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DIFFERENCE IN EXPRESSION OF THE PLASMINOGEN ACTIVATION SYSTEM IN SYNOVIAL TISSUE OF PATIENTS WITH RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS

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SUMMARY

Proteolytic joint destruction in inflammatory and non-inflammatory arthropathy is believed to be mediated, at least in part, by the plasminogen activation (PA) system. To further investigate possible involvement of the PA system, we quantified immunoreactive urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator (t-PA), both plasminogen activator inhibitors (PAI-1 and PAI-2) and u-PA-receptor (u-PAR) in synovial tissue extracts of 14 patients with rheumatoid arthritis (RA) and 12 with osteoarthritis (OA). u-PA, PAI-1, PAI-2 and u-PAR concentrations were significantly higher in RA than in OA patients. t-PA antigen levels were significantly lower in RA than in OA synovial tissue extracts. Immunohistochemistry was performed to compare the distribution and staining intensity of these components in samples of RA and OA synovial tissue. Intense immunostaining of u-PA, u-PAR, PAI-1 and, to a lesser degree, PAI-2 was observed predominantly in the synovial lining of RA patients. In OA patients, u-PA, PAI-1, PAI-2 and u-PAR were barely detectable. t-PA immunostaining was restricted to the endothelial side of vascular walls in both groups. We conclude that the observed increase of u-PA, u-PAR and PAI expression, distributed mainly in the synovial lining area of proliferative and invasively growing synovial tissue in RA patients, supports a pathogenic role for the PA system in destructive arthritis. Depressed t-PA-mediated plasminogen activation might contribute to delayed intra-articular fibrin removal.

KEY WORDS: Urokinase, Plasminogen activation, Immunohistochemistry, Rheumatoid arthritis, Osteoarthritis.

RHEUMATOID arthritis (RA) is a chronic inflammatory disease, characterized by the destruction of articular cartilage and bone. Proteolytic degradation of the extracellular matrix in inflammatory arthritis is considered to be mediated by proteinases like aspartic, serine, cysteine and metalloproteinases [1]. Hyperplastic inflamed synovial tissue overgrows and invades the articular cartilage, and may be involved in the destruction of bone, tendons and ligaments by the production of proteolytic enzymes [2, 3].

Several studies suggest an important pathogenic role of the plasminogen activation (PA) system in destructive joint disease [4–6], but detailed knowledge of the mechanism and components involved is lacking. The central enzyme, plasmin, is a broad-spectrum serine protease, involved in fibrinolysis and thrombolysis as well as in the degradation of extracellular matrix that is required for normal and pathological forms of cellular invasiveness [7]. Plasmin is able to degrade extracellular matrix directly [8] and by activation of latent matrix metalloproteinases [9, 10]. It is produced as inactive plasminogen which is converted into its active form by limited proteolysis of a single peptide bond by plasminogen activators. Two types of plasminogen activators have been characterized: tissue-type plasminogen activator (t-PA), generally viewed as being important in fibrin dissolution, and urokinase-type plasminogen activator (u-PA), considered to be responsible for plasmin generation in processes involving tissue remodelling. Other identified proteins of the PA system include two plasminogen activator inhibitors, PAI-1 and PAI-2 [7], and a cell surface u-PA binding protein, the u-PA receptor (u-PAR). This receptor binds and localizes pro-u-PA as well as u-PA on the cell surface [11].

In patients with RA, a positive correlation between PA and PAI concentrations in synovial fluid and several markers of disease activity has been demonstrated [12–14]. Increased levels of u-PA and PAI in synovial fluid of patients with inflammatory joint disease, compared with corresponding plasma levels, are indicative of local plasminogen activation [15]. Synovial fibroblasts, chondrocytes, and endothelial, mononuclear and polymorphonuclear cells are capable of synthesizing u-PA, t-PA, PAI-1 and PAI-2. These cells could be responsible for the local production of PA and PAI in RA [16–19]. It may be envisaged that local plasminogen activation, in the proliferative and invasively growing synovial membrane, promotes the degradation of joint cartilage and bone.

In the present study, concentrations of several components of the PA system, i.e. u-PA, t-PA, PAI-1, PAI-2 and u-PAR, have been determined in extracts of synovial tissue of patients suffering from RA or OA requiring surgery. Furthermore, the distribution of these components has been investigated by immunohistochemical staining of synovial tissue sections. In

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order to investigate a possible relationship between the level of synovial plasminogen activation and joint destruction, a comparison is made between the findings in destructive inflammatory arthritis, e.g. RA, with those in non-inflammatory, degenerative joint disease, i.e. OA. The results of this study support the pathogenic importance of the PA system in destructive joint disease.

MATERIALS AND METHODS

Tissue sampling and extraction

Specimens of synovial tissue were obtained from 14 RA patients and 12 OA patients who required joint surgery for severe disease. All RA patients, who fulfilled the established criteria [20], were operated on at the Orthopaedic Department of the University Hospital, Leiden. Patients with advanced OA, corresponding to grade 3–4 in the Kellgren classification system [21], were operated on at the Department of Orthopaedics of Rijnland Hospital, Leiderdorp. Specimens of synovial tissue were immediately frozen in liquid nitrogen and stored at −80°C until use.

For quantitative u-PA, t-PA, PAI-1, PAI-2 and u-PAR determination, tissue samples were homogenized in 1 ml 0.1% (v/v) Tween 80, 0.1 M Tris–HCl buffer (pH 7.5) per 60 mg wet tissue, as described previously [22]. The homogenates were centrifuged twice at 8×10^3 *g* for 2.5 min, and the supernatants collected and used in the assays. Protein concentrations were determined by the method of Lowry *et al.* [23].

Quantitative assays

u-PA antigen was measured with an enzyme-linked immunoassay that was developed in our laboratory and performed according to Koolwijk *et al.* [24]. The monoclonal antibodies used in this ELISA recognize all forms of u-PA (pro u-PA, active u-PA and the u-PA/PAI complex) with comparable efficiency. The detection limit is around 0.5 ng/ml. To assess u-PA activity, scu-PA and active tcu-PA were measured separately, using a biological immunoassay as described by Dooijewaard *et al.* [25].

t-PA antigen was determined using the commercial ELISA Imulyse t-PA (Biopool, Umeå, Sweden). This method measures free t-PA antigen and t-PA/PAI complexes with the same sensitivity. The detection limit is ~1.5 ng/ml.

PAI-1 antigen was measured with Innotest PAI-1 ELISA (Innogenetics, Antwerpen, Belgium), using monoclonal mouse anti-human PAI-1 antibodies. This assay recognizes all forms of PAI-1 with the same sensitivity, with a detection limit of ~5 ng/ml. PAI-2 antigen was measured with Tintelize PAI-2 ELISA (Biopool, Umeå, Sweden), using monoclonal mouse anti-human PAI-2 antibodies. This assay recognizes all forms of PAI-2, including the low-molecular-weight form and the glycosylated high-molecular-weight form. The detection limit of this assay is ~6 ng/ml.

u-PAR antigen was measured with Imubind u-PAR ELISA (American Diagnostica Inc., Greenwich, CT, USA), using polyclonal rabbit anti-human u-PAR. This assay recognizes soluble, native u-PAR as well as u-PAR/u-PA and u-PAR/u-PA/PAI-1 complexes. The detection limit is ~0.1 ng/ml. All enzyme immunoassays were performed in duplicate.

TABLE I

Comparison of u-PA, t-PA, PAI-1, PAI-2 and u-PAR concentrations in synovial tissue of patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Results are given as median and interquartile range (25–75%). The median concentrations of u-PA, PAI-1, PAI-2 and u-PAR antigen in RA synovial tissue are significantly higher than those in OA synovial tissue. The median t-PA antigen concentration is significantly lower in RA than in OA synovial tissue (Mann–Whitney *U*-test)

Parameter (ng/mg protein)	RA ($n = 14$)	OA ($n = 12$)	P
u-PA	6.9 (4.4–7.7)	0.03 (0–0.77)	0.0001
t-PA	3.8 (3.0–6.1)	17 (10–28.8)	0.02
PAI-1	15.5 (6.5–30)	3.3 (2.4–5.5)	0.002
PAI-2	0.1 (0–0.32)	0 (0–0)	0.01
u-PAR	2 (1.1–3.5)	0.8 (0.6–1.4)	0.01

Antibodies

Monoclonal antibodies against human u-PA (#3698), polyclonal goat anti-human t-PA antibodies (#387), monoclonal anti-human PAI-1 (#380) and monoclonal anti-human u-PAR antibodies were purchased from American Diagnostica Inc. (Greenwich, CT, USA). Goat polyclonal antibodies against human PAI-2 were a gift from E. Schüler (Behring Werke AG, Marburg, Germany). The characterization of these antibodies, including their positive and negative controls, has been described in previous work [26, 37].

Immunohistochemistry

Tissue samples of five RA and five OA patients were frozen in isopentane and stored at −80°C. Cryostat sections (4 μm) were air dried overnight at room temperature and stored at −80°C until use. Sections were fixed for 10 min in acetone at −20°C before incubation with the primary antibody.

With monoclonal antibodies, a three-step avidin–biotin–peroxidase complex method was applied (Vectastain Elitekit, Vector Laboratories, Burlingame, CA, USA), as described by de Vries *et al.* [26]. Polyclonal antibodies were applied to the sections, washed and incubated with peroxidase-labelled rabbit anti-goat immunoglobulin. Bound antibodies were

TABLE II

Immunohistochemical expression of u-PA, t-PA, PAI-1, PAI-2 and u-PAR in synovial tissue of patients with rheumatoid arthritis (RA) and osteoarthritis (OA)

	u-PA	t-PA	PAI-1	PAI-2	u-PAR
RA	++	+	++	±	++
OA	±	+	±	−	±

++ = strong expression, + = moderate expression, ± = weak expression, − = no expression.

visualized by using 3-amino-9-ethylcarbazole as a substrate for peroxidase, as described previously [26].

Calculations and statistical analysis

Antigen concentrations were expressed as nanograms of antigen per milligram of tissue protein. Differences between the median of the measured synovial tissue concentrations of u-PA, t-PA, PAI-1 and PAI-2 in the RA and the OA group were evaluated with the non-parametric Mann–Whitney *U*-test for unpaired parameters, utilizing the standard software package 'Solo' (BMDP Statistical Software, Los Angeles, CA, USA). Differences were considered significant at $P \leqslant 0.05$.

RESULTS

Quantitative assays

Enzyme-linked immunoassays were performed on homogenates of RA and OA synovial tissue samples to measure u-PA, t-PA, PAI-1, PAI-2 and u-PAR antigen

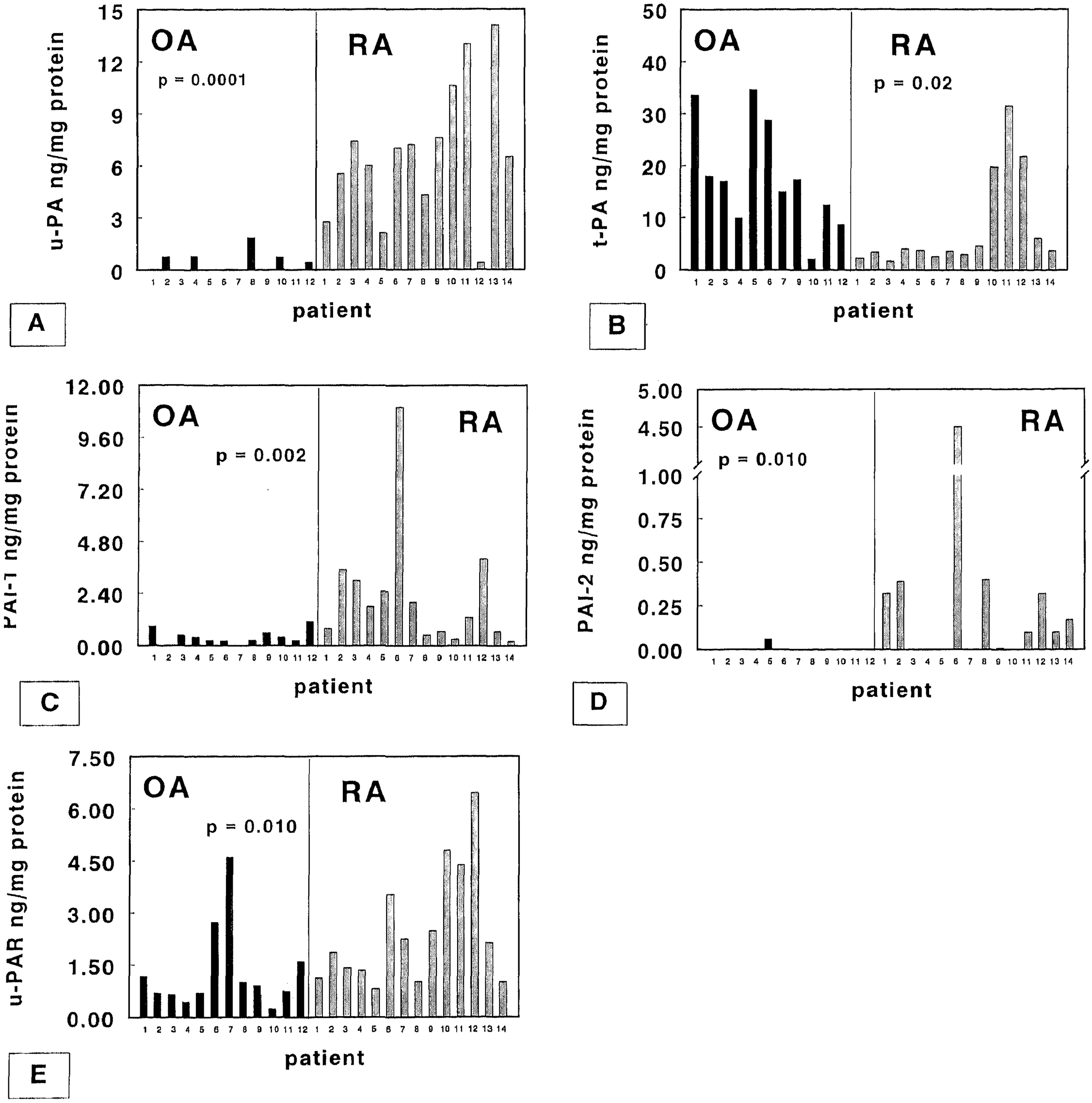


FIG. 1.—Antigen concentration in synovial tissue samples of 14 patients with rheumatoid arthritis and 12 patients with osteoarthritis. Antigen was measured in tissue extracts by enzyme immunoassay and expressed as ng/mg tissue protein. (A) u-PA antigen; (B) t-PA antigen; (C) PAI-1 antigen; (D) PAI-2 antigen; (E) u-PAR antigen. Statistical evaluation is summarized in Table I.

levels. The results in RA patients were compared with those in OA patients.

u-PA antigen could be detected in all RA patients and in five out of 12 OA patients. When present, the u-PA concentration was considerably higher in RA than in OA synovial tissue samples (Fig. 1A). Statistical analysis revealed a significantly higher median value in RA compared with the median value in OA (Table I).

scu-PA and active tcu-PA were determined with a bioimmunoassay. The levels of plasmin-activatable scu-PA and active tcu-PA in samples of synovial tissue of RA and OA patients were measured separately. Plasmin-activatable scu-PA could not be detected in synovial tissue from either RA or OA patients, although active tcu-PA was found (data not shown).

t-PA levels were variable in both groups, but could be detected in all available samples (Fig. 1B). In OA patient number 8, t-PA could not be measured because of a shortage of synovial tissue. The median concentration of t-PA antigen in the RA group was significantly lower than that in the OA group (Table I).

In all RA patients, and in 10 out of 12 OA patients, PAI-1 antigen was found in the synovial tissue (Fig. 1C). The median PAI-1 antigen level was significantly higher in the RA group than in the OA group (Table I).

PAI-2 related antigen was found in synovial tissue of eight out of 14 RA patients and could hardly be detected in one out of 12 OA tissue samples. PAI-2 levels in RA synovial tissue were highly variable (Fig. 1D). The difference between both groups appeared to be statistically significant (Table I).

In all samples of RA and OA patients, the presence of u-PAR antigen was detectable (Fig. 1E). The median u-PAR level was significantly higher in the RA than in the OA group (Table I).

Immunohistochemistry

In order to investigate the localization of PA, PAI and u-PAR, immunohistochemistry was performed on sections of synovial tissue samples from five RA and five OA patients. The degree of expression of the various parameters is summarized in Table II and shown in Fig. 2. Marked expression of u-PA was seen in all RA synovial tissues, especially in the synovial lining cell area and in giant cells (Fig. 2A), but also in some plasma cells in inflammatory cell infiltrates. Less intense immunostaining was observed in some blood vessels, especially in the media of arterioles.

In the OA synovial tissue samples, hardly any u-PA could be detected (Fig. 2B), but when it was expressed, it was restricted to the synovial lining cell area.

t-PA was observed in the endothelial cells of capillaries in both groups (Fig. 2C and D). Modest expression was seen in the RA synovial lining area. No extravascular t-PA could be detected in OA synovial tissue.

Strong PAI-1 immunostaining was seen in all RA synovial tissue samples. It was confined to the lining cell area (Fig. 2E) and capillaries. Hardly any PAI-1 was observed in OA synovial tissue samples (Fig. 2F).

In only a few RA patients, PAI-2 was observed in parts of the synovial lining area (Fig. 2G). No PAI-2 could be detected in any sample of OA patients (Fig. 2H).

Substantial u-PAR expression, predominantly in the lining cell area, was observed in synovial tissue of all RA patients (Fig. 2I). In OA synovial tissue, no expression or only very weak expression of u-PAR was seen, mainly in association with mononuclear cells in the interstitium (Fig. 2J).

DISCUSSION

In the present study, we investigated the expression and localization of several components of the PA system in samples of synovium obtained from patients with RA and OA. As this enzyme system is believed to be involved in extracellular proteolysis leading to joint destruction, a comparison is made between these two patient groups, to further investigate a relationship between synovial tissue plasminogen activation and joint destruction.

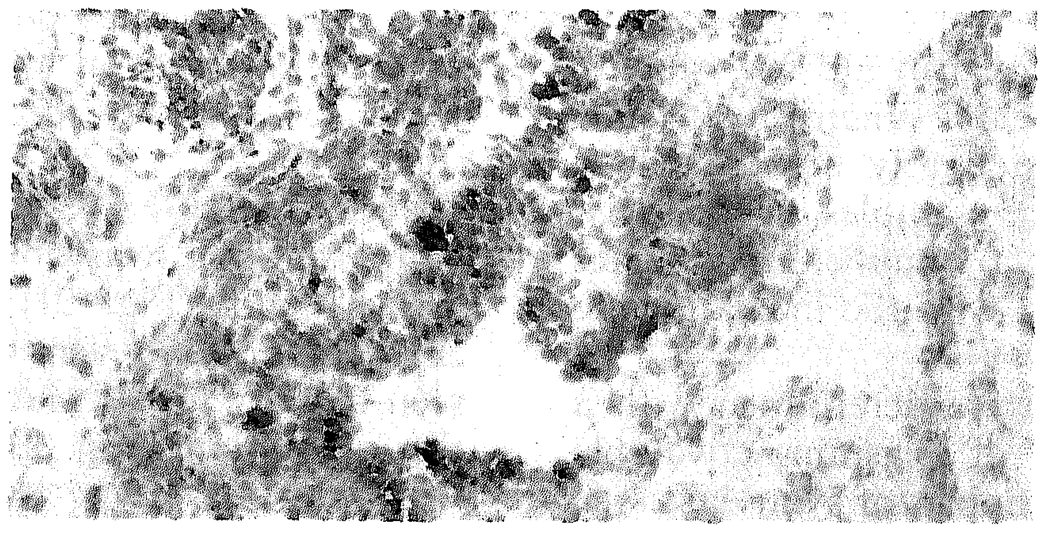
RA synovial tissue homogenates were found to contain variable but significantly higher concentrations of u-PA, PAI-1, PAI-2 and u-PAR than OA synovium homogenates (Figs 1 and 2, Table I). Immunohistochemically, the picture was more homogeneous. u-PA, PAI and u-PAR appeared to be located mainly in the RA synovial lining. t-PA antigen levels in RA synovial tissue were lower than in OA synovial tissue. It was located predominantly in vessel walls and perivascularly.

The increase in u-PA, PAI-1, PAI-2 and u-PAR in tissue extracts of inflamed synovium presumably finds its origin in an enhanced local production. Increased levels of u-PA, PAI-1 and, in some severe cases, PAI-2 in synovial fluid of inflamed joints compared with plasma [13–15] are indicative for generation within the joint. Furthermore, cell and tissue culture studies have demonstrated that synovial fibroblasts, but also chondrocytes, monocytes/macrophages and endothelial cells, are capable of synthesizing u-PA and PAI. *In vivo*, their production may be influenced by cytokines such as interleukin-1, tumour necrosis factor-α and granulocyte-macrophage colony-stimulating factor [16–19].

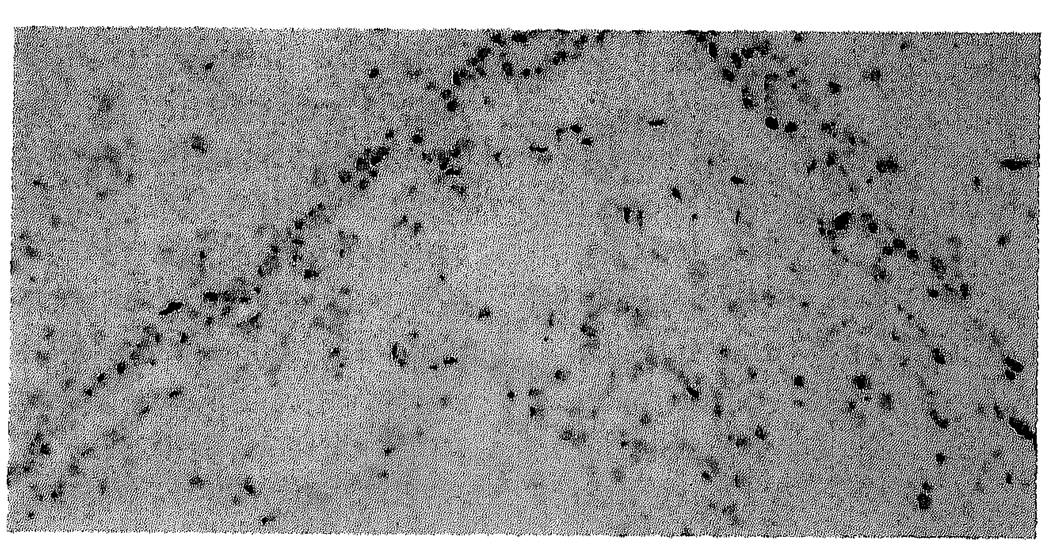
A local increase of u-PA at the expense of t-PA in RA synovial tissue compared with OA synovial tissue is in line with previous observations in synovial fluid [15] and in inflammatory bowel disease [27]. The altered ratio of t-PA (high fibrin affinity) to u-PA (low fibrin affinity) could reflect a shift from fibrin(ogen) degradation towards extracellular matrix degradation. This might result in protraction of fibrin removal on the one hand and enhanced proteolytic degradation of joint bone and cartilage on the other hand [15].

The quantitative ELISA data and the immunohistochemical analyses show a clear difference between RA and OA synovial tissue. This obvious pattern points towards an increased expression of all those enzyme system components believed to be involved in

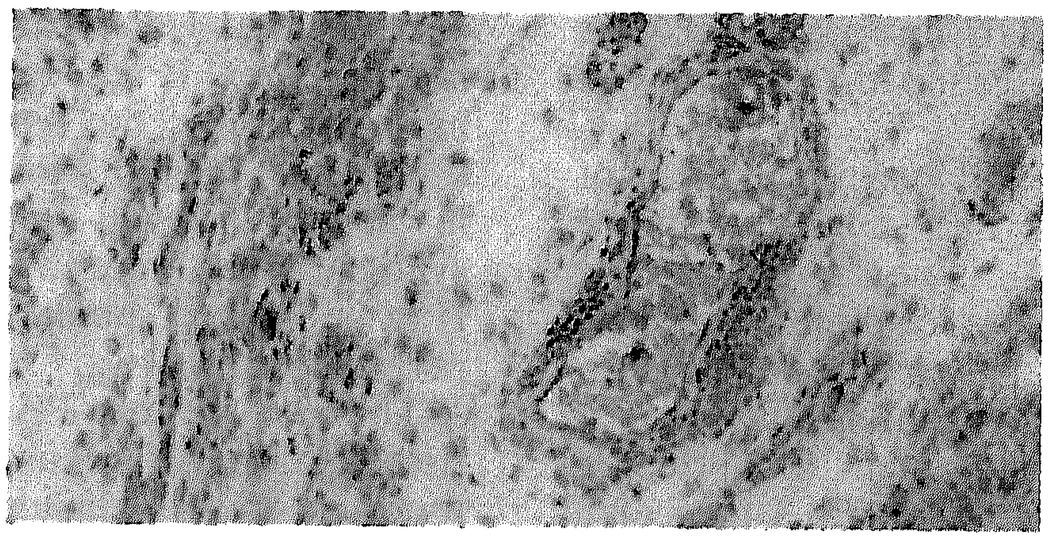
(A)



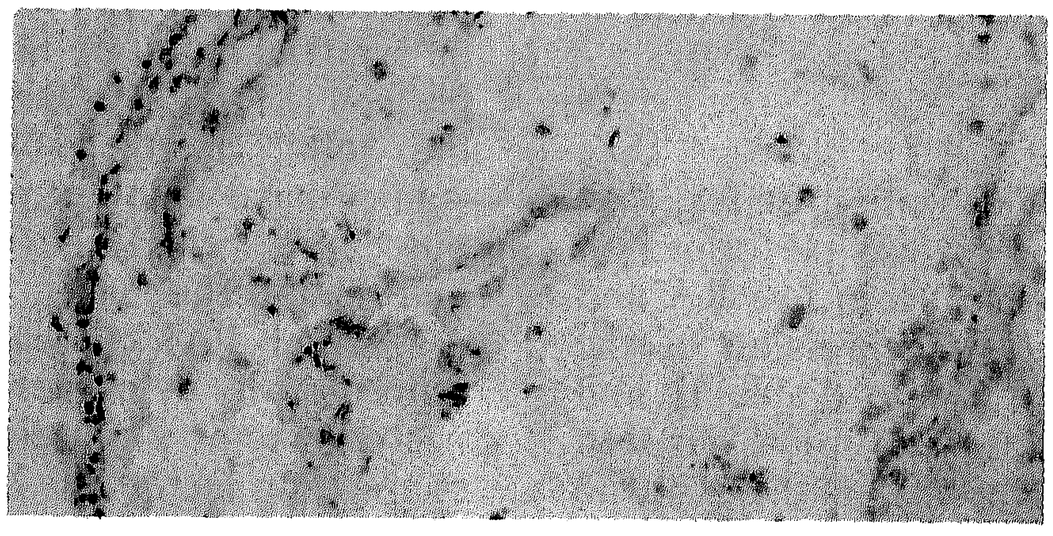
(B)



(C)



(D)



(E)

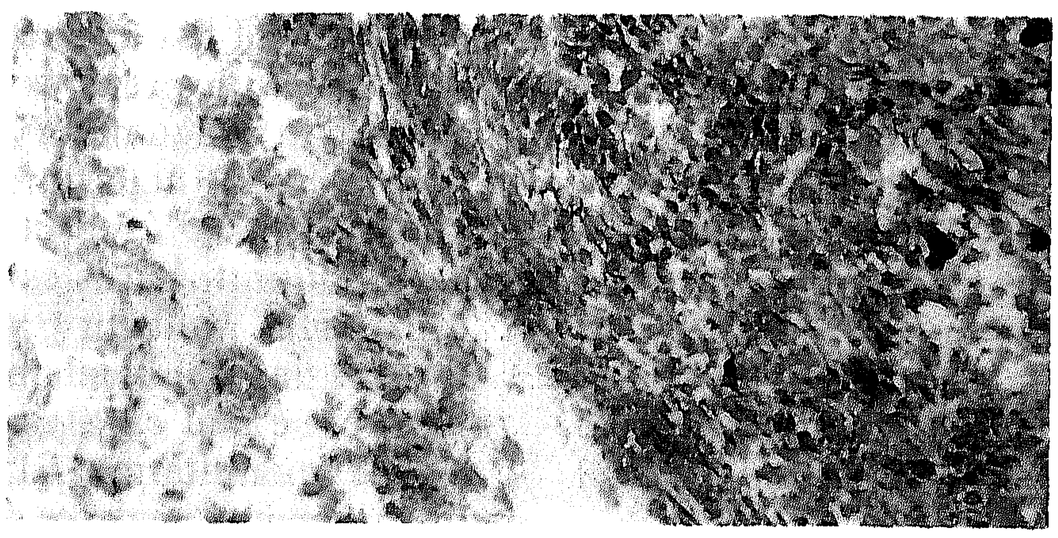


FIG. 2. (A–E).

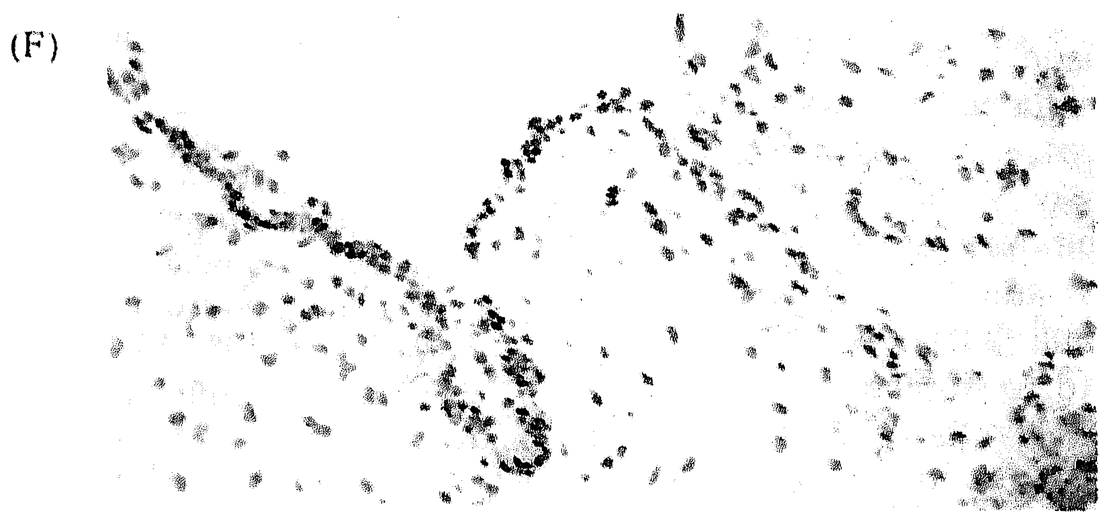


FIG. 2.—Immunohistochemical staining for components of the plasminogen activation system in rheumatoid arthritis (RA) and in osteoarthritis (OA). Immunoreactivity for u-PA: (A) distinct expression in synovial lining cells and giant cells in RA; (B) hardly any expression in OA. Immunoreactivity for t-PA: (C, D) positive staining in endothelial cells in synovial blood vessels in RA and OA. Immunoreactivity for PAI-1: (E) marked staining of synovial lining cells in RA; (F) no staining in OA. Immunoreactivity for PAI-2: (G) focal expression in synovial lining cells in RA; (H) no expression in OA. Immunoreactivity for u-PAR: (I) distinct staining of synovial lining cells in RA; (J) no staining of synovial cells in OA. Magnification: 250×.

tissue remodelling. A comparison between neoplastic tissue and invasively growing inflamed synovial tissue has been made before [7]. Indeed, increased u-PA and PAI-1 levels in tissue extracts or sections have been found in various malignant tumours [26, 28–30], and support the involvement of the PA system in extracellular matrix degradation, but how could an increased u-PA production lead to higher proteolytic activity when its inhibitors PAI-1 and PAI-2 are elevated? The answer may be found in the upregulation of u-PAR expression at the cell surface. First, *in vitro* studies have shown that several cell types are capable of binding u-PA at specific sites of the cell surface, whereas PAI was found at a different location [31]. Second, a differential inhibition of soluble and cell surface receptor-bound u-PA has been demonstrated [32], allowing enzymatic activity of receptor-bound u-PA even in a PAI-rich environment. Third, co-localization of u-PA/u-PAR and plasminogen on the cell surface results in ~100-fold more efficient activation of plasminogen than in the fluid phase [33]. Furthermore, plasmin bound to the cell surface is resistant to α2-antiplasmin [34]. Fourth, the interaction of u-PA with its cell-bound receptor has been shown to strongly enhance the degradation of extracellular matrix [35]. Loss of the surface u-PA activity by blocking the interaction between the receptor and its ligand has been shown to inhibit invasive growth [36]. These phenomena could explain net local proteolytic activity in the presence of increased inhibitor. Our idea about the mechanism of plasmin-mediated joint destruction is that increased u-PA production by inflamed hypertrophic and hyperplastic synovial tissue, overgrowing cartilage in a 'tumour-like' manner, could lead to activation of the readily available plasminogen on the cell surface at sites occupied by u-PAR. This localized formation of active plasmin, capable of degrading extracellular matrix directly and by activation of matrix metalloproteinases, may subsequently result in directed, proteolytic degradation of bone and cartilage.

In conclusion, the increased expression of u-PA, u-PAR, PAI-1 and PAI-2 in arthritic synovium compared with non-inflamed synovial tissue fits in with the concept of a localized, u-PA-mediated, plasmin-dependent degradation of articular structures, finding its origin in inflamed synovial tissue.

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