Screening tumor-targeting bacteriophage particles by pre-clearing phage display

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Abstract Phage display technique provides a powerful approach for the discovery of new tumor-specific peptides. However, the peptides isolated through this technique usually did not possess high tumor-specific property. A pre-clearing step was introduced to increase the efficiency of biopanning by removal of particles that could interact with ubiquitously expressed cellular receptors in the non-target organs. The randomized Ph.D-CX_7C phage library (Phage III) was first pre-cleared in normal mice to reduce vasculature- or organ-targeting phages to get the pre-cleared phage library, and then the tumor-targeting bacteriophage particles (Phage I) were screened from pre-clearing phage library in S180 tumor-bearing mice. The biodistribution results of ^{99m}Tc-labeled phages in mice bearing S180 tumor show that the uptake of ^{99m}Tc-labeled Phage I in tumor is high but low in normal organs, and the tumor-to-liver and tumor-to-spleen ratios of ^{99m}Tc-labeled Phage I are higher than those of ^{99m}Tc-labeled Phage II (tumor-specific phages screened from the original CX7C library) and Phage III (unscreened phages from the original CX7C library). It indicates that the yield of tumor-targeting bacteriophage particles could be improved and the non-specific binding in organs becomes weak. Consequently, the pre-clearing phage display method could improve the yield of positive hits by reducing the non-target organ accumulation of bacteriophage particles.

Key words *In vivo* phage display, Pre-clearing phage library, Tumor-targeting, Radio-labeling

1 Introduction

Phage display technique has been an important type of high-throughput screen for seeking tumor- and organ-targeting peptides during the past two decades. However, a problem in the development of *in vivo* phage display is the high background noise, which is resulted mostly from the pretended clones with inherent undesirable properties such as fast growing capabilities, high infectivity, or non- specific binding properties[1]. Many methods were used to enhance the selectivity in phage display, such as building special libraries[2], prolonging circulation time[3], target presentation and so on. Nicklin *et al*.[4] used filamentous phage display *in vitro* to isolate the peptide SIGYPLP, which appeared only once without pre-clearing but 45 times with pre-clearing. Landon *et al*.[5] pioneered an *in vivo* selection scheme using a pre-cleared phage library which showed the advantage of improved yield of positive hits in selection *in vivo*. The phage libraries were first pre-cleared in non-tumor-bearing mice to reduce vasculature- or organ-targeting phages. Using the same method, Newton *et al*.[6] discovered 19 phage clones that were unique from preselected phage display. However, other researchers using the pre-clearing of phage library reported unaltered results after a short time of pre-clearing circulation, hence the need of further studies on detailed pre-clearing step, including circulation time, number of selection rounds, and right objects as control.

This work was aimed at analyzing pharmacokinetics of phage particles and optimizing the circulation time of pre-clearing step. To probe the practical utility of the pre-clearing approach, we investigated the binding specificity of radiolabeled

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Received date: 2011-10-10

phage particles (phage I) which were selected from S180 tumor *in vivo* after a pre-clearing step.

2 Materials and methods

2.1 Reagents and equipments

All reagents were of analytical grade and used without further purification. Deionized water was used. The Ph.D-CX_7C phage display peptide library kit was from NEB (New England Biolabs, Beijing, China). Other reagents were from Gaoxintaige Biotechnology (Beijing, China). Polyethylene glycol/NaCl and LB medium (10 g bacto-tryptone, 5 g yeast extract and 5 g NaCl in 1 liter of distilled water) were prepared according to procedures recommended in the NEB product manual. PBS-P1 is a mixture containing the protease inhibitor (phenylmethylsulphonyl fluoride) (1 mmol·L^{-1}), aprotinin (20 μg·mL^{-1}) and leupeptin (1 μg·mL^{-1}) in phosphate-buffered saline (PBS, pH 7.4). NHS-MAG_3 (N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycinate) was synthesized in our laboratory.

The ^{99m}Tc-pertechnetate was eluted from a ^{99}Mo-^{99m}Tc generator (China Institute of Atom Energy, Beijing). Radioactivity of the tissue samples was measured by an auto γ counter (Packard, USA) Radioactivity of ^{99m}Tc-labeled products was determined by an FT-630 γ well counter (Beijing Nuclear Instrument Factory, China). A high-speed refrigerated centrifuge from Hitachi Japan was used. T10 basic Disperser/Homogenizer (IKA, Germany) was used to homogenize the tissue samples.

Murine S180 cell line was kindly provided by College of Life Sciences, Peking University. Kunming mice (20−22 g, male) were from Breeding Center of Zoology, Center of Health Science, Peking University.

2.2 Pharmacokinetic analysis of phage particles

Aliquots (200 μL, 10^{11} PFU (plaque forming unit)·mL^{-1} in PBS) of Ph.D-CX_7C phage display peptide library dilute solution were injected into the tail vein of normal mice. The blood samples were harvested from the tail vein post-injection at 2, 5, 20, 40, 60, and 100 min, and 4 and 7 h. The phage contents in each sample were determined by plaque assay.

2.3 Pre-cleared phage libraries

The randomized Ph.D-CX_7C phage library was first pre-cleared in normal mice to reduce vasculature- or organ-targeting phages[5,6]. Aliquots (100 μL, 10^{10} PFU·mL^{-1} in PBS) of Ph.D-CX_7C phage display peptide library were injected into the tail vein of mice. After the phage particles circulated for 1 h, blood samples were drawn from the orbit of eye and mixed with 4 mL of PBS-P1. The blood samples were centrifuged at 4000 rpm for 10 min at 4°C. The supernatants were stored for plaque assay analysis and *Escherichia coli* 2738 bacterial culture solution was added for amplification. The amplified and purified phage particles (10^{10} PFU·mL^{-1}) were injected into mice and recovered again as described above for another two rounds. The amplified and purified phage particles (10^{12} PFU·mL^{-1}) of the last round were collected as pre-cleared phage libraries.

2.4 Phage selection

S180 cells were injected subcutaneously into the right flanks of Kunming mice. When the tumors grew up to 0.5−1.0 cm in diameter (7−10 days), the mice bearing S180 tumor were used to carry out biopanning and biodistribution. Biopanning procedures were performed according to the NEB product manual and the methods reported before[3,7,8]. Aliquots (100 μL, 10^{10} PFU·mL^{-1} in PBS) of the randomized Ph.D-CX_7C phage library or pre-cleared phage libraries were injected into the tail vein of mice bearing S180 tumor. After circulating the phage particles for 1 h, the mice were killed by cervical dislocation and tumors were quickly removed, put into PBS-P1 solution and washed three times with ice-cold PBS-P1. Tumors were homogenized in 2 mL Tris-HCl solution (pH 8.0) with a T10 Basic Disperser. The phage particles bound to S180 tumors were recovered by adding 1 mL of *Escherichia coli* 2738 bacterial culture solution and incubating at room temperature for 10 min. The recovered phages were titered, amplified and purified[9]. The amplified and purified phage particles (10^{12} PFU·mL^{-1}) were injected into S180 tumor-bearing mice and recovered again as described above for another two rounds.

2.5 Conjugation of phage with MAG_3 chelator

Conjugation procedures were performed according to Ruschowski's report[8] and our previous study[10]. In brief, 2–4 μL of 0.1 M sodium bicarbonate (pH 9.0) was added to 100–200 μL of PBS containing about 10^{10} PFU·mL^{-1} phage particles to make the final pH 8.0. With constantly stirring, 4 μL of a fresh solution of 1 g·L^{-1} NHS-MAG_3 in dry dimethylformamide (DMF) was added. The conjugation mixture was incubated at room temperature for 45 min. The mixture was added with 1/6 volume of 20% PEG/NaCl (2.5 mol·mL^{-1}) solution, and incubated on ice for over 15 min. The PEG precipitate was centrifuged for 15–20 min at 10,000 r·min^{-1} at 4°C. MAG_3-phage particles were suspended in PBS and precipitated once again with PEG/NaCl. The final particles were suspended in PBS and stored at 4°C.

2.6 Radiolabeling of MAG_3-phage particles with ^{99m}Tc[10,11]

^{99m}Tc-pertechnetate generator eluant (3.7×10^7 Bq), 20 μL of sodium tartrate (30 mg·mL^{-1}), and 2 μL of a fresh solution of $SnCl_2{\cdot}2H_2O$ (1 mg·mL^{-1} in 0.01 mol·mL^{-1} HCl) were added to 100 μL of MAG_3-phage particles (10^{10} PFU·mL^{-1}). The labeling mixture was incubated at room temperature for 30–60 min. The ^{99m}Tc-labeled MAG_3-phage particles were purified by precipitation twice with PEG/NaCl as described above. The final phage particles were suspended in PBS. Radiochemical purity was estimated by thin-layer chromatography with saline as solvent and paper (Whatman No.1, VWR, USA). The chromatography strips were cut into 1 cm sections, and the radioactivity was determined in the gamma well counter.

2.7 Biodistribution of radiolabeled phage particles

Kunming mice bearing S180 tumor (five mice for each group) received a separate injection into the tail vein of three kinds of phage particles, (a) the third-round selected phage from pre-cleared phage library (Phage I), (b) the third- round selected phage from randomized CX_7C phage libraries (Phage II), and(c) unscreened phages from the original CX_7C library (Phage III). Aliquots (100 μL, about 10^9 PFU·mL^{-1}, 860 kBq) of ^{99m}Tc-MAG_3-phage were injected via tail vein into tumor-bearing mice (five mice for each group) without anesthesia. After the phage particles circulated for 1 h, the mice were killed by cervical dislocation. The tumor nodules and other organs or tissues such as heart, brain, spleen and liver, and so on were removed, washed and weighed prior to radioactivity counting. The injection solution (100 μL) was taken as a standard for calculating the percent of injected dose per gram of tissue, i.e., %ID·g^{-1}. The ratios of tumor to organ or tissue for each mouse were also calculated. The final results are expressed as the mean±standard deviation.

3 Results

3.1 Construction of a pre-cleared CX_7 C phage library

As high background noise *in vivo* limits efficiency of the original phage display and selection, before screening tumor-specific ligands, we first assessed the pharmacokinetic properties of phage particles to gain a better understanding of the blood clearance rate of chosen phage peptide display libraries. The results were exploited to optimize incubation time of phage particles in animal and improve the yield of target hits by removing non-binding phage.

In Fig.1, the phage shows a half-life in blood of 15 min, followed by a 1-h exponential decrement of the phage contents in blood. These are similar to published results[10,12,13]. Although longer time to recover and analyze binding properties of the phage particles would reduce the noise of *in vivo* selection, we preferred one hour as the optimal incubation time, so as to improve the yield of positive tumor-hits.

To separate the organ-targeting phages from the randomized CX_7C phage library, we did three rounds of clearance selection. The data from plaque assay show that the phages harvested from the blood increase steadily from the first round of panning to the third (Table 1). The increased percent of recovery reveals that the obtained phages are appreciably enriched in the blood and the organ-binding phages are much less in the blood after three rounds of pre-clearing selections. The percent of recovery in the third round is only 3-fold higher than that in the

second round, which indicates more rounds of selection will not effectively improve the blood recovery. The amplified and purified phage particles of the last round were collected as pre-cleared phage library.

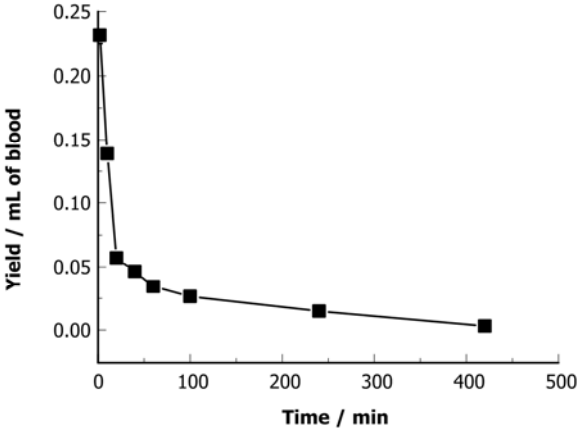

Fig.1 Pharmacokinetic profile of Ph.D-CX_7C phage in blood. 2×10^{10} PFU of Ph.D-CX_7C phage was injected into mice. At the indicated time intervals, 10 μL of blood samples were harvested from the tail vein of each mouse. The numbers of phage particles were determined by plaque assay, and expressed as yield (numbers of phage recovered divided by that of injected) per mL.

Table 1 Analysis of pre-clearing phage display peptide library

Round	Total injected (PFU)	Number of Eluted(PFU)	Recovery Ratios
1	2.2×10^{10}	6.7×10^{6}	3.0×10^{-4}
2	1.0×10^{10}	1.0×10^{8}	1.0×10^{-2}
3	1.3×10^{10}	4.0×10^{8}	3.1×10^{-2}

3.2 In vivo screening for tumor-targeting phage

To screen tumor-specific phage, two CX_7C peptide phage libraries were used for *in vivo* selecting, one was the pre-cleared CX_7C library, and another was the original CX_7C library, which acted as a control to assess the effect of the pre-clearing strategy. A dose of phage (10^{10} PFU) in 100 μL of PBS was injected intravenously into each mouse and then allowed to circulate for one hour before harvesting the tumor. Three rounds of selection were performed, and the phage numbers in the tumor were determined by plaque assay. The number of recovered phage particles did not increase obviously in the following two rounds, being 10^6–10^7. A possible explanation is that the binding sites in tumor are saturated with a mass of phage particles. In fact, the recovery ratio in the second round was 15-fold more than that in the first round, but similar to the ratio in the third round, even though the later was a little lower (data not given). We speculate that although three pre-clearing steps would quicken the process of enrichment in the target, the third round does not improve the recovery ratio. The amplified and purified phage particles of the last round were collected as tumor-specific phages, i.e., Phage I from the pre-cleared CX_7C library, Phage II from the original CX_7C library.

3.3 Biodistribution of ^{99m}Tc labeled tumor-targeting phages in mice

The ^{99m}Tc-labeling efficiency of MAG_3-phages varied between 20% and 50%. With two steps of purification, the radiochemical purity, as determined by strip chromatography with paper and saline as solvent, was routinely greater than 90%.

In order to compare the binding of tumor to the phages obtained from different libraries, the biodistribution of ^{99m}Tc-labeled Phages I, II and III in tumor-bearing mice are given in Table 2. Liver is the highest accumulation of all organs. The uptakes of ^{99m}Tc-labeled phages in heart, muscle and brain are lower. Fig.2 shows percentage injected dose per gram (%ID) of tumor to organ (stomach, spleen, lung and liver) ratio, the accumulation of ^{99m}Tc-labeled Phage I in tumors is higher than that of Phages II and III.

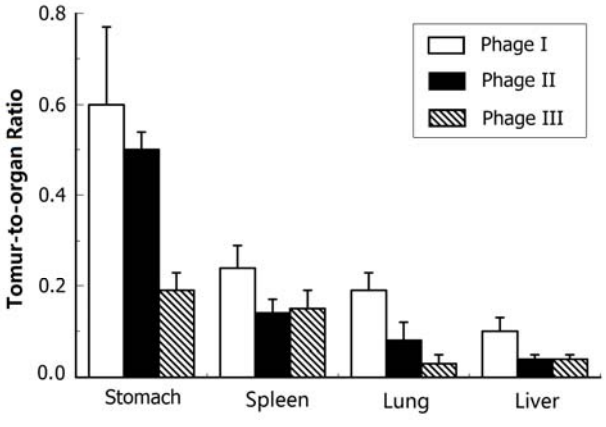

Fig.2 Tumor-to-organ ratios of three ^{99m}Tc-labeled phages in mice bearing S180 tumor xenografts. Uptake (% ID·g^{-1}) in tumor and control organs was measured after 1 h (n=4~5 animals) circulation of the radioligand in the mice. The results are expressed as mean values.

Table 2 shows lower uptake of ^{99m}Tc-labeled Phage I in brain, stomach, spleen, lung and liver. A possible explanation is that the pre-clearing scheme reduces the number of phages targeting vasculature or other organs and improves the specificity or affinity of tumor to some extent.

Table 2 Tumor-to-organ ratios of ^{99m}Tc-labeled phages calculated from % ID·g^{-1} of organs of the tumor-bearing mice (n=4~5)

Tumor-to-organ ratio	^{99m}Tc-labeled Phage I	^{99m}Tc-labeled Phage II	^{99m}Tc-labeled Phage III
Blood	0.31±0.04	0.13±0.03	0.38±0.03
Brain	8.44±0.83	4.21±1.14	7.36±0.89
Small Intestine	0.80±0.29	0.71±0.21	1.63±0.95
Large Intestine	0.77±0.45	0.93±0.38	0.78±0.24
Muscle	3.23±0.58	3.31±0.65	3.08±0.28
Bone	0.12±0.02	0.58±0.18	1.17±0.10
Heart	0.71±0.11	0.51±0.16	0.88±0.27
Stomach	0.60±0.17	0.50±0.04	0.19±0.04
Kidney	0.16±0.05	0.12±0.03	0.31±0.02
Spleen	0.24±0.05	0.14±0.03	0.15±0.04
Lung	0.19±0.04	0.08±0.04	0.03±0.02
Liver	0.10±0.03	0.04±0.01	0.04±0.01

4 Discussion

Panning *in vivo* represents a new application of phage display technique for searching candidates targeting special organs and tumors[8,14]. The panning method may improve binding characteristics and targeting properties of peptides *in vivo*. A number of novel peptides that especially react with organ-specific endothelium and parenchymal makers, tumor-specific vascular endothelial growth factor receptors and cell-binding sites were selected in the past two decades [15–23]. A big problem to most of the peptides is that they do not work as well as the form of phage particles *in vivo*. So, traditional panning *in vivo* should be improved by more effective selection, and the pre-clearing strategy was introduced into biopanning. The experimental results of Nicklin *et al*.[4], Landon *et al*.[5] and Newton *et al*.[6] indicate that specificity and efficiency of the biopanning selection is enhanced by the pre-clearing step.

We used Ph.D.-CX_7C random peptide phage display library panned in Kunming mice bearing S180 xenograft. Before panning *in vivo*, three rounds of pre-clearing selection were performed in normal Kunming mice, and the biopanning efficiency was increased by removing phages that interacted with ubiquitously expressed cellular receptors[4,5,24]. The candidate phage particles were enriched in blood samples collected via orbital bleeds at 1 h after phage administration. Through this procedure, we can assume that the organ-binding phages are much less in the pre-cleared phage library and using this pre-cleared phage library for *in vivo* biopanning would be more efficient and more target-specific.

For rapid investigation of the pre-cleared phage library, S180 tumor bearing mice was used for *in vivo* biopanning and Phage I was isolated as the S180 tumor specific phage library. Afterward, we used ^{99m}Tc-labeled Phages I, II and III for biodistribution and analyzed tumor-to-tissue ratios. In all tissues examined, the tumor-to-tissue ratios of ^{99m}Tc-labeled Phage III for muscle, stomach, lung and liver are less than those of ^{99m}Tc-labeled Phage I and ^{99m}Tc-labeled Phage II, which indicates more non-specific bindings happen in these organs after ^{99m}Tc-labeled Phage III administration. Therefore the high tumor-to-tissue ratios of ^{99m}Tc-labeled Phage III in brain, intestine, bone, heart and kidney could not demonstrate the tumor-specific characteristic. Additionally, except in bone and intestine, the tumor-to-organ ratios of ^{99m}Tc-labeled Phage I for stomach, spleen, lung and liver are significantly higher than those of both ^{99m}Tc-labeled Phages II and III (Fig.2), which

indicates that pre-clearing steps improve tumor-targeting by reducing accumulation of phage particles in the reticuloendothelial system (RES). In other words, the binding specificity to tumor of radiolabeled phage particles which were selected from S180 tumor *in vivo* after a pre-clearing step was increased and enhanced, and the pre-clearing step resulted in a more specific and more condensed phage library. Our preliminary results supported the results in Refs.[4–6] and the pre-clearing approach would be a potential method in phage display. However, to improve efficiency of the pre-clearing approach, further investigation is needed. For instance, the circulation time and number of selection rounds shall be optimized in detailed pre-clearing step. In addition, biopanning shall be performed in different organs or tumor models because the retargeting efficiency of the pre-cleared phage particles may be organ- or tumor model-related.

5 Conclusion

In order to isolate tumor-specific phage particles, Ph.D-CX_7C phage library was first pre-cleared in normal mice. Phage particles recovered from the blood were amplified and injected into S180 tumor-bearing mice for biopanning. Phage particles (Phage I) recovered from the tumor were amplified, radiolabeled with ^{99m}Tc and injected into S180 tumor-bearing mice again for bioevaluation. The biodistribution results indicated that the uptake of ^{99m}Tc-labeled phage I in tumor was higher than that in normal organs. Our study highlights the potential application of pre-clearing step in improving the yield of positive hits by removing non-binding phage and reducing high accumulation background. Further investigations are necessary to determine whether and to what extent the effect of pre-clearing can be improved by optimizing the circulation time and screen in different organs.

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