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Review

Hepatitis E virus cell culture models

Hiroaki Okamoto*

Division of Virology, Department of Infection and Immunity, Jichi Medical University, School of Medicine, Tochigi-Ken 329-0498, Japan

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ABSTRACT

Early studies reported the propagation of hepatitis E virus (HEV) in either primary hepatocytes or several established cell lines, but replication was inefficient. Efficient cell culture systems for HEV in PLC/PRF/5 and A549 cells have recently been established, using inoculum of fecal suspensions with high HEV loads, originally obtained from patients with genotype 3 HEV (the JE03-1760F strain, 2.0×10^7 copies/ml) or genotype 4 HEV (the HE-JF5/15F strain, 1.3×10^7 copies/ml), and many generations were successfully propagated in serial passages of culture supernatant. In addition, a full-length infectious cDNA clone (pJE03-1760F/wt) of the JE03-1760F strain was constructed, which can replicate efficiently in PLC/PRF/5 and A549 cells. A derivative ORF3-deficient mutant revealed that the ORF3 protein of HEV is responsible for virion egress from infected cells and is present on the surface of released HEV particles, which is associated with lipids. Various HEV strains with high loads of $\geq 10^5$ copies/ml in circulating blood were also propagated efficiently in PLC/PRF/5 and A549 cells. This paper reviews the road map toward the development of efficient cell culture systems for a wide variety of HEV strains and introduces the current knowledge on virion egress obtained by cell culture models.

Contents

1. Introduction

Hepatitis E virus (HEV) was discovered in 1983 by immune electron microscopy (Balayan et al., 1983) and was first cloned in 1990 (Reyes et al., 1990). HEV is the causative agent of acute or fulminant hepatitis E, which occurs in many parts of the world, principally as a water-borne infection in developing countries and zoonotically in industrialized countries (Chandra et al., 2008; Colson et al., 2010; Dalton et al., 2008; Emerson and Purcell, 2007; Purcell and Emerson, 2008; Tei et al., 2003; Yazaki et al., 2003). Several species of animals, such as swine, wild boars and deer, are considered to serve as reservoirs of HEV (Meng et al., 1997b; Pavio et al., 2010; Reuter et al., 2009; Sonoda et al., 2004; Takahashi et al., 2003, 2004a), and ingestion of uncooked or undercooked meat and the viscera of these animals may be the major route of HEV infection in industrialized countries, including Japan (Tei et al., 2003; Yazaki et al., 2003). HEV was previously classified in the family *Calciviridae*, but it is now placed in the genus *Hepevirus* within the family *Hepeviridae* (Emerson et al., 2005a). The virion is 27–34 nm in diameter and is believed to be non-enveloped, although HEV particles in the culture supernatant and circulating blood are associated with lipids, as described in detail below. Its genome consists of a single-stranded, positive-sense RNA of approximately 7.2 kilobases (kb) in length, which is capped and polyadenylated (Kabrane-Lazizi et al., 1999; Tam et al., 1991). It contains a short 5′ untranslated region (UTR), three open reading frames (ORFs: ORF1, ORF2, and ORF3), and 3′UTR. ORF1 encodes non-structural proteins including methyltransferase, papain-like cysteine protease, helicase and

* Tel.: +81 285 58 7404; fax: +81 285 44 1557.
E-mail address: hokamoto@jichi.ac.jp

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RNA-dependent RNA polymerase (Agrawal et al., 2001; Koonin et al., 1992). ORF2 and ORF3 overlap, and the ORF2 and ORF3 proteins are translated from a single bicistronic subgenomic RNA of 2.2-kb in length (Graff et al., 2006). The ORF2 protein is the viral capsid protein; the crystal structure of a truncated recombinant ORF2 protein has been elucidated, but the size of the protein in mature virions remains unknown (Guu et al., 2009; Yamashita et al., 2009). The ORF3 protein is a small, phosphorylated protein made of 113 or 114 amino acids (aa), whose function(s) has not been fully defined (Emerson et al., 2010). Four genotypes of HEV that infect humans have been identified (Lu et al., 2006; Okamoto, 2007). Genotypes 1 and 2 were isolated from humans only and are mainly seen in developing countries. Genotypes 3 and 4 are zoonotic, and have been identified in many sporadic cases affecting middle-aged and elderly men in industrialized countries (Lewis et al., 2010; Meng, 2010; Okamoto et al., 2003; Purcell and Emerson, 2008).

HEV transmission studies have mostly been done in nonhuman primates such as macaque species, chimpanzees, and owl monkeys, which have provided important information regarding the biology and pathogenesis of HEV (Purcell and Emerson, 2001). Experimental transmission studies have also been done in pigs, an established reservoir for HEV (Williams et al., 2001). In the swine model, replicative, negative-strand HEV RNA was detected primarily in the small intestines, lymph nodes, colon, and livers, indicating that HEV replicates in tissues other than the liver. The establishment of a practical cell culture system that facilitates the propagation of HEV *in vitro* is critical for virological characterization as well as for studies on the prevention of HEV infection. The propagation and production of HEV *in vitro* have been attempted by many researchers in primary hepatocytes from nonhuman primates (Chimpanzees, cynomolgus macaques, tamarins, and African green monkeys) (Arankalle et al., 1988; Kane et al., 1984; Tam et al., 1996, 1997) and in various continuous cell lines, such as the human normal embryonic liver cells (WRL68), human hepatoma cell lines (PLC/PRF/5, HepG2, and Huh-7 cells), human colon carcinoma cells (Caco-2), human embryo lung diploid cells (2BS), human lung embryonal fibroblast cells (MRC-5), human lung cancer cells (A549), human choriocarcinoma cells (HCCM), African green monkey kidney cells (Vero), and Rhesus monkey kidney from *Macaca mullata* cells (LLC-MK2) (Arankalle et al., 1988; Emerson et al., 2010; Huang et al., 1992, 1995, 1999; Kazachkov Yu et al., 1992; Li et al., 1996; Meng et al., 1996, 1997a; Wei et al., 2000). In addition, Emerson et al. (2005b, 2006a) reported a quantitative cell culture assay for performing both neutralization tests and thermal stability tests by detecting infected HepG2/C3A cells by immunofluorescence microscopy. However, none of these culture systems can provide high-titer infectious HEV in the culture supernatant. This laboratory recently succeeded in establishing efficient cell culture systems for infectious HEV in feces and circulating blood, which has opened up new avenues for analyzing the virus itself that have not been possible since the discovery of the viruses by Balayan et al. (1983) in 1983. This article reviews the establishment and evaluation of cell culture systems for various HEV strains, and summarizes the new knowledge that has evolved by the HEV cell culture models.

2. Propagation of infectious HEV strains of genotypes 3 and 4 in feces and serial passages of their progenies in cultured cells

Each of 21 selected cell lines was inoculated with fecal suspensions with high HEV load (JE03-1760F strain: 2.0×10^7 copies/ml) obtained from a Japanese patient who contracted a domestic infection of genotype 3 HEV. The cell lines were derived from humans [PLC/PRF/5 (CRL-8024), HepG2 (HB-8065), Huh-7 (RCB1366), A549 (CCL-185), MCF (JCRB0314), NUGU-4 (JCRB0834), HEK293 (CRL-1573), HeLa (CCL-13), HT-1080 (JCRB9113), SK-N-MC (HTB-10), and GOTO (JCRB0612)], mice [P19 (CRL-1825), BC3H1 (CRL-1443), L929 (RCB1422), and C2C12 (CRL-1772)], rats [C6 (CCL-107), and IEC-6 (CRL-1592)], monkeys [LLC-MK2 (CCL-7), and CV1 (CRL-10478)], cow [MDBK (CCL-22)], and dog [MDCK (CCL-34)], and were available from either the American Type Culture Collection (ATCC, Manassas, VA) or the RIKEN BRC Cell Bank (RIKEN BioResource Center, Tsukuba, Japan) (Tanaka et al., 2007). Monolayers of cultured cells in a six-well microplate were inoculated with 0.2 ml of the virus stock that had been diluted with phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(−)], and containing 0.2% (w/v) bovine serum albumin, and filtered. After having been inoculated for 1 h at room temperature, the solution was removed and 2 ml of maintenance medium was added and cultured at 35.5 °C in a humidified 5% CO_2 atmosphere. The maintenance medium used for virus culturing was 50% Dulbecco's Modified Eagle Medium (DMEM, GIBCO Cat. No. 12800-017, Invitrogen, Tokyo, Japan) and 50% Medium 199 (GIBCO Cat. No. 31100-027, Invitrogen) containing 2% (v/v) heat-inactivated fetal bovine serum and 30 mM $MgCl_2$ at final concentration. One-half (1 ml) of the culture medium was replaced with fresh maintenance medium every other day since day 2 after inoculation, and the harvested media were stored at −80 °C until testing. The JE03-1760F strain was found to replicate efficiently in two of the 21 cell lines tested; PLC/PRF/5 from human hepatocellular carcinoma and A549 from human lung cancer. HEV progenies were released into culture supernatant in titers reflective of the HEV inoculum. Cultures were inoculated with 1.0×10^5 copies [multiplicity of infection (MOI): approximately 0.1] per well in a six-well plate of PLC/PRF/5 cells, and HEV was first detectable in the culture supernatant 8 days post-inoculation (dpi) and reached 10^8 copies/ml on 50 dpi in all six wells (Fig. 1A). HEV was detectable in culture media of all six wells even when the inoculum size was reduced to 1.0×10^4 copies (MOI: approximately 0.01) per well, although the initial day of appearance was later and highest titer of HEV in the culture supernatant after inoculation decreased. HEV RNA was detectable in only one of the six wells inoculated following inoculation at 1.0×10^3 copies (MOI: approximately 0.001), with less efficient multiplication. A549 cells were seeded at 1.0×10^4 and 1.0×10^5 copies per well, and HEV RNA reached the highest titer of 10^7 copies/ml on 50 dpi in all wells, independent on the titer of seed virus in the inoculum, although it was initially detected on 6 and 4 dpi, respectively. HEV RNA was detectable in two of the six wells inoculated at 1.0×10^3 copies per well, reaching the titer of 10^6 copies/ml on 50 dpi. These results suggest that the highest HEV RNA titer in the culture supernatant during cell culture and the interval between inoculation of seed virus and the appearance of progenies in the culture supernatant differ depending on the cell type inoculated, even when inoculated at the same MOI. Inoculation at higher MOI allows PLC/PRF/5 cells to support replication of JE03-1760F HEV at higher efficiency than A549 cells. On the other hand, A549 cells can support more efficient growth than PLC/PRF/5 cells when inoculated at lower MOI, with an earlier appearance of HEV progenies in the culture medium.

The ORF2 protein in PLC/PRF/5 cells was stained by an immunofluorescence assay (IFA) with a mouse monoclonal antibody against ORF2 protein (anti-ORF2 mAb) (H6225) (Takahashi et al., 2008a). The cultured cells that showed positive staining for ORF2 protein increased over time, thus reflecting a spread in HEV infection in these cells (Fig. 1B). Previous reports suggested that HEV can be cultured in PLC/PRF/5 and A549 cells, although the viral titers in culture media are extremely low, and virus spreading in cultured cells was not seen in any of previously reported cell culture systems for HEV (Huang et al., 1992; Li et al., 1996; Meng et al., 1996, 1997a; Pillot et al., 1987). The first successful propagation of the JE03-1760F strain in PLC/PRF/5 and A549 cells may have been