Establishment of a cell line constitutively expressing E2 glycoprotein of hepatitis C virus and humoral response of hepatitis C patients to the expressed protein

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A Chinese hamster ovary cell line was established which abundantly expresses the second envelope protein (E2) of hepatitis C virus under the control of an exogenous promoter. The expressed E2 protein was found to be a glycoprotein of 58 kDa by immunoprecipitation with sera from patients that had chronic hepatitis C. Using this cell line as antigen in immunofluorescence tests, as high as 93% of patients with non-A non-B hepatitis had antibodies against E2 protein. In Western blots using SDS-denatured E2 protein, however, the detectability of the antibody was drastically reduced to 30%. Immunoprecipitation assays and ELISA, using both native and denatured E2 protein, revealed that antibodies to E2 protein were present in most of the chronic hepatitis C patients and that they reacted only to the native forms.

Introduction

Hepatitis C virus (HCV) is a major causative agent of post-transfusion and sporadic non-A non-B hepatitis (NANBH) throughout the world (Alter, 1988; Choo et al., 1989; Kuo et al., 1989). HCV infection often leads to persistence, resulting in severe chronic liver diseases and, in some cases, this develops into hepatocellular carcinoma (HCC; Miyamura et al., 1990; Saito et al., 1990). However, little is known about whether or how individual HCV proteins are involved in the development of these diseases.

HCV has a positive-stranded RNA genome of 9.4 kb which encodes a precursor polyprotein of about 3000 amino acids (aa) (Kato et al., 1990; Choo et al., 1991; Takamizawa et al., 1991). From the hydropathicity profile of their deduced amino acid sequences and the extensive sequence similarity in the 5' untranslated regions, HCV has been shown to be distinctly related to flaviviruses and pestiviruses (Miller & Purcell, 1990; Takeuchi et al., 1990b; Takamizawa et al., 1991). As in pestiviruses and flaviviruses, the core protein of HCV is thought to be located at the N-terminal end of the polyprotein, followed by two envelope glycoproteins, designated E1 and E2. Because no appropriate cell culture system has yet been developed to support the replication of HCV, expression of HCV genes in cultured cells is important to identify individual HCV proteins and to study their roles in the pathogenesis of HCV infection. These studies also provided information for the development of new diagnostic tests to detect antibodies to HCV antigens (Harada et al., 1991; Matsuura et al., 1992).

E1 and E2 are candidate antigens for future vaccines to hepatitis C, as the NS1 protein of flaviviruses and the E2 protein of pestiviruses, the counterparts of the HCV E2 protein, have been reported to produce neutralizing antibodies (Brandriss et al., 1990; Roehrig et al., 1990; Weiland et al., 1992; Rumenapf et al., 1993). Although several groups have described the E2 protein as a 70 kDa glycoprotein (Hijikata et al., 1991; Grakoui et al., 1993) or a 60-61 kDa glycoprotein (Tomei et al., 1993; Matsuura et al., 1994), the characterization of E2 is still limited and little is known about antibodies against the E2 protein (anti-E2 Ab) present in patients with hepatitis C. In this study, we established a mammalian cell line
expressing the HCV E2 glycoprotein constitutively and abundantly. We found that the expressed glycoprotein had a molecular mass of 58 kDa and that about 90% of chronic NANBH patients had anti-E2 Ab when examined by an immunofluorescence assay. However, only a small portion of them showed anti-E2 Ab when examined by Western blotting. These results suggest that most of the patients’ sera reacted exclusively to the native form of the expressed E2 protein.

Methods

**HCV cDNA cloning.** HCV cDNA covering the C-terminal half of the E2 region was obtained by PCR (Saiki et al., 1985) after reverse transcription of RNA extracted from a serum sample of a healthy carrier, J1 (Kubo et al., 1989; Takahashi et al., 1990a, b). The cDNA was synthesized from an antisense primer S254A (GCTATCGCA-GCATCATCCA; nucleotides (nt) 2554–2535; nucleotide sequence and numbering according to Kato et al. (1990)) and then amplified after the addition of a sense primer J182S (TGCGGTCCAGTGTATTGCTT; GCATCATCCA; nt 2554–2535; nucleotide sequence and number). The E2 region was obtained by PCR (Saiki et al., 1989). A large scale preparation of pilL16SR1325x DNA was achieved from a 1.8 kb partial cosmid pCHmBplneoL (Fig. 1), a derivative of pCHD2L vector pSR816x (Matsuura et al., 1988), containing cDNA 1325 downstream of the SR~ promoter (Takebe et al., 1988). A large scale preparation of pilL16SR 1325x DNA was achieved in vitro packaging of the cosmid (Ikeda et al., 1988).

**Construction of expression vectors.** Plasmid pSR1325x contains cDNA 1325 downstream of the SR~ promoter. To construct expression vectors, plasmid pSR1325x was digested with PstI-XmnI and PvuII. The cDNA fragment was cloned into two expression vectors: 1) pChmBplesL (Fig. 1), a derivative of pCHD2L digested with PstI-XmnI (Ikeda et al., 1988); 2) Ac1325-infected Sf9 cells were used as described previously (Matsuura et al., 1993). Infected cells were disrupted and cell extracts were recovered with Protein A Sepharose CL-4B (Pharmacia) and were divided into six aliquots. Each of the six patient’s sera was added to six tubes of both RIPA-lysate and SDS-lysate.

**S1 mapping.** The procedure of S1 nuclease protection mapping was described previously (Saito et al., 1986; Suzuki et al., 1989). Briefly, 0.1 mg of total cytoplasmic RNA was hybridized at 54 °C with 0.2 μg of 32P-labelled probe DNA digested with nucleases S1 (500 units/ml; Boehringer Mannheim). To prepare the 5’ probe, pSR1325x DNA was digested with Asp718I, the site being at the 3’ end of cDNA 1325, and 5’-labelled using T4 polynucleotide kinase and [γ-32P]ATP. The labelled DNA was digested with XmnI, which cut within the ampicillin-resistance gene. The resulting 4-0 kb fragment was isolated and used as the 5’ probe. The 3’ probe was a 2.4 kb PstI–XmnI fragment, 32P-labelled at the PstI site. The PstI termini were labelled with T4 polymerase, utilizing its 3’-exonuclease activity and subsequently its 3’-polynucleotide activity to add [γ-32P]PCTP.

**ELISA.** To obtain both native and denatured antigens from insect cells, Ac1325-infected Sf9 cells were used as described previously (Matsuura et al., 1993). Infected cells were disrupted and cell extracts precipitated with 33% (w/v) ammonium sulphate. The precipitates were collected by centrifugation. The native-form antigens were prepared by suspending these precipitates in PBS at a concentration of 0.05 A280 units/ml. The denatured antigens were prepared by treating the precipitates with a solution containing 0.5% SDS and 2 mM-mercaptoethanol. The SDS-denatured antigen was then diluted with PBS containing 8 μg/ml to a concentration of 0.05 A280 units/ml. Each well of the ELISA plates (ICN–Flow Laboratories) was coated with 50 μl of the antigen solution for 16 h at 4 °C. Human sera were
analysed for anti-E2 Ab by ELISA using the coated antigens as described (Chiba et al., 1991).

*Western blotting*. CHO cell lines transfected with HCV cDNAs were analysed by Western blotting using a patient’s serum and an avidin-biotin detection method as described previously (Harada et al., 1991). Ac1325-infected Sf9 cells (Matsuura et al., 1993) were used for antibody analysis of NANBH patients’ sera by Western blotting.

**Results**

*Establishment of CHO cell lines expressing the E2 protein of HCV*

To express the E2 protein in mammalian cells, an expression plasmid pSR1325x and its derivative paSR1325x (Fig. 1a, b) were constructed. These plasmids contain HCV cDNA encoding aa 340–742 of the polyprotein and comprising the E2 region under the control of the SRx promoter. To introduce multiple copies of the expression unit into a CHO cell chromosome, we constructed a mulcos′ expression cosmid, pHL16SR1325x. This contained not only the neomycin-resistance gene but also 16 copies of a head-to-tail tandem repeat of the E2 expression unit derived from pSR1325x (Fig. 1a).

Cosmid pHL16SR1325x was transfected into CHO cells and G418-resistant cell clones were screened for E2 expression by indirect immunofluorescence analysis with patients’ serum. One of the typical cell clones, 13L20, was chosen for further analysis because it showed very
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Fig. 2. Immunofluorescence analysis of the HCV E2 protein expressed in the 13L20 cell line. (a) 13L20 cells selected after transfection with pHl16SR1325. (b) LN4, a control cell line, selected after transfection with pChmBpneoL. Fixed cells were analysed by indirect immunofluorescence analysis using patient serum NI (Matsuura et al., 1992).

intense immunostaining (Fig. 2a) compared with control cells (Fig. 2b). The immunofluorescence was detected uniformly in the cytoplasm.

Characterization of the expressed E2 protein

To identify E2 protein expressed in 13L20 cells, [35S]-methionine-labelled cell extract was immunoprecipitated with the patients' serum. A protein with a molecular mass of 58 kDa was detected in abundance (Fig. 3a, lane 2). This protein was also detected in COS-1 cells transiently transfected with pSR1325x (Fig. 3a, lane 4) and by Western blotting in 13L20 cells, although its intensity was much lower (data not shown).

To examine whether the protein is N-glycosylated, the immunoprecipitated protein derived from 13L20 cells was digested with N-glycanase. A protein with a molecular mass of 40 kDa was detected in place of the 58 kDa protein (Fig. 3b). Moreover, the 58 kDa E2 protein was detected when 13L20 cells were labelled with [3H]glucosamine and analysed by immunoprecipitation (data not shown). These results showed that this protein was an N-glycosylated form of the expressed E2 protein and the 40 kDa protein was derived from it by losing the N-glycosylated sugar chains.

S1 mapping of HCV RNAs in CHO cells expressing the E2 protein

To examine expressed E2 mRNA species, total cytoplasmic RNA prepared from the cell line 13L20 and another typical clone 13L18 was analysed by the nuclease S1 protection technique (Fig. 4). Bands with the expected sizes of 1.42 kb and 1.35 kb were detected using the 3' labelled probe and the 5' labelled probe, respectively, and no other bands were detected. These results suggested that both cell lines expressed mRNA covering the entire 1325 cDNA region; no aberrant or abnormally spliced RNA was detected.
Integration of the expression unit into a CHO cell chromosome

The expression cosmid pH16SR1325x contained 16 copies of the E2 expression unit as a tandem array. To examine how many copies of the expression unit were present in 13L20 cells, the genomic DNA was analysed by Southern blotting of three different restriction digests. Using cDNA 1325 as a probe, the main band of full-length expression units was detected with an intensity of three copies per cell, although seven bands of various intensities were also observed (data not shown). This result suggests that the cell line 13L20 contained three copies of a functional expression unit integrated into its chromosomes.

Prevalence of anti-E2 Ab in sera of patients with NANBH

To investigate the prevalence of anti-E2 Ab in NANBH patients, patients' sera were analysed by an indirect immunofluorescence assay using 13L20 cells (Table 1). Anti-E2 Ab was detected in 93% (143 out of 153) of patients with chronic NANBH. The antibody was not detected in any of the sera from 11 patients with alcoholic hepatitis or 50 normal adults. On the other hand, 65%, 93% and 93% of the same set of sera were found to be positive using the conventional HCV antibody assays for anti-C100 (Kuo et al., 1989; Miyamura et al., 1991), anti-p22 (core protein; Harada et al., 1991) and the second generation anti-HCV assay (Ortho Diagnostics), respectively. The result suggests that anti-E2 Ab is detectable in chronic NANBH patients' sera as frequently as anti-core protein antibodies.

Anti-E2 Ab was also detected in all of the 21 sera from HCV carriers without manifestations of liver disease. The antibody was detected in only 14% (three out of 21) of patients with acute NANBH but was detected with high frequency among NANBH patients with chronic

Table 1. Detection of anti-E2 Ab present in NANBH patients' sera using a 13L20 cell immunofluorescence assay

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of patients tested</th>
<th>Number of positives</th>
<th>Percentage positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic NANBH</td>
<td>153</td>
<td>143</td>
<td>93</td>
</tr>
<tr>
<td>CH</td>
<td>45</td>
<td>42</td>
<td>93</td>
</tr>
<tr>
<td>LC</td>
<td>43</td>
<td>39</td>
<td>91</td>
</tr>
<tr>
<td>HCC</td>
<td>65</td>
<td>62</td>
<td>95</td>
</tr>
<tr>
<td>Alcoholics*</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal adults†</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hepatitis C carriers‡</td>
<td>21</td>
<td>21</td>
<td>100</td>
</tr>
<tr>
<td>Acute NANBH</td>
<td>21</td>
<td>3</td>
<td>14</td>
</tr>
</tbody>
</table>

* All sera of these groups were found to be negative for anti-core antibodies.
† Healthy adults with a normal alanine-aminotransferase level.
‡ Healthy adults that were positive for anti-core antibodies.
Reactivity of anti-E2 Ab in patients’ sera

The antibody to E2 protein expressed in 13L20 cells was frequently detected in patients’ sera by immunofluorescence and immunoprecipitation. However, it was detected less frequently using Western blotting. One possible reason may be that the anti-E2 Ab present in sera of hepatitis C patients does not react to SDS-denatured E2 antigen. To examine this possibility, 

Table 2. Reactivity of six patients’ sera to E2 with or without SDS treatment

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>IFA†</th>
<th>RIPA-lysate: SDS-lysate radioactivity‡</th>
<th>Western blotting§</th>
<th>PBS-lysate: SDS-lysate absorbance¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>CH</td>
<td>+ + +</td>
<td>3·9</td>
<td>−</td>
<td>5·3</td>
</tr>
<tr>
<td>F</td>
<td>LC</td>
<td>+ + +</td>
<td>3·4</td>
<td>−</td>
<td>7·4</td>
</tr>
<tr>
<td>A</td>
<td>HCC</td>
<td>+ + +</td>
<td>3·0</td>
<td>−</td>
<td>5·3</td>
</tr>
<tr>
<td>D</td>
<td>CH</td>
<td>+ + +</td>
<td>2·9</td>
<td>+</td>
<td>1·2</td>
</tr>
<tr>
<td>B</td>
<td>HCC</td>
<td>+ + +</td>
<td>1·1</td>
<td>+</td>
<td>0·9</td>
</tr>
<tr>
<td>E</td>
<td>LC</td>
<td>+ + +</td>
<td>1·0</td>
<td>+</td>
<td>0·5</td>
</tr>
</tbody>
</table>

* The six patients analysed were the same as those listed in Fig. 5. Patients’ data were listed in the order of the RIPA/SDS ratio.
† IFA, indirect immunofluorescence assay using fixed 13L20 cells.
‡ Radioactivities of CHO cell-derived gp58 bands in Fig. 4 were measured using a BioImage Analyser BAS2000 (Fuji) and the ratios of radioactivities from RIPA-lysates: SDS-lysates were calculated.
§ Using Ac1325-infected insect cell lysate.
¶ Ratio of A₄₅₀ measurements from ELISA, using Ac1325-infected cell lysates as described in Methods.
33% (51 out of 153) were found to be positive, suggesting that the majority of the patients had anti-E2 Ab reacting only to the native form of the E2 protein.

Detecting of anti-E2 Ab by ELISA

Because ELISA detection of anti-E2 Ab is potentially useful for new blood screening or improved diagnosis of hepatitis C, the E2 protein from Ac1325 was partially purified and two ELISA systems, with or without SDS treatment, were established. The same six sera shown in Fig. 5 were examined. The ratio of $A_{492}$ readings measured by both ELISA systems was calculated (Table 2). These ratios were consistent with those of RIPA-lysates:SDS-lysates assayed by immunoprecipitation (Table 2). The anti-E2 Ab was detected in 71% (50 out of 70) of the chronic NANBH patients’ sera using the native lysate and in 9% (6 out of 70) using the SDS-denatured lysate. All of the sera positive with ELISA using the native lysate were also positive in the immunofluorescence assay. All of the sera positive with ELISA using the SDS-denatured lysate were also positive by Western blotting. Therefore, these results confirm the specificity of the two ELISA systems.

Discussion

We have established CHO cell lines constitutively expressing the HCV E2 protein. The expressed product was identified as a 58 kDa glycoprotein. Using these cell lines, we showed that anti-E2 Ab were present in most of the chronic NANBH patients (93%). The anti-E2 Ab present in patients’ sera reacted well to native E2 but poorly to SDS-denatured E2.

The C-terminal position of the E2 protein is not absolutely clear at present. The location of E2 in the HCV open reading frame was predicted to be from aa 384 to 729 by Takamizawa et al. (1991). Hijikata et al. (1991) reported that in an in vitro translation system the full E2 coding region does not extend past aa 740. Our recent data suggested that the N-terminal position of NS2 lies at about aa 810, although the precise C-terminal position of E2 is not known (Matsuura et al., 1994). The 1325 cDNA used in this study spanned aa residue 340 to 742 and so can be assumed to cover the entire, or at least nearly full-length, E2 region.

The 1325 cDNA also contains some sequence coding for the E1 hydrophobic region, which is necessary for signalase cleavage and subsequent glycosylation of the E2 protein (Inoue et al., 1992). An N-glycosylated 58 kDa protein was produced from cDNA 1325 and N-glycanase treatment yielded a 40 kDa product. Therefore, the 58 kDa protein is considered to be generated from a precursor protein by protease cleavage of its N-terminal region (this does not rule out the possibility that C-terminal cleavage may also occur in this system). The size of E2 protein shown in this study was similar to that found in recent reports (Tomei et al., 1993; Matsuura et al., 1994) but smaller than that seen in earlier reports (gp70; Hijikata et al., 1991; Spaete et al., 1992; Grakoui et al., 1993). This difference may simply be due to aberrant migration on SDS-polyacrylamide gels, although other proteolytic processing or different post-translational modification cannot be ruled out. The E2 protein was present predominantly in the cytoplasm and was not secreted (data not shown), probably because the C-terminal hydrophobic domain was retained in the expressed protein, allowing protein accumulation in 13L20 cells.

The prevalence of anti-E2 Ab in hepatitis C patients is much higher than previously expected (Inoue et al., 1992) and comparable to that of anti-core protein (p22) antibodies (Harada et al., 1991; Watanabe et al., 1991). Previously we reported that the N-terminal half of the E2 protein was antigenic, but the prevalence of antibody against this truncated E2 protein was low (Inoue et al., 1992). The results of the present study suggest that the E2 protein is highly antigenic and possibly useful for screening and diagnostic purposes. We found only one serum positive for anti-E2 Ab but negative for anti-core antibodies. However, this does not necessarily suggest that an anti-E2 Ab assay would improve on present HCV serodiagnosis because, unfortunately, the serum had been stored under conditions inadequate for RT-PCR analysis. Although it is not easy to find such patients, this point should be clarified in future. Recently, the high prevalence of anti-E2 Ab in hepatitis C patients has also been suggested using a limited number of patients’ sera against a baculovirus-expressed E2 protein (Lanford et al., 1993).

By Western blotting, the level of patients with chronic NANBH that were positive for anti-E2 Ab was similar to that of our previous study with a truncated E2 antigen (Inoue et al., 1992). The number of antibody-positive patients in the immunofluorescence assay was as high as 93%. Chronic HCV patients could consequently be classified into two groups. Patients of the first group have antibodies which recognize almost exclusively a native form of the E2 protein. The patients of the other group, about one-third of the patients tested, have antibodies recognizing both native and denatured forms of the antigen. Preliminary retrospective studies have noted no apparent differences in clinical manifestations between the two groups of hepatitis C patients.

Such a phenomenon does not appear to be restricted to HCV. Monoclonal antibodies against the second envelope glycoprotein of hog cholera virus (E2; gp44/48) reacted only with its non-denatured form (Weiland et al.,
1992). It was also reported that when one monoclonal antibody against gp44/48 was used in combination with another monoclonal antibody, neutralizing activity was abolished (Weiland et al., 1992). It is of particular interest to determine whether anti-HCV E2 Ab has neutralizing activity.

The anti-E2 Ab reacting to the native form of E2 was much more easily detected than the antibody reacting to the denatured form. A plausible explanation is that these antibodies readily recognize discontinuous epitope(s) of E2, which may be generated by the formation of higher structures such as homodimers or binding with other protein(s). Interestingly, E2 protein was shown to be coprecipitated with another molecule of itself or E1 protein, suggesting homodimer or heterodimer formation (Spaete et al., 1992; Grakoui et al., 1993; Matsuura et al., 1993, 1994). It was also reported that E2 protein has hypervariable aa regions which are highly heterogenous among HCV isolates and are possibly immunoselective antigens (Weiner et al., 1990; Kato et al., 1993). On the other hand, the anti-E2 Ab that we detected in patients’ sera seemed to recognize conservative epitope(s).

These results suggest that the anti-E2 Ab assay may be diagnostically useful. The biological and pathological significance of anti-E2 Ab present in patients’ sera remains to be clarified.

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References


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