Characterization of an established human hepatoma cell line constitutively expressing non-structural proteins of hepatitis C virus by transfection of viral cDNA

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A human hepatoma cell line constitutively expressing proteins of hepatitis C virus (HCV) was established by transfection with cDNA encoding part of the virus non-structural (NS) genome region. Proteins consistent with authentic processing at NS3/NS4A, NS4A/NS4B and NS4B/NS5A were identified. Pulse-chase experiments indicated that the cleavage between NS3 and NS4A occurred first and cleavage at other sites followed.

Expression of specific surface antigens in response to the presence of HCV proteins was analysed by flow cytometry. A significant increase in CD26 expression was observed in cells expressing the HCV proteins. CD26 plays an important role in cellular signal transduction. Its upregulation in response to the presence of HCV proteins may play a role in viral pathology.

Introduction

Detailed analyses of hepatitis C virus (HCV), the main causative agent of non-A, non-B hepatitis and also known to be closely associated with the development of hepatocellular carcinoma, have long been hampered by the unavailability of in vitro systems to support its replication (Choo et al., 1989; Kuo et al., 1989; Shimizu et al., 1992). Although HCV is thought to replicate principally in hepatocytes, little is known about the tissue-specific processing of the viral proteins and intracellular restrictions on viral gene expression. We have therefore tried to establish human liver cell lines constitutively expressing HCV proteins by transfecting HCV cDNAs in order to study virus-cell interactions during the course of viral replication.

It has, however, been generally difficult to obtain stable transfectants of hepatocytes. The efficiency of hepatocyte colony formation using drug resistance genes is extremely low. We recently found that the EF321 promoter, derived from the gene encoding the human polypeptide chain elongation factor 1α, is useful for obtaining such stable transfectants from human liver cells (Uetsuki et al., 1989; Kim et al., 1993). By the use of this promoter, a human hepatoma cell line constitutively expressing non-structural (NS) proteins of HCV was established. We characterized the proteolytic processing of these proteins. In addition, as the first step to study the interaction of host and viral proteins, surface markers of this clone were analysed.

Methods

Plasmids. A new vector, cdEF321swxneo, was made from charomid 9-36 whose cloning efficiency has previously been shown (Saito et al., 1986b). To construct cdEF321neo, a Swal–Kpnl fragment of 44 kb from pEF321neo (Kim et al., 1990) was introduced into a Smal/Kpnl-digested charomid 9-36. pEF321swxEF was made by replacing a HindIII–HpaI fragment of pEF321-T (Kim et al., 1990) which covers the gene encoding simian virus 40 large T antigen, with a synthetic oligonucleotide (GATTTAAATC) that includes a Swal restriction site. A Swal–HindIII fragment of 2.1 kb from pEF321neo was made blunt with the Klenow fragment of DNA polymerase and inserted into a SnaBI site of pEF321-T which was constructed by filling in at an Asp718 site. To construct cdEF321swxneo, a 24 kb XhoI fragment from pEF321swxEF was cloned into the XhoI-digested cdEF321neo. The plasmid pcEF3269neo was constructed as follows. An HCV cDNA clone spanning nucleotides (nt) 3252 to 7100 (the numbering system is according to Kato et al., 1990) was isolated from healthy carrier J1
(Kubo et al., 1989; Takeuchi et al., 1990a, b). To construct pUC3269, the clone was introduced into a Smal site of the pUC119, in which a BamHI site was replaced with a 10 nt Nol linker. Consequently at the N terminus of the expressed tNS2 protein six extra amino acids (aa) were expected. To terminate readthrough translation, a termination codon was introduced into the clone by site-directed mutagenesis (Kunkel, 1985) and codon 2212 was changed from CAG to TAG. The mutant DNA fragment was recloned into the parent DNA between the EcoRI site at nt 6687 and the Xbal site at nt 7085. To generate the initiator ATG, a Nol-Xbal fragment of 3.9 kb from pUC3269 was ligated with Nol-Xbal-digested pSR816X (Matsuura et al., 1992). A PstI-XbaI fragment of 3.9 kb of this plasmid was made blunt with the Klenow fragment of DNA polymerase I and introduced into the Swal site of cdEF321swxneo. Plasmid pCEF3269neo was constructed by removing the spacer region and cos sites of cdEF3269neo using EcoRV.

Cell culture. HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)-F12 (Gibco) supplemented with 10 % fetal bovine serum, 2 mM-glutamine, 100 U of penicillin per ml and 100 μg of streptomycin per ml. Cells in a 60 mm dish coated with collagen (Corning) were transfected with 10 μg of each plasmid (pCEF321swxneo and pCEF3269neo), respectively (previously linearized with EcoRV according to the method of Chen & Okayama, 1987 with some modifications). Transfectants were selected in DMEM-F12 medium containing 500 μg of G418 (Gibco) per ml. G418-resistant colonies were isolated, replated twice for purification and designated Hepswx and Hep3269neo, respectively.

Antibodies. Immunological reagents used to characterize the expressed HCV proteins were as follows: human serum of a patient with chronic hepatitis C infection (SK); anti-NS5A polyclonal antibodies produced in rabbits immunized with purified recombinant proteins of NSSA (aa 2119 to 2359) expressed in Escherichia coli (Suzuki et al., unpublished results); anti-NS3 monoclonal antibody (MAb), provided by M. Kohara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan); anti-NS4A MAb, provided by A. Takamizawa (Research Foundation for Microbial Diseases of Osaka University, Kagawa, Japan).

Western blotting. For Western blotting, cells were lysed in SDS sample buffer (50 mM-Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol) and sonicated. Lysates from 10⁶ cells were separated by 10-20 % gradient SDS-PAGE, and the radioactivity was measured in a Bio-Image model BAS2000 analyser.

Surface marker analysis. Surface markers were analysed as follows. Cells were trypsinized and washed twice with PBS. For the direct immunofluorescence method, FITC-conjugated MAb s were used. N-1 (CD10), M-CS-2 (CD13), M-CS-1 (CD15) and Nu-TR1 (CD63) were obtained from Nichirei. JML-H14 (CD14) was a gift from A. Hara (JML, Fukuoka, Japan). For the indirect immunofluorescence method, W6/32 (Class I; obtained from Dainihon Seiyaku), Ts145 (CD26; a gift from R. Ueda, Aichi Cancer Center, Nagoya, Japan), K20 (CD29; a gift from M. Wang, Christie Hospital, Manchester, UK), H5C6 (CD63; a gift from J. Hildreth, Johns Hopkins University, Baltimore, Md, USA) and anti-FAS (CD95; a gift from S. Yonehara, JTL, Yokohama, Japan) were used as primary antibodies. FITC-conjugated goat anti-mouse Ig (Tago) was used as secondary antibody. Each primary antibody was classified into CD antigens at the Fifth International Workshop on Human Leukocyte Differentiation Antigens. Cells were incubated with each antibody for 30 min at 4 °C. Stained cells were analysed by the FACScan flow cytometry system (Becton Dickinson Immunocytometry Systems).

Establishment of a human liver cell line constitutively expressing HCV non-structural proteins

The expression plasmid cdEF321swxneo was constructed as shown in Fig. 1(a). To construct pCEF3269neo, HCV cDNA covering from the C-terminal portion of NS2 to the N-terminal half of NSSA (Fig. 1b) was cloned into cdEF321swxneo and then the spacer region and cos sites were removed. The nt sequence of the HCV clone was determined and classified into the type 1b (Aizaki et al., unpublished results). HepG2 cells, originally derived from a human hepatoblastoma, were transfected with 10 μg of each plasmid (pCEF321swxneo and pCEF3269neo), respectively, PBS/0.5 % BSA for 1 h at 37 °C. After incubation the cells were washed and FITC-conjugated goat anti-mouse Ig or goat anti-rabbit Ig (Tago) were added at 1/200 dilution in PBS.

Pulse-chase experiment. For the pulse-chase experiment, cells were labelled with 50 μCi of [35S]label (ICN) for 15 min after 1 h of starvation in methionine and cysteine-free Eagle’s MEM and chased using medium without label for 5, 10, 20, 30, 60, 120 and 240 min. Labelled cells were lysed with TNE buffer. Lysates were immunoprecipitated with the 5K serum and protein A-Sepharose CL-4B was used to collect the immune complexes. The precipitated antigens were separated by 10-20 % gradient SDS-PAGE, and the radioactivity was measured in a Bio-Image model BAS2000 analyser.

Nuclease SI protection. The procedure for nuclease SI protection was described previously (Saito et al., 1986a; Suzuki et al., 1989). An EcoRV/EcoRI fragment of 6.2 kb was derived from pCEF3269neo (Fig. 1a), containing the EF321 promoter (an EcoRV site is located upstream of the N terminus of the EF321 promoter) and almost the entire insert of the HCV cDNA (an EcoRI site at nt 6687). The 5′-end of this fragment (EcoRI site) was labelled with T4 polynucleotide kinase and [32P]ATP. Cytoplasmic RNA (125 μg) extracted from the cells was hybridized at 62 °C for 3 h with 40 ng of 32P-labelled probe DNA, digested with 500 U of nuclease SI (Takara Shuzo) per ml and analysed on a 12 % alkaline gel. X-Omat film (Kodak) was used for autoradiography.
HCV protein expression in a human cell line

Linearized with EcoRV according to the method of Chen & Okayama (1987) with some modifications. Transformants were selected in DMEM containing 500 µg of G418 per ml. Five G418-resistant colonies were cloned and named Hepswx and Hep3269neo, respectively. The expressed HCV proteins were analysed by Western blotting and immunoprecipitation.

As shown in Fig. 2, we identified an uncleaved protein of truncated NS2 (tNS2) and NS3 proteins of approximately Mr 77,000 (77K protein) (Fig. 2a). This value is reasonable since the NS2 cDNA (predicted to encode a 52 aa protein) is not cleaved at the site between NS2 and NS3 (NS2/3). This is consistent with the data of Grakoui et al. (1993a); Hijikata et al. (1993a) and Suzuki et al. (unpublished results). The NS4A protein of Mr 8000 (Fig. 2b) and the N-terminal half of NS5A (240 aa) of Mr 29,000 (Fig. 2b) were also detected. Each processed product was also detected by the serum SK (Fig. 2c) and their sizes were consistent with those reported previously (Grakoui et al., 1993c; Manabe et al., 1994).

Intracellular localization of expressed HCV non-structural proteins was examined by indirect immunofluorescence analysis. As shown in Fig. 3, diffuse cytoplasmic staining of each HCV non-structural protein was observed in Hep3269 cells. No fluorescence was observed in Hepswx cells. Samples were also fixed in ice-cold methanol for 10 min. These two fixation methods gave identical results (data not shown).

The constitutive expression of such processed HCV proteins has been maintained after 30 passages over 6 months (data not shown).

**Proteolytic processing of the HCV non-structural proteins**

The time course of processing of these HCV non-structural proteins was then examined. As shown in Fig. 4, processed tNS2-NS3, NS4A, tNS5A and two unprocessed precursor proteins of tNS2-NS3-NS4-tNS5A, and of NS4A-NS4B-tNS5A were detected. The NS4A and tNS5A proteins appeared when the unprocessed

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**Fig. 1.** (a) Construction of expression vector cdEF321swxneo and pceF3269neo, carrying cDNA of the non-structural region of the HCV genome. Abbreviations: Ap', ampicillin resistance gene; EF, EF321 promoter; n, neomycin resistance gene; cos, the cos (cohesive end)-containing 1780 nt BglII fragment from λ phage Charon 4A; S, spacer fragment; O, replication origin of pBR327; 3269; HCV cDNA fragment spanning nt 3252 to 7100; E, EcoRV; K, KpnI; Sm, SmaI; Sw, SswA; X, XhoI. (b) The structure of the HCV genome is shown at the top. Approximate positions of the proteins used to prepare specific antibodies are indicated by grey boxes. cDNA is shown as an open box. Numbers at both ends of cDNA indicate the nt position of the HCV genome.
proteins disappeared. However, the tNS2–NS3 protein was detected immediately after the chase.

Although non-structural HCV proteins are considered to be generated from the precursor polyprotein by processing, there was an alternative possibility that these proteins are synthesized from aberrantly spliced RNA in this expression system. To examine this possibility, RNA from Hep3269 cells was analysed by a nuclease S1 protection experiment. The predicted size of the band after digestion with nuclease S1 was shorter than the size of the labelled DNA probe because the transcript of the EF321 promoter is spliced. As shown in Fig. 5, no bands were detected except for the expected band of 3-5 kb covering the entire HCV cDNA included in the probe DNA. Each non-structural HCV protein described here was thus generated from the precursor protein by processing.

Analysis of surface markers in Hep3269 cells

To study the interaction of viral and cellular proteins in Hep3269 cells, surface markers, which are characterized as differentiation antigens of leukocytes, were analysed by flow cytometry. As shown in Table 1, CD26 was highly expressed on Hep3269 cells. In contrast, no significant change was found for other surface antigens tested. To investigate the upregulated expression of the CD26 antigen, an immunoprecipitation analysis was performed. As expected, CD26 antigen of \( M_r \) 110000 was detected (Fig. 6). This result was further confirmed by indirect immunofluorescence analysis. Most of the cells showed strong CD26 immunoreaction with anti-CD26 MAb (Fig. 7). Similar results were also obtained in the other four Hep3269 cell clones with the same cDNA insert (data not shown). The fluorescence was mainly located on the cell membrane, supporting the result of flow cytometric analysis. No fluorescence was observed in parent HepG2 cells or a HepG2 clone constitutively expressing structural HCV proteins (data not shown).

Discussion

This is the first report of the establishment of a liver cell line constitutively expressing HCV proteins. We have established other liver cell lines stably expressing structural proteins of HCV by the same methods (Harada et al., unpublished results).

The size of each processed product was consistent with that reported previously. To examine the time course of processing in this system, the cells were analysed by pulse–chase experiment. The appearance of the NS4A and the tNS5A proteins was associated with the disappearance of unprocessed proteins. However, the tNS2–NS3 protein was detected immediately after the
HCV protein expression in a human cell line

Fig. 3. Indirect immunofluorescence analysis of Hep3269 cells. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature and incubated with the anti-NS3 MAb (a, b), anti-NS4A MAb (c, d) or rabbit anti-NS5A Ab (e, f). After incubation the cells were washed and FITC-conjugated goat anti-mouse Ig or goat anti-rabbit Ig was added at 1:200 dilution in PBS.

Fig. 4. Pulse-chase experiment with Hep3269 cells. Cells were labelled with 50 µCi of TRAN35S-label for 15 min and chased for 5 to 240 min before being immunoprecipitated with the SK serum and analysed by 10-20% gradient SDS-PAGE. Mr markers are indicated to the left. Empty arrowheads indicate the estimated sizes of the HCV unprocessed precursor polyproteins. Processed non-structural proteins of HCV are denoted by black arrowheads.

Table 1. Surface marker analysis of Hep3269 cells

<table>
<thead>
<tr>
<th>Surface marker*</th>
<th>Specificity</th>
<th>Hepswx cells</th>
<th>Hep3269 cells</th>
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<tbody>
<tr>
<td>CD10</td>
<td>Neutral endopeptidase</td>
<td>1-0</td>
<td>8-8</td>
</tr>
<tr>
<td>CD13</td>
<td>Aminopeptidase N</td>
<td>26-3</td>
<td>32-4</td>
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</tr>
<tr>
<td>CD15</td>
<td>Lacto-N-fucose pentaosyl III</td>
<td>8-4</td>
<td>24-9</td>
</tr>
<tr>
<td>CD26</td>
<td>Dipeptidyl peptidase IV</td>
<td>1-4</td>
<td>81-6</td>
</tr>
<tr>
<td>CD29</td>
<td>Integrin /β1 chain</td>
<td>92-0</td>
<td>96-6</td>
</tr>
<tr>
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<td>VLA-4 chain</td>
<td>37-0</td>
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<td>CD63</td>
<td>GP-53</td>
<td>18-3</td>
<td>29-3</td>
</tr>
<tr>
<td>CD71</td>
<td>Transferrin receptor</td>
<td>3-2</td>
<td>5-0</td>
</tr>
<tr>
<td>CD95</td>
<td>FAS antigen</td>
<td>15-0</td>
<td>26-8</td>
</tr>
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* Cluster of differentiation antigens of leukocytes.
† Measured by FACScan flow cytometry.
Fig. 7. Indirect immunofluorescence analyses of Hep3269 cells. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature and incubated with Ts145 (anti-CD26) MAb. After incubation the cells were washed and FITC-conjugated goat anti-mouse Ig was added at 1:200 dilution in PBS. (a), Hep3269 cells; (b), Hepsxw cells.

It is known that the NS3 region of the HCV genome has a functional domain of the viral proteinase that cleaves at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B sites, respectively, and the NS3 proteinase acts in cis at its C terminus to process itself from the polyprotein precursor (Grakoui et al., 1993b; Tomei et al., 1993; Failla et al., 1994). The cleavages at NS4A/NS4B and NS4B/NS5A were detected at the same time after the NS3/NS4A was cleaved. This is consistent with the previous notion that they are cleaved in trans by NS3 product. Previous experiments in vitro indicated that the NS5A sequence was necessary for the cleavage at NS4A/NS4B (Hijikata et al., 1993b). Present experiments described above, however, indicated that the partial NS5A sequence (240 aa) was sufficient for this cleavage. To examine whether there was aberrantly spliced RNA in this system, an RNA from the Hep3269 cells was analysed by the S1 nuclease protection method. The results indicated that the non-structural HCV proteins described here were generated from the precursor protein by processing. The serine proteinase responsible for processing the non-structural proteins of HCV is recognized as a possible target for novel antiviral agents. The cell line established in this study provides a useful system for screening compounds targeted against the proteinase.

Although HCV infection is implicated in development of hepatic cirrhosis and hepatocellular carcinoma (Bruix et al., 1989; Colombo et al., 1989; Saito et al., 1990; Nishioka et al., 1991), the mechanism is at present totally unknown. The constitutive expression of HCV proteins in hepatocytes is consequently useful for studying the intracellular events induced by HCV proteins and ultimately the pathogenesis of HCV infection.

As a first approach to study the virus–cell interaction in this system, we analysed surface markers on Hep3269 cells using flow cytometry. It should be noted that significantly upregulated CD26 levels in the clone were observed. CD26 has dipeptidyl peptidase IV (DPP IV, EC3.4.14.5) enzyme activity and is known to cleave N-terminal dipeptides from polypeptides with either L-proline or L-alanine at penultimate positions in its extracellular domain (Ulmer et al., 1990). It is also a T cell activation antigen (Dang et al., 1990) and is directly associated with adenosine deaminase (Kameoka et al., 1993) or Tat of human immunodeficiency virus type 1 (Gutheil et al., 1994). Furthermore, DPP IV has been implicated in hepatocyte/extracellular matrix interaction (Piazza et al., 1989). Although the mechanism remains to be elucidated, it is intriguing that non-structural proteins of HCV expressed in this cell line may be associated with the high expression of CD26 which can mediate signal transduction of hepatocytes. Studies to determine the essential sequence of the HCV genome associated with such upregulation of CD26 are ongoing. Information obtained from these studies may provide clues to understanding the pathophysiology of liver cells infected with HCV.

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