Expression and immunoreactivity of HCV/HBV epitopes

Xin-Yu Xiong, Xiao Liu, Yuan-Ding Chen

Abstract

AIM: To develop the epitope-based vaccines to prevent Hepatitis C virus (HCV)/Hepatitis B virus (HBV) infections.

METHODS: The HCV core epitopes C1 STNPKPQRKTKRTNRPRPQD (residuals aa2-21) and C2 VKFPGGGQIVGVVYLPRR (residuals aa22-40), envelope epitope E GHRMAWDMMMNWSP (residuals aa315-328) and HBsAg epitope S CTTPAQGNSMFPSCCCTKPTDGNC (residuals aa124-147) were displayed in five different sites of the flock house virus capsid protein as a vector, and expressed in E. coli cells (pET-3 system). Immunoreactivity of the epitopes with anti-HCV and anti-HBs antibodies in the serum from hepatitis C and hepatitis B patients were determined.

RESULTS: The expressed chimeric protein carrying the HCV epitopes C1, C2, E (two times), L3C1-I2E-L1C2-L2E could react with anti-HCV antibodies. The expressed chimeric protein carrying the HBV epitopes S, I3S could react with anti-HBs antibodies. The expressed chimeric proteins carrying the HCV epitopes C1, C2, E plus HBV epitope S, L3C1-I2E-L1C2-L2E-I3S could react with anti-HCV and anti-HBs antibodies.

CONCLUSION: These epitopes have highly specific and sensitive immunoreaction and are useful in the development of epitope-based vaccines.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Key words: HCV; HBV; Epitope-based vaccine; Recombinant; Immunoreactivity

Xiong XY, Liu X, Chen YD. Expression and immunoreactivity of HCV/HBV epitopes. World J Gastroenterol 2005;11(41): 6440-6444

INTRODUCTION

hepatitis C virus (HCV) and Hepatitis B virus (HBV) are the causative agents of hepatitis C and B. Exposure to HCV or HBV causes acute hepatitis, leading to chronic hepatitis, liver cirrhosis, hepatocellular carcinoma and even death. The worldwide prevalence is estimated to be around 170 million individuals (3%) infected with HCV and 350 million individuals (7%) infected with HBV[6,9]. HCV and HBV infections are social and economic issues.

HCV is a member of the Flaviviridae family, possessing a linear single-stranded RNA genome of 9.4 kb[3]. The HCV genome contains a single open reading frame (ORF) encoding a polyprotein that is cleaved into the mature viral core, envelope and non-structural proteins[4]. The core protein is the most conserved and contains highly conserved epitopes[5]. The envelope protein E1 is the most variant region and contains the major neutralizing epitopes[6,11]. HBV is a double-stranded circular DNA virus[12]. The surface antigen of HBV (HBsAg), the major antigen protein, consists of large, middle and small proteins encoded by ORF S and preS, and have been successfully used as a hepatitis B vaccine. Since HCV and HBV infections share a similar route, i.e., mainly infected individuals through serum or seral products, it is very important to develop HCV/HBV covalent vaccines to simultaneously protect individuals from HCV and HBV infections.

It has been previously demonstrated that the epitope-presenting system based on the flock house virus (FHV) capsid protein is useful in displaying foreign epitopes[13]. In the present study, epitopes derived from the HCV core, envelope protein and HBsAg were displayed in this system, immunoreactivities were determined, and the possibility to develop epitope-based vaccines was discussed.

MATERIALS AND METHODS

Epitopes and epitope-presenting system

The epitopes studied in this study were derived from the HCV core region residues aa2-21C1 STNPKPQRKTNRNRPQD (C1) and aa22-40, VKFPGGGQIVGVVYLPRR (C2), the envelope region residues aa315-328, GHRMAWDMMMNWSP (E), and the HBsAg residues aa124-147 CTTPAQGNSMFPSCCCTKPTDGNC (S).

The epitope-presenting system was developed using the
FHV capsid protein as a vector (FHV-RNA2 system) The FHV capsid protein expressed in the recombinant system could self-assemble into virus-like particles (VLPs). Six sites on the vector protein outer surface could be chosen for insertion of foreign epitopes which have little influence on the protein structure (Figure 1). The epitopes were inserted into L3 (C1), I2 (E), L1 (C2), L2 (E) and I3 (S), by means of genetic recombination engineering on plasmid pET-3. Four recombinant plasmids were constructed, pET-Wt carrying the bare vector protein gene, pET-I3S carrying the vector gene and the epitope S, pET-L3C1-I2E-L1C2-L2E carrying the vector gene and the epitopes C1, C2, E, and pET-L3C1-I2E-L1C2-L2E-I3S carrying the vector gene and the epitopes C1, C2, E and S.

Expression of chimeric proteins carrying epitopes

The recombinant plasmids were transformed into E. coli BL21(DE3) cells. The chimeric proteins carrying the epitopes were expressed under the control of T7 promotor in TB/ampicillin media at 37 °C. Cells were harvested by centrifugation at 3 000 r/min for 10 min at 4 °C (Beckman rotor type J-20). Cell lysis was accomplished by ultrasonication and centrifuged at 10 000g for 30 min. The chimeric proteins in the deposit were dissolved in 8 mol/L urea and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To purify the expressed chimeric proteins, the band containing the chimeric proteins was cut-off from the gel and the proteins were recovered by further electrophoresis procedures. The purified chimeric proteins were stored at -20 °C and used as an antigen in ELISA and Western blot tests.

Serum samples

Serum samples used in this study were collected from patients with hepatitis C (anti-HCV positive or HCV-RNA positive) or hepatitis B (anti-HBs positive), from the Kunming Infectious Disease Hospital, Kunming, China.

ELISA

Recombinant chimeric proteins carrying HCV/HBV epitopes purified by PAGE were diluted to 5 mg/L in carbonate/bicarbonate buffer (pH 9.6) and used as coating antigens in ELISA test. One hundred microliters of the protein solution was added to each well of 96-well microtiter plates. The plates were incubated overnight at 4 °C and then blocked with 2.5 g/L bovine serum albumin in 10 mmol/L of phosphate-buffered saline containing 0.01% Tween 20 (PBS-T), at 37 °C for 1 h and washed five times with (PBS-T). Sera from hepatitis C or B patients were diluted to the ratio 1:10 and 100 μL was added to each well of the blocked plates. The mixture was incubated at 37 °C for 1 h. After washing, 100 μL of horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G (Sigma BioSciences, St. Louis, MO, USA) was added to each well, and the plates were incubated for 1 h at 37 °C. After incubation and washing, ortho-phenylenediamine dihydrochloride (OPD, Sigma BioSciences, St. Louis, MO, USA) was added, and the color was measured at 455 nm with a Titertek plate reader.

When the cut-off value was (SS-NC)/NC ≥ 2, it was defined as positive. In the formula, SS is the OD value of the serum sample, NC is the value of the negative control.

Western blot

In the Western blot test, the expressed chimeric proteins were separated in 10% SDS-PAGE and transferred onto the nitrocellulose membrane. After incubation with the patient’s serum (in 1 : 400 dilution) and horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G, the immobilized antigens (epitopes) were detected with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma BioSciences, St. Louis, MO, USA).

RESULTS

Expression and purification of chimeric proteins

The chimeric proteins Wt, I3S, L3C1-I2E-L1C2-L2E and L3C1-I2E-L1C2-L2E-I3S were expressed in inclusion body form in transformed cells with the corresponding recombinant plasmids pET-Wt, pET-I3S, pET-L3C1-I2E-L1C2-L2E-I3S, and analyzed by 10% SDS-PAGE (Figure 2). The results showed that the chimeric proteins were highly expressed.
The contents of chimeric proteins were estimated possessing about 35.1% (Wt), 35.5% (I3S), 34.2% (L3C1-I2E-L1C2-L2E), and 39.7% (L3C1-I2E-L1C2-L2E-I3S) of the full cell proteins. After purification, the chimeric proteins possessed about 43.8 kD (Wt), 46.5 kD (I3S), 49.7 kD (L3C1-I2E-L1C2-L2E) and 52.5 kD (L3C1-I2E-L1C2-L2E-I3S) of molecular weight, respectively as expected (Figure 3).

**ELISA test**

Using purified chimeric proteins as a coating antigen, 66 anti-HCV+/HCV-RNA+ and 24 anti-HCV+/HCV-RNA- serum samples from hepatitis C patients were determined (Table 1). The results showed that the chimeric proteins carrying HCV epitopes L3C1-I2E-L1C2-L2E and L3C1-I2E-L1C2-L2E-I3S could react with anti-HCV antibodies with a high specificity and susceptibility. The reactivity rates of chimeric proteins L3C1-I2E-L1C2-L2E and L3C1-I2E-L1C2-L2E-I3S were 95.5% and 95.5% with anti-HCV+/HCV-RNA+ sera, 29.2% and 37.5% with anti-HCV+/HCV-RNA- sera, respectively. No serum samples reacted with the expressed vector protein Wt. Few serum samples reacted with chimeric protein I3S, implying that the patients were co-infected with HBV.

**Western blot test**

Using sera from patients with hepatitis C (anti-HCV positive by ELISA kit) or B (anti-HBs positive by ELISA kit) as detecting antibody, the chimeric proteins carrying HCV epitopes L3C1-I2E-L1C2-L2E and L3C1-I2E-L1C2-L2E-I3S could be recognized by anti-HCV antibody (Figure 4A), and the chimeric protein carrying HBV epitopes S, I3S and L3C1-I2E-L1C2-L2E-I3S could be recognized by anti-HBV antibody on Western blot (Figure 4B). Ten serum samples from hepatitis C or B patients were detected respectively and the results in each group were similar.

**DISCUSSION**

Due to the host defense mechanism and virus genome RNA instability, HCV seems to escape immune pressure by mutation and results in high genetic heterogeneity [15,16]. The humoral immune response to neutralizing antibodies appears to be restricted and isolate-specific. HCV isolates obtained can be classified into at least six major clades (clades 1 to 6) and more than 70 subtypes [3]. Development of HCV vaccines is largely hampered for these characteristics of the virus.

Recent studies indicate that when a virus epitope is present in an appropriate vector system, the epitope can be displayed on the exposed surface of the vector protein with high immunogenicity [17]. Sequencing and immunological analysis showed that the residues aa1-40 in HCV core region are the most conserved and the residues aa315-328 in E1 region are highly conserved too [18]. HBsAg is the major antigenic protein of HBV. Mature HBsAg can self-assemble into VLPs and induce effective immune response. The residues aa120-160 of the large protein S exposed on the outer surface of VLPs are defined as a determinant. This determinant induces cross neutralizing antibodies which protect infections against different HBV subtypes [19-21]. Further studies indicate that the “a” determinant is mainly located within a double-looped structure formed by disulfide bridges between cysteines at 124, 137 and at 139-147 [22,23]. The epitope S (aa124-147) studied in this report contains the key residues of the “a” determinant.

Table 1: Detection of immunoreaction of expressed proteins as antigen with anti-HCV antibodies in serum from hepatitis C patients by ELISA

| Protein                  | Anti-HCV+/HCV-RNA (%) | Anti-HCV+/HCV-RNA (%) |
|--------------------------|------------------------|------------------------|
| Wt                       | 0                      | 0                      |
| I3S                      | 2/66 (3.1)             | 1/24 (4.1)             |
| L3C1-I2E-L1C2-L2E        | 63/66 (95.5)           | 7/24 (29.2)            |
| L3C1-I2E-L1C2-L2E-I3S    | 63/66 (95.5)           | 9/24 (37.5)            |

**Figure 3** Expression of purified expressed proteins. M: protein molecular mass standard; lane 1: recombinant vector protein Wt; lane 2: chimeric protein I3S; lane 3: L3C1-I2E-L1C2-L2E; lane 4: L3C1-I2E-L1C2-L2E-I3S.

**Figure 4** Western blot of expressed proteins using anti-HCV+ (A) and anti-HBsAg+ (B) sera as detecting antibodies. M: protein molecular mass standard; lane 1: recombinant vector protein Wt; lane 2: chimeric protein I3S, lane 3: L3C1-I2E-L1C2-L2E; lane 4: L3C1-I2E-L1C2-L2E-I3S.
determinant. The epitopes displayed in the FHV-RNA2 system in this study had a high reactivity to specific anti-HCV or anti-HBV antibodies, indicating the importance of these HCV and HBV epitopes.

HCV/HBV epitopes, multiple-presented in a fusion form, cross react with HCV and HBV antibodies. DNA immunization with fusion genes encoding different regions of the HCV E2 fused to the HBsAg gene elicits immune responses to both HCV and HBV. The antibody responses induced by the same epitopes are also demonstrated. These results suggest that these epitopes contribute to the development of epitope-based vaccines.

Since HCV and HBV have a similar infection route with a high co-infection rate, development of HCV/HBV covalent vaccines is of great importance. Previous studies on HIV-[123], HCV-[20] and rotavirus-[27-29] single epitopes and the present study demonstrated that the FHV-RNA2 system can be used to study the foreign epitope characteristics and to develop epitope-based vaccines. Since no sustainable cell culture system can be used, whether the antibodies elicited by these epitopes neutralize HCV and HBV infectivity or protect individuals against HCV and HBV infection remains to be studied.

REFERENCES

1 Koff RS. Hepatitis B vaccines: recent advances. Int J Parasitol 2003; 33: 517-523
2 El Khouri M, dos Santos VA. Hepatitis B: epidemiological, immunological, and serological considerations emphasizing mutation. Rev Hosp Clin Fac Med Sao Paulo 2004; 59: 216-224
3 Robertson B, Myers G, Howard C, Brettin T, Bukh J, Gaschen B, Gojobori T, Maertens G, Mizokami M, Nainan O, Netesov S, Nishioka K, Shin T, Simmonds P, Smith D, Stuyver L, Weiner A. Classification, nomenclature, and database development for HCV and related viruses: proposals for standardization. International Committee on Virus Taxonomy. Arch Virol 1998; 143: 2493-2503
4 Lindenbach BD, Rice CM. Flaviviridae: the viruses and their replication. In: D. M. Kipke, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.). Fields virology, 4th ed. Philadelphia, Pa. Lippincott Williams & Wilkins, 2001; 991-1042
5 Hadlock KG, Lanford RE, Perkins S, Rowe J, Yang Q, Levy S, Pileri P, Abrignani S, Fong SK. Human monoclonal antibodies that inhibit binding of hepatitis C virus E2 protein to CD81 and recognize conserved conformational epitopes. J Virol 2000; 74: 10407-10416
6 Bukh J, Miller RH, Purcell RH. Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Semin Liver Dis 1995; 15: 41-63
7 Keck ZY, Op De Beeck A, Hadlock KG, Xia J, Li TK, Dubuisson J, Fong SK. Hepatitis C virus E2 has three immunogenic domains containing conformational epitopes with distinct properties and biological functions. J Virol 2004; 78: 9224-9232
8 Habersetzer F, Fournillier A, Dubuisson J, Rosa D, Abrignani S, Wychowski C, Nakano I, Trépo C, Desgranges C, Inchauspé A. Classification of human monoclonal antibodies specific to the hepatitis C virus glycoprotein E2 with in vitro binding neutralization properties. Virology 1998; 249: 32-41
9 da Silva Cardoso M, Siemonet K, Sturm D, Krone C, Moradpour D, Kubanek B. Isolation and characterization of human monoclonal antibodies against hepatitis C virus envelope glycoproteins. J Med Virol 1996; 55: 28-34
10 Burioni R, Piasant F, Manzin A, Rosa D, Delli Carri V, Bugli F, Sforlòsi L, Abrignani S, Varaldo PE, Fadda G, Clementi M. Dissection of human humoral immune response against hepatitis C virus E2 glycoprotein by repertoire cloning and generation of recombinant Fab fragments. Hepatology 1998; 28: 810-814
11 Allander T, Drakenberg K, Beyene A, Rosa D, Abrignani S, Houghton M, Widell A, Grillner L, Persson MA. Recombinant human monoclonal antibodies against different conformational epitopes of the E2 envelope glycoprotein of hepatitis C virus that inhibit its interaction with CD81. J Gen Virol 2000; 81: 2451-2459
12 Gust ID, Burrell CJ, Coulepis AG, Robinson WS, Zuckereman AJ. Taxonomic classification of human hepatitis B virus. Interobserver 1986; 25: 14-29
13 Tisinetzky SG, Scodegger EA, Evangelisti P, Chen Y, Shiappacassi M, Porro F, Bizik F, Zacchi T, Lunazzi G, Miertus S. Immunoreactivity of chimeric proteins carrying the HIV-1 epitope IGPGRAF. Correlation between predicted conformation and antigenicity. FEBS Lett 1994; 355: 1-4
14 Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol 1990; 185: 60-89
15 Chen YD, Liu MY, Yu WL, Li QJ, Peng M, Dai Q, Liu X, Zhou ZQ. Hepatitis C virus infections and genotypes in China. Hepatobiliary Pancreat Dis Int 2002; 1: 194-201
16 Chen YD, Liu MY, Yu WL, Li QJ, Peng M, Dai Q, Wu J, Liu X, Zhou ZQ. Sequence variability of the 5′ UTR in isolates of hepatitis C virus in China. Hepatobiliary Pancreat Dis Int 2002; 1: 541-552
17 Chen Y, Dai C. [Current status and strategy of research on epitope-based vaccine] Zhongguo Ya Fang Yi Xue Za Zhi. 1999; 33: 315-316
18 Bukh J, Purcell RH, Miller RH. At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proc Natl Acad Sci U S A 1993; 90: 8234-8238
19 Gerin JL, Alexander H, Shih JW, Purcell RH, Dapolito G, Engle R, Green N, Sutcliffe JG, Shinnick TM, Lerner RA. Chemically synthesized peptides of hepatitis B surface antigen duplicate the d/y specificities and induce subtype-specific antibodies in chimpanzees. Proc Natl Acad Sci U S A 1983; 80: 2365-2369
20 Moynihan JS, D’Mello FI, Howard CR. 48-mer synthetic peptide analogue of the hepatitis B virus “a” determinant induces an anti-HBs antibody response after a single injection. J Med Virol 2000; 62: 159-166
21 Stirk HJ, Thornton JM, Howard CR. A topological model for hepatitis B surface antigen. Intervirology 1992; 33: 148-158
22 Berting A, Hahnen J, Kröger M, Gerlich WH. Computer-aided studies on the spatial structure of the small hepatitis B surface protein. Intervirology 1995; 38: 8-15
23 Carman WF, Korula J, Wallace L, MacPhee R, Mims L, Decker R. Fulminant reactivation of hepatitis B due to escaped detection by monoclonal HBsAg ELISA. Lancet 1995; 345: 1406-1407
24 Li Q, Dong C, Wang J, Che Y, Jiang L, Wang J, Sun M, Wang L, Huang J, Ren D. Induction of hepatitis C virus-specific humoral and cellular immune responses in mice and rhesus by artificial multiple epitopes sequence. Viral Immunol 2003; 16: 321-333
25 Chen YD, Xiong X, Liu X, Li J, Wen YL, Chen Y, Dai Q, Cao Z, Yu W. Immunoreactivity of HCV/HBV epitopes displayed in an epitope-presenting system. Mol Immunol 2006; 43:346-442 in press
26 Buratti E, Di Michele M, Song P, Monti-Bragadin C, Scodegger EA, Baralle FE, Tisminetzky SG. Improved reactivity of hepatitis C virus core protein epitopes in a conformational antigen-presenting system. Clin Diagn Lab Immunol 1997; 4: 117-121
Chen YD, Liu MY, Zou YJ, Peng M, Dai CB. Induction of neutralizing antibodies by SA11 Vp4-specific epitopes. Zhongguo Bing du xue 1997; 12: 125-131

Chen YD, Liu MY, Zhao W, Dai CB. Broadly Immunological Reactivities primed by epitopes corresponding to the cleavage region of SA11 Vp4. Zhongguo Bing du xue 1998; 13: 57-63

Liu X, Li JQ, Xiong XY, Chen YN, Peng M, Dai Q, Wen YL, Chen YD. [Protective efficacy of recombinant rotavirus epitope-based vaccine in mice] Zhongguo Yixue Kexueyuan Xue bao 2005; 27: 216-22