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Research Article

The Identification of Three Sizes of Core Proteins during the Establishment of Persistent Hepatitis C Virus Infection *in vitro*

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Similar to Hepatitis C virus (HCV) infection in humans, HCVcc infection can also result in persistent and chronic infection. The core protein is a variable protein and exists in several sizes. Some sizes of core proteins have been reported to be related to chronic HCV infection. To study the possible role of the core protein in persistent HCV infection, a persistent HCVcc infection was established, and the expression of the core protein was analysed over the course of the infection. The results show that there are three sizes of core proteins (p24, p21 and p19) expressed during the establishment of persistent HCVcc infection. Of these, the p21 core protein is the mature form of the HCV core protein. The p24 core protein is the phosphorylated form of p21. The p19 core protein appears to be a functional by-product generated during the course of infection. These three core proteins are all localized in the cytoplasm and can be encapsidated into the HCV virion. The appearance of the p19 and p24 core proteins might be related to acute HCVcc infection and chronic infection respectively and may play an important role in the pathology of a HCV infection.

Hepatitis C virus; Core protein; Persistent infection; Phosphorylation

Hepatitis C virus (HCV), the major causative agent of non-A and non-B hepatitis, is estimated to infect 2% of the world's population (Alter M J, 1997). Viral infection persists in approximately 80% of infected individuals, causing chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Pawlotsky J M, 2004). No vaccine against HCV is currently available and therapeutic approaches remain limited (Houghton M, et al., 2005).

The HCV core protein, which is derived from the N terminus of the viral polyprotein, is not only a component of the nucleocapsid but also has properties that enable it to modulate a number of cellular processes. Several studies suggest that the HCV core protein can regulate cell signal transduction, including MAPK-, JAK-STAT-, NF-κB-, AP-1- and SRE-associated pathways, resulting in the regulation of apoptosis and the suppression of host immunity (Lai M M, et al., 2000; McLauchlan J, 2000). Several studies suggest that the expression of the core protein affects mitochondrial function and lipid metabolism (Moriya K, et al., 2001; Okuda M, et al., 2002). Furthermore, transgenic mice that express the HCV-core protein exhibit certain features of human infection, such as steatosis, insulin resistance and ultimately cancer (Moriya K, et al., 1997; Moriya K, et al., 1998). These results suggest that the core protein might play an important role in the pathogenesis and carcinogenesis of an HCV infection.

The amino acid sequence of the core protein is well conserved among different HCV isolates and genotypes compared to other HCV proteins (Suzuki R, et al., 2005). A signal sequence in its C-terminal region facilitates channeling of the nascent HCV polyprotein to the endoplasmic reticulum (ER) membrane and is the substrate for processing by two host proteases (Kopp M, et al., 2010). After cleavage by signal peptidase (SP), a 23-kDa core protein (191aa, p23) is released and is further processed by signal peptide peptidase (SPP), an integral membrane protease, to yield a 21-kDa core protein (177aa, p21) (Hussy P, et al., 1996). The cleavage by SPP promotes the release of core protein from the ER membrane, and

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then it is trafficked to lipid droplets, which are reported to be the site of HCV particle assembly (McLauchlan J, et al., 2002; Okamoto K, et al., 2008). The expression of a 16-kDa core protein (p16) was found in HCV infected patients, indicating that p16 might be related to chronic HCV infection, but the exact C-terminus of the p16 core protein has not been determined (Yeh C T, et al., 2000). Recently several groups have found that the substitution of amino acid (aa) 70 and/or 91 in the core region of HCV genotype 1b (HCV-1b) is associated with a poor response to pegylated interferon and ribavirin as well as an important predictor of hepatocarcinogenesis (Akuta N, et al., 2010; Akuta N, et al., 2012). Frameshift proteins and internal initiation minicore proteins have also been reported although their functions remain unclear (Eng F J, et al., 2009; Walewski J L, et al., 2001).

The development of an cell culture system for HCV (HCVcc) is a major breakthrough in the field (Heller T, 2005; Wakita T, et al., 2005; Zhong J, 2005). As well HCV infection in human, persistent infection can be established in HCVcc *in vitro* (Zhong J, et al., 2006), which facilitates our understanding of the details of the pathophysiology of persistent HCV infection. To study the possible function of the core protein in persistent HCV infection, its expression was detected during the establishment of HCVcc persistent infection and three sizes (~19 kDa, ~21 kDa and ~24 kDa) of core proteins were identified. Moreover, the three core proteins can all be encapsidated into HCV virions. Of the three resulting core proteins, the p21 core protein was the mature form of the HCV core protein. The p19 core protein might be the cleavage product of the p21 core protein, and the p24 core protein was the phophorylated form of p21. The phosphorylation of the core protein may play an important role in the establishment of persistent HCVcc infection.

MATERIALS AND METHODS

Cell and plasmid

All experiments described in this study were performed using Huh-7.5.1 cell line kindly provided by Dr F. V. Chisari (Zhong J, 2005). The plasmid pJFH1 containing the full-length genomic cDNA sequence of the HCV genotype 2a strain JFH1 was a kind gift from Takaji Wakita (Wakita T, et al., 2005).

Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 10% fetal calf serum (FCS, Gibco) (complete DMEM) in 5% CO2 at 37 ºC.

In vitro transcription and electroporation of HCV RNAs

In vitro transcription and electroporation were performed as described previously (Zhong J, 2005). In brief, linearised plasmid DNA was used as template for *in vitro* RNA transcription using a MEGAscript^{TM} T7 kit (Ambion) according to the manufacturer's protocol. The RNA concentration and integrity were determined by measuring the OD_{260} and denaturing agarose gel electrophoresis respectively. Then the RNA was introduced into Huh-7.5.1 cells by electroporation. Trypsinised Huh7.5.1 cells were washed twice with serum-free Opti-MEM (Invitrogen) and then resuspended in serum-free Opti-MEM at 1×10^7 cells/mL. Ten micrograms of RNA were mixed with 0.4 mL of the cells in a 4-mm cuvette (Bio-Rad), and a Gene Pulser II^{TM} apparatus (Bio-Rad) was used to deliver a single pulse at 0.27 kV, 100 ohms, and 960 μF. Transfected cells were immediately transferred to 24 mL complete DMEM and then plated in two T75 Costar flasks. Cells were routinely passaged twice a week at a dilution of 1:6 depending on confluency.

Western blot

Cells harvested in lysis buffer (50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl，1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitors cocktail (Roche) were denatured by boiling for 5 min. Samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were blocked with TBS (20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl) containing 5% skim milk powder for 1 h at room temperature (RT). Then the membranes were incubated with mouse monoclonal antibodies (MAbs) against the core protein (C7-50, Abcam) at a dilution of 1:1000 for 1 h at RT, followed by three washes with TBS-T (TBS containing 0.1% (v/v) Tween-20) buffer. Then the membranes were incubated with HRP-conjugated goat anti-mouse IgG at a dilution of 1:10000 in TBS for 1 h at RT, followed by three more washes with TBS-T. Proteins were then detected using the Supersignal® West Pico chemiluminescent substrate (Pierce) and an AlphaEase® FC Imaging System (Alpha Innotech Corporation).

Indirect immunofluorescence

Cells seeded on glass coverslips or 96-well plates were washed with PBS and fixed with 4% paraformaldehyde for 20 min at RT. After washing three times with PBS, cells were incubated in blocking buffer(PBS containing 3% BSA, 0.3% Triton X100, and 10% FCS) for at least

30 min and then incubated in binding buffer (PBS containing 3% BSA, 0.3% Triton X-100) with MAbs against the core protein at a dilution of 1:500 for 1 h at RT. Following three washes with PBS, cells were incubated with Fitc-conjugated goat anti-mouse IgG (Thermo) at a 1:100 dilution with binding buffer for 1 h at RT. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Stained samples were then examined with a Leica TCS SPII confocal microscope.

Concentration and Purification of HCV

To concentrate and purify HCV virions, sucrose density-gradient ultracentrifugation analysis was performed as described elsewhere (Wakita T, et al., 2005). Pooled supernatant from cells 20-33 days post- transfection were centrifuged at 4,000 rpm for 5 min to remove cellular debris and then pelleted through a 10% sucrose cushion at 28,000 rpm for 4 h using a SW28 rotor in an L8–80M ultracentrifuge (Beckman). The pellet was resuspended in 1 mL of TNE buffer (50 mmol/L Tris-HCl, pH 8-100 mmol/L NaCl, 1 mmol/L EDTA) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis), loaded onto a 10-60% sucrose gradient (2 mL each gradient), and centrifuged at 120,000 rpmor 16 h at 4 °C in a SW41Ti rotor (Beckman). 2 mL fractions were collected from the top of the gradient and were then analysed by Western- blotting to detect the core protein.

RT-PCR and sequencing of core gene

Total RNA was isolated from Huh7.5.1 cells persistently transfected with JFH-1 or from 1 mL clarified cell culture supernatant using TRIZOL (Invitrogen). The core gene fragment was reverse transcribed and amplified using an RNA LA PCR™ Kit (Takara) with sense (5'-gcctgagggcgcttgcgag-3') and antisense (5'-ccacgtag agagcagagcag-3') primers corresponding to 290-310 nt in the 5'UTR and 1153-1172 nt in the E1 region, respectively. The PCR products were then ligated into a T vector (Takara) for sequencing. The sequencing results were analysed by the DNAstar software package.

Core protein ELISA

Release of the HCV core protein was quantified using an HCV antigen enzyme-linked immunosorbent assay (ELISA) system according to the manufacturer's instructions (Jinda, Hunan, China). Briefly, clarified cell culture supernatants were applied to plates coated with a mixture of mouse anti-core monoclonal antibodies (MAb). Antigen was detected by the addition of a second MAb cocktail conjugated to horseradish peroxidase (HRP). After washing and developing the plates, the absorbance was measured at 490 nm.

Dephosphorylation assay

Cells were lysed by freezing and thawing for five times in 2× lambda protein phosphatase buffer (Sigma) containing a protease inhibitor cocktail (Roche). Then the lysed cells were centrifuged at 12 000 rpm for 10 min to remove cell debris. The supernatant were divided into two groups. One group was digested with λpp in 1× lambda protein phosphatase buffer (Sigma) containing protease inhibitor cocktail (Roche) and 2 mmol/L $MnCl_2$ at 37 ºC for 1 hr. The other group was used as a control and incubated in 1× lambda protein phosphatase buffer (Sigma) containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche) at 37 ºC for 1 hr. Dephosphorylation was detected by Western blot as described above.

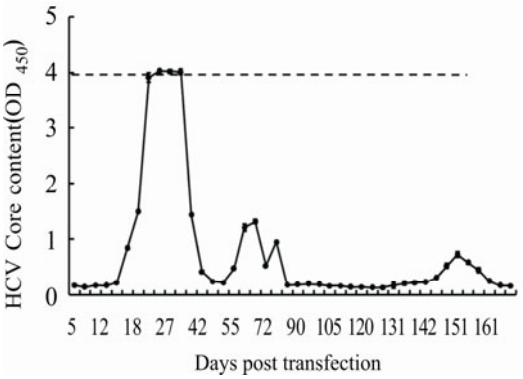
RESULTS

Three different sizes of core protein were identified in the establishment of persistent infection of HCV *in vitro*.

It was previously reported that an *in vitro* persistent HCV infection can be established by transfecting cells with JFH1 genomic RNA followed by sustained culture (Zhong J, et al., 2006). Here, we electroporated JFH1 RNA into Huh7.5.1 cells and cultured the transfected cells for more than 150 days. At the time indicated (normally when the cells were nearly confluent and ready to split), the culture supernatants were collected to quantify the core protein. The result showed that the core protein level in the supernatant was low during the first few days following transfection and then increased rapidly by day 15 p.t., reaching the highest levels (OD450$\geqslant$4 (which is the highest value the machine can detect)) by day 20 p.t.. The core protein in the supernatant was maintained at the maximum measurable levels for 17 days and then decreased quickly by day 37 p.t.. Later on the core protein was present at a low level for 18 days and then increased to a slightly higher level by day 55 p.t.. Finally, the core protein decreased to a basal level by day 84 p.t. and remained at this level until the end of the studied period (Fig. 1A). This result is in accordance with a previous related report (Zhong J, et al., 2006), indicating that we established persistent HCV infection *in vitro*.

To study the possible role of the core protein in persistent HCV infection, the expression of the core protein at different stages of infection was analysed. At the

A



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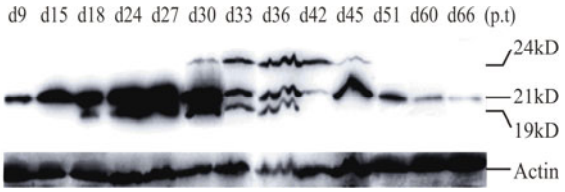

Fig. 1. The identification of three sizes of core proteins during the establishment of HCVcc persistent infection. HCVcc persistent infection was established by transfecting Huh7.5.1 cells with the JFH1 RNA genome followed by sustained culture. At the indicated time points, supernatants (A) and cells (B) were collected and subjected to ELISA and Western-blotting as described in Materials and Methods. The cut-off value of the ELISA is 0.22. The dotted line indicates the maximum detectable OD_{450} of the instrument. The actin band indicates the amount of proteins loaded in each well.

indicated time points, cells were collected and lysed for Western blotting. As shown in Fig. 1B, only one band of the core protein with a size of 21 kDa could be seen in the first few days (before d18) after transfection. In the early stage of CPE (d18 to d27), in addition to the 21-KDa core protein, a new band of 19 kD was detected. During later stages of CPE (d27 to d36), another band of the core protein with a size of approximately 24 kDa appeared and the 19 kDa band disappeared gradually. In the early stage of persistent infection (d37 to d51), the 24 kDa core protein disappeared gradually. In the later stage of persistent infection (after d51), only the 21 kDa core protein remained. Similar results were also obtained after establishing persistent infection by infecting Huh7.5.1 cells with HCV virus stock (data not shown). These results indicate that there were three sizes of core proteins (p19, p21 and p24) that appeared to be present in the HCVcc system when proceeding from acute infection to persistent infection.

All three sizes of core proteins exhibited cytoplasmic localization

It has been reported that the core protein is usually located in the cytoplasm, and the truncated core protein is located in the nucleus (Suzuki R, et al., 1995; Suzuki R, et al., 2005). Because there were three sizes of core proteins during the persistent HCVcc infection, we sought to determine whether the localization of these core proteins differed. To detect the localization of the core protein during persistent HCV infection, cells at the indicated times were collected and applied to IFA. As shown in Fig. 2, although the core protein positive cell numbers differed significantly during different stages of infection, the core protein was primarily located in the cytoplasm over the course of the entire infection. Moreover, the core protein was primarily located surrounding the nucleus, the exception was that the core protein was dispersed through the cytoplasm during the

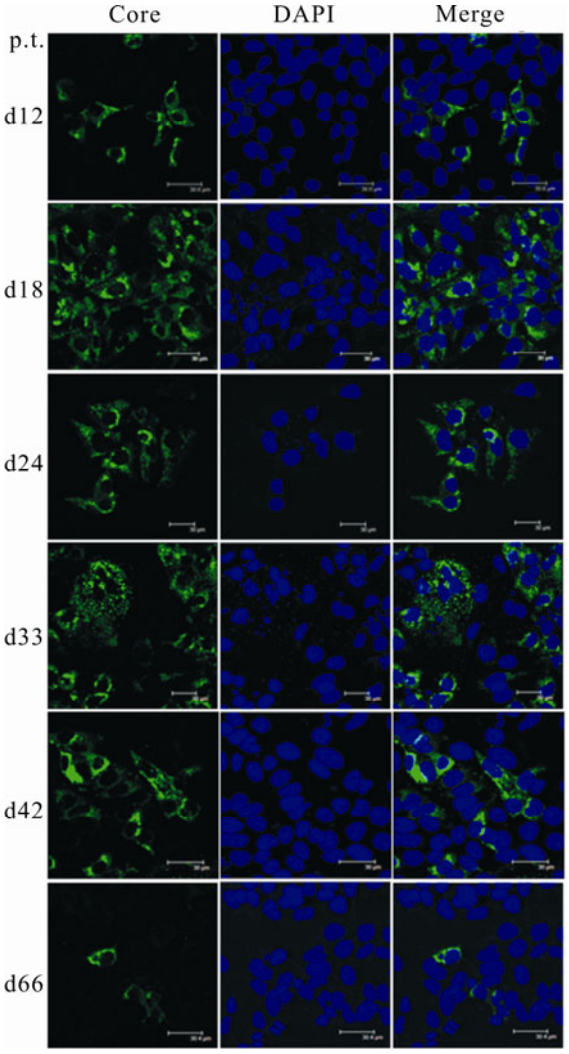

Fig. 2. The localizations of the three core proteins during HCVcc persistent infection. Cells transfected with the JFH1 genomic RNA at the indicated time points were fixed, permeabilised and treated with monoclonal antibodies against the core protein, followed by a secondary antibody (TRITC-conjugated goat anti-mouse IgG). The localization patterns of the core protein are shown in green. Nuclei were stained with DAPI (blue). The merged images are also shown.

later stage of CPE (d33). This exception may be due to the appearance of the p24 core protein. Nuclear fragmentation was also seen when CPE occurred (DAPI staining of the cells at d18 p.t. in Fig. 2), which is in accordance with the microscopy observation that the cells infected with HCV stopped growing and began dying during CPE. This finding suggests that the cell death occurring during the CPE stage may be cell apoptosis. All these results indicate that there was no significant difference in the localization of the three different sized core proteins.

All three core proteins can be encapsidated into virions

Next, the virion assembly capacity of these three core proteins was determined. To study which core protein can be encapsidated into HCV virions, supernatants from cells at d20 to d33 post transfection were collected and centrifuged through a sucrose density gradient. Six fractions were collected from low to high density (from 10% to 60% sucrose) and were used for Western-blotting. As shown in Fig. 3, most HCV virion sedimented in the 20-30% sucrose fraction and contained the three core proteins. Some HCV virion sedimented in the 50% sucrose fraction and contained all three core proteins but the percentage of p19 core protein was much higher than that in the 20-30% sucrose fraction. The HCV virion that sedimented in the 60% sucrose fraction contained only the p21 and p19 core proteins, and the percentage of the p21 core protein was much higher than that in the 20-30% sucrose fraction. This phenomenon may have occurred because there were some p21 core protein encapsidated without lipid membrane, which would have a higher density and would precipitate in the bottom of the centrifugal tube, thus, the percentage of the p21 core protein would be much higher than that in the 20-30% sucrose fraction. These results suggested that all three core proteins could be capsidated into HCV virions, although the percentages of three core proteins were different in different densities.

The p24 core protein is the phosphorylated form of the p21 core protein

It has been reported that the core protein can be phosphorylated by PKA and PKC *in vitro* (Blaney J E, Jr., et al., 2002; Shih C M, et al., 1995). To confirm whether the p24 core protein was the phosphorylated form of the p21 core protein, cells at d36, d42 and d45 p.t. were collected and subjected to a dephosphorylation assay. As shown in Fig. 4, in all three samples, the amount of p24 core protein was significantly decreased after the cell lysates were treated with λPP compared with the untreated samples. At the same time point, the amount of the p21 core protein increased slightly. These results indicate that the p24 core protein was the phosphorylated form of the p21 core protein.

The appearance of the p19 core protein was not caused by the a mutation in the core gene

It has been reported that the occurrence of one truncated core protein (p16) is related to nucleotides 9-11 of the core gene, which normally codes for Lys (Yeh C T, et al., 2000). Moreover, the appearance of the p16 core protein may be related to the pathology of HCV (Yeh C T, et al., 2000). Therefore, we sought to determine if the occurrence of the p19 core protein product was due to mutations in the core gene sequence. Cells and supernatants at different stages of infection were collected, and RNA was extracted from these samples. Then, RT-PCR was performed, and the PCR products were inserted into a T-vector for sequencing. The sequencing results showed that any nucleotide substitutes were primarily those that resulted in no change in the amino acid sequence. Although there were some nucleotide substitutions that resulted in one amino acid change, there was no

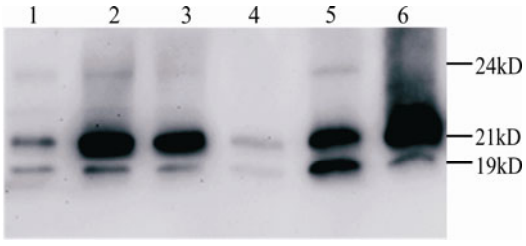

Fig. 3. All three forms of core protein can be encapsidated into HCV virions. Supernatant from cells 20-33 days post-transfection were applied to sucrose density-gradient ultracentrifugation. Six fractions (lane 1 to lane 6) corresponding to 10-60% sucrose in 10% intervals were collected from top of the sucrose gradients. The core proteins in the fractions were analysed by Western-blotting.

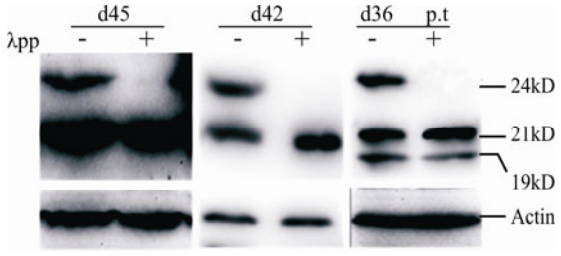

Fig. 4. The p24 core protein is the phosphorylated form of the p21 core protein. Cells transfected with the JFH1 RNA genome and maintained until the indicated time points were lysed and subjected to dephosphorylation assay described in the Materials and Methods. The actin band indicates the amount of protein loaded in each well. λpp, lambda protein phosphatase.

consensus change between samples. These results indicate that the appearance of the p19 core protein was not caused by a mutation in the core gene.

DISCUSSION

Similar to natural HCV infection, HCVcc also initially causes acute infection and then proceeds to persistent infection (Zhong J, et al., 2006). HCVcc infection can partially mimic HCV natural infection in humans and is a useful model to study the detailed interaction between virus and host cells as well as the HCV life cycle, especially the events that occur in the chronic and persistent stages.

Here we found that there were three sizes of core proteins (p24, p21 and p19) produced during the establishment of persistent HCVcc infection. Among them, the p21 core protein is the mature form of the HCV core protein and the p24 core protein is the phophorylated form of the p21 core protein. The expression of the p19 core protein is not caused by the core gene sequence mutation and appears to be a functional by-product during the course of infection. These three core proteins were all localized in the cytoplasm, although the exact cytoplasm localization patterns were slightly different. Moreover, these core proteins can all be encapsidated into HCV virions, although the percentage of each core protein in different fractions after sucrose gradient centrifugation was different.

The HCV NS5A protein has been reported to be cleaved by caspase-like protease(s) and this cleavage can be enhanced by the presence of apoptotic stimuli and inhibited by the caspase inhibitors (Sauter D, et al., 2009). Both N-terminal and C-terminal truncated forms of NS5A have been observed in previous reports. The cleavage of NS5A can impair HCV RNA replication (Sauter D, et al., 2009). Here we found that the p19 core protein occurred at the CPE stage, when cells began undergoing apoptosis (implicated by DAPI staining, see Fig. 2). It is well known that there are many proteases that are activated when apoptosis occurs. Because the production of the p19 core protein was not caused by a core gene mutation, and it appeared when apoptosis began, the presence of the p19 core protein might be caused by the cleavage of the p21 core protein. This cleaved core protein may serve as a defensive protein against HCV infection through regulating the RNA replication or virion production of HCV.

Phosphorylation is one of the major mechanisms by which the activities of protein factors can be regulated. The phosphorylation of the core protein has been observed both in insect cells and in human liver-derived cell lines by PKA and PKC, although the efficiency is much lower in human cell lines (Shih C M, et al., 1995). Moreover, the phosphorylation of the core protein can modulate the trans-suppression activity of the core protein (Shih C M, et al., 1995). Recently it was reported that the phosphorylation of the NS5A protein can regulate production of the HCV virions, suggesting that the phosphorylation is indeed a strategy for the virus to modulate its life cycle (Garcia-Sastre A, et al., 2008). Here we found that the phosphorylation of the core protein occurred during the establishment of persistent HCVcc infection and the efficiency increased during the first few days. The phosphorylation disappeared when the persistent infection became established, implying that the phosphorylation of the core protein may be related to the establishment of persistent HCVcc infection.

Virus infections always induce a host immune response and result in a battle between host and virus. If the host "beats" the virus, the virus will be eradicated. If the virus "beats" the host, the virus will replicate quickly and finally lyse the host cells to release large numbers of virions. If there a balance is established between the virus and the host, then persistent infection occurs. The establishment of a persistent infection is a consequence of the virus developing various strategies to protect itself from the host, and vice versa. Here, the appearance of a cleaved p19 core protein and a phosphorylated p24 core protein may be reflection of the competition between HCV and host cells. Further studies are required to determine which protease(s) and kinase(s) participate in processing the core protein and what the functions of these processed core proteins are. Determining the functions of each form of the HCV core protein will contribute to a better understanding of the virus life cycle as well as its pathogenesis.

Acknowledgements

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