Journal of Controlled Release 130 (2008) 140–145

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

A peptide-based carrier for intracellular delivery of proteins into malignant glial cells *in vitro*

Chunxiao Wu [a], Seong Loong Lo [a], Jerome Boulaire [a], Michelle Li Wen Hong [b], Hui Min Beh [b], Doreen Siu Yi Leung [a], Shu Wang [a,b,*]

[a] *Institute of Bioengineering and Nanotechnology, Singapore 138669, Singapore*
[b] *Department of Biological Sciences, National University of Singapore, Singapore 117543, Singapore*

ARTICLE INFO

Article history:
Received 30 November 2007
Accepted 11 May 2008
Available online 24 May 2008

Keywords:
Phage display
Peptide carrier
Glioma
Targeted protein delivery

ABSTRACT

Aiming at identification of novel peptides that can be employed for effective targeting of malignant gliomas, we used a 12-mer peptide phage display library and cultured human malignant glioma cells for phage selection. Several common phage clones emerged after 4 rounds of biopanning against the U87MG glioblastoma cell line. The most abundant phage clone VTW, expressing a sequence of VTWTPQAWFQWV, bound to U87MG cells 700-fold more efficiently than the original unselected library. The VTW phage also bound strongly to other human glioma cell lines, including H4, SW1088 and SW1783, but very weakly to normal human astrocytes and SV40-immortalized human astroglial cells. When compared to other non-glial tumor cells, the phage showed 400- to 1400-fold higher binding efficiency for U87MG cells. After linked to positively charged lysine peptides, the VTW peptide became water soluble and was able to deliver biologically active, hydrophilic beta-galactosidase into U87MG cells, with up to 90% of the cells being stained intensively blue. This peptide carrier did not show obvious protein delivery activities in the human astrocytes. Our results provide a proof of principle to the concept that peptides identified through phage display technology can be used to develop protein carriers that are capable of mediating intracellular delivery of hydrophilic macromolecules in a tumor cell-specific manner.

1. Introduction

Glioblastoma multiforme (GBM) is the most frequent malignant primary brain tumors in adults [1]. Current treatments typically use surgical excision, followed by radiotherapy and/or chemotherapy. Due to the infiltrative nature of GBM in the brain, complete removal of the tumors is almost impossible and most patients die within two years after diagnosis. Thus, there is certainly a need to improve the efficacy of therapies for this fatal disease.

Selective targeting of tumor cells has been explored to enhance the effectiveness of tumor therapy by eradicating tumor cells specifically, while limiting side effects in non-target cells. The surface molecules of tumor cells are commonly used as targets for tumor recognition. These molecules include fibroblast growth factor receptors (FGFR) and epidermal growth factor receptors (EGFR) that are over-expressed on the surface of tumor cells [2–4]. In addition to these well-known surface receptors, there are many other over-expressed molecules that are associated with tumor cell plasma membranes and can be used for tumor targeting. To screen, identify and select these molecules, phage display technology has been used [5]. This technology uses libraries of bacteriophages that display peptides or polypeptides on their surface. After incubation with target cells followed by washing, the phages that express sequences with a high affinity for specific cell surface molecules will be captured by target cells and selected through recovery. Typically, a phage display library is subjected to several rounds of biopanning against targeted cells to enrich the selected phages. This method does not require prior knowledge on the targeted surface molecules and is likely to identify valuable novel ligands for tumor targeting. Also, the method allows peptide or polypeptide libraries interact with living cells, thus to enrich for the macromolecules that recognize and bind to the native conformations of the targeted surface molecules. Many cancer specific peptides have been identified using this technology and successfully linked to anticancer drug for cancer treatment trials [5]. Although widely used, chemical conjugation of a targeting peptide to therapeutic cargos suffers from the possible limitation of altering the biological activity of the cargos, especially those peptide- or protein-based pharmatherapeutics. Many delivery systems have been developed to circumvent the problem and to maintain the activity and stability of delivered cargos [6]. Carrier systems that are able to carry therapeutic cargoes without the need of chemical cross-linking would be attractive [7].

Several glioblastoma targeting peptides with various specificity and selectivity have been identified by using phage display technique

* Corresponding author. Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos #04-01, Singapore 138669, Singapore. Tel.: +65 6824 7105; fax: +65 6478 9083.
E-mail address: swang@ibn.a-star.edu.sg (S. Wang).

0168-3659/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.jconrel.2008.05.015

[8–10]. Since glioblastomas are histologically and genetically heterogeneous, it is unlikely that one peptide can efficiently target all types of glioblastomas. With more malignant glioma-specific peptides available, highly efficient personalized treatment of this type of brain tumors will become possible. Moreover, while tumor-targeted gene delivery has been widely investigated, tumor-targeted protein delivery is less well explored. The objective of this work was to identify new peptides that can be used for glioma targeting. We are particularly interested in the possibility of using the identified peptides for targeted protein delivery. The commonly used human malignant U87MG glioblastoma cell line was employed for initial biopanning selection. Selected phages were investigated for their binding efficiency and specificity in different types of cells. A peptide derived from one of the selected phages that showed a particularly high binding efficiency for U87MG cells was used to design and develop peptide-based carriers for intracellular delivery of proteins.

2. Materials and methods

2.1. Cell lines

Human U87MG, H4, SVGp12, HepG2, and NTERA-2 cl.D1 cells from ATCC (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Human SW1088 and SW1783 (ATCC) were maintained in RPMI medium containing 10% FBS and antibiotics. Normal human astrocytes (NHA) were purchased from Cambrex Corporation (Walkersville, Maryland. USA) and maintained in astrocyte medium (AGM Basal Medium supplemented with SingleQuot) according to the recommendation of the supplier (Cambrex).

2.2. Phage biopanning

The Ph.D.-12 phage display peptide library was purchased from New England Biolabs (Beverly, MA, USA) and used for biopanning against human glioma U87MG cells. The library was diluted with DMEM containing 1 mg/ml BSA to a concentration of 2×10^{11} plaque formation units (pfu) per 3 ml and pre-incubated in a 60 mm tissue culture dish for 1 h at room temperature on a rocker to deplete plastic binding phages. This depletion step was repeated two more times using new empty dishes. For biopanning experiments, U87MG cells were seeded on a 60 mm tissue culture dish one day before experiments and reached 50% confluence on the day of biopanning. Cells were first incubated in serum-free DMEM for 1 h at 37 °C, followed by 1-hour incubation of DMEM containing 1 mg/ml bovine serum albumin (BSA). The phages collected from the above depletion experiments were then added and incubated with cells for 1 h at 37 °C. After seven washes with DMEM containing 1 mg/ml BSA and three washes with PBS, cells were lysed with 0.8 ml of 100 mM Triethlyamine for 8 min. The lysate was neutralized with 0.8 ml of 1 M Tris–HCl (pH 7.4). Recovered phages were tittered and amplified following the instruction manual provided by New England Biolabs. After totally four rounds of biopanning, phage clones were randomly picked out from the titer plates and sequenced.

The binding specificity of selected phages to different cell lines was evaluated by comparison to the original Ph.D.-12 library. The binding assays are similar to the biopanning procedure describe above, except that 1×10^{10} pfu of phages was used and depleted once in an empty dish. The specificity of each phage is defined by its selectivity ratio, i.e. the recovered phage titer of selected phage divided by the recovered phage titer of the Ph.D.-12 library.

2.3. Peptides and cell binding assay

Hydrophilic ten-mer lysine homopeptide (10K), 16-mer lysine peptide with a linker SIPVKFNKP (16K), lysine peptides with a targeting sequence, including VTWTPQAWFQWVGGGS-10K (10K-VTW), 16K-SIPVKFNKPVTWTPQAWFQWV (16K-VTW) and 10K-TWSPEAW, were prepared using a conventional solid-phase, chemical synthetic method (GL Biochem, Shanghai, China) and have the purity over 98%.

To assess the binding specificity to target cells, 10K-VTW peptides were labeled with Cy3 dye (GE Healthcare, Freiburg, Germany) following the manufacture's instruction. The final dye/peptide ratio was 0.4 after dialysis. The labeled peptides were mixed with plasmid DNA to neutralize the positive charges of the peptide before incubating with cells. U87MG and SVGp12 cells seeded on a 24-well plate were used for the assays. After 1-hour incubation with the labeled peptide/plasmid complexes at 37 °C, cells were washed twice with PBS, fixed with 4% paraformaldyde and observed under a fluorescent microscope. For a competition assay in U87MG cells, 10K peptides were linked to TWSPEAW, a peptide derived from the human interleukin 11 receptor alpha chain (IL-11RA) and used as a VTW homologue peptide for competition. 10K-VTW and 10K-TWSPEAW peptides were then used to form DNA complexes separately in two tubes as described above. The two types of the complexes were co-incubated with cells for 1 h before observation under a fluorescent microscope. For flow cytometry analysis, U87MG cells were dissociated into individual cells by trypsin treatment and re-suspended in serum-free DMEM. After 1-hour incubation with peptide/plasmid complexes at 37 °C, cells were washed with PBS and analyzed on a FACSCalibur flow cytometer (BDIS, San Jose, USA).

2.4. Protein delivery

A 119 kDa subunit of β-galactosidase from Active Motif (CA, USA) was used as a reporter protein. To prepare β-galactosidase/peptide complexes, 2 μg of peptide in 20 μl of PBS was added to 1 μg of β-galactosidase (in 20 μl of PBS), followed by 30-minute incubation. Human glioma cells U87MG and human immortalized astrocytes SVGp12 were grown to 50–70% confluency in 48-well plates. The cells were incubated with the complexes first in serum-free medium for 4 h, followed by serum-containing medium for 20 h. After the 24-hour incubation, the cells were washed extensively, fixed and stained using a β-galactosidase staining kit (Active Motif). The bright-field images were taken with inverted microscope (Olympus Singapore Pte Ltd, Singapore). For enzymatic analysis of protein delivery efficiency, the cells were treated with trypsin or PBS for 10 min at room temperature on orbital shaker 24 h after transfection, followed by addition of serum-containing medium to inactivate trypsin. The cells were pelleted, washed with PBS, and lysed in 50 μl of 1× Reporter Lysis Buffer (Promega, Singapore) for 5 min. The cell lysates were mixed with 50 μl of β-galactosidase enzyme assay 2× buffer (Promega, Singapore) in 96-well plate and incubated at 37 °C for 1 h. One hundred fifty microliter of 1 M sodium carbonate was added to stop the reaction. The absorbance at 420 nm (A_{420}) was then measured with microplate reader (Bio-rad, Singapore).

To assess intracellular delivery efficiency, 16K and 16K-VTW peptides were used to prepare DNA/peptide complexes. Testing cells were incubated with the freshly prepared complexes in the presence of 0.1 mM of chloroquine for 3 h at 37 °C. Cells were further incubated in normal serum-containing DMEM for 24 h. Luciferase activity assays were used to evaluate the expression levels of the reporter gene. For luciferase assays, cells were washed once with PBS and freeze-thawed in reporter cell lysis buffer (Pormega, Madison, WI, USA). Ten μl of lysate was used to measure luciferase activity on a luminometer (Berthold Lumat LB 9507, Germany). The relative light units (RLU) were normalized to the protein concentration of the cell lysate measured with a protein assay kit.