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Identification of a novel cell-penetrating peptide targeting human glioblastoma cell lines as a cancer-homing transporter

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ABSTRACT

Cell-penetrating peptides (CPPs) as a novel biomedical delivery system have been highly anticipated, since they can translocate across biological membranes and are capable of transporting their cargo inside live cells with minimal invasiveness. However, non-selective internalization in various cell types remains a challenge in the clinical application of CPPs, especially in cancer treatment. In this study, we attempted to identify novel cancer-homing CPPs to target glioblastoma multiforme (GBM), which is often refractory and resistant to treatment. We screened for CPPs showing affinity for the human GBM cell line, U87MG, from an mRNA display random peptide library. One of the candidate peptides which amino-acid sequence was obtained from the screening showed selective cell-penetrating activity in U87MG cells. Conjugation of the $p16^{INK4a}$ functional peptide to the GBM-selective CPP induced cellular apoptosis and reduced phosphorylated retinoblastoma protein levels. This indicates that the CPP was capable of delivering a therapeutic molecule into U87MG cells inducing apoptosis. These results suggest that the novel CPP identified in this study permeates with high affinity into GBM cells, revealing it to be a promising imaging and therapeutic tool in the treatment of glioblastoma.

1. Introduction

Glioblastoma multiforme (GBM, WHO grade IV astrocytoma) is the most common malignant brain tumor originating in the central nervous system in adults. Despite advances in surgical resection, chemotherapy, and radiotherapy combined with adjuvant therapy, the median survival in patients with GBM is generally less than 12 months after the time of diagnosis because of its rapid progression and invasive nature [1]. Thus, there is an urgent need for more effective therapeutic strategies for refractory GBM.

Recently, cell-penetrating peptides (CPPs), also referred to as protein transduction domains (PTDs), which have the ability to permeate across the plasma membrane and can facilitate the efficient cellular internalization of biomolecules, have attracted attention as peptide-based delivery systems [2,3]. To date, CPPs such as the human immunodeficiency virus type1 (HIV-1) transcriptional activator TAT protein [4], the Antennapedia (Antp) homeodomain of *Drosophila* [5], and poly-arginine ((Arg)*n*, *n* = 4–16) [6,7] have been the most widely studied with respect to enhancing the intracellular delivery of CPP-conjugated molecules. Since these peptides could efficiently deliver a variety of biological macromolecules, including proteins, peptides, DNAs, RNAs and nanoparticles into various living cells with minimal cytotoxicity, the use of CPPs as a delivery system to directly introduce biologically active molecules into cells has been expected [2,8,9]. However, from a clinical point of view, non-selective internalization of CPPs into various cells is the limiting factor for cell-type or tissue specific targeting applications such as cancer treatments [4,10]. Development of target-selective CPPs may contribute to improving therapeutic efficacy and reducing side effects on normal tissues [11,12]. Accordingly, the purpose of the present study was to identify novel CPPs targeting GBM as selective transporters.

mRNA displayed peptides comprise a genotype (mRNA/cDNA) template and phenotype (nascent protein) that is encoded by its mRNA, and are linked by a covalent bond through the puromycin linker [13]. The *in vitro* cell-free protein synthesis system boasts a diversity of approximately 10^{12}–10^{13} individual sequences, each

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containing 10 contiguous random amino acids that are encoded by a synthetic cDNA library (templates), which is greater than that of phage display technology ($<10^9$) [14]. The amino acid sequence of an mRNA displayed polypeptide can be identified easily by nucleic acid sequencing [15,16]. Thus, mRNA display technology provides a means of screening for useful physiologically active peptides and novel functional proteins. Here, we aimed to investigate novel CPPs with an affinity for the U87MG human GBM cell line using an mRNA display random peptide library *in vitro*. In this article, we present a novel CPP as a potential tool for GBM selective intracellular delivery.

2. Materials & methods

2.1. Peptide synthesis

All peptides in the present study were synthesized chemically by SIGMA–ALDRICH (Tokyo, Japan). Peptide purity was 90% or greater, which was confirmed by high-performance liquid chromatography analysis and mass spectroscopy. Peptides were dissolved in distilled water to generate 1 mM stock solutions.

2.2. Cell culture

The human glioblastoma (GBM) cell line U87MG used in the present study was purchased from the American Type Culture Collection (USA). The other cell lines used for *in vitro* assays are shown in Table 1. All human cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C with 5% CO_2. Primary cultured neurons were obtained from the hippocampus of 18-embryonic-day fetal C57BL6/J mice and maintained in neurobasal medium supplemented with 2% B-27 (Invitrogen), 1% penicillin/streptomycin, and 0.5 mM L-glutamine.

2.3. Fluorescence cellular imaging and quantitative analysis

Cells were seeded at a density of 3×10^5 cells per 35 mm glass bottom dish and incubated with 10 μM of FITC-labeled peptides in complete medium for 2 h at 37 °C. For fluorescence microscopy imaging, cells were washed twice with fresh medium, and cell fluorescence was immediately analyzed using confocal laser scanning microscopy (CLSM) (Olympus Tokyo Japan, FLUOVIEW FV-1000) without fixation. Fluorescence intensities at the region of interest (ROI) of 3 cells per microscopic image were measured by Meta Morph software Version 6 (Olympus), and experiments were conducted in triplicate. Background fluorescence intensity was subtracted from all experiments. For fluorescence-activated cells sorting (FACS) analysis, the cells were washed twice with phosphate-buffered saline (PBS) and collected by trypsinization. Detached cells were resuspended in FACS buffer (PBS, 2% FBS), then samples (1×10^4 cells) were immediately subjected to flow cytometric analysis (MILLIPORE Guava Easy Cyte Plus) using guava soft version2 (MILLIPORE) without fixation.

Table 1
Cell lines of histologically different origins, including human GBM, were used in the cell-penetration assay. Primary cultured mouse neurons were used as a non-neoplastic counterpart.

Cell line	Origin (histological type)
U87MG	Brain (glioblastoma)
U118MG	Brain (glioblastoma)
HeLa	Uterus (squamous cell carcinoma)
MSTO-211H	Lung (adenocarcinoma)
NCI-H226	Lung (adenocarcinoma)
A549	Lung (adenocarcinoma)
PANC-1	Pancreas (epithelioid carcinoma)
HepG2	Liver (hepatoblastoma)
Caco-2	Colon (adenocarcinoma)
HEK293	Non-neoplastic, embryonic kidney
Neuron	Brain (mouse hippocampal neuron)

2.4. RT-PCR

Total RNA was extracted with TRIzol (Invitrogen) from the human glioblastoma cell lines U87MG and U118MG, and HeLa cells. cDNA was synthesized from the RNA product using an oligo (dT) primer and cDNA synthesis kit (TAKARA) according to the manufacturer's instructions. Reverse transcription-PCR was performed with Ex-Taq polymerase (TAKARA) under the following amplification conditions: denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The sense/antisense primer sequences for human $p16^{INK4a}$ were 5′-TTCCTGGACACGCTGGTGGTG-3′ and5′-GGCATCTATGCGGG CATGGTTA-3′, respectively. Actin was used as internal standard gene.

2.5. Detection of apoptotic cells

U87MG cells were seeded at a density of 5×10^5 cells per 60 mm dish and incubated with 20 μM of peptide1NSΔ-p16 MIS or peptide1NSΔ-p16 V95E in complete medium for 4 h at 37 °C, respectively. After treatment, the cells were washed twice with PBS and collected by trypsinization. Then, the cells were resuspended in 100 μl of binding buffer (0.5 M HEPES pH 7.4, 1 M NaCl, 1 M KCl, 1 M $MgCl_2$, 0.2 M $CaCl_2$) containing 5 μl FITC-Annexin V (BD Pharmingen) and 5 μl Propidium iodide (PI) (SIGMA–ALDRICH), and incubated under darkness for 15 min according to the manufacturer's instructions. The cells were immediately subjected to flow cytometric analysis at 1×10^4 cells per sample.

2.6. Western blotting

U87MG cells were seeded at a density of 3×10^5 cells to 35 mm well plate and incubated with 20 μM of peptide1NSΔ-p16 MIS or peptide1NSΔ-p16 V95E in DMEM under a serum free condition for 24 h at 37 °C, respectively. After treatment, the cells were washed with complete medium and further incubated at 37 °C for 4 h. Then, the cells were lysed with 2× SDS sample buffer, and extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using an 8% SDS–PAGE gel and transferred onto a nitrocellulose membrane (BIO-RAD). After blocking with Blocking One (NACALAI TESQUE), the membrane was sequentially probed with the following antibodies: primary antibodies were rabbit polyclonal anti-Ser 807/811 phosphorylated pRB antibody 1:1000 (CST, Cell Signaling Technology), and anti-actin monoclonal antibody 1:3000 (Chemicon); secondary antibodies were anti-rabbit antibody 1:3000 (CST), and anti-mouse antibody 1:3000 (Millipore). After washing with Tris-buffered saline Tween solution (TBS-T), signals were detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare) and Versa Doc (BIO-RAD). Quantifications were carried out by densitometric analysis using Quantity One software (BIO-RAD).

2.7. Statistical analysis

Statistical significance was calculated using Statcel 3 software (OMS publishing Inc.). A student's *t*-test was used for data analysis and p value <0.05 was considered statistically significant. All values