Expression of hepatitis C virus hypervariable region 1 and its clinical significance

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INTRODUCTION
Hepatitis C virus (HCV) is the major etiologic agent of blood transfusion-associated and sporadic non-A non-B hepatitis worldwide. About 70% of the infections become chronic, among which a significant proportion eventually develops cirrhosis and hepatocellular carcinoma. Despite recent success after the combination therapy with Interferon-α and ribavirin[1-3], about 60% of patients still fail to respond. Thus, the development of HCV vaccine is especially important, but it remains an urgent challenge due to the high mutation rate of HCV.

Multiple lines of evidence indicate that one of the principal neutralization determinants corresponds to the hypervariable region 1 (HVR1), which is located in the amino-terminus of E2 of HCV (nt1150-1230). Zibert et al[4] found that an early appearance of antibodies directed to HVR1 is associated with acute self-limiting infection of HCV, while the persistence of HVR1 antibodies is associated with chronic HCV infection. Antibodies against HVR1 have been shown to block adsorption to susceptible cells in vitro[5,6]. Animal antibodies raised against this region have provided effective prophylaxis in chimpanzee challenge experiments[7-9], but attempts to develop a HVR1 vaccine against HCV were hampered by the frequent mutations of HVR1. Although anti-HVR1 antibodies react with HVR1 proteins specifically, a single fraction of antibodies has potentiality to react with more than one HVR1 protein sharing a similar amino acid sequence[10]. Theses findings suggest that HVR1 may play an important role in the prevention of HCV infection.

Our previous study[11] analyzed the variability of HCV envelope region in 12 dominant strains from different cities of China and predicted the immunogenicity with computer programs, demonstrating that genotypes and epidemic areas should be considered when identifying the cross-reactive epitopes for vaccine design. In this study, we selected four HCV strains of two genotypes from three regions (Shanghai, Beijing and Shandong) of China according to the results of the variant analysis and immunogenicity prediction of the envelope region in Chinese HCV strains. The gene fragments of HVR1 were amplified from four corresponding pGEMT-E2 plasmids and sub-cloned into pQE 40 vectors respectively to construct recombinant expression plasmids which expressed HVR1 as fusion proteins with DHFR. The purified DHFR-HVR1 proteins were then used to detect the anti-HVR1 antibodies in 70 serum samples of patients with chronic hepatitis C.

RESULTS
Four DHFR-HVR1 fusion proteins were successfully expressed in E.coli (320-800 ug fusion proteins per 100 ml culture). Each fusion protein (SH1b, BJ1b, SD1b and SD2a) reacted with 72.8% (51/70), 60% (42/70), 48.6% (34/70), and 58.6% (41/70) of the anti-HCV antibodies in 70 serum samples of patients with chronic hepatitis C.

CONCLUSION: The selected HVR1 fusion proteins expressed in E. coli can broadly react with HCV-infected patients’ sera. The intensity and/or quality of the immune response against HCV may be a critical factor determining the response to interferon treatment. With the evolution of virus strains, anti-HVR1 antibodies can not neutralize all the quasispecies. A polyclonal and high immunogenic vaccine comprising a mixture of several HVR1 sequences that cover the reactivity of most HCV isolates may be useful.

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Methods

Baseline viral load (copies/ml) 4.37

E2/NS1 regions was cloned from sera of the patients and (aa384-411) were amplified by PCR. The primers for HCV sequenced as described before. Known duration of infection (Yr) 10.6 9.9

Baseline ALT (IU/ml) 41 40

Sex (F/M) 5/8 2/5

Number 13 7

Age (Yr) 41 40

Table 1 Characteristics of chronic hepatitis C patients treated with IFN-α

|          | Responders | Non responders |
|----------|------------|---------------|
| Number   | 13         | 7             |
| Age (Yr) | 41         | 40            |
| Sex (F/M)| 5/8        | 2/5           |
| Known duration of infection (Yr) | 10.6 | 9.9 |
| Baseline ALT (IU/ml) | 185 | 126 (P = 0.1660) |
| Baseline viral load (copies/ml) | 4.37×10^6 | 2.92×10^6 (P = 0.6908) |

Results

Construction of recombinant expression plasmids

Part of E2/NS1 regions was cloned from sera of the patients and sequenced as described before[11]. From these plasmids pGEMT-E2, DNA fragments containing HVR1 (nt1150-1233, aa384-411) were amplified by PCR. The primers for HCV strain Shanghai 1b (SH1b), sense: nt1150-1161, 5’TTAGATCTGCAACCTACAGC3’, anti-sense: nt1225-1233, 5’CCCAAGCTTAGATTTTCTG3’; the primers for strain Beijing 1b (BJ1b), sense: nt1150-1161, 5’TTAGATCTGGCCACCTATACG3’, anti-sense: same as SH1b; the primers for strain Shandong 1b (SD1b), sense: nt1150-1159, 5’TTAGATCTGAGACCCGTG3’, anti-sense: same as SH1b, and the primers for Shandong 2a (SD2a), sense: nt1150-1159, 5’TTAGATCTGAGACCCCG3’, anti-sense: nt1225-1233, 5’CCCAAGCTTAGATTTTCTG3’. The PCR products were purified and ligated into the Hind III, Bgl II sites of the expression vector pQE40 which allows fusion of HVR1 encoding sequences downstream to the murine dihydrofolate reductase (DHFR) with a N-terminal 6xHis tag. The recombinant plasmids were identified by digestion with Hind III and Bgl II. The inserts were then sequenced to ensure that the DNA encoded the authentic HCV sequence. The identified plasmids were named as pQE40-HVR1-SH1b, BJ1b, SD1b and SD2a respectively.

Expression and purification of the fusion proteins

The recombinant plasmids and pQE40 vector were transformed to E.coli strain TG1. DHFR-HVR1 fusion proteins were expressed by induction with 1 mmol/l isopropyl-β-D-thiogalactoside (IPTG) in 100 ml of LB/ampicillin media cultured at 37 °C with vigorous shaking. After 6 h of induction, cells were harvested by centrifugation at 4 °C and 5 000 g for 30 min.

Harvested cells were re-suspended in 10 ml of 8M urea/20mM β-ME/PBS pH8.0, and cell disruption was performed using an ultrasonic sonication method. After centrifugation at 20 000 g and 4 °C for 30 min, the supernatant was saved for purification on Ni2+-nitrilotriacetate (NTA)-agarose (Qiagen) according to manufacturer’s instructions at room temperatures. Denatured crude extract was used to bind to Ni2+-NTA-agarose pre-equilibrated in 8M urea/20mM β-ME/PBS pH8.0 for 2 h. The gel matrix were then washed with the same solution pH6.3 and gel-bound proteins were eluted with that of pH4.3.

The purified fusion proteins were run on 12 % sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) for identification. For protein visualization and quantification, gels were stained with Coomassie brilliant blue (Sigma). The purity and yield of recombinant proteins were calculated from densitometric scanning results by comparing with known quantity of BSA (Bio-Rad) run on the same gel.

ELISA

The plates were coated with four purified fusion proteins respectively or combined at of 0.2 µg/ml for 1 hour at 37 °C and then overnight at 4 °C in carbonate buffer pH 9.5. After blocking with 1 % BSA for 1 hour at 37 °C, sera were dispensed in wells at a dilution of 1:20 and incubated for 45 min at 37 °C, followed by washing. HRP conjugated goat anti-human IgG (Sino-American Biological Company) diluted 1:8 000 was then added and plates were incubated for 45 min at 37 °C. After washing, the color was developed using TMB according to standard procedures, and the optical density values were measured at 450nm (OD450) in automatic photometer (Wellscan K3, Labsystem Company).

Statistical analysis

The results were analyzed by the t test. In all analyses, a P value less than 0.05 was considered statistically significant.

RESULTS

Construction of the recombinant plasmids

Four recombinant plasmids expressing HVR1 fused with DHFR were constructed as described in methods. Obtained clones were digested with Hind III and Bgl II, 95bp fragments of HVR1 coding sequences could be detected from each of them (Figure 1). Automatic sequencing confirmed that the inserted HVR1 fragments corresponded to reported data (Figure 2). The reading frames of the recombinant proteins were correct.

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ments in recombinant pQE40-HVR1 plasmids. Dashes represent nucleotides identical to those of HVR1-SH1b.

CONSENSUS ETHVTTGAVG HTTSGFTSLF TSGPSQK
HVR1-SH1b A-YT-A-S-N-R-...-S-SQ-...-R-SA-
HVR1-BJ1b G-YT-A-Q-R-A-Q-L-...-R-SA-
HVR1-SD1b -R-...-Q5 Y-LA-L-...-A-
HVR1-SD2a G-...-I-A RAA-S-V-L-PDAK...

**Figure 2B** Deduced amino acid sequence of the HVR1 fragments in pQE40-HVR1. Sequences are shown with a single-letter amino acid code where residue is different from the consensus sequence of genotype 1b defined by Hattori et al., and with a dash where residue is identical.

**Expression and purification of the fusion proteins**

The proteins expressed in transformed E. coli were analyzed by SDS-PAGE. The fusion proteins migrated as an approximately 28 kDa band, approximately 3 kDa larger than DHFR (Figure 3A), in accordance with the fusion of 28 aa HVR1. Figure 3B shows the SDS-PAGE result of four purified fusion proteins and DHFR. The concentrations estimated by BSA grades are 0.4-1.0 µg/µl, so about 320-800 µg of purified protein can be obtained from every 100 ml of bacteria culture.

**Figure 3A** SDS-PAGE of the fusion proteins expressed in E. coli strain TG1. M: protein molecular marker. Lane 1 is vector pQE40 before induction, Lane 2 is one of the recombinant plasmids before induction. Lane 3-7, are recombinant plasmids pQE40-SH1b, BJ1b, SD1b, and SD2a after 4 hours of induction with 1 mmol/l IPTG respectively.

**Figure 3B** Results of the purified proteins. M is protein marker. Lane 1 is plain DHFR. Lane 2-5 are the fusion proteins SH1b, BJ1b, SD1b, and SD2a serially.

**Detection of anti-HVR1 Ab in HCV infected patients’ sera**

Anti-HVR1 antibodies were detected in 70 serum samples from 50 patients with chronic hepatitis C (sera were tested before and after IFN therapy for 20 patients) using the four DHFR-HVR1 fusion proteins respectively. None of the healthy blood donors, who were anti-HCV negative, was anti-HVR1 positive. Anti-HCV seronegative healthy donors had mean OD450 value of 0.06. Sera were scored positive showing OD value >0.2 (cut off = 3 time mean neg + 10 %) in at least two experiments.

Each fusion protein (SH1b, BJ1b, SD1b and SD2a) reacted with 72.8 % (51/70), 60 % (42/70), 48.6 % (34/70), and 58.6 % (41/70) of the anti-HCV positive patients respectively. SH1b was the most broadly reactive fusion protein. 91.4 % (64/70) of the tested sera reacted positively with one or more fusion proteins, and among these, 89.1 % (57/64) can react with more than one fusion proteins, 20.3 % (13/64) samples of these sera were shown to react with all four fusion proteins.

The reactivity of sera was compared between responders and non-responders in 20 patients who received interferon therapy (Table 2). The reactive rates of sera with the four fusion proteins were higher in non-responders than in responders before interferon therapy, but there was no statistical significance. 57.1 % (4/7) of non-responders reacted with all four DHFR-HVR1 fusion proteins, while only 13.3 % (2/15) of responders react with all of them (Figure 4). With the three fusion proteins (SH1b, BJ1b, and SD2a), the ODs of the serum reactivity of the non-responders were higher than those of responders, and the difference had statistical significances for the four mixed proteins between the two groups of patients (P<0.05) (Figure 5).

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suggested that other factors, such as the heterogeneity of virus population and replication in PBMC, might also influence the effectiveness of therapy. Like most RNA viruses, HCV circulates in the human host as a complex population of different but closely related viral variants, commonly referred to as quasispecies. It has been suggested that a reduction in genetic diversity leading to an increasingly homogeneous viral population in the envelope genes, and especially in the HVR1 of the E2 gene, is likely to be the result of a more successful and balanced cellular and humoral immune response, which can be observed in IFN therapy responders with viral clearance. It was also reported that the broad reactivity of serum anti-HVR1 antibodies correlated with viral loads and response to IFN in genotype-1b-infected patients. But Delporto et al. reported that the frequency of anti-HVR1 T cell response was significantly higher in patients who recovered after IFN therapy than that in those who did not, while no difference in the anti-HVR1 antibody reactivities were detected. In our study, the reactive rates of the four HVR1 fusion proteins with patients’ sera were higher in non-responders than those in responders, although there was no statistical difference, which might be due to insufficient number of patients. Meanwhile, 57.1 % (4/7) of non responders reacted with all four HVR1 fusion proteins, while only 15.3 % (2/13) of responders reacted with all of them. These facts suggested that the genetic diversity of HCV was greater in non responders than that in responders. The broad cross-reactivity of anti-HVR1 anti-bodies causes the inefficiency of neutralizing activity as proposed by the theory of “viral antigenic sin”. According to this theory, after the exposure to the first immunodominant and cross-reactive virus strain, patients produce not a new antibody to the second related virus strain, but an antibody to the original antigen, which is inefficient to neutralize the new variant. The findings that the serum reactive rates with the four HVR1 fusion proteins were higher in non responders than in responders may be interpreted by this theory. On the other hand, the O.D. values of anti-HVR1 antibodies were higher in responders than those in non responders. This reflected the immune status of these patients and implied that the pre-therapy immune response is a major factor determining eventual virus elimination as suggested by others.

In conclusion, the selected HVR1 fusion proteins expressed in E. coli can broadly react with HCV-infected patients’ sera. The intensity and/or quality of the immune response against HCV could be a critical factor determining the response to treatment. With the evolution of virus strains, anti-HVR1 antibodies could not neutralize all the quasispecies. A polyvalent and high immunogenic vaccine combining a mixture of several HVR1 sequences that cover the reactivity of most HCV isolates might be useful.

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