Short Communication

Mode of swine hepatitis E virus infection and replication in primary human hepatocytes

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Received 28 May 2014
Accepted 8 August 2014

The aim of this study was to investigate the infection and replication of swine-derived hepatitis E virus (HEV) in primary cultured human hepatocytes (PHCs). Hepatocytes were cultured from the resected normal livers of patients with metastatic tumours. These cultured hepatocytes were infected with swine-derived genotype 3 or 4 HEV. Viral replication was monitored using reverse transcriptase-quantitative PCR. The amount of HEV RNA increased in the culture media and cells following infection. Immunofluorescence staining implied that the spread of HEV infection in hepatocytes was attributed mainly to cell-to-cell transmission via the cell membrane. The sequences of the inoculated and propagated HEV were determined to examine whether sequence variation occurred during infection. Sequence analysis showed that there were no differences between inoculated and propagated HEV, demonstrating that in vitro infection and replication of swine HEV in PHCs occurred without sequence variation.

Hepatitis E virus (HEV) causes acute viral hepatitis E in many parts of the world (Emerson et al., 2004a, b; Huang et al., 2005; Shukla et al., 2011). HEV infection appears to account for >50 % of the acute viral hepatitis cases observed amongst young to middle-aged adults and is associated with an unusually high mortality rate in infected pregnant women of up to 20 % (Datta et al., 1987). HEV transmission is thought to occur mainly through drinking water in developing countries, and through the ingestion of uncooked or undercooked meat and the viscera of animals (Okamoto et al., 2003; Tei et al., 2003). HEV isolates are categorized into four genotypes (Zhai et al., 2006). Genotypes 1 and 2 are restricted to humans. Genotypes 3 and 4 are indicated to undergo zoonotic transmission; swine serve as a reservoir for these genotypes (Meng et al., 1997) and other mammals, such as rats and rabbits, might also act as reservoirs (Li et al., 2009; Ma et al., 2010).

The in vitro propagation of HEV has been attempted in various cell lines and in primary cultured hepatocytes from non-human primates (Kamar et al., 2012). Tanaka et al. (2007) demonstrated an efficient HEV propagation system in a human hepatocarcinoma cell line (PLC/PRF/5) and in a human lung cancer cell line (A549) using a genotype 3 HEV strain (JE03-1760F) obtained from a faecal specimen of a Japanese patient with hepatitis E. However, the HEV obtained from this propagation system was found to contain nucleotide sequences that were different from those of the strain used originally for the development of the HEV propagation system (Lorenzo et al., 2008). To the best of our knowledge, there has been no report of in vitro infection and replication of HEV, including strains obtained from swine, in primary cultured human hepatocytes (PHCs). The present study aimed to obtain direct evidence and to characterize swine-derived HEV infection of human hepatocyte cells using a primary culture system. We also investigated mutational events of the propagated HEVs in PHCs.

Isolates from the following four different HEV clusters were used: G3Ip, G3US, G3SP and G4IP (Takahashi et al., 2003; Lu et al., 2006) (Table 1). These viruses were obtained from the faeces of naturally infected swine in Japan. HEV was purified from faecal samples (10 g) from experimentally infected swine, according to previously...
described procedures (Yunoki et al. 2008). The obtained virus solutions were aliquoted and stored at −80 °C as purified HEV stocks until use.

Total HEV RNA was extracted from each sample using the RNeasy Mini kit (Qiagen) and measured using reverse transcriptase-quantitative PCR (RT-qPCR), as described previously (Jothikumar et al., 2006; Urayama et al., 2010). Based on the standard curve, a viral genome copy number <50 (10¹⁻²) ml⁻¹ was found to be below the lower limit of detection. The HEV infectivity in the samples was assayed according to Huang et al. (1999) with minor modifications, including the use of A549 cells, as described previously (Takahashi et al., 2012).

Liver resection samples were obtained from three patients undergoing partial hepatectomy for metastatic liver tumours at Tsukuba University Hospital. The samples were negative for human immunodeficiency virus and hepatitis B/C virus by serological examination. Hepatocytes were isolated from the normal liver samples using a modified two-step collagenase perfusion procedure, as described previously (Guguen-Guillouzo, 1992). Freshly isolated hepatocytes were seeded in rat-tail-collagen-coated six-well plates (BD Biosciences), on rat-tail-collagen-coated four-well chamber glass slides (BD Biosciences) or on 15 mm rat-tail-collagen-coated glass coverslips in 35 mm Petri dishes at a density of 1.2 × 10⁶ viable cells cm⁻². Adhesion was performed overnight in William’s Medium E supplemented with 200 μM L-glutamine, 10% FBS, 100 μM dexamethasone, 1 μg insulin ml⁻¹ and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO₂ atmosphere (Rumin et al., 1996).

The collection of human hepatocytes and experiments using the hepatocytes were conducted in accordance with informed patient consent after the consideration and approval of a research ethics committee.

As hepatocytes have not been shown to proliferate in culture, passage of the cells was not performed in the present study. The culture medium was replenished at the intervals specified below until the cultures were harvested on the indicated days. To determine if the cultured hepatocytes maintained the characteristics of hepatocytes, albumin release in the culture medium was measured. To obtain this measurement, the culture medium that was removed when the medium was replenished was subjected to ELISA analysis using the Human Albumin ELISA Quantification Set (Bethyl Laboratories). The amount of albumin in the cultures was consistent with previous reports for hepatocyte cultures (Weiss et al., 2002), demonstrating that the hepatocytes in these cultures maintained the characteristics of hepatocytes (data not shown).

Cells cultured in a six-well plate were washed three times with PBS(−) and treated with virus solution (1 ml) that was prepared by diluting the virus stock with William’s Medium E containing 2% FBS as indicated below; the plates were incubated for 1 h at 37 °C in a humidified 5% CO₂ atmosphere. Then, 2 ml William’s Medium E containing 200 μM L-glutamine, 10% FBS, 100 μM dexamethasone, 1 μg insulin ml⁻¹ and 1% penicillin/streptomycin (maintenance medium) was added to the culture, followed by incubation under the same conditions for an additional 2 h. After HEV infection, the cultured cells were washed four times with 8 ml William’s Medium E and replenished with 3 ml maintenance medium before further incubation under the same conditions. The media of the cultured cells were refreshed at the intervals indicated in the respective experiments. At the specified time, the media and cultured cells were collected separately and used for the determination of HEV RNA levels with RT-qPCR. We determined whether HEV infection induced a cytopathic effect in PHCs by monitoring lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) (markers of hepatocyte lysis) activity in the culture medium.

As a preliminary experiment, 0.33 ml of each virus stock solution was used to inoculate a well of cultured cells and the infected cells were examined for the production of progeny virus in the cultures. As shown in Fig. 1(a), all of the virus isolates examined were shown to propagate in the infected cells. HEV was not detected in the mock-infected cells (data not shown). These results indicated that all of the HEV isolates propagated in PHCs.

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Isolation</th>
<th>HEV genome [log₁₀(copies) ml⁻¹]†</th>
<th>HEV infectivity [log₁₀(copies) ml⁻¹]‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3JP</td>
<td>swJR-P5</td>
<td>3.39 × 10⁷</td>
<td>3.16 × 10⁴</td>
</tr>
<tr>
<td>G3US</td>
<td>swJB-M8</td>
<td>5.89 × 10⁶</td>
<td>–</td>
</tr>
<tr>
<td>G3SP</td>
<td>swJB-E10</td>
<td>1.26 × 10⁶</td>
<td>3.98 × 10⁴</td>
</tr>
<tr>
<td>G4JP</td>
<td>swJB-H7</td>
<td>1.58 × 10⁶</td>
<td>6.31 × 10⁴</td>
</tr>
</tbody>
</table>

*Genotypes (G) and clusters of isolates were grouped as described by Takahashi et al. (2003) and Lu et al. (2006).
†For swJB-M, the specific primer sets and probes (HE86, HE87 and FAM-labelled probe FHE100) were designed by Urayama et al. (2010). For the other isolates, the primer set and probe (HE86, HE87 and FAM-labelled probe FHE88) were designed by Jothikumar et al. (2006).
‡Infectivity titre is given as log dilution non-detectable end-point per ml.
Fig. 1. Amount of HEV RNA propagated in PHCs. (a) Amount of HEV RNA in the culture medium of PHCs inoculated with four HEV isolates (genotypes 3 and 4). The amount of HEV RNA in the removed media was measured. Two independent infection experiments (Exp. 1 and 2) were performed for four virus isolates. The abscissa represents the period of days after inoculation (day 0: the day of inoculation), whereas the ordinate represents the amount of HEV RNA on a log scale. The lower limit of detection in the HEV RNA measurement was 1.7 and is shown as a dotted line. (b) Amount of HEV RNA in PHC cultures at various incubation times following infection with HEV G3JP. The human hepatocytes in the culture wells were infected at 4.8×10^6 and 4.8×10^5 viral genome copies per well. The amounts of viral RNA in the medium and the cells were added together for each respective culture well and plotted. The abscissa, ordinate and dotted line are the same as in (a).
For precise characterization of HEV infection of human hepatocytes, the G3JP HEV, which showed the highest increase of virus titre in culture medium at an early phase of infection (Fig. 1a), was selected and used for further analyses. Varying amounts of HEV (4.8 × 10⁶, 4.8 × 10⁵, 4.8 × 10⁴ and 4.8 × 10³ viral genome copies per well) were used to infect PHCs. The infected and mock-infected cells were cultured, and the medium was refreshed every 2–3 days.

Fig. 2. Immunofluorescence staining. (a) Immunofluorescence staining of infected and mock-infected PHCs using an antibody against the HEV ORF2 protein. Fluorescence staining of all of the cell clusters was observed across the entire 15 mm glass coverslips at 1, 2 and 3 weeks after inoculation. Although no ORF2 proteins were detected in the mock-infected cells, the number of cells in each cluster appeared to increase with time after infection. ‘DAPI’ shows the nuclei of the cells in blue, ‘FITC’ shows the ORF2 protein of HEV in green and ‘DAPI+FITC’ shows the superimposed images. (b) Graph showing the result of the immunofluorescence staining in (a). Although the number of fluorescence-stained clusters across the entire 15 mm glass coverslips showed no difference between the time points, the number of fluorescence-stained cells was found to increase significantly with time after infection. Data of the stained cells are expressed as the mean. Bar, SD. The numbers of stained cells were 5.8, 9.4 and 17.3 at 1, 2 and 3 weeks after inoculation, respectively. The numbers of stained cell clusters were 15, 19 and 18 at 1, 2 and 3 weeks after inoculation, respectively. Statistical analyses were carried out with the Mann–Whitney U-test. A P value <0.05 was considered significant. *P<0.05 versus 1 week after inoculation.
days. The cells and medium were collected separately, and the HEV RNA levels were analysed. The amounts of viral RNA in the medium and in the cells were added together for each well to obtain the total virus amount in the culture wells.

In all of the inoculated cells, the total virus RNA amounts detected were decreased at day 2 and then increased until the end of the infection experiment. The results of the infection at $4.8 \times 10^6$ and $4.8 \times 10^5$ viral genome copies per well are shown in Fig. 1(b); the results at $4.8 \times 10^4$ and $4.8 \times 10^3$ viral genome copies per well are not shown. These findings were confirmed by repeated experiments (data not shown). The decrease in the viral RNA observed on day 2 (Fig. 1b) may be the result of the disintegration of the virus that was not involved in the establishment of the infection.

PHCs were inoculated with HEV on 15 mm rat-tail-collagen-coated glass coverslips in 35 mm Petri dishes at a density of $4.8 \times 10^5$ viral genome copies per dish, and cultured for 7, 14 and 21 days. After the culture medium was removed, the HEV-infected or mock-infected PHCs on glass coverslips were fixed with 3% paraformaldehyde in PBS for 10 min and permeabilized in 0.5% Triton X-100 in PBS for 5 min. The coverslips were then soaked in 1% non-fat dried milk in PBS and incubated in a solution containing a rabbit antibody against the HEV ORF2 protein at room temperature for 1 h (Li et al., 1997). After being washed twice with PBS, the cells on the coverslips were stained with a FITC-conjugated goat antibody against rabbit IgG, followed by incubation with 3 μM DAPI. The coverslips were mounted on glass slides and the cells were observed under a fluorescence microscope (Carl Zeiss).

As the immunofluorescence staining in Fig. 2(a) shows, fluorescence-stained cell clusters were observed in the infected cell culture at all of the time points examined. In addition, the number of cells in the clusters appeared to increase with time after the infection. The number of stained cells in the clusters and the number of stained clusters in the cultures were counted across the entire 15 mm rat-tail-collagen-coated glass coverslip. As shown in Fig. 2(b), the number of cells was found to increase significantly with the time after infection ($P<0.05$), whereas the number of clusters appeared to show no differences between the time points. These observations were similar to the spread of HEV infection observed when an infectious HEV cDNA clone was added to cultured cells (Yamada et al., 2009). These observations led us to hypothesize that the spread of HEV infection in human hepatocytes was the result of cell-to-cell transmission through the cell membrane rather than infection of HEV through the culture media.

To examine the possible base changes that occur during the process of swine-derived HEV infection, total RNA obtained from the culture media of PHCs infected with G3p HEV at $4.8 \times 10^4$ viral genome copies per well on day 12 after infection was subjected to RT-PCR for HEV RNA sequence analysis. Total RNA was extracted from the culture media using the QIAamp Viral RNA Mini Kit (Qiagen) followed by ethanol precipitation. The total RNA obtained was subjected to RT-PCR for amplification of the HEV sequences covering the entire HEV genome, following the previously described procedure (Urayama et al., 2010). The amplified HEV fragments were electrophoresed in an agarose gel and isolated from the gels using the QIAEX II Gel Extraction kit (Qiagen). The resulting fragments were sequenced as described previously (Urayama et al., 2010). The HEV genome sequence was compared with the sequence of the HEV used for inoculation. This comparison demonstrated that no base differences were observed between inoculated and propagated HEVs (data not shown), suggesting that swine G3p HEV is able to propagate in PHCs without mutating. Lorenzo et al. (2008) reported that, when HEV obtained from human faeces was used to infect cultured cell lines, the viral genome in later passages was mutated from the viral genome used for infection. This possibility should be further examined in the future by extending the culture period of the PHCs.

Although some portions of the cells were infected with the virus based on observations from the immunofluorescence staining (Fig. 2a), cell rounding and fusion were not observed in the infected cultures during a thorough inspection using a phase-contrast microscope (data not shown). This observation is consistent with the fact that there were no significant increases in LDH and ALT activities (markers of hepatocyte lysis) in the culture medium of the infected cells compared with that of the mock-infected cells (data not shown). These observations imply that HEV infection of human hepatocytes would be a persistent infection. This implication is consistent with the report that HEV infection might evolve into chronic hepatitis in immunocompromised patients (Kamar et al., 2008). However, to confirm this implication, additional experiments using cells, most of which are shown to be infected with HEV, should be performed.

In conclusion, we demonstrated that the propagation of swine HEV in PHCs occurs without mutational events during the course of HEV infection, verifying that HEV is a zoonotic virus. Furthermore, this study implies that HEV infection spreads by the cell-to-cell transmission of virions in human hepatocytes.

**Acknowledgements**

We thank Dr T. C. Li, National Institute of Infectious Diseases, Tokyo, Japan, for providing the rabbit antibody against the HEV ORF2 protein.

**References**


