

Screening and identification of mimotope of gastric cancer associated antigen MGb1-Ag

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Abstract

AIM: Using a monoclonal antibody against gastric cancer antigen named MGb1 to screen a phage-displayed random peptide library fused with coat protein pIII in order to get some information on mimotopes.

METHODS: Through affinity enrichment and ELISA screening, positive clones of phages were amplified. 10 phage clones were selected after three rounds of biopanning and the ability of specific binding of the positive phage clones to MGb1-Ab were detected by ELISA assay (DNA sequencing was performed and the amino acid sequences were deduced) By blocking test, specificity of the mimic phage epitopes was identified.

RESULTS: There were approximately 200 times of enrichment about the titer of bound phages after three rounds of biopanning procedures. DNA of 10 phage clones after the third biopanning was assayed and the result showed that the positive clones had a specific binding activity to MGb1-Ab and a weak ability of binding to control mAb or to mouse IgG. DNA sequencing of 10 phage clones was performed and the amino acid sequences were deduced. According to the homology of the amino acid sequences of the displayed peptides, most of the phage clones had motifs of H(x)Q or L(x)S. And these 10 phage clones could also partly inhibit the binding of MGb1-Ab to gastric cancer cell KATO-III. The percentage of blocking was from (21.0±1.6) % to (39.0±2.7) %.

CONCLUSION: Motifs of H(x)Q and L(x)S selected and identified show a high homology in the mimic epitopes of gastric cancer associated antigen. There may be one or more clones which can act as candidates of tumor vaccines.

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INTRODUCTION

Filamentous bacteriophages have been used extensively in recent years for the display large repertoires of peptides on their surface^[1-3]. These peptides can be expressed by cloning random oligonucleotides at the end of the genes encoding the phage coat proteins^[4]. Phage display system has many advantages such as efficacy of biopanning, ability of amplification and linkage of the displayed peptide with its encoding DNA sequence within the phage particle, enabling quick and simple elucidation of binding sequences. Numerous papers have shown that this approach is successful with an extraordinarily wide range of protein targets, such as structural proteins, signal transduction proteins, receptors, serum proteins, oncoproteins and so on. It is also possible to select phage display peptides that mimic the original characteristics of antigen binding to specific antibodies without previous knowledge of the antigen structure^[5-9]. In this respect, phage display technology has been well established as an important experimental approach in the development of novel vaccines and drugs. Antigens and epitopes play important roles in immune responses to tumors. To select the possible mimotopes of gastric cancer, a random phage display peptide library constructed on pIII was screened by biopanning with MGb1-Ab, a monoclonal antibody against gastric cancer, as a selective molecule.

MATERIALS AND METHODS

Materials

Monoclonal antibody The hybridoma cell line which secretes mouse mAb MGb1 (its immunoglobulin type is IgG) against gastric carcinoma -associated antigens was established by our laboratory. This hybridoma was prepared by taking human gastric carcinoma cell line KATOIII as antigen, and the immunized mouse spleen cells were fused with SP2/0 according to routine procedures for mAb preparation^[10-15]. The mAb in ascitic fluid was prepared by intraperitoneal injection of hybridoma cells into Balb/c mouse and collected at the aseptic environment. Then the mAb was purified by saturated ammonium sulfate precipitation and DEAE-52 anion exchange chromatography^[16,17].

Random peptide library and bacteria The library used was purchased from New England Biolabs Company, containing approximately 2.8×10^9 different phage clones, which composed of the genome of the filamentous phage. The phages in this library were engineered to express a recombinant form of gene III containing a degenerated DNA insert encoding random 7-mer peptide. The recombinant gene III was under the control of LacZ promoter. And all the sub-major coat proteins pIII were recombinants. The ER2537 strain of *E.coli*

was used for culture of phage. Bacteria were cultured in LB medium without any antibiotics.

Methods

Amplification of phages Infections were carried out by incubating phages for 10-15 min at room temperature with ER2537 at the number ratio of 3-5:1 (bacteria: phages). And the bacteria were cultured for another 4.5 h at 37 °C. The supernatants from the culture where phages existed were collected. The culture supernatants of infected bacteria were collected, where bacteria secreted phages. The solution of 200 g·L⁻¹ PEG 2.5 mol·L⁻¹ NaCl was added into the supernatant at the volume ratio to supernatant of 1:5. And the mixture was incubated for 1 h at 4 °C. After centrifuged at 10 000 g, the precipitated phages were pelleted, and then resuspended in Tris-buffered saline (TBS). The protocols of PEG precipitation were repeated. Phages from a culture supernatant volume of 5 mL were usually resuspended in a final volume of 150 µL TBS. The titer of the phages was then assayed.

Assay of phage titer The prepared phages were 10-fold serial diluted in LB with the suggested dilution ranges: 10⁸-10¹¹ for amplified phage culture supernatants and 10¹-10⁴ for unamplified biopanning eluates. A single colony of ER2537 in 5 mL LB was cultured with shaking until mid-log phase. 200 µL of bacteria culture reached mid-log phase was dispensed into microfuge tubes and then 10 µL of diluted phages per tube was vortexed quickly and incubated at room temperature for 1-5 min. Bacteria infected with phages were transferred to a culture tube containing 3 mL 45 °C LB/agarose at the top and immediately poured onto a pre-warmed LB/IPTG/X-gal plate and spread evenly after vortexed. Plates were cooled for 5 min, inverted and incubated overnight at 37 °C. Plaques on plates were counted.

Biopanning Three rounds of biopanning were carried out with mAb-MGb1. During the first round, a ELISA well was used as the solid phase. It was coated by incubating overnight with aliquots of mAb (100 mg·L⁻¹) in the coating buffer (carbonate-bicarbonate buffer, pH9.6) at 4 °C in a humid atmosphere, then washed with TBS for 6 times, blocked in TBS-10 g·L⁻¹ BSA. One aliquot of the library containing 2×10¹¹ pfu phages in 100 µL TBS-0.5 g·L⁻¹ BSA was added to the mAb coated well, incubated for 1 h at 37 °C. Unbound phages were removed and the well was washed 6 times with TBS-1 g·L⁻¹ Tween-20. Eluting buffer (0.2 mol·L⁻¹ glycine with its pH adjusted to 2.2 by HCl) was added for 10 min, then removed and neutralized by adding of Tris-HCl (pH 9.0). Phages eluted from each round were used to infect exponential phase ER2537. After cultured for 4.5 h, phages in the supernatant were precipitated by PEG. The second and third rounds of biopanning were followed as described above except that unbound phages were washed 20 times with TBS-0.5 % Tween-20. After neutralization in the third round of biopanning, the eluted phages were planted on the LB medium directly and cultured overnight at 37 °C.

ELISA to identify positive phage clones ELISA was carried out as routine procedures^[18-26]. ELISA wells were coated by incubating overnight with mAb-MGb1 (100 mg·L⁻¹) in the coating buffer (carbonate-bicarbonate buffer, pH9.6) at 4 °C in a humid atmosphere, washed 6 times with TBS, blocked with TBS-50 g·L⁻¹ BSA. Single clone of phage expressing the recombinant form of gene III containing the peptide insert was incubated in LB for 4.5 h. The coated wells were incubated for 1h at 37 °C with supernatant from such cultures and then the horseradish peroxidase (HRP) labeled mouse-anti-M13 antibody diluted to a volume ratio of 1:5 000 in blocking buffer was added. TMB was used as a substrate for HRP and the absorbance of each well was read at 450 nm.

Extraction of single strand DNA of phages and DNA

sequencing Single strand DNA was prepared from 1.5 mL overnight cultures by extraction and purification by using a single strand of M13 extraction and purification kit (Huashun Bio. Com., China). The culture supernatant of infected bacteria containing phages at the volume of 1.2 mL was transferred to sterile 1.5 mL Eppendorf tubes. 200 µL precipitate buffer was added. The mixture was cooled on ice for 15 min. After centrifugation for 5 min at 10 000 g, the supernatant was discarded and the precipitate was preserved for DNA extraction. Phages were lysed in 500 µL lysis buffer for 2 min at room temperature. The product was transferred to a column containing resin that could absorb DNA. After centrifugation for 15 s, DNA absorbed in resin was washed twice with ethanol and eluted with 20 µL Tris buffer. Single strand DNA of phages was extracted and 5 µL of product was run on a 10 g·L⁻¹ agarose gel in 1×TAE. DNA bands were detected by ethidium bromide staining and visualized by UV light photography. Sequencing procedures were carried out by using a auto-sequence apparatus ABI PRISM310 (PE Com. U.S.A.). 100 ng of such single strand DNA was used as a template. The oligonucleotide 5' - CCC TCA TAG TTA GCG TAA CG-3', complementary to the genomic DNA of the phage and the downstream of the insert, was used as a primer.

Blocking test ELISA wells were coated with KATOIII (1×10⁵ cells per well), washed 6 times with PBS, blocked for 2 h. MAb MGb1 was mixed with 1×10¹⁰ pfu individual phages for 1 h at 37 °C and then the mixture was added into the KATOIII coated wells, incubated for another 1 h. The HRP labeled goat-anti-mouse IgG diluted to a volume ratio of 1:5 000 in the blocking buffer was added then. TMB was used as a substrate for HRP and the absorbance of each well was read at 450 nm^[27].

RESULTS

Biopanning

The ratios of output/input phages in three biopanning rounds are listed in Table 1. The ratio of the last round was about 100 times higher than that of the first round. It indicated that the specific phages were enriched.

Table 1 Yields of washed phages

| Round | Input phages(pfu) | Output phages(pfu) | Yields/% |
|-------|----------------------|---------------------|----------------------|
| 1 | 2.0×10 ¹¹ | 5.8×10 ⁴ | 2.9×10 ⁻⁵ |
| 2 | 2.0×10 ¹¹ | 3.2×10 ⁵ | 1.6×10 ⁻⁴ |
| 3 | 2.0×10 ¹¹ | 4.0×10 ⁶ | 2.0×10 ⁻³ |

Selection of phage clones

MGb1 mAb with specificity to gastric carcinoma was used to screen the library of phages containing random 7-mer peptide inserts. After three rounds of biopanning, some individual phage clones were isolated and screened by ELISA to identify those of interests which bound strongly to the antibody, and 10 clones of them which gave a clear positive signal and had the highest absorbance at 450 nm were selected.

Detection of antigenic specificity of the final-eluted phage by sandwich ELISA

MGb1 mAb was immobilized on the ELISA wells in which the selected phage clones were added. HRP labeled anti-M13 mAb was then used to detect the captured phages. As negative control, anti-endotoxin mAb and mouse IgG were used to replace the coating and the binding antibodies. The results revealed that the selected phages could react to mAb MGb1 specifically, and had weak ability of binding to unrelated antibodies such as anti-endotoxin mAb and mouse IgG (Figure 1).

Extraction of single strand DNA of phages

The length of all phage clones was the same as M13 single strand DNA marker, which indicated that single strand DNA of phages was prepared well and there were no other kinds of phages contaminated (Figure 2).

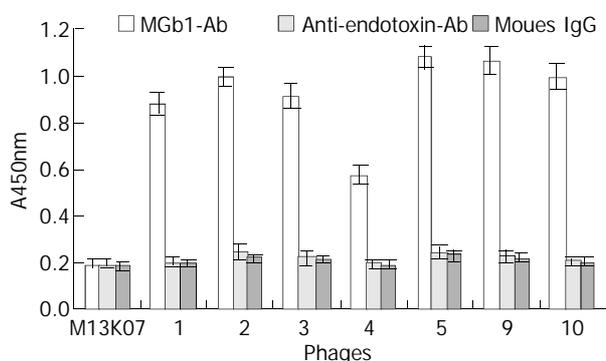


Figure 1 Binding of phages to MGb1-Ab by ELISA at the third round.

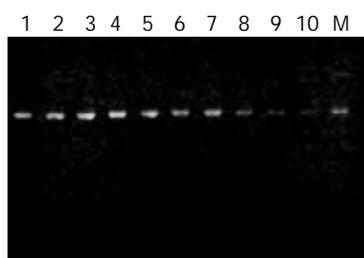


Figure 2 Electrophoresis of single strand DNA of phage clones (lanes 1-10: Single strand DNA of phage clones; lane M: M13 single strand DNA marker).

Identification of motifs amongst the sequences of phage clone inserts

The selected 10 clones were sequenced and motifs could be identified amongst the deduced amino sequences of phage clone inserts for mAb MGb1. According to the homology of amino acid sequences of the displayed peptides, some preserved mimotope information was obtained. Most phage clones had motifs of H(x)Q and L(x)S (Figure 3).

K 5: VPQQKFR
 K 7: VPQQKFR
 K 2: HSQISY
 K10: QPTHOLT
 K1: QHQLPSD
 K3: SLLSTPQ
 K6: SLLSTPQ
 K8: SLLSTPQ
 K9: LRPTLSC
 K4: LPMRPIV

Figure 3 Amino acid sequences of inserts in phage clones.

Blocking test

If the selected clones contained epitopes or mimotopes of the native antigen, then they should be presented to block the binding of mAb MGb1 to gastric cancer cell-line KATOIII. To test this hypothesis, 7 of representative phage clones were used in a blocking test. Wild-type phage M13 was used as a negative control (whose absorbance at 450 nm was represented by B) to ensure that blocking of binding of the mAb to gastric cancer cell-line KATOIII was due to the phage insert (whose

absorbance at 450 nm was represented by A) rather than the mere physical presence of phages. The blocking percentage of each phage was $(25.6 \pm 1.3) \%$, $(34.6 \pm 2.5) \%$, $(32.6 \pm 1.8) \%$, $(21.0 \pm 1.6) \%$, $(39.0 \pm 2.7) \%$, $(36.3 \pm 3.2) \%$, $(29.5 \pm 1.9) \%$, respectively (Figure 4), compared with wells to which no phage was added (whose absorbance at 450 nm was represented by C), which was calculated using the formula: blocking % = $[(C-B)-(C-A)]/A \times 100 \%$.

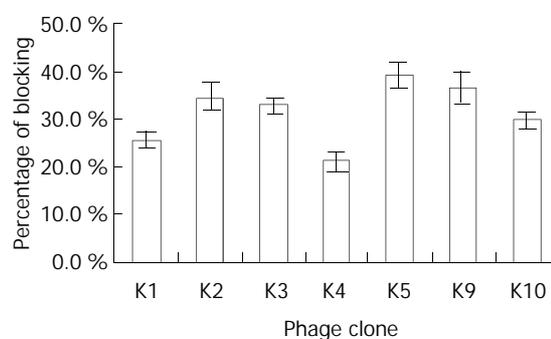


Figure 4 The blocking percentage of phage display peptides to MGb1-Ab by ELISA.

DISCUSSION

Gastric cancer is one kind of malignant tumors with complicated mechanism and is the leading cause of death in China^[27-51]. Biotherapy is a new way for human to combat gastric cancer^[52-63]. Tumor antigens play important roles in the induction of immune responses^[64-66], which are predominantly focused on specific antigenic sites, known as epitopes. Identification of B-cell epitopes can be used to design peptide vaccines against tumor and is actively pursued in many laboratories. Since antibodies recognize B-cell epitopes that are mainly located on the surface of molecules, the native conformation of the antigen is a critical parameter for this interaction to occur. Early studies on the antigenicity of globular proteins suggested that antibodies recognized amino acids which either constituted a short linear stretch along the polypeptide chain (continuous or linear B-cell epitopes), or were brought together by the juxtaposition of the polypeptide chains when the protein was in its native conformation (discontinuous or conformational B-cell epitopes)^[67].

Considerable attention has been paid to the difficulties in identifying the structural characteristics of B-cell epitopes. A number of empirical approaches have been employed to predict B-cell epitopes from the primary amino acid sequence of the protein including identification of regions of hydrophilicity, solvent accessibility, protrusion, atomic mobility and secondary structures. But many of them identify linear B-cell epitopes instead of discontinuous epitopes. B-cell epitopes are entities that can be defined only by their mutual complementarity. Phage display random peptide library offers a convenient way to select phage display peptides that mimic the original characteristics of antigen binding to specific antibodies^[68,69]. One of the advantages of screening random peptide libraries to identify B-cell epitopes is that there is no need to know the amino acid sequence of the protein against which the antibody has been elicited. Since peptide libraries provide a rich source of mimic antigens, identification of B-cell epitopes has been greatly simplified.

In the present study, to select the possible mimotopes of gastric cancer, a random phage display peptide library constructed on pIII was screened by biopanning using MGb1-Ab as a selective molecule. After three biopanning procedures, there was a remarkable enrichment in the titer of bound phages.

Then, 10 phage clones were selected from the third biopanning and ELISA. Their single strand DNA was sequenced and the amino acid sequences were deduced. According to the homology of amino acid sequences of the displayed peptides, some preserved epitope information was obtained. Most phage clones had motifs of H(X)Q or L(X)S. The results of ELISA showed that the positive phage clones had a specific binding activity with MGb1-Ab and could inhibit the binding of MGb1-Ab to gastric cancer cell line KATO-III.

Since the binding site of an antibody is not unique for a single antigen, several mimotopes with different amino acid sequences can be recognized by binding to different subsites within the binding site^[70-72]. To date, little is known about the structure and amino acid sequence of parental antigen MGb1, so we can not tell the exact characteristics of the peptides selected from the 7-mer random peptide library. Research should be done to further characterize the mimotopes recognized by mAb MGb1.

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