Abstract. The receptor for the urokinase-type plasminogen activator (uPA), uPAR, facilitates tumor cell invasion and metastasis by focusing on several ligands, including uPA, integrins and vitronectin. With computational prediction algorithms and structure-based drug design, we identified peptides containing the Gly-Lys-Gly-Glu-Gly-Glu-Gly-Lys-Gly sequence (peptide H1), which strongly interacts with uPAR. The aim of the present study was to investigate the effect of allosteric inhibition at the uPAR interface using a novel synthetic peptide and its function on ovarian cancer cell invasion. The molecular and functional mechanisms of H1 were determined by complementary biochemical and biological methods in the promyeloid U937 cell line as well as ovarian cancer cell lines, including serous carcinoma SKOV3 and clear cell carcinoma TOV21G. The effects of H1 treatment on cancer cell invasion were evaluated in vitro. H1 inhibited cancer cell invasion, without affecting cell viability, accompanied by the suppression of extracellular signal-regulated kinase (ERK)-1 phosphorylation and then matrix metalloproteinase (MMP)-9 expression. H1 failed to block the interaction of uPA-uPAR protein-protein interaction in cells, but antagonized the uPA function. H1 failed to disrupt the uPA-uPAR complex, but abolished the invasion of ovarian cancer cells at least through suppression of the ERK-MMP-9 signaling pathway. Further studies are needed to confirm our observations and to describe the underlying molecular mechanism.

Introduction

Epithelial ovarian cancer has the highest mortality rate among all the gynecologic tumors (1). Recent advances in molecular technologies have made possible the creation of personalized medicine, but peritoneal dissemination is associated with poor overall survival of ovarian cancer patients (1-3). Despite some improvements in treatment, current therapy is not considered curative as the mechanism underlying the peritoneal dissemination remains unclear. To overcome a poor clinical prognosis, understanding the molecular and functional mechanisms involved in the ovarian cancer cell survival, proliferation, invasion, dissemination and metastasis is of great physiological and clinical importance.

Members of the plasminogen activator system including urokinase-type plasminogen activator (uPA) and its receptor uPAR are overexpressed on the surfaces of a wide range of invasive cancer cells, including those of the prostate, brain, breast and colon in addition to ovarian cancer (2). uPAR may be a more aggressive phenotype in a broad range of human various aggressive cancer types, including ovarian cancer (3). uPAR is a glycosyl phosphatidylinositol-anchored cell surface receptor that interacts with uPA and other molecules, such as integrins and vitronectin (VN). Accumulating evidence shows that the uPA-uPAR system, such as uPAR activation and its downstream signaling, is associated with cancer progression, invasion, metastasis and peritoneal dissemination in a variety of tumor types (2,3). uPAR activates diverse signaling pathways, including phosphorylation of extracellular signal-regulated kinase (ERK) and then stimulation of matrix metalloproteinase (MMP)-9 expression (4,5).

Notably, multiple approaches to inhibit the uPA-uPAR system may suppress cancer cell invasion and induce cell death. Indeed, various treatment strategies including selective inhibitors of uPA activity (6-13), specific antagonistic peptides that block uPA-uPAR protein-protein interaction (13-24), shRNA-uPAR-mediated silencing (5,25-32), or inhibitory antibodies against uPA or uPAR (33-36) have been investigated in promising preclinical and clinical trials. Although uPAR promotes cancer progression independently of protease activation, scavenging the active uPA or blocking its function leads to reduced tumor progression and may show be promising for prolonging patient survival (37). Anti-uPAR antibodies preventing uPA binding to uPAR can potentially lead to the suppression of cancer invasion. Functional analysis using shRNA-uPAR transfected to downregulate uPAR revealed a critical role for uPAR in cancer invasion and metastasis. The synthetic peptides that prevent uPA-uPAR interaction are a promising template for designing novel peptide-based small
compounds to provide an effective strategy to treat ovarian cancer (17).

Furthermore, uPAR interacts with not only uPA, but also several molecules, such as VN, integrins, caveolin-1, G protein-coupled receptors (formyl peptide receptor 2), low density lipoprotein receptor-related protein 1 and epidermal growth factor receptor (38,39). Integrins are the important transmembrane binding partners associated with uPAR, leading to integrin-mediated intracellular signaling. Integrins also serve as VN receptors and binding of VN to integrins results in the uPA-mediated cell invasion via uPAR (40). uPAR-integrin signaling to ERK increases the expression of MMPs through API transcription factors (39-41).

Computer simulations can provide a dry lab experience that may fulfill some of the objectives of the in vitro wet labs. We reported previously that structure-based computational docking study using ab initio molecular orbital calculations has predicted co-binding of the omega-loop of amino-terminal fragment of uPA with the positively charged 46, 61 and 98 Lys residues of uPAR (42,43). Molecular dynamics computer simulation by structure-based drug design revealed that the single 9-residue amino acid peptide with the Gly-Lys-Gl y-Glu-Gly-Lys-Gly sequence (peptide H1) has a large binding energy to uPAR that may block the binding between uPAR and some ligands, uPA or VN (42,43). In the present study, we synthesized H1 as a potent uPAR inhibitor and control peptide (an amino acid sequence-shuffled peptide).

The aims of this study were to investigate whether cancer cells depend on the function of uPAR for their invasion and whether H1 specifically inhibits cell invasion in vitro.

Materials and methods

Materials. The materials used in the present study and the manufacturers providing them are as following: anti-total ERK1/2, anti-phospho-p44/42 ERK, anti-MMP-9, anti-β-actin (no. 4967) (all from Cell Signaling Technology, Inc., Danvers, MA, USA), the horseradish peroxidase-conjugated secondary antibodies from Sigma-Aldrich (St. Louis, MO, USA), human uPA from Chemicon International, Inc. (Temecula, CA, USA) and American Diagnostica (Lexington, MA, USA) fluorescein isothiocyanate (FITC)-conjugated uPA from Abcam (cat. no. ab9152; Cambridge, UK) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) powder from Roche Diagnostics (Indianapolis, IN, USA). Plastic ware was purchased from Costar (Cambridge, MA, USA). The protein content was determined using a bichinonic acid assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Plastic ware was purchased from Costar (Cambridge, MA, USA). The protein content was determined using a bichinonic acid assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Linear peptides, H1 (Gly-Lys-Gly-Glu-Gly-Gly-Lys-Gly-Gly) and H2 (Glu-Gly-Gly-Lys-Gly-Gly-Lys-Gly-Gly; an amino acid sequence-shuffled control peptide), were synthesized and HPLC-purified as described (Peptide Institute, Inc., Osaka, Japan) (44).

Flow cytometry. Promyeloid leukemia U937 cells, stimulated for 48 h with phorbol-12-myristate-13-acetate (PMA; 1 mmol/l), were used to investigate binding of H1 and H2 peptides to cell-associated uPAR. Cells (2.5x10^7) were washed several times with phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA) and then subjected to acidic buffer (50 mmol/l glycine/HCl, pH 3.0, for 1 min) to remove receptor-bound endogenous uPA, followed by a neutralization step with 0.5 mol/l HEPEs/NaOH, pH 7.5 (45). The cells were subsequently incubated with increasing amounts of H1 peptide or H2 peptide (0 or 1,000 nM) in the presence of 2 µg of FITC-labeled uPA for 30 min. The cells were washed again with PBS/0.1% BSA. The cell-associated fluorescence was determined.

Cell culture. Human ovarian carcinoma cell lines SKOV3 (serous-type) and TOV21G (clear cell-type) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium/F12, supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin (all from Invitrogen Life Technologies, Carlsbad, CA, USA) in a humidified incubator with 5% CO2 and 95% air at 37°C.

Western blot analysis. Cells were washed 3 times with ice-cold PBS and extracted in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1% Nonidet 40, 0.5 mM ethylenediaminetetraacetic acid, 100 µg/ml phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 100 µm sodium vanadate, 1 mM dithiothreitol, 1 µg/ml aprotinin and 150 mM NaCl for 30 min. The equal amount of protein was separated on 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). The membrane was incubated with phospho-ERK, total ERK, uPAR, or MMP-9 (46). Proteins were visualized using an enhanced chemiluminescence kit (Pierce Biotechnology, Inc.). The blots were quantified by densitometry with the Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

MTS cell viability assay. The effects of synthetic peptides on cell viability were estimated using an MTS assay kit (Promega Corp., Madison, WI, USA) (47). After the H1 or H2 treatment, MTS solution was added to each well for 2 h at 37°C in 5% CO2. The absorbance at 490 nm was recorded using a GloMax-Multi+Multiplate Multimode Reader (Promega Corp.).

Transwell invasion assay. Transwell plates (24-well) (Costar Inc., Corning, NY, USA) were coated with 30 µg/ml of Matrigel at 4°C overnight. After 16 h of serum starvation, 5x10^4 cells in 250 µl of 0.1% PBS medium containing 10 nM uPA in the presence of the indicated H1 peptide or H2 control peptide were added to the upper chamber and incubated at 37°C for 22 h. A total of 500 µl of 10% PBS medium containing the same amount of compounds was simultaneously added to the lower chamber. Non-migrated cells on the top of the transwell were scrapped off with a cotton swab and the number of migrated cells was counted in ten separate fields and averaged across two independent experiments with each concentration in triplicate. We confirmed some results of cell invasion experiments by a novel protocol utilizing the IncuCyte ZOOM instrument (Essen Bioscience, Ann Arbor, MI, USA).

Statistical analysis. The bar graphs are the means ± standard deviation from at least three independent experiments. Comparison of mean values between 2 groups was evaluated using a Student’s t-test.
by the t-test. For all statistical methods, a P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of H1 peptide. We synthesized peptides H1 and H2. H2 peptide (control) is the amino acid sequence-shuffled H1 peptide. We employed a flow cytometry assay using FITC-conjugated uPA to assess whether H1 blocks the uPA-uPAR protein-protein interaction in U937 cells. Free uPA protein significantly inhibited the FITC-labeled uPA protein binding to the uPAR on the U937 cells in a dose-dependent manner, but both H1 and H2 failed to disrupt the interactions between FITC-labeled uPA and cellular uPAR (Fig. 1).

H1 impairs invasion of ovarian cancer cells in vitro. We then determined whether H1 inhibits ovarian cancer cell invasion. Cells were serum-starved for 16 h and then treated with 10 nM uPA at 37°C for the indicated times. SKOV3 and TOV21G cells were treated with 10 nM uPA in the presence or absence of increasing concentrations of H1 or H2 for 22 h. H1 was effective in suppression of the invasion in a dose-dependent manner in cancer cells with IC50 values of approximately 100 nM (Fig. 2). H2 showed no effect on cell invasion. H1 has also similar inhibitory effects on TOV21G cells (data not shown).

Cell viability. We performed MTS assays in SKOV3 and TOV21G with increasing concentrations of H1 or H2 (1,000 nM) for 24 h. H1 and H2 peptides did not affect the viability and growth of SKOV3 cells (data not shown). Similar results were obtained after treatment with each peptide in TOV21G cells.

H1 abolishes uPA-induced ERK phosphorylation and subsequent activation of MMP-9 overexpression. To investigate the underlying mechanism of action for the cell invasion inhibition of H1, we selected SKOV3 cell lines for further investigation. SKOV3 cells were serum-starved for 16 h and then pretreated with different concentrations (100 and 1,000 nM) of H1 or H2. After 30 min, the cells were incubated for another 5 min with 10 nM uPA. Phosphorylated and total ERK were detected by western blot analysis. H1, but not H2, significantly suppressed the uPA-induced phosphorylation of ERK in a dose-dependent manner (Fig. 3A).

Next we examined whether H1 was able to suppress the uPA-induced MMP-9 expression through inactivation of the ERK pathway. Treatment for 24 h of SKOV3 cells with H1 resulted in dose-dependent suppression of MMP-9 expression, starting at a concentration of 100 nM (Fig. 3B). H2 did not reduce the expression of MMP-9.

Discussion

In a previous in silico study, we designed small molecule inhibitors of the uPAR-ligand interaction by molecular docking and molecular dynamic simulation studies (43). Compound H1 was selected and identified using ab initio molecular simulation method (42,43). Since our previous studies were fully dependent on computational prediction algorithms, the function of H1 was confirmed by wet lab
experiments. Our results demonstrated that H1 significantly inhibited the uPA-dependent cell invasion, possibly though suppression of ERK-activated MMP-9 expression (Fig. 2). The inhibition of cell invasion occurs at high nano-molar concentrations. Importantly, the amino acid sequence-shuffled H2 peptide exhibited no effect on uPA-dependent cell invasion. The compound did not affect cell viability and its potency is independent of the inhibition of cell growth. This may provide promising evidence for the therapeutic potential of H1 against ovarian cancer cells. Of note, H1 failed to inhibit uPA binding to the uPAR, but mitigated the uPAR-dependent signaling pathway. We suggested that H1 and uPA would bind at distinct sites on uPAR molecule. However, H1 did not block the binding of VN to uPAR protein (data not shown).

Several researchers have identified, synthesized and preclinically examined several compounds acting as potential inhibitors of the uPA-uPAR interaction. The following are currently promising anti-invasive/metastatic agents: protease inhibitors (8,13), small molecular peptides (13-17,24), antibodies (34,36) and siRNA/shRNA (5-25,32). Some of these inhibitors (8,13), small molecular peptides (13-17,24), antiangiogenesis (32) and siRNA/shRNA (5-25,32) have been evaluated in vivo pharmacokinetic and efficacy studies in an animal cancer metastasis model. These promising findings demonstrate the therapeutic potential of this synthetic H1 peptide against ovarian cancer and require further preclinical investigations. However, the effect on invasion of this active peptide was inconsistent with its ability to inhibit the interaction between uPA and uPAR.

In conclusion, H1 is a promising template for the development of orally bioavailable compounds with greater efficacy on cancer cell invasion. Further studies are needed to evaluate the molecular mechanism of H1 peptide.

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References


