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• VIRAL HEPATITIS •

Preparation of human single chain Fv antibody against hepatitis C virus E2 protein and its identification in immunohistochemistry

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Abstract

AIM: To screen human single chain Fv antibody (scFv) against hepatitis C virus E2 antigen and identify its application in immunohistochemistry.

METHODS: The phage antibody library was panned by HCV E2 antigen, which was coated in microtiter plate. After five rounds of biopanning,56 phage clones were identified specific to HCV E2 antigen. The selected scFv clones were digested by SfiI/NotI and DNA was sequenced. Then it was subcloned into the vector pCANTAB5E for expression as E-tagged soluble scFv. The liver tissue sections from normal person and patients with chronic hepatitis B and chronic hepatitis C were immunostained with HCV E2 scFv antibody.

RESULTS: The data of scFv-E2 DNA digestion and DNA sequencing showed that the scFv gene is composed of 750 bp. ELISA and immunohistochemistry demonstrated that the human single chain Fv antibody against hepatitis C E2 antigen has a specific binding character with hepatitis virus E2 antigen and paraffin-embedded tissue, but did not react with liver tissues from healthy persons or patients with chronic hepatitis B.

CONCLUSION: We have successfully screened and identified HCV E2 scFv and the scFv could be used in the immunostaining of liver tissue sections from patients with chronic hepatitis C.

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INTRODUCTION

Hepatitis C virus (HCV) has been identified as the major etiological agent of post-transfusion non-A non-B hepatitis[1-10], responsible for most cases of non-A non-B hepatitis. Hepatitis C is a disease of clinical importance because of its high infection rate in blood donors and its persistence as chronic infections which may lead to cirrhosis and hepatocellular carcinoma in the long term[11-26]. The variability of the HCV genome has difficulties in serological detection and vaccine design. Recent advance in phage technology offers a means of cloning human anti-HCV antibodies of a defined specificity that may have potential therapeutic use[27]. We now report the generation of phage display antibody using phage antibody library. From this library we obtained specific single-chain Fv antibody that recognizes the viral envelope protein E2, using HCV E2 protein as the immobilized antigen and proceeding immunohistochemistry.

MATERIALS AND METHODS

Materials

Humanized scFv antibody phage library in which the variable region coding gene of VL and VH were amplified by polymerase chain reaction (PCR) with degenerate primers and connected with a glycine linker [(Gly4Ser)3] was widely used in the screen and identification of humanized scFv to various antigens[28-34]. The recombinant HCV E2 protein was purchased from Virostat Co, USA. Phage M13K07 was purchased from Pharmacia Co., Sweden. Other reagents used in this experiment are all domestic products of analytical grade.

Biopanning

The phage library was amplified in 37 ℃. The host *E. coli* TG1was infected with phage M13K07 and incubated at 37 ℃ for 12 hours, the phage in the supernatant was harvested and concentrated by PEG. Culture plate (Nunc) was coated with recombinant HCV E2 protein at the concentration of 80 mg/L. The coating buffer was 0.05 mol/L $NaHCO_3$, pH 9.6. The plate was blocked with BSA at the concentration of 20 g/L for 2 hours and the concentrated phages were added to the well of the plate, incubated at the room temperature for 90 min. The plates were washed 20 times with PBST and PBS buffer respectively. The bound phage was eluted by the 0.1M of triethylamine, and neutralized with 1M Tris buffer (pH 7.4). Recovered phages were used to infect the host *E. coli* TG1 at the log phase growth and HCV E2 protein-binding phages were amplified. The procedure of absorption-elution-amplification was repeated 5 times.

Identification of phage clones

After 5 rounds of biopanning, 56 phage clones were selected randomly. The clones grew in 400 μl 2×TY-AMP-Glu at 37 ℃ overnight. The culture was transformed to another Eppendorf tube when its A600 nm reached 0.5. The culture was continued at 30 ℃ overnight after adding helper phage. ELISA for determining the supernatants was repeated at least two times. The cross-reaction of the supernatants to the BSA antigen was conducted. According to the ELISA results to the HCV E2 and BSA, one clone with high reaction to HCV E2 and low reaction to BSA was selected.

Sequencing analysis

The plasmid DNA was prepared using Wizard plus minipreps DNA Purification System (Promega Co., USA) and sequenced using ABI automated DNA sequencing machine.

Expression of human HCV E2-scFv in E.coli

The selected HCV E2 scFv clone was subcloned as SfiI/NotI fragments into the vector pCANTAB5E for expression as E-tagged soluble scFv. DNA digestion and electrophoresis confirmed the recombinant vector pCANTAB5E-E2-scFv. Competent *E. coli* XL1-Blue was transformed with pCANTAB5E-E2-scFv and transformed XL1-Blue was induced by IPTG for 20 h. The *E. coli* was harvested by centrifugation at 10 000rpm. The culture supernatant was rendered for ELISA test according to the standard procedure. In ELISA detection, Nunc plate was coated with 1μg/well of recombinant HCV E2 antigen and blocked with 2 % bovine serum albumin (BSA) at 37 ℃ for 2 h. The supernatants from induced and non-induced transformed *E. coli* were added and incubated at 37 ℃ for 2 h. The plate was washed with PBS buffer, and 100 μl of HRP/anti-E Tag 1:4000 ratio diluted in PBS buffer containing 1 % BSA was added, and incubated at 37 ℃ for 1 h. The substrate solution was added and A450nm value was measured.

Immunohistochemical identification of scFv in liver tissue

Paraffin-embedded liver tissue slices were from patients with positive anti-HCV antibodies and HCV-RNA. After deactivating endogenous hyperoxidase, these slices were submersed in the methanol solution with 0.5 % H_2O_2 in the room temperature. Fifty min later, they were washed with PBS buffer for 3 times,5 % BSA was added and slices were stored overnight at 4 ℃. Self-made scFv primary anti- HCV E2 single-chain antibodies were diluted at 1:100 ratio and added on to the slice. They were kept in the 37 ℃ incubator for 1 h, then 4 ℃ refrigerator overnight. HRP-sheep anti-M13 antibodies (diluted to 1:200) solutions were dropped on to the tissue sections, incubated at 37℃ for 40 min. After washing 3 times with PBS buffer, DAB solutions (9 mg DAB, 13.5 ml Tris.cl, 1.5 ml $CoCl_2$, 15 μl 30 % H_2O_2) were dropped on to the tissue sections at room temperature. After ten minutes, the slices were washed with PBS buffer for 3 times again, and 1 % heamatin solution was used to stain the cell nucleus. Gradient ethanol was utilized to dehydrate and dimethylbenzene to clear the sections, then neutral resin to envelope them. The resultant slices were observed under microscope. The controls were set as follows: 1) PBS buffer instead of anti- HCV E2 scFv; 2) HBsAg, HBcAg double-positive liver tissue sections; and 3) Normal liver tissue sections.

RESULTS

Screening and identification phage clones

Using HCV E2 protein as immobilized antigen, the humanized scFv phage library was biopanned. After 5 rounds of biopanning, 56 phage clones were selected randomly. ELISA and cross-reaction of these clones to BSA confirmed their specificity to HCV E2. Among the 56 phage clones, 16 showed good reactivity to the recombinant HCV E2 protein with high A value in the ELISA. In the cross-reaction screen, 6 among the 16 showed low cross-reaction with BSA. The combined results indicated that 1 of the 6 showed the highest reaction to HCV E2 protein and lowest reaction to BSA. One clone has been selected for further DNA digestion and sequence analysis. The DNA sequence digestion was made by SfiI/NotI in Figure 1. Its nucleic acid sequence and deduced amino acid sequence about HCV-E2-scFv fragment are shown in Figure 2.

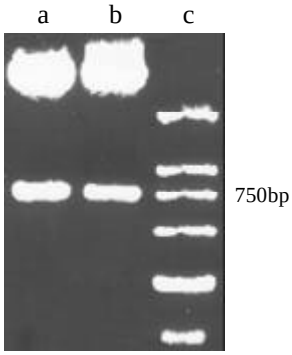

Figure 1 Restriction map of HCV E2-scFv by SfiI/NotI digestion. A,B: HCV E2-scFv; C: DNA Marker

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ATG GCC CAG GTG CAG CTG GTG CAG TCT GGG GCT GAG GTG AAG AAG CCT GGG GCC
 M  A  Q  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  A
TCA GTG AAG GTT TCC TGC AAG GCT TCT GGA TAC ACC TTC ACT AGC TAT GCT ATG
 S  V  K  V  S  C  K  A  S  G  Y  T  F  T  S  Y  A  M
CAT TGG GTG CGC CAG GCC CCC GGA CAA AGG CTT GAG TGG ATG GGA TGG ATC AAC
 H  W  V  R  Q  A  P  G  Q  R  L  E  W  M  G  W  I  N
GCT GGC AAT GGT AAC ACA AAA TAT TCA CAG AAG TTC CAG GGC AGA GTC ACC ATT
 A  G  N  G  N  T  K  Y  S  Q  K  F  Q  G  R  V  T  I
ACC AGG GAC ACA TCC GCG AGC ACA GCC TAC ATG GAG CTG AGC AGC CTG AGA TCT
 T  R  D  T  S  A  S  T  A  Y  M  E  L  S  S  L  R  S
GAA GAC ACG GCC GTG TAT TAC TGT GCA AGA TCG AGT GGG CCG ATG CAT CGT GAG
 E  D  T  A  V  Y  Y  C  A  R  S  S  G  P  M  H  R  E
TGG GGC CAA GGT ACC CTG GTC ACC GTG TCG AGA GGT GGA GGC GGT TCA GGC GGA
 W  G  Q  G  T  L  V  T  V  S  R  G  G  G  G  S  G  G
GGT GGC TCT GGC GGT GGC GGA TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT
 G  G  S  G  G  G  G  S  S  E  L  T  Q  D  P  A  V  S
GTG GCC TTG GGA CAG ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC
 V  A  L  G  Q  T  V  R  I  T  C  Q  G  D  S  L  R  S
TAT TAT GCA AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC
 Y  Y  A  S  W  Y  Q  Q  K  P  G  Q  A  P  V  L  V  I
TAT GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC AGC
 Y  G  K  N  N  R  P  S  G  I  P  D  R  F  S  G  S  S
TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA GAT GAG GCT
 S  G  N  T  A  S  L  T  I  T  G  A  Q  A  E  D  E  A
GAC TAT TAC TGT AAC TCC CGG GAC AGC AGT GGT AAC CAT GTG GTA TTC GGC GGA
 D  Y  Y  C  N  S  R  D  S  S  G  N  H  V  V  F  G  G
GGG ACC AAG CTG ACC GTC CTA GGT GCG GCC GCA GAA CAA AAA CTC ATC TCA GAA
 G  T  K  L  T  V  L  G  A  A  A  E  Q  K  L  I  S  E
GAG GAT CTG AAT GGG GCC GCA TAG
 E  D  L  N  G  A  A  *
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Figure 2 Nucleic acid and deduced amino acid sequences of scFv for HCV E2 protein GenBank accession number for this sequence is AF317001

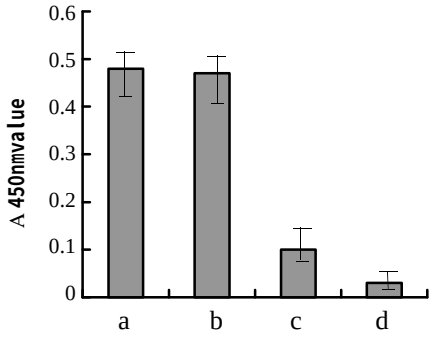

Figure 3 Absorbances of HCV-E2-scFv binding to E2 antigen by ELISA. (a). supernant from induced XL1-blue transformed with pCANTAB5E-E2-scFv; (b). posive control; (c). supernant from non-induced XL1-blue transformed with pCANTAB5E-E2-scFv, d. negtive control

Expression of human HCV-E2-scFv in E. coli.

The expressed HCV-E2-scFv antibody from *E. coli* XL1-blue transformed by pCANTAB5E and induced by IPTG was confirmed by ELISA as shown in Figure3. The recombinant HCV E2 antigen was taken as the positive control. The protein from induced and non-induced *E. coli* XL1-blue transformed by expression vector was positive. But the protein derived from the *E. coli* non-transformed by pCANTAB5E- HCV E2- scFv was negative. These results indicated that the soluble form of human HCV E2- scFv antibody has been successfully expressed in this procedure.

Immunostaining of HCV E2 antigen of liver tissue sections

The different sections from liver tissues of healthy persons and patients with chronic hepatitis B or C were immunostained. The positive immunostaining was seen only in the liver tissue section of patients with chronic hepatitis C, but not in the liver tissue of normal person and patients with chronic hepatitis B as seen in (Figure 4A). The HCV E2 antigen was mainly located in the cytoplasm of the hepatocytes infected by HCV virus (Figure 4B).

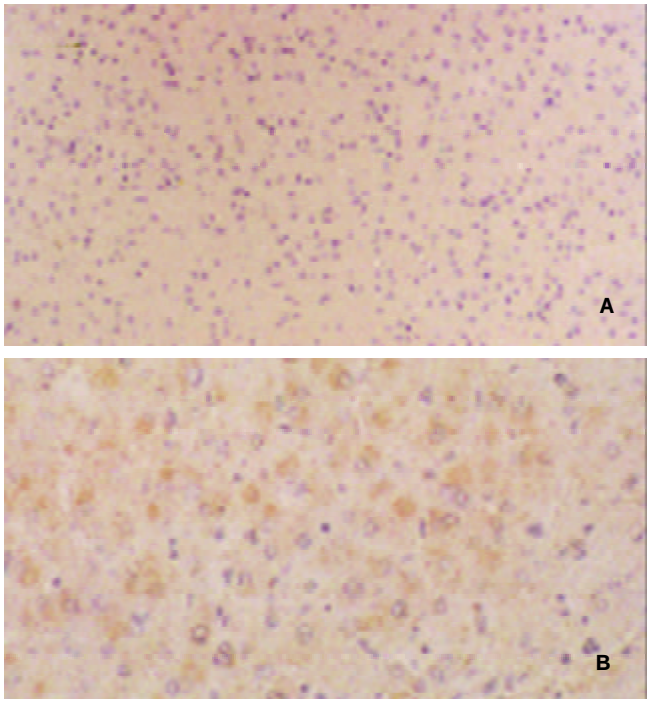

Figure 4 A. Immunohistochemistry of liver tissue from health person. B. Immunohistochemistry of liver tissue from patients with chronic hepatitis C E2 antigen was detected in the cytoplasm of some liver cells

DISCUSSION

HCV is the etiological agent responsible for most cases of non-A non-B hepatitis. It is a blood-related disease of clinical importance since a large number of cases developed chronic infections which may lead to cirrhosis and hepatocellular carcinoma. The variability of the HCV genome has posed serious problems in serological detection of HCV and vaccine design[27-38]. The possibility of using neutralizing antibodies in passive immunization is also hampered by the existence of genotypes and quasispecies[39-50]. To circumvent this, it is possible to envisage the use of cross reactive antibodies with a broad reactivity directed against functionally conserved surface domains common to various genotypes. There has been *in vitro* and *in vivo* evidence that HCV infection elicits a neutralizing antibody response in humans. The virus envelope protein is the primary target for the host immune system. Despite the high variability of the HCV envelope sequence, certain domains of biological importance, e.g. ligands required in viral attachment to host cell receptors, have to be preserved. Antibody specific for the envelope E2 protein has been shown to be cross-reactive HCV E2 and to be able to block viral attachment to cultured human fibroblast cells. However, attept to obtain human monoclonal anti -HCV antibodies is an urgency.

Phage library is a powerful tool for the selection of important and useful antibody specificities espeacially for getting humanized scFv[51-53]. It has many advantages. First, it is the only method to get specific antibody by passing the immunization step. It can mimic the maturation procedure of human antibody in vivo, so it is possible to get high affinity antibody from this selection. Second, the scFv with a low molecular weight, can make it potentially applicable in the clinical diagnosis and treatment of both infectious disease and cancer. Finally, it has no Fc fragment, so the backgroud is very low.

The phage technology offers a means of cloning human anti-HCV antibodies of a defined specificity that may be potential for therapeutic and diagnostic use. In this report, we have obtained anti-HCV E2 scFv by affinity selection and purified recombinant hepatitis C virus E2 as coating antigen from synthetic phage display antibody library. After 5 rounds of selection, 56 clones were evaluated by enzyme-linked immunosorbent assay (ELISA). One phage clone was selected from 56 clones according to the highest A450 nm value in the ELISA identification and lowest cross-reaction to the bovine serum albumin (BSA). The coding fragments of scFv were sequenced. The affinity and specificity of scFv were evaluated by ELISA and immunohistochemistry. HCV E2-scFv DNA digestion and sequence data showed that the scFv gene is composed of 750 bp. ELISA and immunohistochemistry demonstrated that the human single chain Fv antibody against HCV E2 antigen has a specific binding activity with hepatitis C virus E2 antigen. This study illustrated the feasibility of using antibody-engineering technology with the phage display library that may be useful for future therapeutic and detection purpose.

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