Deflazacort modulates the fibrinolytic pattern and reduces uPA-dependent chemioinvasion and proliferation in rheumatoid arthritis synoviocytes

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Objective. Extracellular fibrinolysis, controlled by the cell-associated fibrinolytic system (urokinase plasminogen activator, uPA; uPA receptor, uPAR; plasminogen activator inhibitor type-1, PAI-1), is involved in cartilage damage generation and in rheumatoid arthritis (RA) synovitis. Since steroids reduce the rate of radiological progression of RA, we planned to evaluate in healthy and RA synoviocytes the effects of the steroid deflazacort on uPA, uPAR and PAI-1 expression, and subsequent phenotypic modifications in terms of uPA/uPAR-dependent invasion and proliferation.

Methods. uPA, uPAR and PAI-1 levels were studied by ELISA, RT-PCR (uPAR) and zymography (uPA) in synoviocytes from four RA patients and four healthy controls. Chemoinvasion was assessed by the Boyden chamber invasion assay, using Matrigel as the invasion substrate. Proliferation was evaluated by cell counting. Both invasion and proliferation were measured upon treatment with deflazacort 5 μM with or without parallel stimulation with uPA 500 ng/ml or in the presence of monoclonal anti-uPA and anti-uPAR antibodies.

Results. Invasion and proliferation of RA synoviocytes require a proper functional balance of the fibrinolytic system. Both deflazacort and monoclonal antibodies against uPA and uPAR reduced expression and activity of the system, thus inhibiting invasion and proliferation. In RA synoviocytes, deflazacort induced higher PAI-1 and lower uPA and uPAR levels, as well as a decrease in uPA enzymatic activity. The levels of uPAR mRNA were concomitantly reduced, as was uPA-induced chemooinvasion. All these effects were also shown in controls, though to a lesser extent.

Conclusions. Deflazacort might control RA synovial proliferation and invasion by differential modulation of single members of the fibrinolytic system.

Key words: Deflazacort, Rheumatoid Arthritis, Fibrinolysis, uPA, Synoviocytes, Steroids.
and synoviocytes, where their production can be regulated by a variety of cytokines produced in diseased joints [8, 11].

Upon isolation, RA synoviocytes recover their constitutive environment-independent phenotype, which has been suggested to resemble the premature phenotype of primordial synoviocytes [12]. Whatever the case, in the RA joint the abundance of uPA triggers a multi-enzyme cascade leading to ECM destruction and cell progression in the underlying tissues. Serine proteases are involved in cartilage degradation both by direct activation of latent MMPs, which have a main role in cartilage breakdown, and by inducing a potent pro-angiogenic stimulus that influences the synovial pannus growth.

uPA/uPAR interaction induces plasmin-dependent events, such as chemoinvasion, and also plasmin-independent events, such as chemotaxis and chemokinesis [13], proliferation [14, 15], differentiation and autocrine secretion of uPA [16]. Our group has shown that, in healthy synoviocytes, uPA/uPAR interaction determines dose-dependent chemotaxis, chemoinvasion and proliferation [17].

The final evolution of RA is erosion of bone and cartilage and joint ankylosis. The aim of using disease-modifying anti-rheumatic drugs (DMARDs) is to interrupt this trend, particularly in the early phases of the disease, in order to prevent bone erosions.

The hypothesis that steroids may have an effect on joint destruction has been re-evaluated. Several papers have addressed this problem and have supported the theory that steroids, in early RA, may significantly reduce the rate of radiological progression [18, 19]. Indeed, the withdrawal of these drugs led to a significant deterioration of the disease with an overshoot of radiographic progression [20].

Deflazacort (DFZ) is an oxazoline derivative of prednisolone with anti-inflammatory and immunosuppressive activity. In general, DFZ appears less active than other steroids on the parameters associated with the development of corticosteroid-induced osteoporosis [21].

DFZ is commonly used in the treatment of RA, for its efficacy and safety [21]. However, the effects of DFZ on synoviocytes are not clear and little is known about the relationships between the fibrinolytic system and DFZ in healthy and RA synoviocytes.

Our aim was to study the effects of DFZ on RA synoviocytes to understand if the drug may modulate the membrane-bound fibrinolytic system and if it may interfere with uPA-dependent cellular proliferation and invasion, in order to evaluate the potential future use of this drug in the control of RA-associated bone erosion and cartilage breakdown.

### Materials and methods

#### Patients

Patients with RA, whose demographic and clinical characteristics are shown in Table 1, and healthy controls matched for sex and age were used as a source of synoviocytes. Synovial tissue was obtained from four RA subjects undergoing surgery for synoviectomy or joint replacement, and from four controls undergoing orthopaedic surgery for knee traumatic events.

Before synoviectomy, in order to rule out potential confounding effect of treatment on synoviocytes, the DMARDs and corticosteroids were washed out for 6 weeks and only non-steroidal anti-inflammatory drugs and paracetamol were allowed. The design of the study, which conformed to standards currently applied in Italy, was approved by the local ethical committee and written informed consent was obtained from patients and controls enrolled in this study according to the Declaration of Helsinki.

#### Synovial cell cultures

Synovia was removed from knee joints, cut and subjected to a mild proteolytic treatment (0.05% trypsin, 0.5 mM EDTA in phosphate-buffered saline, for 10 min at 37°C with gentle shaking). Trypsin was neutralized with fetal calf serum (FCS) (Celbio, Milan, Italy) and cells were plated in culture dishes with RPMI 1640 (Cambrex BioScience, Milan, Italy) supplemented with 10% FCS, 2 mM glutamine (Cambrex) and penicillin–streptomycin (Cambrex). The cell monolayers were used within the 7th passage in culture. The cells were considered type B fibroblast-like synovial cells if negative on staining with anti-CD68, anti-CD14, anti-CD11b and anti-CD11c (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and positive by staining for uridine diphosphoglucose dehydrogenase (UDPGD), and if they had a spindle-shaped, fibroblast-like morphology.

DFZ (Guidotti, Pisa, Italy) was dissolved in acetic acid 100 mmol and further diluted with culture medium. Samples were analysed both in basal conditions and after treatment with DFZ (48 h). In preliminary experiments we tested the effects of different doses of DFZ, in order to identify the maximal dose without lethal effects. Thus, the concentrations of 3 μM and 5 μM DFZ were chosen for further experiments and used in this study. Such concentrations are in agreement with those reported for other corticosteroids in vitro [22].

#### Proliferation assay

Cell growth was quantified in subconfluent cell monolayers. Synoviocytes were seeded in 24 multi-well plates (15 000 cells/well) with 10% FCS in RPMI 1640. After 48 h incubation, cells were washed three times with serum-free medium and incubated in 0.2% FCS medium for an additional 48 h. Then, cells were incubated for 48 h in 10% FCS medium, or in 0.2% FCS medium, or in 0.2% FCS with uPA (Serono, Rome, Italy) 500 ng/ml with without uPA or uPAR antagonists. These were anti-human uPA monoclonal antibody (mAb) 5B4 and anti-uPAR mAb 3936 (mAb 3936) (American Diagnostica, Montreal, Canada), which were used at 1.5 μg/ml. The mAb 5B4 and 3936 both sterically impeded uPA/uPAR interaction. In preliminary experiments we tested the effects of different doses of uPA, in order to identify which dose could be more efficacious in inducing

### Table 1. Demographic and clinical characteristics of RA patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>RA duration (yr)</th>
<th>Rheumatoid factor (UI)</th>
<th>ERS (mm/h)</th>
<th>CRP (mg/dl)</th>
<th>DAS28</th>
<th>Sharp/van der Heijde score</th>
</tr>
</thead>
<tbody>
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<td>36</td>
<td>10</td>
<td>Negative</td>
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<td>10</td>
<td>7.34</td>
<td>60</td>
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<td>34</td>
<td>4</td>
<td>Positive (40 UI)</td>
<td>34</td>
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<td>5</td>
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<td>4</td>
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<td>48</td>
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<td>50</td>
<td>8</td>
<td>Negative</td>
<td>30</td>
<td>3</td>
<td>5.25</td>
<td>52</td>
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DAS28, disease activity score on 28 joints [30]; Sharp/van der Heijde score, Modified Sharp/van der Heijde score [31].
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Synoviocyte proliferation. Thus, the concentration of 500 ng/ml was chosen.

Each experiment was performed in triplicate. At the end of incubation cells were counted.

Samples were analysed both in basal conditions and after treatment with DFZ 3 μM and 5 μM (48 h).

Migration assays

The Boyden chamber procedure was used to evaluate cell migration [17]. The method is based on the passage of cells across porous filters against a concentration gradient of the migration effector. A 48-well micro-chemotaxis chamber (Neuroprobe, Gaithersburg, MD, USA) was used. The two wells were separated by a polyvinyl pyrrolidine-free polycarbonate filter, 8 µm pore size (Neuroprobe). To evaluate chemoinvasion, the filter was coated with Matrigel (50 µg/filter) (Becton Dickinson, Bedford, MA, USA). Test solutions were dissolved in serum-free medium and placed in the lower wells. Cell suspension (50 µL, 12,500 cells) was added to the upper and/or lower well. uPA 100 ng/ml and/or DFZ (3 and 5 μM) was added to the lower well.

In the experiment with neutralizing antibodies, the anti-uPA mAb 5B4 (1.5 µg/ml) was placed in the lower wells, while the anti-uPAR mAb 3936 (1.5 µg/ml) was incubated with the cell suspension. Irrelevant mouse IgGs were used at the same concentration in both the upper and lower well, to verify the specificity of the effect. The chamber was incubated at 37°C for 5 h, and the filter was then removed and fixed with methanol. Non-migrating cells on the upper surface of the filter were removed with a cotton swab. Cells were stained with Diff-Quick (Mertz-Dade, Dade International, Milan, Italy) and counted by light microscope (40×) in 10 random fields for each well. Mobilization was measured by the number of cells moving across the Matrigel and the filter pores and spread on the lower surface of the filter. Each experiment was performed in triplicate. Mean values of migrated cells for each experimental point were calculated.

Samples were analysed both in basal conditions and after treatment with DFZ (48 h).

DFZ 3 μM and 5 μM were used for migration assays.

Analysis of uPA, uPAR and PAI-1 levels

Samples were analysed for uPA, uPA and PAI-1 by commercially available enzyme-linked immunoassay kits (Imubind; American Diagnostica) according to the manufacturer’s instructions. Briefly, synoviocytes were seeded in six multi-well plates (25,000 cells/well) with 10% FCS in RPMI 1640. After 48 h of incubation, cells were washed three times with serum-free medium and incubated in 0.2% FCS medium for an additional 48 h. Then, cells were treated with DFZ 3 and 5 μM for 48 h. At the end of incubation, cells were detached, counted and lysed, as suggested by the manufacturer. The lysates were replaced in their original well and incubated for 1 h at 4°C to allow exhaustive extraction of undetached material. Cell extracts were centrifuged and stored at –80°C till uPAR analysis. Culture media were collected, centrifuged and stored 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.

The sensitivity levels were: 10 pg of uPA/ml of sample; 0.1 ng of uPAR/ml of sample; 1 ng of PAI-1/ml of sample.

Statistics

The non-parametric Mann-Whitney test for independent samples was used to compare results from healthy and RA synoviocytes for the levels of uPA, uPAR and PAI-1. The results were expressed as mean ± s.d.

Zymography was evaluated by densitometric comparison between lysis areas produced by uPA. The proliferation induced by uPA and DFZ was evaluated by the two-tailed t-test for independent samples and by analysis of variance (ANOVA) with Bonferroni correction.

Migration was expressed as the mean ± s.e.m. of the percentage of the healthy basal response, which was considered as 100%.

Results

uPA-dependent synoviocyte proliferation

uPA induced dose-dependent proliferation in both healthy (H) and RA synoviocytes, reaching a maximum at 500 ng/ml (Fig. 1)
Proportionally, uPA increased cell proliferation in H and RA synoviocytes, without reaching a significant difference between the two cell lines at any uPA dose.

These results showed that uPA exerted a proliferative effect both on healthy and RA synoviocytes, and indicated 500 ng/ml uPA as the minimal dose providing the maximal proliferative effect. This dose was used in the following experiments on uPA-dependent proliferation.

Effects of DFZ on synoviocyte proliferation

DFZ inhibited in a dose-dependent manner cell proliferation in both H and RA synoviocytes (P < 0.001 for any dose in both cell lines), reaching a maximum at 5 μM (Fig. 2A).

DFZ induced a percentage decrease in cell proliferation in H and RA synoviocytes, that, for any DFZ dose, did not differ significantly between the cell lines from either source.

These results indicated that DFZ exerted an antiproliferative effect on both H and RA synoviocytes and that DFZ was able to induce this effect even at 3 μM.

Effects of DFZ on uPA-dependent proliferation

In both normal and RA synoviocytes, DFZ significantly reduced uPA-dependent and 10% FCS-challenged proliferation (P < 0.001 in both case), until reaching the basal values (P > 0.05) (Fig. 2B).

As shown in Fig. 2B, RA synoviocytes were as prone as normal synoviocytes to spontaneous (0.2% FCS) proliferation and to proliferation induced by 10% FCS, as well as 500 ng/ml uPA (P > 0.05 in all cases).

In particular, serum-dependent proliferation (10% FCS) did not significantly differ from uPA-dependent proliferation in both H and RA lines.

The proliferative effect elicited by 500 ng/ml uPA in 0.2% FCS was significantly reduced by the mAb antagonists of uPA and uPAR in H and RA cells (P < 0.001) (Fig. 2B). This indicates that uPA/uPAR interaction is required for the proliferative effect of uPA on normal and RA synoviocytes.

DFZ blocked the effect of uPA by reducing the specific interactions between uPA and uPAR; in fact DFZ reduction of uPA-challenged proliferation was not different from the reduction of uPA proliferation induced by 5B4 and 3936 (Fig. 2B) and was not increased by cotreatment with them, indicating a common target for DFZ and anti-uPA/uPAR antibodies (data not shown).

Alternatively, our data do not exclude the possibility that DFZ, as well as antibody-dependent antagonization of uPA/uPAR interaction, could affect ligand–receptor downstream signalling events, which are known to be critical for uPAR-dependent activity.

uPA, uPAR and PAI-1 levels in normal and RA synoviocytes

uPA released in the culture medium by RA synoviocytes was significantly lower than uPA produced by normal cells (3.57 ± 0.20 vs 11.19 ± 0.33 ng/10⁶ cells, P < 0.05) (Fig. 3A).
Moreover, RA synoviocytes released into the culture medium significantly higher amounts of PAI-1 than healthy ones (7.62 ± 0.30 vs 3.67 ± 0.46 µg/10⁶ cells, P < 0.05) (Fig. 3C).

Effects of DFZ on uPA, uPAR and PAI-1 levels in normal and RA synoviocytes

DFZ significantly reduced uPA levels in H and RA synoviocytes (P < 0.0001) (Fig. 3A). DFZ reduced uPA levels dose-dependently: DFZ 5 µM was more efficacious than DFZ 3 µM in H (P < 0.05) and RA synoviocytes (P < 0.01). DFZ-dependent reduction of uPA levels at 3 and 5 µM was significantly higher (P < 0.05) in healthy than in RA synoviocytes.

Zymographic assays performed on culture medium from cells treated with DFZ 3 and 5 µM confirmed the data obtained with uPA. DFZ reduced uPA enzymatic activity in a dose-dependent manner, both in H and in RA synoviocytes (data not shown).

DFZ significantly (P < 0.0001) reduced uPAR levels in cell lysates from H and RA synoviocytes (Fig. 3B). DFZ 5 µM was more efficacious than DFZ 3 µM in reducing uPAR levels in RA synoviocytes (P < 0.01), but not in healthy ones (P > 0.05). The effects of DFZ 3 and 5 µM were significantly higher (P < 0.05) in RA than in healthy synoviocytes.

DFZ significantly increased (P < 0.0001) PAI-1 levels in healthy and RA synoviocytes (Fig. 3C). This was true both for DFZ 3 and DFZ 5 µM (healthy synoviocytes, P < 0.01 and P < 0.001, respectively; RA, P < 0.001). DFZ 5 µM was more efficacious than DFZ 3 µM in increasing PAI-1 levels, both in healthy and in RA synoviocytes (P < 0.001).

Effects of DFZ on uPAR gene expression

To confirm the data obtained on the protein, we performed RT-PCR analysis of the specific uPAR mRNA, which provided the data shown in Fig. 4. The expression of the uPAR gene was normalized with respect to GAPDH constitutive gene expression. Gene expression resembled protein expression: healthy lines showed less uPAR than RA lines and treatment with DFZ decreased uPAR expression dose-dependently both in H and RA synoviocytes.

The effects of DFZ 3 and 5 µM were significantly higher (P < 0.05) in RA than in healthy synoviocytes.

uPA-dependent synoviocyte chemoinvasion

uPA-dependent chemoinvasion was dose-dependent (Fig. 5A), with a maximal effect at 100 ng/ml for both healthy and RA synoviocytes. Basal migration was more pronounced (more than 40%) in RA than in healthy synoviocytes. This difference was maintained at each concentration of uPA in the lower well of the migration chamber. The increase in invasion observed after 5 h of incubation with 100 ng/ml uPA was counteracted by the incubation of invasive cells with mAb antagonists of uPA and uPAR, indicating that uPA/uPAR interaction is required for the invasive effect of uPA on normal and pathological synoviocytes (Fig. 5B).

Effects of DFZ on synoviocyte chemoinvasion

DFZ inhibited in a dose-dependent manner cell invasion in both normal and RA synoviocytes, reaching a maximum at 5 µM (Fig. 5B)

DFZ induced a proportional decrease in invasion in synoviocytes from both sources, but such a decrease did not differ significantly between healthy and RA cells at any DFZ dose.
DFZ significantly reduced uPA-induced chemoinvasion in healthy and RA synoviocytes. DFZ 5 μM reduced chemoinvasion to basal values.

When DFZ was incubated in the upper chamber with the cells, this drug caused similar effects on chemoinvasion. In fact, DFZ was able to inhibit migration induced by uPA in a dose-dependent way, with maximal action at the concentration of 5 μM, and this decrease in mobility was proportionally similar in H and RA synoviocytes (data not shown).

DFZ blocked uPA-dependent chemoinvasion reducing uPAR expression and, therefore, the specific interactions between uPA and uPAR. DFZ reduction of uPA-dependent migration was similar to the inhibition caused by the antagonist antibodies (Fig. 5B).

**Discussion**

This is the first study on the effects of DFZ on RA synoviocytes and on their fibrinolytic pattern and function. DFZ is a corticosteroid widely used in the treatment of RA for its safety and efficacy on inflammation [21]. Our data clearly show that DFZ acts as an inhibitor of the fibrinolytic system in RA synoviocytes but not in healthy ones. Indeed, the drug inhibits cell proliferation and invasion in a dose-dependent manner, reduces uPA and uPAR levels, increases PAI-1 levels and blocks uPA activity by reducing uPAR and thereby decreasing the specific interactions between uPA and uPAR. A few studies on another corticosteroid, hydrocortisone, have addressed the relationship between corticosteroid activity and the fibrinolytic system in different cell model systems. In particular, addition of 1 μM hydrocortisone was shown to reduce uPA production and to increase PAI-1 synthesis in cultured mouse keratinocytes [25]. Additionally, hydrocortisone was shown to be able to inhibit the basic fibroblast growth factor (b-FGF)-induced increase in uPA activity in rat mammary myoepithelial cells [26].

Despite increasing evidence of the significance of corticosteroids in RA, their aetiopathological role and potential long-term effect on RA progression remain unclear. The link between the mechanisms of action of glucocorticoids and the fibrinolytic system is still to be elucidated in RA synoviocytes.

In RA, the formation and invasiveness of synovial pannus, supported by angiogenesis, is linked to serine proteinases, mainly uPA [7], present in high quantity in the RA joint and produced by monocytes, chondrocytes and synoviocytes themselves under the effects of a variety of cytokines [8, 11].

The uPA/uPAR/PAI-1 system is an organizer of cell-ECM contacts and covers the full range of activities required to promote cell invasion and to disrupt cell attachment sites. We have recently shown that RA synoviocytes display a fibrinolytic machinery (uPA, uPAR and PAI-1) addressed towards an invasive pattern [10]. DFZ inhibits RA synoviocyte proliferation...
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FIG. 5. uPA/uPAR-dependent chemoinvasion in healthy (H) and RA synoviocytes. (A) cell invasion of Matrigel-coated filters by H and RA synoviocytes as a function of uPA concentration in the lower well of the migration chamber. (B) Percentage increase in Matrigel invasion in H and RA synoviocytes treated with DFZ or neutralizing antibodies: Basal, invasion of H and RA synoviocytes in the presence of 0.2% FCS in the lower well; C+ invasion stimulated by conditioned medium of A431 cell line, used as a source of chemotactic agents; DFZ 3 and DFZ 5, spontaneous invasion in the presence of deflazacort 3 and 5 μM in the lower well; uPA, invasion challenged with 100 ng/ml of uPA in the lower well; uPA + DFZ 3 and uPA + DFZ 5, invasion challenged with 100 ng/ml uPA in the presence of DFZ 3 and 5 μM in the lower well; uPA + SB4 and uPA + 3936, invasion challenged with 100 ng/ml uPA in the presence of 1.5 μg/ml of mAbs SB4 and 3936 in the lower and upper well, respectively. In both panels, each point represents the mean ± s.d. of three experiments performed in triplicate on four normal and four RA synovial cell lines. $P < 0.01$ vs basal; # $P < 0.001$ vs uPA, both H and RA.

The authors have declared no conflicts of interest.

References