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Hepatitis C virus infection of mouse hepatoma cell expressing human CD81 or LDLR

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Summary. – Hepatitis C virus (HCV) infection represents a serious public health problem worldwide. Development of new therapeutics against HCV has been hampered by the lack of a small-animal model. Until now, it has been unclear which host factors influence the HCV infection. It was known that human CD81 (hCD81) or low-density lipoprotein receptor (hLDLR), the putative HCV receptors, induce concentration of viral particles on the cell surface. In this study, recombinant plasmids containing hCD81 or hLDLR genes were transfected to mouse hepatoma Hepa 1-6 cells and transgenic cell lines expressing hCD81 (hCD81/1-6 cell line) or hLDLR (hLDLR/1-6 cell line) on their surface have been established. HCV infection of these cell lines showed that the virus was bound, entered the cell, and replicated inside the cell. This finding is essential for the development of mouse model for the study of HCV replication *in vivo.*

Keywords: Hepatitis C virus; hepatoma cells; transgenic cells; CD81; LDLR

Introduction

HCV (the genus *Hepacivirus,* the family *Flaviviridae*) is an enveloped positive-strand RNA virus that chronically infects 170 million people worldwide (Safioleas and Manti, 2007). Except humans, a chimpanzee is the only animal susceptible to the HCV infection (Kolykhalov *et al*., 1997). Chimpanzee is an excellent animal model for the development of effective drugs and vaccines against HCV. However, the ethical issues, limited availability, and very high costs severely limit the use of chimpanzees as the laboratory animals. Therefore, development of a small-animal model for HCV research is essential. The mouse is widely used in the medical research because of its availability, low costs, and peak effectiveness. However, HCV is not able to infect mouse liver cells (McCaffrey *et al.*, 2002). Much effort has been taken to develop a suitable mouse model including engraftment human liver fragment or hepatoma cells infected with HCV *in vitro* to the severe combined immunodeficiency mouse or athymic nude mouse. Until now, some success has been achieved, because HCV could be detected in serum of infected mice, and HCV replication took place in the human liver cells or human hepatoma cells (Ilan *et al.*, 2002; Mercer *et al.*, 2001). However, limited liver donors and low rate of engraftment survival make this mouse model incapable to meet the requirement for an effective experimental animal. Therefore, a new way of development of the effective mouse models still needs to be explored.

Recently, it was demonstrated that replicon derived from the HCV genotype 2a consensus sequence was able to replicate in mouse hepatoma cells, albeit infectious particles produced in Huh-7 cells were not able to establish detectable HCV RNA replication in naive mouse cells. This suggested that HCV entry may be an obstacle in HCV infection of mouse cells (Uprichard *et al*., 2006). Apparently, a lack of HCV receptors on the mouse liver cells was a reason for their resistance to HCV infection (Masciopinto *et al*., 2002).

In this study, we attempted to establish transgenic mouse hepatoma cell lines expressing hCD81 or hLDLR, the putative receptors for HCV in humans (Monazahian *et al*., 1999; Pileri *et al*., 1998). By infecting these cell lines with HCV

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Abbreviations: hCD81 = human CD81; HCV = Hepatitis C virus; hLDLR = human low-density lipoprotein receptor; p.i. = post infection

and studying the virus binding to the cell surface and its replication in the cell, we tried to demonstrate that hCD81 and/or hLDLR actually enabled the HCV binding, entry, and replication in the mouse cells.

Materials and Methods

Cells. Mouse hepatoma Hepa 1-6 cells (a generous gift from Shoujun Yuan) were cultured in RPMI-1640 containing 10% fetal bovine serum.

HCV-positive human serum. HCV-positive sera were obtained from Beijing 302 hospital. The pooled serum samples contained 10^6 copies/ml of HCV RNA plus-strand as determined by Light Cycler TM (Roche).

Plasmid constructs. hCD81 and hLDLR genes from human hepatoma HepG2 cells (serum-free cultured) were amplified by RT-PCR and inserted into pcDNA3 vector under the control of mouse albumin enhancer and promoter to produce plasmid constructs pcDNA3-AlbEP-hCDE81 and pcDNA3-AlbEP-hLDLR, respectively (Fig. 1a) (Hu *et al*., 1992; Herbst *et al*., 1989, 1990; Izban and Papaconstantinou, 1989; Gorski *et al*., 1986).

Establishment of transgenic cell lines. Mouse hepatoma Hepa 1-6 cells were transfected with the plasmid constructs using Lipofectamine 2000 (Invitrogen). After 2 weeks of selection with 0.75 mg/ml G418, the hCD81 gene-containing Hepa 1-6 cells (hCD81/1-6 cells) and the hLDLR gene-containing Hepa 1-6 cells (hLDLR/1-6 cells) were further cultured in a medium containing 0.25 mg/ml G418. Mouse hepatoma Hepa 1-6 cells transfected with the empty plasmid pcDNA3 (pcDNA3/1-6) were used as a negative control.

RT-PCRs for hCD81 and hLDLR mRNAs. Total RNA was isolated from transgenic cells with Trizol Reagent (Invitrogen), digested with DNase I (Promega), and extracted with phenol: chloroform. The cDNA was synthesized from total RNA with SuperScript II reverse transcriptase, RNase inhibitor, and random oligo$(dT)_{15}$ primer (all Invitrogen). The PCR was performed using DNA polymerase (Promega), reverse primer specific to the SV40 poly(A) sequence (5'-CGAAGAACTCCAGCATGAGA-3'), forward primers for hLDLR (5'-CCGGTCGACAGAGGCTGCGAG CATGG-3') or hCD81 (5'-CCCAAGCTTGCCGCCATGGGAGT GGAG-3'). The size of the amplicons was 1.4 kb (hCD81) and 0.86 kb (hLDLR).

Flow cytometry. 10^5 hCD81/1-6 cells were collected and treated with a mouse monoclonal antibody JS-81 to hCD81 (BD Pharmingen) for 1 hr at 37°C, then stained with FITC-labeled goat anti-mouse IgG in darkness for 30 mins at 4°C. The stained cells were assayed in flow cytometry. The pcDNA3/1-6 transgenic cells were used as a negative control.

Strand-specific nested RT-PCRs for HCV RNA. Total RNA was isolated from HCV-positive serum with Trizol LS Reagent, dissolved in 15 µl of DEPC-water, and stored at -20°C. Strand-specific RT step (10 µl) was performed at 42°C for 60 mins with heat-denatured total RNA (5 µl), 25 pmoles of either the 5NTRR324-304 primer for the plus-strand or the 5NTRTF primer for the minus-strand, and Superscipt II reverse transcriptase (25 U). The reaction was stopped at 70°C for 15 mins. The 1st PCR mixture (10 µl) consisted of RT products (1 µl), outer primer (10 pmoles), and Taq DNA polymerase (0.25 U). The 2nd PCR mixture (20 µl) contained the first PCR products (2 µl), inner primer (20 pmoles), and Taq DNA polymerase (0.5 U). The cycling conditions for both PCRs were identical: denaturation at 94°C/2 mins, 35 cycles of 94°C/30 secs, 55°C/30 secs, and 72°C/40 secs, and final elongation at 72°C/10 mins. The products of the 2nd PCR were analyzed in 2% agarose gel containing ethidium bromide. All used primers are shown in Table 1 (Rumin *et al*., 1999; Shindo *et al*., 1994).

HCV-binding of cells. Hepa 1-6, transgenic pcDNA3/1-6, hLDLR/1-6 and hCD81/1-6 cells were incubated with 100 µl of serum containing 10^5 copies/ml HCV RNA plus-strand for 3 hrs at 37°C. Then, the cells were washed 3 times with medium, trypsinized, and total RNA was isolated using Trizol method. Total RNA was analyzed in strand-specific RT-PCR assay. PCR-amplified product was analyzed by agarose gel electrophoresis.

HCV infection of cells. Hepa 1-6, pcDNA3/1-6 cells and transgenic Hepa 1-6 cells stably expressing hCD81 or hLDLR were infected with HCV-positive serum at 37°C for 24 hrs. Then, the cells were washed 3 times and cultured in standard media with FCS.The infected cells and their supernatants were harvested separately each day until day 7 post infection (p. i.). Collected cells were sub-cultured on day 3 and 5 p.i. Total RNA was isolated using Trizol method and dissolved in 15 µl of DEPC-water and stored at -20°C until used. Each of 5 µl RNA were used for strand-specific

Table 1. Primers used in strand-specific nested RT-PCRs for HCV and PCR for β-actin

Primer	Sequence(5'-3')	Strand specificity
5NTRF (nt 1–21)	GGCGACACTCCACCATAGATC	forward outer primer of +RNA
5NTRF (nt 28–48)	CTGTGAGGAACTACTGTCTTC	forward inner primer of +RNA
5NTRR (nt 324–304)	GGTGCACGGTCTACGAGACCT	RT primer of +RNA reverse outer primer of +RNA and -RNA
5NTRR (nt 284–264)	CCCTATCAGGCAGTACCACAA	reverse inner primer of +RNA and -RNA
5NTRTF (T+nt 40–55)	TGTCATGGTGGCGAATAAGCC ATGGCGTTAGTA T	RT primer of-RNA,Tag sequence and HCV sequence
5NTRT	TGTCATGGTGGCGAATAA	forward inner and out primer of +RNA and -RNA, unrelated HCV sequences, just a tag
ACTINF858-880	CTCTTTTCCAGCCTTCCTTCTTG	forward primer of mouse β-actin
ACTINR1215-1238	GGGTGTAAA ACGCAGCTCAGTAAC	reverse primer of mouse β-actin

Table 2. Detection of plus and minus strands of HCV RNA in HCV-infected transgenic cells

Cells	Time p.i.									
	3 hrs		day 1		day 3		day 5		day 7	
	+RNA	-RNA	+RNA	-RNA	+RNA	-RNA	+RNA	-RNA	+RNA	-RNA
Hepa 1-6	+	–	+	–	–	–	–	–	–	–
pcDNA/1-6	+	–	+	–	–	–	–	–	–	–
hCD81/1-6	+	–	+	–	+	+	+	+	–	–
hLDLR/1-6	+	–	+	–	+	–	+	+	–	–

(+) = present; (–) = absent.

RT and mouse β-actin were reverse transcribed using oligo$(dT)_{15}$ primer. At the time of harvest, medium was removed and the cells were washed 3 times with PBS. Total RNA was purified from 10^4 hepatoma cells using Trizol reagent. The precipitated RNA was dissolved in 50 µl diethyl DEPC-treated water and quantified. RNA was analyzed in strand-specific RT-PCR assay. PCR-amplified product was analyzed by agarose gel electrophoresis.

Immunofluorescence assay. Infected cells were grown on coverslips, washed twice with PBS, fixed with cold acetone for 15 mins, and permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 30 mins. The first incubation was performed with rabbit polyclonal antibody against HCV core antigen diluted 1:50 (Zhongshan). After washing the FITC-labeled antibody against mouse immunoglobulins diluted 1:100 was added (Zhongshan). After washing and mounting, the slides were observed under a fluorescent microscope AX80TF (Olympus).

Results

Establishment of transgenic cell lines

The cloned hCD81 and hLDLR genes were confirmed by DNA sequencing. The hCD81 and hLDLR sequences perfectly aligned with hCD81 and hLDLR sequences in the GenBank (Acc. Nos. gi|21237760|ref|NM004356.2| and gi|21629647|gb|AY114155.1|, respectively). The genes hCD81 and hLDLR under control of mouse liver-specific albumin enhancer and promoter were successfully inserted at Aat II and Sac I sites of pcDNA3 vector. Next, we showed that hCD81 or hLDLR genes transcribed their mRNA in the transfected Hepa 1-6 cells (Fig. 1b). Also, hCD81 molecules were expressed on the surface of transgenic hCD81/1-6 cells (Fig. 1c). The hLDLR gene expression was indirectly proved by fusing in-frame with the GFP gene in vector pEGFP-C1. Upon transfection into Hepa 1-6 cells, hLDLR were located mainly on cell membrane and cytoplasm as shown by a laser confocal microscopy.

HCV binding and replication in transgenic cell lines

As little as 10 copies of HCV RNA plus-strand can be detected in serum by using the strand-specific nested RT-PCR (Fig. 2a). HCV particles binding assay performed prior to the HCV infection showed that on the surface of the mouse hepatoma Hepa 1-6 cells were molecules able to bind HCV particles (Table 2). The following results of HCV infection showed the presence of HCV RNA plus-strand in hepatoma Hepa 1-6 cells until 2 days p.i. (data not shown). This result confirmed that HCV binding molecules existed also on the surfaces of mouse hepatoma Hepa 1-6 cells. However, HCV did not enter the cell because we were not able to detect HCV RNA in mouse hepatoma Hepa 1-6 cells 2 days p.i. (Table 2). In general, it was possible that HCV could bind to the mouse liver cells also *in vivo*.

HCV RNA plus-strand detection in transfected hCD81/1-6 and hLDLR/1-6 cells was attributed to the presence of HCV specific receptors.

HCV minus-RNA strands were amplified in hCD81/1-6 cells on day 3 p.i. and in both cell lines hCD81/1-6 and hLDLR/1-6 on day 5 p.i. (Fig. 2b). Since the appearance of the minus-strand intermediator was the indication of HCV genome replication, we concluded that hCD81 or hLDLR expressed on the surfaces of transgenic hepatoma Hepa 1-6 cells were able to bind HCV particles and the binding led to endocytosis and subsequent replication of the HCV. Moreover, the expression of HCV core protein was positive in transgenic cells hCD81/1-6 and hLDLR/1-6, while negative in the control pcDNA3/1-6 cells (Fig. 2c). These results suggested that the attachment of HCV to control pcDNA3/1-6 cells was not specific, while hCD81 and hLDLR transfection made the HCV attachment specific. Thus, the hCD81/1-6 or hLDLR/1-6 cells were permissive to the infection with serum-derived HCV particles.

Discussion

Valuable studies by Uprichard *et al.* (2006) showed that HCV replicon clones can efficiently replicate in mouse cells. In mouse hepatoma cells, there might be a block for endocytosis of HCV particles. We decided to study the ability of transgenic mouse cells that expressed hLDLR or hCD81 to bind and replicate HCV particles.

In our study, we found that there were non-specific receptors on the surface of mouse hepatoma Hepa 1-6 cells able to bind HCV, but the virus did not enter the mouse hepatoma

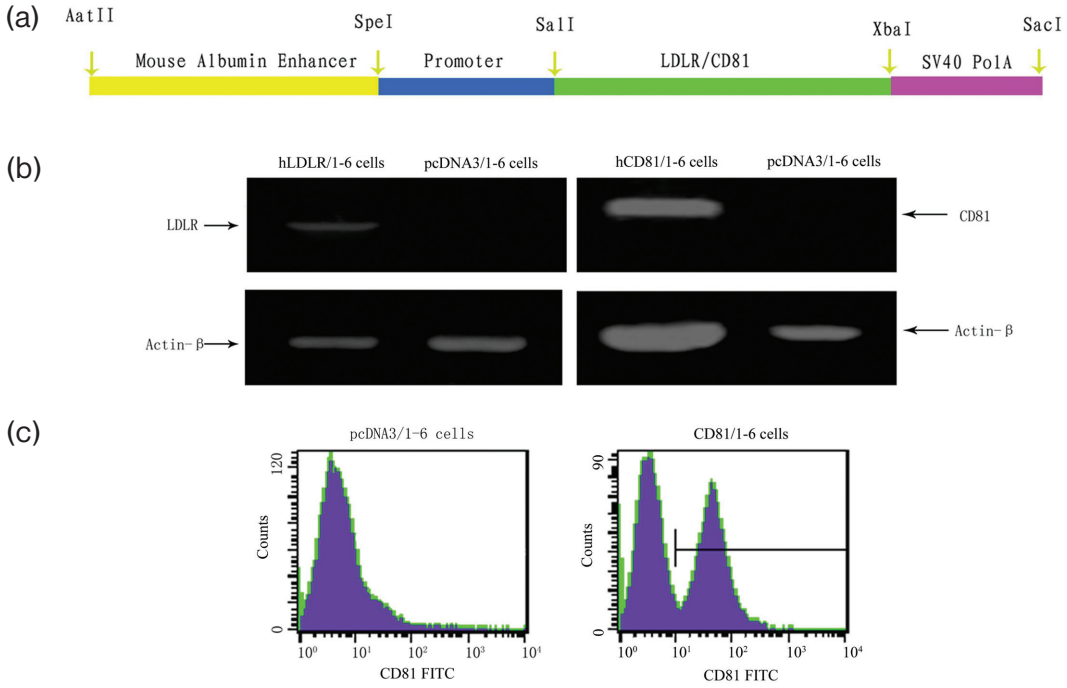

Fig. 1

Expression of hCD81 and hLDLR genes in transgenic cells

(a) Partial structure of recombinant plasmids pcDNA3-AlbEP-hCDE81 and pcDNA3-AlbEP-hLDLR. (b) Expression of hCD81 and hLDLR genes in transgenic hCD81/1-6 and hLDLR/1-6 cells, respectively, detected by RT-PCR including negative control (pcDNA3/1-6). (c) Flow cytometry of hCD81/1-6 cells and pcDNA3/1-6 cells.

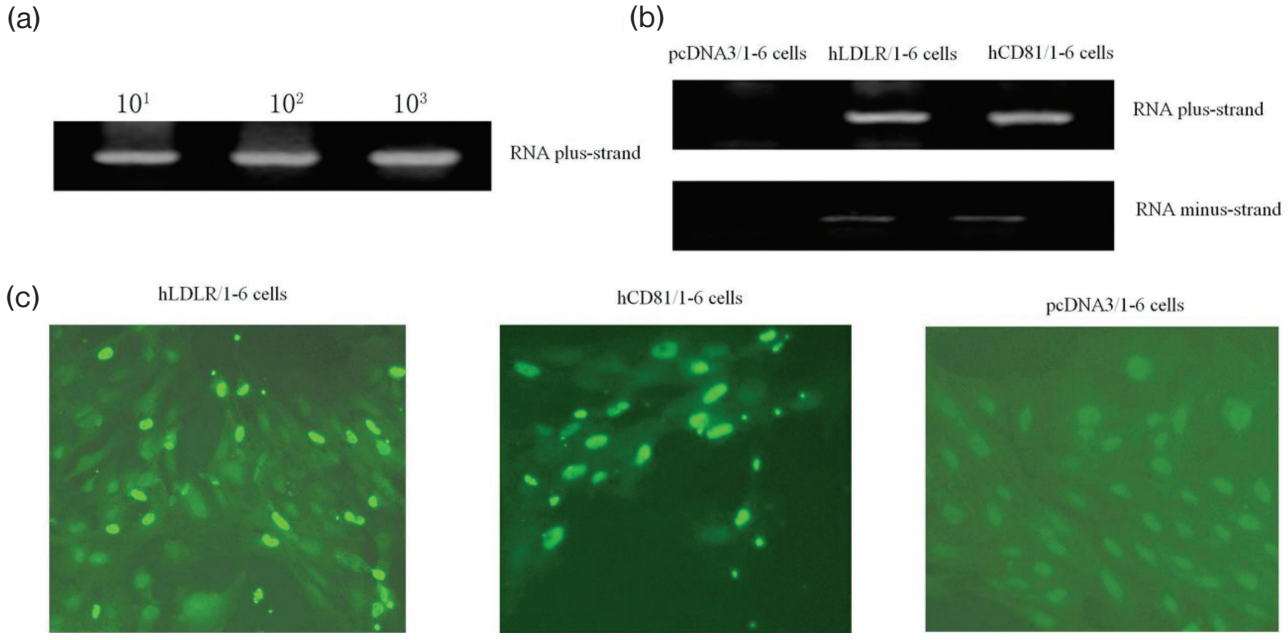

Fig. 2

HCV infection of transgenic cells

(a) Sensitivity of the nested RT-PCR for plus-strand of HCV RNA. The numbers of plus-strand copies/ml are indicated on the top. (b) Expression of plus- and minus-strands of HCV RNA in transgenic cells infected with HCV as detected by strands-specific nested RT-PCR including negative control (pcDNA3/1-6 cells). (c) immunofluorescence assay of HCV core antigen in HCV-infected transgenic cells including negative control (pcDNA3/1-6 cells).

cells after binding. The presence of hCD81 or hLDLR on the surface of mouse hepatoma Hepa 1-6 cells resulted in the endocytosis of HCV (Agnello *et al*., 1999). After the entry into cells, HCV RNA plus-strand began to translate viral proteins including RNA-dependent RNA polymerase. Then, minus-strand RNA was transcribed by RNA-dependent RNA polymerase from the template of RNA plus-strand.

However, we do not know yet whether hCD81 and hLDLR molecules can bind HCV directly. Another question is, whether transgenic cells are able to express the desired receptors on their surface during several passages in the absence of selection pressure. Nevertheless, the transgenic cells can be used for detailed study of the interaction of HCV and mouse hepatoma cells.

In conclusion, we proved that HCV could infect and replicate in transgenic mouse hepatoma cell expressing hCD81 or hLDLR. Currently, we have the possibility to obtain transgenic mouse that can be infected with HCV *in vivo,* what may represent a starting point for the establishment of the mouse model for investigation the HCV replication.

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