Biochemical and Biophysical Research Communications 372 (2008) 157–161

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hepatitis C virus infection in mouse hepatoma cells co-expressing human CD81 and Sip-L

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ARTICLE INFO

Article history:
Received 25 April 2008
Available online 12 May 2008

Keywords:
Permissiveness
Interferon
Stibogluconate
Animal model
Nude mice
Epstein-Barr virus nuclear antigen-1
Hepatitis C virus
Membrane-type 1 matrix metalloproteinase
Submergence induced protein-like
Replication

ABSTRACT

Although human CD81 has been shown to be essential for hepatitis C virus (HCV) infection, non-hepatic cells or transgenic animals expressing human CD81 alone did not support HCV replication. Co-expression of other cofactors was thus necessary for HCV replication. Previously, a hepatic factor named Sip-L was found to support HCV replication in an otherwise non-permissive cell line. To understand the species specificity of hepatic factors required for HCV replication, mouse hepatoma cells co-expressing human CD81 and Sip-L (Hepa1-6-CD81-Sip-L cells) were subjected for HCV infection assay. It was discovered that Hepa1-6-CD81-Sip-L cells were permissive for HCV infection and replication. An animal model was thus established by subcutaneous injection of the permissive cells into nude mice to generate tumors. Viral passages could be achieved in these animals. The antiviral effects of interferon and sodium stibogluconate administrated as a single agent or in combination were demonstrated in this animal model.

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Hepatitis C virus (HCV) is a blood-borne virus, the major etiologic agent of non-A, non-B hepatitis worldwide [1–5]. HCV infection results in chronic hepatitis, whereby severe sequels, such as cirrhosis, hepatic failure and hepatocellular carcinoma, may occur [4,5]. HCV is a positive, single-stranded RNA virus with a genome of 9.6-kb in length [6]. With the help of an internal ribosome entry site, a polyprotein of about 3000 amino acid residues is translated. The long open reading frame is followed by a poly U homopolymer plus a highly conserved sequence-element over the 3′-terminus, which is required for HCV replication [7]. Investigation of HCV replication cycle was impeded by the lack of efficient cell culture systems as well as small animal models [8,9]. However, significant advance has been made using heterologous systems, including functional cDNA clones, the replicon system, functional HCV pseudoparticles, and recently, the production of JFH-1 based recombinant infectious HCV particles [6,10–13].

To enter the cell, HCV E2 binds with high affinity to CD81, a tetraspanin located on the surface of hepatocytes [14]. However, CD81 alone is not sufficient to mediate cell entry and other cofactors appear to be required [15–17]. By use of a molecular screening strategy, we have previously identified a hepatic factor, named submergence induced protein-like factor (Sip-L), capable of supporting HCV infection and replication in an otherwise non-permissive cell line [18]. Sip-L is a member of the Cupin superfamily, wherein a diversity of function has been assigned to the member [19]. Translation from an upstream in-frame initiation codon results in an amino-terminus extended form of Sip-L, which has recently been found to be a binding protein of the cytoplasmic tail of membrane-type 1 matrix metalloproteinase (MT1-MMP) [20]. The MT1-MMP has been shown to participate in many membranous activities including endocytosis. It is therefore possible that Sip-L assists HCV replication by facilitating cell entry of the virus. In this study, we showed that co-expression of human CD81 and Sip-L in mouse hepatoma cells rendered the cells permissive for HCV infection and replication.

Materials and methods

DNA plasmid construction. The Epstein-Barr virus nuclear antigen-1 (EBNA-1) assisted extrachromosomal replication system was established as described previously and the plasmid, pDR2-Sip-L, which was identical to the cDNA clone 61.31 obtained from our previous screening study, was used to express Sip-L in culture

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doi:10.1016/j.bbrc.2008.05.018

cells [18]. To obtain pIRES-CD81, the coding region of human CD81 flanked by primers 5′-GCGCCGCCATGGGAGTGGAG-3′ (nt 231–250, sense) and 5′-CTCAGTACACGGAGCTGTTC-3′ (nt 950–931, antisense) was amplified by PCR, blunt ended, and inserted into pIRESbleo (Clontech). Nucleotide sequences of all constructions were verified using an automatic DNA sequencer (CEQ 2000; Beckman Instruments, Inc., Fullerton, Calif.).

Cell lines, transfection, and establishment of stable transformants. The mouse hepatoma cells, Hepa1-6, were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Transformants were selected by addition of 250 μg of G418 per ml or 150 μg of hygromycin B per ml or 300 μg of Zeocin per ml into the culture medium when needed. To obtain Hepa1-6EBNA cells, pCMVEBNA, encoding EBNA-1 (Clontech), and pSV2neo were cotransfected into Hepa1-6 cells and selected by G418 to obtain stable transformants expressing EBNA-1. Subsequently, the cells were transfected with pDR2-Sip-L and selected by hygromycin to obtain Hepa1-6-Sip-L cells. Hepa1-6-CD81 cells were established by transfection of pIRES-CD81 into Hepa1-6EBNA cells and selected by Zeocin. Hepa1-6-CD81-Sip-L cells were established by transfection of pIRES-CD81 into Hepa1-6-Sip-L cells and selected by Zeocin.

HCV infection assay and reverse transcription (RT)-PCR. The HCV infection assay was performed according to the procedure described previously [18,21]. As a control, β-actin mRNA were detected simultaneously. A fragment of the RT-PCR product of HCV-RNA was inserted into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). This plasmid (pTOPO-HCV) served as a positive hybridization control. The primers and probes used for these procedures were described previously [18,22]. The procedure for minus-strand-specific RT-PCR for detection of minus strand HCV-RNA was described elsewhere [23]. When needed, intracellular HCV-RNA was quantitatively measured using a published in house assay [24].

Immunofluorescence analysis. Cells were grown on cover slips. On the seventh days of HCV infection, cells were fixed and subjected for immunofluorescence analysis. To detect HCV core protein, the primary antibody used was monoclonal mouse anti-HCV core antibody C7-50 (Affinity BioReagents, Golden, CO). To detect human CD81, the primary antibody used was monoclonal mouse anti-human CD81 antibody (Leinco Technologies, Inc., Saint Louis, MO). The secondary antibody used was fluorescein isothiocyanate-conjugated AffiniPure goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). To visualize the nuclei, cells were simultaneously stained with DAPI (200 ng/mL). Immunofluorescence microscopy was performed using a Nikon Eclipse E600 inverted microscope.

Animal models. Male athymic BALB/c nude mice were obtained from National Animal Experimental Center, Taiwan (Taipei, Taiwan). The mice were maintained in specific pathogen-free conditions and used when 4 weeks old. Cells were harvested by trypsinization, 10^6 cells with viability >95% were injected subcutaneously into the backs of nude mice. Formation of the tumors was monitored daily until day 20. Tumor volume (V) was calculated as $V = (1/2)ab^2$, where a is the longest diameter and b is the shortest diameter of the tumor. After 20 weeks, mice were sacrificed and tumors were resected for study. The tumors were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles, IN) and stored at −70 °C for immunohistochemistry.

Results

Hepa1-6 cells co-expressing CD81 and Sip-L supported HCV replication

To understand whether mouse hepatoma cells (Hepa1-6) expressing human CD81 and Sip-L supported HCV replication, three cell lines were established: Hepa1-6-CD81, Hepa1-6-Sip-L, and Hepa1-6-CD81-Sip-L. The expression of CD81 was confirmed by Western blot as well as immunofluorescence analysis (Fig. 1A and B). The expression of Sip-L was verified by Northern blot analysis (Fig. 1C). These three cell lines were then submitted for HCV infection assay. The results indicated that only Hepa1-6-CD81-Sip-L cells were permissive for HCV infection (Fig. 2A). The viral RNA could also be detected in the culture medium (Fig. 2A). Minus-strand HCV-RNA was also detected in infected Hepa1-6-CD81-Sip-L cells (Fig. 2B). Furthermore, HCV core protein could be detected by Immunofluorescence analysis (Fig. 2C). The positive cells were usually distributed in clusters, resembling foci formation.

Suppression of HCV replication by antiviral agents

After inoculation of the Hepa1-6-CD81-Sip-L cells, intracellular HCV-RNA was assayed every 2 days. It was found that HCV-RNA was detectable on day 3–9 after infection and a peak level was reached on day 7 (Fig. 3A). For the convenience of our experiments, cells were harvested on day 5 in the subsequent antiviral assays. Three antiviral agents were tested for their anti-HCV effect. One hour after inoculation, various concentrations of interferon-alpha, cyclosporine-A, and sodium stibogluconate were added into the culture medium. Intracellular HCV-RNA concentration was measured on day 5 by use of a quantitative assay (Fig. 3B) [24].

Establishment of an animal model for antiviral assays

To establish an animal model for evaluation of antiviral agents, Hepa1-6-CD81-Sip-L cells (5×10^5) were infected with 10^4 copies of HCV using HCV-positive serum. One day after infection, cells were trypsinized, washed and injected (10^6 cells) subcutaneously into nude mice. Mouse blood (200 μL) was collected via tail bleeds every 2 days. The mice carrying the tumors were allowed to grow for 20 days. The tumors varied from 1.1 to 2.2 cm^3 in size. HCV-RNA was detected in the mouse plasma (50 μL) on days 2–6 and days 16–18 (weakly positive) after injection (Fig. 3C). A peak level was reached on day 6. The mice had to be sacrificed on day 20 because of the occurrence of tumor necrosis (Fig. 3D). The tumor was resected and subjected to frozen section and immunofluorescence analysis. A few cells positive for HCV core protein were still present (Fig. 3E), indicating that the weakly positive signals of HCV-RNA on day 16 and 18 were not a result of contamination.

Viral passage between the nude mice carrying Hepa1-6-CD81-Sip-L cells

To understand whether HCV could be passed between nude mice carrying Hepa1-6-CD81-Sip-L cells, uninfected cells (10^6) were injected subcutaneously into one side of the mouse back, while 50 μL of HCV-positive mouse plasma were injected subcutaneously into the other side of the mouse back simultaneously. Mouse blood (200 μL) was collected via tail bleeds on the seventh day after injection. The tumors were 0.24–0.45 cm^3 in size. The mouse plasma was then used to inject another mouse carrying uninfected Hepa1-6-CD81-Sip-L cells (Fig. 4A). The passage was performed for two consecutive times. HCV-RNA was detectable in the plasma samples derived from the mice receiving consecutive viral passages (Fig. 4B).

Antiviral assays conducted in the mice model

To perform antiviral assays in the mice model, HCV-infected Hepa1-6-CD81-Sip-L cells were trypsinized, washed and injected subcutaneously into nude mice as described in the previous sections. Mouse blood was collected on days 1, 4, 6, 9, and 12. Mice

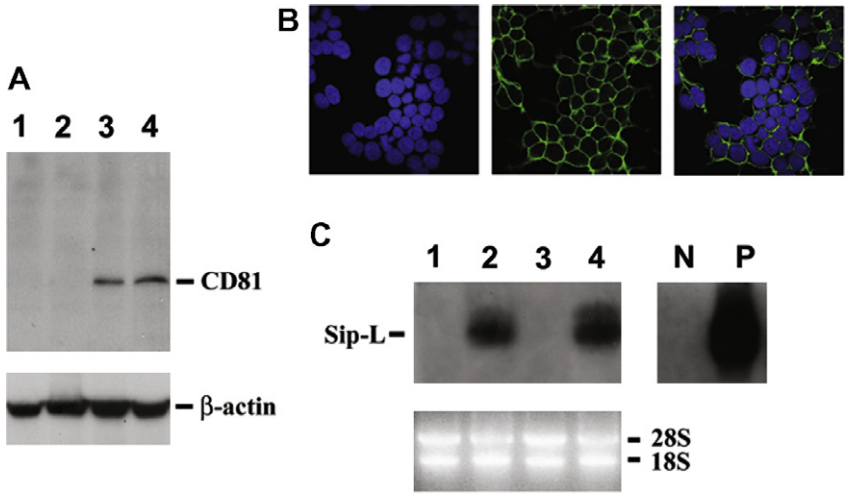

Fig. 1. Expression of CD81 and *Sip-L* in mouse hepatoma cells (Hepa1-6). (A) Expression of CD81 was examined by Western blot. Four cell lines were assayed, respectively: the naïve Hepa1-6 (lane 1), Hepa1-6-Sip-L (lane 2), Hepa1-6-CD81 (lane 3), and Hepa1-6-CD81-Sip-L (lane 4). The amounts of β-actin (bottom) were used as loading controls. (B) Immunofluorescence analysis was performed for Hepa1-6-CD81-Sip-L cells. Nuclei were stained using DAPI (left panel); CD81 was visualized by FITC-conjugated secondary antibody (middle panel); and the two pictures were merged for subcellular localization (right panel). (C) Expression of *Sip-L* was examined by Northern blot. Four cell lines, the same as those in (A), were used (lanes 1-4). rRNA (28 and 18S, bottom) as loading controls; N, 20 pg of pDR2 as negative hybridization control; P, 20 pg of pDR2-Sip-L as positive hybridization control.

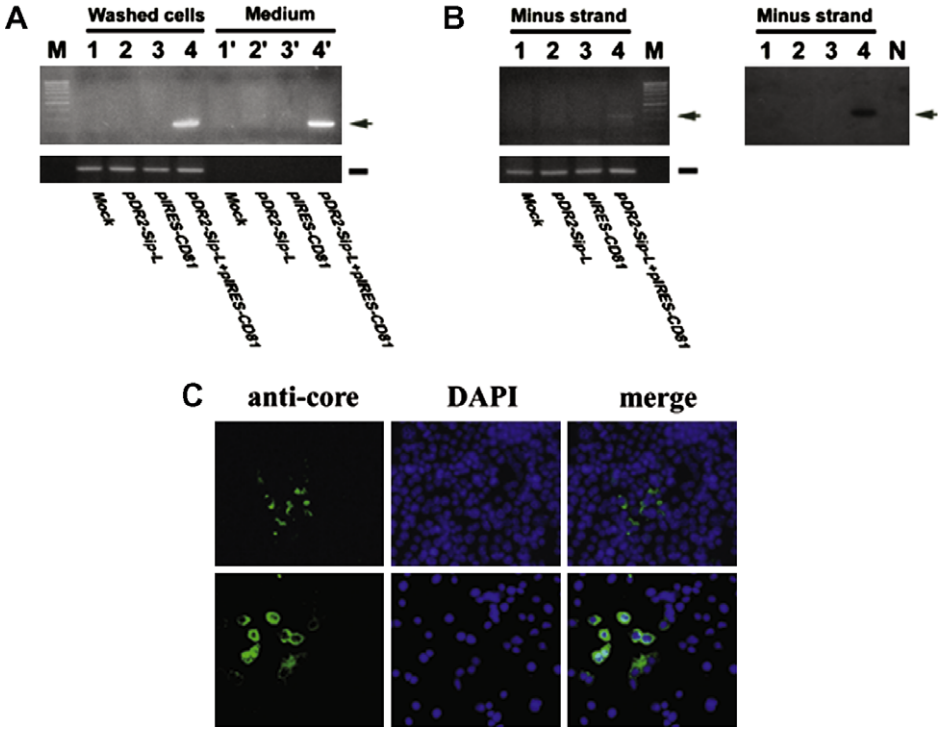

Fig. 2. HCV infection assays. (A) Four cell lines were subjected for HCV infection assays: the na Hepa1-6 (lane 1 and 1′), Hepa1-6-Sip-L (lane 2 and 2′), Hepa1-6-CD81 (lane 3 and 3′), and Hepa1-6-CD81-Sip-L (lane 4 and 4′). The cells were inoculated with 10^4 copies of HCV using HCV-positive serum (genotype 1b) [21]. HCV-RNA in the trypsinized and washed cells (lanes 1–4) and the culture medium (lanes 1′–4′) was detected by RT-PCR. M, molecular weight marker; Arrowhead, position of the RT-PCR product of HCV-RNA; Short line, the RT-PCR product of β-actin RNA as controls. (B) Detection of minus-strand HCV-RNA. The cell lysates from the four infected cell lines were subjected for minus-strand specific RT-PCR (left panel). The products were also submitted for Southern blot analysis (right penal). N, 10^7 copies of HCV-RNA from the serum samples (plus-strand) were used for RT-PCR in parallel as a PCR control of minus-strand specificity. (C) Immunofluorescence detection of HCV core protein in Hepa1-6-CD81-Sip-L cells. HCV core protein was visualized by FITC-conjugated secondary antibody (left panel); nuclei were stained by DAPI (middle panel); and the two pictures were merged to show the subcellular localizations (right panel).

were divided into 5 groups (2 in each group). In the negative control group, no cells were injected but 10^4 copies of HCV were injected subcutaneously. In the positive control group, HCV-infected Hepa1-6-CD81-Sip-L cells (using 10^4 copies HCV) were injected but no drug was given. For the remaining groups of mice, 10^4 or 10^5 copies of HCV were used to infect Hepa1-6-CD81-Sip-L cells (Fig. 4C). In the interferon-treated group, 50,000 IU of interferon-alpha was given on days 2, 4, 6, 9, and 11. In the stibogluconate-treated group, 20 μg/g (body weight-based) of sodium stibogluconate was given on days 2–7, 9, and 10 (Fig. 4D). In the

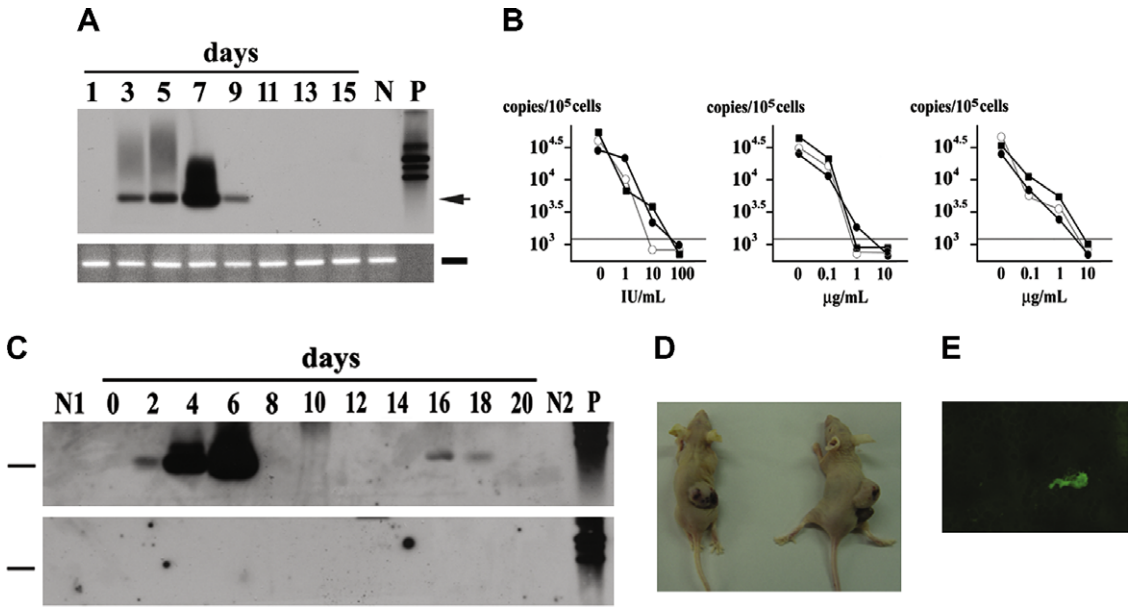

Fig. 3. Antiviral assays using Hepa1-6-CD81-Sip-L cells. (A) After inoculation, cells were harvested every 2 days and HCV-RNA was detected. Arrowhead, position of the RT-PCR product of HCV-RNA; Short line, the RT-PCR product of β-actin RNA as controls; N, 10 pg of pCR2.1-TOPO as a negative hybridization control; P, 10 pg of pTOPO-HCV as a positive hybridization control. (B) Antiviral assays. Different concentrations of interferon-alpha (IFN: 0, 1, 10, 100 IU/mL), cyclosporine-A (CS-A: 0, 0.1, 1, 10 μg/mL), and sodium stibogluconate (SSG: 0, 0.1, 1, 10 μg/mL) were added to the culture medium of HCV-infected Hepa1-6-CD81-Sip-L cells during the infection assays. Cells were harvested on day 5. HCV-RNA was detected by an in-house quantitative assay [24]. Three independent experiments were performed for all quantitative assays (solid squares, solid circles, and empty circles). (C) Detection of HCV-RNA in the tail blood of nude mice subcutaneously injected with HCV-infected Hepa1-6-CD81-Sip-L cells (upper panel) and HCV-inoculated Hepa1-6 cells (lower panel). The tail blood was collected every 2 days up to 20 days for the detection of HCV-RNA. Short lines, positions of the RT-PCR products of HCV-RNA; N1, the supernatant of the second wash of cells as a contamination control; N2, 10 pg of pCR2.1-TOPO; P, 10 pg of pTOPO-HCV. (D) The nude mice carrying tumors of the HCV-infected Hepa1-6-CD81-Sip-L cells (left) and HCV-inoculated Hepa1-6 cells (right) on the 20th days. (E) Immunofluorescence staining of HCV core protein in the frozen section of the HCV-infected Hepa1-6-CD81-Sip-L tumor on the 20th days.

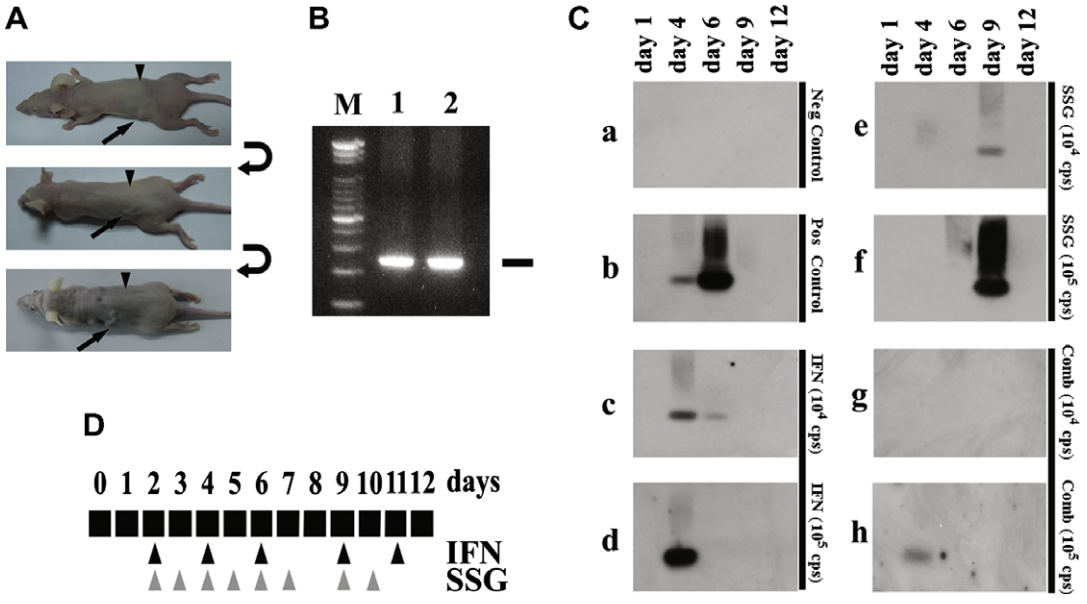

Fig. 4. Viral passages and antiviral assays in the animal model. (A) Uninfected Hepa1-6-CD81-Sip-L cells were injected into one side of the back of nude mouse and HCV-positive mouse plasma was injected into the other side of the back. Tail blood was collected on the 7th day for HCV-RNA assay and viral passage. (B) HCV-RNA in the tail blood from the first and second passages was detected by one step RT-PCR. Short line, the position of the product of RT-PCR for HCV-RNA. (C) To performed antiviral assays, 5 groups of mice were included. (a) Negative control group, no cells were injected but 10^4 copies of HCV were subcutaneously injected; (b) Positive control group, HCV-infected (10^4 copies of HCV used) Hepa1-6-CD81-Sip-L cells were injected but no drug given; (c and d) IFN-treated group, the same as the positive control group but IFN was given as scheduled; (e and f) SSG-treated group, SSG was given as scheduled; (g and h) Combination-treated group, both IFN and SSG were given in parallel as scheduled. In group c, e, and g, 10^4 copies of HCV were used to infect Hepa1-6-CD81-Sip-L cells; In group d, f, and h, 10^5 copies of HCV were used. (D) Schedule for the administration of interferon and/ or sodium stibogluconate.

combination-treated group, both drugs were given together following the same schedule. The results showed that no HCV-RNA could be detected in any of the blood samples obtained from the negative control group (Fig. 4C, panel a). HCV-RNA was detectable on days 4 and 6 with a peak observed on day 6 in the positive control group (Fig. 4C, panel b). In the interferon-treated group, the

HCV-RNA peak on day 6 diminished (Fig. 4C, panel c and d), while HCV-RNA remained detectable on day 4. In the stibogluconate-treated group, HCV-RNA was not detectable on day 4 and 6, but a positive signal appeared on day 9, suggesting a rapid relapse when drug skipped (Fig. 4C, panel e and f). Finally, in the combination-treated group, HCV-RNA was only barely detectable on day 4 in one of the mice (Fig. 4C, panel h).

Discussion

The molecular basis of species specificity in HCV infection was not clearly understood. Transgenic mice expressing CD81 could not support HCV infection [25]. In this study, it was found that in addition to other cellular factors already provided by the mouse hepatoma cells, only human CD81 and Sip-L are required for successful HCV infection and replication. The function of human CD81 in HCV infection was clear. It provides a docking site for HCV to initiate cell entry [14]. On the other hand, the function of Sip-L in HCV infection was poorly understood. It has been discovered that an isoform of Sip-L is the binding protein of MT1-MMP, a membranous type of MMP, which involved several membranous activities including endocytosis [20]. It is therefore reasonable to assume that both CD81 and Sip-L function in facilitating cell entry of HCV. Once the virus successfully enters the cells, other factors required for HCV replication are not species specific.

In this study, we took advantage of the fact that Hepa1-6 cells are highly tumorigenic so that an animal model for antiviral assays could be established. Previously, it has been shown that interferon and stibogluconate synergistically suppressed HCV replication in the replicon system [26,27]. In this study, it was demonstrated that when stibogluconate was given alone, HCV replication relapsed rapidly to a detectable level when one dose was missed. This observation is consistent with the fact that stibogluconate has a very short half-life [28]. However, when the two drugs were combined, HCV can be suppressed very efficiently with no relapsed of viremia. This result raised the possibility of using a short induction-course of stibogluconate in the initial stage of interferon-based anti-HCV treatment in patients carrying very high titer of HCV.

In summary, we discovered that mouse hepatoma cells co-expressing human CD81 and Sip-L supported HCV infection and replication. Based on this finding, an animal model was established for antiviral assays.

Acknowledgement

This study is supported by a grant from the Program of Genomic Medicine, National Science Council, Taiwan (95-3112-B-182-008).

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