Rheumatoid synovial fibroblasts constitutively express the fibrinolytic pattern of invasive tumor-like cells

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Abstract

Objective

In rheumatoid arthritis (RA) the synovial membrane proliferates and invades the underlying tissues. The cell-associated fibrinolytic system (urokinase-type plasminogen activator, uPA; uPA receptor, uPAR; plasminogen activator inhibitor-type 1, PAI-1) is pivotal in cell invasion and proliferation. For this reason, the expression and the role of such enzymatic system was investigated in synovial fibroblasts (SF) of normal and RA patients.

Methods

In SF obtained from RA patients and control subjects, uPA, uPAR and PAI-1 were measured by ELISA of cell lysates and culture medium and by RT-PCR of mRNAs. uPA was also studied by zymography. Proliferation was measured by cell counting and cell invasion with the Boyden chamber.

Results

RA-SF over-express uPAR and PAI-1 and are more prone than the normal counterpart to spontaneous and uPA-challenged invasion and proliferation, which are counteracted by antagonists of the fibrinolytic system.

Conclusions

RA-SF display the fibrinolytic pattern and behaviour of invasive tumor-like cells. Antagonists of the fibrinolytic system are able to revert growth and invasion of both normal and RA-SF.

Key words

Rheumatoid arthritis, synovial fibroblasts, invasion, proliferation, uPA, uPAR, PAI-1, fibrinolytic system.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterised by hypertrophic synovitis and bone erosion. Cells involved in the inflammatory cascade in synovial tissue produce factors that contribute to the pathogenesis of the disease. These cells initiate and/or perpetuate the articular damage. In this respect, the activity of extracellular proteases, involved in the growth and invasion of the synovial pannus, is critical in the onset and maintenance of the disease.

Over-expression of cell-associated proteases characterises many diseases, including neoplastic cell invasion into surrounding tissues (1), tumor- or inflammation-associated angiogenesis (2) and the breakdown of the articular cartilage (3).

Two classes of such proteases, the cell-associated fibrinolytic system, which consists of the urokinase-type plasminogen activator (uPA), the uPA receptor (uPAR) and plasminogen activator inhibitors (PAIs), and the matrix metalloproteinase system (MMPs), co-operate in tuning cell invasion and proliferation activities (4).

In tumor invasion, activation of fibrinolytic system opens a path to invading cells in the extra-cellular matrix (ECM) (5). As a general rule, uPAR is over-expressed on the surface of invasive cells, while uPA may be produced by the cancer cell itself and by tumor-associated stromal and inflammatory cells. The cell-associated fibrinolytic system (6) co-works with the MMPs, also present in the fluid phase and at the surface of normal and neoplastic cells (7).

In RA, the hypertrophic synovial pannus behaves like a local tumor, invading the joint cavity and eroding the cartilage and the bone (8). This process is supported by MMPs and serine proteinases (9), present in high quantity in arthritic joints. There are evidences that serine-proteinases present in the synovial membrane (10), are produced by synovial fibroblasts (SF) and by inflammatory cells (11-13), and released into the joint cavity (12). SF also express uPAR on their membrane (11,12) and pick-up uPA either by autocriny or by paracriny. Additionally, RA synovial tissue shows higher levels of PAI-1 than osteoarthritis synovium (13) and cultured RA synoviocytes produce a higher amount of PAI-1 with respect to osteoarthritis and normal synoviocytes (11,14). In this respect, it is important to underline that PAI-1 may alternatively promote or suppress cellular adhesiveness (recently reviewed in 4). Since cell migration may be envisaged as alternating phases of cell attachment and detachment, PAI-1 seems to be endowed with all the requirements to favour cell migration.

The plasminogen activator/plasmin system is involved in the inflammatory remodelling of connective tissues occurring in arthritic joints (15). Plasminogen activator activity is detected in cultures of human monocytes, chondrocytes, and synoviocytes and can be regulated by a variety of cytokines produced in diseased joints (9).

In addition to its function in the plasminogen activation process, uPA/uPAR interaction induces plasmin-dependent events, such as chemoinvasion, and plasmin-independent events, such as chemotaxis and chemokinesis (16), proliferation (17,18), differentiation and autocrine secretion of uPA (19). The peculiar uPA arthritogenic activity and induction of the release of IL-6, IL-1β and TNF-α may be blocked by a synthetic uPA inhibitor (20). Recently, our group has shown, in normal SF, that uPA/uPAR interaction determines chemotaxis, chemoinvasion and proliferation in a dose-dependent fashion (21). Moreover, there is evidence not only that uPA/uPAR interaction stimulates cancer cell proliferation (9, 22), but also that high expression of uPAR alone regulates the rapid growth of cells by interacting and activating α5β1-integrins, which trigger a signalling cascade that culminates in very strong and persistent ERK activation (23).

Taken together, all the data related to the uPA/uPAR system in SF point to a pro-migratory and proliferative role of uPAR, which can be enhanced by loading SF uPAR with uPA produced also by other cells of the microenvironment. Since the fibrinolytic pattern and the

function of the fibrinolytic components of normal and RA SF isolated from their microenvironment has not yet been investigated, the aim of our work was to study in RAand control isolated human SF the constitutive (microenvironment-independent) expression of the components of the cell-associated fibrinolytic system (uPA, uPAR and PAI-1), and the uPA-dependent and uPAR-dependent effects on cell invasion and proliferation.

Materials and methods

Patients

Synovial tissue was obtained from 4 RAsubjects (1 male, 36 years old; 3 females, 34, 35 and 36 years old) undergoing surgery for knee synoviectomy or replacement, and from 4 controls (2 males, 30 and 34 years old; 2 females, 32 and 33 years old) undergoing arthroscopy for knee traumatic events.

Synovial cells cultures

Synovium was removed from knee joints, cut and subjected to a mild proteolytic treatment (0.05% trypsin, 0.5 mM EDTA in phosphate buffer saline, for 10 min at 37°C under gentle shaking). Trypsin was neutralized with fetal calf serum (FCS) (Celbio, Milano, Italy) and cells were plated in culture dishes with RPMI 1640 (Cambrex Bio Science, Milano, Italy) supplemented with 10% FCS, 2 mM glutamin (Cambrex) and penicillin-streptomycin (Cambrex). Cell monolayers were used within the 7th passage in culture. The cells were considered type B fibroblast-like synoviocytes if negative by staining with anti-CD69, anti-CD14, anti-CD11b and anti-CD11c (Santa Cruz Biotechnology, Santa Cruz, California, USA), positive by staining for the enzyme uridine-diphospho glucose dehydrogenase (UDPGD), and if they had a spindle-shaped, fibroblast-like morphologic appearance.

Analysis of uPA, uPAR and PAI-1 levels

Samples were analyzed for uPAR, uPA and PAI-1 by commercially available enzyme-linked immunoassay kits (IMUBIND, American Diagnostica, Montreal, Canada) according to the manufacturer instructions. Briefly, synoviocytes were seeded in 6 multi-well plates (25,000 cells/well) with 10% FCS in RPMI 1640. After 48 h of incubation, cells were washed 3 times with serum-free medium and incubated in 0.2%FCS medium for additional 48h. At the end of incubation, cells were detached, counted and lysed by a lysis buffer, as suggested by the manufacturer. The lysates were replaced in their original well and incubated for 1h at 4°C, to allow exhaustive extraction of undetached material. Cells extracts were centrifuged and stocked at –80°C till uPAR analysis. Culture mediums were collected, centrifuged and supernatants were stocked at –80°C till uPA and PAI-1 determination. The results were correlated to the standard curve, within the range of linearity. Each sample was evaluated in triplicate and with two different dilutions.

Analysis of uPA enzymatic activity

uPA enzymatic activity was evaluated by zymography. Culture medium samples were collected as previously described and concentrated ten times by centrifugation at 8000 rpm for 30 min in centricon tubes (Amicon Division, Beverly, MA, USA) with 30 kDa molecular weight cut-off pores. The samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis (10%) under non-reducing conditions and migrated proteins were transferred onto 0.45 μm pore-size nitrocellulose filter (BioRad, Richmond, California, USA) in a 0.04 M phosphate buffer (pH 6.5), under a current of 0.4 A during a 2-hour run. The nitrocellulose filter was removed and placed on an indicating layer containing casein and plasminogen. After overnight incubation at 37°C, uPA digestion of plasminogen showed clear bands of lysis in the cloudy casein background, corresponding to the position of plasminogen activators in the polyacrylamide gel. uPA 0.1U/ml was used as positive control. In some zymograms, amiloride was incorporated into the underlay at a concentration of 1 mmol/L to specifically inhibit uPA. In order to be sure to load comparable amounts of culture medium in the zymography analysis, the media to be subjected to western blot were added with 250 μg/ml actin (Sigma Aldrich, cat. A 3653) as an internal standard, and the transferred proteins were quantified and normalized to the corresponding actin protein revealed with an anti-actin monoclonal antibody (Sigma Aldrich, cat. A5060).

Proliferation assay

Cell growth was quantified in sub-confluent cell monolayers. SF were seeded in 24 multi-well plates (15,000 cells/well) with 10% FCS in RPMI 1640. After 24h incubation to allow adhesion, cells were washed 3 times with serum-free medium and incubated in 0.2% FCS medium for additional 48h. Medium was then removed and cells were incubated for 48 h in 10% FCS medium alone; 0.2% FCS medium alone; 0.2% FCS with different concentrations of uPA (50, 100, 250, 500, 1000 ng/ml) (Serono, Roma, Italy) alone or with uPA or uPAR antagonists. These are anti-human uPA monoclonal antibody 5B4 (Mab 5B4) and anti-uPAR monoclonal antibody 3936 (mAb 3936) (American Diagnostica, Montreal, Quebec, Canada), which were used at 1.5 μg/ml. mAb 5B4 impeded sterically uPA/uPAR interaction, as well as mAb 3936, developed against the uPA-binding site of uPAR. Each experimental point was performed in triplicate. At the end of incubation cells were counted.

Migration assay

The Boyden chamber procedure was used to evaluate cell migration (16). A 48-well micro-chemotaxis chamber (Neuroprobe, Gaithersburg, MD, USA) was used. The two wells were separated by a polyvinyl-pyrrolidone (PVP)-free polycarbonate filter (Neuroprobe), 8 m pore size. To evaluate chemoinvasion, the filter was coated with Matrigel (Becton-Dickinson Labware, Two Oak Park, Bedford, MA, USA) (50 μg/filter). Test solutions were dissolved in serum-free medium and placed in the lower wells. 50 μl of cell suspension (12,500 cells) were added to each upper well. In the experiment with neutralizing antibodies, the anti-uPA mAb 5B4 was placed in the lower wells, while the anti-uPAR mAb 3936 was

incubated with the cell suspension. The chamber was incubated at 37°C for 5 hours. Cell migration was measured as previously described (16). Each experimental point was performed in triplicate.

uPA, uPAR and PAI-1 genes mRNA quantitation by reverse transcriptase-polymerase chain reaction

Messenger RNA levels of uPA, uPAR and PAI-1 genes in basal conditions were determined by an internal standard-based quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. GAPDH was chosen as the internal standard gene because of its expression at a constant per-cell level.

Total RNA was extracted according to the method of Chomczynski and Sacchi (24). RT-PCR and analysis of PCR products were performed as previously described (25).

Statistics

Non-parametric Wilcoxon test for independent samples was used to compare results from control and RA synoviocytes for the levels of uPA, uPAR and PAI-1. The results were expressed as mean ± standard deviation (SD).

Zimography was evaluated by densitometric comparison between lysis areas produced by uPA. The proliferation induced by uPA was evaluated by 2-tailed t-test for independent samples and by analysis of variance (ANOVA).

Migration was expressed as the mean ± SD of % of basal response considered as 100%.

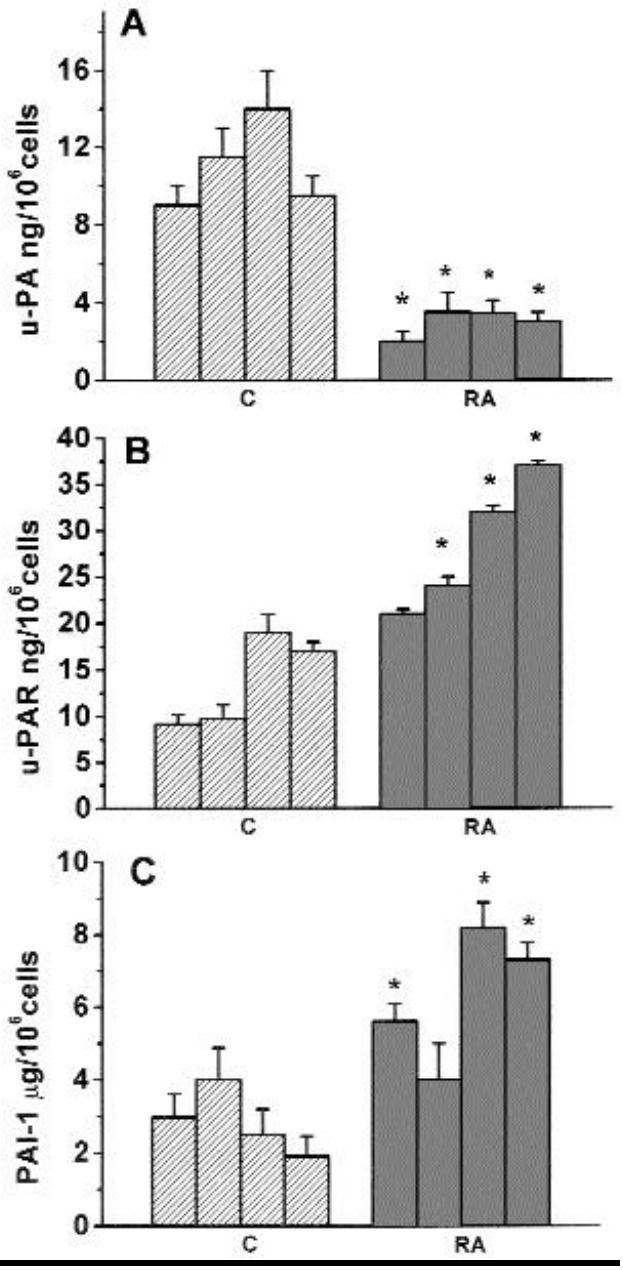

Fig. 1. Fibrinolytic pattern of control and RAsynovial fibroblasts. C refers to 4 different synovial cell cultures from control individuals, RA refers to synovial cell cultures from 4 different rheumatoid arthritis patients. **A**: uPA antigen was quantified on aliquots of the culture medium by ELISA assay and reported as ng/10^6 synovial cells. **B**: uPAR was quantified by ELISAassay on aliquots of cell lysates and reported as ng/10^6 synovial cells. **C**: PAI-1 was measured in aliquots of the culture medium, and reported as µg/10^6 synovial cells. In all panels, each point represents the mean ± SD of three experiments performed in triplicate on each synovial fibroblast line. In panel A, * indicates that each value is significantly different from the lowest value of the control counterpart ($p < 0.05$). In panels B and C, * indicates that each value is significantly different from the highest value of the control counterpart ($p < 0.05$).

Results

uPA, uPAR and PAI-1 levels in control and RA synovial fibroblasts and RT-PCR for uPA, uPAR and PAI-1 gene expression

In the culture medium of RA-SF, uPA was significantly lower than in control cells (2.6 ± 1.4 vs 10.9 ± 2.1 ng/10^6 cells, p = 0.01) (Fig. 1A). On the contrary, RA-SF released into the culture medium significantly higher amounts of PAI-1 compared to control SF (6.3 ± 1.1 vs 2.9 ± 0.7 µg/10^6 cells, p = 0.01) (Fig. 1B). Higher levels of uPAR were found in cell lysates of RA-SF (28.5 ± 4.8 vs 13.0 ± 3.0 ng/10^6 cells, $p < 0.05$) (Fig. 1C).

Zimographic assay (Fig. 2), performed on aliquots of the culture medium, confirmed the data obtained with antibody-based determination of uPA. Zymography showed the presence of a single Mr 54000 lysis band. The amiloride sensitivity of the caseinolytic activity allowed its identification as uPA(not shown). Culture mediums of RA-SF displayed a lower activity than mediums obtained from control SF.

The analysis of uPA, uPAR and PAI-1 expression by RT-PCR provided the data shown in Fig. 3 A-O. The expression of each gene of the fibrinolytic system was normalized with respect to GAPDH gene expression. Gene expression was similar to protein expression: control lines showed less uPAR and PAI-1 and more uPA than RA. Moreover, the extent of mRNAexpression in each cell line paralleled the extent of protein level.

uPA-dependent and constitutive synovial fibroblast proliferation

The effects of uPA on proliferation were evaluated in control and RA-SF. Cells were counted 48 h after plating in the absence or in the presence of

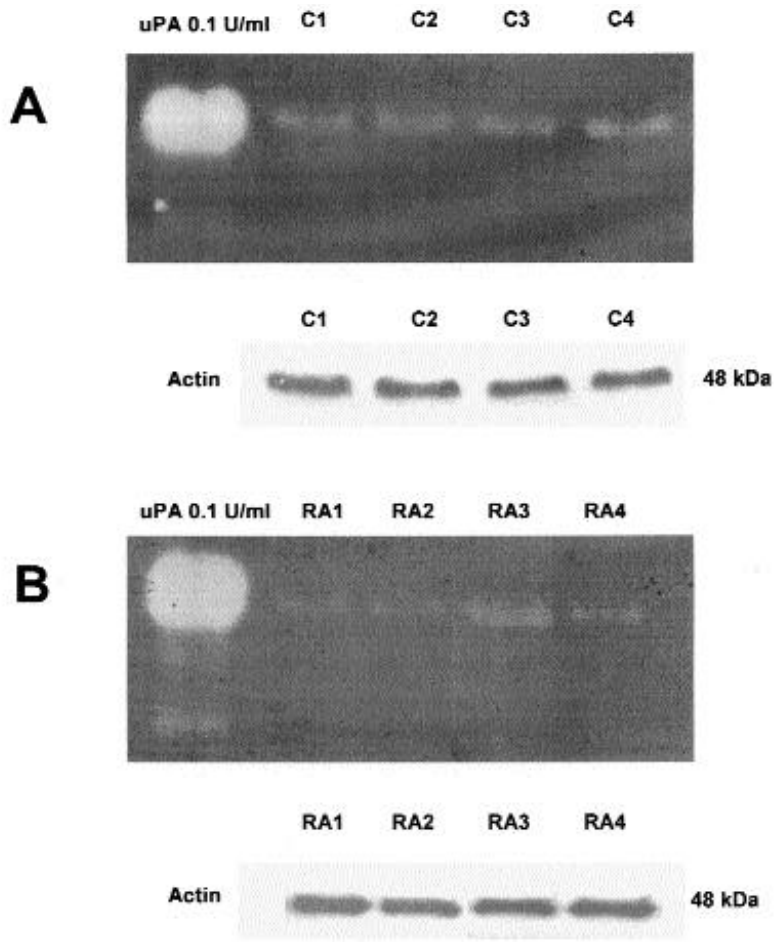

Fig. 2. Zymography of plasminogen activation activity in control and RAsynoviocytes. 30 µl of centrikon-concentrated culture medium from four different cultures of control synovial fibroblasts (panel **A**) and from four different cultures of RAsynovial fibroblasts (panel **B**) were subjected to SDS-polyacrylamide electrophoresis and transferred onto nitrocellulose, as described in the text. Actin was previously added to the culture medium, as an internal standard to verify comparable amounts of loaded proteins, as described under Materials and Methods. 30 µl of standard uPA (0.1 U/ml) was used as internal reference activity. uPAactivity results into clear lysis bands in the cloudy casein background.

increasing concentrations of uPA. uPA induced a dose-dependent cellular growth both in control and RA–SF, reaching a plateau at 250 ng/ml (Fig. 4A). Proportionally, uPA induced a higher percent increase of cell proliferation in RA than in control SF. These results indicate that uPA exerts a growth-promoting effect and that the minimal dose that stimulates a maximal growth is 500 ng/ml uPA both on control and RA-SF. This uPA concentration was used in further experiments to show uPA-dependent proliferation in the absence and in the presence of mAb antagonists of uPA/uPAR interaction. It is noteworthy that 48h after plating under no stimulation condition, even if the number of seeded cells was identical (15×10^3 cells/well) RAcells showed a greater capacity of spontaneous proliferation than control (Fig. 4B). RA-SF were more prone to spontaneous proliferation (0.2% FCS) and to induced proliferation (500 ng/ml uPA) than control SF. Cell growth elicited by 500 ng/ml of uPA in 0.2% FCS was strongly reduced by monoclonal antibodies antagonists of uPA (mAb 5B4), but even more by the anti-uPAR mAb 3936 (Fig. 4B), which reduced SF proliferation much below the level of control unstimulated SF, indicating that 1) uPAR *per se* is required for constitutive proliferation of both normal and RA-SF, and 2) uPA/uPAR interaction potentiates proliferation of both control and RAsynoviocytes.

uPA-dependent synovial fibroblast chemoinvasion

Invasion of the surrounding ECM and of the bone is a characteristic of proliferating synovial cells in RA. Therefore, to mimic this condition, the invasion of control and RA-SF was evaluated. SF were challenged with increasing concentrations of uPA (5-250 ng/ml) placed in the lower well of Boyden chambers in which the filters were coated with the natural basal membrane Matrigel (50 µg/filter) (Fig. 5A). The data show that uPA-dependent chemoinvasion was dose-dependent, with a maximal effect at 100 ng/ml for both control and RA-SF. Moreover, basal migration (invasion of Matrigel in the absence of chemoattractancts in the lower well of the migration chamber) was more pronounced (more than 40%) in RA than in control SF. This difference was maintained at each concentration of uPA in the lower well of the migration chamber. Increase of invasion observed after a 5-hour incubation with 100 ng/ml uPA, was counteracted by incubation of invasive cells with monoclonal antibodies antagonists of uPA (mAb 5B4) and of uPAR (mAb 3936), indicating that both uPAR *per se* and uPA/uPAR interaction is required for the invasive effect of uPA on normal and pathologic synovial cells (Fig. 5B).

Discussion

This study shows that isolated control and RA-SF differentially express uPA, uPAR and PAI-1 and that uPA/uPAR interaction stimulates SF chemoinvasion and proliferation. Identification of the complete machinery of the uPA/uPAR system in RA-SF, extends the results previously obtained by our group in control SF (21) and confirms observations reported by other groups (12, 13).

Compared to their normal counterpart, RA-SF display a fibrinolytic pattern characterized by over-expression of uPAR and PAI-1 and a parallel reduced expression and activity of uPA. Apparently, these data are not in agreement with previous observations (12,13), indicating an over-expression not only of uPAR and PAI-1, but also of uPA in

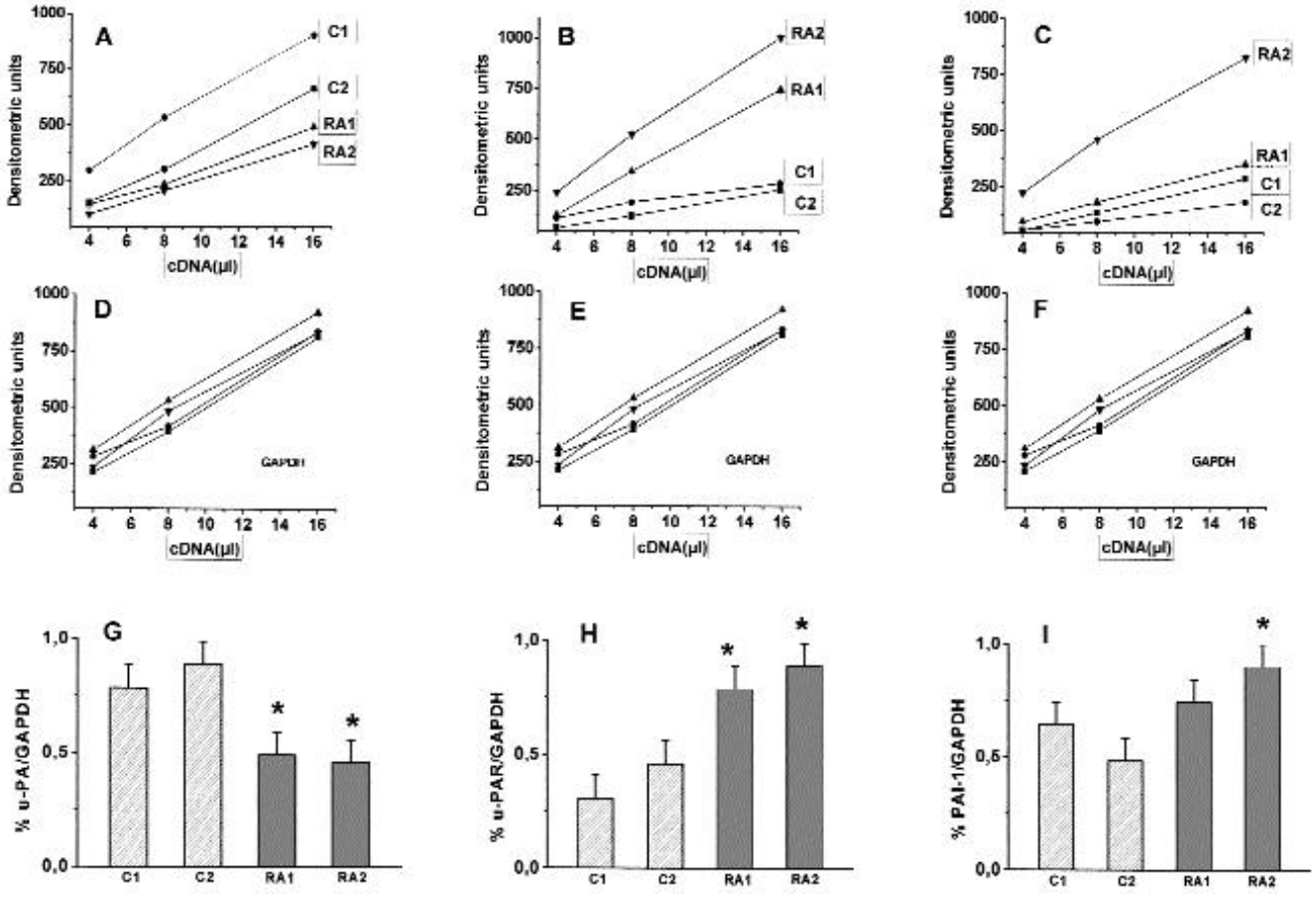

Fig. 3. RT-PCR analysis of uPA, uPAR, PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAin control and RAsynovial fibroblasts. For the RT reaction, 1 µg of total RNAisolated from two lines of control synovial fibroblasts (C1 and C2) and from two lines of RAsynovial fibroblasts (RA1 and RA2) were used. Panels A and D refer to analysis of uPA mRNA. The final quantification of uPA mRNAis shown in panel G. Panels B and E refer to the analysis of uPAR mRNA. The final quantification of uPAR mRNAis shown in panel H. Panels C and F refer to the analysis of PAI-1 mRNA. The final quantification of PAI-1 mRNAis shown in panel I. The upper line of panels (A, B and C) show the densitometric scanning of increasing volumes (µl) of amplified cDNA. The second line of panels (D, E and F) shows the densitometric scanning of increasing quantities of GAPDH amplified cDNA. The data reported in panels G, H and I indicate the relative amounts of uPA, uPAR and PAI-1 cDNA, as a ratio with the related GAPDH cDNAand represent the mean ± SD of two experiments performed in duplicate on each cell line. * indicates that values are significantly different from the corresponding value of the control or RAcounterpart ($p < 0.05$).

synovial tissue of RA patients. However, data on the up-regulation of all the components of the RA-SF-associated fibrinolytic system were performed on tissue specimens, not on isolated synovial cells cultured as monolayer. Under *in vivo* conditions, uPA expression up-regulation in RA-SF may be the product of the complex cytokine cross-talk triggered by the inflammatory environment of the arthritic joint. This consideration does not mean that uPA has not a pathogenic role in RA-associated joint lesions, but that RA-SF have either the possibility to pick-up uPA produced by other cells, or to bind on their membrane uPA produced by the same synovial cells under the effect of inflammatory cytokines. Therefore, it is possible that in the inflammatory environment SF pick-up uPA by autocriny or by a paracrine mechanism, since uPA is also produced by monocytes and chondrocytes. Indeed, the production of uPA is stimulated both in resident and inflammatory cells by growth factors and cytokines, present in high concentration in rheumatoid synovium. Granulocyte-macrophage colony stimulating factor (GM-CSF) increases uPA activity and uPA m-RNA level of human monocytes *in vitro* (26). IL-1, mainly produced by monocytes, induces uPA activity in human synoviocytes (27,28). Previous observations not only reported an over-expression of uPA in synovial tissue of RA patients, but also hypothesized that increased uPA might be associated with the clinical severity of the disease (29), and descrided an association between uPA gene 3'-UTR T/C polymorphism and RAto support, from a genetic point of view, the hypothesis that uPA is associated with RA(30).

To our knowledge, no studies are available on the constitutive expression of fibrinolytic molecules in isolated human normal and RA-SF. In a study performed to analyse the gene expression profile of RA-SF cultures, authors observed over-expression of platelet-derived growth factor receptor-α (PDGFR-α), plasminogen activator inhibitor-1 (PAI-1) and of stromal cell-derived factor 1A (SDF1A), speculating that such an aberrant gene expres-

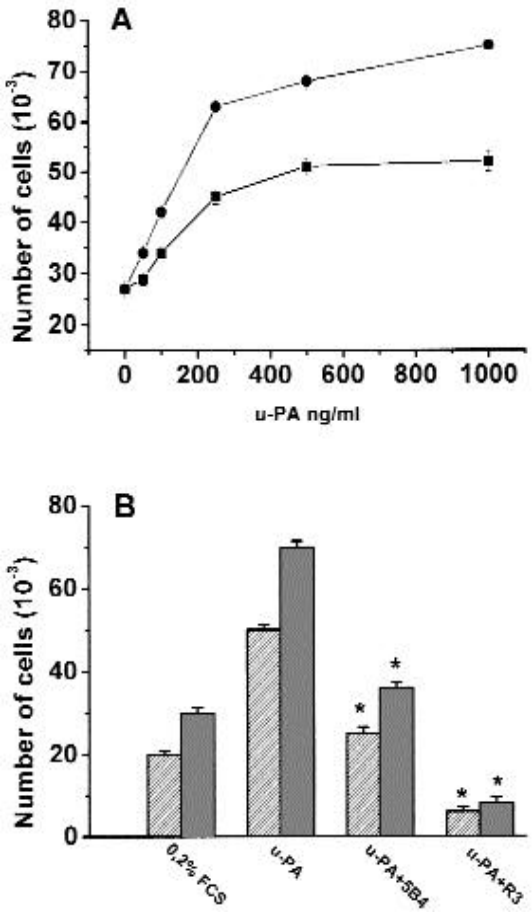

Fig. 4. uPA/u-AR-dependent proliferation in control and RA synoviocytes. **A**: proliferation of control (■) and RA (●) synoviocytes as a function of uPA concentration. **B**: proliferation of normal (hatched column) and RA(grey column) synovial fibroblasts under control conditions (0.2% FCS), under the activity of 500 ng/ml uPA in 0.2% FCS, and in the presence of mAb 5B4 and mAb 3936 (R3), that impair uPA/uPAR binding (see text for details). In both panels, each point represents the mean ± SD of three experiments performed in triplicate on 4 normal and 4 RAsynovial fibroblast lines. * indicates that values are significantly different from the corresponding value obtained in the presence of agonist (uPA) ($p < 0.05$).

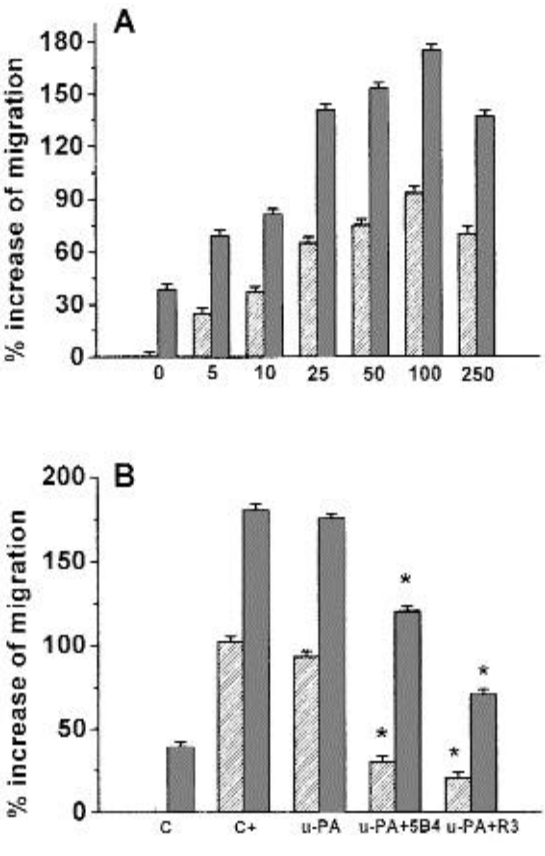

Fig. 5. uPA/uPAR-dependent chemoinvasion in control and RAsynovial fibroblasts. **A**: cell invasion of Matrigel-coated filters of control (black column) and RA (grey column) synovial fibroblasts, as a function of uPA concentration in the lower well of the migration chamber. **B**: percent increase of Matrigel invasion in the following conditions: C, invasion of control and RA synovial cells in the presence of 0.2% FCS in the lower well; C+, invasion stimulated by conditioned medium of transformed human cells (medium of A431, human epidermoid carcinoma cell line); uPA, invasion challenged with 100 ng/ml of uPA in the lower well; uPA + 5B4 and uPA + 3936 (R3), invasion challenged with 100 ng/ml uPA in the presence of 1.5 μg/ml of mAbs 5B4 and 3936 (R3) in the lower well, respectively. In both panels, each point represents the mean ± SD of three experiments performed in triplicate on 4 normal and 4 RA synovial fibroblast lines. * indicates that values are significantly different from the corresponding value obtained in the absence of antagonist mAbs ($p < 0.05$).

sion profile suggests that RA-SF may have retained the premature phenotype of primordial synovial cells (14). This situation is strongly suggestive of what happens in many human tumors. Experimental model systems with animal tumor metastasis and the finding that high levels of uPAR and PAI-1 in many tumor types predict poor patient prognosis, strongly suggest a causal role for the uPA system up-regulation in cancer cell invasion (and metastasis), a situation which is similar to RA synovial pannus invasion and growth. High levels of uPA, uPAR and PAI-1 in extracts from primary tumors are associated with poor relapse-free and overall survival (31). Studies aiming to target the production of components of the fibrinolytic system to the various cells of the tumor microenvironment have shown that only in a few tumors (i.e. squamous cell carcinoma) uPA is produced by malignant cells, while it is usually produced by tumor-associated fibroblasts in colon adenocarcinoma and mammary ductal carcinoma (6, 32). These studies do not rule out the possibility that uPA up-regulation in tumor cells themselves may be an epigenetic event.

Although the demonstration of a lower expression of uPA at the mRNA level in RA-SF suggests the presence of constitutive alterations of uPA gene expression, transcriptional or post-transcriptional mechanisms in the regulation of uPA production cannot be ruled out even under our experimental conditions.

The increased expression of PAI-1 in RA synoviocytes is in agreement with previous data showing higher levels of PAI-1 in RA synovial tissues (13) and higher production of PAI-1 in cultured RA-SF (11).

The increase in PAI expression and production is a common feature of invasive pathologies, including cancer. As for uPA, also PAI-1 does not seem to be produced by cancer cells [with some exceptions, such as squamous cell carcinoma (6)] but by other cells of the tumor microenvironment, such as endothelial cells (6). In RA, elevated PAI-1 could act as an ECM-stabilising molecule by its ability to block extra-

cellular proteolysis. This is an important requisite to provide cells with a substrate favouring cell movement. At the same time, PAI-1 is able to promote cell detachment, mainly by competition with vitronectin for uPAR binding sites (4). Therefore, cell migration can be envisaged as being regulated by "stop and go" signals provided by the alternating prevalence of ECM degradation and stabilisation.

The events described above are related to plasmin generation and are due to the cross-talk between the surface-activated plasmin and both receptor-bound and fluid phase MMPs zymogens. MMPs activation in the cell surrounding, ultimately causes cartilage destruction and bone erosions.

The increased production and overexpression of uPAR in RAmay depend on the need to activate the fibrinolitic pathway in order to degrade and invade ECM, as well as to promote interaction between uPAR and vitronectin, which provides the adhesive grip necessary for cell locomotion, events required in all the invasive pathologies (4).

In tumors, uPAR has always been found associated to invasive cells. As underlined in the introduction, uPAR delivers within the cell messages independent of uPAbinding, which result in cell proliferation. uPAR-mediated transduction is amplified by uPA binding and triggers other events, which solely rely on uPA/uPAR interaction, such as cell chemotaxis/chemokinesis. Within a tissue and in the *in vitro* Matrigel invasion assay, uPA-dependent chemoinvasion is triggered by transductions which activate the movement-related intracellular machinery and is matched with the generation of plasmin which is required to open a path to the invasive cell. Also synovial cell proliferation may constitutively depend on uPAR and may be potentiated by uPA on both normal and RA-SF. By their very nature these events are critical in the natural history of joint lesions.

These effects (uPAR/uPA-dependent invasion and proliferation, PAI-1 production) were already demonstrated by our group on control synoviocytes, enlarging the range of putative roles of the uPA/uPAR system in diseased joints (21). Our data are also in agreement with the recently reported proinflammatory and arthritogenic capacity of uPA in experimental arthritis (20). Our data, as well as data from other laboratories (12, 13), strongly suggest that RA-SF express a cell-associated fibrinolytic machinery which is substantially different from control synovial cells, and exploit all the properties of the cell-associated fibrinolytic system to express the disease-related phenotypic pattern, which consists in high invasivity and high proliferation with respect to normal SF. Since a strict cooperation among all the members of the fibrinolytic system is required to obtain the synovial RA phenotype, the up-stream inhibition of uPAR might provide an anti-invasive strategy in RA, where pannus invasion is a pivotal pathogenetic event.

Various attempts with different compounds have been done to inhibit cell surface associated uPA and uPAR in order to inhibit cell invasion (33). Our group demonstrated that inhibition of uPAR gene expression by antisense oligodeoxyribonucleotides (aODN) blocks the uPA-dependent proliferation, chemoinvasion, and chemotaxis of human control SF *in vitro* (21), while Jin T *et al*. have shown the ability of a synthetic uPA inhibitor to reduce inflammation (20). These results indicate that uPA inhibiting strategy and in particular the use of aODNs, might be considered to control cell invasion in RA, both administering the drug systemically or by intra-articular injection.

In conclusion, RA-SF express more pronounced phenotipic effects related to uPA/uPAR-dependent proliferation, chemotaxis and invasion. This confirms that the RA synovium behaves like a local invasive tumor which invades and proliferates exploiting the fibrinolytic machinery and that the components of this system may be a potential target for new RAtherapies.

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