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DETECTION OF ANTIBODY TO HEPATITIS C E2/NS1 PROTEIN IN PATIENTS WITH TYPE C HEPATITIS

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SUMMARY: Putative E2/NS1 sequence of hepatitis C virus was expressed in E. coli as a fusion protein with maltose binding protein. Approximately 80 kDa protein was obtained containing 38 kDa E2/NS1 protein. The antibody to this protein was detectable in the same serum from which the sequence was amplified. It was also detectable in none of 7 acute hepatitis, in 2 of 12 chronic persistent hepatitis, in 3 of 25 chronic active hepatitis, and in 2 of 4 cirrhosis. It was detectable in none of 10 normal subjects. In 3 cases who were positive for the antibody before the interferon treatment, it became undetectable after the treatment. Thus, it seems that the antibody is not a neutralizing antibody and is related to active viral replication.

Hepatitis C virus (HCV) is a major etiologic agent of non-B chronic liver disease and hepatocellular carcinoma (1,2). Recently, hepatitis C virus was molecularly cloned and the sequences were reported (1,3-5). The studies on HCV sequences revealed that HCV was single stranded RNA virus encoding a large polyprotein, and was distantly related to flaviviruses and pestiviruses (1,3-5). From similarity to these viruses, HCV is supposed to encode structural proteins (core and envelope) near its amino-terminal end and 5 non-structural (NS) proteins following them. The glycosylated protein designated as E2/NS1 protein lies immediately downstream of the envelope protein in the polyprotein (3-5). The biological role of HCV E2/NS1 protein is not revealed yet. We amplified the putative E2/NS1 sequence and expressed the encoded protein as a

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fusion protein with maltose binding protein (MBP) and examined the presence of antibody to this protein in patients with hepatitis C by western blot.

Materials and Methods

A serum sample was obtained from a 53 years old female patient with chronic hepatitis who is positive for anti-C-100-3 antibody. Nucleic acids were extracted from 100μl of serum as described (6). The extracted nucleic acids were reverse transcribed to cDNA, then the sequence encoding hepatitis C virus amino acids 364-702 was amplified by nested PCR method (7). The sequence includes 7 amino acids of envelope 1 protein (amino acid 364-370), 13 amino acids of signal peptidase recongition site (amino acid 371-383), and 319 E2/NS1 protein (amino acid 384-702)(5). Putative E2/NS1 protein is supposed to start from amino acid 384 and end at around amino acid 729 (5,8), so approximately 95% of E2/NS1 protein is included in the sequence. The primers used for the amplification are as follows: outer primers [sense primer; 5'-TGGGACATGATGATGAACTG-3' (nucleotides 1287-1306) and antisense primer; 5'-CTATCAGCAGCATCATCCA-3' (nucleotides 2535-2553)] and inner primers [sense primer; 5'-ATGGTGGGGAACTGGGCTAA-3' (nucleotides 1429-1438) and antisense primer; 5'-TATAGGTATTGCACGTCCA-3'(nucleotides 2419-2437)]. The amino acid and nucleotide numbers are based on the report by Kato et al. (4).

The amplified DNA segment was inserted to expression vector p-mal-C (New Engl Biolabs, MA, USA) at its Stu I site (9). The vector DNA with inserted DNA was transfected to E. coli TBI and selected by ampicillin (9). The E. coli clone with the vector correctly inserting the amplified DNA was incubated at 37°C for 2 hours in the presence of IPTG to express the E2/NS1 protein as a fusion protein with MBP. Then 5 μl of the E. coli lysate expressing E2/NS1 protein was applied to 12.5% SDS-PAGE, then blotted to nitrocellulose filter (NCF). The Western blot was performed as described (10). Briefly as follows: The strips of NCF were treated with buffer containing 2 % gelatine for 2 hours at room temperature, then incubated with 100-fold diluted sera from patients with various liver diseases. The filters were washed with tris buffered saline with 0.5% tween 20 (TTBS), then incubated with biotinylated-anti-human IgG,

followed by incubation with peroxidase labelled avidin-biotin complex, washed with TTBS between each steps. Reaction with diamino benzidine was performed to detect the signal (10).

Patients and serum samples

Sera from 48 patients with liver disease who are positive for HCV RNA (7 acute hepatitis, 12 chronic persistent hepatitis, 25 chronic active hepatitis, 4 cirrhosis) and 10 normal subjects were examined for the presence of anti-E2/NS1 antibody. The serum from which the sequence was amplified was also examined. Serial serum samples from 3 patients who were positive for the antibody and who were treated successfully by interferon (IFN) were also examined (11). HCV RNA was detected as reported (6). Anti-C-100-3 was detected by a commercial kit (2).

Results

Expression of HCV E2/NS1 protein

By amplifying the extracted nucleic acid from a patient serum, approximately 1.0 kilobase putative E2/NS1 sequence was obtained. After ligating the sequence to expression vector p-mal-c, and expressing the sequence as a fusion protein, approximately 80 KDa protein including 38 KDa E2/NS1 protein and 42 KDa maltose binding protein was obtained.

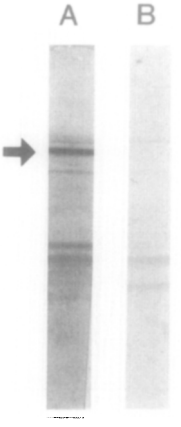

Fig.1. Detection of antibody against the E2/NS1 protein expressed as a fusion protein with maltose binding protein. A: The serum from which this E2/NS1 protein was made. B: HCV RNA negative serum. Arrow indicates the position of 80 KDa protein.

Table 1. Prevalence of anti-E2/NS1 antibody in patients with HCV infection

	AH	CPH	CAH	LC	normal
positive for anti-E2	0	2	3	2	0
number	7	12	25	4	10
(%)	0	17	12	50	0

AH; acute hepatitis, CPH; chronic persistent hepatitis, CAH; chronic active hepatitis, LC; cirrhosis of the liver.

Detection of anti-E2/NS1 antibody

The antibody to this protein was detected in the same serum used for amplification of the sequence (Fig.1). It was also detected in 7 of 48 (15%) patients with HCV RNA positive patients. None of 7 patients with acute hepatitis, 2 of 12 patients with chronic persistent hepatitis, 3 of 25 patients with chronic active hepatitis, and 2 of 4 patients with cirrhosis were positive for the anti-E2/NS1 antibody. None of 10 normal subjects were positive for the antibody (Table 1). In three patients who were positive for the anti-E2/NS1 antibody before interferon treatment, the antibody became undetectable after the treatment (Fig. 2).

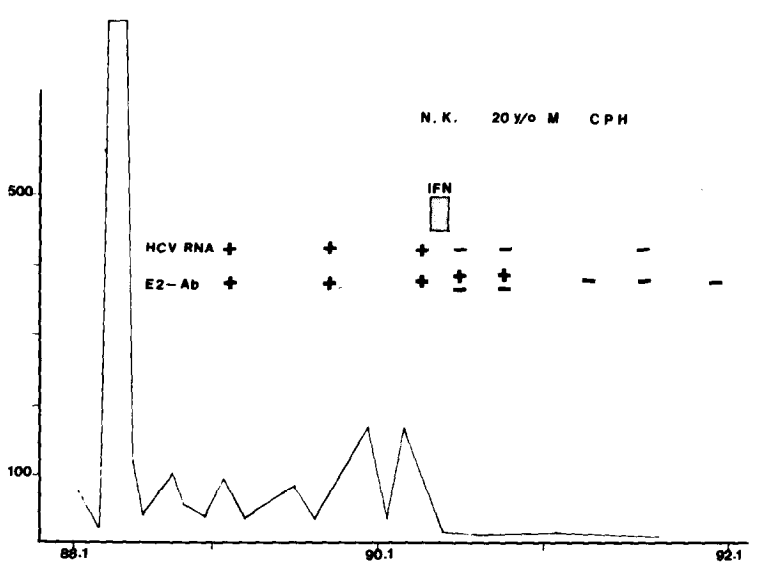

Fig.2a. Sequential analysis of HCV-RNA and anti-E2/NS1 antibody in a patient with chronic persistent hepatitis who was treated with IFN alpha.The solid line indicates the movement of ALT (IU/L).

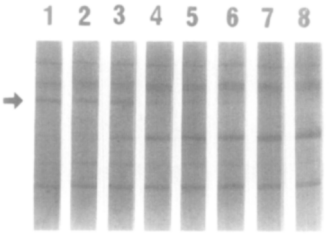

Fig.2b. Detection of anti-E2/NS1 antibody in serial serum samples from the patient. Lane 1; 12 months before the IFN treatment, lane 2; 6 months before the treatment, lane 3; just before the treatment, lane 4; at the end of the treatment (4 weeks after the beginning of the treatment), lane 5; 4 months after the end of treatment, lane 6; 10 months after the treatment, lane 7; 13 months after the treatment, lane 8; 18 months after the treatment. The 80 KDa E2/NS1 band indicated by an arrow gradually decreased after the IFN treatment. The fourth band which increased after the IFN treatment was considered to be a non-specific band as discussed in the text.

Discussion

The coding sequence of E2/NS1 protein of hepatitis C virus is partially determined. Because of the presence of sequences for signal peptidase, amino acid around 390 is supposed to be a starting site and amino acid around 729 is a ending site for HCV E2/NS1 protein (5). Recent translation study of the putative structural sequence and direct sequence of the translated proteins revealed amino acid 384 is the starting site (8). The protein we expressed includes majority (95%) of the reported HCV E2/NS1 sequence, so the protein we expressed must be applicable for the detection of antibody to E2/NS1 protein.

Our examination revealed the presence of E2/NS1 antibody in sera from chronically infected patients. This indicates that E2/NS1 protein is produced in relation to the active viral replication. The disappearance of this antibody by IFN treatment suggests that this protein was produced during active viral replication probably indicating the role in viral formation.

The biological role of the HCV E2/NS1 protein is not fully understood. From the analogy to other flaviviruses and pestiviruses, the production of the protein during viral replication is probably related to the organization of the viral membrane (12). The E2 protein of pestiviruses is supposed to be a component of the viral membrane (13). The NS1 protein of the flaviviruses is not a component of the virion, it is believed to elicit neutral antibodies (14). As the HCV E2/NS1 protein is heavily glycosylated compared to

flaviviruses and the hydrophobicity profile is more similar to pestiviruses than flaviviruses, HCV E2/NS1 protein might be a component of the virus membrane as in pestiviruses(13). This HCV E2/NS1 protein might play a role in organization of viral membrane and could be a candidate for the vaccine (13).

The evidence that the antibody to HCV E2/NS1 was detectable in patients with chronic hepatitis C fabors that the antibody is not a neutralizing antibody and the epitope(s) of the antigen does not exist on the surface of the viral membrane. However, the presence of hypervariable regions was reported in E2/NS1 sequence, and the epitope of the E2/NS1 protein might exsist in these regions (15). If this is the case, the situation could occur that the virus exists in the presence of neutralizing antibody by changing the epitope and by escaping the immunological attack by the host. So the presence of antibody does not necessarily mean that this antibody is not a neutralizing one. However, the antibody was also detected in the same serum from which we made the E2/NS1 protein. The coexistence of HCV and the E2/NS1 antibody indicates the absence of epitope of E2/NS1 protein on the surface of the virus membrane and that the antibody is not functioning as a neutralizing antibody. If the E2/NS1 protein exists as a component of the viral membrane as in pestiviruses, the epitope may not exist on the surface but inside the membrane. The evidence that the antibody did not increase after disappearance of the virus by IFN treatment would also fabor that this is not a neutralizing antibody. The intensity of the fourth band in figure 2b (42 KDa) increased after the IFN treatment as if it were reflecting the presence of a neutralizing antibody. However, when E. coli clone with plasmid p-mal-c with E2/NS1 sequence and clone without plasmid were examined side by side with the same serum as used for lane 8 in figure 2b, the same 42 KDa band was detected (data not shown), so this fourth band was considered to be a non-specific one.

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