Mouse Thymus Targeted Peptide Isolated by In Vivo Phage Display Can Inhibit Bioactivity of Thymus Output In Vivo

Yang Yu, Zizheng Wang and Tongxin Du

J Biomol Screen 2008; 13; 968 originally published online Oct 31, 2008;
DOI: 10.1177/1087057108326537

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YANG YU,[1] ZIZHENG WANG,[2] and TONGXIN DU[3]

Heterogeneity of the vasculature in different organs has been well documented by the method of in vivo phage display. Using this technology, several peptide ligands that home to tissue-specific vascular endothelial cell have been isolated. Such peptide ligands directed against specific vascular surface molecules can be used as targeted therapeutic compounds or imaging agents to the vasculature of the specific organ in vivo. In this study, the authors perform in vivo selection in mice using a phage display random peptide library and separated phage peptides homing to mouse thymus by 3 rounds of in vivo panning. Sequence analysis showed that CHAQGSAEC is the dominant peptide sequence. Immunohistochemistry confirmed that the phage peptide CHAQGSAEC can bind specifically to thymus blood vessels in mice. Furthermore, phage peptide CHAQGSAEC and free peptide CHAQGSAEC can inhibit the bioactivity of thymus output in vivo. These results indicate the feasibility of the targeted peptide for possible function as a kind of tool to inhibit thymus bioactivity or as a targeted compound for targeted medicine. (*Journal of Biomolecular Screening* 2008:968-974)

Key words: in vivo, phage display, phage peptide, thymus, thymus output

INTRODUCTION

PHAGE DISPLAY IS A TECHNIQUE IN WHICH PEPTIDES or proteins are expressed as a fusion with coat proteins of a bacteriophage, resulting in display of the fused peptides on the surface of the phage, whereas the DNA encoding the fusion peptides is within the phage. Phage display has been used to create a physical linkage between a vast library of random peptides and the DNA sequences, allowing rapid identification of peptide ligands for a variety of target molecules by an in vitro or in vivo selection process. In its general form, selection is carried out by incubating a library of phage-displayed peptides with the immobilized target molecule, washing away the unbound phage peptides, and eluting the specifically bound phage peptides. The eluted phage peptides are then amplified and taken through additional cycles of binding-eluting-amplification to enrich the pool of specifically binding phage peptides. After several rounds of selection, peptides with high specificity to the target molecule can be isolated.[1]

In vivo phage display is performed within living organisms without preconceived notions about target molecules, and it can identify peptide ligands homing to any organs of interest in an unbiased and internally controlled process.[2] The procedure involves intravenous administration of a phage display random peptide library, which lets phage peptides circulate for a certain period to allow their distribution in the vascular system. This is followed by perfusion of the vascular system and washing of the organs to remove nonspecifically bound phage peptides, after which clones homing to vascular beds of the interested organ are recovered by host *Escherichia coli* infection and amplified for subsequent rounds of selection.

The approach of in vivo phage display has documented heterogeneity of the vasculature in some organs in mice, including prostate,[3] breast,[4] white adipose tissue,[5] and pancreas.[6] All results of these studies showed evidence that organ-specific molecular heterogeneity of the vasculature is a universal phenomenon.[7] Importantly, research in humans has confirmed that the vascular endothelium expresses differential molecules depending on its tissue localization.[8]

The applications of in vivo phage display have made considerable progress in the development of screening methods using phage display random peptide libraries to isolate novel peptide ligands homing to specific organs, which might lead to the development of peptidomimetic drugs or imaging agents.[9,10] In general, these targeted compounds are more effective and less toxic than the untargeted parental compounds. Furthermore, peptides have several advantages over antibodies as drug candidates, including a) lower

[1]Department of Gene Detection, [2]Department of Nuclear Medicine, and [3]Department of Immunology, Nanjing Clinical Nuclear Medicine Center, Nanjing First Hospital affiliated to Nanjing Medical University, Nanjing, Jiangsu, China.

Received Jul 23, 2008, and in revised form Aug 1, 2008. Accepted for publication Aug 4, 2008.

Journal of Biomolecular Screening 13(10); 2008
DOI: 10.1177/1087057108326537

manufacturing costs (synthetic vs. recombinant production), b) higher activity per mass, c) lower royalty stack than antibodies because of a simpler intellectual property landscape during discovery and manufacturing, d) greater stability (lengthy storage at room temperature acceptable), e) less chance of unintended interaction with the immune system (assuming the peptide contains no known immune system signaling sequence), and f) better organ penetration.[11]

The thymus gland is a central lymphoid organ in which bone marrow–derived T cell precursors undergo differentiation, eventually leading to migration of positively selected thymocytes to the peripheral lymphoid organs. Although the function of the thymus for the maturation of the T cell may be halted after puberty, it may serve as a place to preserve and keep capturing the unwanted autoreactive T cell repertoire for the whole life, which leads to proliferation or malignancy in the thymus during the lifetime. Of great importance, a neoplastic transformation such as thymoma may trigger the self-specific unwanted autoreactive inmate T cells. In humans, the association between autoimmune diseases and thymoma is well recognized, particularly the frequent association of thymoma with myasthenia gravis (MG).[12] Therefore, isolation of thymus-specific peptides may provide a tool to study its functional abnormality.

In this study, we use a phage display random peptide library to perform in vivo selection in mice, isolating specific peptides homing to mouse thymus by 3 rounds of panning. Depending on the analysis of T cell receptor excision circle (TREC) expression, we also found a peptide that can inhibit bioactivity of thymus output.

MATERIALS AND METHODS

Reagents

The Ph.D.-C7C™ phage display peptide library kit used for this experiment was purchased from New England Biolabs (Beverly, MA). It contains a heptapeptide phage display library, *E. coli* ER2738 host strain, and a sequencing primer. The library is based on a combinatorial library of a random heptapeptide fused to the minor coat protein (pIII) of the M13 phage. The random peptide sequence is flanked by a pair of cysteine residues. The disulfide-constrained cyclic heptapeptides are expressed at the N-terminus of pIII, with the first cysteine preceded by an alanine residue and the second cysteine followed by a short spacer (Gly-Gly-Gly-Ser) and then the wild-type pIII sequence. The supplied library consists of approximately 1.2×10^9 random peptide sequences and 2.0×10^{12} plaque-forming units (pfu). The diversity of the library was confirmed by sequencing the native library by the manufacturer. Phage peptides of 2.0×10^{11} pfu were used for each round of in vivo biopanning. All manipulations of the phage display library were performed according to the manufacturer's instructions.

Animals

Female BALB/c mice ages 6 to 8 weeks were purchased from the Experimental Animal Center of Nantong University. All animal experiments were conducted according to standard procedures approved by Nanjing Medical University.

In vivo panning of phage display random peptide library

In each round of in vivo panning, a female BALB/c mouse was anesthetized with Avertin (0.015 mL g^{-1}) and injected intravenously (tail vein) with 2.0×10^{11} pfu of the phage display random peptide library (Ph.D.-C7CO™ Phage Display Peptide Library Kit, New England Biolabs) diluted in 200 μL Tris-buffered saline (TBS). After 15 min of phage peptides circulation in vivo, the mouse was kept in the anesthetic condition and perfused through the left ventricle with 50 mL TBS to remove unbound phages and nonspecific binding phages. After that, thymus and kidney (as control organ) were removed, weighed, and homogenized in ice-cold 1 mL TBS-PI (TBS containing the protease inhibitors phenyl methyl sulphonyl fluoride [1 mM], aprotinin [20 μg mL^{-1}], and leupeptin [1 μg mL^{-1}]). Last, thymus and kidney were washed 3 times with ice-cold TBS-PI containing 1% bovine serum albumin (BSA), and binding phage peptides were rescued by infection with ER2738 bacteria for amplification. For the next round of in vivo selection, clones recovered from the previous round were scraped from the plate to grow, and amplified phage peptides were purified and titered. Phage peptides of 2.0×10^{11} pfu recovered from the previous round were injected into another mouse for the next round of in vivo panning as described above. Three rounds of in vivo selection were completed.

In each round of in vivo selection, phage peptides were infected, amplified, purified, and titered according to the manufacturer's instructions.

Analysis of peptide sequences

After 3 rounds of in vivo panning, the phage peptides recovered from thymus and kidney in mice were titered, and 11 (more than 10 were needed according to the manufacturer's instructions) monoclonal phage peptides recovered from each organ were picked out from 1 plate (a plate having no more than 100 plaques according to the manufacturer's instructions) for nucleotide purification and sequencing. Sequencing templates were prepared by rapid purification according to the manufacturer's instructions, and the M13-96 primer, 5′-CCC TCA TAG TTA GCG TAA CG-3′, was used for sequencing.

The peptide sequences were analyzed to identify peptide frequency in the corresponding organ.

Immunohistochemistry and competitive binding experiment

The dominant phage peptide CHAQGSAEC was dealt with in the in vivo thymus binding assay. Three BALB/c mice 6 to 8

weeks were anesthetized, injected with 2.0×10^{11} pfu of phage peptide CHAQGSAEC, and perfused as described above. After that, thymus and control organs (heart, brain, liver, lung, kidney) were removed and washed with ice-cold TBS-PI. All tissues was dissected and embedded in the optimal cutting temperature compound (OCT, Sakura, Torrance, CA) for frozen section. Bound phage peptides were detected by mouse anti-M13 antibody (4 °C overnight, 1:100, GE) and horseradish peroxidase (HRP)–goat antimouse IgG($F(ab')_2$) antibody (37 °C 1 h, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA). After diaminobenzidine exposure, sections were counterstained with hematoxylin before mounting for microscopy. Wild-type phage with no peptide insert and phage peptide CEMQTSTAC recovered from kidney were used as control.

Cyclic heptapeptide CHAQGSAEC was synthesized from Invitrogen (Carlsbad, CA), and it was purified by high-performance liquid chromatography and confirmed by mass spectrometry.

For the competitive binding experiment, synthetic soluble peptide CHAQGSAEC (100 μM/L, 200 μL) was injected into 3 mice for a 15-min in vivo circulation before corresponding phage peptide (phage peptide CHAQGSAEC, 2.0×10^{11} pfu diluted in 200 μL TBS) injection to confirm that the phage peptide CHAQGSAEC specifically was binding to the thymus. Immunohistochemistry was carried out as described above.

Real-time PCR to evaluate activity of thymus output

The thymus-homing synthetic peptide CHAQGSAEC (100 μM/L, 200 μL), corresponding phage peptide (2.0×10^{11} pfu diluted in 200 μL TBS), and phage peptide CEMQTSTAC recovered from kidney (2.0×10^{11} pfu diluted in 200 μL TBS) were injected into 15 BALB/c mice. Then, peripheral blood was acquired by eyeball removal at 15 min, 1 h, 3 h, 6 h, and 24 h from different mice (3 mice representing each time point of the study). DNA was extracted to carry out real-time PCR to evaluate bioactivity of thymus output by TREC expression level.

DNA was extracted using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI). Real-time PCR was carried out using LightCycler1.2 (Roche, Nutley, NJ) in the presence of SYBR Premix Ex Taq (Takara, Shiga, Japan). The reaction conditions of real-time PCR were adopted according to the manufacturer's instructions. Primers were used for the detection and quantification of mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH; reference) and TRECs (target). Specific primers for mouse GAPDH were 5′-GTC TCC TGC GAC TTC AGC-3′ for forward and 5′- TCA TTG TCA TAC CAG GAA ATG AGC-3′ for reverse; primers for TRECs were 5′-CAG AGG GGT GCC TCT GTC A-3′ for forward and 5′-GGA CCC CTC ACA AAG TGT CG-3′ for reverse. The mouse GAPDH and TRECs primers were flanked by 107-bp and 142-bp regions, respectively. For each PCR product, the melting curve was determined to confirm specificity.

Table 1. Increased Selectivity of Thymus-Targeting Phage Peptides after 3 Iterative Rounds of Panning in BALB/c Mice

	First Round of In Vivo Panning	*Second Round of In Vivo Panning*	*Third Round of In Vivo Panning*
Weight of thymus, g	0.0283	0.0228	0.0257
Phage output from thymus, pfu	1.0×10^3	2.0×10^7	6.0×10^{10}
Recovered colonies from thymus, pfu g^{-1}	3.53×10^4	8.77×10^8	2.34×10^{12}
Weight of kidney, g	0.3113	0.2430	0.3019
Phage output from kidney, pfu	8.0×10^5	2.0×10^5	3.0×10^5
Recovered colonies from kidney, pfu g^{-1}	2.57×10^6	8.23×10^5	9.94×10^5

Phage peptides of 2.0×10^{11} pfu were used for each round of in vivo biopanning.

The comparative threshold cycle number ($2^{-\Delta\Delta Ct}$) method was used for quantification after a validation experiment demonstrated that efficiencies of the target (TRECs) and reference (GAPDH) were approximately equal. Ct values defined the threshold cycle of PCR, at which amplified products were detected. ΔCt was the difference in the threshold cycles for mouse TRECs and GAPDH. Results were represented as fold changes ($2^{-\Delta\Delta Ct}$) in TREC expression, which were calculated from ΔCt values of TRECs at different incubation times relative to ΔCt values of time 0. Comparison was made between time 0 and later time points after treatment with the free peptide, corresponding phage peptide, or control phage peptide. The above assays of thymic activity evaluation were performed in triplicate on different occasions. All data were analyzed for the comparison of group means using 1-way analysis of variance; $p < 0.05$ was considered statistically significant.

RESULTS

In vivo panning of phage display random peptide library

Mice thymus-homing phage peptides were isolated from a cyclic heptapeptide phage display library by the in vivo phage display. After 3 iterative rounds of panning in BALB/c mice, titering of phage peptides showed that thymus-targeting phages increased from 3.53×10^4 pfu g^{-1} to 2.34×10^{12} pfu g^{-1}, whereas no increase was observed in kidney (**Table 1**). These results suggested there was a markedly increased selectivity of phage peptides in mouse thymus.

Analysis of peptide sequences

After 3 rounds of in vivo panning, the recombinant phage particles were selected to carry out sequence analysis. Only

Table 2. Analysis of Peptide Sequences after 3 Iterative Rounds of Panning in BALB/c Mice

Organ	*Phage Peptide Sequence*	*Peptide Frequency, %*
Thymus	CHAQGSAEC	100.0
Kidney	CEMQTSTAC	9.1
Kidney	CHSSPTLYC	9.1
Kidney	CQSEVSQLC	9.1
Kidney	CPLITAAFC	9.1
Kidney	CLGNKAHTC	9.1
Kidney	CNKGAGKYC	9.1
Kidney	CSPDLPQRC	9.1
Kidney	CGTNKPWNC	9.1
Kidney	CMPNTLREC	9.1
Kidney	CTPRADRHC	9.1
Kidney	CTQHNPHQC	9.1

peptide sequence CHAQGSAEC was identified from the thymus (**Table 2**). From these results, we conclude that there are enrichments of thymus-homing phage peptides after 3 rounds of in vivo panning. In contrast, there were not any identical sequences among phage peptides recovered from kidney, the control organ (**Table 2**).

Immunohistochemistry and competitive binding experiment

Phage peptide CHAQGSAEC was chosen to confirm its tropism for the thymus by immunohistochemistry. It showed localization to the vasculature of mouse thymic tissue (**Fig. 1A, B**), whereas in control organs (heart, brain, liver, lung, and kidney) of 3 mice injected with phage peptide CHAQGSAEC, staining was not detectable (**Fig. 1C-G**). In contrast, the wild-type phage with no peptide insert and phage peptide CEMQTSTAC recovered from kidney were undetectable in blood vessels of the thymus in the other 2 mice (**Fig. 1H, I**). In addition, injection with synthetic soluble peptide CHAQGSAEC could inhibit phage peptide CHAQGSAEC in binding to the vasculature of the thymus in the other 3 mice (**Fig. 1J**). These results suggest that phage peptide CHAQGSAEC could bind mouse thymus specifically.

Real-time PCR to evaluate bioactivity of thymus output

To explore the bioactivity of peptide CHAQGSAEC on mouse thymus, we performed the real-time quantitative PCR for the specific detection and quantification of mouse TRECs to evaluate the activity of the thymus output. For each PCR product, the melting curve was determined to confirm specificity. The dimer of TREC primers appeared in a tube without templates, whereas only specific products appeared in a regular reactive tube (**Fig. 2**).

Fold changes in expression of TRECs, which were calculated from the arithmetic formula $2^{-\Delta\Delta Ct}$, showed that compared with phage peptide CEMQTSTAC recovered from kidney, phage peptide CHAQGSAEC and free peptide CHAQGSAEC can downregulate the expression of TRECs at different reactive time points ($F = 103.4$, $p < 0.05$; **Fig. 3**). These results suggest that CHAQGSAEC could inhibit the bioactivity of thymus output.

DISCUSSION

Here, we describe peptide sequence CHAQGSAEC—identified by in vivo screening of the phage display random peptide library—that is capable of homing selectively to the vasculature of the mouse thymus and inhibiting bioactivity of thymus output.

Two research groups have isolated thymic stromal cell-specific antibodies using thymic tissue or thymic epithelium with the phage display,[13,14] but many of these antibodies recognize epitopes that are located on the surface of cell, and these phenotypes of cells are unstable when the cells are removed from their microenvironment.[15] The in vivo method, which can mimic protein-protein interactions in living organisms, may solve this problem.

The method of in vivo phage display has several important advantages. First, phage-displayed peptides isolated by this functional screening can home selectively to a site of interest. Second, in vivo panning includes an inherent "blocking step": peptides that recognize plasma and ubiquitous cell surface proteins are depleted from the selected pool. This counterselection can make peptide ligands show selectivity to the vasculature of a particular organ. Third, in vivo phage display has been shown to select peptides that bind to unknown endothelial molecules, some of which may be expressed selectively in the vasculature of specific tissues.[16,17] In addition, several advantages of peptides over antibodies, as mentioned earlier, may helped for the drug candidates in vivo.

The thymus in adults has a high value in research. Although the function of the thymus may be halted after puberty, it may serve as a place to preserve the autoreactive T cell repertoire for the whole life, which is confirmed by the frequent association of thymoma with MG. Moreover, the high degree of molecular conservation in the thymus microenvironment between mouse and human has been revealed.[18]

In this study, we have perfused the mice through the heart after the injected phage peptides had circulated. We used 15 min for circulation of phage peptides in vivo because we hoped that phage peptides would circulate during the longer period of time, compared with 5 to 10 minutes carried out by other research groups, and this may be not only stable in the circulation but also penetrative in the target organ. Moreover, the perfusion with a large volume of TBS can increase the stringency

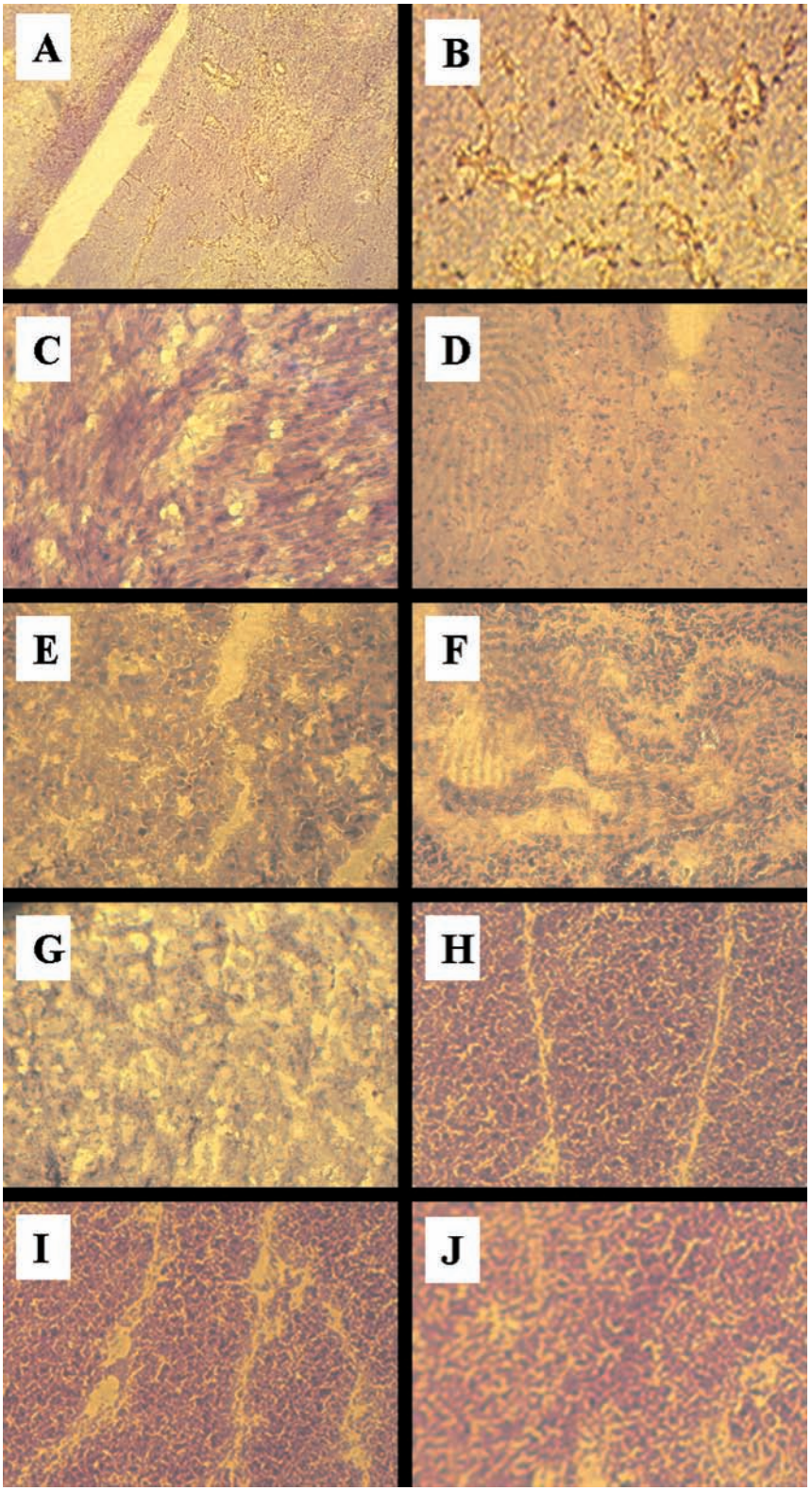

FIG. 1. Phage peptide binding assay by immunostaining. (**A, B**) Phage peptide CHAQGSAEC showed localization to the vasculature of mouse thymic tissue. (**C-G**) Control organs, such as heart, brain, liver, lung, and kidney, of mice injected with phage peptide CHAQGSAEC; staining was not detectable. (**H**) Wild-type phage with no peptide insert was undetectable in blood vessels of the thymus. (**I**) Phage peptide CEMQTSTAC recovered from kidney was undetectable. (**J**) Injection with the corresponding synthetic soluble peptide CHAQGSAEC can inhibit phage peptide CHAQGSAEC in binding to the vasculature of the thymus. (**A**) 100×; (**B-J**) 400×.

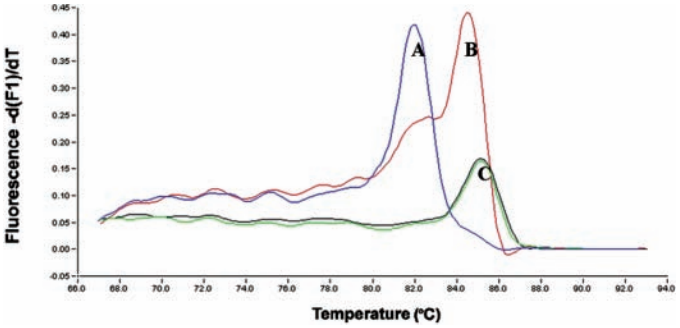

FIG. 2. The melting curves were determined to confirm specificity of PCR products. (**A**) Dimer of T cell receptor excision circle (TREC) primers in a tube without templates. (**B**) PCR product of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in a regular reactive tube. (**C**) PCR product of TRECs in a regular reactive tube.

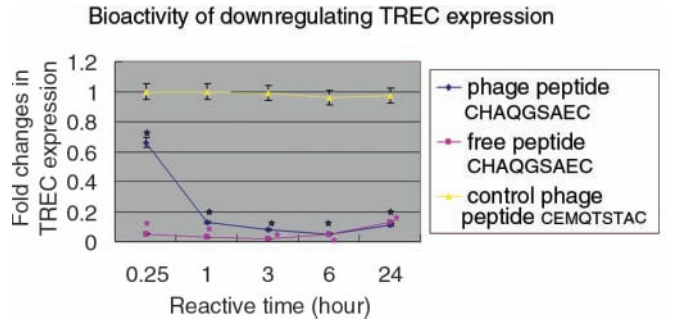

FIG. 3. Examination of phage peptide and free peptide effect on T cell receptor excision circles (TRECs). Peptide effect on the thymus was evaluated by real-time PCR after injection into mice. Time courses after treatment with the free peptide, corresponding phage peptide, or control phage peptide are shown. Fold changes ($2^{-\Delta\Delta Ct}$) in TREC expression were calculated from ΔCt values of TRECs at different incubation times relative to ΔCt values of time 0. Comparison was made between time 0 and later time points. ΔCt values of TRECs were obtained after subtraction of Ct values for mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH; $p < 0.05$).

of the screen and reduce the nonspecific background of phage recovery. Thus, screening for peptides that home specifically to the thymus may be possible.

We completed 3 iterative rounds of panning in BALB/c mice. In fact, after 2 rounds of in vivo panning, the recombinant phage particles were selected to carry out sequence analysis. Two individual phage peptides homing to the thymus were identified, which shared the consensus motif GSA. The frequency of peptide CHAQGSAEC is 76.9%, and the frequency of peptide CLGSARATC is 23.1%. After 3 rounds of in vivo panning, there remained only 1 peptide sequence, CHAQGSAEC. From this phenomenon, we think the peptide sequence flanking GSA also affected its binding. Peptide CHAQGSAEC may have more powerful binding ability upon selection and washing. These results of sequence analysis also confirmed the enrichments of thymus-homing phage peptides round by round, which were suggested by the counters of the plaque-forming unit. After that, binding specificity was confirmed by direct binding assays and competitive binding experiments in different mice.

We also performed a primary bioinformatics study with the found CHAQGSAEC peptide (data not shown) and discovered some clues about proteins expressed on the cell surface. But we believe that they should be confirmed by extracting potential candidate proteins in our following study.

In the evaluation of thymus output activity, the level of TRECs in the peripheral blood was used as a measurement of recent thymic output activity. As an immunological organ, the thymus requires a large diversity of immunoglobulins (Ig) and T cell receptors (TCRs) to screen the body for pathogens. Such diversity is achieved by rearranging preexisting segments of the Ig or TCR genes randomly. This process involves the excision of intervening segments of DNA, the ends of which are subsequently ligated to form TRECs. The level of TRECs in peripheral blood provides a surrogate measure of thymic activity. Measurement of TRECs in peripheral blood has been used to study thymus output in mice and humans.[19] In this study, we used a real-time quantitative PCR assay for the specific detection and quantification of mouse TRECs.

In conclusion, this is the report of the in vivo selection of a phage display random peptide library in mice for the thymus-homing peptide. Specific binding of phage peptide CHAQGSAEC was justified, which indicates nonrandom thymus accumulation. Moreover, peptide isolated from this study can inhibit the bioactivity of thymus output. This study indicates the feasibility of this peptide for possible function as a kind of tool to inhibit thymus bioactivity or as a targeted compound for targeted medicine.

ACKNOWLEDGMENTS

We thank Wei Qu, Yan Li, and Yan Su (Nanjing Clinical Nuclear Medicine Center, Nanjing First Hospital affiliated to Nanjing Medical University) for technical assistance with the animal experiments.

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Address correspondence to:
Zizheng Wang
Department of Nuclear Medicine
Nanjing Clinical Nuclear Medicine Center
Changle Road 87, Nanjing, Jiangsu, China

E-mail: zzwang136@yahoo.com.cn