Blockage of urokinase receptor reduces in vitro the motility and the deformability of endothelial cells

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Abstract The binding of urokinase (u-PA) to its cell surface receptor (u-PAR) is critical for tumor cell invasion. Here, we report that the disruption of this binding by an u-PAR antagonist ATF-HSA inhibits in vitro the motility of endothelial cells in a dose-dependent manner. This inhibition was also observed when the cells were first stimulated with potent angiogenic factors, including bFGF or VEGF. [^{3}H]thymidine incorporation assay demonstrated that ATF-HSA did not affect the cell proliferation. ATF-HSA was more potent than plasmin inhibitors, suggesting that it exerts its effects not solely by inhibiting the remodeling of the extracellular matrix. In fact, analysis of the cell shape change during migration revealed for the first time that its effect is related to a decrease in cell deformability. These results suggest that u-PAR antagonist may be a new approach to control angiogenesis.

Key words: Urokinase receptor antagonist; Endothelial cell; Migration; Angiogenesis; Metastasis

1. Introduction

The urokinase-type plasminogen activator (u-PA) and its receptor (u-PAR), a 55 kDa glycoprotein linked to the cell membrane by a glycosylphosphatidylinositol anchor, plays an important role in cell migration and tissue remodeling. Migrating cells distribute u-PAR selectively on the leading edge of the membranes and concentrate u-PA secreted either by themselves or by neighbouring stroma cells. The binding of u-PA to its receptor in turn greatly potentiates plasminogen/plasmin conversion on the cell surface [1]. This is an important observation because plasmin is a wide range serine protease which can directly degrade some of the components of the extracellular matrix, such as fibronectin or laminin, but can also promote local degradation of the stroma by converting inactive zymogens into active metalloproteinases [2,3]. It was also recently reported that u-PA/u-PAR is involved in cell adhesion by mediating cell attachment to vitronectin, a process which can occur with or without u-PA [4,5]. Furthermore, this attachment can be upmodulated by the binding of either u-PA or its amino-terminal fragment (ATF) which is implicated in the binding to

u-PAR [4,6]. In addition, u-PA activates the hepatocyte growth factor which is involved in angiogenesis [7]. Therefore, the u-PA/u-PAR system controls cell migration at multiple levels. For these reasons, u-PAR antagonists are viewed as potent antimetastatic agents.

The involvement of u-PA/u-PAR in endothelial cell migration is suggested by the demonstration that migrating endothelial cells secrete u-PA and express u-PAR and that u-PA secretion and u-PAR expression can be upregulated by stimulating the endothelial cells with basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) [8–12]. This is further confirmed by a recent study using u-PA$^{-/-}$ deficient mice which revealed a reduced reendothelialization after vascular trauma in these mice [13]. This finding further strengthens the relationship between fibrinolysis system and angiogenesis. Because angiogenesis controls the processes of tumor development and metastasis [14], it is important to evaluate to what extent disruption of u-PA-binding to u-PAR can inhibit endothelial cell migration.

We previously reported the design of a yeast-derived u-PAR antagonist in which the amino-terminal part of u-PA, ATF, was associated to human serum albumin (ATF-HSA). Apart from specifically displacing urokinase from its cell surface receptor, the hybrid molecule could also inhibit pro-urokinase-dependent plasminogen activation in the presence of u-PAR-bearing cells as well as in vitro tumor cell invasiveness [15]. In this study, we addressed the effect of the blockage of u-PAR by ATF-HSA or anti-u-PAR antibodies on the migration of endothelial cells and compared its effect with that of aprotinin, a potent inhibitor of plasmin. VEGF and bFGF were included in this study to stimulate the migration of endothelial cells and we found that u-PA/u-PAR disruption also reduced the motility of stimulated endothelial cells. The mechanisms by which the ATF-HSA molecule exerts its effects are discussed.

2. Materials and methods

2.1. Materials

Monoclonal (#3936) and rabbit polyclonal antibodies raised against u-PAR were purchased from America Diagnostica (Greenwich, CT). Both types of antibodies inhibit the binding of u-PA to u-PAR. Aprotinin was purchased from Bayer Pharma (Puteaux, France). The ATF-HSA chimeric protein is a *Kluyveromyces*-secreted u-PAR antagonist which has been previously described and characterized [15]. It is a genetic conjugate comprising human serum albumin (HSA), followed by the 1–135 amino-terminal fragment (ATF) of human urokinase.

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Control *Kluyveromyces*-secreted HSA was purified as for the ATF-HSA hybrid.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords and cultured in 10-cm culture dishes with medium 199 supplemented with 20% FCS, 2 mmol/l L-glutamine, 10^5 IU/l penicillin and 100 mg/l streptomycin (Gibco, Paisley, UK). HUVEC were used at passage 1 after the cells reached confluence.

2.3. Migration assay of endothelial cells

The migration assay was performed essentially as previously described [8]. After reaching confluence, HUVEC were removed from half of culture space by a cell scraper to stimulate their migration in a Rose chamber. The cells were then washed with the culture medium and fresh culture medium was added. The following agents were were perfused into the closed chamber to stimulate or inhibit cell migration: basic fibroblast growth factor (bFGF) (R & D systems, Abingdon, UK), vascular endothelial growth factor (VEGF, provided by Dr. J. Plouet, UPR 9006, CNRS, Toulouse, France), aprotinin, ATF-HSA, or antibodies specific to u-PAR. Cell motility was recorded by microcinematographic techniques. The displacement of the cell migration frontier was recorded every 5 min for 2 days. The migration rate of the cells was determined by measuring the displacement of the migration edges during this incubation period.

2.4. Cell deformability analysis

Quiescent confluent endothelial cells have a typical oval shape. Upon entering into a migrating phenotype, their shape becomes irregular due to the emission of pseudopods. The length of perimeter of the cell is indeed proportional to the cell migration rate. In addition, the faster the cells migrate, the more frequently the cells change their shape. These principles allowed us to determine the cell deformability during migration by continuous shape-recording using microcinematography. We determined the shape factor (SF) of individual cells by measuring the ratio of the cell perimeter to the cell surface (SF = perimeter^2/4Πsurface; SF = 1 for a circle, while higher values are obtained when the cells emit their pseudopods). SF is, therefore, indicative of the deformability of the cells during migration. Following microcinematography, the images were processed in a Samba 2005 analyser (Alcatel TITN, Grenoble, France). SF determination was performed for randomly selected endothelial cells incubated in the presence of ATF-HSA (100 nmol/l) or recombinant HSA (control, 100 nmol/l) for a duration of 1-3 h with 10-min intervals. Statistical studies was performed using Student's *t*-test.

2.5. Incorporation of [³H]thymidine

Cells were cultured in the presence or absence of bFGF (20 μg/l), with or without 500 nmol/l ATF-HSA for 24 h. 1 mmol/l of [³H]methyl thymidine (Amersham) was then added and the cells were further incubated for 18 h. Samples were collected using a Skatron harvester (Skatron, Lier, Norway) and incorporated [³H]thymidine was measured in a liquid scintillation counter. Experiments were done in triplicate.

3. Results

3.1. Cell motility

In this study, wound creation was used to induce cell migration. Cell migration rate was calculated with the data of the shifting of migration edges. The effect of bFGF and VEGF in endothelial cell motility was first evaluated. As expected, these two well-known angiogenic factors were able to accelerate endothelial cell migration in a dose-dependent manner. At optimal concentration (20 μg/l), each angiogenic factor can increase cell motility by 2-fold. The amplitude of this effect was maximal after 24 h for bFGF, while it was 36 h for VEGF at the optimal concentration. These conditions were thus retained when bFGF and VEGF were used throughout this study.

The effect of specifically blocking the binding u-PA to its cell surface receptor for endothelial cells mobility was then investi-
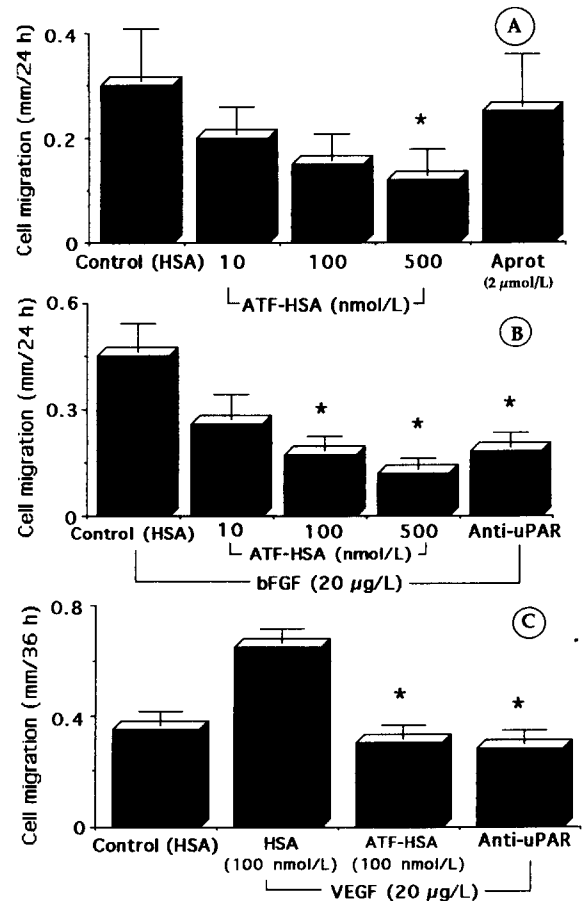

Fig. 1. Inhibition of HUVEC migration by blockage of the u-PAR. (A) Inhibition of HUVEC migration in the absence of angiogenic factor by ATF-HSA at the indicated concentrations and aprotinin at 2 μmol/l. After wound creation, ATF-HSA and aprotinin (2 μmol/l) were added to the culture and the displacement of migration frontier was recorded after 24 h. (B) Inhibition of HUVEC migration in the presence of bFGF (20 μg/l) by ATF-HSA hybrid and a monoclonal antibody specific to u-PAR (10 mg/l). The displacement of the migration frontier was recorded after 24 h incubation. (C) Inhibition of HUVEC migration in the presence of VEGF (20 μg/l) by ATF-HSA (100 nmol/l) and by a polyclonal antiserum raised against u-PAR (1:100 dilution). The displacement of migration frontier was recorded after 36 h incubation ($n \geq 4$, mean ± S.E.M.). *$P < 0.05$.

gated. As shown in Fig. 1, the ATF-HSA molecule inhibited the migration of endothelial cells in a dose-dependent manner, even when migration was stimulated with optimal concentrations of bFGF (Fig. 1A,B) or VEGF (Fig. 1C). HUVEC migration was also significantly inhibited when monoclonal or polyclonal antibodies directed against u-PAR at saturable concentrations were used, while recombinant HSA and irrelevant immunoglobulins had no effect. Aprotinin, a potent plasmin inhibitor, also inhibited endothelial cell migration but showed limited effect even at a very high concentration (2 μmol/l), which efficiently blocks plasmin activity (K_i = 1nmol/l) (Fig. 1A).

3.2. Cell proliferation

The cell migration rate is usually associated with the cell proliferation rate, since most growth factors stimulate both cell proliferation and migration. For this reason, the effect of ATF-HSA on endothelial cell proliferation was also evaluated using
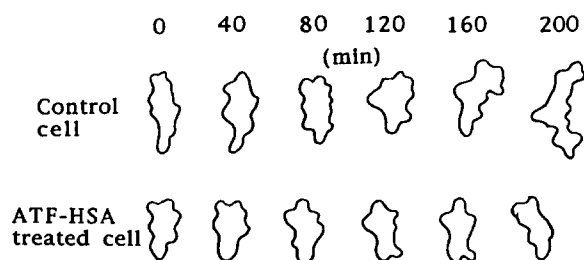
Fig. 2. Cell shape change in control endothelial cells and ATF-HSA-treated endothelial cells. HUVEC cells were cultured to confluence. A wound was then created to induce cell migration. HSA (control cells) or ATF-HSA was infused at 100 nmol/l into the Rose chamber. After recording the cell migration, cell images was transfered to an image-analyser. The two series of the images given by image-analyser are showed here to represent the difference of the dynamic shape change of HSA- (control) or ATF-HSA-treated cells during a period of 200 min migration. Each series contains 6 images of the contour of one cell during its migration, but recorded at different times after wound creation as indicated.

[^{3}H]thymidine incorporation assay. The results showed that ATF-HSA had no effect on endothelial cell proliferation: [^{3}H]thymidine incorporation was at same rate either in the presence or in the absence of ATF-HSA (2200 ± 50 cpm vs. 2150 ± 120 cpm, $n = 6$, mean ± S.D.). Stimulatory effect of bFGF was also not influenced by the presence of the u-PAR antagonist (6450 ± 250 cpm vs. 6300 ± 190 cpm for control, $n = 6$, mean ± S.D.). Thus, ATF-HSA-mediated inhibition of cell migration was not due to an inhibition of cell proliferation.

3.3. Cell deformability

Using microcinematography recording of the migrating endothelial cells, we observed that the cells became rigid in the presence of ATF-HSA. As shown in (Fig. 2), after wound creation, the shape of HSA-treated cells quickly became irregular with the formation of pseudopods on moving cells while the shape change of ATF-HSA-treated cells was inhibited. This inhibition of the cell shape change or the reduction of cell deformability prompted us to accurately quantify this morphological change by analysing the cell contours recorded by a microcinematography with an image-analyser. The quick shape change of control cells was indicated by the increase in SF values following the wound creation [2.3 ± 0.3 ($t = 0$ min) vs. 2.7 ± 0.2 ($t = 60$ min), $n = 10$, $P < 0.02$]. In contrast, the SF of ATF-HSA-treated cells did not increase as for the control cells [2.3 ± 0.15 ($t = 0$ min) vs. 2.2 ± 0.2 ($t = 60$ min), $n = 10$, $P > 0.05$]. No significant difference in SF is apparent for HSA- or ATF-HSA-treated cells before the induction of cell migration by wound creation. This difference in cell deformability was observed throughout the duration of the microcinematographic record, although we only presented the data from a limited period of time (Fig. 3).

4. Discussion

Receptor-bound urokinase is crucial for tumor cell and endohelial cell migration [1–3,11,12,16]. In this study, we investigated the effect of blockage of u-PAR by a yeast-secreted ATF-HSA hybrid molecule in the in vitro migration of endothelial cells by microcinematography.

We demonstrated that the motility of unstimulated endothe-

lial cells can be inhibited in a dose-dependent manner by disrupting the u-PA/u-PAR interaction with the ATF-HSA molecule. This dose-dependent inhibitory effect was also observed with both bFGF- and VEGF-stimulated endothelial cells. This dose–effect range of ATF-HSA for inhibiting endothelial cell migration is in good agreement with our previous results which showed a requirement of about 100 nmol/l to significantly inhibit plasminogen activation by urokinase on cell surfaces and tumor cell migration [15]. The inhibitory effect was also achieved by the use of both polyclonal and monoclonal antibodies raised against u-PAR and which inhibit the binding of u-PA. These results indicated that the u-PA/u-PAR system is likely a crucial determinant for the motility of endothelial cells.

A previous study reported a proliferative effect induced by ATF-binding to u-PAR on an osteoblastic cell line [17]. Therefore, we have investigated the effect of ATF-HSA on the proliferation of endothelial cells using [^{3}H]thymidine incorporation method. Since, basal and bFGF-stimulated cell proliferation were not modified by the addition of ATF-HSA, we concluded that the inhibitory effect exerted by the ATF-HSA molecule on endothelial cell migration was not due to an inhibition of cell proliferation.

The mechanisms involved in the inhibitory effect mediated by the ATF-HSA hybrid or the antibodies against u-PAR can be multifactorials. First, modulation of cell surface u-PA activ-
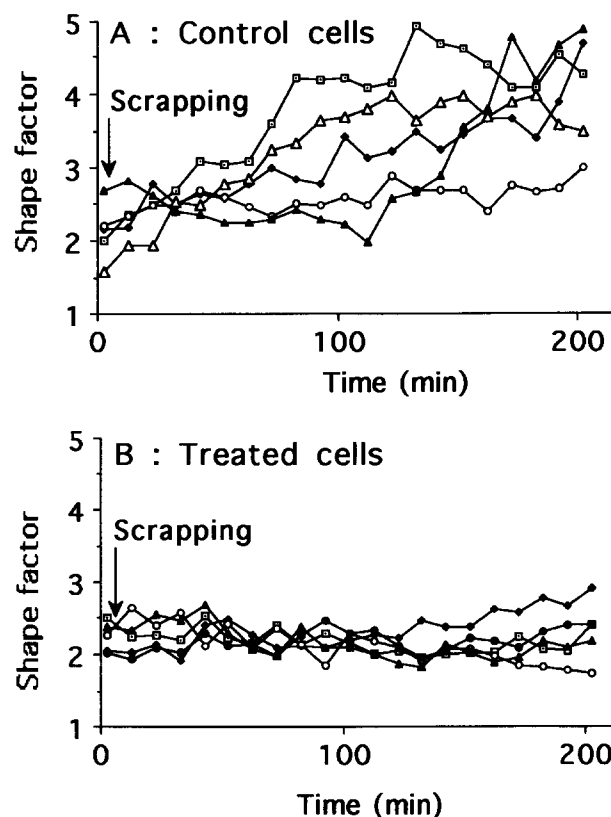

Fig. 3. SF evolution in ATF-HSA-treated endothelial cells HUVEC cells were cultured to confluence. A wound was then created and HSA (A) or ATF-HSA (B) was infused at 100 nmol/l into a closed Rose chamber and the migration frontier was photographed at 10-min interval. The recorded cell shapes within the scope were treated with a Samba 2005 Image-analyser. Each curve represents the SF values of a randomly selected individual cell in the migration frontier.

ity may be involved. It has previously been shown that plasminogen activation by scu-PA was greatly enhanced when both molecules were bound to their respective surface receptors [1]. Bound u-PA could activate plasminogen which in turn could promote the direct degradation/remodeling of the extracellular matrix as this proteolytic process is essential for cell mobility [2]. This u-PA activity is involved also in the in situ activation or liberation of angiogenic factors, such as HGF and bFGF [7,12]. However, the inhibition of plasmin generation by our hybrid molecule is certainly not the only mechanism to explain the ATF-HSA-induced inhibition of endothelial cell migration because aprotinin inhibits plasmin with K_i about 1 nmol/l, while at 2 μmol/l its effect was less efficient than the u-PAR antagonists on the inhibition of cell motility. Second, modulation of cell adhesion by u-PAR antagonists may also be involved as it has been shown that both u-PA and u-PAR binds to vitronectin [4,5]. Interestingly, u-PAR affinity for vitronectin is modulated by the binding of u-PA or its amino-terminal fragment [4]. This cell attachment may be limited in time when u-PA is bound to u-PAR because the complex u-PA/u-PAR can be internalized [18]. In such model, the saturation of u-PAR by the anti-u-PAR antibodies or the ATF-HSA hybrid would decrease cell motility by disrupting a continuous loop of adhesion and detachment during cell movement. This interpretation is in good agreement with our result of a decreased deformability of endothelial cells in the presence of ATF-HSA or anti-u-PAR antibodies. Cell deformability during migration was directly observed with microcinematography technique. This method allowed us to determine SF evolution during cell movement in order to describe a dynamic process of membrane extension and retraction. The significant reduction by ATF-HSA of the rate of SF change during endothelial cell movement supports the hypothesis that u-PA/u-PAR interaction is involved in the adhesion and deadhesion process of cell movement [4]. However, the list of the biological events induced by u-PA/u-PAR interaction are still growing. For example, a mechanism of u-PAR-mediated mechanical force transfer has been recently suggested by the study in which an increased stiffness of cell membranes of myoblasts induced by u-PAR specific antibodies was observed [19]. u-PAR-mediated transmission of signals has also been suggested [20]. Most importantly, the recent demonstration that u-PAR, β_2-integrin and Src-kinase form a single complex on cell surface suggested a u-PAR-mediated coordinated action of extracellular proteolysis, adhesion and cell activation at the right time and place during cell movement [21]. In that respect, our results imply that u-PAR antagonists could interfere with a variety of actions that occur during cell movement. Further studies are needed to illustrate these speculations.

To produce clinical benefits, antimetastatic drugs would likely have to inhibit both tumor invasion and angiogenesis [13]. The ATF-HSA molecule of this study can disrupt the binding of u-PA to u-PAR and inhibits both tumor cell invasiveness [15] and endothelial cell migration, even when the latter were stimulated with major tumor-associated angiogenic factors, such as bFGF or VEGF [22]. This inhibitory effect on endothelial cell migration is particularly interesting for angiogenesis which is an absolute requirement for both tumor growth and metastasis, while u-PAR is not ubiquitously present on the tumor cells.

Because of specificity of species for u-PAR recognition by u-PA, the antiangiogenic property of human ATF-HSA could not be studied in vivo with available angiogenesis model. Further studies with u-PAR antagonist of non-human origin will answer whether uPAR blockage will exhibit antitumoral and antiangiogenic effects in vivo.

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