Pregnancy serum facilitates hepatitis E virus replication in vitro

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Hepatitis E virus (HEV) infection causes high mortality in pregnant women. However, the pathogenic mechanisms of HEV infection in pregnant women remain unknown. In this study, the roles of pregnancy serum in HEV infection were investigated using an efficient cell culture system. HEV infection was exacerbated by supplementing with pregnancy serum, especially in the third trimester of pregnancy. Oestrogen receptors (ER-α and ER-β) were activated in cells supplemented with pregnancy serum and were significantly inhibited during HEV infection. Type I IFN, especially IFN-β, showed delayed upregulation in HEV-infected cells supplemented with the serum in the third trimester of pregnancy, which indicated that delayed IFN-β expression may facilitate viral replication. Results suggested that pregnancy serum accelerated HEV replication by suppressing oestrogen receptors and type I IFN in the early stage of infection.

INTRODUCTION

Hepatitis E virus (HEV) is considered as one of the most common causes of acute hepatitis worldwide (Aggarwal & Krawczynski, 2000). The major routes of viral transmission are via faecal–oral contact, contaminated water and food (Rein et al., 2012). HEV infections cause acute hepatitis and fulminant hepatic failure, but most infections are asymptomatic (Aggarwal & Krawczynski, 2000). However, pregnant women are exceptionally susceptible to HEV infection, especially those in the third trimester of pregnancy. HEV infection causes a mortality rate above 20% in pregnant women and aggravates in the second or third trimester of pregnancy (Labrique et al., 2012; Navaneethan et al., 2008). A very high mortality rate of 65.8% has also been reported in HEV-infected pregnant women suffering from fulminant hepatitis failure (Labrique et al., 2012). Pregnancy is an important risk factor of HEV infection and higher viral loads have been found in pregnant than in non-pregnant women (Jilani et al., 2007). Furthermore, high hormone levels and low immunity status during pregnancy may influence viral replication.

Pregnant women are more severely affected by some viral infections, including influenza virus (Pazos et al., 2012), severe acute respiratory syndrome coronavirus (Secznyska et al., 2014; Wong et al., 2003), rhinovirus (Forbes et al., 2012), hepatitis B virus (HBV) (Lapiński et al., 2010) and HEV (Labrique et al., 2012; Navaneethan et al., 2008). Pregnant women infected with HEV in the third trimester of pregnancy may suffer from premature delivery, stillbirth and mortality. Increased morbidity caused by HEV infection is associated with the third trimester of pregnancy and correlates well with the highest levels of oestrogen. Although oestrogen levels during pregnancy have been associated with reduced levels of immune-mediated morbidity, severe morbidity is a hallmark of HEV infection during pregnancy. Oestrogen enhances the ability to produce inflammatory mediators and cytokines upon Toll-like receptor activation, by stimulating oestrogen receptors (ER)-α in macrophages (Biswas et al., 2005; Calippe et al., 2010). In the present study, the relationship between pregnancy serum and HEV infection was investigated in A549 cells.

Innate immune system is the first line of defence against invading pathogens in the host (Hochhaus & Burchert 2010; Weber et al., 2004). HEV causes an acute, self-limiting disease, although chronic HEV infection has been reported (Kamar et al., 2008; Versluis et al., 2013). Acute viral infection of susceptible host cells initiates a type I IFN response that predominantly consists of IFN-α and -β (IFN-α/β) signalling via IFN-α receptors (Weber et al., 2004). Hepatic damage in HEV infected patients is mediated by the immune system and not by the direct replication of HEV (Aggarwal & Jameel, 2011). Innate immune gene expression is more attenuated in HEV-infected than in hepatitis C
virus-infected chimpanzees, indicating that HEV infection elicits weak effects on the innate immune system (Yu et al., 2010). Dong et al. (2012) reported that HEV infection inhibits IFN-α expression by regulating STAT1 phosphorylation in A549 cells. However, it remains unclear whether weakened immune status and high oestrogen levels in the serum of pregnant women influence HEV replication in vitro.

Inefficient cell culture systems have greatly impeded further HEV analyses. Nevertheless, an efficient cell culture has recently been developed in A549 (human lung cancer) and PLC/PRF/5 (human hepatocellular carcinoma) cell lines (Okamoto, 2010). Infectious HEV progenies inoculated with HEV infected faecal specimens are secreted in high titres into A549 or PLC/PRF/5 cell culture media (Okamoto, 2010; Takahashi et al., 2012). Thus, studies can be performed to determine whether the serum of pregnant women accelerates HEV replication in an efficient cell culture.

In the present study, the roles of pregnancy serum in HEV infection were investigated using an efficient A549 cell culture system.

RESULTS

Pregnancy serum facilitates HEV replication in A549 cells

HEV virion was secreted into the cell supernatant and detected by reverse transcription nested PCR (RT-nPCR) in A549 cells supplemented with serum from healthy women, either pregnant or non-pregnant, at 12, 24, 36, 72 h and 6 days post-inoculation (Fig. 1a).

To determine whether only the serum of pregnant women facilitated HEV replication, HEV-infected cells supplemented with the serum of healthy non-pregnant women, children, pregnant women or FBS were compared. Six days post-inoculation, replication of HEV at the protein level was analysed by Western blot, which indicated that HEV ORF2 protein (71 kDa) expressed in the cells supplemented with the serum of pregnant women (first, IgG+ and third trimester) was significantly increased compared with that supplemented with serum of healthy non-pregnant women or children (Fig. 1b, c). We further confirmed that HEV-infected cells supplemented with serum from the third trimester of pregnancy were more conducive to viral replication than those in the first trimester of pregnancy (Fig. 1b, c).

It was reported that anti-HEV immune serum had no significant neutralization function (Arankalle et al., 1998). HEV infection was observed in cells supplemented with pregnancy serum that reacted positively to anti-HEV IgG antibody; furthermore, higher levels of ORF2 protein were observed when compared with cells supplemented with serum of pregnant women and which reacted negatively to anti-HEV IgG antibody (Fig. 1c).

HEV infection inhibits oestrogen receptor expression

The hormonal changes throughout the pregnancy period are significant and may be associated with increased viral replication. Higher HEV titres were secreted in the cell culture supplemented with the serum of pregnant women than in that supplemented with the serum of healthy non-pregnant women, children or with FBS. Hormones may constitute the most significant difference between the serum of non-pregnant and pregnant women. The level of oestrogen is remarkably increased in pregnant women. The results of sex hormone determination indicated that oestrogen (E2) in mixed serum from the first and third trimesters of pregnancy was 21.8- and 165.5-fold higher than that of FBS, respectively (Fig. 2d). Oestrogen in mixed serum of the third trimester of pregnancy was 6.9-fold higher than the upper limits of referenced human serum in the third trimester of pregnancy.

Oestrogen plays important physiological roles in maintaining life, sexual function and immune regulation through binding to its specific receptor (ER-α or ER-β). In the present study, cells supplied with serum from the third trimester of pregnancy induced persistent ER-α and -β expression. This result indicated that significantly increased oestrogen levels during pregnancy activate the expression of oestrogen receptors (Fig. 2a, b). Interestingly, cells infected with HEV significantly inhibited the expression of both ER-α and -β. Reduced activation of ERs may decrease the products of inflammatory mediators and cytokines, thereby suppressing the innate immunity of host cells. As a result, HEV replication is initiated and promoted.

HEV infection suppresses type I IFN expression

The low immunity status of pregnant women is possibly related to HEV susceptibility and severity (Jilani et al., 2007). In the first 12 h of HEV replication, IFN-α showed a low level of upregulation in cells supplemented with human serum (healthy non-pregnancy and pregnancy serum), but levels were inhibited in cells supplemented with FBS. Subsequently, IFN-α was activated from 36 h post-inoculation, especially in cells supplemented with serum from the third trimester of pregnancy (Fig. 3a). Devhare et al. (2013) reported that IFN induction required live HEV infection, the higher level of IFN indicated indirectly that cells supplemented with serum from the third trimester of pregnancy were more beneficial for HEV replication.

IFN-β showed delayed upregulation in the early stage of replication, but increased from 36 h post-inoculation which is consistent with a previous study (Devhare et al., 2013). However, HEV-infected A549 cells supplemented with serum from the third trimester of pregnancy showed a persistent inhibition of IFN-β in the first 72 h and had increased only slightly by 6 days post-inoculation (Fig. 3b).
This result indicated that HEV in cells supplemented with serum from the third trimester of pregnancy is more beneficial to maintaining transcription of the IFN-β gene at low levels throughout the infection, which promotes HEV replication.

**DISCUSSION**

Pregnant women are at increased risk of severe illnesses such as influenza A virus and HEV infections, which lead to high mortality during pregnancy (Kourtis et al., 2014). In HEV-endemic areas (India, South-east Asia and Africa), HEV infection is a major cause of maternal death and fetal loss. Abnormalities in liver function are common in normal pregnancy (notable increase in alkaline phosphatase and decrease in serum albumin), but increases in serum bilirubin and aminotransferase suggest that pre-existing liver disease, liver disease related to pregnancy, or liver disease unrelated to pregnancy are exacerbated (Than & Neuberger, 2013). Therefore, pregnant women show high mortality rates when infected with HEV.

During pregnancy a large number of hormones play an important role; an epidemiological survey has revealed that oestrogen (E2) levels in the serum are significantly higher in pregnant women infected with HEV than in uninfected pregnant women and non-pregnant women (Navaneethan et al., 2008). In the present study, the level of E2 in mixed serum from the third trimester of pregnancy was
significantly higher than that in the first trimester of pregnancy, healthy non-pregnant women or FBS. In the meantime, more HEV virion replicated in cells supplemented with serum in the third trimester of pregnancy, which contained the highest oestrogen level. Thus, the high level of oestrogen may be associated with efficient HEV replication.

Oestrogen binds to its specific receptor (ER-α and ER-β) and activates the corresponding response element of target genes, regulating gene expression at the transcriptional level (mRNA) and thereby playing an important physiological role in maintaining life, sexual function and immune regulation. In this study, Western blot analysis showed that only cells supplemented with the serum of pregnant women (first or third trimester of pregnancy) induced ER-α and ER-β expression. Furthermore, HEV infection significantly inhibited ER-α and -β expression, suggesting that oestrogen and its receptor ERs are involved in HEV infection. ER-α mediated oestrogen plays a major biological function in the liver. Clinical and animal-based experiments have demonstrated that abnormal ER-α expression is closely related to hepatocyte proliferation and liver cirrhosis (Shimizu, 2003; Yan et al., 2011). For instance, HBV X protein can inhibit ER transcription. The
DNA of HBV can easily integrate with the ER gene of a host cell, resulting in abnormal shear of ER transcription; thus, the regulatory function of ER related to cell growth is altered and liver cancer is induced (Wang et al., 2012).

Oestrogen and ERs in the liver protect hepatocytes from oxidative stress, inflammatory injury and cell death. Women are more likely to eradicate hepatitis C virus than men after they are exposed to initial infection. However, postmenopausal women exhibit increased rates of fibrosis compared with those of reproductive age because they have lost the protective effects of oestrogen (Baden et al., 2014). The evident increase in oestrogen levels in pregnant women, especially in the third trimester, facilitates HEV infection; by contrast, HEV replication reduces ER expression.

E2 concomitantly inhibits PI3K activity and Akt phosphorylation (Biswas et al., 2005). Inhibition of the PI3K-PKB-mTOR signal pathway has been shown to facilitate HEV replication (Zhou et al., 2014). Weakened immunity and increased nutritional demands during pregnancy are associated with the downregulation of PI3K-PKB-mTOR signalling and promotion of HEV infection (Zhou et al., 2014).

Immunological alterations with advancing pregnancy may impair virus clearance, resulting in increased severity of infectious diseases. Meanwhile, numerous viruses can counteract the IFN system of the host by modulating the production of IFNs to inhibit their functions. In the present study, IFN-α was inhibited in the first 12 h post-inoculation and showed a low-level increase until 36 h. It was reported that HEV inhibits IFN-α signalling through the regulation of STAT1 phosphorylation (Dong et al., 2012). Furthermore, HEV infection significantly inhibited IFN-β expression in the early stage of replication, especially in cells supplemented with the serum of women in the third trimester of pregnancy. This delayed upregulation of IFNs in the early stage of infection facilitates HEV replication, and may explain the higher viral titres found in women in their third trimester of pregnancy compared with first trimester or non-pregnant women with HEV infection.

In conclusion, the present study demonstrated that pregnancy serum, especially in the third trimester, facilitates HEV infection in vitro.

**METHODS**

**Ethical statement.** All sera were collected from patients during hepatitis E outbreaks for epidemic investigations in 2012 in Kunming City, China. This study was granted local research ethical approval to recruit pregnant women, healthy adult, non-pregnant women and children. All patients were made aware of and gave approval to
participate in this study. Patients with hepatitis A virus, HBV, hepatitis C virus or human immunodeficiency virus were excluded.

**Virus.** Swine faecal samples containing HEV genotype 4 (GenBank accession no. KJ155502) were obtained from a village in Southwestern China (Huang et al., 2011). Faecal suspension (10%) was centrifuged at 12,000 g at 4 °C for 10 min, filtered through 0.22 μm microfilters and treated with penicillin and streptomycin for 1 h. The suspension was then stored in liquid nitrogen until use. The viral genomic titres consisted of 1.0 × 10^{8} copies determined using real-time quantitative PCR (qPCR), as previously described (Huang et al., 2013).

**Cell cultures.** Human lung epithelial A549 cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) FBS, 100 U penicillin ml^{−1} and 100 μg streptomycin ml^{−1} at 37 °C under 5% CO_{2}.

**Serum preparation and sex hormone examination.** Serum was obtained from asymptomatic pregnant women, non-pregnant women and children for HEV sero-epidemiology investigation, as described in our previous study (Huang et al., 2013). Thirty sera from women in the first or third trimester of pregnancy that were negative for HEV RNA, HEV IgG and HEV IgM were separately mixed, filtered with a 0.22 μm microfilter and heat-inactivated at 56 °C for 30 min. Sera of pregnant women in their third trimester, healthy non-pregnant women and children positive for anti-HEV IgG antibody served as control.

Oestrogen (E2) of the mixed serum and FBS (GIBCO) was determined using a UniCel DxI 800 Immunoassay System (Beckman). Sera in the third trimester of pregnancy served as reference value.

**Viral inoculation and passages.** Cells were planted in six-well microplates 24 h before inoculation and supplemented with 10% FBS (FBS group), 10% mixed serum from healthy non-pregnant women (healthy non-pregnant women group), 10% mixed serum from children (children group), 10% mixed serum from women in the first trimester of pregnancy (first trimester group), 10% mixed serum from women in the third trimester of pregnancy (third trimester group) and 10% mixed serum from women in the third trimester of pregnancy positive for anti-HEV antibody (IgG^+ group). The protocol for HEV inoculation in A549 cells has been previously described (Huang et al., 1999; Okamoto, 2010; Tanaka et al., 2007). In brief, monolayer cells were washed three times and inoculated with 0.2 ml of the filtered virus inoculum for 1 h. The solution was removed after inoculation and fresh maintenance medium containing 2% FBS, 2% healthy non-pregnant human serum, 2% child serum, 2% first trimester of pregnancy serum or 2% third trimester of pregnancy serum (with or without IgG antibody) was added separately. Next, 30 mM MgCl_{2} (final concentration) was added. The cell culture supernatant was either collected for HEV replication detection or used for secreted IFN determination.

**Detection of HEV RNA in cell supernatant by RT-nPCR.** Cells were collected and freeze-thawed three times. Total RNA was extracted using Trizol according to the manufacturer’s directions. Reverse transcription (RT) analysis was performed using AMV Reverse Transcriptase XL (Takara) according to the manufacturer’s directions. HEV RNA (ORF2) in the cell supernatant supplemented with serum from either healthy non-pregnant women or pregnant women (third trimester) was detected by RT-nPCR as described previously (Huang et al., 2002).

**Determination of type I IFN by real-time qPCR.** Cell supernatant was collected at different time points after HEV inoculation. Total RNA was extracted and cDNA was synthesized by RT assay as described above. Changes in IFN-α and IFN-β in HEV-infected cell supernatants supplemented with different sera were analysed using SYBR green-based qPCR assays. The primers and PCR protocol used were as per a previous study (Devhare et al., 2013). In brief, synthesized first-strand cDNA (2 μl) was added as a template. Real-time qPCR was performed under the following conditions: 95 °C for 30 s, followed by 39 cycles of 95 °C for 5 s and 60 °C for 31 s. The housekeeping gene (GAPDH) served as a loading control. Real-time qPCR was performed using an ABI PRISM 7300 Real-Time PCR System. Fold changes in mRNA transcripts of IFN-α and IFN-β were calculated using the formula 2^{(ΔΔC_{T} \text{ of gene} / \text{C_{T}} \text{of GAPDH})}, where Ct is the threshold cycle.

**Analysis of change in protein expression by Western blot.** Cells were harvested at different infection times, 12, 36, 72 h and 6 days post-inoculation, and lysed with lysis buffer. An equivalent amount of total protein was separated by 10% SDS—PAGE and transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with 5% skimmed milk and the membrane was incubated separately with primary antibodies, including HEV ORF2 (ABR, 1:1000 dilution), ERz and ERβ (Abgent, 1:1000 dilution) at 4 °C overnight. HRP-conjugated IgG was used as a secondary antibody (Promega, 1:1000 dilution). The GAPDH protein served as a loading control. The bands were exposed to X-ray film using an Immobilon ECL kit (Millipore).

**Statistical analyses.** All experiments were performed at least three times. Data are presented as mean ± SD. Statistical analysis was performed on Western blot using GraphPad Prism software and P-values were calculated using Student’s t-test to determine the significance of differences between two or more groups, with a 0.05 level of probability (P<0.05) considered statistically significant.

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