Hepatitis C Virus E2 Protein Purified from Mammalian Cells Is Frequently Recognized by E2-specific Antibodies in Patient Sera*

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The envelope protein of hepatitis C virus (HCV) is composed of two membrane-associated glycoproteins, E1 and E2. To obtain HCV E2 protein as a secretory form at a high level, we constructed a recombinant chines hamster ovary (CHO) cell line expressing a C-terminal truncated E2 (E2t) fused to human growth hormone (hGH), CHO/hGHE2t. The hGHE2t fusion protein was purified from the culture supernatant using anti-hGH mAb affinity chromatography at approximately 80% purity. The purified hGHE2t protein appeared to be assembled into oligomers linked by intermolecular disulfide bond(s) when density gradient centrifugation and SDS-polyacrylamide gel electrophoresis were employed. When the purified fusion protein was used for testing its ability to bind to antibodies specific for HCV by enzyme-linked immunosorbent assay, the protein was recognized by antibodies in sera from 90% of HCV-positive patients. Treatment of hGHE2t protein by β-mercaptoethanol, but not by heat and SDS, significantly reduced its reactivity to the antibodies of patient sera, suggesting that intermolecular and/or intramolecular disulfide bonds are important for its ability to recognize its specific antibody and that the E2 protein contains discontinuous antigenic epitope(s).

Hepatitis C virus (HCV) is a major causative agent of post-transfusion and sporadic non-A, non-B hepatitis throughout the world (1, 2). In most cases, the virus appears to cause a persistent infection. Previous studies indicate that the development of chronic liver diseases, cirrhosis, and hepatocellular carcinoma is associated with chronic HCV infection (3).

Comparative analyses of the genomes from several HCV strains indicate that HCV is a member of the family Flaviviridae, which includes flaviviruses and pestiviruses (4). The HCV genome is a 9.5-kilobase positive-strand RNA from which a single polypeptide is expressed and processed by cellular and viral proteinases to produce the putative viral structural and nonstructural proteins (4–6). It was previously shown that structural proteins were composed of the core protein of 18–22 kDa and two glycosylated envelope proteins, E1 of 31–35 kDa and E2 of 58–74 kDa (5, 7–11). Although some lymphocyte cell lines have shown to support the limited replication of HCV, there has not been in vitro cell culture system efficiently enough to be used for viral propagation and for detailed virological studies (12). Expression studies using recombinant cDNA templates are the only means for identifying individual HCV proteins and to study their roles in the pathogenesis of HCV infection.

The hydrophobicity profile of HCV polyprotein suggested that the HCV E2 protein corresponds to the flavivirus NS1 glycoprotein and the major pestivirus envelope protein gp53/gp55 (E2; gp53 in bovine viral diarrhea virus and gp55 in hog cholera virus), which were reported to induce protective immunity in experimental animals (13, 14). HCV envelope proteins are of considerable interest, because experimentally challenged chimpanzees were either protected or shown to ameliorate disease following vaccination with recombinant E1/E2 subunits (15). It was recently reported that HCV E2 protein expressed in Chinese hamster ovary (CHO) cells binds to target cells at a high affinity. In addition, antibody which neutralizes the binding of E2 to target cell appears to correlate with protection from HCV infection (16). These results suggest that HCV E2 protein is a key viral antigen for a hepatitis C vaccine.

The HCV envelope protein expressed in cells infected with recombinant baculovirus and vaccinia virus was used for detection of envelope-specific antibody in patient sera (17–19). However, the purification of HCV envelope protein at a high yield was thought to be a difficult task. When HCV envelope proteins were purified from HeLa cells infected with recombinant vaccinia virus, approximately 1.5 mg of partially purified E1/E2 protein was obtained from a 120-liter culture of infected HeLa cells (15). Since the yield of purified E1/E2 protein appeared to be extremely low, presumably due to the membrane association of the HCV envelope protein, biochemical and immunological studies with E1 and E2 protein has been hampered so far.

In this study, we established two recombinant CHO cell lines expressing a hGH and a secretory hGHE2t fusion protein, consisting of human growth hormone (hGH), thrombin recognition sequence, and a C-terminal truncated E2 (E2t) region. The fusion protein was shown to be a dimer and higher order oligomer, and purified to greater than 80% purity by using immunoaffinity chromatography from the culture supernatant.
Prevalence of anti-E2 antibody in patients’ sera and antigenic character of the purified hGHE2t protein were further identified by enzyme-linked immunosorbent assay (ELISA).

EXPERIMENTAL PROCEDURES

Patients and Plasma—Sera were obtained from blood donors and patients who visited to Korea Cancer Center hospital and Asan Medical Center, located in Seoul, Korea, from 1993 to 1995. Sera were obtained from 24 blood donors who were healthy adults with high alanine aminotransferase levels and negative for anti-HCV antibody assay (hGCV ELISA 3.0, Green Cross Corporation, Korea). Uninfected recombinant antigens of core and NS5 purified from bacterial cells and five immunodominant peptides (21–28 amino acids) of NS4 and NS5. When hGCV ELISA 3.0 was compared with Ortho HCV 3.0 (Ortho, Neckegadum, Germany) using 990 blood samples, the results of hGCV ELISA 3.0 showed 99.6% consistency with that of Ortho HCV 3.0. Four samples showing discrepant results were re-tested by using RIBA 3.0 (Chiron Corporation) and hGCV BLOT 3.0 (Genelabs Diagnostics, Singapore). One of four samples was shown to be false positive with hGCV ELISA 3.0, while two others were with Ortho HCV 3.0. The last one is underdetermined. The diagnosis of chronic hepatitis and liver cirrhosis was made by persistent liver enzyme abnormalities for longer than 6 months duration, physical findings, ultrasonography, and computed tomography. Evidence of portal hypertension and/or liver biopsy. For the diagnosis of hepatocellular carcinoma, serum α-fetoprotein, imaging studies, and histological confirmation were performed in all cases. HCV seropositive subjects which showed at least 4-fold elevations in serum transaminases for longer than 6 months were defined as chronic HCV. Sera from 115 patients with chronic renal failure who were on maintenance hemodialysis were used. As a negative control, we used 62 blood samples from healthy individuals who had normal alanine aminotransferase levels and were negative for hGCV ELISA 3.0.

HCV cDNA Cloning—HCV cDNA covering E1 and E2 regions was obtained by PCR after reverse transcription of RNA extracted from a serum sample of an HCV-positive patient (20). The cDNA was synthesized from an antisense primer 5′-GGG TCT AGA ACA TCA GGG ACC AGC GGC TGG-3′ and 5′-GCC TCT AGA ACA TCA GGG ACC AGC GGC TGG-3′, and then amplified after the addition of an antisense primer E1N 5′-TAT TCC ATG TGG GAC TGG GGC-3′. The complete nucleotide sequence of the clone was determined and classified into type 1b.

Construction of Plasmids—To construct pSK-IRES, internal ribosome entry sequence (IRES) of encephalomyocarditis virus (EMCV) was amplified by PCR from pTM1 (21) using primers 5′-GGG TGA ATT CCG CCG TCT AGA ACA TCA GGG ACC AGC GGC TGG-3′ and 5′-GCC TCT AGA ACA TCA GGG ACC AGC GGC TGG-3′ and the amplified product was inserted into Smal site of pBluescript SK+ (Strategene). pMT3 was constructed by inserting the EcoRI DNA fragment containing IRES of pSK-IRES into EcoRI site of pMT2 (22). The DNA fragments encoding amino acid residues 364–693 and 364–740 were obtained by PCR amplification using E2N (5′-CCA TAT CCA CCG GGG GCC AGC GGC TGG-3′ and 5′-CCA TAT CCA CCG GGG GCC AGC GGC TGG-3′), and then amplified after the addition of an antisense primer E1N 5′-TAT TCC ATG TGG GAC TGG GGC-3′. The amplified DNA fragment of the clone was determined and classified into type 1b.

Purification and Characterization of HCV E2 Protein

Purification of hGHE2t and the C-terminal Truncated E2 Protein—Anti-hGH mAb (25 mg) was added to ascites fluid by saturated ammonium sulfate precipitation method (25). dhfr-CHO cells transfected were maintained as described previously under selective conditions in α-minimal essential medium containing dialyzed 5% fetal calf serum (Life Technologies, Inc.) (26). CHO cell lines expressing recombinant HCV E2 protein were initially screened using immunoblotting and ELISA with serum from an HCV-positive patient and anti-hGH rabbit polyclonal antibody. Five sera were subjected to five subsequent rounds of methotexate (Sigma) selection.

Antibodies—Serum of a patient with chronic hepatitis C was assessed as a positive by using hGCV ELISA 3.0 (Green Cross Corporation, Korea), anti-hGH rabbit polyclonal antibody was raised against the purified hGCV protein expressed in Leuconostoc mesenteroides (27). Goat anti-rabbit antibody (Cappel, Cochran, and diagnostic) (Ig) conjugated with horseradish peroxidase were purchased from DAKO diagnostics Ltd (Denmark). Anti-hGH mAb was purified from a hybridoma obtained from the American Type Culture Collection.

Immunoprecipitation and Immunoblot Analysis—COS-7 cells were transfected and metabolically labeled with 75 μCi of 35S-Express label (NEP Life Science Products) as described previously (27). The labeled cells were lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.5% SDS) and then centrifuged at 15,000 × g for 10 min. Portions of each lysate and culture supernatant were incubated with either serum from an HCV-positive patient or anti-hGH rabbit polyclonal antibody. Immunocomplexes were collected by using Staphylococcus aureus Cowan I (Cowan I) (Oxoid) (28). Immunoprecipitates were solubilized and analyzed by SDS-polyacrylamide gel electrophoresis.

Immunoblot analysis was done according to the method as described previously (27). After transfer of proteins onto a nitrocellulose membrane, blots were treated with the block solution containing 5% nonfat milk in TBS buffer (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. Either serum from an HCV-positive patient or anti-hGH rabbit antibody (diluted 1:1000 in block solution) as the primary antibody and either goat anti-human or anti-rabbit IgG conjugated with horseradish peroxidase (diluted 1:10000 in block solution) as the secondary antibody were used to detect HCV E2 protein and developed by enhanced chemiluminescence (ECL, Amersham Corp.).

Analysis of Directive Linkages—The presence of inter- and intramolecular disulfide linkages was analyzed by the method of Allor and Barber (29). Detection of HCV proteins was performed by immunoblotting.

Sedimentation through Sucrose Density Gradients—The purified hGCV, the hGHE2t fusion protein (20 μg in 200 μl), and molecular mass standards (180 μg in 200 μl; Pharmacia) were layered onto 4.8 ml of 5–40% linear sucrose density gradients containing 100 mM NaCl and 50 mM Tris-HCl, pH 7.5. Centrifugation was performed at 45,000 rpm for 9 h in a Beckman type 55 rotor at 4 °C. Gradients were fractionated by puncturing the bottom of the tube and collecting 15 fractions of 330 μl each. Gradient fractions were analyzed by immunoblotting.

ELISA—Microliter plates (Immulon 2, Dynatech) were coated with either the culture supernatant from transfected cells or the purified hGHE2t protein (400 ng/ml). A portion of the purified protein was treated with heat, 0.2% SDS, 100 mM β-mercaptoethanol (β-ME) with or without heat, and N-glycosidase F at 37 °C for 2 h, and then washed with PBS containing 0.05% Tween 20. The wells were incubated with 1% bovine serum albumin and then followed by incubation with sera from HCV-positive patients at 37 °C for 2 h. After aspiration of the unbound material and washing of the wells, wells were incubated with goat anti-human Ig coupled with horseradish peroxidase. The reaction was developed by the addition of tetramethylbenzidine.

Purification of hGHE2t and the C-terminal Truncated E2 Protein—Anti-hGH mAb (25 mg), purified from ascites fluid by saturated (NH4)2SO4 precipitation, was coupled to 3 g of CNBr-activated Sephase-4B (Pharmacia) according to the manufacturer’s instructions. When CHO cells expressing hGHE2t protein were grown to subconfluent monolayers, culture medium was changed with serum-free medium (Life Technologies, Inc.). After additional 72-h incubation, the medium was harvested and applied to the hG2H antibody column equilibrated with PBS. The column was washed extensively with PBS and 0.5 mM NaCl in PBS, and the bound hGHE2t protein was eluted with 3 mM Na2SO4 in 10 mM sodium phosphate, pH 7.2. The protein was immediately dialyzed against PBS. For the purification of C-terminal truncated E2 protein, the purified hGHE2t fusion protein was dialyzed against thorin digestion buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2, 0.1% β-ME) and digested with thrombin (Boehringer Mannheim) for 2 h, and then applied to an hG2H antibody column. The C-terminal truncated E2 protein was purified by collecting the unbound protein.
Deglycosylation of Purified Protein—The purified hGHE2t protein was adjusted to 0.2% SDS and 100 mM β-ME, and boiled for 2 min. Treatment of N-glycosidase F was performed in the buffer containing 20 mM sodium phosphate, pH 8.0, 10 mM EDTA, 0.1% SDS, and 100 mM β-ME for 1 h at 37 °C.

Reverse Transcription PCR—HCV RNA was extracted from serum by proteinase K digestion-phenol/chloroform extraction method as described previously (30). Reverse transcription and amplification using an antisense primer 300A (5′-AC-3′) and a sense primer 80S (5′-ATC ACT CCC CTG TGA GGA ACT AC-3′) were performed as described previously (20), and amplified products were analyzed by electrophoresis on 1.5% agarose gels.

RESULTS

Construction and Identification of Various Recombinant Plasmids Expressing HCV E2 as a Secretory Form—Biochemical and immunological studies of HCV envelope proteins in virion have been limited due to a lack of an in vitro cell culture system allowing virus propagation. In addition, the purification of a native HCV envelope protein without denaturation appears to be difficult because HCV envelope proteins are membrane-associated. To express and purify HCV E2 protein at a high level, we designed several expression vectors for establishment of a recombinant CHO cell line. pMT3-E2 and pMT3-E2t plasmids were designed to express the full-length and the C-terminal-truncated E2 gene, which encompass the indicated regions, respectively. The DNA fragment encoding the recognition sequence of thrombin (Leu-Val-Pro-Arg-Gly-Ser) was inserted between the hGH region and HCV E2 to generate pMT3-hGHE2 and pMT3-hGHE2t, respectively. EMCV IRES, the hGH gene, and the E2 region of HCV are designated as thick black bars, open boxes, and hatched boxes, respectively. The gene cloned was expressed under the control of adenovirus major late promoter. Numbers in parentheses indicate the number of amino acids encoded by the DNA fragment.

To determine whether the expressed E2 protein can be secreted, the culture supernatant of transfected COS-7 cells was also immunoprecipitated with sera from an HCV-positive patient. Neither E2t nor E2 protein was detected in culture supernatant. In contrast, a significant amount of hGHE2t and hGHE2 fusion proteins identified by sera from an HCV-positive patient were also detected when the same lysate was precipitated with anti-hGH rabbit antibody (data not shown). These results indicate that E2t, E2, hGHE2t, and hGHE2 proteins expressed in COS-7 cells were immunoreactive with circulating antibodies in HCV-infected individuals.

To determine the resulting constructs were capable of expressing immunologically relevant protein, the recombinant constructs were tested by the transient transfection assay in COS-7 cells. As shown in Fig. 2, E2t, E2, hGHE2t, and hGHE2 proteins were expressed as molecular masses of 45–54, 52–62, 64–74, and 72–82 kDa, respectively, when cell lysates were specifically precipitated with sera from an HCV-positive patient (Fig. 2, lanes 2, 3, 5, and 6). In contrast, any specific protein bands were not detected in cell lysates transfected with either pMT3 or pMT3-hGH as a control (Fig. 2, lanes 1 and 4). The corresponding bands of hGHE2t and hGHE2 fusion proteins identified by sera from an HCV-positive patient were also detected when the same lysate was precipitated with anti-hGH rabbit antibody (data not shown). These results indicate that E2t, E2, hGHE2t, and hGHE2 proteins expressed in COS-7 cells were immunoreactive with circulating antibodies in HCV-infected individuals.

2 K. J. Lee, Y.-A. Suh, Y. G. Cho, and Y.-C. Sung, unpublished data.
Establishment of Recombinant CHO Cell Lines Expressing hGHE2t and hGH—pMT3-hGHE2t and pMT3-hGH as a control were transfected into dhfr CHO cells, and then positive cell lines were screened using ELISA and immunoblotting with anti-hGH rabbit antibody. After subsequent rounds of methotrexate selection, the recombinant CHO/hGHE2t and CHO/hGH cell lines were adapted at a medium containing up to 20 and 1 \( \mu \text{M} \) methotrexate, respectively. At this concentration of methotrexate, the expression level of hGHE2t and hGH protein was shown to be 7 and 2 \( \mu \text{g/ml} \) by an anti-hGH ELISA kit (Boehringer Mannheim), respectively. The hGHE2t protein was, as expected, detected as the molecular mass of 70–86 kDa by sera from an HCV-positive patient in immunoblot analysis (data not shown). These results suggest that the hGHE2t protein produced in CHO cells as well as COS-7 cells is a soluble secretory protein which is immunologically relevant.

Purification of the hGHE2t Protein Expressed in CHO Cells—To purify hGHE2t fusion protein, the hGH mAb affinity column was prepared by coupling the hGH mAb to the activated Sepharose-4B. Culture supernatants of recombinant CHO/hGHE2t and CHO/hGH cells were applied to the hGH mAb affinity column and washed serially with PBS and 0.5 \( \mu \text{M} \) NaCl in PBS, and then hGHE2t protein was eluted with 3 \( \mu \text{M} \) NaSCN. By repeating this simple immunooaffinity chromatography twice, approximately 4.0 mg of hGHE2t protein with about 80% purity were obtained from 1 liter of culture of the recombinant CHO/hGHE2t cells. The hGHE2t protein purified from recombinant CHO cell line showed a broad band of 70–86 kDa on SDS gel stained with Coomassie Brilliant Blue and was identified with sera from an HCV-positive patient (Fig. 3, A and B, lane 1). The E2t protein was obtained after thrombin digestion, followed by hGH mAb affinity chromatography. The purified E2t protein showed a smaller molecular mass (45–65 kDa) and a broader band than expected in SDS-polyacrylamide gel electrophoresis analysis (Fig. 3, A and B, lane 2). Sequencing of the amino-terminal residues resulted in Val\(^{387}\) and Cys\(^{607}\) at the amino terminus. The predicted amino acid sequences around the cleavage sites of thrombin are Leu-Val-Pro-Arg-Gly-Ser-Pro-His-Met-Arg\(^{386}\)-Gly-Ser-Pro-Val\(^{387}\) and Leu\(^{603}\)-Thr\(^{604}\)-Pro-Arg\(^{605}\)-Gly-Ser-Pro-Val\(^{387}\) and Cys\(^{607}\) of the purified hGHE2t fusion protein, respectively. It is likely that the smaller and broader E2t bands are caused by partial thrombin cleavage of E2t at other unexpected cleavage sites. The hGHE2t fusion protein was used to further characterize the HCV E2 protein for the following reasons. First, the fusion protein appears to be cleaved by thrombin at unexpected sites, resulting in a smaller E2 protein. Second, the binding activity of the E2 protein to an E2-specific antibody was shown to be highly sensitive to beta-ME, which should be included in the buffer of thrombin cleavage (see below).

The Secreted E2 Protein Forms Oligomeric Complexes—Although it was reported that E1 and E2 protein form a heterodimer, it is still controversial that this interaction is mediated by either intermolecular disulfide bonds and/or noncovalent association (10, 27, 31). In addition, a homo-oligomeric complex formation of E1 or E2 protein remained to be elucidated. Therefore, we characterized the intermolecular association of purified hGHE2t fusion protein by the method of Allore and Barber (29), which detects disulfide linkages by band shift caused by diffusion of beta-ME from reduced samples into adjacent lanes containing nonreduced samples. Under nonreducing conditions, additional high molecular mass bands were observed (Fig. 4, A, lanes 5–7). These high molecular mass bands disappeared in nonreducing samples adjacent to reduced lanes (lanes 4 and 8) as well as lanes containing reducing agents (lanes 1–3 and 9–11), indicating that they were gener-
markers are indicated at the left.

...such as dimer, trimer, and tetramer forms. The HCV E2 protein as a secreted form appears to be a mixture of monomer and oligomers. Our results suggest that the HCV E2 protein may exist as monomer, dimer, trimer, and tetramer forms (Fig. 4). Taken together, our results suggest that the HCV E2 protein as a secreted form appears to be a mixture of monomer and oligomers such as dimer, trimer, and tetramers.

**Prevalence of Anti-E2 Antibodies in Sera of Patients**—To investigate the prevalence of anti-E2 antibody in chronic non-A, non-B hepatitis and hemodialysis patients in Korea, patients' sera were analyzed by an ELISA using the hGHE2t protein (Table I). The diluted patients' sera was incubated with the protein coated in microtiter plates and detected with anti-human Ig coupled with horseradish peroxidase. It was found that anti-hGH antibody was not detected in any patient's serum, whereas the purified hGH protein was detected with anti-hGH rabbit antibody. Molecular mass markers are indicated at the left on the gel.

**DISCUSSION**

In this report, we established a recombinant CHO cell line expressing HCV E2 as a secretory form. The hGHE2t fusion protein was produced at a high level (7 mg/liter) and purified to greater than 80% purity using simple immunoaffinity chromatography (4 mg/liter). The purified hGHE2t protein was recognized by sera from 90% of HCV-positive patients and 9% of hemodialysis patients. Reactivity of the purified protein to anti-E2 antibody from patients' sera appeared to be reduced by the diagnostic assay kit of HCV infection for patients with chronic renal failure.

The binding activity of the purified E2 protein to patients' sera in various conditions—The major antigenic epitope(s) of HCV envelope proteins is thought to be conformation-dependent, because the E2 protein is highly glycosylated and recognized more frequently in a native form than in a denatured form by sera from HCV-positive patients. To examine the character of antigenic epitopes, the hGHE2t fusion protein was treated with denaturing and/or reducing agents and analyzed by ELISA using 19 HCV-positive patients' sera. Heat, SDS, and β-ME were used at maximal concentrations that do not inhibit ELISA. The protein denatured with either 0.2% SDS or boiling for 5 min appeared to have the binding activity to patients' sera as efficiently as did non-denatured protein (Table II). In contrast, treatment with either β-ME or β-ME/heat showed decreased reactivity in about 80% of patients' sera. These results suggest that intermolecular and/or intramolecular disulfide linkage(s) of hGHE2t protein molecules are important for the preservation of the antigenic determinant.

To investigate the prevalence of anti-E2 antibody in chronic non-A, non-B hepatitis and hemodialysis patients, we established a recombinant CHO cell line expressing HCV E2 as a secretory form. The hGHE2t fusion protein was produced at a high level (7 mg/liter) and purified to greater than 80% purity using simple immunoaffinity chromatography (4 mg/liter). The purified hGHE2t protein was recognized by sera from 90% of HCV-positive patients and 9% of hemodialysis patients. Reactivity of the purified protein to anti-E2 antibody from patients' sera appeared to be reduced by the diagnostic assay kit of HCV infection for patients with chronic renal failure.
the treatment with reducing agent, but not with denaturing agents, such as 0.2% SDS and boiling.

The hydrophobicity profile of HCV polyprotein showed that the C-terminal region of the E2 protein is hydrophobic (4). It was previously reported that the full-length E2 protein remained membrane-associated, presumably due to the putative transmembrane domain, and that deletion of the C-terminal hydrophobic region appeared to facilitate the secretion of the transmembrane domain, and that deletion of the C-terminal region of the E2 protein is hydrophobic (4). It

| Diagnosis                  | No. of patient tested | No. of positive (%) (anti-E2 antibody) | No. of positive (%) + (anti-HCV) | Percent positive (anti-E2 antibody/anti-HCV) |
|----------------------------|-----------------------|----------------------------------------|----------------------------------|---------------------------------------------|
| Anti-HCV (−)/high ALT      | 24                    | 0 (0)                                  | 0 (0)                            | 0                                           |
| Chronic NANBH              | 83                    | 69 (83)                                | 77 (93)                          | 90                                          |
| Chronic hepatitis          | 34                    | 26 (76)                                | 30 (88)                          | 87                                          |
| Liver cirrhosis            | 24                    | 19 (79)                                | 22 (92)                          | 86                                          |
| Hepatocellular carcinoma   | 25                    | 24 (96)                                | 25 (100)                         | 96                                          |
| Normal adults              | 62                    | 0 (0)                                  | 0 (0)                            | 0                                           |
| Hemodialysis patients      | 115                   | 6                                      | 6                                | ND                                          |
|                           | 0                     | 0                                      | 1                                | ND                                          |
|                           | 4                     | 0                                      | 0                                | 75                                          |

a ELISA for determination of anti-HCV was performed using HCV ELISA 3.0 (Green Cross Corporation, Korea).

b Healthy adults with a high alanine aminotransferase (ALT) levels were found to be negative for anti-HCV antibody assay.

c Non-A, non-B hepatitis.
d Healthy adults with normal ALT levels and negative for anti-HCV antibody assay.

* Patients with chronic renal failure and maintenance on hemodialysis.

### Table II

Reactivity of patients’ sera to the purified hGHE2t protein under various conditions

Sera from 19 patients with various levels of anti-E2 antibody were tested for the reactivity to hGHE2t protein at various conditions. The purified hGHE2t protein was treated with 0.2% SDS (SDS-treated), heat by boiling (heat-treated), 100 mM β-ME (β-ME-treated), boiling in the presence of 100 mM β-ME (β-ME/heat-treated), and N-glycosidase F (Degly) pretreated with β-ME/heat. The treated hGHE2t protein was coated into microtiter plates, and ELISA was performed as described under “Experimental Procedures.” The numbers represent the value of optical density measured at 450 nm.

| Diagnosis                  | Patients | No. of patient tested | No. of positive (%) | Percent positive (%) |
|----------------------------|----------|-----------------------|---------------------|---------------------|
|                            |          |                       |                     |                     |
| Chronic hepatitis          | N6       | 2.760                 | 2.614               | 2.803               |
|                            | N5       | 2.185                 | 2.259               | 1.840               |
|                            | N1       | 0.610                 | 0.673               | 0.338               |
|                            | N9       | 0.609                 | 0.644               | 0.250               |
|                            | N36      | 0.230                 | 0.242               | 0.119               |
| Liver cirrhosis            | 345      | 2.450                 | 2.377               | 1.999               |
|                            | 146      | 2.313                 | 2.313               | 1.808               |
|                            | 662      | 2.208                 | 2.317               | 1.580               |
|                            | 322      | 2.032                 | 2.208               | 1.775               |
|                            | 115      | 0.404                 | 0.379               | 0.769               |
|                            | N114     | 0.238                 | 0.164               | 0.175               |
|                            | 188      | 0.177                 | 0.213               | 0.159               |
| Hepatocellular carcinoma   | 158      | 2.685                 | 2.561               | 2.757               |
|                            | N83      | 2.422                 | 2.422               | 2.937               |
|                            | N4       | 2.315                 | 2.315               | 2.296               |
|                            | 163      | 2.118                 | 1.896               | 2.456               |
|                            | 15       | 2.011                 | 2.071               | 1.502               |
|                            | 172      | 0.257                 | 0.262               | 0.387               |
|                            | N2       | 0.155                 | 0.172               | 0.263               |

The major glycoprotein of pestiviruses was shown to form disulfide-linked heterodimers (E1 and E2) and homodimers (E0 and E2) (13, 33). The association mode of HCV E1 and E2 is still a matter of controversy. It was reported that a fraction of E1 and E2 present in lysates of cells infected with recombinant vaccinia virus was associated via disulfide linkage (27). In contrast, others demonstrated that E1 and E2 proteins expressed in insect and HeLa cells are noncovalently associated (5, 10). Our results showed that the secreted hGHE2t protein appeared to form a homodimer and higher order oligomer, which are linked by intermolecular disulfide bond(s). A homo-oligomerization is likely to be innate property of E2 protein, because it was not observed in hGH protein. Oligomerization of envelope protein in other enveloped RNA viruses, such as vesicular stomatitis virus G protein and simian and human immunodeficiency virus envelope protein, has also been observed (34–36). It was reported that oligomerization was shown to be required for intracellular transport and cell surface expression.
of many viral glycoproteins (37) and that the preM and the E protein of West Nile virus are present as heterodimers in cell-associated virus, whereas the E protein of extracellular virus has a tendency to oligomerize into a trimer (38). Although the actual arrangement of E1 and E2 proteins in the virion is not known, our data support that the processing and maturation of envelope proteins of HCV would be similar to those of pestiviruses, in which the homo-oligomeric complex of envelope proteins, E0 and E2, is formed by an intermolecular disulfide bond.

When anti-E2 antibody levels of patient sera were determined by ELISA using the purified hGHE2t protein, a wide range of anti-E2 antibody levels were observed in chronically infected patients (range, 420 to 44320; mean 4352; n = 28) (data not shown). This result is well consistent with the previous results (15). However, the correlation between the titer of anti-E2 antibody and specific liver disease was not observed. It is very interesting to note that several cases of hemodialysis patients’ sera were determined as positive with both anti-E2 antibody detection and reverse transcription PCR analysis, even though assay using HCV ELISA 3.0 was shown to be negative. Since immune responses might be down-regulated in immunosuppressed patients such as patients with chronic renal failure, it is possible that the antibody response to intracellular protein is weakly generated, not enough to be detected by the current anti-HCV diagnostic test. In contrast, the antibody response to envelope protein would be elicited because the E2 glycoprotein on the surface of the infected cell and the virion might be taken up by antigen-presenting cells and/or can act as a T cell-independent antigen owing to its heavy glycosylation. In this regard, the E2 protein of HCV has the potential to be used for the detection of HCV infection in immunocompromised patients.

It was previously reported that E2-specific antibodies from patients with hepatitis C were able to bind to the native E2 protein much better than to the denatured E2 protein (10). In addition, monoclonal antibodies against the envelope protein of hog cholera virus (E0; gp44/48) appeared to react only with its native form, suggesting that these antibodies appear to recognize discontinuous epitope(s) of E0 which may be generated by the formation of higher structures (38). In contrast, our purified hGHE2t protein denatured with SDS or boiling did not show any significant differences in the reactivity to anti-E2 antibody of patients’ sera. The discrepancy might be due to the denaturation condition and/or the E2 protein used. The reactivity of the purified hGHE2t protein to anti-E2 antibody was, however, decreased by treatment of a reducing agent, suggesting that the purified hGHE2t protein has a discontinuous antigenic epitope(s) generated by disulfide linkage(s).

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Purification and Characterization of HCV E2 Protein