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Identification of a Transferrin Receptor-binding Peptide from a Phage-displayed Peptide Library

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Using a phage peptide library approach, we have isolated a peptide ligand that binds to transferrin receptor on the surface of human melanoma cell, B16F10. The library was first screened twice by recovering internalized phages and was further screened three times by competitively eluting transferrin receptor-specific phages with human transferrin among the phages bound to the cell surface. The peptides displayed by the selected phages were fused to translocation and catalytic domain of *Pseudomonas* exotoxin to prepare recombinant toxins. After estimating cytotoxicity of each recombinant toxin toward B16F10 cell, seven clones were selected. Sequence analysis revealed that one of the clones displayed a peptide which had a significant sequence homology with human transferrin. The peptide was chemically synthesized and was shown to be functional in delivering cytotoxic agents into B16F10 cell via interaction with transferrin receptor.

Key words : Melanoma, B16F10, transferrin receptor, peptide library

Introduction

Traditional anti-cancer agents effectively eliminate rapidly dividing tumor cells, but are often toxic to normal cells as well. To resolve such problems, there has been much effort to find tumor-specific antigens that might be used as a receptor to which therapeutic molecules are targeted. Among others, transferrin receptor (TfR) has been identified as one of the successful target molecules with a density up to 100-fold higher than the average expression of normal cells, its ability to internalize, and its central role in the cellular pathology of human cancer [6-8]. In fact, transferrin (Tf) and anti-TfR antibody have been successfully used to deliver cytotoxic agents into cancer cells [4,9,10,14].

However, Tf or antibody has some drawbacks such as instability at extreme conditions, immunogenic property, poor tissue penetration and difficulty in site-directed chemical modifications. Small peptides that could be selectively recognized and internalized by tumor cells should overcome some of these limitations. Recently, the advances in combinatorial peptide library technology have made it possible to identify specific peptide ligands for tumor cell surface markers both *in vitro* and *in vivo* [1,12,13]. Such peptide libraries include virtually all possible sequences of short peptides that can mimic conformational structures of both continuous and discontinuous epitopes. Biopanning of a peptide library against known cell surface proteins or tumor cells have resulted in identification of numerous specific binding peptides.

Here, we describe a novel procedure to identify a peptide ligand capable of binding to and delivering therapeutic molecules into the B16F10 melanoma cell from a phage-displayed peptide library. Three kinds of biopanning method were used for this purpose. In the first step, the library phages were added to B16F10 cell and only the internalized phages were collected. Secondly, TfR-specific phages were selected by the competitive elution with human Tf. Finally, recombinant toxins targeted by the selected peptides were prepared and toxicity of each recombinant toxin toward the B16F10 cell was estimated. One of the selected peptides had significant sequence homology with human Tf and ability to deliver cytotoxic agents into B16F10 cell.

Materials and Methods

Peptide library and cell line

The peptide library used in this experiment was constructed with pCANTAB5E vector (GE Healthcare, Sweden) and consisted of 12 random amino acids flanked by two cysteine residues [5]. The library contains approximately 10^8 independent clones with a titer of 10^{11} CFU/ml. The melanoma cell line, B16F10 was provided by Dr. F. C. Szoka (UCSF,

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San Francisco, USA). The B16F10 cell was cultured in a medium consisting of MEM Eagle supplemented with 10% FBS, 2.2 g/l sodium bicarbonate, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids.

Biopanning of internalized phages

B16F10 cell was grown to around 90% saturation in a 35 mm culture dish and washed three times with serum-free medium. Phage-peptide library was added to the dish in 4 ml serum-free medium containing 0.1 mM chloroquine. The culture dish was incubated for 1 hr at 37°C. Surface-bound phages as well as unbound phages were removed by washing the dish three times with Tris-buffered saline (TBS) and three times with ice-cold 0.1 M glycine-HCl, pH 2.2, thereby leaving only internalized phages. The internalized phages were released by scraping the cells in 0.5 ml of 30 mM Tris-Cl, 1 mM EDTA, pH 8.0. The recovered phages were amplified with *Escherichia coli* (*E. coli*) XL-1 blue and M13KO7 as a host and a helper phage. The biopanning procedure was repeated once more. The number of added phages in each biopanning step was 10^{10}-10^{11} CFU.

Biopanning of TfR-specific phages

The phages amplified after second round of the above biopanning were added to a 35 mm culture dish containing B16F10 cell. The temperature was maintained below 4°C in order to suppress internalization of phages. Unbound phages were removed by washing seven times with cold TBS. The TfR-specific phages were eluted with 2 ml of 10^{-6} M human Tf (Sigma-Aldrich, USA) in TBS. The recovered phages were amplified as described above. Three rounds of successive biopanning yielded a phage pool that might contain phages capable of interacting with TfR in a cell surface and getting into the cell.

Preparation and analysis of recombinant toxins

Phagemid DNA was purified from *E. coli* XL-blue cells infected with phages obtained after third round biopanning of TfR-specific phages. Gene III of the phagemid was substituted with a gene encoding for domains II and III of *Pseudomonas* exotoxin (translocation and catalytic domain, PE40) by using *Bam*HI and *Eco*RI restriction enzyme sites. *E. coli* BL21 (DE3) pLysS was transformed with the recombinant vectors and spread on a LB-agar plate containing ampicilin. Thirty clones were randomly selected and grown separately in 4 ml LB media for 4 hr at 37°C. Isopropyl β-D-1-thiogalactopyranoside was added at a concentration of 0.25 mM and the culture media were further incubated for 4 hr at 30°C. The cells were harvested, resuspended in 0.5 ml TBS and homogenized by sonication. The supernatant was collected after centrifugation and sterilized by filtration through 0.2 μm membrane. Expression of the recombinant toxin was confirmed by the Western blot analysis with anti-*Pseudomonas* exotoxin antibody (Sigma-Aldrich). The supernatant containing recombinant toxin was added to B16F10 cell grown in a 96-well microplate and the microplate was incubated in a CO_2 incubator for 12-14 hr. Cell number was estimated with the cell assay kit (Promega, USA). Extract of *E. coli* BL21 (DE3) pLysS devoid of the recombinant vector was used as a control. After initial screening, clones showing high toxicity were chosen and the cytotoxicity test was repeated three times. Finally, seven clones were selected and their DNA sequences were analyzed.

Cell binding assay

Avidin protein (Sigma-Aldrich) was labeled with fluorescein isothiocyanate (FITC) (Sigma-Aldrich) according to the manual. The concentration of FITC-avidin was adjusted to 0.1 mg/ml with PBS. The dodecameric peptide representing the 3T14 sequence was synthesized at Peptron (Korea). Amino terminus of the peptide was conjugated with biotin. FITC-avidin was mixed with the biotinylated peptide at a molar ratio of 1:30 to prepare labeled peptides. Free peptides were removed by dialysis three times against 2 l PBS. Protein concentration was determined with BCA protein assay kit (Pierce Biotechnology, USA). B16F10 cell was cultured on a sterilized cover slip contained in a 35 mm culture dish. The cell was washed with warm PBS and fixed with 4% formaldehyde in PBS for 1 min. The cover slip was rinsed three times with PBS and placed within a humidified chamber. Appropriate amount of the labeled peptide was mixed with 1% bovine serum albumin (BSA) in PBS and the mixture was added to the chamber. The chamber was incubated for 2 hr at room temperature. The cover slip was washed three times with PBS and attached to a slide glass in the presence of 20% glycerol. The cell on the cover slip was observed with a fluorescence microscope.

Liposome targeting test

Dried lipid film was prepared with 10.4 mg egg phosphatidyl choline, 3.48 mg cholesterol and 1 μg 1,2-dioleoyl-sn-Glycero-3-phosphoethanolamine-N-biotinyl

(Biotin-PE) (Avanti Polar Lipids, USA) dissolved in 1 ml of 1:2 (v/v) mixture of chloroform and methanol. The film was hydrated and suspended in 2 ml of 0.1 M N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0, 0.2 M sucrose, 0.1 M cytosine β-D-arabinofuranoside (Sigma-Aldrich) for 2 hr at 37°C. The mixture was sonicated until the white opaque suspension became hazily transparent. The liposome suspension was centrifuged at 12,000x *g* for 10 min. All of the centrifugation steps were carried out at room temperature. The supernatant was collected and mixed with two volumes of 0.1 M HEPES, pH 7.0, 0.2 M glucose. The liposome was pelleted by centrifugation at 12,000x *g* for 30 min and suspended in 1 ml PBS. The biotin group exposed on the liposome surface was conjugated to avidin by adding the liposome suspension slowly to 1 ml solution of 66 μg/ml avidin in PBS. The mixture was centrifuged for 10 min at 12,000x *g*. The pellet was washed with PBS, resuspended in 3 ml PBS and divided into two tubes. The biotinylated 3T14 peptide was added to one tube to attach the peptide to the liposome surface via avidin protein. Liposome in the remaining tube was used as a control. Cytotoxicity of the two liposome preparations was tested as described above.

Results and Discussion

Biopanning

Recovery yields in the biopanning of internalized phages were 0.0031% and 0.04% in the first and second round respectively. The relatively high yields indicate that the chloroquine protected internalized phages from degradation. When the second round phages were amplified and subjected to the biopanning of TfR-specific phages, yield was lowered to about 0.01%, reflecting that only a fraction of internalized phages were specific to TfR. After third round biopanning of TfR-specific phages, however, the yield increased as high as 0.15%, due to the amplification of the phages that survived in the biopanning process.

In a typical biopanning process, a selective pressure is applied to a peptide library until small number of enriched sequence is obtained. However, enrichment of a specific phage does not depend only on the affinity of the phage-displayed peptide but also on the biological properties of the phage such as growth rate and stability. Therefore, it could be anticipated that good binders might be found by analyzing sufficient number of phage clone before the phage population converged on a small number of enriched phages. In order to estimate affinity of peptides displayed on each phage clone, we constructed recombinant expression vectors comprising sequences for PE40 and peptides of the phages obtained after third round biopanning of TfR-specific phages. Significant amount of recombinant toxins was detected in the soluble extracts of *E. coli* strains transformed with the recombinant vectors as judged by Western blot analysis. Among the randomly selected thirty clones, seven clones were selected by their consistent cytoxicity toward B16F10 cell in the repeated experiments.

Sequence analysis

DNA sequences encoding for the random peptides of the selected seven clones are summarized in Table 1. Most striking feature was that stop codon or single base insertion was observed in all of the seven sequences. Although the host *E. coli* strain has a *sup*E genotype by which TAG stop codon is translated into glutamine, two sequences still contain TAA or TGA stop codons and single base insertion. Cárcamo *et al.* reported a high occurrence of peptide-encoding genes that do not have an open reading frame (ORF) when a library of long peptides displayed on the filamentous phage was used in biopanning experiments against protein targets such as interleukins and mouse double minute 2 (MDM2) [3]. The reading frame was interrupted by one or more non-suppressed stop codons, or a post-transcriptional frameshift was needed to account for the expression of the fusion protein consisting of the random peptide and phage coat protein. In contrast, all of the selected clones appeared to have normal ORF in biopanning against monoclonal or polyclonal antibodies.

Expression of fusion proteins from the defective genes is

Table 1. DNA sequences of selected seven clones. Sequence encoding for the random dodecameric peptide is flanked by two cysteine codons. The stop codons are denoted by underlines

Name	Sequence
3T3	TGCGCTCGTTGTGAGAGTTAGTTGGTGTTGGTTATTGAGTGC
3T5	TGCAGTGGTAAGAGTCCTTTTCTTTAGTAGCGGTGTGTTTGC
3T7	TGCACGTTGTTGGATGATCTTGCGTGGTAGGGGATTAGGTGC
3T14	TGCTTTGGTTAGTCTTCGTTGCCTCGTGATGGTCCGAATTGC
3T18[a]	TGCAAGTCGCATTTGGAGTGTGCTGCCTAATTGTTTTGGTTGC
3T22	TGCACGATGGCGTCTACTACGAATTTTTCGACGCTGTAGTGC
3T24[a]	TGCGTTAAAGTCTGCTTGTGATCCGTGTCATACTAGTAGGTGC

[a]Two sequences, 3T18 and 3T24 have 37 bases instead of normal 36 bases between two flanking cysteine codons.

enabled by the ability of ribosomes to follow alternative paths during translation, such as shifting of reading frame and reading through stop codons [2,11]. It may well be possible that biopanning against difficult targets leads to selection of low-abundance clones because there are no available clones with regular ORF [3]. However, there is no reason to postulate that MDM2 and interleukins are difficult targets whereas antibodies are not. Another possibility is that some peptide sequences are toxic to the *E. coli* host and must be expressed at very low levels [3]. This is more plausible explanation because the clones with ORF defects were observed only in the biopanning against proteins with certain biological functions.

BLAST search was performed with the peptide sequences deduced from the DNA sequences in a hope to find homology with hTf. The amber stop codon was translated into glutamine and the sequences containing ORF defects were excluded. Among the five sequences that were subjected to the BLAST search, only the peptide 3T14 showed a significant homology with residues 28-35 of mature hTf protein (Table 2). The Tf molecule is divided into two related lobes, designated as the N-lobe (336 amino acids) and C-lobe (343 amino acids), which are linked by a short spacer sequence. The residues 28-35 are mainly placed in a loop connecting first α-helix and second β-sheet strand of the N-lobe. The α-helix and β-sheet are held together by a disulfide bond and the loop projected outwardly. But, it is not clear whether the loop is participated in the interaction with TfR.

Table 2. Amino acid sequence comparison of 3T14 peptide with human Tf

Name	Sequence
3T14	C F G Q **S S L P** R **D G P** N C
human Tf (19-41)	C Q S F R D H M K **S** V **I P** S **D G P** S V A C V K
	α-helix loop β-sheet

Cell binding activity

The 3T14 peptide was conjugated to the fluorescein-labeled avidin (FITC-avidin) protein. The resulting 3T14-FITC-avidin complex was added to B16F10 cell and binding of the complex was analyzed with a fluorescence microscopy. Control experiment was concurrently performed with FITC-avidin protein lacking the 3T14 peptide. As shown in Fig. 1, fluorescence intensity associated with the B16F10 cell was much higher in the experiment with 3T14-FITC-avidin complex than in the control experiment with the non-targeted FITC-avidin. Moreover, the binding of 3T14-FITC-avidin complex was significantly lowered by the addition of free 3T14 peptide or Tf. These results suggest that the binding of 3T14-FITC-avidin complex to B16F10 cell was mediated by the specific interaction between the 3T14 peptide and TfR in the cell surface.

Liposome targeting

In order to test the capability of the 3T14 peptide to deliv-

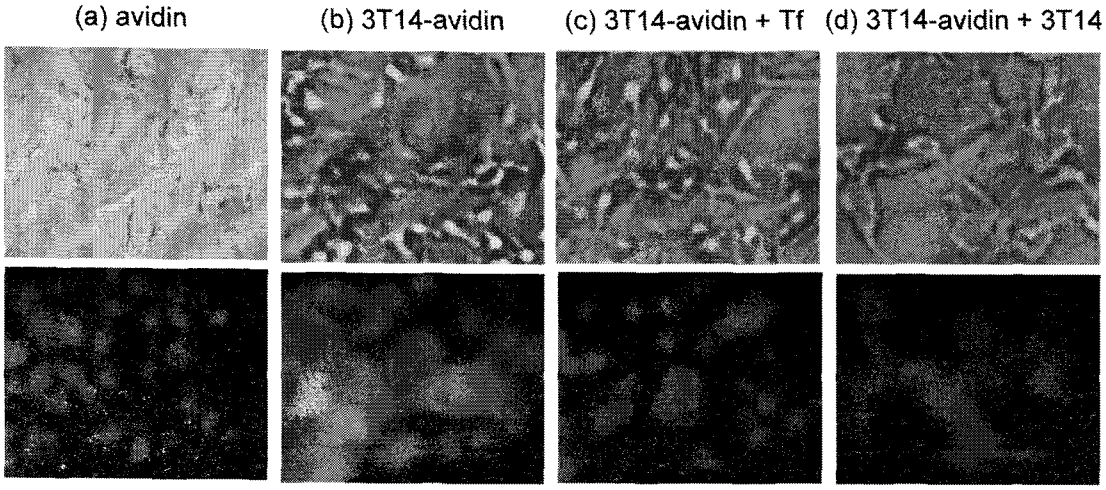

Fig. 1. Binding of the 3T14 peptide to B16F10 cell. Biotinylated 3T14 peptide was conjugated to the fluorescently labeled avidin protein (FITC-avidin). FITC-avidin protein or 3T14-FITC-avidin complex was added to B16F10 cell fixed on a slide glass and fluorescence associated with the cell was observed. Upper and lower rows show the cells observed with a light microscope and a fluorescence microscope respectively. The cell was treated with (a) FITC-avidin, (b) 3T14-FITC-avidin, (c) 3T14-FITC-avidin in the presence of human Tf and (d) 3T14-FITC-avidin in the presence of free 3T14 peptide.

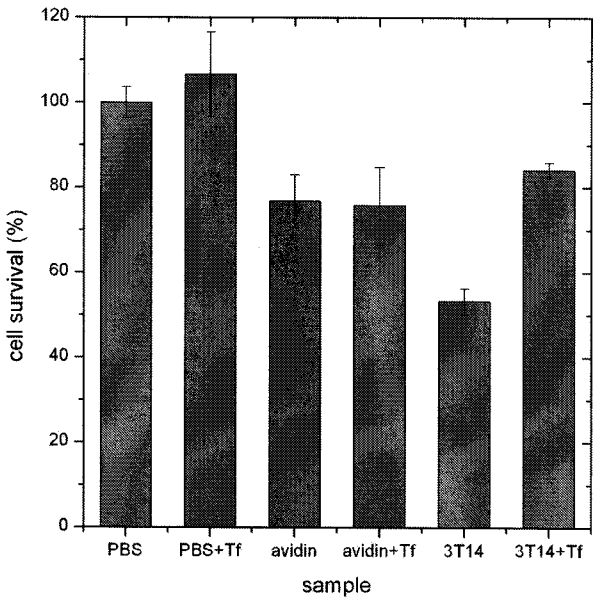

Fig. 2. Cytotoxic activity of liposomes targeted by the 3T14 peptide toward B16F10 cell. The 3T14 peptide was conjugated via avidin protein to the surface of liposome containing cytosine β-D-arabinofuranoside. The liposome was added to B16F10 cell grown in a 96-well microplate in the presence or absence of human Tf and cell survival was estimated (columns 3T14 and 3T14+Tf). Two kinds of control experiment were simultaneously performed by adding PBS (columns PBS and PBS+Tf) or control liposome which had only avidin on its surface (columns avidin and avidin+Tf). Each data point represents the mean from triplicated determinations, and the error bar represents the standard deviation.

er therapeutics, the peptide was conjugated via avidin protein to the surface of liposome containing cytotoxic drug, cytosine β-D-arabinofuranoside. The targeted liposome was efficient in killing B16F10 cell while a little cytotoxicity was observed with the control liposome which had only avidin on its surface (Fig. 2). Furthermore, cytotoxic activity of the targeted liposome was reversed by the addition of Tf, proving that the activity is based on the interaction of the liposome with TfR. In contrast, death of the cell by the control liposome was not affected by the addition of Tf. Nonspecific interaction of avidin with the cell surface may account for the partial cytotoxic activity of the targeted liposome in the presence of Tf as well as the activity of control liposome. Thus, the 3T14 peptide might be useful for the targeted drug delivery to cancer cells overexpressing TfR.

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초록 : 파지-펩타이드 문고로부터 트랜스페린 수용체에 결합하는 펩타이드 탐색

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펩타이드 문고 기술을 이용하여 흑색종 세포주인 B16F10에 결합하는 펩타이드 리간드를 검색하였다. 먼저 세포 내부로 들어간 파지들을 선택하는 방법으로 두 번 검색한 후 표면에 결합한 파지들 가운데 트랜스페린 단백질을 이용하여 트랜스페린 수용체에 결합한 파지들만을 선별적으로 용출시키는 방법으로 세 번 검색하였다. 다음으로 이 두 가지 방법을 통해 선별된 파지들에 표현된 펩타이드들을 *Pseudomonas* exotoxin의 전이 영역과 촉매 영역에 융합시킨 재조합 독소들을 만들었다. B16F10 세포에 대한 각 재조합 독소의 활성을 측정하여 일곱 개의 클론을 선택한 후 염기서열을 분석하였다. 그 결과 그 가운데 한 클론에서 표현하는 펩타이드의 아미노산 서열이 사람의 트랜스페린과 유사한 서열을 갖는 것으로 확인되었다. 그 펩타이드를 화학적으로 합성한 후 항암제를 포함하는 리포솜에 붙여 실험한 결과 트랜스페린 수용체를 통해 치료물질을 전달할 수 있는 가능성을 지닌 것으로 평가되었다.