t Hart BA, Breedveld FC, Tak PP: **Asymptomatic synovitis precedes clinically manifest arthritis.** *Arthritis Rheum* 1998, **41**:1481-1488.

41. Sitrin RG, Pan PM, Harper HA, Todd RF 3rd, Harsh DM, Blackwood RA: **Clustering of urokinase receptors (uPAR; CD87) induces proinflammatory signaling in human polymorphonuclear neutrophils.** *J Immunol* 2000, **165**:3341-3349.
42. Braat EA, Jie AF, Runday HK, Beekman B, Rijken DC: **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.** *Ann Rheum Dis* 2000, **59**:315-318.
43. Juliano RL, Haskill S: **Signal transduction from the extracellular matrix.** *J Cell Biol* 1993, **120**:577-585.
44. Laskin DL, Soltys RA, Berg RA, Riley DJ: **Activation of alveolar macrophages by native and synthetic collagen-like polypeptides.** *Am J Respir Cell Mol Biol* 1994, **10**:58-64.
45. Olman MA, Hagood JS, Simmons WL, Fuller GM, Vinson C, White KE: **Fibrin fragment induction of plasminogen activator inhibitor transcription is mediated by activator protein-1 through a highly conserved element.** *Blood* 1999, **94**:2029-2038.
46. West DC, Hampson IN, Arnold F, Kumar S: **Angiogenesis induced by degradation products of hyaluronic acid.** *Science* 1985, **228**:1324-1326.

Correspondence

Maria Bokarewa, Department of Rheumatology, Guldhedsgatan 10, S-41346 Göteborg, Sweden. Tel: +46 31 3424692; fax: +46 31 823925; e-mail: maria.bokarewa@rheuma.gu.se