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Bladder tumor-targeted delivery of pro-apoptotic peptide for cancer therapy

Hyun-Kyung Jung [a,b], Soyoun Kim [a,b], Rang-Woon Park [a,b,c], Jae-Yong Park [c,d], In-San Kim [e,f], Byungheon Lee [a,b,c,*]

[a] Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Daegu, Republic of Korea
[b] CMRI, School of Medicine, Kyungpook National University, Daegu, Republic of Korea
[c] BK21 Plus KNU Biomedical Convergence Program, Department of Biomedical Science, School of Medicine, Kyungpook National University, Daegu, Republic of Korea
[d] Department of Internal Medicine, School of Medicine, Kyungpook National University, Daegu, Republic of Korea
[e] Center for Theragnosis, Biomedical Research Institute, Korea Institute of Science and Technology, Seoul, Republic of Korea
[f] KU-KIST School, Korea University, Seoul, Republic of Korea

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ABSTRACT

The overall prognosis of conventional chemotherapy for the treatment of bladder cancer is poor and reduction of its systemic side effects remains an unsolved issue. Targeted therapy for bladder cancer could improve therapeutic efficacy and reduce side effects. This study investigated a hybrid peptide (named Bl*d*-1-KLA) composed of the CSNRDARRC peptide (Bld-1), which binds to bladder tumor cells, and the D-KLAKLAKKLAKLAK (KLA) peptide, which disrupts mitochondrial membrane and induces apoptotic cell death, as a bladder cancer-targeted therapeutic agent. Bld-1-KLA selectively bound to HT1376 bladder tumor cells and efficiently internalized into the cells but not to other types of tumor and normal cell lines. Bld-1-KLA exerted cytotoxic effects selectively to HT1376 cells (LC_{50} = 41.5 μM), but not to other types of cells. Pretreatment of cells with Bld-1 inhibited the binding and cytotoxicity by Bld-1-KLA in HT1376 cells. It induced apoptosis of bladder tumor cells, while Bld-1 or KLA alone showed much lesser effect on apoptosis, and was co-localized in mitochondria. Bld-1-KLA was stable up to 24 h in serum. *In vivo* fluorescence imaging showed that homing of Bld-1-KLA in the tumor in HT1376 tumor-bearing nude mice was greater than that of the control peptide-KLA after intravenous injection. Treatment of tumor-bearing mice with Bld-1-KLA, compared to the control peptide-KLA, induced apoptosis of tumor cells and inhibited tumor growth more efficiently. No significant side effects on body weight and the liver and myeloid function were observed in mice treated with Bld-1-KLA. These results suggest that Bld-1-KLA is a promising therapeutic agent for targeted therapy of bladder cancer.

1. Introduction

Bladder cancer is the ninth most common cause of cancer worldwide [1]. Most cases of bladder cancer are superficial tumors that are confined to the epithelial cell layer of the bladder and are treated by transurethral resection [2]. However, the tumors that invade muscle layer of the bladder may spread to distant sites (metastasis). Metastatic bladder tumors have been treated with cisplatin-based combined chemotherapy regimens, but only about 10% to 15% of the patients show long-term disease-free survival [3]. Development of targeted therapeutics for metastatic bladder tumors remains an unmet medical need, which could improve the therapeutic efficacy besides reducing systemic side effects.

Mitochondria play an essential role in apoptotic cell death. This has led to the development of anticancer agents that disrupt mitochondrial functions and induce apoptosis in tumor cells [4,5]. A typical example is the cationic and amphipathic peptide KLAKLAKKLAKLAK (KLA). It is a naturally occurring antibacterial peptide that binds to and disrupts the negatively charged bacterial membrane; however, it generally does not disrupt zwitterionic, eukaryotic plasma membrane and hence is not cytotoxic to eukaryotic cells [6]. On the other hand, it triggers mitochondrial membrane disruption and results in the release of cytochrome c and induction of apoptosis after internalization into eukaryotic cells [7].

To facilitate internalization into cells, KLA has been fused with various cell-penetrating peptides (CPPs), such as Tat peptide and poly-arginine. CPPs efficiently transport proteins across the cell membrane and show significant antitumor activity [8–11]. For example, poly-

* Corresponding author at: Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, 680 Gukchaebosangro, Junggu, Daegu, 700-842, Republic of Korea.
E-mail address: leebh@knu.ac.kr (B. Lee).

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arginine-mediated introduction of KLA into HT-1080 fibrosarcoma cells resulted in a much lower LC_{50} as than that associated with anticancer chemotherapeutic agents such as cisplatin [10]. Although the intracellular delivery of CPP-fused KLA is very efficient, there is a drawback: it shows non-specific internalization into both normal and tumor cells. Fusion of KLA with homing peptides that selectively bind to target cells, can overcome the issue of non-specific internalization. Examples of homing peptides include cyclic CNGRC and RGD-4C peptides, which home to tumor cells and angiogenic endothelial cells through binding to aminopeptidase N and αvβ3 integrin, respectively [7,12,13], the YCDGFYACYMDV Her-2-binding peptide [14], SMSIARL prostate tumor-targeted peptide [15], and CKGGRAKDC adipose tissue vasculature-targeted peptide [16]. Another approach is the fusion of KLA with antibodies, such as anti-CD19 antibody, to target B cell leukemia [17]. In addition, KLA was also delivered to tumors by loading into nanoparticles, based on the enhanced permeability and retention effect of leaky tumor vasculature [18], or by loading into targeted nanoparticles labeled with tumor-homing peptides [19].

Screening of phage-displayed peptide libraries has been used to identify peptides that selectively bind to target cells (such as tumor cells, angiogenic endothelial cells, and apoptotic cells) or target proteins (on the cell surface or in serum) [20–22]. Using phage display, we have previously identified a Bld-1 peptide, CSNRDARRC, which binds to bladder tumor cells [23]. When systemically administered, Bld-1 homed to bladder tumor subcutaneously implanted in nude mice or carcinogen-induced orthotopic bladder tumor in rats [23]. When instilled into the lumen of tumor-bearing rat bladder, Bld-1 bound to the tumor lesion while it showed little binding to neighboring normal tissue. Therefore, this study employed Bld-1 as a targeting ligand for selective delivery of KLA to bladder tumors and examined the possibility of the Bld-1-KLA hybrid peptide as a bladder tumor-targeted pro-apoptotic peptide for targeted therapy of bladder cancer.

2. Materials and methods

2.1. Cell lines and cultures

HT1376 (human bladder tumor) cell line was cultured in α-MEM medium. A549 (human lung tumor), BEAS-2B (normal bronchial epithelial cells), and Jurkat (human T lymphocytes) cell lines were cultured in RPMI medium. HUVECs (human umbilical vein endothelial cells) were cultured in ECM medium containing 1% endothelial cell growth supplement. JEG-3 and BeWo (human trophoblastic cells) were cultured in DMEM medium. All cells were obtained from American Type Culture Collection (ATCC). All culture media were supplemented with 10% fetal bovine serum (FBS, GE Healthcare Life Sciences, Pittsburgh, PA) and 1% antibiotics (penicillin 100 IU/ml, and streptomycin 100 μg/ml). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Design and synthesis of peptides

All peptides were synthesized using the standard Fmoc method and purified by high-performance liquid chromatography to >90% purity by Peptron Inc. (Daejeon, Korea). Peptides were conjugated with fluorescein isothiocyanate (FITC) or with FPR675 near-infrared fluorescence (NIRF) dye (BioActs, Incheon, Korea) at the amino terminus. Lyophilized peptides were reconstituted in dimethyl sulfoxide to prepare a 100 mM stock and diluted with phosphate-buffered saline (PBS) to get working concentrations. The amino acid sequence and molecular weight (MW) for peptides were as follows: Bld-1, CSNRDARRC (MW = 1080); KLA, $_D$(KLAKLAK)$_2$ (MW = 1524); Bld-1-KLA, CSNRDARRC-GG-$_D$(KLAKLAK)$_2$, (MW = 2680); control peptide, CNSSSVDKC (MW = 942); and Control-KLA, CNSSSVDKC-GG-$_D$(KLAKLAK)$_2$ (MW = 2542). The KLA peptide was synthesized in the *D*-enantiomer form to avoid degradation by proteases as previously described [7]. The GG sequence was used as a bridge to impart peptide flexibility and minimize potential steric interactions between targeting peptide and pro-apoptotic peptide. The NSSSVDK sequence from T7 phage capsid protein was flanked with cysteine at both amino and carboxy terminals and used as control in this study.

2.3. Cellular binding and uptake of peptides

FITC-labeled peptides were used for both cell binding and intracellular uptake assays at a concentration of 10 μM. For binding assays, cells were incubated with peptides at 4 °C for 1 h. Subsequently, cells were fixed with 4% paraformaldehyde (PFA) and then incubated with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining and observed under a fluorescence microscope (Carl Zeiss, Jena, Germany). For quantitative analysis of cell binding, cells were harvested, incubated with peptides at 4 °C for 1 h, and analyzed using a flow cytometer (BD Biosciences, San Jose, CA).

For analysis of intracellular uptake, cells were incubated with FITC-labeled peptides at 37 °C for 1 h, fixed with PFA, and counterstained with DAPI for nucleus. Cells were mounted with ProLong® Gold Antifade Reagent (Thermo Fisher Scientific, Waltham, MA). *Z*-section images were taken using a confocal microscope (Carl Zeiss). For trafficking of intracellular localization, cells were incubated with FITC-labeled peptides at 37 °C for 1 h, fixed with PFA, and co-stained with 100 nM of LysoTracker dye (Thermo Fisher Scientific) and MitoTracker dye (Thermo Fisher Scientific) at 37 °C for 30 min for the staining of lysosomes and mitochondria, respectively. Images were taken for FITC-peptide, LysoTracker (577/590 nm), MitoTracker (644/665 nm) and merged using a confocal microscope.

For the competition of cell binding and uptake, cells were pretreated with non-fluorescent Bld-1 at a concentration of 100 μM at 4 °C for 1 h and then incubated with FITC-Bld-1-KLA at a concentration of 10 μM at 4 °C for 1 h.

2.4. Cytotoxicity and apoptosis assays

Unlabeled peptides were used for both apoptosis and cytotoxicity assays. For cytotoxicity assays, cells (3000/well in 96-well plates) were incubated with peptides at the indicated concentrations at 37 °C for 1 h following which peptides were removed and cells were washed. For the competition of cytotoxicity, cells were pretreated with Bld-1 (100 μM) at 37 °C for 1 h or with leptin (160 ng/ml, Sigma-Aldrich Corp. St. Louis, MO) at 37 °C for 24 h and then incubated with Bld-1-KLA at 37 °C for 1 h. After treatment, a fresh culture medium containing 2% FBS was added to all the wells. Cells were then incubated for an additional 24 h at 37 °C. Subsequently, 10 μl of Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) solution was added to each well. After 3 h, absorbance at 450 nm was measured using a microplate reader (BioRad, Hercules, CA).

For the analysis of apoptosis, cells (20,000/well in 4-well chamber slides) were incubated with peptides for 16 h at 37 °C. Subsequently, cells were stained with Alexa594-conjugated annexin V (Life Technologies, Carlsbad, CA) for 15 min at 24 °C, fixed with PFA, and stained with DAPI for nuclear counterstaining. The percentages of fluorescent (annexin V-bound) cells were quantified using MacBiophotonics Image J software.

2.5. Serum stability assays

Serum stability of peptides was evaluated as previously described [24]. Briefly, serum was retrieved from mouse blood and subjected to filtration through a 0.22 μm filter. PBS solution (50 μl) containing 100 μg of a peptide was incubated with an equivalent volume of filtered serum at 37 °C for the indicated time. Subsequently, samples were diluted and fractionated by C18 reverse-phase fast protein liquid chromatography (FPLC) with a linear gradient of acetonitrile. Each fraction