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Blockage of the urokinase receptor on the cell surface: construction and characterization of a hybrid protein consisting of the N-terminal fragment of human urokinase and human albumin

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Abstract Receptor-bound urokinase is likely to be a crucial determinant in both tumor invasion and angiogenesis. We report here that a yeast-derived genetic conjugate between human serum albumin and the 1–135 N-terminal residues of urokinase (u-PA) competitively inhibits the binding of exogenous and endogenous u-PA to its cell-anchored receptor (u-PAR). This hybrid molecule (ATF-HSA) also inhibits in vitro pro-urokinase-dependent plasminogen activation in the presence of u-PAR bearing cells. These effects are probably responsible for the observed in vitro inhibition of tumor cell invasion in a reconstituted basement membrane extract (Matrigel).

Key words: Hybrid protein; Urokinase receptor antagonist; Plasminogen activation; Tumor metastasis

1. Introduction

The urokinase receptor (u-PAR), a 55 kDa glycoprotein linked to the cell membrane by a glycosylphosphatidylinositol anchor, plays a central role in cell migration and tissue remodelling [1,2]. For example, migrating cells distribute u-PAR selectively on the leading edge of the membranes, and thus concentrate u-PA secreted either by themselves or by neighboring stromal cells [3,4]. Importantly, u-PA binding to its specific receptor greatly potentiates plasminogen activation on the cell surface [5]. Generated 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 its ability to convert inactive zymogens into active metalloproteinases [6]. Furthermore, the u-PA/u-PAR is also likely involved in neovascularization, an essential process for tumor cell proliferation and metastasis [7,8].

Therefore, therapeutic molecules aimed at disrupting the u-PA/u-PAR interaction might be effective in anticancer therapy by targeting both tumor cell invasiveness and angiogenesis. For example, monoclonal antibodies directed against u-PA or u-PAR, or small peptides derived from the receptor-binding domain (epidermal growth factor-like domain) of u-PA have proven to be effective in decreasing the invasiveness of cancer cells in vitro [9–11]. In addition, Wilhem et al. recently reported that a soluble form of u-PAR consisting of its 277 aminoterminal residues could be secreted by CHO cells and that this recombinant molecule could inhibit both the proliferation and the in vitro invasion of cells derived from a human ovarian cancer [12]. Most importantly, with the view of designing a long-lasting antagonist of the u-PA/u-PAR interaction, Crowley et al. constructed a chimeric molecule consisting of the cell binding domain of u-PA genetically linked to the constant region of an immunoglobulin [13]. This hybrid molecule, referred to as an immunoadhesin [14,15], was efficient in inhibiting the dissemination of human tumor cells in a metastasis experimental model.

Another way to enhance the bioavailability of an otherwise unstable peptide is based on its genetic fusion to human serum albumin [16]. The pharmacokinetic issue of bioavailability is important because antiproteolytic strategies aimed at controlling disease progression will probably require long-term treatment before any clinical benefit is apparent. As a first step to study the efficacy of a long-term displacement of u-PA from its cell surface receptor during the metastatic process, we designed a genetic conjugate between human serum albumin (HSA) and the receptor binding, non-catalytic aminoterminal fragment of u-PA (ATF) [17]. In this work we report the construction and the isolation of this chimeric molecule which can be efficiently secreted by *Kluyveromyces* yeasts and purified and its efficacity on cell biology. ATF-HSA is a potent antagonist of the u-PA/u-PAR interaction on the surface of cancer cells and is a promising agent as indicated by its remarkable in vitro efficacy in inhibiting cancer cell invasiveness. The physiological implications of this effect are discussed.

2. Materials and methods

2.1. ATF-HSA protein expression and purification

The gene encoding the ATF-HSA genetic conjugate (748 residues) was generated by PCR amplification of the HSA and the human u-PA cDNAs. Briefly, this composite gene is contained within a *Hin*dIII restriction fragment that can be described from its 5′ to 3′ end by the succession of: (i) a *Hin*dIII–*Bgl*II fragment comprising the 22 nucleotides (nt) of the 5′ untranslated region (from the CAP site to the translation initiation codon) of the *Saccharomyces cerevisiae* PGK gene, immediately followed by a 72 nt sequence encoding the 24-residue HSA proper region. This secretion signal is known to be fully functional in *Kluyveromyces lactis* [16,18] due to the presence of KEX1 maturase activity (the homologue of the KEX2 activity of *S. cerevisiae*);

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(ii) genetically linked to a *Bam*HI–*Aha*II restriction fragment encoding the first 135 N-terminal residues of mature human u-PA (ATF), immediately followed by a four Gly amino acid spacer; (iii) genetically linked to an *Aha*II–*Hin*dIII restriction fragment coding for the 585 residues of mature HSA. This *Hin*dIII fragment was then cloned in an *E. coli–K. lactis* shuttle vector to generate plasmid pYG1477 (Fig. 1). This plasmid is isogenic to plasmid pYG107 except that the *Hin*dIII fragment encodes the ATF-HSA gene instead of HSA [18]. Plasmid pYG1477 is thus a multicopy pKD1-derived expression plasmid which can stably propagate in *Kluyveromyces* yeasts [19,20]. It was transferred in *K. lactis* strain MW98–8C (a cir° strain kindly supplied by H. Fukuhara, Orsay, France), and transformants were selected at 28°C on YPD (yeast extract 1%, bactopeptone 2%, glucose 2%) plates containing 200 mg/l of Geneticin (G418). The transformants were then grown in shake flasks for 72 h in YPL (same composition as YPD except that 2% lactose was used instead of glucose) to allow for maximal expression of the ATF-HSA protein from the LAC4 promoter of pYG1477. SDS-PAGE analysis of the culture supernatants indicated that the hybrid protein (80 kDa) is by far the most abundant protein, and is therefore easily recovered and purified. For that purpose, the cells were sedimented by centrifugation and the supernatant was diafiltrated against a Tris 50 mM pH 7.3 buffer. It was then concentrated using a MINI-ULTRASETTE device (Filtron Tec. Corp.) equipped with a 10 kDa cut-off membrane, and loaded on a 7 ml anion exchange D Zephyr column (Sepracor) from which the chimeric protein was eluted with a 0–1 M NaCl gradient. Fractions containing the protein were then pooled, dialyzed and passed on a pseudo-affinity Trisacryl Blue column (Sepracor) from which it was eluted by a one step 3.5 M NaCl gradient. After extensive dialysis against pure water, the protein was freeze-dried and stored at −70°C. Purity was estimated by SDS-PAGE analysis.

2.2. Cell culture

Human breast-adenocarcinoma cell line MDA-MB-231 was cultured in DMEM medium containing 10% FCS, while monoblast cell line U937 was cultured in RPMI-1640 medium containing 10% FCS as described [21,22].

2.3. Inhibition of u-PA binding to u-PAR

Recombinant single chain-urokinase (scu-PA, provided by Grunenthal GmbH, Aachen, Germany) was radioiodinated by the chloramine T procedure [23]. U937 cells were used in the binding assay. Cell-bound

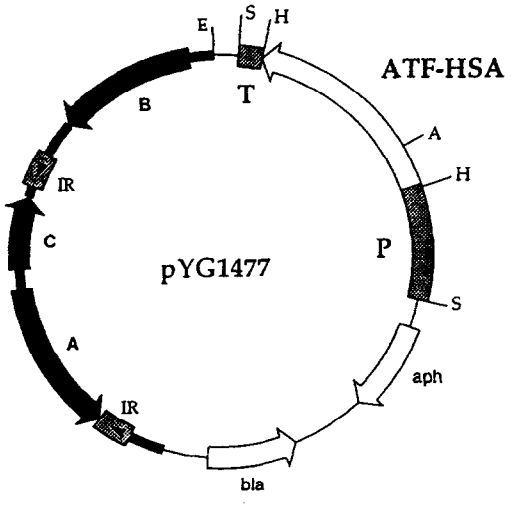

Fig. 1. Construction of the ATF-HSA expression plasmid. pYG1477 is a multi-copy pKD1-derived plasmid which can stably propagate in *K. lactis* yeast. Secretion of the ATF-HSA hybrid is directed by the prepro secretion leader from HSA. Relevant restriction sites are indicated: E, *Eco*RI; S, *Sal*I; H, *Hin*dIII; A, *Aha*II. Abreviations used: P, *K. lactis* LAC4 promoter; T, *S. cerevisiae* PGK transcription terminator; A, B, and C refer to the three open reading frames from plasmid pKD1, and IR to its inverted repeats. bla and aph refer to the selective markers allowing selection of *E. coli* transformants with ampicillin and *K. lactis* transformants with geneticin, respectively.

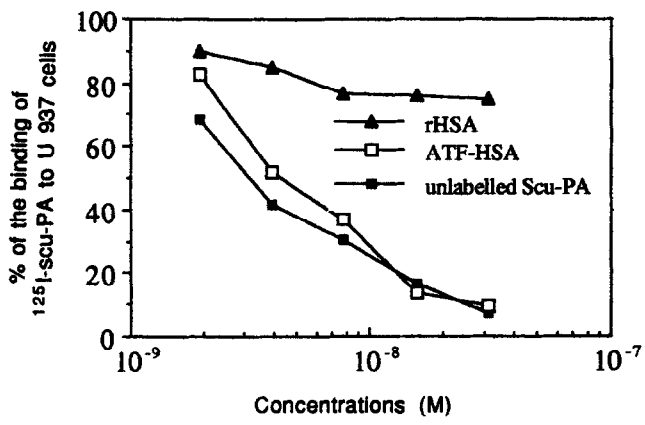

Fig. 2. Inhibition of [^{125}I]scu-PA binding to U937 cells. 2 nM of [^{125}I]scu-PAwas added to the acid-eluted U937 cell suspension with or without ATF-HSA, rHSA or unlabeled scu-PA at the indicated concentrations. After 2 h incubation at 4°C with gentle agitation, the cells were placed on a 20% sucrose-PBS solution and centrifuged to remove unbound [^{125}I]scu-PA. Cell-bound [^{125}I]scu-PA was counted with a gamma counter.

urokinase was first removed by a 3 min exposure at room temperature to a 0.05 M glycine-HCl (pH 3.0)/0.1 M NaCl solution. The cell suspension was then neutralized by addition of 0.2 volumes of a 0.5 M HEPES(pH 7.5)/0.1 M NaCl solution, and centrifuged. Cells were resuspended at a concentration of 2.5×10^6/ml in PBS containing 0.5% bovine serum albumin (BSA), and incubated for 2 h at 4°C with 2 nM [^{125}I]scu-PA together with different concentrations of the ATF-HSA genetic conjugate, recombinant human serum albumin (rHSA) (Rhone Poulenc Rorer) or unlabelled scu-PA with gentle agitation. An urokinase inhibitory tripeptide Glu-Gly-Arg-Ketone (0.1 mM) and plasmin inhibitor aprotinin (100 nM) were included in the incubation buffer to avoid any enzymatic reaction. 100 ml aliquots of the cell suspensions were then layered on 0.5 ml of a 20% sucrose-PBS buffer, and the cells were sedimented for 5 min at $12{,}000 \times g$. The radioactivity associated with the cells was counted in a gamma counter. All tests were performed in triplicate.

2.4. Zymogram assays

Zymography was performed essentially as described [8] with a mixture of 1 ml of an 8% commercial instant nonfat dry milk solution, 1.5 ml of PBS (with 0.9 mM Ca^{2+} and 1 mM Mg^{2+}), 1.5 ml of a 2.5% agar solution in water, and 40 μl of a 4 mg/ml plasminogen solution in a 100 mm culture dish. The gel was formed in the absence or presence of 1 mM amiloride (Sigma, USA), a specific inhibitor of u-PA. After overnight culture in the presence or the absence of different concentrations of ATF-HSA or recombinant ATF (kindly given by Prof. M. Soria, Milano, Italy), 25×10^3 washed U937 or MDA-MB-231 cells were laid down onto the gels. After a 4 h incubation at 37°C, the zymogram was then analyzed for areas of lysis.

2.5. Inhibition of plasminogen activation in the presence of cell-associated u-PAR

10^6 U937 or MDA-MB-231 cells were first incubated for 3 min in a 0.05 M glycine-HCl(pH 3.0)/0.1 M NaCl solution, as described above. Following neutralization, the cells were incubated with 0.08 nM of scu-PA at 37°C in the presence of different concentrations of ATF-HSA for 10 min. Glu-plasminogen at 0.4 μM (Kabi Vitrum, Sweden) was then added and the mixture was incubated at 37°C for different time periods to generate plasmin. At the end of the incubation period, u-PA activity was neutralized by the tripeptide Glu-Gly-Arg-ketone (0.1 mM; kindly provided by Pr. Lijnen, Leuven, Belgium), and plasmin generated was evaluated by its amidolytic activity on the plasmin-sensitive chromogenic substrate S-2251 (Kabi Vitrum, Sweden). The paranitoanilin released from the substrate was measured by its absorbance at 405 nm. Plasmin generation was calculated by comparison with a standard curve established with purified plasmin (Kabi Vitrum, Sweden).

2.6. Cell invasion assay

The assay was performed essentially as described [24]. Briefly, 6.5 mm^2 polycarbonate filters of 1.2-μm pore size (transwell, Costar) were coated with 50 μg of basement membrane extracts (kindly provided by Dr. Foidart, Liège, Belgium) and dried. The lower chambers of the transwell units were filled with fibroblast conditioned medium supplemented with 2 mg/ml of BSA, while the upper chambers of the units were seeded with 2×10^5 MDA-MB-231 cells in serum free DMEM culture medium containing 0.2 mg/ml of BSA. The inhibitors were then added into the upper chambers and the transwell units were incubated for 24 h in a culture chamber. The cells and the matrigel on the upper side of the filters were then carefully removed so that the cells which traveled through the matrigel and adhered on the inferior side of the filters could be stained by May-Grünwald and Giemsa coloration, and counted with a microscope.

3. Results and discussion

Previous work first indicated that u-PA binds to its receptor via residues 12–32 of its EGF domain [17]. ATF, a fragment of u-PA composed of the EGF domain followed by the kringle domain of u-PA, is a more efficient antagonist than the 12–32 peptide [17, and data not shown]. However, the purification of natural ATF is time-consuming and yield limited quantity. In addition, the in vivo unstability of small polypeptides in general renders the ATF unsuitable for the use in vivo experiments. Therefore, we have constructed a genetic conjugate composed of the 135 N-terminal residues of u-PA associated to HSA. HSA was chosen as a pharmacological carrier for the following reasons: (i) it is a long-lasting neutral carrier which can be secreted by *Kluyveromyces*yeasts to very high levels [18], (ii) it can escape the vascular compartment allowing access to cancer tissue, and (iii) it has previously been shown to increase the half-life of a 179-residue peptide in rabbits by 140-fold [16]. The composite ATF-HSA gene is expressed from the LAC4 promoter of pYG1477, a multicopy pKD1-derived expression plasmid (Fig. 1) which can be stably propagated in *Kluyveromyces* yeasts (data not shown). SDS-PAGE analysis of the culture supernatants indicated that the hybrid protein (80 kDa) is by far the most abundant protein (120/150 mg/l in shake-flasks cultures), enabling easy recovery and characterization. After isolation the purity of the chimeric molecule was more than 90%.

To evaluate the biological activity of this chimeric molecule, we first tested its ability to inhibit exogenous u-PA binding to U937 cells which are known to express abundant u-PAR on their cell surface. In this assay, cell bound u-PA was first eluted from the cell surface by an acid treatment. The purified ATF-HSA fusion protein was then compared with recombinant scu-PA for its ability to compete for the binding of [^{125}I]scu-PA to the cell surface. The results show that 50% inhibition of the binding of labeled scu-PA (2 nM) required 3.1 nM scu-PA, or 4.2 nM ATF-HSA (Fig. 2), while no inhibition was observed with the recombinant HSA as control. We therefore concluded that ATF-HSA is a potent inhibitor of scu-PA binding to u-PAR. These results also indicate that the ATF moiety of the hybrid is fully accessible to the cell surface u-PAR.

Because binding of u-PA to its receptor may occur in an autocrine fashion in cells which synthesize both u-PA and u-PAR (especially in endothelial cells and some cancer cells such as U937 or MDA-MB-231 cells) and consequently exhibit high endogenous u-PA activity on their surface, we tested in a second time if ATF-HSA could inhibit the binding of endogenous u-PA. For this purpose, MDA-MB-231 cells were cultured overnight in the presence of ATF-HSA at different concentrations, and u-PA activity was then measured in a zymogram assay [8]. As shown in Fig. 3, the ATF-HSA hybrid protein can inhibit endogenous u-PA activity in a dose-dependent manner. In the presence of amiloride, lysis was totally inhibited (not shown) demonstrating that lysis was only dependent on u-PA. Moreover, since the hybrid-mediated inhibition was highly efficient when compared to ATF, we again concluded that genetic coupling had not significantly reduced the ability of the ATF moiety to interact with u-PAR. Identical results were also obtained with U937 cells (data not shown). Taken together, these experiments clearly demonstrate that our fusion protein efficiently competes with both endogenous and exogenous u-PA for binding to u-PAR.

It has previously been shown that plasminogen activation by scu-PA was greatly enhanced when both molecules were bound

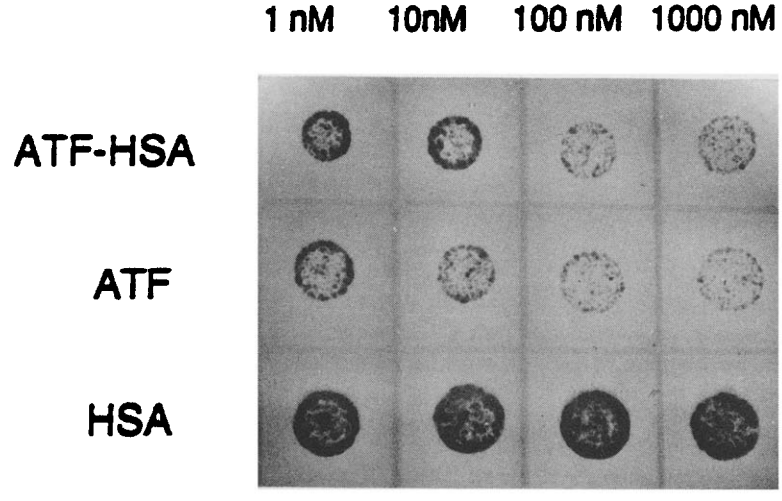

Fig. 3. Inhibition of endogenous u-PA binding to MDA-MB-231 cells. The cells were incubated for 16 h with the indicated concentrations of ATF-HSA, rATF or rHSA. The cells were then suspended with 0.05% EDTA solution, washed 3 times with culture medium and 2.5×10^4 cells of each sample were applied to a thin layer of agar containing casein and plasminogen. After 4 h incubation at 37°C, the agar gel was photographed under dark-field illumination.

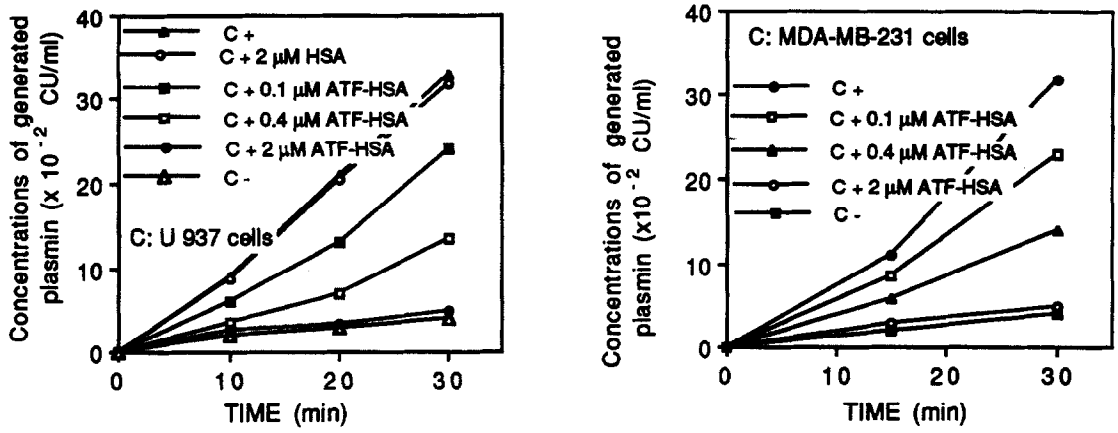

Fig. 4. Inhibition of plasminogen activation in the presence of cell-associated u-PAR. 10^6 acid-eluted U937 or MDA-MB-231 cells were mixed with 0.08 nM scu-PA with or without the indicated concentrations of ATF-HSA or control rHSA. After 10 min, glu-plasminogen was added into the mixture, and the reaction was allowed to proceed until the addition of 0.1 mM of the u-PA inhibitor Glu-Gly-Arg-ketone at the indicated times. The cells were then removed and the concentration of generated plasmin was assayed in the presence of plasmin sensitive substrate S-2251.

to their respective surface receptors [5]. The neutralization of this u-PAR potentiating effect by the ATF-HSA hybrid was therefore investigated. We first confirmed that plasmin generation was considerably enhanced when U937 cells or MDA-MB-231 cells were added to a mixture of plasminogen and scu-PA (Fig. 4). Interestingly, we found that this enhancement was also inhibited in a dose-dependent manner by the addition of ATF-HSA. This effect is specific to the ATF moiety of the hybrid since rHSA again had no effect in this assay.

The construction of this ATF-HSA by genetic engineering technique will facilitate a series of in vivo studies. The antimetastatic effect of in vivo administration of uPAR antagonist is mostly expected and proven effective in some experimental models [11,13] The ability of the ATF-HSA molecule to inhibit tumor cell invasiveness in vitro on a reconstituted extracellular matrix (Matrigel)was checked, and we found that the invasion of MDA-MB-231 cells was inhibited by approximately 50% at 120 nM, and by 80% at 750 nM. Its anticancer effect remains to be tested in vivo. Furthermore, since the expression of uPAR is not limited to cancer cells, other potential applications such as anti-inflammatory or anti-angiogenic effects as well as relevant side effects could also be expected. Thus, the construction of this molecule with satisfying pharmaceutical properties is a critical step of the work aimed to explore the usefulness of uPA:uPAR antagonist in a number of clinical applications.

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