［平成21年度］

B．研究方法

TLR3発現ヒト培養細胞の可溶化物を出発材料とし、poly(I:C)の取り込みに関与する分子をpoly(I:C)-Sepharoseを用いて精製し、プロテオーム解析を行った。同定した分子は、siRNAによるノックダウンを行い、poly(I:C)の取り込みと活性への関与を査定した。

C．研究結果

質量分析により、poly(I:C)-Sepahroseに特異的に結合する127個の蛋白を同定した。その中から、膜蛋白ならびに膜に結合する性質を有する蛋白3個を選択し、siRNAによるノックダウンで機能査定を行った。HEK293細胞を用いたレポータージーンアッセイで、TLR3を介したIFN-β promoterの活性化に必要な新規分子Raftlinを同定した。HeLa細胞やヒト単球由来樹状細胞でRaftlinをノックダウンすると、poly(I:C)刺激によるIFN-β産生は非常に減弱することが判明した。

RaftlinをノックダウンしたHeLa細胞やヒト単球由来樹状細胞では、Texas Red標識したpoly(I:C)の細胞内への取り込みが起きないことが共焦点レーザー顕微鏡での観察より明らかとなった。一方、poly(I:C)同様、クラスリン依存的経路でエンドサイトーシスされるトランスフェリンの運搬には関与していなかった。Raftlinはpoly(I:C)刺激により細胞質から細胞膜へリクルートされクラスリンと相互作用し、その後エンドソームのTLR3と共局在することから、クラスリン複合体と協調してpoly(I:C)取り込みレセプターの細胞内への運搬に関わっていることが明らかとなった。

［平成２２年度］

B．研究方法

TLR3のリガンド認識機構を考慮し、19種類のRNA誘導体を合成した。*In vitro assay*として、1. TLR3を介したIFN-β promoterの活性化、2.細胞質内MDA5経路の活性化、3. マウス骨髄系樹状細胞(BMDC)活性化によるNK細胞活性化、4.BMDC活性化によるサイトカイン産生について検討した。また、*in vivo assay*として、1.マウス腹腔内投与後の炎症性サイトカイン産生の経時的測定、2. B16メラノーマ細胞を用いたマウス移植がんモデルにおけるアジュバント効果の査定を行った。

C．研究結果

19種類のRNA誘導体のうち、7種類の誘導体について*in vivo*におけるアジュバント効果を調べた結果、3種類のRNA誘導体（#13, #18, #19)がB16メラノーマ細胞を用いたマウス移植がんモデルにおいて、poly(I:C)とほぼ同等のがん退縮効果を示した。マウス腹腔内投与後のIL-6, TNF-α, IL-10産生は、#18, #19では全く検出されず(10 pg/ml以下)、#13ではIL-6, TNF-α産生はpoly(I:C)投与の50%、IL-10は10%程度であった。各RNA誘導体をHEK293細胞の細胞質に直接導入し、RIG-I, MDA5を介したIFN-β promoter の活性化を測定したところ、いずれの誘導体においても活性化は見られなかった。一方、HEK293細胞でのTLR3を介したIFN-β promoter 活性化は、#13でpoly(I:C)と同等の活性化が見られたが、#18, #19は殆ど活性化しなかった。#18, #19はBMDC刺激で、TLR3-TICAM-1依存的にIL-6, TNF-α, IL-12産生を誘導することがTICAM-1ノックアウトマウスを用いた実験で明らかになった。また、BMDC活性化によるNK細胞活性化は、3種のRNA誘導体いずれにおいても誘導された。

D．考察

合成dsRNAのpoly(I:C)は古くからタイプI IFN誘導剤、NK細胞活性化剤として、in vivoおよびin vitroの実験で用いられてきた。Poly(I:C)は樹状細胞のエンドソームに局在する1型膜タンパク質のTLR3と細胞質RNAヘリケースのMDA5を活性化し、TLR3依存的にIL-12産生を、MDA5依存的にタイプI IFN産生ををを誘導することがこれまでの研究より明らかとなっている。これらのサイトカンはいずれもNK細胞活性化に重要であるが、本研究における新規分子INAMを介したNK細胞活性化経路の発見により、poly(I:C)によるNK細胞活性化にTLR3シグナルが非常に重要であることが明らかになった。アジュバント機能として重要なCTL誘導においては、poly(I:C)はTLR3-TICAM-1シグナル依存的に抗原のクロスプライミングによるCTL活性化を誘導することを現在明らかにしており、今後分子レベルで解析する予定である。

アジュバントは細胞外から投与されその機能を発揮するが、アジュバントの取り込み機構に関する研究は殆どなされていない。Poly(I:C)の取り込みに関して、これまでにマウスマクロファージではCD14、ヒト上皮細胞ではScavenger receptor A が取り込みに関与することが報告されている。しかし、骨髄系樹状細胞はCD14を発現していないこと、Scavenger receptorの阻害剤でpoly(I:C)刺激によるIFN-β産生が変化しないことから、樹状細胞においてはこれらと

異なる取り込みレセプターが存在すると考えられる。本研究で同定したRaftlinは取り込みレセプターそのものではなく、取り込みレセプターと協調して取り込みに関与すると考えられる。Raftlinはpoly(I:C)刺激後、細胞質から細胞膜にリクルートされクラスリンと相互作用するが、poly(I:C)がTLR3エンドソームに到達すると相互作用しないことから、細胞膜でクラスリン-AP-2複合体によるcargoの選択、配送にクラスリンおよび取り込みレセプターと相互作用することで関与すると考えられる。今回の研究は、アジュバントの機能発現にアジュバントの取り込みが重要であること示したものである。今後、樹状細胞に発現する取り込みレセプターを同定し、poly(I:C)の細胞内分配とエフェクター活性の関連を明らかにする予定である。

これまでの一連の研究から、骨髄系樹状細胞のTLR3シグナルの重要性が明らかとなり、副作用の少ないTLR3リガンドの開発が課題となっている。細胞外からTLR3のみを活性化できるRNA誘導体はこれまで報告がなく、本研究の化合物がはじめてである。新規RNA誘導体は、以下の特徴を有している。① poly(I:C) 同様細胞外からエンドソームTLR3にターゲットされる。② TLR3を活性化しTICAM-1を介してシグナルを伝達する。③細胞質RIG-I, MDA5経路を活性化しない。④B16メラノーマ細胞を用いたマウス移植がんモデルにおいて、poly(I:C)と同等の抗がん活性を示す。⑤マウス生体内投与での炎症性サイトカイン産生量はpoly(I:C)投与より少ない。

In vivoとin vitroにおけるサイトカイン産生誘導が異なる理由として、in vivoにおいてRNA誘導体を取り込む細胞群がBMDCと異なるDCサブセットもしくは他の細胞集団の可能性、in vivo でのRNA誘導体の分解、消費が考えられる。RNA誘導体の抗がん活性はタイプI IFNやサイトカイン産生能とパラレルではないことから、異なるシグナル伝達系路もしくはシグナル伝達の場を介して誘導されると考えられる。今後、新規RNA誘導体によるCTL活性化能、in vivoデリバリー、担当細胞群および抗がん免疫シグナルの同定を行い、次世代アジュバントとして確立する予定である。

E．結論

1. 骨髄系樹状細胞のエンドソームTLR3シグナルによるNK細胞活性化の分子メカニズムを明らかにした。

2. Poly(I:C)の取り込みに必須の分子Raftlinを同定した。

3. TLR3-TICAM-1経路のみ活性化し、過度のサイトカイン産生を誘起せずアジュバント効果を示す新規RNA誘導体を得た。

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H．知的財産権の出願・登録状況

1．特許取得

1．発明の名称：I型インターフェロンの発現調節剤、PCT/JP2008/001648、発明者：瀬谷司、松本美佐子、押海裕之、出願日：2008年6月25日、出願人：北海道大学

2．発明の名称：M161Agからなるサイトカイン誘発剤、登録番号：2,320,656、発明者：瀬谷司、松本美佐子、登録日：2009年8月4日、出願人：JST

2．実用新案登録 なし

Ⅲ．研究成果の刊行に関する一覧表

III. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
H. Ohtani *et al.*	Molecular evolution of immunoglobulin superfamily genes in primates.	Immunogenetics	In press		2011
A. Watanabe *et al.*	Raftlin is involved in the nucleocapture complex to induce poly(I:C)-mediated TLR3 activation.	J. Biol. Chem.	In press		2011
M. Matsumoto *et al.*	Antiviral responses induced by the TLR3 pathway.	Rev. Med. Virol.	In press		2011
R. Sawahata *et al.*	Failure of mycoplasma lipoprotein MALP-2 to induce NK cell activation through dendritic cell TLR2.	Microbes Infect.	In press		2011
A. Saito *et al.*	Improved capacity of a monkey-tropic HIV-1 derivative to replicate in cynomolgus monkeys with minimal modifications.	Microbes and Infection	13	58-64	2011
M. Yabu *et al.*	IL-23-dependent and –independent enhancement pathways of IL-17A production by lactic acid.	Int. Immunol.	23	29-41	2011
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Ⅳ．研究成果の刊行物・別冊

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Characterization of infectious hepatitis C virus from liver-derived cell lines

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ABSTRACT

The efficient production of infectious HCV from the JFH-1 strain is restricted to the Huh7 cell line and its derivatives. However, the factors involved in this restriction are unknown. In this study, we examined the production of infectious HCV from other liver-derived cell lines, and characterized the produced viruses. Clones of the Huh7, HepG2, and IMY-N9, harboring the JFH-1 full-genomic replicon, were obtained. The supernatant of each cell clone exhibited infectivity for naïve Huh7. Each infectious supernatant was then characterized by sucrose density gradient. For all of the cell lines, the main peak of the HCV-core protein and RNA exhibited at approximately 1.15 g/mL of buoyant density. However, the supernatant from the IMY-N9 differed from that of Huh7 in the ratio of core:RNA at 1.15 g/mL and significant peaks were also observed at lower density. The virus particles produced from the different cell lines may have different characteristics.

Hepatitis C virus (HCV) is an enveloped virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family. HCV is a human pathogen and HCV infection is a major cause of chronic hepatitis, liver cirrhosis and hepatic carcinoma. The main therapy for HCV is treatment with pegylated-interferon and rivabirin. However, these agents show little effect for patients that have a high titer of HCV-RNA, genotype 1. Thus, it is necessary to develop new, more effective therapies and preventive treatments to counteract HCV infection. It was discovered that a genotype 2a strain of HCV, JFH-1, can efficiently replicate in the Huh7 cell line [1], and an *in vitro* culture model of infectious HCV has also been successfully developed using the JFH-1 genome [2–4]. Recently, it has become possible to produce various chimeric HCV by replacement of the JFH-1 structural protein region with that of other strains. The HCV particles produced from such chimera are expected to lead to the development of a HCV vaccine, and new anti-HCV pharmaceuticals.

The infectious HCV-derived JFH-1 genome was developed using the human hepatoma Huh7 cell line [5]. Although the sub-genomic replicon RNA of JFH-1 can autonomously replicate, not only in Huh7 cells, but in other human liver [6], non-hepatic [7], and mouse [8] cells, infectious HCV production has been restricted to Huh7-derived cells. In this study, we undertook a comparative study of infectious HCV particles produced from different cell lines including Huh7. Infectious HCV particles were successfully produced into the culture media and characterized.

Materials and methods

Cell culture. Huh7, Huh7.5.1 ([3], a generous gift from Dr. Francis V. Chisari), HepG2, and IMY-N9 cells were cultured at 37 °C in 5% CO_2. The HepG2 cells were cultured in modified Eagle's medium containing 10% fetal bovine serum. All of the other cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, as described previously [6].

Plasmids. The pFGR-JFH1 and pFGR-JFH1/deltaE12 plasmids, encoding the full-genomic replicon, and envelope-deleted replicons, respectively, were generated as previously described [9].

RNA synthesis. RNA synthesis was performed as described previously [2]. Briefly, the pFGR-JFH1 plasmid was digested with XbaI and then treated with Mung Bean nuclease (New England Biolabs, Beverly, MA). The digested plasmid DNA fragment was then purified and used as a template for RNA synthesis. HCV-RNA was synthesized *in vitro* using a MEGAscript™ T7 kit (Ambion, Austin, TX). The synthesized RNA was treated with DNaseI, followed by acid phenol extraction to remove any remaining template DNA.

Establishment of replicon cells. Cell lines harboring FGR-JFH1 replicons were produced as described previously [9]. Briefly, trypsinized cells were washed with Opti-MEM I™ reduced-serum medium (Invitrogen, Carlsbad, CA) and resuspended at 7.5×10^6 cells/mL with Cytomix buffer [1]. RNA (10 μg), synthesized from pFGR-JFH1, was mixed with 400 μL of cell suspension and transferred to an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybrid, Middlesex, UK). The cells were then pulsed at 260 V and 950 μF with the Gene Pulser II™ apparatus (Bio-Rad,

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Hercules, CA). Transfected cells were immediately transferred to 10-cm culture dishes, each containing 8 mL of culture medium. G418 (0.8–1.0 mg/mL) (Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 16–24 h after transfection. Culture medium, supplemented with G418, was replaced twice per week. Three weeks after transfection, sparsely grown G418-resistant colonies were independently isolated using a cloning cylinder (Asahi Techno Glass Co., Tokyo, Japan), and were expanded.

Preparation of supernatants from FGR-JFH1 replicon cells. Culture media was collected from Huh7, IMY-N9, and HepG2 cell lines harboring the FGR-JFH1 replicon and was passed through a 0.45-μm filter. Filtrated culture media was then pooled and concentrated 50-fold using Amicon Ultra-15 (100,000 Molecular weight cut off; Millipore, Bedford, MA), and stored at −80 °C until use.

Assay of infection of naïve Huh7 cells. Infection of naïve Huh7 cells were assayed by immunofluorescence and colony formation assays. For the immunofluorescence assay naïve Huh7.5.1 cells were seeded at 1×10^4 cells/well in an 8-well chamber slide (Becton Dickinson, Franklin Lakes, NJ), cultured overnight and then inoculated with diluted culture media containing infectious HCV particles (1×10^6 HCV-RNA copies). At 72 h after inoculation, the cells were fixed in acetone/methanol (1:1) for 10 min at −20 °C, and the infected foci were visualized by immunofluorescence as follows.

An anti-core HCV protein monoclonal antibody 2H9 [2] was added to the cells at 50 μg/mL in BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan). After incubation for 1 h at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) diluted in BlockAce. The cells were then washed, treated with DAPI solution (Sigma, Saint Louis, MO) at 0.1 μg/mL and examined by Biozero fluorescence microscopy (Keyence, Osaka, Japan).

Colony formation assays were performed as described previously [9]. Briefly, naïve Huh7 cells were inoculated with culture supernatants from replicon-expressing cell lines for 2 h, and then cultured with complete medium. Inoculated cells were cultured for 3 weeks in medium supplemented with G418 (0.3 mg/mL). Cell survival was assessed by staining with crystal violet.

Titration of infectivity. The infectivity titer of the culture supernatants was determined on Huh7.5.1 cells by end point dilution and immunofluorescence as described above. Briefly, each sample was serially diluted 10-fold in DMEM-10% FBS and 100 μL was used to inoculate Huh7.5.1 cells. Infection was examined 72 h post-inoculation by immunofluorescence using a mouse monoclonal anti-core antibody and secondary anti-mouse IgG-Alexa 488 conjugated antibodies. Infectious foci were counted and the titer was calculated and expressed as focus forming units per mL (FFU/mL).

Sucrose density gradient analysis. Concentrated cell supernatants were layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer containing 10 mM Tris, pH7.5, 150 mM NaCl, and 0.1 mM EDTA. Gradients were centrifuged in an SW41 rotor (Beckman Coulter, Fullerton, CA) at 35,000 rpm for 16 h at 4 °C, and fractions (400 μL each) were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100 μL drop from each fraction following a gradient run.

Quantification of HCV-core protein and RNA. The level of the HCV-core protein in culture supernatants or sucrose density gradient fractions, was assayed using an immunoassay as described elsewhere [10]. Viral RNA was isolated from harvested culture media, or sucrose density gradient fractions, using the QiaAmp Viral RNA Extraction kit (Qiagen, Tokyo, Japan). The copy number of HCV RNA was determined by real-time detection reverse transcription-polymerase chain reaction (RTD-PCR), using an ABI Prism 7500fast sequence detector system (Applied Biosystems, Tokyo, Japan) [11].

Results

Production of infectious HCV from human liver-derived cell lines

We first determined if it was possible to produce infectious HCV from cell lines other than Huh7. We selected the HepG2 and IMY-N9 cell lines to establish human liver-derived cell lines that enable replication of the JFH-1 genome [6]. Since full-genomic JFH-1 did not transiently replicate in these cells (data not shown), we established FGR-JFH1 replicon cells that stably replicate the JFH-1 genome. In the culture media obtained from these full-genomic replicon cells, HCV-RNA titers were detected by RTD-PCR. The titer of HCV-RNA was highest in the supernatant from an IMY-N9 cell clone and lowest from a HepG2 cell clone (Table 1). When naïve Huh7.5.1 cells were inoculated with culture supernatants from the replicon cells, infected cells could be detected by immunofluorescence using an anti-HCV-core protein antibody (Fig. 1A). These data suggested that HepG2 and IMY-N9 cells are able to produce infectious HCV.

We then compared the specific infectivity of the replicon containing culture supernatants from the different cells. Specific infectivity was calculated by dividing the infectious titer, calculated by immunofluorescence of infectious foci, of the culture media by the titer obtained for HCV-RNA. Using these calculations the culture media from Huh7 and HepG2 cells showed almost the same specific infectivity whereas that from IMY-N9 cell was relatively higher (Table 1). Thus the infectious HCV in the culture media might differ according to the cell line from which it was obtained.

To clarify the differences observed in specific infectivity, we next examined the ability of the various cellular supernatants to induce colony formation. For this assay naïve Huh7 cells were inoculated with culture media of the same HCV-RNA titer as that of the FGR-JFH1 virus and were cultured in G418-containing medium. Cell survival was assayed by staining with crystal violet, and the number of colonies formed was counted. Consistent with the specific infectivity results, the supernatant of the IMY-N9 replicon cell showed higher colony formation compared with that of Huh7 and HepG2 replicon cells (Fig. 1B and C). Thus IMY-N9 cells produce infectious HCV with a relatively higher infectivity than the other cell lines suggesting that the supernatant derived from the different replicon producing cells may differ.

Characterization of the FGR-JFH1 virus from different liver-derived cells

To further characterize potential differences between the viruses produced by the different cell lines we next characterized the FGR-JFH1 virus in the media of the different cell lines by sucrose density gradient analysis. Concentrated cell supernatants were layered on top of a preformed continuous 10–60% sucrose gradient and centrifuged. Twenty-four fractions were collected and the HCV-core protein and RNA was assayed in each fraction. The peak fraction of the HCV-core protein and that of the RNA coincided at a density of 1.15 g/mL in all supernatants. However, the supernatant of the IMY-N9 cells showed different profiles for both the HCV-core protein and RNA compared to those of Huh7. Thus the IMY-N9 cells had a different ratio of

Table 1
Infectivity of the supernatant of replicon cell lines.

Producing cell	HCV-RNA (copies/mL)	Infectious titer (FFU/mL)	Specific infectivity (FFU/RNA copy)
Huh7	$1.36 \pm 0.02 \times 10^8$	$1.30 \pm 0.32 \times 10^4$	9.56×10^{-5}
IMY-N9	$2.80 \pm 0.04 \times 10^8$	$3.75 \pm 0.38 \times 10^4$	1.34×10^{-4}
HepG2	$8.80 \pm 0.75 \times 10^7$	$7.70 \pm 1.41 \times 10^3$	7.96×10^{-5}

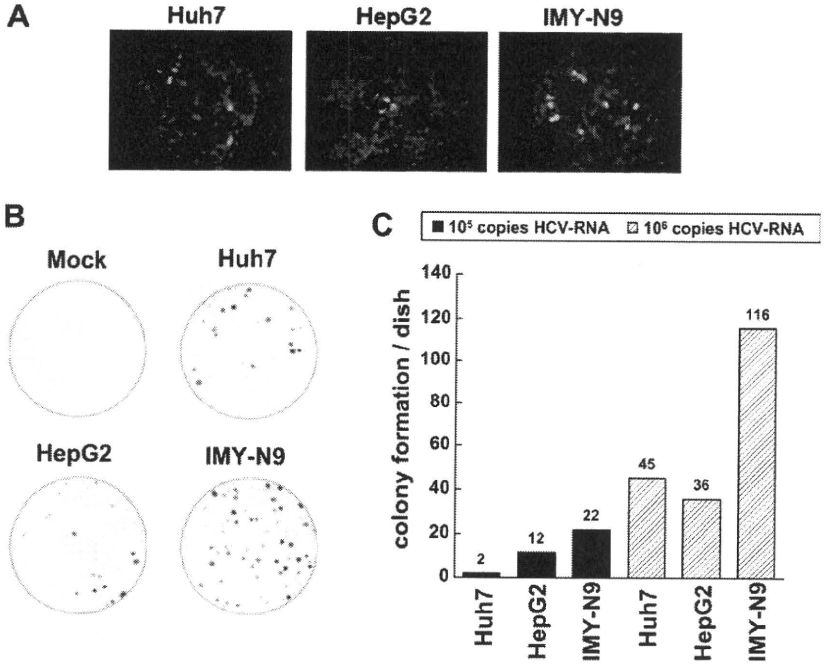

Fig. 1. Naïve Huh7 cell infection assay of JFH-1 full-genomic replicon cell culture supernatants. (A) JFH-1 full-genomic replicon (FGR-JFH1) cells were established in Huh7, HepG2, and IMY-N9 cell lines. Supernatants derived from Huh7 (*left*), HepG2 (*middle*), and IMY-N9 (*right*) cells (1×10^6 HCV-RNA copies) were inoculated into naïve Huh7.5.1 cells (1×10^4) for 48 h, and infected cells were then detected by immunofluorescence using an anti-core antibody (clone 2H9) (green). (B) Naïve Huh7 cells (5×10^5) were inoculated with mock, Huh7, HepG2, and IMY-N9-derived supernatants (10^6 HCV-RNA copies per 10-cm dish) of FGR-JFH1 cells for 2 h. Inoculated cells were cultured for 3 weeks in complete medium supplemented with G418 (0.3 mg/mL), and G418-resistant cells were stained using crystal violet. (C) The number of G418-resistant colonies obtained in (B) was calculated when 10^5 or 10^6 copies of HCV-RNA were tested. Mean values of colony number were indicated in duplicate experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HCV-core protein and RNA at a density of 1.15 g/mL (RNA/Core ratio; Huh7: 511, IMY-N9: 133 copies/fmol) and also showed a secondary peak at lower density (approximately 1.05 g/mL). For all supernatants the peak of infectivity exhibited at a density of 1.10 g/mL that was slightly lower than that of the HCV-core protein and RNA peaks. Furthermore infectivity was barely detectable in the lower density fractions (Fig. 2) suggesting that the HCV-core protein and RNA that was detected at lower density was irrelevant for infectivity of the different supernatants.

We considered the possibility that the core protein and RNA in the lighter fractions may be due to cellular debris containing a replication complex. To determine if this might be the case we therefore analyzed the supernatants from Huh7 and IMY-N9 envelope-deleted replicon cells (FGR-JFH1/deltaE12). The HCV-core protein and RNA were detected in the supernatants of these cells although the titers were very low. These supernatants were not infective for naïve Huh7 cells (data not shown). Furthermore, analysis of the concentrated supernatants of these cell lines by sucrose density gradient analysis detected both the HCV-core protein and RNA, and the major peaks of HCV-RNA were detected in the lower density (approximately 1.10 g/mL) fractions (Fig. 3). However, the profiles of HCV-core protein and RNA did not coincide for either cell line.

Discussion

Infectious HCV can be produced in cell culture by using the JFH-1 genome. This system permits investigation of various aspects of the HCV life cycle such as the steps of entry into cells, replication, and secretion. Infectious HCV derived from JFH-1 is robustly produced in Huh7 cell lines [2,3], and the infectious particles have been characterized. However the difficulty in robustly producing infectious HCV from other cell lines prevents a comparative study of HCV production among different cell lines. In this study, we compared infectious HCV production in Huh7 with that of other cell lines, and characterized the viruses produced.

First, we established Huh7, IMY-N9, and HepG2 FGR-JFH1 replicon cells. These cell lines were able to replicate the JFH-1 subgenomic replicon [6]. The HCV-core protein and RNA were detected in all of the supernatants and all of these supernatants showed infectivity for naïve Huh7. Infectivity was evaluated by transient infection and colony formation assays. These assays indicated that the infectious supernatant from IMY-N9 cell had higher infectivity than the other cell lines for naïve Huh7 cells.

Next, we characterized each supernatant by sucrose density gradient analysis, which revealed both similarities and differences among the infectious supernatants. All samples showed typical peaks at 1.15 g/mL buoyant density for HCV-core protein and RNA, and infectious fractions showed an almost identical buoyant density of 1.10 g/mL. However, the supernatant from the IMY-N9 cells showed a difference in the core/RNA ratio at a density of 1.15 g/mL and higher secondary peak of HCV-core protein and RNA at a lower density (approximately 1.05 g/mL). Since the fractions at lower density did not correlate with infectivity, it is believed that the component at lower density does not contain infectious HCV particles but rather cellular debris that contains HCV proteins, RNA, and lipids [12]. HCV can associate with lipoprotein [13,14], and is secreted with VLDL [15]. Thus, the observed differences in the HCV-producing cells may derive from differences in lipoprotein synthesis. However, it is also possible that the components migrating at lower density contain virus particles. The deletion mutant of FGR-JFH1 (FGR-JFH1/deltaE12) did replicate in Huh7 and IMY-N9 cells, and these replicon cells secreted the HCV-core protein into the culture media, although at low levels. HCV-RNA was also detected in the same culture medium, and the profile of this HCV-RNA differed from that of the HCV-core protein in sucrose density gradient analysis. Thus, the

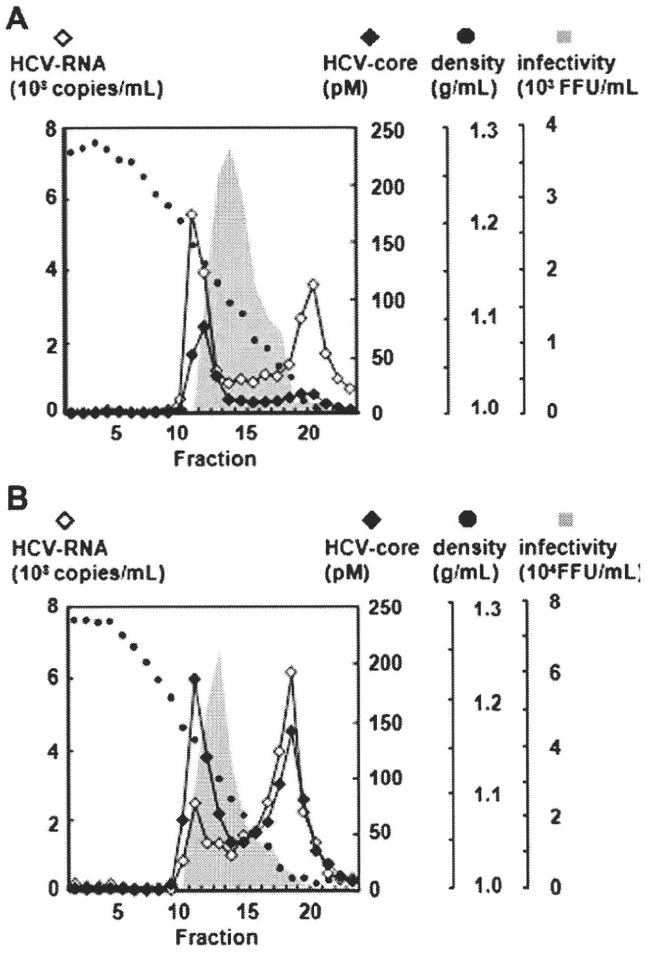

Fig. 2. Density gradient analysis of infectious HCV derived from Huh7 and IMY-N9 cells. Concentrated supernatants of Huh7 cells (A) and IMY-N9 cells (B) were layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer. The gradients were centrifuged in a SW41 rotor at 35,000 rpm for 16 h at 4 °C, and fractions (400 μL each) were collected from the bottom of the tube. The buoyant density (closed circles), HCV-core protein (closed diamonds), HCV-RNA (open diamonds) and infectivity for naïve Huh7.5.1 cells (shown in gray) was detected in each fraction as described in Materials and methods.

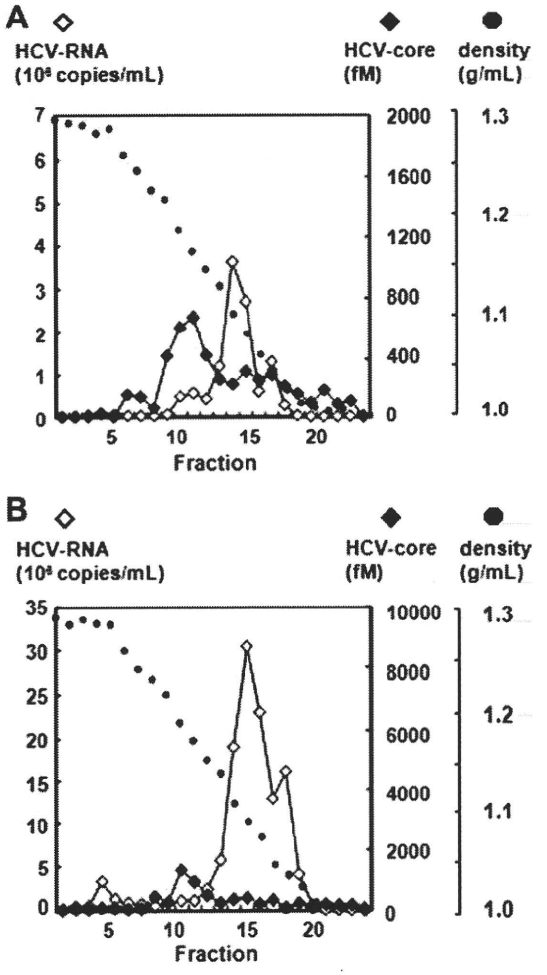

Fig. 3. Density gradient analysis of supernatants derived from Huh7 and IMY-N9 cells transfected with FGR-JFH1/deltaE12 RNA. Concentrated supernatants from Huh7 (A) and IMY-N9 (B) cells were analyzed by sucrose density gradient as described in the legend to Fig. 2. The buoyant density (closed circles), HCV-core protein (closed diamonds) and HCV-RNA (open diamonds) was analyzed in each fraction.

peak fractions containing the HCV-core protein and RNA from the supernatant of FGR-JFH1/deltaE12 cells were different from the peak fractions from that of FGR-JFH1 cells. Therefore it is possible that all of the peaks of HCV-core protein and RNA observed in the supernatant of FGR-JFH1 replicon cells may correlate to virus particles with different densities. However, the reason why they centrifuge at different densities is unclear. Interestingly, the supernatants from cells transfected with envelope-deleted replicon RNA exhibit non-identical HCV-core protein and RNA profiles on a sucrose density gradient. Envelope-deleted replicon RNA may have a decreased ability to form nucleocapsids although a detailed examination is necessary to establish this point.

We previously developed a method for infectious HCV production using the FGR-JFH1 [9], and have now succeeded in producing infectious HCV in the supernatant of cultured liver-derived cell lines harboring FGR-JFH1 RNA. Infectious HCV particles are useful for vaccine production and are considered good antigens for the generation of useful antibodies. Selection of an appropriate cell line is important for the production of HCV particles for vaccine development. The technique used in this study seemed to be appropriate for producing infectious HCV in various cell lines [8].

A second advantage of using HepG2 and IMY-N9 cells for the production of virus particles is that these parental cell lines, unlike the Huh7 cell line, do not express the CD81 molecule on the cell surface, however, the expression on cell clones used in this study was not confirmed. This means that the FGR-JFH1 replicon of these cell lines may have a single cycle of HCV production, encompassing replication, assembly, budding and secretion, and do not show HCV permissiveness. These cells should therefore be useful for the discovery of drugs targeted against HCV assembly and secretion.

Acknowledgments

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Hepatitis C Virus JFH-1 Strain Infection in Chimpanzees Is Associated With Low Pathogenicity and Emergence of an Adaptive Mutation

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The identification of the hepatitis C virus (HCV) strain JFH-1 enabled the successful development of infectious cell culture systems. Although this strain replicates efficiently and produces infectious virus in cell culture, the replication capacity and pathogenesis *in vivo* are still undefined. To assess the *in vivo* phenotype of the JFH-1 virus, cell culture–generated JFH-1 virus (JFH-1cc) and patient serum from which JFH-1 was isolated were inoculated into chimpanzees. Both animals became HCV RNA-positive 3 days after inoculation but showed low-level viremia and no evidence of hepatitis. HCV viremia persisted 8 and 34 weeks in JFH-1cc and patient serum–infected chimpanzees, respectively. Immunological analysis revealed that HCV-specific immune responses were similarly induced in both animals. Sequencing of HCV at various times of infection indicated more substitutions in the patient serum–inoculated chimpanzee, and the higher level of sequence variations seemed to be associated with a prolonged infection in this animal. A common mutation G838R in the NS2 region emerged early in both chimpanzees. This mutation enhances viral assembly, leading to an increase in viral production in transfected or infected cells. *Conclusion:* Our study shows that the HCV JFH-1 strain causes attenuated infection and low pathogenicity in chimpanzees and is capable of adapting *in vivo* with a unique mutation conferring an enhanced replicative phenotype. (HEPATOLOGY 2008;48:732-740.)

Hepatitis C virus (HCV) infects approximately 170 million people worldwide and is a major causative agent of chronic liver diseases including cirrhosis and hepatocellular carcinoma.[1,2] However, the underlying biological mechanisms of pathogenesis and persistence are still not well understood. No vaccine protecting against HCV infection is currently available.[3] Therapy for HCV-related chronic hepatitis remains problematic, with limited efficacy, high cost, and substantial adverse effects.[1,4,5] Understanding the biology of this virus and the development of new therapies has been hampered by a lack of appropriate model systems for replication and infection of this virus.

Abbreviations: ALT, alanine aminotransferase; ELISpot, enzyme-linked immunosorbent spot; FFU, focus-forming unit; HCV, hepatitis C virus; HVR, hypervariable region; IFN-γ interferon gamma; JFH-1cc, cell culture generated JFH-1 virus; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription polymerase chain reaction; SFU, spot-forming unit; WT, wild-type.

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Recent progress with a unique HCV genotype 2a strain, JFH-1, isolated from a case of fulminant hepatitis in Japan, has led to the development of a robust HCV infectious cell culture system.[6-9] This JFH-1 strain can replicate efficiently, produce the infectious viral particles, and show robust infection *in vitro*. However, in our previous report, the inoculation of cell culture–generated JFH-1 virus (JFH-1cc) induced only transient and attenuated infection in a chimpanzee.[8] The observed low virulence of this strain *in vivo* was unexpected but consistent, with an inverse relationship between *in vivo* and *in vitro* properties of cell culture adaptive mutations in the HCV replicon system.[10]

In this study, we performed an extensive analysis of the *in vivo* replication and pathogenicity of the JFH-1 strain by inoculating chimpanzees with JFH-1cc and patient serum from which the JFH-1 strain was isolated. Furthermore, we analyzed viral sequences during the infection to identify mutations that might represent *in vivo* adaptive mutations with unique phenotypes.

Materials and Methods

Cell Culture. Huh7 derivative cell lines Huh7.5 and Huh7.5.1 were provided by Charles Rice (Rockefeller University, New York, NY) and Francis Chisari (Scripps Research Institute, La Jolla, CA), respectively.[7,9] The Huh7 derivative clone Huh7-25 that lacks CD81 expression was reported previously.[11]

Inocula. The production of JFH-1cc has been reported previously.[12] Briefly, the full-length JFH-1 RNA was synthesized by *in vitro* transcription with linearized pJFH-1 plasmid and MEGAscript kit (Ambion, Austin, TX).[8] Ten micrograms full-length JFH-1 RNA was transfected into 3.0×10^6 Huh7 cells by electroporation, and the culture medium with JFH-1cc was harvested 5 days after transfection. The culture medium was passed through a 0.45-μm filter unit. The case of fulminant hepatitis C from which the JFH-1 strain was isolated has been reported previously.[6] An aliquot of acute-phase serum (point A as indicated by Kato et al.[6]) was used in this study. To determine the HCV RNA titers in these inocula, total RNA was extracted from 140 μL of these samples by QIAamp Viral RNA Kit (QIAGEN, Valencia, CA), and copy numbers of HCV RNA were determined by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR), as described previously.[13]

Infection Study in Chimpanzees. Housing, maintenance, and care of the chimpanzees used in this study conformed to the requirement for the humane use of animals in scientific research as defined by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention. Chimpanzee 10273 (CH10273, female, age 5, 20 kg) was inoculated intravenously with 100 μL serum (9.6×10^6 copies) from the fulminant hepatitis patient mixed with 400 μL Dulbecco's modified Eagle's medium culture medium. Chimpanzee 10274 (CH10274, female, age 5, 22 kg) was inoculated intravenously with 500 μL Dulbecco's modified Eagle's medium culture medium containing JFH-1cc (1.4×10^7 copies). Serum and liver biopsy samples of these animals were obtained at baseline and weekly after inoculation.

Measurement of HCV RNA, anti-HCV, and Alanine Aminotransferase. HCV RNA in chimpanzees was quantitatively measured by nested RT-PCR with a sensitivity of detection of approximately 50 IU/mL (COBAS Amplicor; Roche Molecular Systems, Pleasanton, CA) and was quantified using Amplicor Monitor (Roche Molecular Systems). Serum samples were tested for anti-HCV (ORTHO version 3.0 enzyme-linked immunosorbent assay test system, Ortho-Clinical Diagnostics, Raritan, NJ). Serum alanine aminotransferase (ALT) values in chimpanzee's sera were established using a commercially available assay kit in accordance with the manufacturer's instructions (Drew Scientific, Dallas, TX). Cutoff values representing 95% confidence limit for the upper level of normal ALT activity were calculated individually for each chimpanzee using 10 pre-inoculation enzyme values obtained over a period of 4 to 6 weeks, and were 73 U/L in CH10274 and 76 U/L in CH10273.

HCV Sequencing. The total RNA was extracted from 280 μL chimpanzee sera collected at appropriate time points by the use of QIAamp viral RNA kit, and complementary DNA was synthesized by use of Superscript III (Invitrogen, Carlsbad, CA). The complementary DNAs were subsequently amplified with TaKaRa LA *Taq* DNA polymerase (Takara Mirus Bio, Madison, WI). Five separate fragments were amplified by nested PCR covering the entire open reading frame and a part of the 5'UTR of the JFH-1 strain as follows; nt 128-1829, nt 1763-4381, nt 4278-6316, nt 6172–7904, and nt 7670-9222. The sequence of each amplified fragment was determined directly. The fragment encompassing hypervariable region 1 (HVR-1) (nt 128-1829) was cloned into the pGEM-T easy vector (Promega, Madison, WI) and 10 clones from each time point were sequenced.

T-Cell Proliferation and Interferon-γ Enzyme-Linked Immunosorbent Spot Assays. The cryopreserved peripheral blood mononuclear cells (PBMCs) were used for immunological analysis. Standard T-cell proliferation assay was performed as described previously.[14] Cells were stimulated with recombinant HCV genotype 2a core or NS5a protein (Fitzgerald Industries Interna-