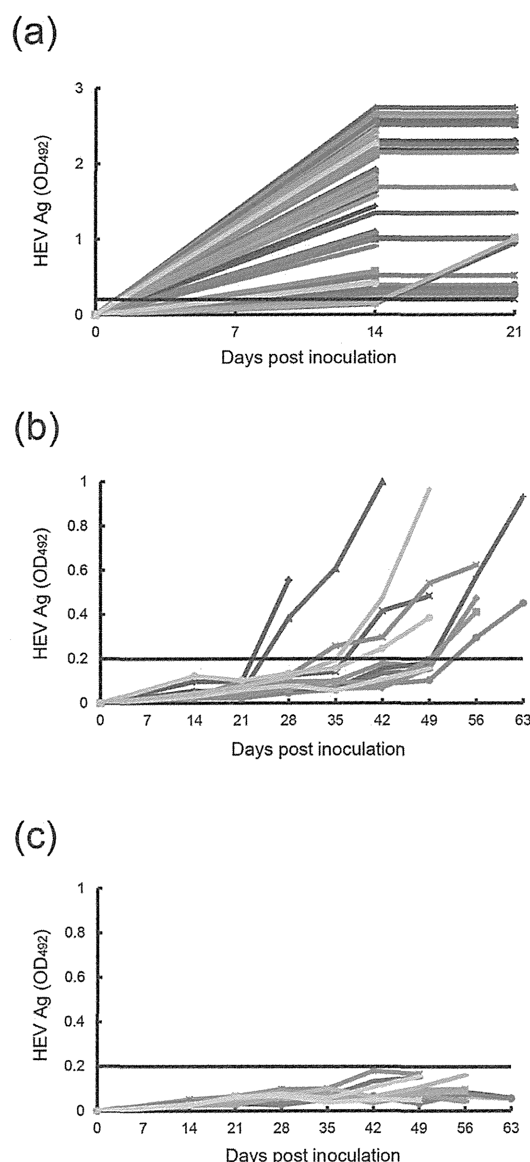

Fig. 1. Three groups of permissiveness of PLC/PRF/5 subclones for HEV infection. (a) All Group A clones showed HEV antigen production (as measured by ELISA OD values) until three weeks post infection; these clones were defined as highly permissive for HEV infection. (b) All Group B clones yielded sub-threshold (defined as ELISA ODs under 0.2) antigen production until three weeks post-infection. (c) Group C (non-permissive subclones) clones maintained sub-threshold antigen production throughout the duration of the study (through Day 63). HEV Ag, HEV antigen.

which was established from the G3-HEV83–2-27 strain, was used for experimental transfection as described previously (38). Starting on Day 2 post-transfection, the spent media were replaced with maintenance media every three or four days. To monitor virus production by the transfected cells, HEV antigen production was determined by ELISA analysis. To quantify the activity of HEV replication in each of four representative subclones, transfection by the G3-HEV83–2-27 strain was compared between the permissive and non-permissive PLC/PRF/5 subclones. The replication patterns of the four clones were very similar (Fig. 2b).

The binding affinities of the four subclones for S-VLPs (20, 21) were tested using flow cytometry. The S-VLPs were obtained from a baculovirus expression system using a part of ORF2 that encoded a truncated capsid protein that lacked the normal N- and C-termini (20, 21). PLC/PRF/5 subclones were washed once with flow cytometry buffer (2% FCS in PBS) and then incubated for 30 min at 37 °C with 50 ng/mL S-VLPs per 100 μL flow cytometry buffer. These subclones were washed and stained for 30 min at 37 °C with 0.1 μL polyclonal anti-G3-HEV-LP antibody per 100 μL flow cytometry buffer (21). After washing with flow cytometry buffer, the subclones were incubated for 30 min at 37 °C with secondary antibodies conjugated to FITC (Dako, Carpinteria, CA, USA). The cells were washed and analyzed using a FACSCalibur (BD Biosciences) and FlowJo software (Treestar Inc., Ashland, OR, USA). For each subclone, flow cytometric analysis was performed following incubation with S-VLPs, rabbit polyclonal anti-G3-HEV-LP antibody, or rabbit preimmune serum, followed by incubation with fluorescently-labeled secondary antibody. In comparison with the negative controls, all subclones showed similar S-VLP binding affinity regardless of permissiveness, approximately 30% to 60% of cells exhibiting fluorescent labeling (Fig. 2c).

Thus, in this study, we have shown that PLC/PRF/5 cells uniquely present limited permissiveness, suggesting that they are a mixture of permissive and non-permissive subclones for HEV infection and that PLC/PRF/5 subclones have various degrees of permissiveness to HEV virus infection, ranging from strong permissiveness to complete non-permissiveness, as expected from the limited permissiveness of the parental cell line. Although the permissiveness to infection differed among the PLC/PRF/5 subclones, these efficiencies did not correlate with the efficiency of intracellular virus replication. Additionally, non-permissive subclones retained the ability to bind HEV-LPs. We infer that these non-permissive subclones may have some deficiencies in the entry steps of HEV infection after attachment.

We have shown that the PLC/PRF/5 subclones exhibit various sensitivities for HEV infection. Akazawa *et al.* reported a similar phenomenon with the expression of CD81 (a member of the tetraspanin family that plays a critical role in hepatitis C virus infection [40]), which they reported correlates with the permissiveness of Huh7

© 2014 The Societies and Wiley Publishing Asia Pty Ltd

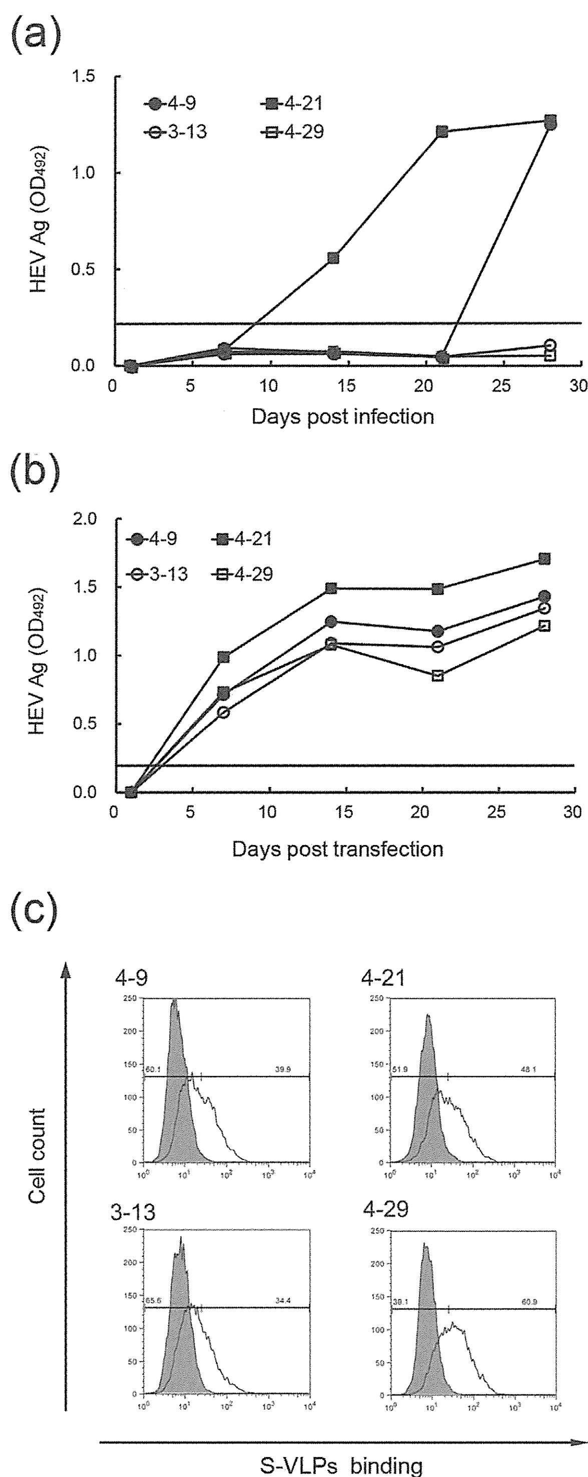

Fig. 2. Characterization of PLC/PRF/5 subclones. Time courses of (a) HEV infection and (b) transfection with HEV infectious clone. Black lines in (a) and (b) indicate threshold of susceptibility to HEV antigen, defined by OD value of 0.2 at 492 nm. (c) Characterization of binding to S-VLPs. Shaded histograms are negative controls; unshaded histograms are S-VLP binding samples. Value on the right-hand side of each graph indicates percentage fluorescence-positive cells.

subclones for HCV infection (39). Based on their findings, those researchers suggested that some unknown receptors may be involved in the permissiveness of Huh7 subclones for HCV infection.

Hepatitis E virus replication efficiencies differed slightly among the PLC/PRF/5 subclones, their efficiencies correlating modestly with permissiveness to infection; moreover, almost all the remaining subclones of PLC/PRF/5 cells showed similar responses for HEV infection and replication (data not shown). We observed strong antigen secretion in clones that exhibited rapid increases in HEV antigen production, indicating the efficacy of genome replication and probably reflecting differences in host factors related to HEV life cycle machinery. Akazawa *et al.* showed that subclones lacking CD81 expression were non-permissive for HCV infection; however, the same subclones still exhibited reduced HCV replication, implying slight differences in HEV replication in permissive subclones and non-permissive subclones.

S-VLPs bound these subclones at different percentages, suggesting that they are impaired in HEV entry, that is, in steps occurring after attachment. Heparan sulfate proteoglycans are required for cellular binding of the HEV ORF2 capsid protein and for viral infection (41). This first attachment factor is also used by other viruses (42–44). On the other hand, forced expression of the receptor for enterovirus 71 reportedly increases the permissiveness of low-permissiveness subclones (45). Considered in combination with the findings of the present study, these data suggest that differences in S-VLP binding (ranging from 30% to 60%) may reflect differences in strength of expression of unknown receptor(s) and co-receptor(s).

PLC/PRF/5 cells showed limited permissiveness during long-term culturing for HEV infection. Therefore, we cloned and characterized the PLC/PRF/5 cells in an attempt to clarify the reason(s) for the unique characteristics of HEV infection. The observed diversities of viral propagation phenotypes among the subclones imply potential applications for the various subclones in further characterizing the hepatitis E viral life cycle.

In conclusion, we have shown that PLC/PRF/5 cells have limited permissiveness, indicating that they could be considered quasispecies. To investigate this variability, we cloned and characterized subclones from our cell line, and showed different susceptibility among subclones. Our data suggest that determination and comparison of strength of gene and protein expression in permissive and non-permissive subclones may be useful for identification of host factors that contribute to the attachment and entry steps in the HEV life cycle.

© 2014 The Societies and Wiley Publishing Asia Pty Ltd

ACKNOWLEDGMENTS

This work was supported in part by grants-in-aid from the Ministry of Health, Labour, and Welfare, Japan, and from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

DISCLOSURE

None of the authors has any conflict of interest associated with this study.

REFERENCES

1. Chandra V., Taneja S., Kalia M., Jameel S. (2008) Molecular biology and pathogenesis of hepatitis E virus. *J Biosci* **33**: 451–64.
2. Acharya S.K., Dasarathy S., Kumer T.L., Sushma S., Prasanna K.S., Tandon A., Sreenivas V., Nijhawan S., Panda S.K., Nanda S.K., Irshad M., Joshi Y.K., Duttagupta S., Tandon R.K., Tandon B.N. (1996) Fulminant hepatitis in a tropical population: clinical course, cause, and early predictors of outcome. *Hepatology* **23**: 1448–55.
3. Khuroo M.S. (1991) Hepatitis E: the enterically transmitted non-A, non-B hepatitis. *Indian J Gastroenterol* **10**: 96–100.
4. Khuroo M.S., Teli M.R., Skidmore S., Sofi M.A., Khuroo M.I. (1981) Incidence and severity of viral hepatitis in pregnancy. *Am J Med* **70**: 252–5.
5. Meng X.J., Purcell R.H., Halbur P.G., Lehman J.R., Webb D.M., Tsareva T.S., Haynes J.S., Thacker B.J., Emerson S.U. (1997) A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci USA* **94**: 9860–5.
6. Sonoda H., Abe M., Sugimoto T., Sato Y., Bando M., Fukui E., Mizuo H., Takahashi M., Nishizawa T., Okamoto H. (2004) Prevalence of hepatitis E virus (HEV) Infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan. *J Clin Microbiol* **42**: 5371–4.
7. Okamoto H. (2007) Genetic variability and evolution of hepatitis E virus. *Virus Res* **127**: 216–28.
8. Li T.C., Chijiwa K., Sera N., Ishibashi T., Etoh Y., Shinohara Y., Kurata Y., Ishida M., Sakamoto S., Takeda N., Miyamura T. (2005) Hepatitis E virus transmission from wild boar meat. *Emerg Infect Dis* **11**: 1958–60.
9. Yazaki Y., Mizuo H., Takahashi M., Nishizawa T., Sasaki N., Gotanda Y., Okamoto H. (2003) Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. *J Gen Virol* **84**: 2351–7.
10. Takahashi M., Nishizawa T., Sato H., Sato Y., Jirintai Nagashima, Okamoto H. (2011) Analysis of the full-length genome of a hepatitis E virus isolate obtained from a wild boar in Japan that is classifiable into a novel genotype. *J Gen Virol* **92**: 902–8.
11. Takahashi K., Terada S., Kokuryu H., Arai M., Mishiro S. (2010) A wild boar-derived hepatitis E virus isolate presumably representing so far unidentified genotype 5. *Kanzo* **51**: 536–38.
12. Emerson S.U., Anderson D., Arankalle A., Meng X.J., Purdy M., Schlauder G.G., Tsarev S.A. (2005) Hepevirus. In: Fauquet M.A. M.C.M, Desselberger J.M.U, Ball L.A., eds. Virus Taxonomy: Eighth Report of the ICTV. London: Elsevier/Academic Press, pp. 853–857.
13. Takahashi M., Tanaka T., Takahashi H., Hoshino Y., Nagashima S., Jirintai Mizuo, Yazaki Y., Takagi T., Azuma M., Kusano E., Isoda N., Sugano K., Okamoto H. (2010) Hepatitis E Virus (HEV) strains in serum samples can replicate efficiently in cultured cells despite the coexistence of HEV antibodies: characterization of HEV virions in blood circulation. *J Clin Microbiol* **48**: 1112–25.
14. Tam A.W., Smith M.M., Guerra M.E., Huang C.C., Bradley D.W., Fry K.E., Reyes G.R. (1991) Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* **185**: 120–31.
15. Agrawal S., Gupta D., Panda S.K. (2001) The 3' end of hepatitis E virus (HEV) genome binds specifically to the viral RNA-dependent RNA polymerase (RdRp). *Virology* **282**: 87–101.
16. Magden J., Takeda N., Li T., Auvinen P., Ahola T., Miyamura T., Merits A., Kaariainen L. (2001) Virus-specific mRNA capping enzyme encoded by hepatitis E virus. *J Virol* **75**: 6249–55.
17. Koonin E.V., Gorbalenya A.E., Purdy M.A., Rozanov M.N., Reyes G.R., Bradley D.W. (1992) Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. *Proc Natl Acad Sci USA* **89**: 8259–63.
18. Graff J., Torian U., Nguyen H., Emerson S.U. (2006) A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. *J Virol* **80**: 5919–26.
19. Yamashita T., Mori Y., Miyazaki N., Cheng R.H., Yoshimura M., Unno H., Shima R., Moriishi K., Tsukihara T., Li T.C., Takeda N., Miyamura T., Matsuura Y. (2009) Biological and immunological characteristics of hepatitis E virus-like particles based on the crystal structure. *Proc Natl Acad Sci USA* **106**: 12986–91.
20. Li T.C., Takeda N., Miyamura T., Matsuura Y., Wang J.C., Engvall H., Hammar L., Xing L., Cheng R.H. (2005) Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus. *J Virol* **79**: 12999–3006.
21. Li T.C., Yamakawa Y., Suzuki K., Tatsumi M., Razak M.A., Uchida T., Takeda N., Miyamura T. (1997) Expression and self-assembly of empty virus-like particles of hepatitis E virus. *J Virol* **71**: 7207–13.
22. Emerson S.U., Zhang M., Meng X.J., Nguyen H., St Claire M., Govindarajan S., Huang Y.K., Purcell R.H. (2001) Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a cis-reactive element. *Proc Natl Acad Sci USA* **98**: 15270–5.
23. Tanaka T., Takahashi M., Kusano E., Okamoto H. (2007) Development and evaluation of an efficient cell-culture system for Hepatitis E virus. *J Gen Virol* **88**: 903–11.
24. Kane M.A., Bradley D.W., Shrestha S.M., Maynard J.E., Cook E.H., Mishra R.P., Joshi D.D. (1984) Epidemic non-A, non-B hepatitis in Nepal. Recovery of a possible etiologic agent and transmission studies in marmosets. *JAMA* **252**: 3140–5.
25. Arankalle V.A., Ticehurst J., Sreenivasan M.A., Kapikian A.Z., Popper H., Pavri K.M., Purcell R.H. (1988) Aetiological association of a virus-like particle with enterically transmitted non-A, non-B hepatitis. *Lancet* **1**: 550–4.
26. Huang R.T., Li D.R., Wei J., Huang X.R., Yuan X.T., Tian X. (1992) Isolation and identification of hepatitis E virus in Xinjiang, China. *J Gen Virol* **73**: 1143–8.

© 2014 The Societies and Wiley Publishing Asia Pty Ltd

27. Huang R., Nakazono N., Ishii K., Li D., Kawamata O., Kawaguchi R., Tsukada Y. (1995) Hepatitis E virus (87A strain) propagated in A549 cells. *J Med Virol* **47**: 299–302.
28. Huang R., Li D., Wei S., Li Q., Yuan X., Geng L., Li X., Liu M. (1999) Cell culture of sporadic hepatitis E virus in China. *Clin Diagn Lab Immunol* **6**: 729–33.
29. Kazachkov Yu, Balayan A., Ivannikova M.S., Panina T.A., Orlova L.I., Zamyatina T.M., Kusov N.A. (1992) Hepatitis E virus in cultivated cells. *Arch Virol* **127**: 399–402.
30. Tsarev S.A., Tsareva T.S., Emerson S.U., Yarbough P.O., Legters L.J., Moskal T., Purcell R.H. (1994) Infectivity titration of a prototype strain of hepatitis E virus in cynomolgus monkeys. *J Med Virol* **43**: 135–42.
31. Meng J., Dubreuil P., Pillot J. (1997) A new PCR-based seroneutralization assay in cell culture for diagnosis of hepatitis E. *J Clin Microbiol* **35**: 1373–7.
32. Tam A.W., White R., Yarbough P.O., Murphy B.J., Mcatee C.P., Lanford R.E., Fuerst T.R. (1997) In vitro infection and replication of hepatitis E virus in primary cynomolgus macaque hepatocytes. *Virology* **238**: 94–102.
33. Wei S., Walsh P., Huang R., To S.S. (2000) 93G, a novel sporadic strain of hepatitis E virus in South China isolated by cell culture. *J Med Virol* **61**: 311–8.
34. Yamada K., Takahashi M., Hoshino Y., Takahashi H., Ichiyama K., Tanaka T., Okamoto H. (2009) Construction of an infectious cDNA clone of hepatitis E virus strain JE03–1760F that can propagate efficiently in cultured cells. *J Gen Virol* **90**: 457–62.
35. Kwon H.M., Leroith T., Pudupakam R.S., Pierson F.W., Huang Y.W., Dryman B.A., Meng X.J. (2011) Construction of an infectious cDNA clone of avian hepatitis E virus (avian HEV) recovered from a clinically healthy chicken in the United States and characterization of its pathogenicity in specific-pathogen-free chickens. *Vet Microbiol* **147**: 310–9.
36. Cordoba L., Feagins A.R., Opriessnig T., Cossaboom C.M., Dryman B.A., Huang Y.W., Meng X.J. (2012) Rescue of a genotype 4 human hepatitis E virus from cloned cDNA and characterization of intergenotypic chimeric viruses in cultured human liver cells and in pigs. *J Gen Virol* **93**: 2183–94.
37. Zhu Y., Yu X., Zhang Y., Ni Y., Si F., Yu R., Dong S., Huang Y., Li Z. (2013) Infectivity of a genotype 4 hepatitis E virus cDNA clone by intrahepatic inoculation of laboratory rats. *Vet Microbiol* **166**: 405–11.
38. Shiota T., Li T.C., Yoshizaki S., Kato T., Wakita T., Ishii K. (2013) The hepatitis E virus capsid C-terminal region is essential for the viral life cycle: implication for viral genome encapsidation and particle stabilization. *J Virol* **87**: 6031–6.
39. Akazawa D., Date T., Morikawa K., Murayama A., Miyamoto M., Kaga M., Barth H., Baumert T.F., Dubuisson J., Wakita T. (2007) CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. *J Virol* **81**: 5036–45.
40. Levy S., Todd S.C., Maecker H.T. (1998) CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu Rev Immunol* **16**: 89–109.
41. Kalia M., Chandra V., Rahman S.A., Sehgal D., Jameel S. (2009) Heparan sulfate proteoglycans are required for cellular binding of the hepatitis E virus ORF2 capsid protein and for viral infection. *J Virol* **83**: 12714–24.
42. Fry E.E., Lea S.M., Jackson T., Newman J.W., Ellard F.M., Blakemore W.E., Abu-Ghazaleh R., Samuel A., King A.M., Stuart D.I. (1999) The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. *EMBO J* **18**: 543–54.
43. Hilgard P., Stockert R. (2000) Heparan sulfate proteoglycans initiate dengue virus infection of hepatocytes. *Hepatology* **32**: 1069–77.
44. Mcclain D.S., Fuller A.O. (1994) Cell-specific kinetics and efficiency of herpes simplex virus type 1 entry are determined by two distinct phases of attachment. *Virology* **198**: 690–702.
45. Yamayoshi S., Yamashita Y., Li J., Hanagata N., Minowa T., Takemura T., Koike S. (2009) Scavenger receptor B2 is a cellular receptor for enterovirus 71. *Nat Med* **15**: 798–801.

© 2014 The Societies and Wiley Publishing Asia Pty Ltd

Hepatology Research 2014 doi: 10.1111/hepr.12390

Case Report

Rare case of transfusion-transmitted hepatitis E from the blood of a donor infected with the hepatitis E virus genotype 3 indigenous to Japan: Viral dynamics from onset to recovery

Takeshi Matsui,[1,2] Jong-Hon Kang,[1] Keiji Matsubayashi,[3] Hajime Yamazaki,[1] Kazumasa Nagai,[1] Hidekatsu Sakata,[3] Kunihiko Tsuji[1] and Hiroyuki Maguchi[1]

[1]*Center for Gastroenterology, Teine Keijinkai Hospital, Sapporo,* [2]*Department of Virology, Nagoya City University Graduate School of Medical Sciences, Nagoya,* [3]*Japanese Red Cross Hokkaido Block Blood Center, Hokkaido, Japan*

Aim: The transfusion transmission of hepatitis E can occur even in non-endemic areas in the world as autochthonous hepatitis E has been increasingly reported in developed countries where the hepatitis E virus (HEV) is not prevalent. We investigated the post-transfusion transmission of hepatitis E in a patient by molecularly confirming its presence, and characterized the viral kinetics of HEV in this case.

Methods: A Japanese man underwent re-thoracotomy for hemostasis followed by platelet transfusion. After the transfusion, the blood donor was found to be HEV positive. The donated blood was re-examined and was found to contain HEV. Throughout the prospective follow up of the patient, we analyzed the viral kinetics, chronological anti-HEV antibody level changes and disease progression during the entire course of HEV infection from transfusion until the end of viremia.

Results: Sequence analysis of the strains isolated from both the donor and the patient who contracted acute hepatitis E showed an identical match for 326 nucleotides in open reading frame 1. Two strains belonged to HEV genotype 3 indigenous to Japan.

Conclusion: To the best of our knowledge, this is the first detailed report on the entire natural course of hepatitis E from viral transmission, then clearance, to replication preceding liver injury caused by HEV genotype 3, which is responsible for autochthonous infection in developed countries. The findings provide valuable insights into the mechanism of the transfusion transmission of HEV and subsequent viral dynamics.

Key words: genotype, hepatitis E virus, hepatitis E virus RNA, transfusion-transmitted hepatitis

INTRODUCTION

HEPATITIS E IS a disease that is endemic in the Asian, African and Latin American regions. Because the main transmission route of the hepatitis E virus (HEV) is fecal–oral, hepatitis E has been regarded as a water-borne disease in these regions.[1,2] HEV is a recognized zoonotic disease and, in industrialized countries, several species of animals (e.g. wild boar, deer and swine) serve as reservoirs of the infection in humans via food-borne transmission.[3] In contrast to this zoonotic route of infection, reports on the instances of transfusion transmission of HEV are few, regardless of whether HEV is prevalent or not. Post-transfusion hepatitis E can occur by infection with HEV genotypes indigenous to communities. However, actual cases of transfusion transmission of HEV have rarely been confirmed molecularly.[3–7] There are four major HEV genotypes in humans. Genotypes 1 and 2 have been isolated only from humans in prevalent areas, whereas genotypes 3 and 4 are zoonotic and have been identified in many sporadic cases affecting middle-aged and elderly men even in industrialized countries.

We previously reported a case of acute hepatitis E caused by transfusion of blood containing HEV

Correspondence: Dr Takeshi Matsui, 1-jo 12-chome, Maeda, Teine-ku, Sapporo 006-8555, Japan. Email: mtake0402@gmail.com, mtake@tkeijinkai.gr.jp
Received 7 January 2014; revision 7 July 2014; accepted 7 July 2014.

© 2014 The Japan Society of Hepatology

genotype 4, including the changes in HEV loads and the entire clinical course of hepatitis E.[5] In the present case, we encountered a patient who received transfusion of blood that contained HEV genotype 3. Being a rare case of transfusion transmission of HEV, we prospectively analyzed the viral kinetics and chronological changes in anti-HEV antibody levels, as well as disease progression during the entire course of HEV infection from transfusion until the end of viremia.

CASE REPORT

A JAPANESE MAN in his 70s and who had never traveled abroad suffered acute myocardial infarction and was brought to a nearby hospital in August 2005. He underwent percutaneous transluminal coronary angioplasty and coronary artery bypass graft. Following surgery, the patient developed hemorrhagic cardiac tamponade. Re-thoracotomy was performed for hemostasis followed by platelet transfusion. The transfused blood had a normal alanine aminotransferase (ALT) level and passed all currently required blood screening tests at the Japanese Red Cross Hokkaido Block Blood Center. After blood transfusion, however, the blood donor was found to be HEV positive. Following re-examination of the blood donated, the transfused platelet was found to contain $10^{6.8}$ copies of the RNA of HEV genotype 3.

The patient was closely followed up to determine the onset of HEV infection. Moreover, the HEV loads were continuously monitored in a series of sera. On day 10 post-transfusion of the HEV-contaminated blood, HEV RNA was detected in the blood sampled from the patient. On day 54 post-transfusion, the patient showed an elevated serum ALT level of 109 IU/L and an aspartate aminotransferase (AST) of 70 IU/L despite being asymptomatic. Acute hepatitis A, B or C infection was excluded by serological and virological examinations. Moreover, an infection marker was not found for cytomegalovirus or Epstein–Barr virus in sera on admission. The patient had no history of new drug administration at least 6 months before the onset of liver injury. Notably, his liver injury was diagnosed as a consequence of acute hepatitis E. Because HEV genotype 3 appeared to be more remotely related to the severity of disease than HEV genotype 4,[8] a wait-and-see approach without treatment against HEV was taken. The aminotransferase levels reached 704 IU/L for AST and 972 IU/L for ALT on day 65 post-transfusion. However, these levels immediately returned to the normal levels on day 85. The patient was discharged on day 76 (Fig. 1).

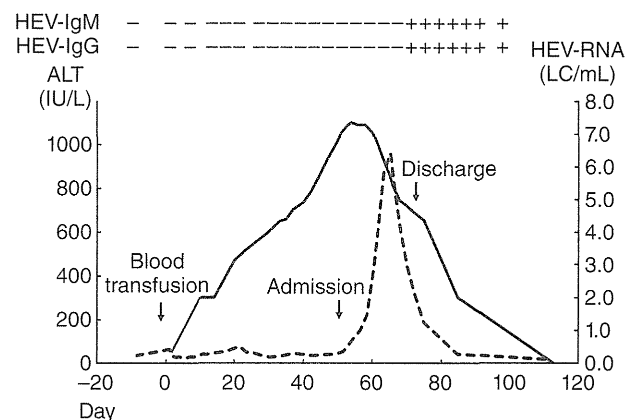

Figure 1 On day 10 after the platelet concentrate transfusion, hepatitis E virus (HEV) RNA was first quantified at 2.0 log copies/mL, and this level reached a peak of 7.4 log copies/mL on day 54. The immunoglobulin (Ig)M and IgG class antibodies against HEV became detectable on day 60. The alanine aminotransferase (ALT) level increased to 972 IU/L after the peak HEV RNA level. Thereafter, the level immediately decreased and normalized on day 85. ------, ALT; ———, HEV RNA.

Informed consent was obtained from the patient regarding the publication of his case and for a description of the assay of virological and serological markers using the sampled sera. This study was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Serum HEV RNA was quantified as reported previously.[9] For reverse transcription polymerase chain reaction (RT–PCR) to detect HEV RNA, the following oligonucleotides were designed to detect 75 nucleotides (nt) in the open reading frame (ORF)2 region of all HEV genotypes: forward, 5′-CGGCGGT GGTTTCTGG-3′, and reverse 5′-AAGGGGTTGGTT GGATGAATA-3′, and mixed probe with a 5′-reporter dye (6-carboxyfluorescein, FAM) and a 3′-quencher dye (6-carboxy-tetramethylrhodamine, TAMRA) and FAM-5′-TGACAGGGTTGATTCTCAGCCCTTCG-3′-TAMRA, FAM-5′-TGACCGGGTTGATTCTCAGCCCTTC-3′-TAMRA and FAM-5′-TGACCGGGCTGATTCTCAGCC CTT-3′-TAMRA (Sigma-Aldrich Japan, Tokyo, Japan). Nucleic acid was also extracted from the serum, saliva and fecal suspension in saline, as previously described.[5,8] Twenty microliters of a nucleic acid sample was used for each reaction. Each 50-μL reaction mixture contained 25 μL of 2 × QuantiTect Probe RT–PCR kit Master Mix (QuantiTect Probe RT–PCR kit; QIAGEN, Hilden, Germany), 0.5 μL of QuantiTect

© 2014 The Japan Society of Hepatology

Probe RT Mix, 400 nM each of forward and reverse primers and 67 nM each of the three probes. The RT–PCR mixture was incubated at 50°C for 30 min and at 95°C for 15 min, followed by 50 cycles at 94°C, 15 s at 60°C, 1 min utilizing a thermocyler (Applied Biosystems 7500 Real Time PCR System; Applied Biosystems, Tokyo, Japan). Quantitation standards of HEV RNA were obtained by *in vitro* transcription (MAXIscript T7 high-yield transcription kit; Ambion, Austin, TX, USA) of cDNA of the HEV ORF2 region that was cloned into a plasmid (pCRII-TOPO; Invitrogen, Carlsbad, CA, USA). HEV viral load was determined from standard curves generated using 10^1–10^7 copies of HEV RNA per reaction. Samples were tested for immunoglobulin (Ig)M and IgG class antibodies against HEV using a commercial enzyme-linked immunoassay kit (Viragent HEV-Ab; Cosmic, Tokyo, Japan). A phylogenetic tree was constructed using the neighbor-joining method based on the 326-nt partial sequence of the ORF1 region in 84 HEV isolates using the methods described previously.[10] HRC-HE34 C1, HRC-HE34 C2, HRC-HE34 C3, HRC-HE34 C4 and HRC-HE34 C5 were isolated from the donor. Furthermore, JWH-Sap06 C1, JWH-Sap06 C2, JWH-Sap06 C3, JWH-Sap06 C4 and JWH-Sap06 C5 were separated from the patient in this study. Bootstrap values are indicated for the major nodes as a percentage obtained from 1000 re-samplings of the dataset. HRC-HE34 C2 from the donor was 100% identical to JWH-Sap06 C2, JWH-Sap06 C3, JWH-Sap06 C4 and JWH-Sap06 C5 from the patient. There were conformities of more than 98% between other strains isolated from the donor and patient (Fig. 2, Table 1). These strains belonged to HEV genotype 3, which is regarded as indigenous to Japan. HEV RNA load and anti-HEV antibody levels were measured prospectively for the blood sample series post-transfusion. On day 10 post-transfusion, serum HEV RNA was quantified as 2.0 log copies/mL for the first time, and reached 7.4 log copies/mL on day 54. Viremia lasted until day 85 post-transfusion. Thereafter, HEV RNA was undetectable on day 115 (Fig. 1). HEV loads in the saliva and feces were also assessed prospectively from day 61 to day 75.[8] Fecal HEV RNA peaked on day 65 and then decreased. A small amount of HEV RNA was transiently detected in the saliva from day 66 to day 72 (Fig. 3). Although IgM and IgG class antibodies against HEV were not detected in the plasma sampled at 8 days before blood transfusion, these antibodies became detectable on day 60 post-transfusion. These detections were consistent with the peak of HEV RNA. Aminotransferase levels reached a late peak for the highest titration of HEV RNA and IgM and IgG class antibodies against HEV (Fig. 1).

DISCUSSION

TRANSFUSION-TRANSMITTED HEPATITIS E has been reported to occur in highly prevalent regions of HEV infection.[1,11–13] However, only a few of these reports have described the molecular confirmation of viral transmission. Thus far, there have been only five reports that described HEV transmission via blood transfusion in non-endemic countries (Table 2).[9,14–16] In the present study, the PCR products for the HEV RNA derived from both the blood donor and the patient who developed hepatitis E indicated complete nucleotide sequence identity, and the strains isolated from both were closely located in the phylogenetic tree of HEV genotype 3. These findings strongly demonstrated that the hepatitis E of the patient was caused by the transfusion of blood from the donor (Fig. 2).

In the present case, it was confirmed soon after the transfusion that the blood given was infected with HEV. This allowed the prospective observation of the kinetics of HEV load, the subsequent appearance of anti-HEV class antibodies and a series of chronological changes in the serum aminotransferase levels throughout the natural course of the acute infection of HEV genotype 3. Viremia was immediately observed on day 10 post-transfusion, followed by a gradual increase in the HEV load that reached a peak after 45 days.

In 2008, Matsubayashi *et al.* reported a case of post-transfusion hepatitis E in a male patient in whom $10^{5.4}$ copies of HEV genotype 4 was transfused (Table 2).[5] In their previous prospective monitoring of changes in HEV RNA, the quantified level had shown an abrupt increase and demonstrated a peak level of 7.2 log copies/mL just 22 days following viral emergence in sera.[5] The present case was distinct from the previous case in terms of background characteristics such as underlying disease and immunological conditions. Notably, the duration from viral appearance to viral peak load was longer in the present case (i.e. 45 days) than in the previously reported case (i.e. 22 days).

Hepatitis E virus genotype 4 infection is significantly associated with disease progression to fulminant hepatitis compared with HEV genotype 3 infection. As serum viral load may reflect the extent of viral replication in the liver, an exponential increase in the quantified serum HEV level in HEV genotype 4 infection may be associated with the induction of an active immune response

© 2014 The Japan Society of Hepatology

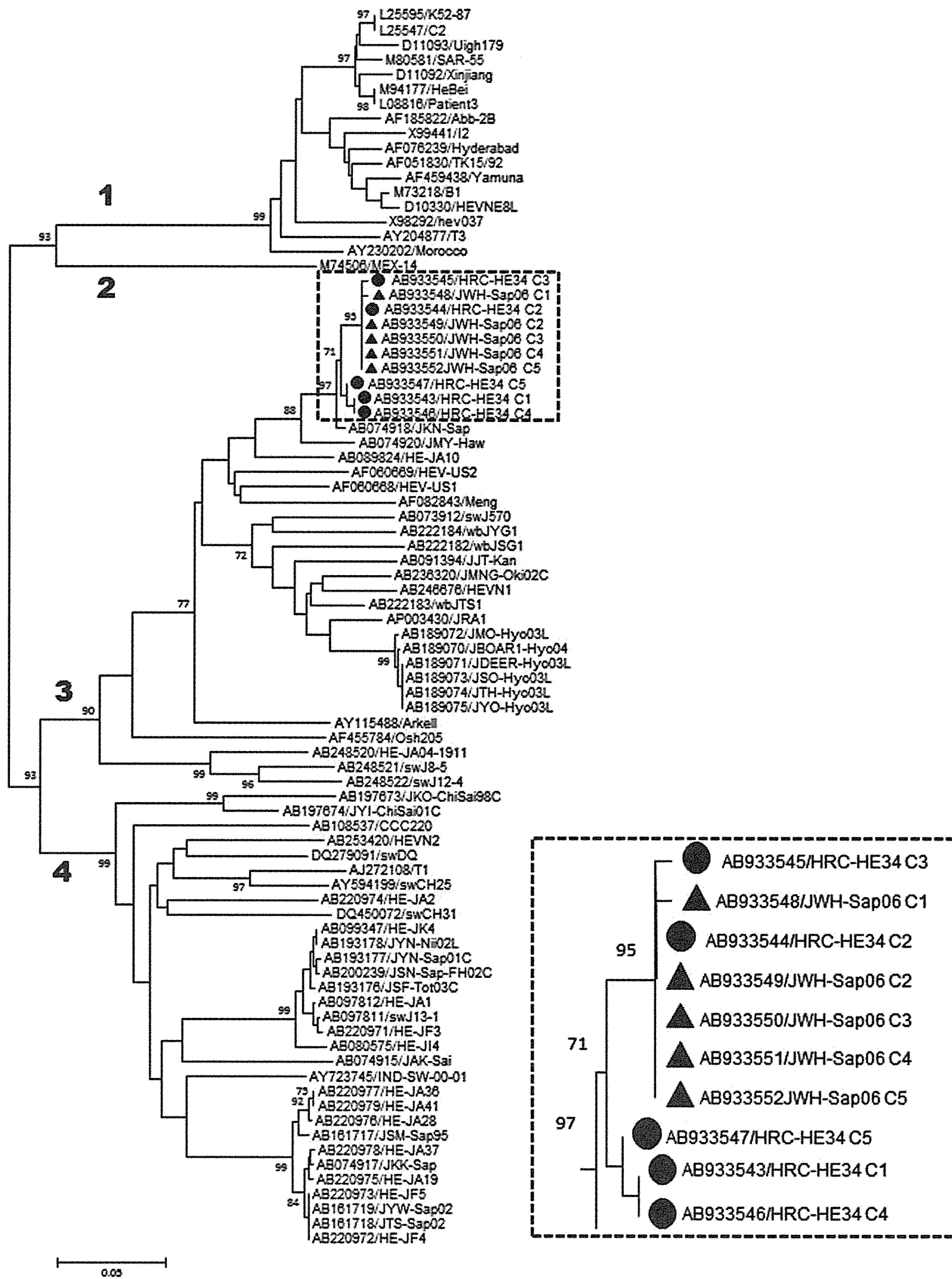

Figure 2 Phylogenetic tree constructed using the neighbor-joining method based on the 326-nucleotide partial sequence of the open reading frame 1 region of 84 hepatitis E virus (HEV) isolates. HRC-HE34 C2 isolated from the donor was 100% identical to JWH-Sap06 C2, JWH-Sap06 C3, JWH-Sap06 C4 and JWH-Sap06 C5 separated from the patient. Bootstrap values are indicated for the major nodes as a percentage obtained from 1000 re-samplings of the dataset.

© 2014 The Japan Society of Hepatology

Table 1 Nucleotide similarity (%) of the isolates recovered from the donor and the patient

Isolate name	JWH-Sap06 C1	JWH-Sap06 C2	JWH-Sap06 C3	JWH-Sap06 C4	JWH-Sap06 C5	HRC-HE34 C1	HRC-HE34 C2	HRC-HE34 C3	HRC-HE34 C4
JWH-Sap06 C1									
JWH-Sap06 C2	99.7								
JWH-Sap06 C3	99.7	100.0							
JWH-Sap06 C4	99.7	100.0	100.0						
JWH-Sap06 C5	99.7	100.0	100.0	100.0					
HRC-HE34 C1	98.2	98.5	98.5	98.5	98.5				
HRC-HE34 C2	99.7	100.0	100.0	100.0	100.0	98.5			
HRC-HE34 C3	99.4	99.7	99.7	99.7	99.7	98.2	99.7		
HRC-HE34 C4	98.2	98.5	98.5	98.5	98.5	100.0	98.5	98.2	
HRC-HE34 C5	98.5	98.8	98.8	98.8	98.8	99.7	98.8	98.5	99.7

The numbers of base differences per sequence from between sequences are shown. The analysis involved 10 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 326 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

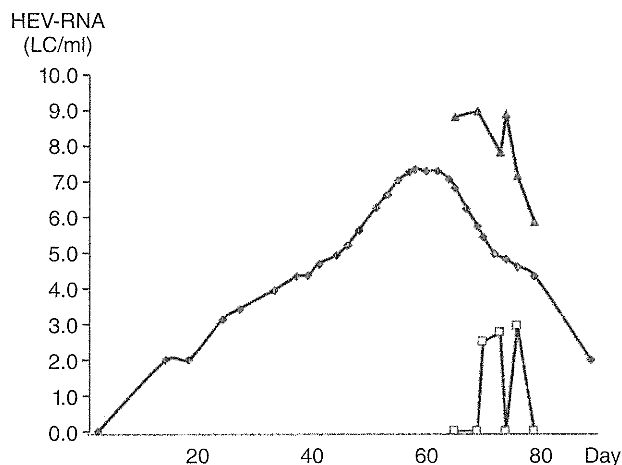

Figure 3 Levels of fecal hepatitis E virus (HEV) RNA peaked on day 65, and then decreased. A small amount of HEV RNA was transiently detected in the saliva from day 66 to day 72. —◆—, sera RNA in sera log cp/mL; —□—, saliva RNA in saliva log cp/mL; —▲—, feces RNA in stool log cp/g.

around virus-infected hepatocytes. Further evaluation of the relationship between the replication velocity and genotypes of HEV is warranted. In the present case, the appearance of anti-HEV class antibodies and the rapid decrease in the serum viral load were observed almost simultaneously. From day 58 post-transfusion, an abrupt decrease in the quantified HEV level, coincident emergence of IgG and IgM class anti-HEV antibodies, and changes in the aminotransferase levels occurred. These dynamic changes in infection markers of HEV were followed by a striking increase and a rapid decrease in aminotransferase levels which were recognized to reflect a series of clinical courses of acute hepatitis. Despite a return of the aminotransferase level to the normal range, the HEV RNA was still detectable until day 85. These present observations provide valuable information regarding the chronological relationships between viral kinetics and the specific immune response in transfusion-transmitted HEV infection.

To date, five molecularly confirmed cases of transfusion-transmitted HEV infection have been reported from non-endemic countries.[3–7] Interestingly, two cases were from the northern island of Hokkaido, Japan,[13,15] consistent with previous reports suggesting the high prevalence of HEV in Hokkaido.[17,18] In addition, all three cases from Hokkaido had been diagnosed at the stage of transition towards the establishment of regional measures for HEV screening in blood donors. To prevent the transfusion transmission of HEV on the

© 2014 The Japan Society of Hepatology

Table 2 Molecularly confirmed cases of transfusion-transmitted HEV infection from non-endemic countries

Case no.	Age (years)	Sex	Peak ALT (IU/L)	HEV genotype	Blood supply content	Incidence area
1	60	Male	2050	3	FFP	UK
2	21	Male	–	3	RBC	Japan (Kanto)
3	7	Male	2001	3	RBC	France
4	67	Male	1595	4	FFP	Japan (Hokkaido)
5	69	Male	673	4	PC	Japan (Hokkaido)
Present case	72	Male	972	3	PC	Japan (Hokkaido)

ALT, alanine aminotransferase; FFP, fresh frozen plasma; HEV, hepatitis E virus; PC, platelet; RBC, red blood cell.

island, the Japanese Red Cross Hokkaido Block Blood Center has taken a series of gradual but concrete measures from 2004. Previously, donated blood was checked for HEV RNA or anti-HEV IgM only when serum transaminase levels increased even in the presence of either hepatitis B virus (HBV) or hepatitis C virus (HCV) infection. Recently, multiple blood samples have been screened for HEV using nucleotide amplification tests. Moreover, retrospective surveys also have been undertaken for HEV positive donors. With the establishment of a screening system for HEV, it is expected that the transfusion-related transmission of HEV can be prevented, even in cases of HBV or HCV infection, particularly in Hokkaido, Japan.

In conclusion, the present case allowed the characterization of the entire natural course of acute HEV genotype 3 infection, which is responsible for autochthonous infection in developed countries. Moreover, viral transmission, clearance and replication preceding hepatitis were prospectively clarified, which provide valuable insights into the mechanism of transfusion transmission of HEV.

ACKNOWLEDGMENT

THE AUTHORS ARE indebted to Dr Edward Barroga, Associate Professor and Senior Medical Editor of the Department of International Medical Communications of Tokyo Medical University for reviewing and editing the manuscript.

REFERENCES

1 Wang CH, Flehmig B, Moeckli R. Transmission of hepatitis E virus by transfusion? *Lancet* 1993; **27** (341): 825–6.
2 Pavio N, Meng XJ, Renou C. Zoonotic hepatitis E: animal reservoirs and emerging risks. *Vet Res* 2010; **41**: 46.
3 Matsubayashi K, Nagaoka Y, Sakata H *et al.* Transfusion-transmitted hepatitis E caused by apparently indigenous hepatitis E virus strain in Hokkaido, Japan. *Transfusion* 2004; **44**: 934–40.
4 Tamura A, Shimizu YK, Tanaka T *et al.* Persistent infection of hepatitis E virus transmitted by blood transfusion in a patient with T-cell lymphoma. *Hepatol Res* 2007; **37**: 113–20.
5 Matsubayashi K, Kang JH, Sakata H *et al.* A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis E virus via zoonotic food-borne route. *Transfusion* 2008; **48**: 1368–75.
6 Boxall E, Herborn A, Kochethu G *et al.* Transfusion-transmitted hepatitis E in a "nonhyperendemic" country. *Transfus Med* 2006; **16**: 79–83.
7 Colson P, Coze C, Gallian P, Henry M, De Micco P, Tamalet C. Transfusion-associated hepatitis E, France. *Emerg Infect Dis* 2007; **13**: 648–9.
8 Inoue J, Nishizawa T, Takahashi M *et al.* Analysis of the full-length genome of genotype 4 hepatitis E virus isolates from patients with fulminant or acute self-limited hepatitis E. *J Med Virol* 2006; **78**: 476–84.
9 Mizuo H, Suzuki K, Takikawa Y *et al.* Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. *J Clin Microbiol* 2002; **40**: 3209–18.
10 Takahashi K, Kang JH, Ohnishi S, Hino K, Mishiro S. Genetic heterogeneity of hepatitis E virus recovered from Japanese patients with acute sporadic hepatitis. *J Infect Dis* 2002; **1** (185): 1342–5.
11 Wang JT, Lin JT, Sheu JC, Wang TH, Chen DS. Hepatitis E virus and posttransfusion hepatitis. *J Infect Dis* 1994; **169**: 229–30.
12 Arankalle VA, Chobe LP. Retrospective analysis of blood transfusion recipients: evidence for post-transfusion hepatitis E. *Vox Sang* 2000; **79**: 72–4.
13 Khuroo MS, Kamili S, Yattoo GN. Hepatitis E virus infection may be transmitted through blood transfusions in an endemic area. *J Gastroenterol Hepatol* 2004; **19**: 778–84.
14 Schlauder GG, Dawson GJ, Erker JC *et al.* The sequence and phylogenetic analysis of a novel hepatitis E virus isolated

© 2014 The Japan Society of Hepatology

from a patient with acute hepatitis reported in the United States. *J Gen Virol* 1998; **79** (Pt 3): 447–56.

15 Worm HC, Wurzer H, Frosner G. Sporadic hepatitis E in Austria. *N Engl J Med* 1998; **19** (339): 1554–5.

16 Takahashi K, Iwata K, Watanabe N *et al.* Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. *Virology* 2001; **15** (287): 9–12.

17 Takahashi M, Tamura K, Hoshino Y *et al.* A nationwide survey of hepatitis E virus infection in the general population of Japan. *J Med Virol* 2010; **82:** 271–81.

18 Miyashita K, Kang JH, Saga A *et al.* Three cases of acute or fulminant hepatitis E caused by ingestion of pork meat and entrails in Hokkaido, Japan: zoonotic food-borne transmission of hepatitis E virus and public health concerns. *Hepatol Res* 2012; **42:** 870–8.

© 2014 The Japan Society of Hepatology

Journal of General Virology (2014), **95**, 2677–2682 DOI 10.1099/vir.0.068452-0

Short Communication

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

Yukio Oshiro,[1] Hiroshi Yasue,[2] Kazuhiro Takahashi,[1] Shinji Hattori,[3] Shoji Ideno,[3] Takeru Urayama,[3] Mitsuru Chiba,[1] Suguru Osari,[4] Tadasuke Naito,[5] Kaoru Takeuchi,[4] Kyosuke Nagata[4] and Nobuhiro Ohkohchi[1]

Correspondence
Yukio Oshiro
oshiro@md.tsukuba.ac.jp

[1]Division of Gastroenterological and Hepatobiliary Surgery and Organ Transplantation, University of Tsukuba, Tsukuba, Japan

[2]Animal Genome Research Unit, National Institute of Agrobiological Sciences, Tsukuba, Japan

[3]Japan Blood Products Organization, Kobe, Japan

[4]Department of Infection Biology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan

[5]Kawasaki Medical School, Okayama, Japan

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: $G3_{JP}$, $G3_{US}$, $G3_{SP}$ and $G4_{JP}$ (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

068452 © 2014 The Authors *Printed in Great Britain*

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 ($10^{1.7}$) $ml^{-1}$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 1.2×10^{6} 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 % CO_2 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 % CO_2 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 1. HEV genome and infectivity

Genotype*	Isolation	Viral titre	
		HEV genome [log_{10}(copies) ml^{-1}]†	HEV infectivity [log_{10}(copies) ml^{-1}]‡
$G3_{JP}$	swJR-P5	3.39×10^{7}	3.16×10^{4}
$G3_{US}$	swJB-M8	5.89×10^{6}	–
$G3_{SP}$	swJB-E10	1.26×10^{7}	3.98×10^{4}
$G4_{JP}$	swJB-H7	1.58×10^{7}	6.31×10^{3}

*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.

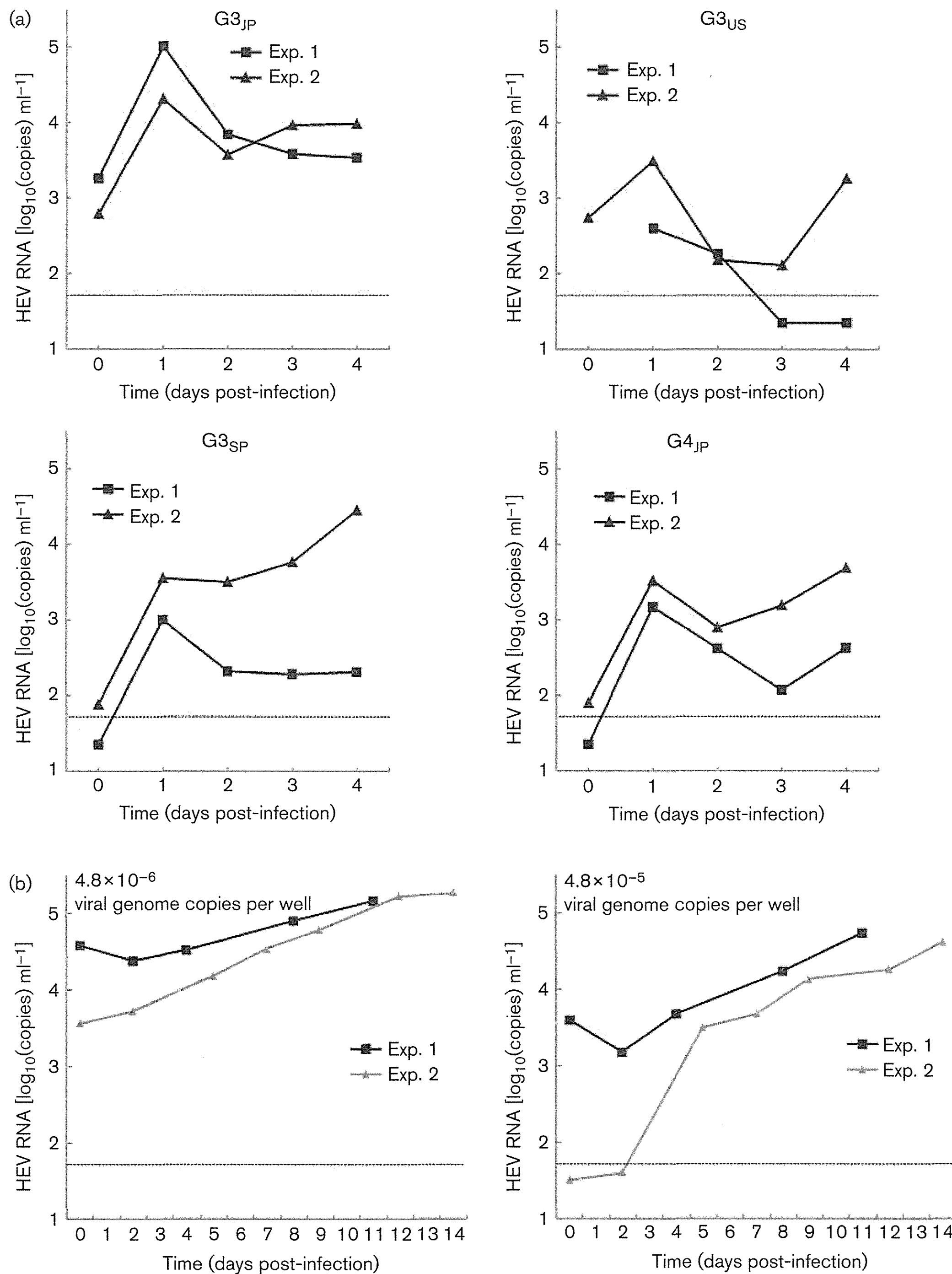

Fig. 1. Amount of HEV RNA propagated in PHCs. (a) Amount of HEV RNA in the culture medium of PHCs inoculated with four HEV isolates (genotypes 3 and 4). The amount of HEV RNA in the removed media was measured. Two independent infection experiments (Exp. 1 and 2) were performed for four virus isolates. 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 $G3_{JP}$. 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).

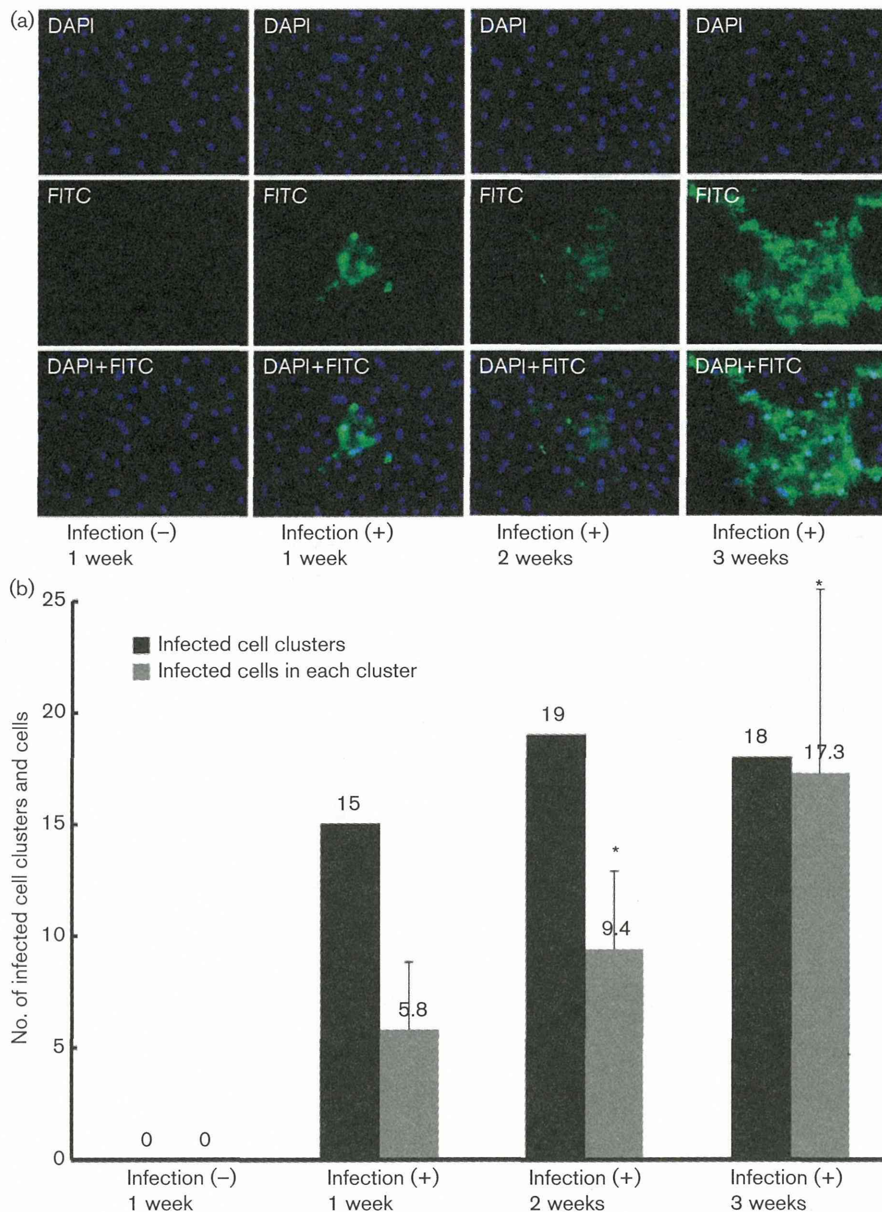

Fig. 2. Immunofluorescence staining. (a) Immunofluorescence staining of infected and mock-infected PHCs using an antibody against the HEV ORF2 protein. Fluorescence staining of all of the cell clusters was observed across the entire 15 mm glass coverslips at 1, 2 and 3 weeks after inoculation. Although 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.

For precise characterization of HEV infection of human hepatocytes, the $G3_{JP}$ 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 (4.8×10^6, 4.8×10^5, 4.8×10^4 and 4.8×10^3 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. 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 4.8×10^6 and 4.8×10^5 viral genome copies per well are shown in Fig. 1(b); the results at 4.8×10^4 and 4.8×10^3 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 4.8×10^5 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 $G3_{JP}$ HEV at 4.8×10^3 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 $G3_{JP}$ 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

Datta, R., Panda, S. K., Tandon, B. N., Madnagopalan, N., Bose, S. L., Acharya, S. K. & Nayak, N. C. (1987). Acute sporadic non-A, non-B viral hepatitis of adults in India – epidemiological and immunological studies. *J Gastroenterol Hepatol* **2**, 333–345.

Emerson, S. U., Nguyen, H., Graff, J., Stephany, D. A., Brockington, A. & Purcell, R. H. (2004a). *In vitro* replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. *J Virol* **78**, 4838–4846.

Emerson, S. U., Anderson, D., Arankalle, A., Meng, X. J., Purdy, M., Schlauder, G. G. & Tsarev, S. A. (2004b). Hepevirus. In *Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses*, pp. 851–855. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. London: Elsevier.

Guguen-Guillouzo, C. (1992). Isolation and culture of animal and human hepatocytes. In *Culture of Epithelial Cells*, pp. 197–223. Edited by R. I. Freshney. New York: Wiley-Liss.

Huang, R., Li, D., Wei, S., Li, Q., Yuan, X., Geng, L., Li, X. & Liu, M. (1999). Cell culture of sporadic hepatitis E virus in China. *Clin Diagn Lab Immunol* **6**, 729–733.

Huang, Y. W., Haqshenas, G., Kasorndorkbua, C., Halbur, P. G., Emerson, S. U. & Meng, X. J. (2005). Capped RNA transcripts of full-length cDNA clones of swine hepatitis E virus are replication competent when transfected into Huh7 cells and infectious when intrahepatically inoculated into pigs. *J Virol* **79**, 1552–1558.

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. J. & Hill, V. R. (2006). A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *J Virol Methods* **131**, 65–71.

Kamar, N., Selves, J., Mansuy, J. M., Ouezzani, L., Péron, J. M., Guitard, J., Cointault, O., Esposito, L., Abravanel, F. & other authors (2008). Hepatitis E virus and chronic hepatitis in organ-transplant recipients. *N Engl J Med* **358**, 811–817.

Kamar, N., Bendall, R., Legrand-Abravanel, F., Xia, N. S., Ijaz, S., Izopet, J. & Dalton, H. R. (2012). Hepatitis E. *Lancet* **379**, 2477–2488.

Li, T. C., Yamakawa, Y., Suzuki, K., Tatsumi, M., Razak, M. A., Uchida, T., Takeda, N. & Miyamura, T. (1997). Expression and self-assembly of empty virus-like particles of hepatitis E virus. *J Virol* **71**, 7207–7213.

Li, W., Sun, Q., She, R., Wang, D., Duan, X., Yin, J. & Ding, Y. (2009). Experimental infection of Mongolian gerbils by a genotype 4 strain of swine hepatitis E virus. *J Med Virol* **81**, 1591–1596.

Lorenzo, F. R., Tanaka, T., Takahashi, H., Ichiyama, K., Hoshino, Y., Yamada, K., Inoue, J., Takahashi, M. & Okamoto, H. (2008). Mutational events during the primary propagation and consecutive passages of hepatitis E virus strain JE03-1760F in cell culture. *Virus Res* **137**, 86–96.

Lu, L., Li, C. & Hagedorn, C. H. (2006). Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. *Rev Med Virol* **16**, 5–36.

Ma, H., Zheng, L., Liu, Y., Zhao, C., Harrison, T. J., Ma, Y., Sun, S., Zhang, J. & Wang, Y. (2010). Experimental infection of rabbits with rabbit and genotypes 1 and 4 hepatitis E viruses. *PLoS ONE* **5**, e9160.

Meng, X. J., Purcell, R. H., Halbur, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S., Haynes, J. S., Thacker, B. J. & Emerson, S. U. (1997). A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci U S A* **94**, 9860–9865.

Okamoto, H., Takahashi, M. & Nishizawa, T. (2003). Features of hepatitis E virus infection in Japan. *Intern Med* **42**, 1065–1071.

Rumin, S., Gripon, P., Le Seyec, J., Corral-Debrinski, M. & Guguen-Guillouzo, C. (1996). Long-term productive episomal hepatitis B virus replication in primary cultures of adult human hepatocytes infected *in vitro*. *J Viral Hepat* **3**, 227–238.

Shukla, P., Nguyen, H. T., Torian, U., Engle, R. E., Faulk, K., Dalton, H. R., Bendall, R. P., Keane, F. E., Purcell, R. H. & Emerson, S. U. (2011). Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus–host recombinant. *Proc Natl Acad Sci U S A* **108**, 2438–2443.

Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F. & Okamoto, H. (2003). Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. *J Gen Virol* **84**, 851–862.

Takahashi, H., Tanaka, T., Jirintai, S., Nagashima, S., Takahashi, M., Nishizawa, T., Mizuo, H., Yazaki, Y. & Okamoto, H. (2012). A549 and PLC/PRF/5 cells can support the efficient propagation of swine and wild boar hepatitis E virus (HEV) strains: demonstration of HEV infectivity of porcine liver sold as food. *Arch Virol* **157**, 235–246.

Tanaka, T., Takahashi, M., Kusano, E. & Okamoto, H. (2007). Development and evaluation of an efficient cell-culture system for hepatitis E virus. *J Gen Virol* **88**, 903–911.

Tei, S., Kitajima, N., Takahashi, K. & Mishiro, S. (2003). Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet* **362**, 371–373.

Urayama, T., Sapsutthipas, S., Tsujikawa, M., Yamashita, A., Nishigaki, H., Ibrahim, M. S., Hagiwara, K., Yunoki, M., Yasunaga, T. & other authors (2010). Full-length sequences of one genotype 4 and three genotype 3 hepatitis E viruses in fecal samples from domestic swine in Japan. *Open Vet Sci J* **4**, 11–19.

Weiss, T. S., Jahn, B., Cetto, M., Jauch, K. W. & Thasler, W. E. (2002). Collagen sandwich culture affects intracellular polyamine levels of human hepatocytes. *Cell Prolif* **35**, 257–267.

Yamada, K., Takahashi, M., Hoshino, Y., Takahashi, H., Ichiyama, K., Tanaka, T. & Okamoto, H. (2009). Construction of an infectious cDNA clone of hepatitis E virus strain JE03-1760F that can propagate efficiently in cultured cells. *J Gen Virol* **90**, 457–462.

Yunoki, M., Yamamoto, S., Tanaka, H., Nishigaki, H., Tanaka, Y., Nishida, A., Adan-Kubo, J., Tsujikawa, M., Hattori, S. & other authors (2008). Extent of hepatitis E virus elimination is affected by stabilizers present in plasma products and pore size of nanofilters. *Vox Sang* **95**, 94–100.

Zhai, L., Dai, X. & Meng, J. (2006). Hepatitis E virus genotyping based on full-length genome and partial genomic regions. *Virus Res* **120**, 57–69.

J Gastroenterol (2014) 49:1557–1566
DOI 10.1007/s00535-013-0927-6

The Japanese Society of Gastroenterology

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Proliferation of mouse liver stem/progenitor cells induced by plasma from patients with acute liver failure is modulated by $P2Y_2$ receptor-mediated JNK activation

Ting Wang · Yasuhiro Takikawa · Asako Watanabe · Keisuke Kakisaka · Kanta Oigawa · Yasuhiro Miyamoto · Kazuyuki Suzuki

Received: 18 March 2013 / Accepted: 9 December 2013 / Published online: 22 December 2013
© Springer Japan 2013

Abstract

Background We recently reported that acute liver failure plasma (ALF-P) promotes the proliferation of mouse liver oval cells (OCs) through c-jun N-terminal kinase (JNK) activation. The aim of this study was to investigate the mechanism by which ALF-P induces JNK activation and OC proliferation.

Methods OCs and primary hepatocytes were exposed to ALF-P or normal control plasma (NC-P). Cell proliferation and activation of JNK and other JNK signaling molecules were detected subsequently. Next, we determined the effects of extracellular adenosine triphosphate (ATP) and ATP receptors on ALF-P-stimulated cell growth. Finally, the relationship between the tumor necrosis factor alpha (TNFα) and ATP receptor pathways was investigated.

Results Cell proliferation accompanied by JNK activation was only observed in ALF-P-stimulated OCs. ALF-P stimulated the activation of SEK1/MKK4 and ATF2, but not c-Jun. Both PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid) treatment and $P2Y_2$ (G-protein-coupled) small interfering RNA (siRNA) transfection blocked the effects of ALF-P on cell proliferation and JNK activation. However, ATP levels in ALF-P were significantly lower than that in NC-P, and ATP did not stimulate the proliferation of OCs. On the other hand, TNFα stimulated JNK activation and proliferation of OCs. TNFα receptor antagonist partly inhibited the ALF-P-stimulated proliferation of OCs. Moreover, PPADS significantly inhibited TNFα-stimulated cell proliferation, induced apoptosis, and inhibited the activation of JNK. However, our data showed no significant difference in plasma TNFα levels between the NC-P and ALF-P samples.

Conclusions JNK activation induced by $P2Y_2$ receptor crosstalk with the TNFα signaling pathway is important in mediating the effects of ALF-P on the proliferation and survival of OCs.

Keywords ALF-P · JNK · OCs · P2Y · TNFα

Abbreviations

ALF	Acute liver failure
ALF-P	ALF plasma
NC-P	Normal control plasma
HPCs	Hepatic progenitor cells
OC	Oval cell
JNK	c-jun N-terminal kinase
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AFP	Alpha-fetoprotein
Alb	Albumin
CK19	Cytokeratin 19
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
TAT	Tyrosine aminotransferase
G6Pase	Glucose-6-phosphatase
IL	Interleukin
IRB	Institutional review board
HGF	Hepatocyte growth factor
TUDC	Tauroursodeoxycholate
EGF	Epidermal growth factor

Electronic supplementary material The online version of this article (doi:10.1007/s00535-013-0927-6) contains supplementary material, which is available to authorized users.

T. Wang (✉) · Y. Takikawa (✉) · A. Watanabe · K. Kakisaka · K. Oigawa · Y. Miyamoto · K. Suzuki
Division of Hepatology, Department of Internal Medicine, Iwate Medical University, Morioka, Iwate, Japan
e-mail: tingwang@iwate-med.ac.jp

Y. Takikawa
e-mail: ytakikaw@iwate-med.ac.jp

IFN	Interferon
TGFβ	Transforming growth factor beta
TNF	Tumor necrosis factor
TNFR	TNF receptor
AP-1	Activating protein 1
ATP	Adenosine triphosphate
PPADS	Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
PI	Propidium iodide
ATF2	Activating transcriptional factor 2
RT-PCR	Reverse transcriptase-polymerase chain reaction
siRNA	Small interfering RNA
TPE	Total plasma exchange
RLUs	Relative light units

Introduction

In spite of its vigorous capacity for regeneration in response to resection of more than 50 % of its mass, the liver becomes disabled in the case of acute liver failure (ALF). ALF is a fatal clinical syndrome characterized by the sudden initiation of irreversible hepatocyte death, which leads to hepatic encephalopathy and finally to multi-organ failure [1, 2]. Although liver transplantation is currently the best option for improving the survival rate, the rapid progression and variable course of ALF limit its use [2]. As an alternative to transplantation, developing effective methods to improve the regenerative capacity of the failing liver is regarded as an ideal goal that could save patients' lives.

Hepatic progenitor cells (HPCs), also named oval cells (OCs) in rodents, have attracted a great deal of attention because they appear only when the regenerative capacity of the liver is blocked [3, 4]. Because they generate hepatocytes and biliary epithelial cells [5, 6], HPCs are generally recognized as a major resource for pathological liver repair. However, clinical studies have provided evidence that extensive HPC activation is negatively correlated with the survival rate of ALF patients and is a sign of disease severity [7]. The findings indicate that in ALF, rather than differentiating into functional hepatocytes, HPCs continue proliferating, which might impair liver regeneration. Investigating the mechanism underlying the proliferation of HPCs in ALF is therefore of clinical significance.

Plasma or serum is usually used to evaluate the in vivo microenvironment. The cytotoxicity of plasma or serum from patients with ALF has been demonstrated. The cytotoxic effects include inhibiting the proliferation of hepatocytes, interfering with protein synthesis, and causing cell death [8–11]. Conversely, ALF plasma (ALF-P) was not toxic to the function of primary rat hepatocytes in three-dimensional culture [12]. Our previous study showed that ALF-P stimulated the proliferation of OCs. This effect was blocked by a specific c-Jun N-terminal kinase (JNK) inhibitor (SP600125), which indicates that JNK activation is required for the ALF-P-stimulated proliferation of OCs [13]. Although the relationship between JNK and the proliferation and death of hepatocytes is well-described [14, 15], the upstream molecular mechanism that leads to the activation of JNK signaling in OCs stimulated by ALF-P remains unknown.

Extracellular adenosine triphosphate (ATP), an important signaling molecule in the inflammatory and cellular stress responses, was recently reported to affect liver regeneration by activating JNK signaling [16]. ATP exerts its function through P2 receptors. Mammalian P2 receptors consist of ion channels (P2X) and G-protein-coupled (P2Y) subtypes. P2Y receptors control glycogen metabolism and proliferation-associated responses, such as increased [Ca^{2+}] and mitogen-activated protein kinase cascades, in primary human hepatocytes [17]. Among the members of the P2Y receptor family, the $P2Y_2$ receptor mediates the ATP-activated JNK pathway and consequently contributes to hepatocyte proliferation in vitro and cell cycle progression in rat liver after partial hepatectomy [16]. However, ATP and the $P2Y_2$ receptor promote cell death in mice with acute liver injury [18, 19]. These findings appear to reflect dual roles of ATP receptors in the regulation of cell growth under pathological conditions.

Tumor necrosis factor (TNF) signaling via TNF receptor (TNFR)-1 is one of best-studied pathways leading to JNK activation. The initial TNFR-1-mediated JNK activation is transient and associated with cell survival and proliferation through activating protein 1 (AP-1), whereas sustained JNK activation is closely related to TNFα-induced programmed cell death in the liver [14, 15, 20–22]. Moreover, TNFα takes a central role in the pathogenesis of ALF. The levels of circulating TNFα are increased in ALF patients and are associated with a poor prognosis [23–25].

Notably, a close relationship exists between extracellular ATP and TNFα in the development of ALF. TNFα mediates hepatic apoptosis during ALF, and the apoptosis itself is a highly ATP-dependent process. ATP depletion upon massive cell injury induces the expression of inflammatory cytokines including TNFα. However, little is known about the roles of ATP, TNFα, and their pathways in the regulation of OCs in ALF.

Here, we demonstrate that one of the P2Y receptors, $P2Y_2$ subtype, activates JNK and plays an important role in mediating the proliferative and anti-apoptotic effects of

ALF-P in OCs. The present study also provides evidence that $P2Y_2$ receptor signaling in ALF-P is ATP-independent and closely linked with the TNFα receptor signaling pathway.

Materials and methods

Patients

Three patients with ALF in maximum coma grades 2, 3, and 5 were studied. The etiology of the three patients were B hepatitis in two cases and non-A and non-B hepatitis in one. The patients consisted of two males and one female with an age range of 53–68 years. None of the patients survived. The laboratory parameters were shown as total bilirubin of 11.4 (range 4.6–14.9) mg/dL, aspartate aminotransferase (AST) 2534 (626–5496) IU/L, alanine aminotransferase (ALT) 1307 (1173–1480) IU/L, total bile acid 217.5 (177.0–318.0) μM/L, prothrombin time 27.7 (22.4–32.4) %, alpha-fetoprotein (AFP) 738.4 (2.0–2149.0) ng/mL, and hepatocyte growth factor (HGF) 2.06 (0.58–3.70) ng/mL. Control samples were taken from three normal subjects, one male and two females with an age range of 35–45 years. Blood in the acute phase of the disease was taken on ice into endotoxin-free heparinized vacutainers. The blood was centrifuged at 1,500g for 10 min. The resulting plasma was stored in aliquots at −80 °C. Approval for the study was obtained from the institutional review board (IRB, H19-87) of Iwate Medical University, Morioka, Japan, and informed consent was obtained from the patients' relatives.

Oval cells

A cell line of mouse liver stem/progenitor cells was established and provided by Professor Atsushi Miyajima and Dr. Minoru Tanaka of Tokyo University [26].

Isolation and primary culture of mouse hepatocytes

Mouse hepatocytes were isolated by the two-step collagenase perfusion method using 0.025 % collagenase [27]. The hepatocytes were plated in collagen 1-coated dishes. After the cells were attached, the medium was changed to serum-free medium for the proliferation assay.

Proliferation assay

Serum-starved oval cells or primary hepatocytes (3×10^4 cells/mL) were treated with 30 % ALF-P, 30 % normal control plasma (NC-P), recombinant murine tumor necrosis factor α (rmTNFα: R&D systems, USA), or the adenosine triphosphate receptor agonists (ATP: R&D systems, USA; ATPγS and 2MeSATP: Sigma-Aldrich, Germany) with and without the relative inhibitors (R-7050: Calbiochem, USA; pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADs), NF023, and Apyrase: Sigma-Aldrich, Germany; AG1478 and SB431542: Santa Cruz, USA) for the indicated period. The number of viable cells were evaluated by adding the cell count reagent SF (Nacalai Tesque Inc., Japan) directly to the cells [28]. Absorbance was measured at 450 nm by a microplate photometer (Immuno Mini NJ-2300: InterMed, Japan).

Evaluation of apoptosis and live/dead cell staining

Oval cells were seeded at a density of 5×10^4 cells/mL into sterile culture plate and left overnight for adherence. After serum starvation, cells were treated with 30 % ALF-P, 30 % NC-P, or TNFα with and without PPADs and NF023 for 72 h. Apoptotic cells were stained by the 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Dojindo, Japan). In addition, Live-dye™ (MBL, USA), a cell-permeable green fluorescent dye and a non-cell-permeable red fluorescent dye propidium iodide (PI, MBL, USA) were used for the differential staining of live and dead cells, according to manufacturer's instructions. The cells were scored under inverted fluorescence microscopy (ECLIPSE TE300: Nikon, Japan) at 20× magnification, and were photographed using a digital camera (DXC-S500/OL; Olympus, Tokyo, Japan).

Western blot analysis

Total protein was isolated from the OCs using a total protein extraction kit from BioChain Institute Inc. (Hayward, CA, USA). A total of 20 μg of protein from each sample was separated using 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene difluoride membrane. Immunoblotting was performed using specific antibodies against p-JNK, p-ATF2, p-MKK4, p-c-Jun (Cell Signaling Technology, USA), and β-actin (Santa Cruz Biotechnology, USA). The immunoreactive bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences, UK).

ATP determination assay

The plasma ATP concentration was measured using an ATP assay kit (Toyo-ink, Japan), according to the manufacturer's instructions. In brief, plasma ATP was extracted using the ATP extraction reagent. Luminescence reagents were added to the samples and the relative light units (RLUs) were measured immediately with the Lumitester