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VIRAL HEPATITIS

HepG2 cells support viral replication and gene expression of hepatitis C virus genotype 4 *in vitro*

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Abstract

AIM: To establish a cell culture system with long-term replication of hepatitis C virus (HCV) genome and expression of viral antigens *in vitro*.

METHODS: HepG2 cell line was tested for its susceptibility to HCV by incubation with a serum from a patient with chronic hepatitis C. Cells and supernatant were harvested at various time points during the culture. Culture supernatant was tested for its ability to infect naïve cells. The presence of minus (antisense) RNA strand, and the detection of core and E1 antigens in cells were examined by RT-PCR and immunological techniques (flow cytometry and Western blot) respectively.

RESULTS: The intracellular HCV RNA was first detected on d 3 after infection and then could be consistently detected in both cells and supernatant over a period of at least three months. The fresh cells could be infected with supernatant from cultured infected cells. Flow cytometric analysis showed surface and intracellular HCV antigen expression using in house made polyclonal antibodies (anti-core, and anti-E1). Western blot analysis showed the expression of a cluster of immunogenic peptides at molecular weights extended between 31 and 45 kDa in an one month old culture of infected cells whereas this cluster was undetectable in uninfected HepG2 cells.

CONCLUSION: HepG2 cell line is not only susceptible to HCV infection but also supports its replication *in vitro*. Expression of HCV structural proteins can be detected in infected HepG2 cells. These cells are also capable of shedding viral particles into culture media which in turn become infectious to uninfected cells.

Key words: Hepatitis C virus; *In vitro* propagation; Genomic replication; Gene expression; HepG2 cells

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INTRODUCTION

The lack of an efficient cell culture system or a readily available small animal model has hampered the development of therapies for hepatitis C virus (HCV) infection. The chimpanzee is the only animal that is susceptible to hepatitis viral infections, but its endangered status and financial considerations limit its widespread use in viral hepatitis research. Despite these difficulties, recent introduction of heterologous cDNA expression systems[1] and subgenomic replicons[2] have allowed researchers to study various aspects of the viral life cycle and examine novel antiviral therapies. Also, among the surrogate animal models that have been developed are mouse liver repopulated with human hepatocytes and transgenic mice expressing hepatitis antigens[3-5]. For reasons that are not evident, infection of primary hepatocytes and established cell lines with hepatitis viruses have not only produced poor viral replication and low viral yields but have also suffered from poor reproducibility[6]. The entry of virus into a cell, followed by productive viral replication, depends on both viral and host cell proteins. Only differentiated cells may express the latter. Thus, studies of HCV and HBV infectivity initially used

primary hepatocytes from humans or chimpanzees. One group infects human fetal hepatocytes with HCV-infected serum[7]. The viral replication is quite low and detectable only by RT-PCR amplification. Using this technique, another group showed an increase in the number of HCV+ strands by d 5, indicating that these hepatocytes support viral replication. Similarly, yet another group showed that adult primary human hepatocytes could be infected with HCV in culture conditions that support long-term cultures of hepatocytes for at least 4 mo[8]. Under these culture conditions, viral positive-strand RNA was first detectable by PCR after 10 d of infection, and the viral RNA titer increased in culture media during a 3-mo culture. This group also demonstrated *de novo* synthesis of negative-strand viral RNA. Culture supernatants from HCV-infected hepatocytes could transmit infection to naive hepatocytes, indicating the production of infectious viral particles. However, the efficiency of viral infection is unpredictable and does not correlate with viral RNA titers. Addition of polyethylene glycol to the primary hepatocyte cultures maintained in the presence of 20 g/L dimethylsulfoxide markedly increases the infection of HBV[9] but not HCV[10]. HCV is lymphotropic, and peripheral blood mononuclear cell cultures support HCV replication[11]. However, the level of viral replication is very low[12]. Because primary hepatocytes are difficult to grow in cultures, some researchers have attempted to infect immortalized hepatocytes and hepatoma cell lines. Ikeda and colleagues[13,14] used PH5CH, a nontumorigenic, immortalized human hepatocyte cell line, to assess the infectivity of HCV positive sera. There was an increase in the HCV sense -strand RNA during the first 12 d of culture, and the viral RNA remained detectable for at least 30 d after infection. Nucleotide sequence determination of the HCV genome in the hypervariable region 1 showed that there is a shift toward the limited HVR-1 population, indicating strong selection for HCV variants during the infection[13]. Furthermore, IFNγ inhibits the viral replication in these cells[14]. Recently, Guha *et al*[5] reported that *in vitro* cell culture models can at best demonstrate the infectivity of the virus but are not suitable to study viral life cycle because of the very low levels of viral replication. These systems could be used in evaluating drugs for antiviral activity or inhibition of HCV infection. Also, Horscroft *et al*[15] have summarized the recent development of HCV replicon cell culture system and its use in anti-HCV drug discovery. In the present study, we tested the susceptibility of HepG2 cell line to HCV and established an infection cell model that could support HCV long-term replication *in vitro*. The presence of both sense- and antisense-RNA strands and expression of viral core and envelope proteins in infected cells as well as the ability of these cells to exocytose infectious viral particles into culture media suggests that the current cellular model allows study of HCV life cycle.

MATERIALS AND METHODS

HEPG2 cell culture

Caucasian male *Homo sapiens* (human) hepatocellular carcinoma cell line (HepG2; ATCC, HB-8065, Manassas, USA) was used to establish the *in vitro* HCV replication. HepG2 culturing and infection were carried out according to the protocols described by Seipp *et al*[10]. HepG2 cells were maintained in 75 cm^2 culture flasks (greiner bio-one GmbH, Germany) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose and 10 g/L L-glutamine (Bio Whittaker, a Combrex Company, Belgium) containing 100 mL/L fetal calf serum (FCS; Biochrome KG Berlin Germany), 10 g/L antibiotics (penicillin/streptomycin; Biochrome KG, Berlin, Germany) and 1 g/L antimycotic (fungisone 250 mg/L; Gibco-BRL life Technologies, Grand Island, New Y). After adding all supplements the medium is called complete. The culture medium was renewed by a fresh medium every 3 d, and cells were subcultured (6-10 d).

In summary the medium was discarded, the adherent cell layer was shortly treated with trypsin-EDTA (2.5 g/L; Sigma, Deisenhofen, Germany) to remove the left traces of trypsin inhibitors from the FCS contained in the medium. After discarding, 1.0 mL of fresh trypsin-EDTA was added onto the cells and flasks were kept either at room temperature or at 37℃ (5-15 min) to observe the dettachement of cells from the flask wall. To avoid extended proteolytic effect of trypsin on the dettached cells complete medium was added to inhibit the enzyme activity. Cells were spun down at 400 g for 2 min, resuspended in 1 mL of complete medium, the exact count of cells was recorded in 50 μL aliqout after mixed with equal volume of trypan blue (5 g/L; BiochromKG, Berlin, Germany.) using a hemocytometer (Right Line; Sigma, Deisenhofen, Germany). A total of 3×10^6 cells were suspended in 10 mL complete medium and incubated at 37℃ in 5% CO_2.

Viral inoculation and sample collection

Cells were grown for 48 h to semi-confluence in complete medium, washed twice with FCS -free medium, then inoculated with a serum sample (500 μL sense and 500 μL FCS-free DMEM/3×10^6 cells) obtained from HCV infected patients (RT-PCR and antibody positives). The HCV genotype in the used sera was previously characterized as genotype 4 based on the method described by Ohno *et al*[16]. The viral load in the used serum was quantitated by real time PCR and the average copy number was 290×10^6 copies/L. After 90 min, DMEM containing FCS was added to make the overall serum contents 100 mL/L in a final volume of 8 mL including the volume of human serum used for infection as mentioned above (0.04483 copies/cell). Cells were maintained overnight at 37℃ in 5% CO_2. On the next day, adherent cells were washed three times with culture medium to get rid of the remaining infection serum and incubation was continued in complete medium containing 100 mL/L FCS with regular medium changes. The viral infection in HepG2 cells throughout the culture duration was assessed qualitatively by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of viral antigens, RT-PCR amplification of sense and antisense strands and quantitatively by real time PCR.

Flow cytometric analysis of intracellular staining of HCV core antigen in infected HEPG2 cells

The intracellular staining of HCV core antigen in infected HepG2 cells was quantified by using a fluorescence activated cell sorting (FACS) based assay. Intracellular staining labeling was performed by direct immuno-fluorescence. HepG2 cells (collected after addition of trypsin) were centrifuged and supernatants were removed. Cell pellets were washed 4 times with PBS. For intracellular staining, cells were incubated with 4% paraformaldehyde for 10 min and 0.1% Triton X-100 in Tris buffer (pH 7.4) for 6 min. After washed with PBS, cells were incubated with FITC-labeled $F(ab)_2$ portion of HCV core antibody (at 1:2000 dilution) for 30 min at 4℃. Cells were washed with PBS containing 1% normal goat serum and suspended in 500 μL and analyzed by flow cytometry (FACS Calibure, BD). Mean fluorescence intensity was determined using Cell Quest software (Becton Dickinson).

Flow cytometric analysis of labeled E1 antigen on surface of infected HEPG2 cells

The surface staining of HCV E1 antigen in infected HepG2 cells was quantified by using a fluorescence activated cell sorting (FACS) based assay. Surface labeling was performed by direct immuno-fluorescence. HepG2 cells collected after trypsinization were centrifuged and supernatants were removed. Cell pellets were washed 4 times with PBS. Cells were incubated with FITC labeled HCV E1 antibody (at 1:1500 dilution) for 30 min at 4℃. Cells were washed 3 times with PBS containing 10 mL/L normal goat serum and suspended in 500 μLPBS and analyzed with flow cytometry (FACS Calibure, BD). Mean fluorescence intensity was determined using Cell Quest software (Becton Dickinson).

Western blot analysis of HCV antigens in HEPG2 cells

Uninfected and infected HepG2 cell lysates were subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)[17] through 40 g/L stacking and 160 g/L resolving gels in 0.75 mm-thick vertical slab gels. Cell lysate samples were diluted at 1:25 in PBS, mixed with the sample buffer (0.125 mol/L Tris base, 40 g/L SDS, 2% glycerol, 100 g/L mercaptoethanol, and 1 g/L bromophenol blue as a tracking dye) and immediately boiled for three min. A mixture of reference proteins was run in parallel. Gels were then stained with Coomassie blue. Western blotting was performed as follows: resolved samples separated by SDS-PAGE were electro-transferred onto nitrocellulose membranes (0.45 mm pore size). On the next day, membranes were cut into individual strips each of 0.3 mm width. Strips were washed 3 times with PBS-3 g/L T each for 5 min and blocked against non specific binding at room temperature for 1 h in PBS-3 g/L T-10 g/L bovine serum albumin (BSA). Strips were washed 3 times as above and incubated with diluted first antibody (infected human serum at 1:100, or anti-core/envelope rabbit antibodies at 1:500) in PBS-3 g/L T at room temperature for 2 h. After washed 3 times, strips were incubated with diluted peroxidase-labeled second antibodies (anti-human IgG/IgM mixture at 1:5000 in PBS-3 g/L T for previously treated strips with human sera or anti-rabbit IgG at 1:1000 in PBS-3 g/L T for those treated with rabbit anti-core/envelope antisera. Both antibodies were from Jakson Immuno Research Laboratories; Dianova, Hamburg, Germany) for 2 h at room temperature. Visualization of immune complexes on the nitrocellulose membrane was done by developing the strips with 0.01 mol/L PBS (pH 7.4) containing 50 mg diaminobenzedine (Sigma; Deisenhofen, Germany) and 100 μL of 30 mL/L hydrogen peroxide.

Isolation and extraction of RNA from serum and HEPG2 cells

Isolation and extraction of RNA from serum and HEPG2 cells were performed as reported in our previous study[18]. Briefly, cells were precipitated and washed in the same buffer to remove adherent viral particles before lysis in 4 mol/L guanidinium isothiocyanate containing 25 mmol/L sodium citrate and 0.5% sarcosyl and 0.1 mol/L b-mercaptoethanol. Cellular RNA was extracted using the single-step method described originally by Chomczynski and Sacchi[19].

PCR of genomic RNA strands of HCV

Reverse transcription-nested PCR was carried out according to Lohr *et al*[20] with few modifications. Retrotranscription was performed in 25 mL reaction mixture containing 20 units of AMV reverse transcriptase (Clonetech, USA) with either 400 ng of total PBMC RNA or 3 mL of purified RNA from serum samples (equivalent to 30 mL serum) as template, 40 units of RNAsin (Clontech, USA), a final concentration of 0.2 mmol/L from each dNTP (Promega, Madison, WI, USA) and 50 pmol of the reverse primer P1 (for sense strand) or 50 pmol of the forward primer P2 (for anti-sense strand). The reaction was incubated at 42℃ for 60 min. and denatured at 98℃ for 10 min. Amplification of the highly conserved 5' -UTR sequences was done using two rounds of PCR with 2 pairs of nested primers. The first round amplification was done in 50 mL reaction containing 50 pmol from each of P2 forward primers and P3 reverse primers, 0.2 mmol/L from each dNTP, 10 μL from RT reaction mixture as template and 2 units of Taq DNA polymerase (Promega, USA) in 1 × buffer supplied with the enzyme. The thermal cycling protocol was as follows: 1 min at 94℃, 1 min at 55℃ and 1 min at 72℃ for 30 cycles. The second round amplification was done as the first round, except for use of the nested reverse primer P4 and forward primer P5 at 50 pmol each. A fragment of 172 bp was identified in positive samples. Primer sequences are as follows: P1: 5' ggtgcacggtctacgagacctc 3'; P2: 5' aactactgtcttcacgcagaa 3'; P3: 5' tgctcatggtgcacggtcta 3'; P4: 5' actcggctagcagtctcgcg 3'; P5: 5' gtgcagcctccaggaccc 3'. To control false detection of negative-strand HCV RNA and known variations in PCR efficiency[21,22], specific control assays and rigorous standardization of the reaction were employed as previously described[20]. These specific control assays were cDNA synthesis without RNA templates to exclude product contamination, cDNA synthesis without RTase to exclude Taq polymerase RTase activity, cDNA synthesis and PCR step done with only the reverse or forward

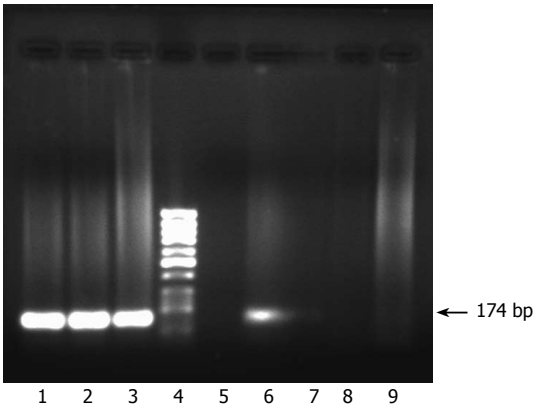

Figure 1 Establishment of an *in vitro* infection experiment of HepG2 cells with serum of a HCV genotype 4-infected patient and monitoring success of infection by nested RT-PCR amplification of viral sense and minus strands. The patient was confirmed to be infected with HCV as demonstrated by nested RT-PCR amplification of viral positive strand in the serum (lane 1), and both viral positive and negative strands (lane 2 and lane 3 respectively) in the peripheral blood mononuclear cells. After infection of the cells with the patient sera (see the detailed method in the materials and methods), cells were carefully washed and nested RT-PCR was carried out on the last wash to make sure that the culture medium contained no more viral RNA and results showed no amplified products (lane 5). At this stage we were sure that any detection of viral RNA within the cells could reflect successful viral adsorption and penetration. Three days after infection, RNA was extracted from both cells and their supernatant and nested RT-PCR for detection of both viral strands was carried out and results showed the presence of the sense strand in the cells (lane 6) but not in the supernatant (lane 7). The antisense strand was neither present in the cells nor in the supernatant (lanes 8, 9). Lane 4 is molecular weight standard DNA marker (Ø-X-174/HaeIII; Q-BIOgene, Germany).

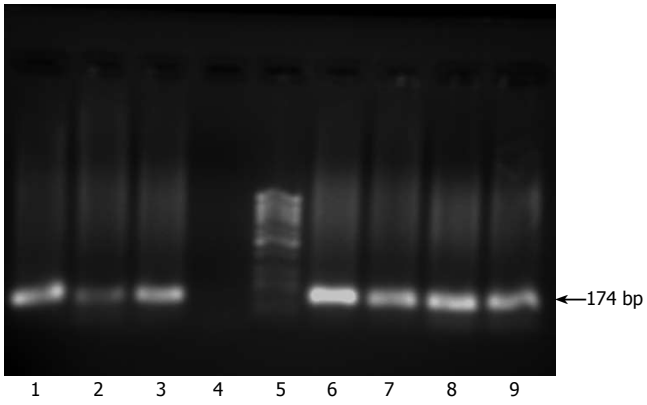

Figure 2 Monitoring of active viral replication at regular time intervals post infection of HepG2 cells. RNA was extracted from infected cells and infectious supernatants and their passages at 1, 2 and 4 wk post infection and nested RT-PCR was carried out for detection of both viral strands. Results showed the presence of both viral strands in RNA extracted from cells 1 wk post infection (lanes 1, 2) but only the sense strand was detectable in the supernatant at this time point (lane 3) while the antisense strand was absent (lane 4). At 2 and 4 wk post infection, both viral strands were detectable in both cells and supernatant. Lanes 6-9 show the presence of both sense and antisense strands in both cells and supernatant at 4 wk post infection. Lane 5 shows molecular weight standard DNA marker (Ø-X-174/Hae Ⅲ; Q-BIOgene, Germany).

primer to confirm no contamination from mixed primers. These controls were consistently negative. In addition, cDNA synthesis was carried out using only one primer followed by heat inactivation of RTase activity at 95℃ for 1 h, in an attempt to diminish false detection of negative-strand prior to the addition of the second primer.

RESULTS

Establishment of HCV HEPG2 cells in culture

Success of infection was monitored by nested RT-PCR amplification of viral sense and antisense (minus) strands (Figure 1). To confirm the infection of HCV in a patient with chronic active hepatitis whose serum was used in infection of HepG2 cells, nested RT-PCR amplification of viral sense strand in the serum (lane 1) and both viral sense and antisense strands (lane 2 and lane 3 respectively) in peripheral blood mononuclear cells were demonstrated. The viral load was quantified in patient's serum as 2.9×10^5 using real time PCR method (results not shown). After infection of the cells with the patient's sera, cells were carefully washed and nested RT-PCR was carried out on the last wash to make sure that the cell wash contained no more viral RNA (lane 5). At this stage we were sure that any detection of viral RNA within the cells could reflect successful viral binding and entry. Three days after infection, RNA was extracted from cells and culture media. Nested RT-PCR was carried out for detection of both viral strands. Results shown in Figure 1 displayed the presence of sense RNA strand in the cells (lane 6) but not in the culture media (lane 7). The negative strand was neither present in the cells nor in the culture media (lanes 8, 9).

Monitoring of active viral replication at regular time intervals after infection of HEPG2 cells

RNA was extracted from infected cells and infectious supernatants and their passages at 1, 2 and 4 wk post infection and nested RT-PCR was carried out for detection of both viral strands (Figure 2). Results showed the presence of both viral strands in RNA extracted from cells 1 wk post infection (lanes 1, 2) but only the positive strand was detectable in the supernatant at this time point (lane 3) while the negative strand was undetectable (lane 4). At 2 and 4 wk post infection, both viral strands were detectable in both cells and supernatant. Lanes 6-9 show the presence of both sense and antisense strands in both cells and supernatants 4 wk post infection. Results at 2 wk were not demonstrated.

Monitoring infection of HEPG2 cells using culture medium from primary infected cells by nested RT-PCR

After incubation of HepG2 cells with infectious medium presumably containing exocytosed viral particles from primary infected cells, *de novo* infected cells were carefully washed to get rid of any viral traces and the last wash was checked for presence of viral RNA using nested RT-PCR which produced no amplified products (Figure 3, lane 1). RNA was extracted from infected cells as well as their culture media at 3 d, 1 wk, 2 wk and 4 wk post co-incubation with the infectious medium and subjected to nested RT-PCR to check the presence of either or both viral strands (Figure 3). After 3 d the cells contained only sense viral strand (lane 2) while the anti-sense strand was undetectable (lane 3). The supernatant contained neither strand (lanes 4, 5). After 1 wk post infection,

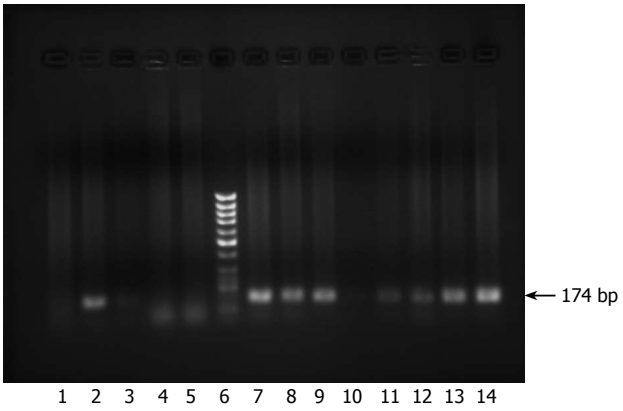

Figure 3 Monitoring infection of HepG2 cells using culture medium from primary infected cells by nested RT-PCR. After incubation of HepG2 cells with infectious medium presumably containing exocytosed virions from primary infected cells, cells were carefully washed to get rid of any viral traces and the last wash was subjected to RNA extraction followed by nested RT-PCR. Results showed no amplification of positive strand products (lane 1). RNA was extracted from infected cells and supernatants at 3 d, 1, 2 and 4 wk post co-incubation with the infectious supernatant and subjected to nested RT-PCR to check for the presence of each viral strand. At 3 d the cells contained only the positive viral strand (lane 2) but the negative strand was undetectable (lane 3). However, the supernatants contained none of the strands, only primers used could be seen (lanes 4, 5). Lane 6 is molecular weight standard DNA marker (Ø-X-174/HaeⅢ; Q-BIOgene, Germany). At 1 wk post infection, both sense and antisense strands were detectable in the RNA extracted from the infected cells (lanes 7, 8), whereas the supernatant contained only the sense strand (lane 9) but not the antisense strand (lane 10). At 2 and 4 wk, RNA extracted from infected cells as well as the supernatants contained both positive and negative strands. Results of amplification for both positive and negative strands for cellular RNA are shown (2 wk, lanes 11, 12) and (4 wk, lanes 13, 14).

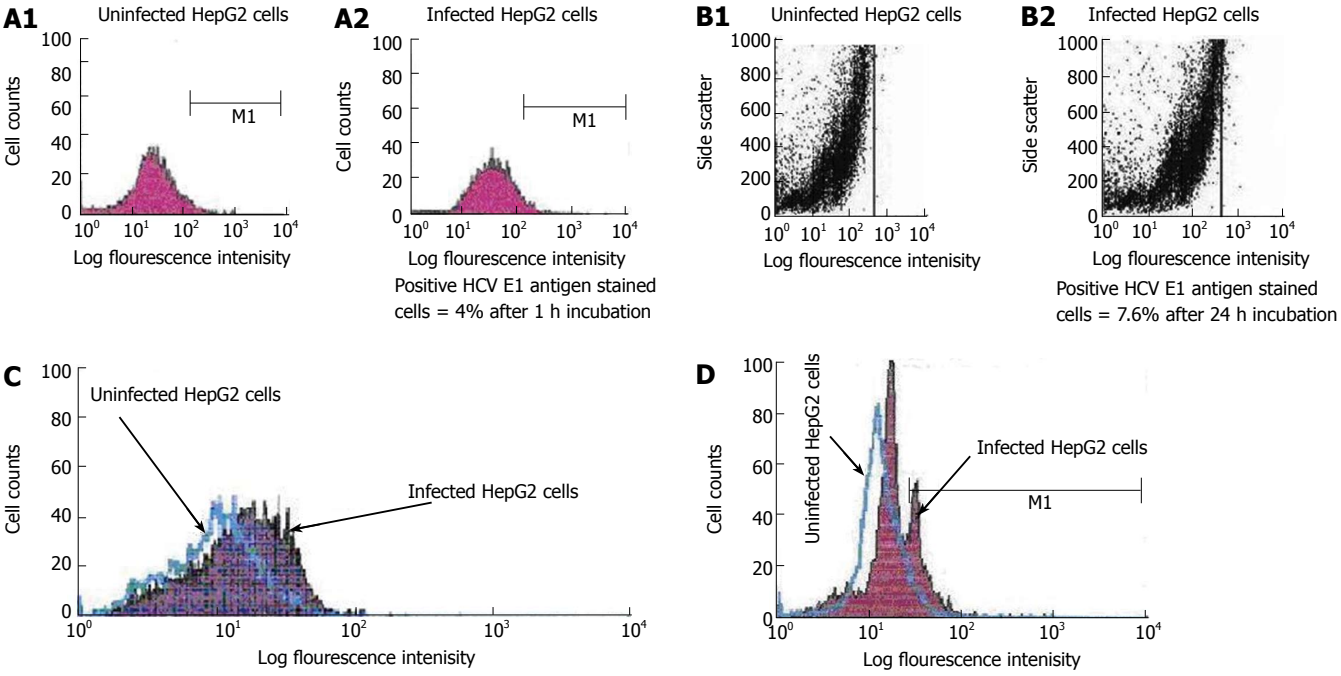

Figure 4 **A**: Single parameter histogram for flow cytometric analysis of surface staining of HCV E1 gene expression on the infected HepG2 cells after one hour incubation. HepG2 cells were incubated with PBS (**A1**) (uninfected) or with HCV positive serum (**A2**) (infected) for 1 h incubation. Cells were harvested and stained with FITIC labeled HCV anti-E1 antibody as described in materials and methods; **B**: Dot histogram for flow cytometric analysis of surface staining of HCV E1 gene expression on the infected HepG2 cells after 24 h incubation. HepG2 cells were incubated with PBS (**B1**) (uninfected) or with HCV positive serum (**B2**) (infected) for 24 h incubation. Cells were harvested and stained with FITIC labeled HCV anti-E1 antibody; **C**: Overlap histogram for flow cytometric analysis of surface staining of HCV E1 gene expression on the infected HepG2 cells after one week incubation. HepG2 cells were incubated with PBS (uninfected) or with HCV positive serum (infected) for one week incubation. Cells were harvested and stained with FITIC labeled HCV anti-E1 antibody; **D**: Overlap histogram for flow cytometric analysis of intracellular staining of HCV core gene expression in the infected HepG2 cells after 3 d incubation. HepG2 cells were incubated with PBS (uninfected) or with HCV positive serum (infected) for 3 d incubation. Cells were harvested and stained with FITIC labeled HCV anti-core antibody Labeled cells were analyzed with flow cytometry (FACS Calibure, Becton Dickinson).

both sense and antisense viral strands were detectable in infected cells (lanes 7, 8), whereas the supernatant contained only the sense strand (lane 9) while the antisense strand was undetectable (lane 10). After 2 and 4 wk post infection, RNA extracted from infected cells as well as their supernatants contained both positive and negative strands. Results of amplification of both positive and negative strands from cellular RNA at 2 and 4 wk are presented in lanes 11-14. Results of the nested RT-PCR on the supernatant at the same time points were not demonstrated since they were identical to those obtained from the cells. However, culture supernatant from infected HepG2 cells was used to infect naïve (uninfected) cultured HepG2 cells and we found that these HepG2 cells were infected as detected by RT-PCR (Data not shown).

Flow cytometric analysis of surface and intracellular staining of HCV antigen expression in infected HEPG2 cells

Flow cytometric analysis showed that HCV core and E1 antigens were detected on surface and inside of the infected HepG2 cells. Figures 4A-C show the percentage of anti E1 positive staining on the surface of HepG2 cells after 1 h (4%) and 24 h (7.6%) and one week (12.5%) of incubation of HepG2 cells with positive HCV serum sample. Core protein was detectable in 5.7% of cells after

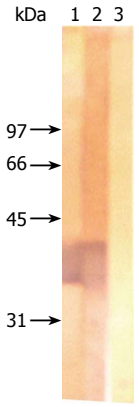

Figure 5 Testing translation of viral E1 in supernatant and lysates of HepG2 cells infected with HCV from 1 mo culture by Western blot analysis. Supernatant (strip 1) and lysates (strip 2) of HepG2 cells infected with HCV were subjected to Western blot analysis, hybridization with the anti-E1 antibody clearly showed the expression of a cluster of immunogenic proteins at molecular weights localized between 31 and 45 kDa. This cluster was undetectable on the strip immobilized with uninfected HepG2 cell lysates (strip 3).

24 h, and increased to 13.5% of cells after 3 d. Figure 4D shows the intracellular staining of core antigen using F(ab)2 portion of the core antibody after infection of 3 d.

Western blot analysis of HCV viral antigen expression in infected HEPG2 cell lysates

When supernatant (Figure 5, strip 1) and lysates (strip 2) of HepG2 cells infected with HCV were subjected to Western blot analysis, hybridization with the anti-E1 antibody could clearly show the expression of a cluster of immunogenic peptides at molecular weights extended between 31 kDa and 45 kDa over 1 mo period. This cluster was undetectable on the strip immobilized with uninfected HepG2 cell lysates (strip 3).

DISCUSSION

Although knowledge of the molecular biology of HCV has progressed rapidly, our understanding of viral replication and pathogenicity is still hampered by the lack of reliable and efficient cell culture systems. To achieve a reliable *in vitro* system we need to obtain a biological status wherein viral-host interactions mimic exactly what happens naturally *in vivo*, since both viral and host factors make up together the overall outcome of the pathogenetic pathways. The reasons for using HepG2 cells in the current study include the great similarity in biosynthetic pathways between primary hepatocytes and HepG2 cells[23]. Also the later cells contain LDL and CD81 receptors which are known to mediate HCV entry into cells[24]. Validity of HepG2 cells in propagating HCV has been reported by other laboratories[25]. The viral component of the model has several alternative strategies. Subgenomic or genomic replicons have been used in elucidating the replicative machinery of the virus[26] but could not mimic the actual viral replication cycle and shedding of the virus to the culture medium. Despite the extremely robust *in vivo* replication rate of HCV using genomic replicons, efforts to propagate the virus in cell culture have been frustratingly unsuccessful[27]. Thus the viral replication but not the biologically relevant infectious viral particles can be demonstrated by such approach. In the present study we utilized infectious serum with native viral particles presumably containing the full length viral RNA genome in infecting HepG2 cells *in vitro*. The recent understanding of the HCV molecular biology demonstrates that both 5' and 3' untranslated regions of the viral RNA genome play a pivotal role in translation of viral proteins *via* interaction with cellular factors including eukaryotic initiation factor 3 eIF3[35], 40S ribosomal subunit[28] and poly pyrimidine tract binding (PTB)[29] protein. Besides, it has been shown that intra genetic viral interactions such as NS4a/NS5a are required for key pathways in HCV life cycle. In the current study, the use of infectious viral particles containing intact RNA genome could guarantee the presence of the necessary elements involved in translation of polyprotein precursor and viral replication. We have presented several lines of evidence that the cell model described herein maintains HCV life cycle. A minor fraction of cells (4%) had a detectable viral envelope on cell surface as early as one hour after incubation. This fraction steadily increased to 7.6% in 24 h and 12.5% after one week. E1 protein reached detectable levels by Western blotting analyses at both intracellular and extra cellular compartments after one month. *De novo* synthesis of RNA minus strand was detected inside HepG2 cells as early as one week post infection and appeared in the medium one week later. However, the detection of the replicative intermediate (antisense strand HCV RNA) is thought to be reasonable for assessment of HCV replication. Because detectable HCV structural proteins in cells after infection may represent the residue of the inoculated virus after releasing the viral genome to cytoplasm, it is necessary to demonstrate that HCV structural proteins detected in the infected cultures are newly synthesized rather than residuals of viral inoculum. Interestingly, the core protein was only detectable in 5.7% of cells after 24 h and increased to 13.5% of cells after 3 d, indicating that such observed increase in core expression reflects part of *de novo* synthesized structural viral proteins. The ability of culture medium to transmit viral particles to new cells later in one month culture with concomitant detection of core (results not shown) and envelope proteins as well as detection of sense and antisense RNA strands suggest that infected HepG2 cells reach a state of equilibrium after one month of infection. Other cellular models for HCV propagation can transmit viral particles to naïve cells[30]. We assume that this approach brings our *in vitro* system to become closer to native viral infection status occurring *in vivo*. The expression of different viral antigens agrees with the earlier reports that liver and blood cells from infected patients do support these expressions[11,31-35]. Our observation that HCV RNA detection was intermittent during early days post infection agrees with previous reports on infection experiments[7,10,33], a finding which has led the investigators to suspect the consistency of viral replication and gene expression in these cell models.

In conclusion, we report an *in vitro* system of cultured HepG2 cells infected with HCV particles. These cells support viral replication and gene expression. The consistent expression of viral proteins and the ability of culture medium to transmit the virus to new cells make this model optimum for studying HCV life cycle, screening for anti HCV drugs and testing the efficacy of therapeutic antibodies.

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