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Urokinase-controlled tumor penetrating peptide

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ABSTRACT

Tumor penetrating peptides contain a cryptic (R/K)XX(R/K) CendR element that must be C-terminally exposed to trigger neuropilin-1 (NRP-1) binding, cellular internalization and malignant tissue penetration. The specific proteases that are involved in processing of tumor penetrating peptides identified using phage display are not known. Here we design *de novo* a tumor-penetrating peptide based on consensus cleavage motif of urokinase-type plasminogen activator (uPA). We expressed the peptide, uCendR (RPARSGR↓SAGGSVA, ↓ shows cleavage site), on phage or coated it onto silver nanoparticles and showed that it is cleaved by uPA, and that the cleavage triggers binding to recombinant NRP-1 and to NPR-1-expressing cells. Upon systemic administration to mice bearing uPA-overexpressing breast tumors, FAM-labeled uCendR peptide and uCendR-coated nanoparticles preferentially accumulated in tumor tissue. We also show that uCendR phage internalization into cultured cancer cells and its penetration in explants of murine tumors and clinical tumor explants can be potentiated by combining the uCendR peptide with tumor-homing module, CRGDC.
Our work demonstrates the feasibility of designing tumor-penetrating peptides that are activated by a specific tumor protease. As upregulation of protease expression is one of the hallmarks of cancer, and numerous tumor proteases have substrate specificities compatible with proteolytic unmasking of cryptic CendR motifs, the strategy described here may provide a generic approach for designing proteolytically-actuated peptides for tumor-penetrative payload delivery.

1. Introduction

Distinct molecular features of tumor vessels can be used for affinity-based (synaphic) delivery of diagnostic and therapeutic cargo using various affinity ligands (e.g. peptides, antibodies). A prototypic tumor-penetrating peptide, iRGD (CRGDKGPDC), combines synaphic targeting with tumor-specific vascular exit and tissue penetration [1]. The activity of iRGD depends on the cell - internalization and tissue-penetration sequence motif R/KXXR/K. We have termed this motif the C-end Rule or CendR motif and the endocytotic pathway it triggers the CendR pathway [1,2]. iRGD is a composite of the tumor-homing RGD motif, which binds to α_v-integrins in angiogenic tumor vessels, and an overlapping cryptic RGDK CendR motif. Proteolytic cleavage of iRGD to unmask the CendR motif in tumors [1] provides a key regulatory step, whereby iRGD loses affinity for integrins, while acquiring NRP-1-binding activity, which induces extravasation and cell and tumor penetration [1,3]. The identity of the iRGD processing protease(s) is not known. During tumorigenesis, extracellular proteolytic machinery is activated at a number of levels (transcriptional activation, increased secretion, upregulation of cell surface receptors for proteases, and downregulation of protease inhibitors) [4,5]. Of the ~600 proteases in the human degradome, many have substrate specificities compatible with C-terminal unmasking of R/KXXR/K required for CendR activation. We hypothesized that it may be possible to design cryptic CendR peptides that are activated by specific tumor proteases to trigger tumor penetration.

Urokinase-type plasminogen activator (uPA), proteolytically converts plasminogen to the broad-spectrum protease, plasmin. In healthy adults, uPA generally occurs with low abundance and has a limited tissue distribution. uPA overexpression is firmly linked to angiogenesis, tumor invasion and metastasis [6,7]. In tumors, high-affinity uPA receptor (uPAR) concentrates active uPA to the surface of tumor cells, macrophages, and angiogenic endothelial cells [8]. uPA and its receptor are key components of a cell surface proteolytic cascade used by both tumor cells and activated capillary endothelial cells for basement membrane invasion, a process required for metastasis and angiogenesis. In response to angiogenic agents, endothelial cells in tumor neovessels

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secrete uPA and upregulate cell surface expression of uPAR [6–8]. uPA has been used for protease-activated systemic drug targeting, and its use is further supported by successful application of uPA-mediated activation of anthrax toxin [9,10], cytotoxic proteins [11], and cytotoxic drug conjugates [12] for tumor therapy.

The aim of the current study was to design a urokinase-activatable tumor-penetrating peptide, which we call uCendR, and validate its activity for cell- and tumor penetrative delivery of payloads. Our study demonstrates the feasibility of rationally designing tumor-penetrating peptides that are activated by specific tumor proteases. These findings expand the versatility and utility of CendR-based platforms in tumor targeting.

2. Materials and methods

2.1. Peptides and proteins

Peptides were purchased from Lifetein Inc. (Somerset, NJ), or synthesized using Fmoc/t-Bu chemistry on a microwave assisted automated peptide synthesizer (Liberty, CEM Corporation, Matthews, NC). Peptides were purified by high-performance liquid chromatography (HPLC) using 0.1% TFA in acetonitrile-water mixtures to 90%–95% purity and validated by Quadrupole-time-of-flight (Q-TOF) mass spectral analysis. Fluorescent and biotinylated peptides were synthesized by using 5(6)-carboxyfluorescein (FAM) or biotin, each with 6-aminohexanoic acid (Ahx) spacer between it and the N-terminus of the peptide. NRP-1 b1b2 protein was purified at the Protein Production and Analysis Facility at the Sanford Burnham Prebys Medical Discovery Institute (La Jolla, CA).

2.2. Silver nanoparticle synthesis, functionalization

Silver nanoparticles (AgNPs) were prepared by citrate reduction as described [13]. An extinction of $1 \times 10^{10}\ M^{-1}\ cm^{-1}$ at the 405 nm Ag plasmon peak was used to quantify the concentration of AgNPs. The synthesis of Ag citrate (20–50 nm diameter) was performed as follows: $AgNO_3$ (450 mg, Sigma) was dissolved in 2.5 L water and vigorously boiled while stirring, and trisodium citrate dihydrate (500 mg, Sigma) in 50 mL water was added. After 20–30 min the solution became greenish brown, at which point the heat was turned off and the solution stirred overnight. UV–Vis showed an absorbance of ~10 at 405 nm, 1 cm path length. Neutravidin (NA) was conjugated to 5 kDa heterobifunctional succinimidyl carboxymethyl ester polyethylene glycol (PEG) ortho-pyridyl disulfide (NHS-PEG-OPSS, Jenkem Technology, Plano, TX) and dialyzed against 0.1× phosphate-buffered saline (PBS) as described [13]. The NA product (3.1 mg/mL, 2 mL, with ~2 PEG-OPSS per NA) was added to 500 mL Ag, sonicated, then 25 mL of 50 mM morpholino sulfonic acid (MES) buffer, pH 6 (adjusted with HNO_3) was added and incubated overnight at 37 °C. The solution was cooled to room temperature and 50 mL of 10× PBS was added, final pH ~7.4. Then 250 μL of Tween 20 (10 wt%) in water was added to reduce adsorption of colloid to plastic. Ag was centrifuged (Thermo Nalgene Cat #3140–0500, 500 mL) at 12 k ×*g* at 4 °C 1 h, and redispersed (bath sonication, Branson model 1510), then combined and spun in a 40 mL tube at 17 k ×*g* 20 min 4 °C. The pellet was redispersed in PBS with 0.005% Tween 20 (PBST). Next, lipoic acid PEG amine (LPN, 5.7 mg of 1000 g/mol, Nanocs, #PG2-AMLA-1 k) was dissolved in 570 μL 0.1 M tris(2-carboxyethyl)phosphine hydrochloride solution pH 7 (TCEP, Sigma) and allowed to reduce the lipoic acid group for at least 1 h. The Ag was treated with 1 mM TCEP for 30 min to reduce the disulfides of the NA-OPSS. Then LPN was added (0.05 mg/mL) and incubated at 37 °C for 1 h. The product was pelleted and redispersed in PBST, filtered using 0.45 μm syringe filter (PVDF, Millipore Cat# SLHV033RS), and stored at 4 °C. Optical density was ~300 at 1 cm, 405 nm Ag plasmon peak. For dye-labeling, 20 μL of 2 mM amine-reactive NHS-Oregon Green 488 (Thermo, Cat#*O*-6147) dissolved in DMSO was added per mL of Ag, followed by 15 μL 7.5% sodium bicarbonate solution (Gibco, Cat#25080). The labeling occurred at room temperature for 1 h then overnight at 4 °C, then washed 3× (7 k ×*g*, room temperature), re-dispersing in PBST. The biotinylated peptides were loaded into the AgNPs, washed by centrifugation and brought up in PBST. Prior to use, Ag was filtered (0.22 μm, Millipore Cat#SLGV013SL).

2.3. In vitro cell uptake with silver

PPC-1 or M21 cells were seeded into 96 well plates and used the following day. Peptide loaded AgNPs (25 μL, 200 O.D.) were incubated in Eppendorf tubes with or without 5 μL of uPA (Calbiochem #672112, stock 10,000 μ/mL) for at least 30 min at 37 °C, then 3 μL was added to cells for 90 min at 37 °C. For etching, 5 μL of etchant (20 mM $K_3Fe(III)(CN)_6$ and 20 mM $Na_2S_2O_3$ in PBS) was added prior to imaging. Imaging was performed as described [13] under dark field illumination to visualize the AgNP core (20× objective, Leica DMIRE2).

2.4. Fluorescence assay with silver

Peptide loaded AgNPs (25 μL, 300 O.D.) were incubated in Eppendorf tubes with or without 5 μL of uPA (Calbiochem #672112, stock 10,000 u/ml) or 5 μL PBS for at least 30 min at 37 °C. Note that for biotin-blocked AgNPs, excess D-biotin in DMSO was added 15 min prior to adding the biotin-Ahx-GGSGRPARSGRSAGGK(Rho)DA-OH. Then, 5 μL AgNP solution was diluted into 200 μL PBS in 96 well plates (black wall, clear bottom) and read on a Tecan plate reader. AgNPs without OR488 label or peptide was used as a background control, and gave negligible signal above blank PBS. Assay conditions were OR488 channel: excitation 485 nm, emission 538 nm, cutoff 515 nm; Rho channel: excitation 544, emission 590, cutoff 570.

2.5. Quantum dots

Streptavidin ITK-605 quantum dots (Invitrogen, Carlsbad, CA) were modified with biotinylated peptides by incubation with a 100-fold molar excess of peptide followed by removal of free peptide by dialysis.

2.6. Mice and cell lines

All animal experimentation was performed according to procedures approved by the Animal Research Committee at the University of Sanford Burnham Prebys Medical Discovery Institute (La Jolla, CA). For tumor injections and before sacrificing, the mice were anesthetized with intraperitoneal injections of xylazine (10 mg/kg) and ketamine (50 mg/kg). BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used for tumor xenografts. For histological analysis, tissues were fixed in 4% paraformaldehyde, cryoprotected in phosphate buffered saline solution containing 30% sucrose, frozen, and sectioned at 10 μm. PPC-1, 4T1, and M21 cell lines were maintained in the Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin.

2.7. Peptide phage display

T7-select phage display system (EMD Biosciences, Gibbstown, NJ) was used for individual peptide-phage cloning according the manufacturer's instructions. Phage was purified by precipitation with PEG-8000 (Sigma, St. Louis, MO) followed by $CsCl_2$ gradient ultracentrifugation and dialysis. The sequences of displayed peptides were determined from the DNA encoding the insert-containing region at the C-terminus of the T7 major coat protein gp10.

For phage binding studies [14], cultured cells were grown to near-confluence and dissociated with trypsin, and mouse organs were dissociated using the Medimachine system (BD Biosciences, San Jose, CA). To measure phage binding, 10^6 cells in binding buffer (DMEM containing