Phage Probes for Cancer Diagnostic and Treatment

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ABSTRACT

Phage particles carrying cell-specific peptides have the potential to serve as nanoprobes for profiling of cancer cell surfaces, allowing parallel development of diagnostics with companion therapeutics. Phage probes derived from a landscape phage display library were assayed for binding to human glioma (brain tumor) cell lines, each with a different morphology and originated from a different patient. These phage probes were bound to the cells in distinctive patterns that were individual for each cell line. Subsequently, we designed and tested anti-cancer peptides with two domains: glioma-binding (derived from phage display) and pro-apoptotic. The resulting bifunctional peptides showed preferential cytotoxicity toward glioma cells compared to normal brain astrocytes. A strategy that incorporates the use of phage nanoprobes for cell-surface molecular profiling of individual tumors followed by cytotoxic treatments formulated on the basis of tumor profiles is discussed.

Keywords: brain tumor, glioma, landscape phage display, bifunctional anti-cancer peptides, personalized treatment

1 INTRODUCTION

Phage display is a powerful discovery tool with a variety of applications including identification of peptides and proteins that specifically recognize and bind to cell-surface targets. The specificity offered by phage display-derived peptides is particularly important for development of anti-cancer treatments since selective cell recognition allows enhancement of drug efficiency and reduction of toxic side effects to normal cells. Cancer cell-specific peptides are used widely as delivery moieties for construction of gene therapy vectors, liposomes, or cytotoxic drugs [1]. Phage particles carrying such peptides, on the other hand, have the potential to serve as nanoprobes for profiling of cell surfaces of cancer specimens, providing an opportunity for development of diagnostics with corresponding anti-cancer therapeutics. Here, we tested phage probes and corresponding synthetic peptides derived from a landscape f8-1/8-mer phage display library [2] for different rat and human glioma cell lines. Due to the fact that landscape phage particles provide thousands of sites for phage-target binding, they are well positioned as very specific cell-recognizing nanoprobes.

2 MATERIALS AND METHODS

2.1 Cells

All cell lines were purchased from American Type Culture Collection (Manassas, VA) and cultured as recommended. Rat cell lines: RG2 (CRL-2433) is a rat cell line isolated from brain tumors induced by a single dose of N-ethyl-N-nitrosourea. The biological characteristics of this tumor closely resemble those of human glioblastoma. CTX TNA2 (CRL-2006) was established from primary cultures of type I astrocytes taken from brain frontal cortex tissue of one-day-old rats. Cells were immortalized with SV40 and are non-tumorigenic in immunosuppressed mice. Human cell lines; Hs 683 (HTB-138) is a human glioma cell line isolated from explant cultures of a glioma taken from the left temporal lobe of a 76 year-old man. A 172 (CRL-1620) is a human glioblastoma cell line isolated from a 53 year-old man. M059K (CRL-2365) is a human malignant glioblastoma cell line isolated from a brain tumor of a 33 year-old man. SW 1088 (HTB-12) is an astrocytoma cell line taken from a 72 year-old man and is hypertriploid.

2.2 Peptides and phage clones

Peptides were synthesized using Fmoc chemistry and purified (95%) by Biomer Technology (Concord, CA). The peptides were produced with amidated (-$CONH_2$) C-terminals for increased peptide stability and quality. Peptide solutions for cell treatments were prepared in Dulbecco's modified Eagle's medium at the concentrations shown in Figure 2. Individual phage clones carrying glioma-targeting peptides (sequences shown in Figure 1) were amplified in bacteria and purified as previously described [3].

2.3 Cell-binding assay

A cell-binding assay was used to evaluate the binding of glioma-specific phage clones to different human glioma cell lines. Briefly, individual phage clones (~ 10^9cfu/25 cm^2 flask) were incubated with the cells for 1 h at room temperature, followed by removal of unbound phage and measurement of cell-associated phage via titering in bacteria [3].

2.4 MTT assay

RG2 glioma cells or CTX TNA2 astrocytes were plated into 96-well plates at a density of 3000 cells/well and incubated for 24 h. Media were removed and replaced with 90 μl of respective media without fetal calf serum for each cell line and 10 μl aliquots of peptide treatments (concentrations are shown in Figure 2) were added for a final volume of 100 μl. The well-characterized cytotoxic compound staurosporine at 1 μM concentration was used as a positive control (OD_{550} ~ 0.1). Negative control was no treatment. Cells were returned to the incubator for 24 h and then tested using an MTT assay. Briefly, 20 μl (5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in PBS per well was added to all wells and then the plate returned to the incubator for 4.5 h. Media were removed from the plates and 150 μl per well of dimethyl sulfoxide added and plates shaken for 30 min. Absorbance was read at 550 nm.

3 RESULTS

To evaluate the ability of phage particles to serve as nanoprobes for cell-surface profiling, phage clones displaying glioma-selective peptides identified for RG2 rat glioma cells in our previous study [4] were assayed for binding to four human glioma cell lines, each with a different morphology and derived from a different patient. It was shown that four tested phage clones were bound to the cells in distinctive patterns that were individual for each cell line (Figure 1). For example, SW 1088 and Hs 683 gliomas bind VGLPEHTQ phage at high numbers (92.7% and 239.8%, respectively). At the same time, these cell lines demonstrate very poor binding with all phage clones containing $D^S/M/_LTK$ consensus sequence. In contrast, $D^S/M/_LTK$-containing phage clones showed appreciable (with the average around 30%) binding to two other human gliomas, M059K and A 172, while VGLPEHTQ phage bound the cells at very low numbers (4.0 and 0.1 % respectively).

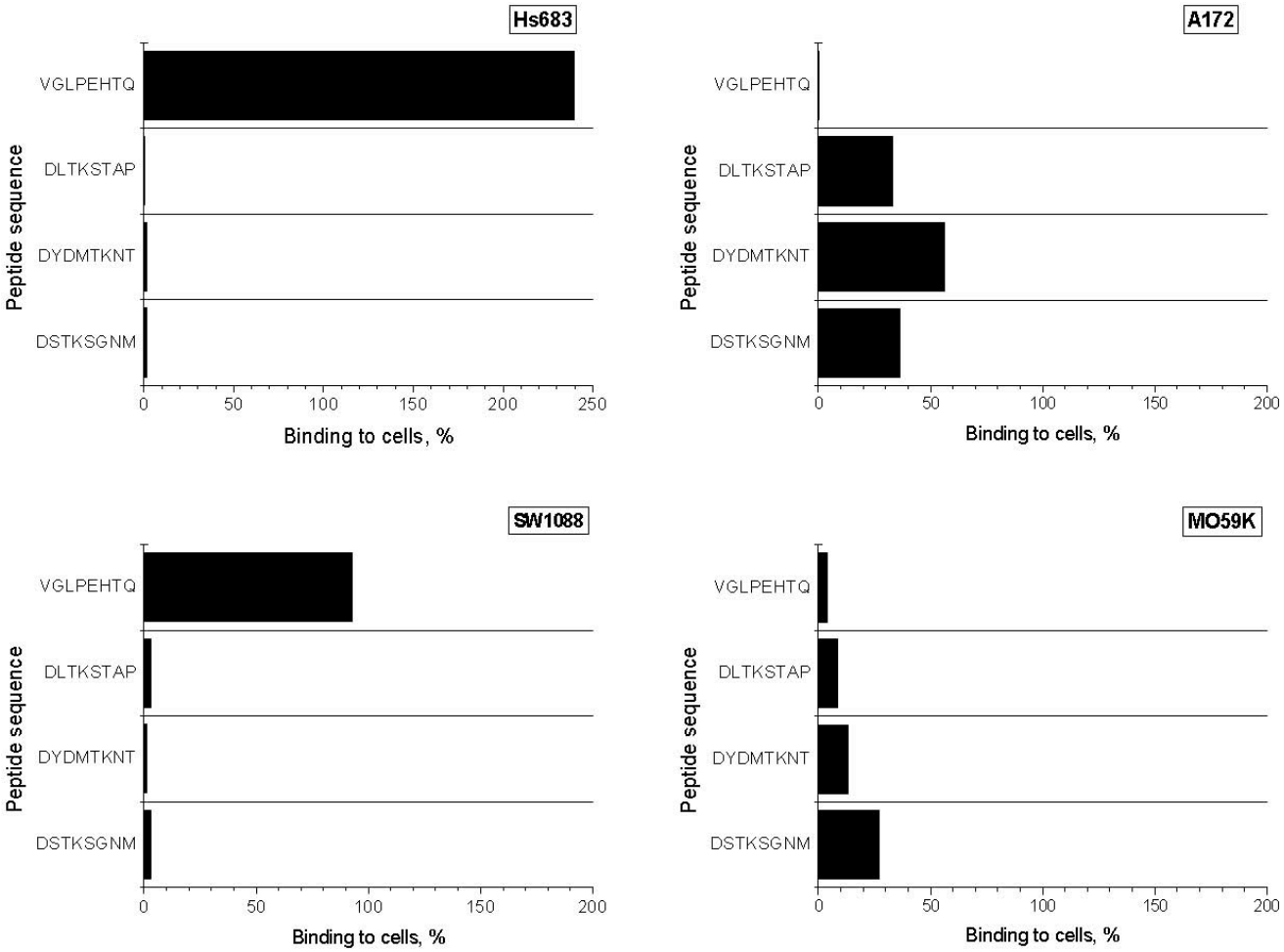

Figure 1: Binding patterns of human glioma cell lines Hs 683, SW 1088, A 172, and MO59K with phage probes selected for RG2 cells. Sequences of peptides displayed on phage probes are shown along Y axis. Phage titers are presented as relative binding (%), assuming that binding to RG2 cells is equal to 100%.

Subsequently, we designed and tested anti-cancer peptides with two domains: (1) glioma-targeting DSTKSGNM or DSTK to perform selective delivery to cancer cells and (2) pro-apoptotic d(KLAKLAK)$_2$ to perform the cell killing function. Two targeting sequences originated from the DSTKSGNM phage clone. One of them is an entire DSTKSGNM peptide expressed by the phage, while the other is a truncated version of this sequence, DSTK, which represents a consensus glioma cell-binding motif. The DSTK peptide was used in cyclic form (C-DSTK-C) for structural stability. A cationic peptide d(KLAKLAK)$_2$ that is known as a potent inducer of apoptosis [5] was used as a cytotoxic functional component of the combined molecules. The choice of the cationic peptide was based on studies showing its potential therapeutic applicability [6-9]. This peptide has no hemolytic activity, preserves its function in serum (stable to enzymatic degradation and does not bind to serum components), and does not cause immunological responses. Two glycine amino acids act as a spacer between the targeting and d(KLAKLAK)$_2$ domains.

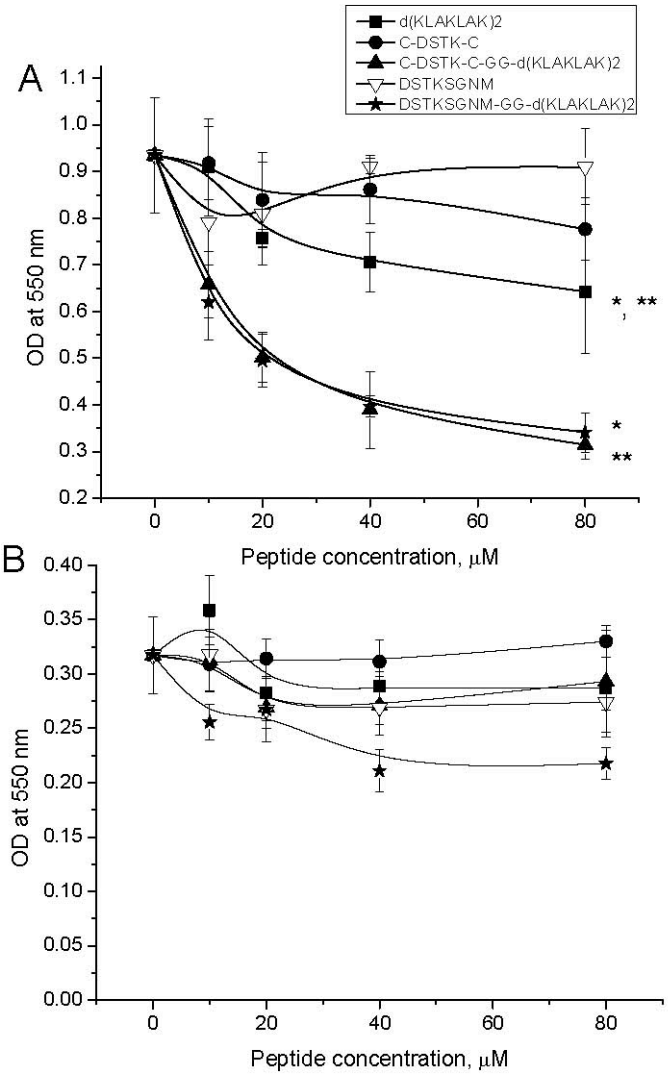

Figure 2: Metabolic activity of normal and cancer cells. RG2 glioma cells (A) and CTX TNA2 astrocytes (B) were treated with different concentrations of anti-glioma peptides as well as control peptides. The metabolic activity of the cells was evaluated 24 h later using the MTT assay. The OD value at zero peptide concentration corresponds to untreated controls. Negative result of bifunctional peptides (DSTKSGNM-GG-d(KLAKLAK)$_2$ or C-DSTK-C-GG-d(KLAKLAK)$_2$) significantly exceeded the effects of the cationic peptide alone (d(KLAKLAK)$_2$). * P=0.005, ** P=0.003.

Since internalization is needed for the dual peptide molecule to be cytotoxic, DSTKSGNM phage clone was tested, prior to peptide cytotoxicity studies, for the ability to be internalized by glioma cells. To determine this, the phage preparation was reacted with RG2 glioma cells. Following the reaction, the cells were fixed and treated with saponin to make cell membranes permeable to anti-phage antibodies to allow staining of phage within the cells. Cells treated with saponin demonstrated bright staining with anti-M13 phage antibodies, indicating intracellular localization of the phage (not shown). The control RG2 cells, without saponin treatment, showed light staining corresponding to the phage bound to cell surfaces. Non-targeting control (no insert library vector) phage did not exhibit any staining. Thus, DSTKSGNM peptide facilitates phage internalization into RG2 rat glioma cells.

To test cytotoxic properties of the bifunctional peptides, RG2 rat glioma cells and normal rat CTX TNA2 astrocytes were treated with increasing peptide concentrations. The metabolic activity of the treated cells as well as control cells (no treatment) was evaluated after 24 h using MTT assay. Figure 2 represents the curves of cellular metabolic responses to the treatments versus peptide concentration. d(KLAKLAK)$_2$ cell-killing peptide alone was not toxic to CTX TNA2 cells at the concentration of 10 μM and demonstrated some insignificant toxicity at higher concentrations including 80 μM. The addition of this peptide resulted in suppression of oxidative metabolism of RG2 glioma cells down to approximately 70% of untreated controls at the highest concentration tested of 80 μM. The effect of d(KLAKLAK)$_2$ on glioma cells was increased considerably by adding to this peptide a glioma-targeting domain, either DSTKSGNM or DSTK. A significant reduction in cellular metabolism to approximately 35% occurred for concentrations of 40 and 80 μM. In contrast, at similar concentrations, C-DSTK-C-GG-d(KLAKLAK)$_2$ was not toxic to normal rat astrocytes. DSTKSGNM-GG-d(KLAKLAK)$_2$ peptide resulted in some toxicity to these cells, but at a lower level than that seen with glioma cells. RG2 glioma cells as well as normal astrocytes were essentially unaffected by targeting peptides alone.

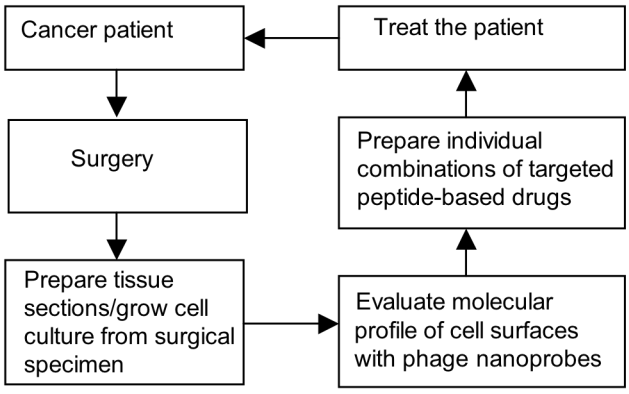

Figure 3: A strategy for development of personalized anti-cancer treatments that matches peptide drugs to the molecular profiles of individual tumors.

Since gliomas are very heterogenous as are many other tumors, knowledge of cell-surface molecular patterns for each patient is an absolute requirement for development of successful targeted anti-cancer therapies with minimal toxic side effects. A strategy that incorporates the use of phage nanoprobes for cell-surface molecular profiling of individual tumors followed by cytotoxic treatments formulated on the basis of tumor profiles is shown in Figure 3. Here, phage that carry cell-targeting peptides are identified using surgical specimens/biopsies from multiple cancer patients (steps for one patient only are shown in the figure) and placed into a bank of phage probes. In parallel, a bank of bifunctional (targeting+cytotoxic) peptide drugs is created. Phage probes are used to establish molecular profiles of individual tumors. Based on molecular profiles, a patient-specific combination of drugs from the bank of peptide drugs is prepared and the patient is treated. This strategy has potential for the development of effective cancer cell-killing agents for each individual patient, with greatly diminished negative results on normal cells.

4 FUTURE DIRECTIONS

Through the development of specific cell surface-binding molecules, phage display can offer innovative solutions for cancer diagnostics and treatment. Such future uses of phage display include: (1) development of phage nanoprobes for profiling of tumors in individual patients followed by personalized treatments based on individual tumor profiles, (2) identification of peptides for cancer stem cell targeting and affinity isolation of corresponding cell-specific biomarkers for anti-cancer immunotherapy, and (3) identification of peptides that cross blood-brain barrier for delivery of clinically significant doses of anti-cancer treatments to malignant brain cells. The latest is unique for brain malignancies that are the focus of our research.

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