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Forum Minireview

Lifestyle-Related Diseases of the Digestive System: A New In Vitro Model of Hepatitis C Virion Production: Application of Basic Research on Hepatitis C Virus to Clinical Medicine

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Abstract. The hepatitis C virus (HCV) is an enveloped virus with a single positive-strand RNA genome of about 9.6 kb. It is a major cause of liver disease worldwide. Clear understanding of the viral life cycle has been hampered by the lack of a robust cell culture system. While the development of the HCV replicon system was a major breakthrough, infectious virions could not be produced with the replicon system. Recently, several groups have reported producing HCV virions using in vitro systems. One of these is a replicon system, but with the special genotype 2a strain JFH-1. Another is a DNA transfection system, with the construct containing the cDNA of the known infectious HCV genotype 1b flanked by two ribozymes. The development of these models further extends the repertoire of tools available for the study of HCV biology, and in particular, they may help to elucidate the molecular details of hepatitis C viral assembly and release. This review discusses the progression of experimental strategies related to HCV and how these strategies may be applied to clinical medicine.

Keywords: life style-related disease, hepatitis C virus (HCV), replication, in vitro system, HCV-like particle, particle

Introduction

The hepatitis C virus (HCV) was discovered in 1989 (1). Many clinical hepatitis cases that were termed non-A, non-B hepatitis were subsequently labeled as hepatitis caused by HCV (2). HCV, an enveloped virus with a positive-strand RNA genome, approximately 9.6 kilobases in length, encodes a single large polyprotein of about 3,000 amino acids within the *Flaviviridae* family. About 200 million people worldwide are reportedly infected with this virus, making HCV infection a major public health problem (3, 4). The majority of HCV-infected patients fail to clear the virus, and in some cases, HCV infection progresses to liver failure and hepatocellular carcinoma (5, 6). Standard therapy for HCV infection consists of administration of interferon (IFN) in combination with ribavirin, but these regimens are effective in no more than approximately 50% of infected persons, at best. Furthermore, there are no effective vaccines against HCV infection because of the lack of a reproducible tissue culture system to propagate the virus and the lack of a convenient animal model for HCV infection, other than the chimpanzee. Thus, establishment of a new model system for viral replication, assembly, and release is urgently needed. Recently, several groups have reported new in vitro models for HCV replication. This review discusses some of the recently described robust in vitro models for hepatitis virus replication.

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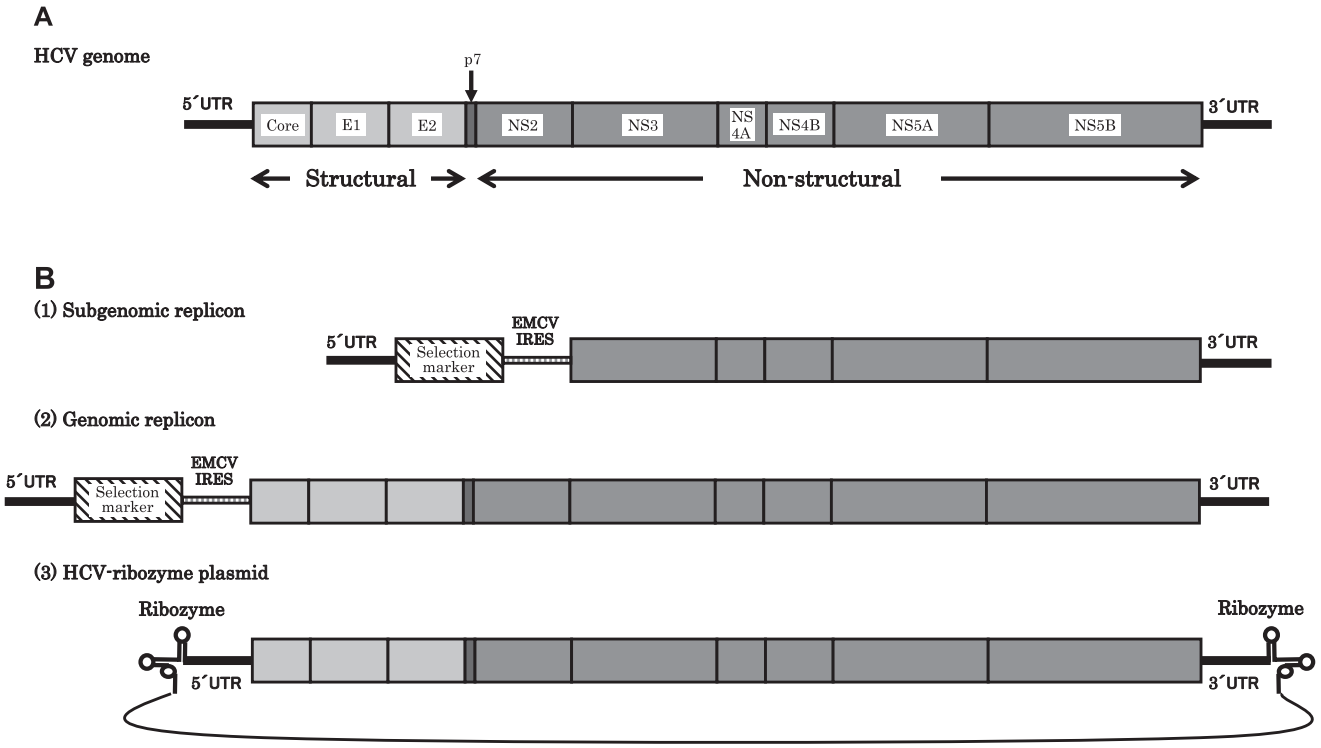

Fig. 1. A: Structure and organization of the HCV genome. B: The structure of (1) the subgenomic HCV replicon, (2) the genomic HCV replicon, and (3) HCV-ribozyme plasmid used for the production of hepatitis C virions. Core, core protein; E1, envelope 1 glycoprotein; E2, envelop 2 glycoprotein; NS, nonstructural protein; UTR, untranslated region; IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus.

Hepatitis C virus

The HCV genome has a long open reading frame (ORF) flanked by 5'- and 3'-untranslated regions (UTR). The ORF is processed into structural (core, E1, and E2), p7, and nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (2, 7) (Fig. 1A). The HCV polyprotein is cleaved co- and post-translationally by cellular and viral proteinases. The structural proteins are located in the amino-terminal one-third, with the remaining two-third constituting nonstructural replicative proteins. The core, E1, and E2 proteins are the major components of the virus particles. The envelope proteins (E1 and E2) are highly glycosylated proteins. Protein p7, located at the carboxy terminus of E2, is a highly hydrophobic polypeptide of unknown function. Most of the NS proteins 2-5B (the term indicates that these proteins are not expected constituents of the virus particle) are required for replication of the viral RNA (8). The N-terminal one-third of the NS3 protein functions as a serine protease, and the remainder has RNA helicase activity. NS4A is a co-factor of the NS3 serine protease. NS5B is the viral RNA-dependent RNA polymerase (RdRp) and is essential for viral replication.

HCV isolates have been classified into six genotypes and various subtypes based on the sequences at the 5'-UTR and NS5b regions (9). Moreover, HCV sequence variability may even be observed within a single HCV-infected individual (termed quasispecies). Among the genotypes, genotypes 1 and 2 are distributed worldwide and are known to be associated with different clinical profiles and therapeutic responses (10). Therapy for patients chronically infected with genotype 1 has mostly been dependent on IFN-based regimens, which are effective in no more than approximately 50% of infected persons, at best (3, 11, 12).

Replication system for HCV

1) Infectious clone

This strategy was one of the important breakthroughs in HCV research. Until it was developed, the lack of an infectious molecular clone for the genetic analysis of HCV function hampered the development of new and more effective treatments. The identified infectious clone was an RNA transcript derived from cloned HCV cDNA, which could initiate infection and cause hepatitis in transfected chimpanzees (13, 14). The correctness of the sequence of the transfected RNA transcript was confirmed by the identical sequence of the virus

recovered from chimpanzees. Genetically stable infectious clones of HCV could be constructed using this strategy, and the infectiousness/lack of infectiousness of other HCV constructs could be easily studied. These studies helped in defining the structure of the functional HCV genome and proved that the HCV alone is sufficient to cause hepatic disease. The problem, however, was that chimpanzees were required, severely restricting the number of laboratories that could use this system; furthermore, the endangered status of these animals makes it necessary to develop more practical models for future studies on HCV replication.

2) Hepatitis C virus-like particles (HCV-LPs)

Antibody-mediated neutralization of HCV has been suggested by a study of patients undergoing liver transplantation for HCV- and HBV-related liver cirrhosis. It was shown that polyclonal immunoglobulins administered for viral decontamination and also contain anti-HCV could reduce the risk of HCV infection in the transplanted liver (15). The role of neutralizing antibodies in protective immunity against HCV infection may be very important. Trials to generate recombinant HCV subunit vaccines have focused on the expression of parts of individual E1 or E2 proteins in the soluble form (16). However, this approach was not successful because the expressed viral proteins were not in the appropriate conformation. Several groups had described synthesis of HCV-LPs in insect cells using a recombinant baculovirus containing the cDNA of the HCV structural core, E1, and E2 proteins (17, 18). Other groups have reported the formation of HCV-LPs in mammalian cells (19, 20). Several studies have demonstrated that HCV-LPs contain E2 in a native conformation, resembling the properly folded E2 in the virion (21 – 23). Some previous studies have shown that antiviral antibodies in acute and chronic HCV infections interact with HCV-LPs. The advantages of HCV-LPs are that they are not infectious and can induce humoral and cellular immune responses and are therefore considered to be potential candidates for vaccine development (24 – 26). HCV-LPs cannot be secreted from insect cells; therefore, it is difficult to study the secretion of these particles using this system.

3) HCV replicon system

The development of subgenomic and genomic replicons, with self-replication of RNAs of the HCV to high levels in the human hepatoma cell line, Huh-7, was a major breakthrough (8, 27 – 29) (Fig. 1B (1)). This system was very useful for understanding the processes involved in viral replication and virus-cell interactions and also a useful means to test therapeutic targets. The replication capability of HCV seemed to be linked to the growth levels of the host cells, with the highest levels of HCV RNA produced from exponentially growing cells and the lowest from resting cells, a finding that has been supported by more than one study (30). Interestingly, HCV replicons seem to require some adaptive mutations for efficient RNA replication in culture (31, 32).

At first generation, HCV replicons were only constructed from molecular clones of genotype 1b. More recently, however, the establishment of HCV replicons originating from HCV genotypes 1a and 2a has also been reported (33, 34) (Fig. 1B (2)), creating opportunities for research on genotype-specific HCV replication.

Although replicons using the full-length HCV genome have been developed, particles had not been described. The replicons have a sequence coding for the selection marker in the HCV sequence, which might be difficult to package in the RNA. This was a one of limitations of the original replicon system.

4) Production of HCV particles in vitro

Recently, some groups have succeeded in producing HCV particles using in vitro systems. One group used DNA transfection. They constructed infectious HCV genotype 1b cDNA between two hammerhead ribozymes that were designed to generate the exact 5' and 3' ends of the HCV when cleaved (35) (Fig. 1B (3)). The point was “Do ribozymes indeed function in cell culture systems?”. They confirmed the sequences at both the 5' and 3' end by the method of rapid amplification of cDNA ends (RACE) (36). RNA extracted from the medium was studied and both ends were cleaved exactly as the ends of HCV sequences. In the culture medium, viral particles were visualized by electron microscopy. The buoyant density of these particles was 1.16 g/ml, which is consistent with the published density of free HCV particles (37). These results indicated that the cell culture medium indeed had HCV particles with HCV-RNA. Unfortunately, the infectivity of these particles was not satisfactory. Therefore, this model system was not useful for studying viral entry and the earliest events in the life cycle of the HCV, but nonetheless expected to be valid for investigating the later parts of the life cycle, specifically viral assembly and release. These steps had been not clear because until this strategy was developed, there had been no means to study the processes involved in the viral assembly and release.

Another group demonstrated that the full-length JFH-1 genome replicates efficiently and supports secretion of viral particles following transfection of RNA into Huh-7 cells (38). The culture supernatant of these cells could infect chimpanzees and be subpassaged to naive Huh-7 cells. This JFH-1 strain came from a fulminant

hepatitis patient and belonged to genotype 2a (39). It was demonstrated that the sequence of JFH-1 showed some deviation from other genotype 2a strains derived from chronic hepatitis patients and that JFH-1 does not require adaptive mutations for replication. One group constructed a chimeric JFH-1 genome containing the core with the nonstructural protein 2 (NS2) region of the HCV strain J6, another genotype 2 strain. This genome, FL-J6/JFH, replicated and produced a high level of infectious viruses in cell culture (40). In this system, HCV replication was inhibited by IFN alpha and several other HCV-specific antiviral compounds (41). Based on these results, these models are considered to represent powerful tools for studying the HCV life cycle and developing novel antiviral strategies against HCV in the future.

Very recently, the production of infectious HCV particles from a highly adapted H77 genome (called H77-S) that contains a total of five adaptive mutations was reported (42). However, the specific infectivity of the H77-S particles was significantly lower (~400-fold) than that of the genotype 2a virus strain described above.

In another newly developed system, cDNA plasmids of various genotypes were constructed and their infectivity and replication levels were evaluated (43). HCV particles of various genotypes could be produced, including 1a (H77), 1b (CG1b), and 2a (J6 and JFH-1), using the HCV-ribozyme system. The constructs also contained the secreted alkaline phosphatase gene as a control to assess transfection efficiency and effects of culture conditions. The cell-culture-generated HCV was infectious in chimpanzees and could be subpassaged to naïve Huh-7 cells. This system has the advantage that it is based on DNA expression plasmids, much easier to manipulate, and contains a reported gene to monitor various culture conditions. Among the four genotypes, the JFH-1 strain replicated significantly and more efficiently than other strains.

The JFH-1 appeared to replicate very efficiently in all the systems, so what are the unique characteristics of the JFH-1 strain? Is it a rare exceptional HCV strain possessing several properties not shared by other genotype 1 isolates? We do not have a satisfactory answer for this question at present; however, this system serves as a valuable tool for studying the replication and pathogenesis of HCV.

5) Animal models

While animal models are not in vitro systems, there have also been some breakthroughs in animal studies, and a few are presented here. For the study of HCV, the chimpanzee is the only useful animal, but it is very expensive and only restricted use of this animal is allowed because of their endangered animal status. A simple small-animal model of HCV infection is, therefore, urgently needed. Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice with engrafted human hepatocytes (44). This model was improved to generate a human hepatocyte chimeric mouse where mouse hepatocytes were extensively replaced with human hepatocytes (45). This mouse model seems to be useful for the study of HCV virology. In fact, very recently, genotype 1a and 2a HCV engineered with a human hepatocyte chimeric mouse system has been reported (46).

How can basic research on HCV be applied to clinical medicine?

The HCV life cycle can be simplified into three steps (Fig. 2): i) cell attachment and entry; ii) replication, including protein translations and processing and RNA replication; and iii) virion assembly and release. The third step of virion assembly and release was unclear until quite recently, as there had been no satisfactory means to study this step. HCV-LPs can be produced, but these particles are not successfully released from the cells. However, currently, production of HCV particles using in vitro systems is proving to be useful for the research on virion release. If we could elucidate this step, the information can be used to design new types of antivirus agents.

For experiments on cell attachment and entry [step (i)], HCV-LPs and HCV virions produced artificially with the JFH-1 replicon or HCV-ribozyme transfection systems are appropriate. HCV-LPs have been reported to bind to several human hepatic (primary hepatocytes, HepG2, Huh7, and NKNT-3) cell lines (47). After binding to cells, HCV-LPs are internalized into the cytoplasm, and this process could be inhibited with anti-E1 or anti-E2 antibodies. HCV-LPs are believed to be good candidates for the development of HCV vaccines; however, satisfactory results have not been obtained until now. The glycosylated form of the envelope proteins in HCV-LPs was different from the E2 expressed in mammalian cells (24), but the particles derived from JFH-1 or DNA transfection are considered to represent authentic virions, which are therefore expected to be good candidates for producing HCV vaccines. Both systems need improvements for producing the particles more efficiently, and a purification technique needs to be established.

HCV replicon systems are very helpful for investigating virus replication. Simply stated, if we want to

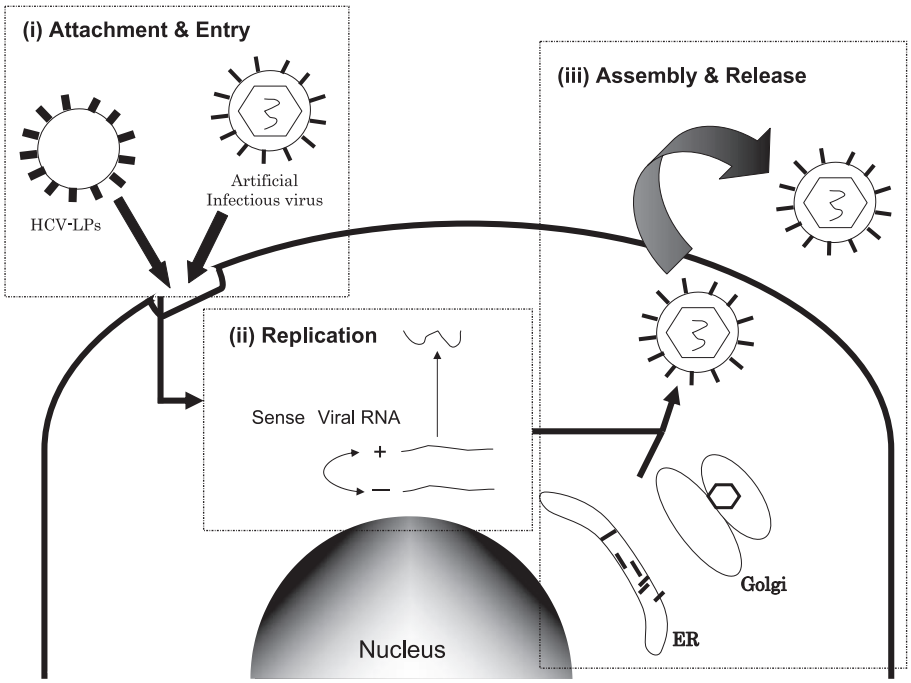

Fig. 2. A cartoon depicting the HCV life cycle. (i) Cell attachment and entry; HCV-LPs or the system of artificial HCV infectious virion production is very useful for studying this step. (ii) replication, including protein translations and processing and RNA replication; the replicon system is very useful for studying this step. (iii) virion assembly and release; the system of artificial HCV infectious virion production is very useful for studying this step. ER: endoplasmic reticulum

examine the ability of an agent to inhibit HCV replication, the agent simply needs to be added to the HCV replicon culture medium and the expression level of the HCV protein evaluated. Many different HCV replicons have been generated, including replicons with reporter genes (such as the firefly luciferase) that allow fast and reproducible screening if large series of compounds are evaluated (48, 49). Recently, protease inhibitors were developed as antiviral agents against HCV and applied to the replicon system to examine their ability to inhibit HCV replication (50, 51).

How can the HCV-ribozyme plasmid transfection system be used? Of course, it can be applied for screening of newly developed drugs too, but the replicon system appears to be more convenient for this purpose. The advantage of the HCV-ribozyme plasmid system is that it is easy to use for recombination. Until today, many replicons derived from different genotype 1 isolates have been developed, but a replicon from another genotype (2a) was established only recently. On the other hand, three different genotypes, that is, the 1a, 1b, and 2a genotypes, have been constructed with the HCV-ribozyme plasmid system (43). Each of the constructs yielded infective particles. This system is expected to enable examination of the characteristics of the viruses in each patient, if their sequences can be incorporated in the HCV-ribozyme plasmid. Long RT-PCR (LRP) amplification of RNA templates is sometimes difficult, but recently, a protocol has been developed for robust amplification of a near full-length HCV genomic sequence from clinical samples, followed by efficient cloning (52, 53). If we can combine these two methods efficiently, we can tailor DNA plasmids for individual cases. It is hoped that the systems would eventually allow tailor-made treatments to be developed for HCV infection.

Conclusion

The subject of this session is ‘From molecular mechanisms to therapeutic strategies’. Development of the HCV in vitro system is one of the representative subjects. Especially, advances in basic research on HCV and development of antiviral therapies have been made with the development of the replicon cell culture model in recent years. There is a fair possibility of success in the development of an HCV vaccine, although several problems still need to be resolved. For instance, one group showed that there was a high incidence of HCV reinfection within injectable drug users (54). This report suggests that protective HCV vaccines should be based on epitopes from multiple genotypes.

Until now, many strategies for HCV basic research have been developed, and they are expected to play an active role in clinical medicine.

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