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Recombinant soluble urokinase receptor as a scavenger for urokinase-type plasminogen activator (uPA)

Inhibition of proliferation and invasion of human ovarian cancer cells

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Abstract

A recombinant soluble human urokinase receptor comprising amino acids 1–277 was cloned and transfected into CHO cells. The mutant protein (rec-uPAR$_{277}$), purified from the CHO cell supernatant by affinity chromatography on immobilized urokinase (uPA), in a four-fold excess, completely abolished the binding of FITC-labeled pro-uPA to the human ovarian cancer cell line, OV-MZ-6. This invasive and tumorigenic cancer cell line expresses uPA, its inhibitor PAI-1, and the high-affinity receptor for uPA, uPAR. Rec-uPAR$_{277}$ significantly reduced the proliferation of OV-MZ-6 cells in a concentration-dependent manner without altering the viability of the cells. Invasion of OV-MZ-6 cells tested in an in vitro Matrigel invasion assay was inhibited by rec-uPAR$_{277}$ up to 75%. In conclusion, these results demonstrate that rec-uPAR$_{277}$ can function as a scavenger for uPA in vitro by inhibiting proliferation and invasion of human cancer cells.

Key words: Urokinase receptor; Invasion; Proliferation; Ovarian cancer

1. Introduction

Urokinase-type plasminogen activator (uPA) is synthesized and secreted by a variety of normal and malignant cells. In solution or upon binding to a GPI-anchored high-affinity receptor on the cell surface (uPAR), uPA activates plasminogen to plasmin which in turn degrades extracellular matrix proteins, such as fibrin and fibronectin [1,2]. The binding of uPA and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), induces internalization of the receptor–uPA–inhibitor complex [3] and may lead to the activation of a phosphotyrosine kinase [4]. It has also been shown that uPA itself exerts cytokine-like activity by increasing the proliferation of the prostate cancer cell line, CCL 20.2, or the HL 60 cell line [5,6].

The uPA–uPAR system is critically involved in the invasion and metastasis of cancer. Cells lacking detectable urokinase expression become invasive upon transformation with a uPA expression plasmid [7]. Furthermore, monoclonal antibodies directed against uPA or uPA peptides comprising residues 17–32 of the epidermal growth factor-like domain of uPA can decrease the invasiveness of cancer cells in vitro [8,9]. In another approach, inhibition of metastasis of cancer cells in nude mice was achieved by introducing an expression plasmid of a proteolytically inactive uPA mutant into cancer cells, thereby blocking the uPA–uPAR interaction on the cancer cell surface in an autocrine fashion [10].

In this report, we tested whether a soluble, truncated uPAR shows the ability to function as a scavenger for uPA by inhibiting cell proliferation and invasion of the human ovarian cancer cell line, OV-MZ-6.

2. Materials and methods

2.1. Cell culture

The human ovarian cancer cell line, OV-MZ-6, established from a patient with cystadenocarcinoma of the ovary as detailed in [11], was cultured in Falcon Easy Access tissue culture flasks (Becton-Dickinson, Heidelberg, Germany). Cells were cultivated in Dulbecco's modified Eagle's medium in the presence of 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin supplemented with 10 mM HEPES, 0.27 mM asparagine and 0.55 mM arginine. The adherently

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Abbreviations: uPA, urokinase-type plasminogen activator; uPAR, urokinase receptor; rec-uPAR$_{277}$, soluble recombinant human urokinase receptor 1–277 lacking the GPI moiety; PAI-1, plasminogen activator inhibitor type 1; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PMSF, phenylmethylsulfonylfluoride.

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growing cells were subcultured using 0.05% EDTA in PBS to avoid destruction of cell surface antigens.

2.2. Recombinant truncated soluble urokinase receptor

The truncated uPAR, comprising amino acids 1–277 (rec-uPAR$_{277}$), was cloned and expressed as described in detail in [12]. Rec-uPAR$_{277}$ was purified from culture supernatants by affinity chromatography as outlined in [12] with slight modifications. 125 mg of PMSF-inactivated uPA was coupled to CH-Sepharose (Pharmacia, Freiburg, Germany) according to the manufacturer's recommendation. Culture medium containing rec-uPAR$_{277}$ was passed over the uPA-Sepharose in the presence of 1 M NaCl, 1 mM PMSF, 1 mM EDTA and 2000 U/ml aprotinin to reduce low affinity interaction of uPA with proteins and to inhibit degradation of uPA by proteases present in the culture supernatants. Bound rec-uPAR$_{277}$ was eluted with 0.2 M acetic acid, pH 2.0 and stored at this pH at −20°C until use. Prior to its use, purified rec-uPAR$_{277}$ was dialyzed against PBS, pH 7.4. Protein content was determined by the bicinchoninc acid method (Pierce, Rockford, IL, USA). The purified protein analyzed by SDS-PAGE as shown in [12] migrates with an apparent molecular mass of 50–60 kDa. The three obvious bands very likely represent different glycosylation variants, since all three variants bind to uPA and, in addition, N-terminal amino acid sequence analysis revealed only a single amino acid sequence. This amino acid sequence was in agreement with the published uPAR sequence [15].

2.3. Detection of uPAR on cancer cells

10^6 cells were suspended in 0.5 ml of 50 mM glycine-HCl, containing 100 mM NaCl, pH 3.0, in order to dissociate receptor-bound uPA from its receptor (30 s, 22°C), and then neutralized by the addition of 0.5 ml of 5 mM HEPES containing 100 mM NaCl, pH 7.5. The cells were washed twice with PBS/0.1% BSA. uPAR antigen was detected on the cancer cell surface by incubating cells with increasing concentrations of monoclonal antibody #3936 (American Diagnostica, Greenwich, CT, USA) (range 0–2000 ng/ml) for 30 min at 22°C. After washing twice with PBS/0.1% BSA and resuspension in 250 μl PBS/0.1% BSA containing 4 μg/ml FITC-goat anti-mouse IgG (Sigma, St. Louis, MO, USA), the reaction mixture was incubated for 30 min at 22°C. Cells were washed as described above and cell-associated fluorescence was measured with the FACScan flow cytofluorometer (Becton Dickinson, Heidelberg, Germany). Autofluorescence and non-specific binding of FITC-antibody in the absence of monoclonal antibody #3936 was subtracted from total fluorescence.

Binding of pro-uPA to cell surface uPAR was performed as described [12]. Briefly, cells were treated with 50 mM glycine-HCl, 0.5 M NaCl, pH 3.0, to dissociate receptor-bound uPA. After centrifugation, 10^6 cells were resuspended in 1 ml PBS/0.1% BSA containing 100 ng FITC-pro-uPA (30 min, 22°C). Cell associated fluorescence was determined without washing the cells (real time analysis) on the FACScan. Non-specific binding of FITC-pro-uPA to cells was determined in the presence of 6 μg/ml (60-fold excess) of parent pro-uPA. The competitor rec-uPAR$_{277}$ was preincubated (30 min, 22°C) at different concentrations (range 0–2 μg/ml) with 100 ng/ml of a fixed concentration of FITC-pro-uPA before addition to the cells.

Cellular DNA was stained with propidium iodide (40 μg/ml) to verify viability of the cells. Cells which stained with propidium iodide were not included in the analysis.

2.4. Proliferation assay

Proliferation was analyzed using the Cell Titer 96 non-radioactive cell proliferation assay purchased from Promega (Madison, WI, USA). The influence of rec-uPAR$_{277}$ on the viability of the cells was determined by staining cells in parallel experiments with Trypan blue.

2.5. Invasion assay

The in vitro invasion of cancer cells was studied in a double-filter assay as described [13]. Briefly, Matrigel (Collaborative Biomedical Products, Bedford, MA, USA) was located between an upper polycarbonate filter and a lower nitrocellulose filter. 10^5 cells in 1 ml cell culture medium, with or without rec-uPAR$_{277}$, were applied on top of the filter sandwich and incubated for 16 h at 37°C. The whole set-up was fixed with glutaraldehyde for 72 h, then disassembled and the filter stained, either with hematoxylin (in the case of the nitrocellulose filters including the Matrigel) or with Giemsa's solution (in the case of the polycarbonate filter). The invasion rate was determined by calculating the ratio of adherent cells on the upper filter and of cells that had invaded into the Matrigel and lower filter.

3. Results and discussion

The present study aimed at investigating whether a soluble recombinant uPAR possesses the capability to inhibit proliferation and/or invasion of cancer cells by acting as a scavenger for uPA. For this purpose, the human ovarian cancer cell line, OV-MZ-6, which expresses uPAR, uPA and its inhibitor, PAI-1, was selected as the target cell line [14]. Cell surface-bound uPAR was detected with a monoclonal antibody (mAb) directed to uPAR (#3936) applying flow cytofluorometry. The binding of mAb #3936 is dose-dependent; saturation is achieved at ~1 μg/ml mAb #3936 (Fig. 1). A 60-fold molar excess of soluble rec-uPAR$_{277}$ completely abolished binding of mAb #3936 to cellular uPAR, demonstrating the specificity of the uPAR–mAb interaction.

uPAR on OV-MZ-6 is functionally active. This was shown by flow cytofluorometry using fluorescently labeled pro-uPA (FITC-pro-uPA). Binding of FITC-pro-uPA (input 100 ng/ml) to uPAR on OV-MZ-6 cells was inhibited by rec-uPAR$_{277}$ (range 0–2 μg/ml) in a concentration-dependent manner (Fig. 2). Under these conditions, 50% of FITC-pro-uPA binding was achieved at an input of 200 ng/ml of rec-uPAR$_{277}$. Since the molecular mass of pro-uPA (54 kDa) and rec-uPAR$_{277}$ (55 kDa) are very close, a 50% inhibition of pro-uPA binding activity is thus affected by a two-fold molar excess of rec-uPAR$_{277}$ over FITC-pro-uPA. Similar results have been demonstrated for the promyelocytic cell line, U-937 [12].

uPA-stimulated proliferation of tumor cell lines is known to be mediated via binding of uPA to uPAR [5,6].

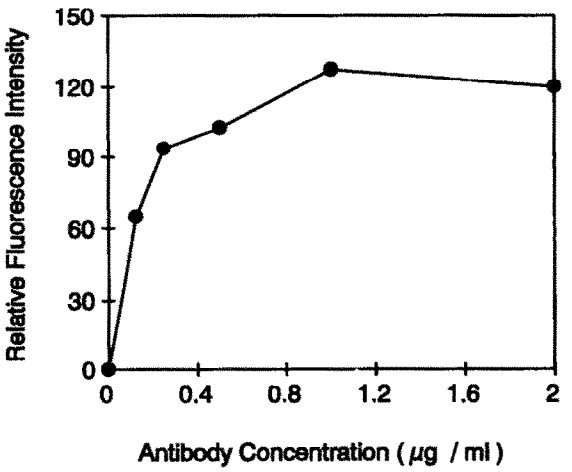

Fig. 1. Detection of uPAR by mAb #3936 (indirect immunofluorescence) on the human ovarian cancer cell line OV-MZ-6, by flow cytofluorometry (FACS) as described in detail in section 2. Autofluorescence of OV-MZ-6 and non-specific binding in the absence of mAb #3936 were subtracted from total binding. Fluorescence is expressed as relative fluorescence mean channels.

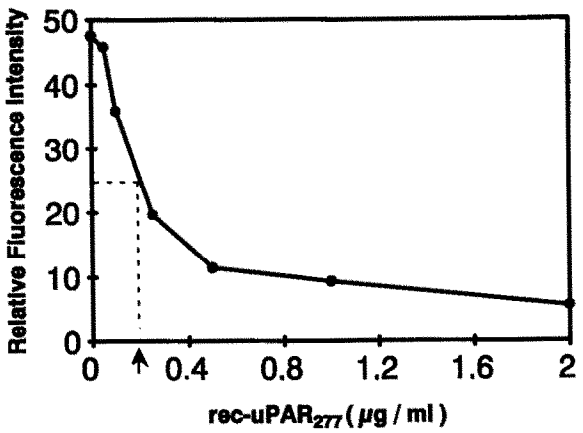

Fig. 2. Rec-uPAR$_{277}$ inhibits binding of fluorescently labeled pro-uPA (FITC-pro-uPA) to the living human ovarian cancer cell line, OV-MZ-6. Binding of FITC-labeled pro-uPA to OV-MZ-6 in the presence of various concentrations of rec-uPAR$_{277}$ was determined by flow cytofluorometry as described in detail in section 2. The arrow indicates 50% inhibition (200 ng/ml rec-uPAR$_{277}$).

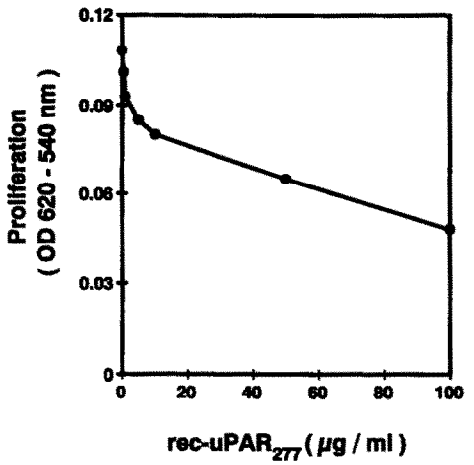

Fig. 3. Effect of rec-uPAR$_{277}$ on the proliferation of the human ovarian cancer cell line, OV-MZ-6. Mean values of three experiments are shown, standard deviation is less than 10% for each data point.

In some cells, enhancement of proliferation is mediated by the active enzyme [16], in others by the amino-terminal fragment of uPA (amino acids 1–158) [17]. This stimulation of the proliferation is inhibited by chemically inactivated uPA or uPA analogues [16,17]. Cells expressing uPA and uPAR show the ability to saturate their own cell surface uPAR in an autocrine fashion [18]. Binding of endogenous uPA to OV-MZ-6 cells triggers cell proliferation. The addition of rec-uPAR$_{277}$ (24 h exposure) significantly reduced proliferation of OV-MZ-6 cancer cells in a dose-dependent manner (Fig. 3). Viability of OV-MZ-6 cells was unaffected by different rec-uPAR$_{277}$ concentrations, as verified by Trypan blue exclusion.

A second biological effect of rec-uPAR$_{277}$ on the uPA–uPAR interaction was demonstrated by an in vitro invasion assay. Rec-uPAR$_{277}$ inhibited invasion of OV-MZ-6 at 10 µg/ml by 50% and at 20 µg/ml by 75%. The adhesion capacity of OV-MZ-6 cells was, however, not significantly altered. At 20 µg/ml rec-uPAR$_{277}$, the invasion of OV-MZ-6 was inhibited by 75% but proliferation only by 29%. This clearly demonstrates that the reduced invasion capacity of OV-MZ-6 in the presence of rec-uPAR$_{277}$ observed in the in vitro invasion assay is an additive effect of inhibition of cell proliferation and invasion. uPA-dependent tumor cell invasion was already shown by others. In vitro tumor cell invasiveness was inhibited significantly by mAb's to uPA [8] or uPA peptides comprising residues 17–32 of the epidermal growth factor domain of uPA [9]. We demonstrate that not only uPA analogues or mAb's are able to influence the invasion of cancer cells but also rec-uPAR$_{277}$.

OV-MZ-6 cells express both uPA and its inhibitor, PAI-1 [14]. Stimulation of tumor cell proliferation might be the result of internalization of the uPAR–uPA–PAI-1 complex. Indeed, in breast cancer patients, elevated uPA–PAI-1 values are associated with increased S-phase, a measure of cell proliferation [19]. The addition of excess rec-uPAR$_{277}$ to OV-MZ-6 resulted in binding of uPA and/or uPA complexed with PAI-1 leading to a reduction of cell surface uPA activity and, thus, to a decrease in cell-associated plasmin activity by preventing activation of plasminogen to plasmin [20]. The degradation of extracellular matrix proteins by plasmin, which facilitates cancer cell invasion, is therefore subsequently reduced.

In conclusion, a soluble recombinant truncated form of the urokinase receptor is able to block binding of uPA to cell surface-bound uPAR. Consequently, rec-uPAR$_{277}$ functions as a scavenger for uPA by inhibiting uPA-induced tumor cell proliferation and invasion of the OV-MZ-6 ovarian cancer cell line into the Matrigel. On the basis of these results we propose that in addition to uPA analogues and mAb's to uPA, rec-uPAR$_{277}$ is a putative candidate for treatment of cancer spread. Nevertheless, inhibition of tumor growth and metastasis in vivo should

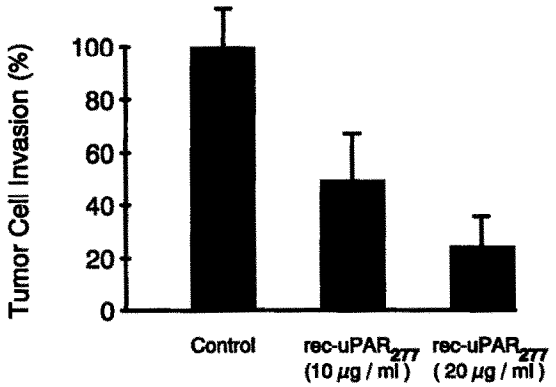

Fig. 4. Inhibition of the invasion of OV-MZ-6 cells by rec-uPAR$_{277}$ determined by an in vitro invasion assay. Mean values ± S.D. of three experiments are indicated.

be demonstrated by animal experiments (e.g. nude mice) which still awaits verification.

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