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ORIGINAL ARTICLE

In vivo phage display screen for peptide sequences that cross the blood–cerebrospinal-fluid barrier

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Abstract There is lack of a barrier between CSF and brain, thus peptide that can cross the blood–cerebrospinal-fluid barrier (BCSFB) will have a greater chance of providing access to the brain. In this study, we screened for a novel peptide sequence that can cross the BCSFB from the systemic circulation using phage display. We applied a 12-mer phage display peptide library (Ph.D.-12) intravenously in rats and recovered phage from the cerebrospinal fluid. A longer circulation time was used according to the biodistributive CSF/blood ratio of the phage particles. Following sequential rounds of isolation, several phages were sequenced, and a peptide sequence (TPSYDTYAAELR, referred to as the TPS peptide) was identified. Clone 12-1, which encoded the TPS peptide, was enriched approximately 53 times greater than the random library phage. After labeling with FITC, the TPS peptide demonstrated significantly greater brain accumulation efficiency. This study demonstrates the feasibility of using in vivo phage display to screen for peptides that can cross the BCSFB from the systemic circulation. In conclusion, the TPS peptide represents a previously unreported promising motif that can be used to design a drug delivery system that can cross the BCSFB.

Keywords Phage display · In vivo screening · Blood–cerebrospinal-fluid barrier (BCSFB) · Peptide

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Introduction

The blood–brain barrier (BBB) is formed by endothelial cells and tight junctions that comprise capillary walls (Abbott et al. 2010) and represents the homeostatic defense mechanism of the brain against pathogens and toxins (Praveen et al. 2004). Ninety-eight percent of small molecule drugs and 100 % of large molecule drugs, including peptides, recombinant proteins, monoclonal antibodies, genes, and short interfering RNAs, cannot cross the BBB (Pardridge 2007). A similar barrier, the blood–cerebrospinal-fluid barrier (BCSFB) is formed by the epithelial cells of the choroid plexuses. The BCSFB acts to protect the brain from harmful substances and to ensure the homeostasis of brain fluids. The capillaries in the choroid plexus and the circumventricular organs do not form tight junctions, and the blood vessels in these structures are freely permeable (Begley 2004). Furthermore, there is lack of a barrier between CSF and brain. Therefore, peptide that can cross the BCSFB will have a greater chance of providing access to the brain.

Many strategies have been employed to circumvent the BCSFB, including opening the BCSFB, an invasive and non-selective procedure that might lead to the unwanted entry of a wide range of potential neurotoxins and other agents, adversely affecting central nervous system (CNS) homeostasis and producing undesirable side effects. In contrast, nanocarriers, such as liposomes and nanoparticles, represent better alternatives, and the number of available nanoparticles and liposomes that are available for therapy has increased. The surface of these liposomes or nanoparticles is usually modified such that the designed constructs can be targeted to the CNS via specific BCSFB mechanisms. Obtaining peptide ligands that can cross the BCSFB might enable the design of drugs that are more selective, resulting in greater therapeutic efficiency, while decreasing systemic toxicity.

Phage display was established by Smith, Scott, and colleagues using filamentous M13-derived bacteriophage in 1985 (Smith 1985; Pande et al. 2010). M13 is a filamentous bacteriophage comprising a single-stranded DNA core encapsulated by a protein coat. M13 is by far the most commonly used phage display system. The biology of M13 has been reviewed several times (Sidhu 2001; Kehoe and Kay 2005; Cabilly 1999). Genes of interest can be fused to those encoding phage coat proteins, resulting in the display of the encoded proteins on the surface of viruses containing the genes of interest. Phage display technology thus physically links the phenotypes of polypeptides displayed on the coat protein to the corresponding genotypes. Phage libraries can be subjected to the selection of the encoded proteins and recovered clones can then be identified by sequencing. Phage display has been used to screen for peptides that bind to specific receptors (Lee et al. 2001; Murai et al. 2003), tumor cells (Lee et al. 2004), or tumor vessels (Arap et al. 1988; Joyce et al. 2003). Strategies for panning cells in vitro (Barry et al. 1996) or tissues in vivo (Pasqualini et al. 1997, 2000) with phage libraries have been described with the aim of obtaining peptide ligands with organ- or tumor-binding specificity. In vivo screening methods have been used to search for tissue-homing peptides (Arap et al. 2002; Kolonin et al. 2006). Wan applied a C7C phage-display library intranasally to rats and recovered phages from the brain tissue; a peptide sequence (ACTTPHAWLCG) was recovered that allowed BBB bypass through the nasal-to-brain passage (Wan et al. 2009). Rooy et al. (2010) selected two 15-amino acid peptides (GLA and GYR) that bound to the murine brain in an in situ brain perfusion model.

The aim of our study is to identify peptides that can cross the BCSFB from the systemic circulation. To achieve this aim, we employed a 12-mer peptide library based on M13. During in vivo phage display selection, library phage was administered intravenously to rats. The library phage was allowed to circulate for some time to allow the clones to diffuse in the vascular system; later, specific phages were recovered from the CSF. The recovered phage was then amplified in *E. coli* and subjected to additional screening cycles to enrich the pool for specific sequences. Typically, phage clones with high affinity were obtained after three to four rounds of selection. The screening of phage-displayed peptide libraries in vivo, therefore, represents a direct and rapid method of identifying novel peptide sequences for drug-targeted delivery.

Materials and methods

Materials

The Ph.D.-12™ phage display peptide library kit, which is based on M13 bacteriophage, was purchased from New England Biolabs (Beverly, MA, USA). *Escherichia coli* ER2738 (an F^+ strain) was used for M13 phage propagation. The displayed peptides were fused at the N-terminus of the minor coat protein pIII. The complexity of the library was ~2.7 × 10^9 transformants. 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Xgal) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Isopropyl-beta-D-thiogalactoside (IPTG) was purchased from EMD Millipore (Germany). Deionized water (Millipore, Bedford, MA) was used throughout.

Adult male Sprague–Dawley rats (280–300 g) and male nude mice (16–20 g) were obtained from the Sino-British Sippr/BK Lab. Animals were maintained at 22 ± 2 °C under a 12 h/12 h light–dark cycle with access to food and water ad libitum. The animal experiments were carried out in accordance with protocols that were evaluated and approved by the Ethical Committee of Fudan University.

Screening of phage libraries in vivo

The appropriate time needed to recover the phages from the cerebrospinal fluid was determined according to a recovery time curve. The titer [in transducing units (TU)/ml cerebrospinal fluid] of the phages recovered from cerebrospinal fluid was plotted against time to construct the recovery time curve. The rats were injected in the tail vein with 10^{12} plaque-forming units (pfu) of Ph.D.-12™ phage-display library suspended in 100 µl TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.5). The phage was allowed to circulate in the rats for 1, 2, 4, 8, 12, and 24 h before cerebrospinal fluid was drawn for phage titering.

Three rounds of screening were performed using adult male Sprague–Dawley rats (280–300 g). In the first round, the rats ($n = 3$) were injected intravenously (iv) with 10^{12} pfu of Ph.D.-12™ phage-display library suspended in 100 µl TBS. The phage was allowed to circulate for some time in vivo. The rats were then anesthetized using 5 % chloral hydrate (0.4 g/kg), and phage-containing supernatant was recovered from the cerebrospinal fluid, amplified by infecting competent bacteria (*Escherichia coli* ER2738), titered and pooled for the next round of screening. Subsequent screening rounds were conducted by intravenously injecting the newly amplified phage (1 × 10^{12} pfu in 100 µl TBS) and repeating the procedures described above.

Titering of phage

Phage titers were determined after each round of screening and amplification by serial dilution in TBS. The phage dilutions (10 µl) were incubated with 200 µl of late log phase *Escherichia coli* ER 2738 bacteria for 10 min at room temperature to allow for infection. The bacteria were

then spread on a LB agar plate containing IPTG and Xgal, and the plates were incubated overnight in an incubator at 37 °C. The number of transducing units was calculated by counting the blue plaques on the plates after 24 h of incubation.

Phage titer $= 10^2 \times a \times b$ (pfu),

where a is the number of blue plaques on the LB plates after overnight incubation and b is the dilution factor.

DNA extraction and sequencing

After isolating bacteriophage clones from the last round of selection, 10 clones were randomly selected and sequenced (ABI3730). The peptide-encoding nucleotide sequences were determined using 96 gIII primers (5′-^{HO}CCC TCA TAG TTA GCG TAA CG-3′) that are included in the Ph.D.-12 phage-display library. The phage-displayed peptide sequences were translated and aligned using the Lasergene program (v7.1).

Peptide synthesis

The TPS peptide (TPSYDTYAAELR) and the FITC-labeled TPS peptide were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. using a standard solid phase FMOC method and were purified to >95 % using high-performance liquid chromatography (HPLC). The TPS peptide was labeled with FITC at the N-terminal end. All peptides were verified using mass spectrometry (LCMS-2010a, Shimadzu, Japan).

In vivo homing of phage to the cerebrospinal fluid

Nude mice were injected in the tail vein with 1 μM (in 200 μl PBS) FITC-labeled TPS peptide (FITC-TPS). Nude mice were intravenously administered PBS and PBS containing the same amount of FITC was designated as controls. One and twelve hours after injection, images were recorded using the Maestro in vivo imaging system (Cambridge Research & Instrumentation, MA; excitation = 495 nm, emission = 525 nm). During the injection and image acquisition process, the mice were anesthetized using 5 % chloral hydrate.

Statistical analysis

Statistical analysis was performed using Student's t test when one group was compared with the control group. p values of <0.05 were considered statistically significant. All statistical analyses were performed using Stata version 8.0, and the data presented are means ± standard deviations, unless otherwise noted.

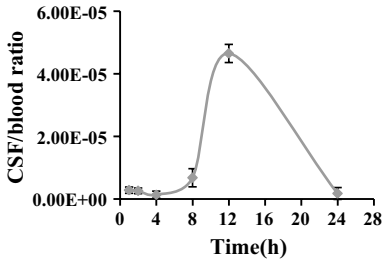

Fig. 1 The time curve of CSF/blood ratio of M13 phages titer. The accumulation of intact infectious phage particles in CSF was analyzed by the ratio of phage particles in CSF and in blood. The accumulation of phages in CSF peaked at 12 h. (mean ± SD, $n = 3$)

Results and discussion

In vivo phage display

Since its introduction by Pasqualini and Ruoslahti (1996), in vivo phage display has been widely utilized and has been proven very effective in selecting phages with high organ specificity upon systemic injection (Smith and Petrenko 1997). Therefore, we selected this technique for screening.

Previous research using phage display biopanning to obtain organ-homing peptides has been performed using very short in vivo circulation times; typically, phage is recovered from various organs after 5–15 min of circulation (Pasqualini and Ruoslahti 1996; Kolonin et al. 2006). However, intravenous administration of phage can lead to high background levels of circulating phage, which affect the screening results. Recovered phage can easily be "overwhelmed" by circulating phage, especially during the first few hours after intravenous injection.

Zou et al. (2004) studied the biodistribution of filamentous phage peptide libraries in mice. This work demonstrated that the accumulation of intact infectious phage particles in tissues [calculated by dividing the titer (in TU/g tissue)] differed within a given organ at a given time according to the titer in their blood (in TU/ml) at that time. We found that the CSF/blood ratio of phage particles reached a maximum at 12 h (Fig. 1). Therefore, we chose 12 h as the optimal time point to recover phages from the cerebrospinal fluid after tail vein administration.

The efficiency of the phage in reaching the cerebrospinal fluid increased after each round of panning, and after three rounds of consecutive biopanning, the amount of recovered phage was increased by approximately 8 times compared with the first round (Fig. 2). Ten phage clones were randomly chosen from the final round of biopanning for DNA sequencing. The sequences of the displayed peptides are shown in Table 1.

A multiple sequence alignment analysis using the Lasergene (version 7.1.0) program revealed that 40 % of the

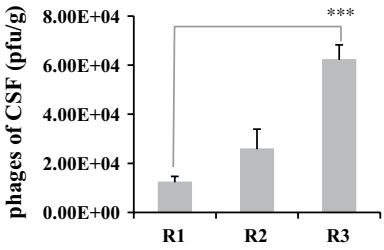

Fig. 2 Three rounds of in vivo screening. Phages were injected iv into SD rats. *R1–R3* referred to round 1–round 3. Rescued phages from the third round had a great augment (mean ± SD, $n = 3$, * $p < 0.01$)

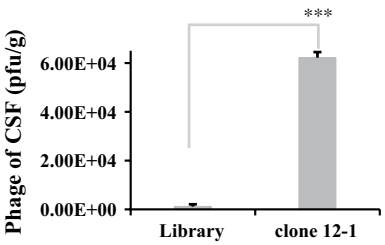

Fig. 3 High efficiency of Clone 12-1 phage homing to CSF. Library phage and phage Clone 12-1 were injected iv into two groups of SD rats (10^{11} pfu in 100 μl TBS), respectively. The library phage was used as control. (mean ± SD, $n = 3$, ***$p < 0.005$)

Table 1 Sequences of individual phage clones

phage clones	sequence	Frequency
12-1	**TPSYDTYAAELR**	4
12-2	KSIALKNTNPHA	1
12-3	SPCVQCSSGLCP	1
12-4	PCLKMGIHTTKR	1
12-5	SVSVGMKPSPRP	2
12-6	YGLHRQAACPLT	1
12-7	**TPSYDTYAAELR**	4
12-8	SVSVGMKPSPRP	2
12-9	**TPSYDTYAAELR**	4
12-10	**TPSYDTYAAELR**	4

A same sequence contained in phage clones are shown in boldface letters

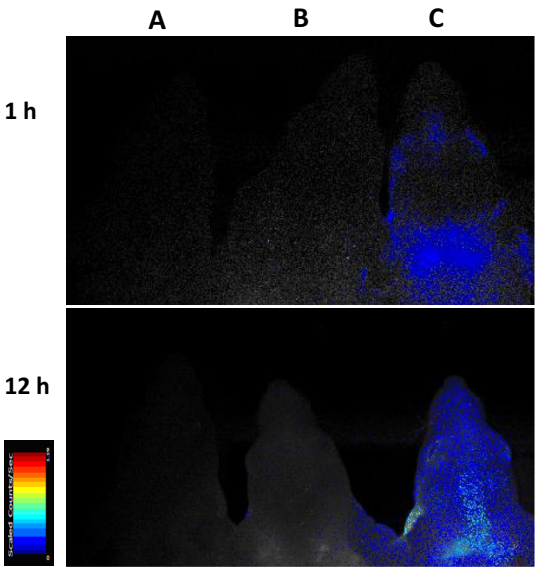

Fig. 4 In vivo distribution of TPS peptide. Optical in vivo imaging of nude mice vein administrated with FITC-TPS (**c**). Nude mouse administrated with PBS (**a**) and PBS containing FITC (**b**) was set as control. Images were taken 1 h and 12 h after injection

phage clones contained the sequence TPSYDTYAAELR (termed the TPS peptide); the phage clone encoding the TPS peptide is termed Clone 12-1. The TPS peptide sequence homologies with previously published proteins having been identified using BLASTP (www.ncbi.nlm.nih.gov), and no putative conserved domains have been detected.

To further evaluate the targeting potential of the TPS peptide, the phage encoding the TPS peptide was systemically injected into the tail vein of SD rats. M13 library phage was used as the control. For both phage types, 10^{12} pfu were injected. Phage was recovered from the CSF 12 h after administration. As shown in Fig. 3, Clone 12-1 was enriched approximately 53-fold more than library phage recovered from the CSF. These results revealed that Clone 12-1 was significantly superior to the library phages with respect to transport efficiency into the CSF.

In vivo optical images

Optical in vivo images were recorded 1 and 12 h after injection. FITC label was obviously accumulated in the brain of the nude mice that were administered FITC-TPS (Fig. 4). Twelve hours after administration, FITC-TPS exhibited significantly greater brain accumulation. Thus, using optical imaging techniques, we demonstrated the CSF-specific targeting capability of the TPS peptide.

The M13 library employed in this study represents 2.7×10^9 unique genotypes encoding random 12-mer peptides that are genomically fused to the pIII coat protein of filamentous M13 phage. The likelihood that a specific phage clone will survive each selection round is increased when the phage is administered at a dose of 10^{12} pfu, which provides 370 copies of each phage clone. When using 10^{11} pfu, only 37 copies of each clone are present.

Previously, we used in vivo screening of a phage-displayed peptide library to isolate peptides that could be used to target drug delivery systems to the brain (Li et al. 2011). A 12-amino acid peptide (termed Pep TGN)

was selected from several rounds of in vivo screening. Pep TGN was covalently conjugated to the surface of poly(ethyleneglycol)-poly(lactic-co-glycolic acid) (PEG-PLGA)-based nanoparticles. The TGN-modified nanoparticles exhibited great potential for targeted drug delivery across the blood–brain barrier. Similarly, the TPS peptide obtained here could be used to construct a similar delivery system that could deliver drugs across the BCSFB. Further studies are currently underway to construct a drug delivery system that can cross the BCSFB using the TPS peptide identified in this study and to evaluate its efficiency of drug delivery.

Conclusions

In this study, we identified a novel peptide sequence, TPSYDTYAAELR, which can cross the BCSFB. Phage Clone 12-1, which includes the peptide TPS, revealed significant superiority over library phage with respect to CSF transport efficiency. To the best of our knowledge, this peptide is a previously unreported CSF-homing motif and might be a useful ligand for the design of a drug delivery system intended to cross the BCSFB.

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Conflict of interest The authors declare that they have no conflict of interest.

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