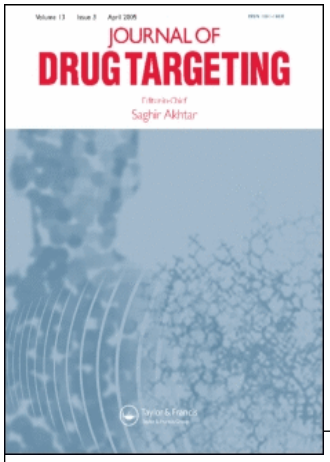

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Identification of oligopeptide binding to colon cancer cells separated from patients using laser capture microdissection

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Abstract

The development of intravascular conjugates that efficiently deliver genes or drugs to tumors is limited by the lack of efficacious targeting ligands. Small targeting peptides, such as those iterated by phage display technology, offer enormous potential for these applications. The majority of reports published to date have focused on the identification of peptides isolated for their ability to bind to human cancer cell lines *in vitro*, and have failed to account for the loss of polarization and de-differentiation of such cells from their *in vivo* state. Here, we report a novel approach for the identification of peptides capable of binding specifically to cancer cells derived from clinically resected human colon cancer. In this strategy, laser capture microdissection (LCM) is performed on a surgically resected colon cancer specimen to separate only cancer cells from the specimen. Subsequently, biopanning was performed on the LCM-selected colon cancer cells to identify peptide sequences that bound specifically to them. A peptide containing the SPT motif was selected as the most promising consensus sequence binding specifically to the LCM-selected colon cancer cells. Phage clones displaying the SPT motif demonstrated 9-fold higher binding to colon cancer cells derived from a patient than insertless phage ($p < 0.05$), while, recovery of the SPT phage from the colon cancer cell lines DLD-1 and HCT-15 was 7-fold higher than that of the control insertless phage ($p < 0.05$). The binding of SPT phage to colon cancer cells from the patient was confirmed by immunofluorescence. Additionally, a synthesized SPT-containing peptide (SPTKSNS) showed binding activity in the absence of mitogenic effects on colon cancer cells *in vitro*. In summary, we have introduced LCM into a biopanning procedure and identified a small peptide that binds preferentially to colon cancer cells derived from a clinically resected sample. This procedure could be applicable for the design of customized cancer cell targeting methodologies using clinical biopsy samples from human subjects.

Keywords: *Colon cancer, phage display, biopanning, tumor targeting, peptide, laser capture microdissection*

Introduction

Colon cancer is one of the most common malignancies worldwide (Kerr et al. 2003), accounting for approximately 530,000 deaths annually (Parkin et al. 2005). Surgery remains the mainstay of treatment for colon cancer and currently offers the only chance of cure. Colon cancer is not uniformly fatal, although the survival rate varies according to the stage of disease. Fatality occurs once the disease becomes recurrent or has metastasized, and a major obstacle to the establishment of effective therapies for multiple metastatic colon cancer is the countless localities of the metastasized tumor deposits, including those not

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visible to the naked eye. Therefore, establishment of a methodology that can selectively target individual and distant metastatic tumors via the bloodstream would facilitate a dramatic improvement in the therapeutic efficacy of treatments for multiple metastatic colon cancer.

Candidate targeting agents have been studied by several groups attempting to confer tumor tropism. Numerous ligands have been evaluated in this context, including a large number of antibodies, both fragments and single-chain Fv molecules (Kashentseva et al. 2002), and growth factors such as fibroblast growth factor 2 (FGF) (Fisher et al. 2001) and vascular endothelial growth factor (VEGF) (Backer and Backer 2001). However, this empirical approach to the identification of targeting ligands has recently been largely superseded by the use of library-based screening systems (biopanning) that have been designed to allow iterative selection of high-affinity ligands by repeated screening and enrichment of living libraries.

The biopanning technique using phage peptide display libraries has been shown to be a powerful tool for identifying specific ligands on target organs and tumors (Maruta et al. 2002; Akita et al. 2006; Shimizu et al. 2007). This technique has usually been performed for purified cell surface markers (Pan et al. 2003; Aggarwal et al. 2006), cultured cell lines (Kelly and Jones 2003; Lee et al. 2004), or tumor-bearing animals (Arap et al. 1998; Newton et al. 2006). However, to identify peptides that bind specifically to cancer cells derived from human subjects, tissue sections from patients would make an ideal selection target. However, because of the associated ethical difficulties and potential risks to the patient, it is not easy to perform reiterative biopanning procedures in human subjects. Accordingly, we have developed human *ex vivo* biopanning methodologies (Maruta et al. 2003; Maruta et al. 2007). These strategies involve direct injection of phage libraries into freshly resected specimens of human colon cancer via a feeding artery, and the phages that become associated with the tumor are subsequently reclaimed from isolated samples. However, it is unclear whether the selected phages have actually bound to cancer cells using this methodology, since tumor tissue is an extremely heterogeneous mixture of multiple cell types including cancer cells, stromal cells, endothelial cells, and inflammatory cells. Thus, the problem of cellular heterogeneity has been a significant barrier to the use of biopanning selection for patient samples.

In the present study, we employed laser capture microdissection (LCM) to select only cancer cells as a binding target, thereby eliminating the possibility of iterating peptide binding to "off-target" cell populations such as non-neoplastic cells and stromal elements. The advent of LCM has allowed small clusters of homogeneous cells to be isolated and removed from the tissue sections under direct microscopic visualization. Using this technique in combination with phage display technology, it is possible to identify a panel of cancer cell-specific peptides that are more likely to target clinically relevant receptors.

Material and methods

Patients contributing to this study

Patients undergoing colectomy for advanced colon cancer at the Shinshu University Hospital were recruited into this study between October 2005 and April 2006. The details of the patients involved are shown in Table I. Approval was obtained from the Shinshu University ethics committee before the study, and written informed consent was obtained from all patients for the use of their tissue specimens.

Cell lines and cell culture

The cell lines, DLD-1, HCT-15, Colo205, Lovo (human colorectal carcinoma), AZ521 (human gastric carcinoma), Panc-1 (human pancreas carcinoma), Huh-7 (human hepatocellular carcinoma), RBE (human cholangiocarcinoma), and HeLa (human cervical carcinoma), were obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Cells were maintained in RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 units per ml penicillin, and 100 mg per ml streptomycin (Sigma) at 37°C in 5% CO_2. The cells were passaged and expanded by trypsinization of the cell monolayers followed by replating every 4 days.

Table I. Summary of the patients contributing to this study.

			Tumor							
Patient	Age	Sex	Location	Histopathology*	Size (cm)	Operation	*T*	*N*	*M*	Stage
C1	83	M	Sigmoid colon	Moderately	4 × 4	Sigmoidectomy	pT4	pN1	pM0	IIIB
C2	45	M	Sigmoid colon	Well	3 × 3	Sigmoidectomy, hepatectomy	pT4	pN1	pM1	IV
C3	79	F	Sigmoid colon	Well	6 × 4	Sigmoidectomy	pT3	pN1	pM0	IIA

* "Moderately" and "well" denote moderately differentiated adenocarcinoma and well-differentiated adenocarcinoma, respectively.

Biopanning system using cancer cells derived from surgically resected colon specimens

We employed the LCM system to select only cancer cells from surgically resected colon specimens for the biopanning procedure. Samples of tumor and non-cancerous colon tissue were obtained at the time of colectomy from patients with operable colon cancer. The sample from the tumor tissue was mounted in Tissue-Tek OCT compound (Sakura Finechemicals, Tokyo, Japan) and frozen, and then sectioned at 10 μm in a cryostat (Leica CM3050; Leica Microsystems, Tokyo, Japan) at −20°C and placed onto prepared membrane slides (PEN membrane covered; PALM Microlaser Technologies GmbH, Bernried, Germany). The parts containing areas of cancer cells were identified using hematoxylin–eosin (HE) staining and then microdissected using a microlaser system (PALM-MB; PALM Microlaser Technologies GmbH). Approximately, 3000 cancer cells were captured from one step of LCM, and a total of 15 LCM steps were performed, yielding approximately 45,000 cancer cells for one round of biopanning.

The PhD-C7C M13 heptapeptide phage display library (New England Biolabs, Beverly, MA, USA) was used for biopanning. Initially, a subtraction step using non-cancerous colon tissue was performed. Samples from non-cancerous tissue were weighed and homogenized using a motor-driven Teflon-on-glass homogenizer (Digital homogenizer; Iuchi, Osaka, Japan). Then, these homogenized samples were washed four times with phosphate-buffered saline (PBS; Dainippon Sumitomo Pharma Co. Osaka, Japan), and finally, a 200-mg sample of non-cancerous tissue was prepared in 5 ml PBS. The phage library of 5×10^{10} plaque forming units (PFU) per 200-mg sample was added, and incubated for 1 h at 37°C with slow shaking. The samples were pelleted in a centrifuge at 2000 rpm for 5 min (GS-15R; Beckman, Palo Alto, CA, USA) and unbound phage was added to the LCM-selected cancer cells followed by incubation for 1 h at 37°C with slow shaking. The samples were washed two times with PBS, 5 ml of 0.2 M glycine–HCl was added as a general buffer for non-specific disruption of binding interactions for 3 min, and fluids were neutralized with 750 μl of 1 M Tris–HCl. After washing with PBS, 3 ml of PBS containing 0.5% Tween 20 (polyoxyethylene (20) sorbitan monolaurate; Kanto Chemical, Tokyo, Japan) was added to the cells. Eluted phages were quantified by titering on X-gal (5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside; Wako, Osaka, Japan) and IPTG (isopropyl-β-D-thiogalactopyranoside; Wako) agar plates containing *Escherichia coli* strain ER2738. Remaining phages were amplified by the addition to a 20-ml early log phase culture of ER2738 for 5 h at 37°C with vigorous shaking (150 rpm). Amplified phages were isolated from the resulting culture using the manufacturer's recommended protocol, concentrated, titered, and used for subsequent rounds of biopanning. In total, three rounds of biopanning were performed.

Isolation and sequencing of phage DNA

After each round of biopanning, individual phage clones were isolated and their total DNA was isolated according to the recommended protocol of the sequencing kit manufacturer (Applied Biosystems, Perkin–Elmer, Foster City, CA, USA). The resulting DNA was used for sequencing analysis using the −96 primer together with a BigDye terminator cycle sequencing kit (Amersham Biosciences, Buckinghamshire, UK). The DNA sequences were determined using an automated ABI PRISM sequencer 3100 Genetic Analyzer (Applied Biosystems). Next, searches for human proteins mimicked by the selected peptide motifs were carried out using online databases through the National Center for Biotechnology Information (NCBI; http:www.ncbi.nlm.nih.gov/BLAST/).

Evaluation of the binding activity of the selected phage to cancer cells derived from surgically resected specimen

To evaluate the ability of selected phage clones to bind to cancer cells, either the selected or insertless (a phage clone displaying no peptide insert) phage was added to colon cancer cells separated from patient C1 by LCM at 5×10^8 PFU. Recovery was quantified by titering multiple dilutions of the homogenate, as described above. In addition, binding of the selected phage clone was assessed in the laser capture microdissected colon cancer cells derived from other patients (C2–3, Table I). Recovery of the selected phage was expressed as a ratio relative to that of the insertless phage from cancer cells in each patient. A phage clone containing an irrelevant heptapeptide was used as a control.

Evaluation of the selected phage clones for binding to human cancer cells in vitro

The human cancer cell lines (DLD-1, HCT-15, Colo205, Lovo, AZ521, Panc-1, Huh-7, RBE, and HeLa) were grown to confluence in 6-well plates, acclimatized to 4°C for 30 min, and washed two times briefly with PBS containing 1% bovine serum albumin (BSA; Sigma). Then, the selected phage clones diluted in 1 ml of RPMI containing 1% BSA at a concentration of 5×10^8 PFU was added to each well at 4°C. After 1-h incubation with gentle agitation, medium containing unbound phage was discarded, and the cells were washed four times with PBS containing 1% BSA. The phage binding to the cells was then evaluated by plaque infection assay, as described above.

Immunofluorescence analysis of binding of the selected phage

The binding activity of the selected phage clone on surgically resected specimens (patient C1) was evaluated by immunofluorescence analysis. A sample of the tumor tissue was mounted on optimal cutting temperature (OCT) compound and frozen, and sectioned at 10 μm in a cryostat at −20°C. After washing two times with PBS, the sectioned sample was incubated with 2×10^{10} PFU of the selected phage clone or control phage (insertless) for 30 min. The sample was then washed four times with PBS. This was followed by the addition of 100 μl of 0.2 M glycine–HCl (pH 2.2) as a general buffer for non-specific disruption of binding interactions for 5 min, and fluids were neutralized with 15 μl of 1 M Tris–HCl (pH 9.1). The sample was then washed with PBS and fixed in ethanol for 3 min. After washing the slides with distilled water, endogenous peroxidase was blocked by incubating the slides in methanol containing 0.3% H_2O_2 for 30 min. The slides were washed two times (10 min each time) and then incubated with Tris-buffered saline (TBS) containing 1% BSA. They were then incubated with rabbit anti-M13 monoclonal antibody (Sigma) diluted 1:600 in blocking buffer for 1 h at room temperature, washed three times (5 min each time) with TBS, and incubated with Alexa 555-conjugated goat anti-rabbit immunoglobulin (DAKO, Carpinteria, CA, USA) diluted 1:400 in blocking buffer for 1 h at room temperature. The slides were then washed three times (5 min each time) with TBS. 4′,6-Diamino-2-phenylindole (DAPI, Sigma) was used as a nuclear counterstain and visualized using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan).

Similarly, the binding activity of the selected phage clone on human colon cancer cell lines (DLD-1, HCT-15) and non-colon cancer cell HeLa was evaluated by immunofluorescence analysis. DLD-1, HCT-15, and HeLa cells were grown to confluence in 8-well chamber slides in RPMI containing 10% FBS. The cells were washed with PBS containing 1% BSA and incubated for 1 h at 4°C in RPMI (containing 1% BSA) containing 1×10^{10} PFU of the selected phage clone. The medium was discarded, and the cells were washed four times with PBS containing 1% BSA, fixed with methanol–acetone (−20°C), and blocked with 10% goat serum for 20 min at room temperature. Immunofluorescence analysis was then performed as described above.

Flow cytometry analysis of binding of the selected phage

The binding activity of the selected phage clone on DLD-1 and HCT-15 cells was evaluated by flow cytometry. DLD-1 and HCT-15 cells were grown to confluence in 6-well chamber slides in RPMI containing 10% FBS. The cells were washed with PBS containing 1% BSA, and incubated for 2 h at 4°C in RPMI (containing 1% BSA) containing 5×10^{11} PFU of the selected phage clone. The medium was discarded, and the cells were washed with PBS containing 1% BSA, fixed for 5 min in 1% formaldehyde, and permeabilized for 30 min in 90% methanol. They were then incubated with rabbit anti-M13 monoclonal antibody (Sigma) diluted 1:1000 in blocking buffer for 1 h at room temperature, washed, and incubated with Alexa 488-conjugated anti-rabbit immunoglobulin (DAKO) diluted 1:500 in blocking buffer for 1 h at room temperature. They were then washed two times and suspended in 500 μl PBS. Flow cytometry was performed using a FACScalibur (Becton Dickinson and company, New Jersey, NJ, USA).

Competitive inhibitory effects of the synthesized peptide on phage accumulation in vitro

To confirm the capacity of the synthesized selected peptide (SPTKSNS) to bind to colon cancer cells, its inhibitory effects on phage association were examined. DLD-1 cells were preincubated with the SPTKSNS peptide (synthesized by Cosmo Bio, Tokyo, Japan) or control peptide at 1 or 10 μM for 60 min at 4°C, and then 5×10^8 PFU of the selected phage diluted in 1 ml RPMI containing 1% BSA was added. The phage was allowed to bind to the cells for 1 h at 4°C with gentle agitation. Medium containing unbound phage was discarded, and the cells were then washed four times for 5 min each time with PBS containing 1% BSA, before the cell-associated phages were recovered by lysing the cells in 1 ml per well of 30 mM Tris–HCl (pH 8.0) containing 10 mM (ethylenediaminetetraacetic acid; Gibco BRL, Grand Island, NY, USA) on ice for 1 h. The number of phages recovered was determined by titering multiple dilutions of the eluted phage as described above.

Evaluation of the mitogenicity of the SPTKSNS peptide in DLD-1 cells

DLD-1 cells were plated onto 96-well plates at 5×10^3 cells per well and incubated at 37°C in RPMI medium containing 10% FBS in either the presence or absence of the SPTKSNS peptide or control peptide at 10 or 100 μM. After 24, 48, 72, and 96 h, the viability of the DLD-1 cells was assessed using the MTS assay, as described previously (Shimizu et al. 2007). The medium was replaced with 120 μl of FBS-free RPMI containing 20 μl of CellTiter®96 AQueous One solution reagent (Promega, Madison, WI, USA), and the culture plates were incubated at 37°C for 2 h. Next, 100 μl of the medium was transferred to a new 96-well plate and the quantity of the formazan product present was determined by measuring the absorbance

at 490 nm using a microplate autoreader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Results are presented as the mean ± SD, and significance of differences was evaluated using Student's *t*-test. The level of significance was set at $p < 0.05$.

Results

Isolation of specific peptide binding to cancer cells derived from surgically resected specimens

Three consecutive rounds of *in vitro* biopanning were performed on the LCM-selected cancer cells using surgically resected specimens derived from patients with colon cancer. After three rounds of selection, 4.1 times more phages were recovered from the sample than when using the naive phage library. In addition, the phage recovery rate after the subtraction step was apparently increased (41 and 80% in the first and third rounds, respectively). After the three rounds of biopanning, 37 phage plaques were isolated, and their DNA was sequenced. We then compared the relative frequencies of each tripeptide motif, and sequencing analysis revealed some homology among different peptide sequences (Figure 1). The sequences containing the PTX and SPX motifs appeared in 27 and 22% of 37 samples, respectively (where X represents any amino acid), and SPT was found in 8%. Additionally, the prevalence of the PTX, SPX, and SPT motifs was markedly higher in the final round of biopanning than in the first two rounds (Figure 1). Therefore, peptides containing the SPT motif (SPTKSNS, 17p; QHASPTN, 5p) were selected as the most promising consensus sequences and studied in greater depth.

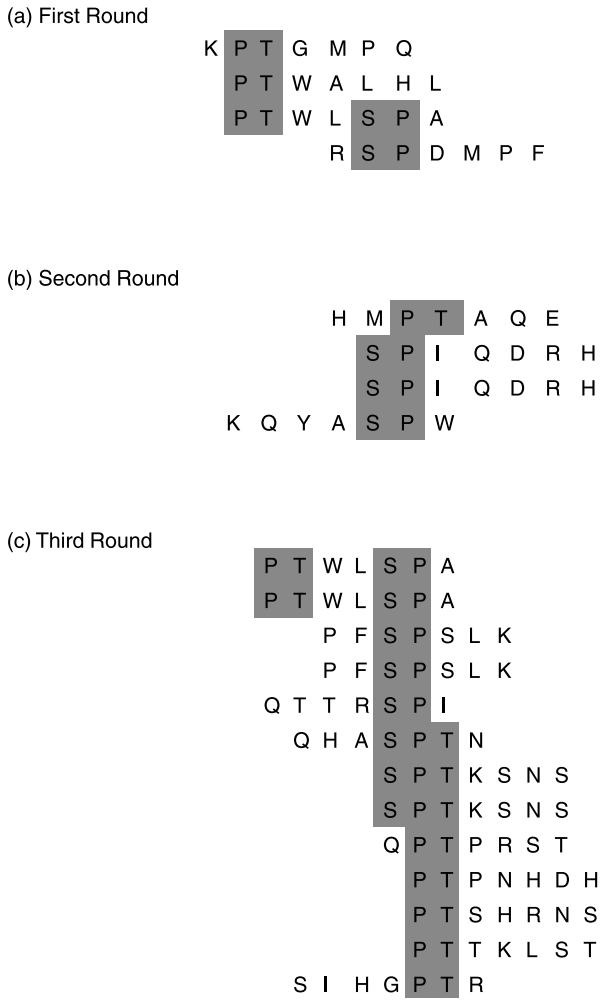

Figure 1. Iteration of consensus oligopeptides capable of binding to tumor tissue after panning rounds 1–3. Sequences deduced from tightly bound phage isolated after the three rounds of biopanning. Only the consensus sequences SPX and PTX are shown (a) after the first round (from 20 samples) and (b) after the second (from 20 samples) and third rounds (from 37 samples).

Binding of the selected phage to cancer cells derived from patients

To assess the tumor-binding activity of phage clones expressing the candidate oligopeptides, homogeneous phage solutions were prepared and incubated with the LCM-selected colon cancer cells from patients. The phage clone expressing SPTKSNS (17p) or QHASPTN (5p) bounds preferentially to cancer cells, showing 9.6 or 8.5 times greater binding relative to that of the insertless phage in patient C1, respectively (Figure 2).

In addition, these SPT motif-containing phage showed significantly more binding to cancer cells than control phage (a phage clone displaying an irrelevant heptapeptide) (Figure 2). By contrast, SPT phage did not show any significant binding activity in patients C2 and C3 (Figure 2).

Binding of the selected phage to human cancer cell lines

The binding of the SPTKSNS phage clones (17p) to confluent cultures of DLD-1, HCT-15, Colo205, Lovo, AZ521, Panc-1, Huh-7, RBE, and HeLa cells was evaluated in comparison with the binding of the insertless phage to each cell line. Recovery of the SPTKSNS phage from DLD-1 and HCT-15 was seven times higher than that of the insertless phage (Figure 3). On the other hand, there was no significant difference between the recoveries of SPTKSNS phage and insertless phage from non-colon caner cell lines.

Immunofluorescence and flow cytometric analysis of phage clone binding to colon cancer

The ability of the phage clone expressing SPTKSNS (17p) to bind to colon cancer was assessed using

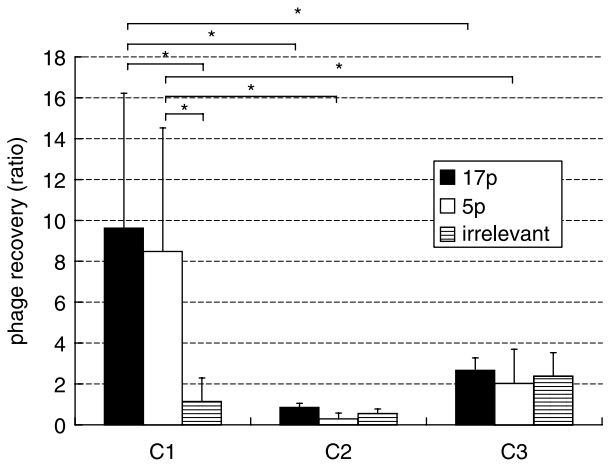

Figure 2. Evaluation of the binding activity of the selected phage to cancer cells derived from surgically resected specimens. Either the selected (17p/5p) or insertless (a phage clone displaying no peptide insert) phage was added to colon cancer cells separated from patients (C1–C3) by LCM at 5×10^8 PFU. Phage binding activities were evaluated by plaque infection assay. Recovery of the phage was expressed as a ratio relative to that of the insertless phage from cancer cells in each patient. A phage clone containing an irrelevant heptapeptide was used as a control. Values represent mean ± SD ($n = 3$), $\star p < 0.05$.

immunofluorescence. The results indicated that SPTKSNS phage particles bound preferentially to cancer cells, compared to control phage (Figure 4b,c). In addition, SPTKSNS phage showed little binding to normal cells (Figure 4e). In addition, SPTKSNS phage showed apparently stronger binding activity to the DLD-1 and HCT-15 colon cancer cell lines than the control phage (Figure 4f–i), but not to non-colon cancer (HeLa; Figure 4j,k). The increased levels of cell association of SPTKSNS phage was confirmed by flow cytometry and demonstrated a significant increase in the fluorescence intensity of DLD-1 and HCT-15 colon cancer cells incubated with SPTKSNS phage in comparison with control phage (Figure 4l,m).

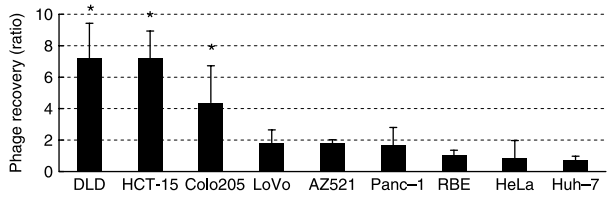

Figure 3. Evaluation of binding of the phage clone expressing SPTKSNS to cultures of non-colon cancer cells (AZ521, Panc-1, Huh-7, RBE, and HeLa), in comparison with colon cancer cells (DLD-1, HCT-15, Colo205, and Lovo). Each cell line was cultured in 6-well plates and incubated with phage expressing SPTKSNS or control phage (insertless). Cells were scraped off, and phage recoveries from each cell line were titered by plaque infection assay. Recovery of each phage was expressed as a ratio relative to that of the control phage from each cell line. Values represent mean ± D ($n = 3$), $\star p < 0.05$ compared with the control.

Competitive inhibitory effects of synthesized peptides on phage accumulation in vitro

To confirm the capacity of the synthesized peptides to bind to colon cancer cells, DLD-1 cells were preincubated with 1 or 10 μM SPTKSNS peptide or control peptide (VRWEMNL) before the addition of 5×10^8 PFU of the selected phage. The inhibitory effects of the synthesized peptides on phage accumulation were examined by titering the phage bound to the cancer cells. Preincubation of cells with the SPTKSNS peptide caused 92% inhibition of the binding activity of the SPTKSNS phage to colon cancer cells, whereas the control peptide had no discernable effect on phage association (Figure 5).

Assessment of possible mitogenicity of the selected peptide

Since growth factor receptors are among those most commonly unregulated within the tumor microenvironment and serve to promote angiogenesis and tumor growth, it was considered important to ascertain whether the iterated peptides could stimulate any mitogenic effect that might preclude their deployment for cancer treatment. The effect of the SPTKSNS or control VRWEMNL peptide on cell growth rate was evaluated using the MTS assay. However, the presence of the SPTKSNS or control peptide had no discernible effect on cell growth (Figure 6).

Discussion

In this study, we attempted to identify cancer-specific cell-binding oligopeptides using a clinically relevant system, whereby cancer cells directly separated from a patient with colon cancer were subjected to biopanning. We employed an LCM system to specifically isolate only cancer cells from the specimen removed from a patient who had undergone colectomy for colon cancer. It is important to use relevant human tissues for biopanning to identify suitable targeting ligands, since there are likely to be substantial differences between antigens expressed in human and murine tumor-associated tissues, and also between clinical human tumors and human tumor cell lines growing as xenografts in mice. For example, expression of the tumor endothelial marker (TEM) 7 antigen is considered to be selectively upregulated within the endothelium of human colorectal adenomas (St Croix et al. 2000), whereas in mouse model systems, expression of TEM 7 is not observed in angiogenic blood vessels but rather in Purkinje cells (Carson-Walter et al. 2001). Indeed, the use of phage libraries to iterate prostate-specific sequences in mice (Arap et al. 2002a) and humans (Arap et al. 2002b) has yielded markedly differing results, bearing little apparent homology to each other, despite the work being performed by the same group. Consequently, it seems

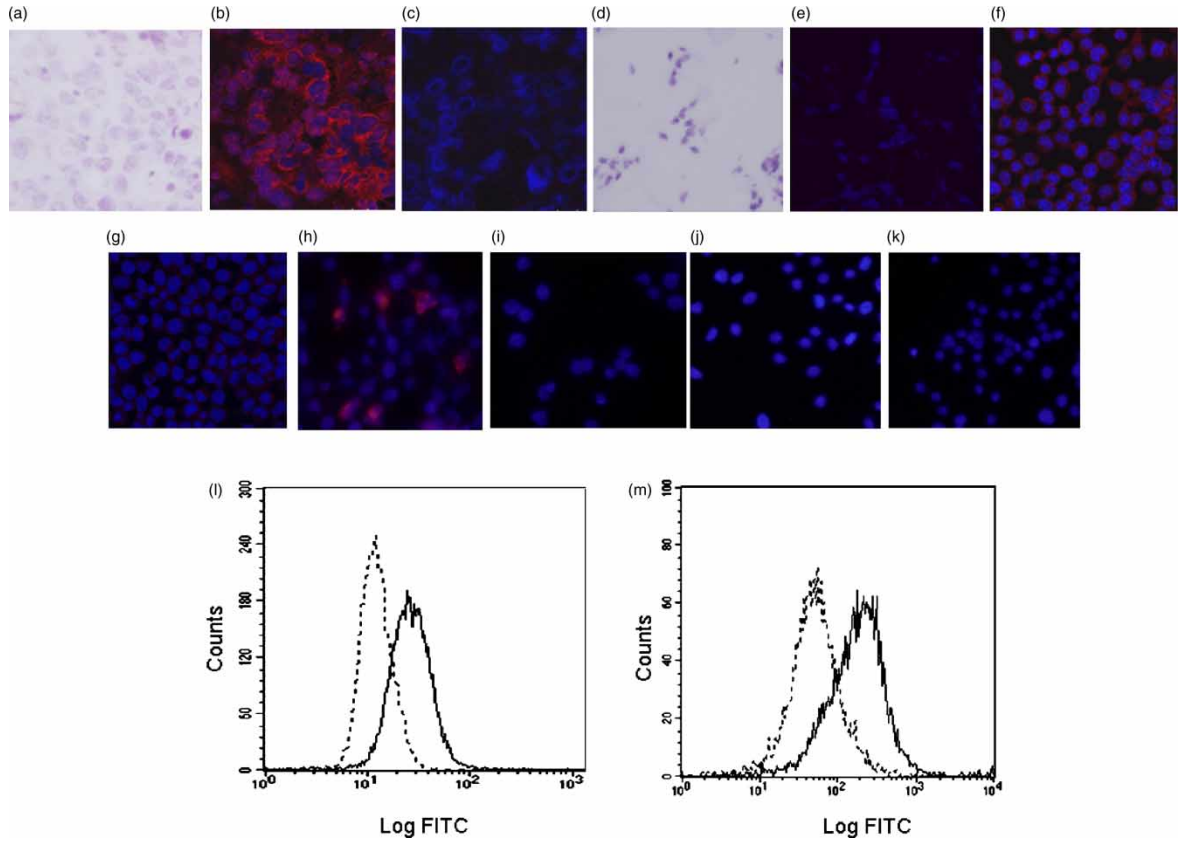

Figure 4. Immunofluorescence analysis of phage clone binding to colon cancer cells. Sample of cancer tissue derived from a patient or DLD-1 was incubated with SPTKSNS or insertless phage. The phage was visualized by fluorescence using mouse anti-M13 phage antibody followed by Alexa 555-conjugated goat anti-rabbit antibody. DAPI was used for nuclear counterstaining, and (a, d) HE staining was used for surgically resected colon cancer or normal tissue. (b, e) Immunostaining for SPTKSNS phage in surgically resected colon cancer or normal tissue. (c) Immunostaining for control phage in surgically resected colon cancer tissue. (f, h, j) Immunostaining for SPTKSNS phage in DLD-1, HCT-15, and HeLa cells. (g, i, k) Immunostaining for insertless phage in DLD-1, HCT-15, and HeLa cells (magnification 400 ×). (l, m) The fluorescence intensity of DLD-1 and HCT-15 cells incubated with SPTKSNS phage (thick line) and control phage (dashed line) was measured by flow cytometry.

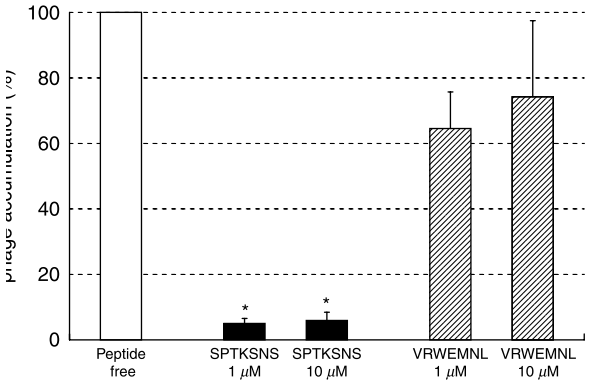

Figure 5. Competitive inhibition of phage accumulation by the synthesized peptides. DLD-1 cells were preincubated with 1 or 10 μM synthesized SPTKSNS peptide or control peptide (VRWEMNL) for 60 min at 4°C, followed by the addition of 5×10^8 PFU of the SPTKSNS phage. The inhibitory effects of the synthesized peptides on phage accumulation were examined by titering the phages bound to the cells. Values represent mean ± SD ($n = 3$), $\star p < 0.05$.

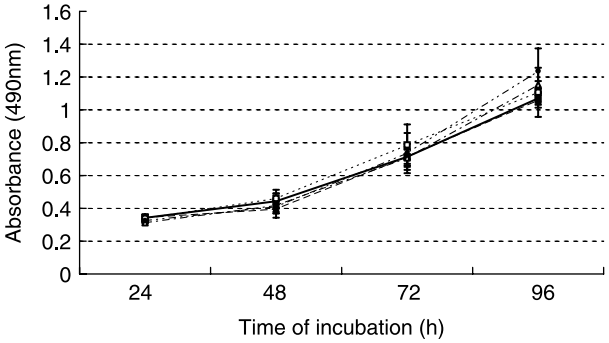

Figure 6. Effect of the SPTKSNS peptide on cell viability. DLD-1 cells were plated at a density of 5×10^3 per well in a 96-well plate and incubated in RPMI medium containing 10% FCS, in either the presence of the SPTKSNS peptide at 10 (▲) or 100 μM (■) or control peptide (VRWEMNL) at 10 (Δ) or 100 μM (□) or without the peptide (●). After 24, 48, 72, and 96 h, the viability of the DLD-1 cells was assessed using the MTS assay, as described in the *Materials and Methods* section. The presence or absence of the SPTKSNS peptide made no significant difference to cell growth.

highly unlikely that targeted delivery can be achieved in humans using ligands iterated with murine model systems, and it is desirable to employ phage panning technology directly in humans or clinical samples of human origin. Because of ethical difficulties and the potential risk of disease transmission by performing biopanning in subsequent patients, reiterative biopanning in human subjects has not generally been considered as a feasible option. Therefore, we have developed the biopanning strategy using resected samples from human patients. In our initial studies, we used whole tumor samples containing multiple cell types, including cancer cells, stromal cells, endothelial cells, and inflammatory cells (Maruta et al. 2003). Because of the heterogeneous nature of such samples, the specific cell types to which the iterated phage clones became bound were unclear. Therefore, to improve selectivity, we applied LCM to selectively separate only cancerous cells from the surgically isolated samples. The cells isolated in this manner will retain many of their clinical characteristics and are likely to be substantially less differentiated than cells that have been extensively passaged in culture, and therefore represent a much more reliable target for biopanning. Indeed, previous studies have demonstrated that after several generations in culture, human cell lines express numerous cell surface epitopes that differ from those present in the original tissue (Arap et al. 2002b). Therefore, it is important to use cancer cells derived directly from patients in the biopanning procedure to reliably identify cancer cell-binding peptides.

A few previous reports have described the successful combined use of phage peptide display library and LCM (Lu et al. 2004; Yao et al. 2005; Ruan et al. 2006). Yao et al. reported phage peptide display combined with LCM to identify peptide ligands for vascular receptors in the islets of Langerhans in the murine pancreas. Lu et al. introduced phage peptide display combined with LCM to identify ligands for oral squamous cell carcinoma xenografts in nude mice. However, these studies were performed on mouse organs or human xenografts grown in mice. As mentioned previously, it is important to use relevant human tissue for biopanning in order to identify targeting ligands. The present report is the first to describe combination of LCM with biopanning using cancerous cells derived directly from a human subject.

In this study, an important priority was that the biopanning was performed on cancer cells derived from a single patient. Given the nature of this strategy, there was a possibility that the peptides iterated in this manner would bind to receptors upregulated uniquely within the tumor of this patient. This may be one reason why the derived peptide, SPTKSNS, bound to cancer cells from the native patient (C1) but failed to bind to cancer cells from other patients (C2 and C3, Figure 1). Interestingly, SPT peptide bound to the DLD-1, HCT-15, and Colo205 cells, but did not bind to Lovo, despite the fact that both cell lines are derived from colon cancer. Therefore, it seems likely that DLD-1 and HCT-15 colon cancer cells have the same receptors as the native patient (C1), whereas Lovo cells lack this receptor. In fact, DLD-1 and HCT-15 cells are derived from the same individual (Dexter et al. 1979).

Differences in biological behavior between cancer patients are important in clinical practice. For example, there is substantial interpatient variability in anticancer drug responses, and a better understanding of the mechanisms defining such differential responses would enable more efficacious dosages to be calculated. Recent studies have demonstrated that interpatient variability in the expression of genes that control tumor drug metabolism enzymes and drug targets greatly influence therapeutic efficacy and adverse responses. For example, 80–90% of an administered dose of 5-fluorouracil (5-FU) is degraded by dihydropyrimidine dehydrogenase (DPD), and it has been reported that DPD-deficient patients with cancer can develop life-threatening complications following treatment with topical 5-FU (Diasio and Johnson 1999; Johnson et al. 1999; Yoshinare et al. 2003). Genetic polymorphisms in the gene encoding thymidylate synthase have also been shown to influence response to 5-FU therapy (Adlard et al. 2002; Watters and McLeod 2003). Consequently, through the use of molecular markers and gene analysis, the concept of tailor-made therapies that adopt appropriate pharmacotherapy based on the expected drug response of each individual cancer patient has been developed. The biopanning procedure described here may contribute to the development of tailor-made cancer targeting methodologies exploiting differences in ligand–receptor binding behavior.

The SPTKSNS peptide showed no significant ability to mediate mitogenesis *in vitro* after binding to colon cancer cells. This is important when applying a novel peptide to actual cancer treatment, as it is undesirable to give potent mitogens to cancer patients. In addition to this safety profile, SPTKSNS has several pharmacological advantages. The majority of targeting ligands in cancer therapy are relatively large proteins and have some pharmacological limitations with serum components and high costs of manufacture, such as single-chain antibody fragments. By contrast, SPTKSNS is a simple peptide with excellent stability and a low manufacturing cost. Furthermore, although it consists of only seven amino acid residues, SPTKSNS is expected to work sufficiently well as a targeting ligand, since peptides containing three amino acid residues, such as RGD, have been reported to provide the minimal framework for structural formation and protein–protein interactions (Arap et al. 2002a). In fact, the competitive inhibition of SPTKSNS phage binding to colon cancer cells by the synthesized SPTKSNS peptide implies that the peptide itself has the ability to bind to colon cancer cells.

In summary, we have introduced LCM to the biopanning strategy and identified a small peptide binding to colon cancer cells derived from an actual patient. This strategy could be applied for tailor-made cancer cell targeting methodology using biopsy samples from actual patients.

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