REVIEW

Advances in Genomic Research on Hepatitis C Virus with a Useful Tool, Replicon System

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Abstract: The research for hepatitis C virus (HCV) has long delayed by missing of *in vitro* culture system. Since the development of replicon system, a replication system of subgenomic HCV RNAs in a hepatoma cell line, has been reported, many virological and clinical findings have been discovered. Recently, in addition of subgenomic replication system, hepatitis C virus full-length RNA replication has been possible, and a few cell culture systems producing viral particles have been produced. These developments enabled us to investigate the life cycle or intracellular circumstance of HCV production. By screening of newly synthesized drugs with this replicon system, several possible medicines have been established and clinical researches are now running. Among them, VX950 and SCH503034 are nearest to clinical use. Other possible agents for reducing viral replication such as cyclophyllin inhibitors, inhibitors of sphingomyelin synthesis, HMG-CoA reductase inhibitors, or RNA-dependent RNA polymerase inhibitors have been also investigated. Furthermore the mechanism for development of hepatocellular carcinoma in the HCV infected liver has been vigorously studied using the HCV replicon system. (Keio J Med 57 (2) : 75–83, June 2008)

Key words: hepatitis C virus, replicon, VX950, lipid raft, Rb

Introduction

Hepatitis C virus (HCV) belongs to the Hepacivirus genus of the *Flaviviridae* family. It is a spherical particle, 55 to 65 nm in diameter, with an envelope, and consists of the genome of a 9.6-kb plus strand RNA. It has short untranslated regions at both the 5'- and 3'- termini, and the middle region, which accounts for about 95% of the whole genome, encodes a precursor protein consisting of roughly 3,010 amino acid residues. The coding regions of viral structural proteins such as core as well as E1 and E2 are located at the N-terminus, while the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B are situated at the middle region near the C-terminus.^{1,2} HCV is categorized according to 6 genotypes and is further classified into several subtypes.

HCV is transmitted through blood and it often causes persistent infection. Post-infection symptoms are often

mild. Infected subjects develop chronic hepatitis from a carrier state, and eventually, liver cirrhosis and hepatocellular carcinoma (HCC) over period of up to 30 or more years. In Japan, it is estimated that there are 1.5 million HCV-infected people, and infection is more common among the elderly than among younger individuals. About 30,000 patients die of HCC associated with HCV infection, and HCC is currently ranked as the fourth leading cause of death. The ideal treatment is eradication of HCV from the body, and a combination of interferon (IFN) and ribavirin (RBV) is the current mainstream regimen.³

Presence of the hypervariable region (HVR) at the N-terminus of the E1/E2/NS1 regions which is responsible for surface structure, is thought to play a role in persistent HCV infection. Nevertheless, heterogeneity of the HVR does not correlate with hepatitis activity.⁴ With regard to HCV eradication by IFN, analysis of the viral

Fig. 1 The structure of HCV genome, that of HCV full-length replicon and subgenomic replicon

sensitivity to IFN revealed the presence of the IFN sensitivity-determining region (ISDR), which influences sensitivity to IFN, at the center of NS5A, a non-structural protein of HCV.^{5,6} Meanwhile, the number of amino acid mutation in the ISDR has been reported to have a significant negative correlation with the amount of HCV RNA.⁷

Since the replicon system has been developed, virological aspects of HCV including its life cycle were uncovered, and development of this system and more recently of a cell culture system has enabled the development of HCV-specific inhibitors and quantitative measurement of antiviral potency. Many of the newly developed inhibitors are under investigation in pre-clinical and clinical trials, and these specific inhibitors will improve treatment opportunities of patients with chronic hepatitis C, especially in difficult-to-treat patients including patients who do not respond to Peg-IFN and RBV combination therapy. In this mini-review, we introduced the HCV replicon system and summarized newly developed virological and clinical discoveries, following to the development of replicon and cell culture systems.

Cells Replicating an HCV Gene-replication Unit (replicon)

In 1989, a group led by Houghton *et al.* at Chiron (a subsidiary of Novartis AG, Basel, Switzerland) succeeded in cloning the HCV genome for the first time.⁸ In 1997, Rice *et al.* successfully established an infectious

clone from cloned cDNA, which induced acute hepatitis in chimpanzees.⁹ Chimpanzees which are the only reliable animal model for HCV infection, are difficult to obtain and expensive to maintain and are therefore not suitable for drug screening studies. As a result, the development of efficient replication systems in cultured cells has been eagerly anticipated.

In 1999, Bartenschlager *et al.* established the first culture system in which HCV subgenomic RNA replicated and maintained itself and in which HCV gene replication was efficiently reproduced.¹⁰ This system was referred to as an HCV replicon system. The structure of HCV replicon RNA is shown in Fig. 1. In HCV subgenomic RNA, a structural protein region encoding the viral particle is replaced with the code sequence of aminoglycoside phosphotransferase (Neor) that detoxifies neomycin harboring cytotoxicity. The internal ribosome entry site (IRES) derived from encephalomyocarditis virus (EMCV) is inserted at the down-stream region of the Neor gene. Due to this sequence, translation starts within RNA, and non-structural protein, which is located downstream and involved in HCV gene replication, is efficiently translated.

RNA synthesized *in vitro* can be introduced into HCC-derived HuH7 cells, and with RNA introduced cells can be selected with neomycin. The HCV genome sequence that can self-replicate in cells, and cells that enable the self-replication, are selected and replicon cells are thus obtained (Fig. 2). To date, similar replicon cells have been established with several different HCV genome se-

Fig. 2 Outline of HCV replicon system. Subgenomic replicon RNAs are produced from plasmid including T7 promoter by *in vitro* transcription using T7 RNA polymerase. These RNAs are transfected into the human hepatoma cell line Huh-7. Only cells in which the replicon self-replicates will carry the gene encoding neomycin phosphotransferase, which inactivates G418, to become resistant to G418.

quences.^{11–13} Thus, the development of the HCV replicon system has enabled the analysis of virus genome replication at the cellular level.¹⁴

Research on the HCV Genome Replication System

Research on HCV genome replication started with analysis of the RNA genome structure in replicon cells. To date, it has been determined that a phenomenon called adaptive mutation occurs in the RNA genome in cultured cells.¹⁵ This is presumed to be a result of selected amino acid mutation in HCV protein so that RNA can efficiently replicate and proliferate in cultured cells. The RNA structure required for genome replication has been identified by artificial introduction of mutation (e.g., various deletion mutations) into HCV subgenome RNA. Furthermore, replication systems are currently indispensable for elucidation of the pathogenesis of HCV infection and development of antiviral drugs. We previously found differences in specific sites in the HCV NS5B region between patients that did or did not respond to IFN-RBV combination therapy,¹⁶ and are now exploring alterations using the replicon system *in vitro*.¹⁷

Full-length HCV RNA Replication System

While the subgenomic replicon *in vitro* system is used to express non-structural proteins necessary for replication, not only non-structural proteins but also structural proteins are actually expressed *in vivo*. The influence of HCV on host cell factors, such as IFN, other cytokines,

and signal transduction, are complex events mediated by full-length HCV proteins. Different full-length replicon RNAs have been produced by some research groups.^{18,19} In the full-length HCV RNA replication system, the presence of double strand RNA (dsRNA), consisting of the HCV RNA plus strand and the minus strand, an intermediate replication product, enables easier evaluation of the influence of HCV, including the effect of dsRNA, on host factors, compared with a replicon system with non-structural regions using a variety of promoters. Since structural proteins are required for the production of viral particles, full-length HCV RNA replication systems have advanced research on *in vivo* properties of the virus, including packaging, budding, and infection^{20–24} (Fig.3).

Structure of the Replication Complex

Intracellular environments for HCV RNA synthesis and replication were examined in the subgenome replicon system, and it was found that replicon HCV RNA was localized in the cell membrane structure, especially at endoplasmic reticulum membrane, and partly in the region surrounded by non-structural protein-like lipid membrane, while most of the non-structural protein was present on the cytoplasmic side of the lipid membrane.²⁴ This suggests that replicon RNA replicates in a structure surrounded by lipid membrane, i.e. lipid raft. Similar results have also been reported with regard to RNA replication of plant viruses such as bromomosaic virus. Intracellular environments surrounded by lipid membrane

Fig. 3 HCV life cycle

- Virus binds to host cell receptors (CD81 and other unidentified candidates, such as low density lipoprotein receptor (LDL-R) and scavenger receptor class-B type-I (SR-BI)) and enters by endocytosis.
- After uncoating nucleocapsid, IRES-mediated translation and polyprotein processing occurs on the endoplasmic reticulum (ER).
- RNA replication: formation of a replication complex on the altered ER membrane, and transcription and synthesis of (-) strand RNA, which serves as a template for progeny (+) strand RNA.
- Packing and assembly: structural protein (E1, E2 and core) and progeny (+) strand RNA assemble.
- Virion maturation and particle release.

are potentially those for the HCV genome replication complex.^{26,27}

JFH-1 Strain Replicon and Production of Infectious Virus Particles

Wakita *et al.* cloned an HCV gene from serum of a patient with fulminant hepatitis, a rare complication of type C chronic hepatitis, and designated it as the JFH-1 strain genotype 2a. Subgenomic²⁸ and full-length replicons²⁹ were constructed from the JFH-1 strain, and production of viral particles in the culture supernatant of HCV RNA replicating cells was confirmed. In addition, an HCV culture system with even higher infection efficiency was established by combination of the JFH-1 strain and cured cells established from replicon cell experiments.^{30,31} However, the onset of fulminant hepatitis is extremely rare in even HCV genotype 2a cases, suggesting that JFH-1 strain was established from a very rare clinical case. The viral particles released from the JFH-1 replicon can infect chimpanzees but do not cause hepatitis. The establishment of JFH-1 strain which forms infectious viral particles opened a black box of HCV virology and biology. The role of CD81 and heparin sulfate proteoglycan on HCV entry into the cell has been demonstrated using this system recently.³¹ On the other hand, in Japan, the most prevalent HCV subtype is genotype 1b, and the virus is most resistant to eradication by IFN in patients

with this genotype (1b). It is anticipated that a replicon that produces infectious viral particles derived from genotype 1b HCV will be established.

Research and Application of Replicons and the Full-length HCV RNA Replication System: Screening of New Drugs with Replicons

Pegylated-IFN (Peg-IFN) in combination with RBV is the current mainstream treatment for chronic hepatitis C, however, such therapy has several limitations which remain to be overcome. Although viral eradication rates have been improved by the combination of Peg-IFN and RBV compared with IFN monotherapy, the treatment is effective in only half of patients infected with genotype 1b HCV with a high viral load. HCV dynamics at the early treatment phase is associated with treatment efficacy Peg-IFN and RBV combination therapy, and early suppression of viral replication is thought to be important.³³ In addition, adverse effects and lower response rates can be a hindrance in the treatment of elderly patients.³⁴ To solve these problems, screening with replicons is being used for the development of new anti-HCV agents to replace Peg-IFN and RBV in clinical practice.

<Development of new treatments targeted at host factors>

Shimotohno *et al.* reported that cyclosporine A (CyA),

an immunosuppressant widely used in clinical practice, suppressed HCV replication in the replicon system.³⁵ This HCV replication suppression is not related with the cytotoxicity of CyA or the function as an immunosuppressant, but is a specific action of the replicon cells against HCV protein. The underlying mechanisms, binding to cyclophyllin and inhibition of peptidylprolyl isomerase enzyme activity, are thought to inhibit HCV replication.^{36,37} It has been reported that CyA is effective against HCV *in vivo*,^{38,39} which has generated clinical interest.⁴⁰

As mentioned previously, the HCV replication complex is created in the endoplasmic reticulum membrane, and an HCV life cycle is formed. It has been suggested that HCV replication can be regulated by targeting several molecules in the lipid raft, consisting of high-fluidity lipids such as saturated fatty acids, cholesterol and sphingomyelin, in the area of high-fluidity portion formed with unsaturated fatty acid bi-layered membrane. One study reported suppression of HCV replicon replication by myriocin, which interferes with the sphingomyelin synthesis pathway.⁴¹ In addition, it was reported that 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors suppress HCV replicon replication.⁴²

<Development of new treatments targeted at viral factors>

The development of new drugs targeted at NS3 encoding serine protease involved in viral replication³⁸ and NS5B encoding RNA polymerase⁴³ is another strategy that has been pursued. These drugs, which are expected to directly exert antiviral activity, referred to as specifically-targeted antiviral therapies (STATCs). It has been reported that serine protease activity encoded by NS3 inhibits not only the processing of viral protein but also IFN signal transduction⁴⁴, and clinical trials are ongoing for clinical application of drugs targeted at this site. VX950^{45,46} and SCH503034^{47,48} are among the NS3 protease inhibitors under development. Since HCV is highly mutable, monotherapy using one of these drugs results in immediate appearance of a resistant clone.^{49,50} Therefore, clinical trials on VX950 with or without IFN and in combination with RBV are ongoing in the U.S. and European countries. Similarly, clinical trials of SCH503034 with and without IFN are underway in patients refractory to a combination of Peg-IFN- α 2b and RBV.

NM283, an RNA-dependent RNA polymerase inhibitor, was found to be effective in chimpanzees and cultured cells. However, since monotherapy with this agent was not fully effective, combination with IFN was required and as a result, adverse effects due to high-dose administration were reported.^{51,52}

Since toll-like receptor 9 agonists likely exert antiviral activity by enhancing intrinsic IFN production, their ad-

ministration in combination with Peg-IFN and RBV improved early virological response rates.⁵³ Moreover, inhibition of HCV replicon with siRNA has been reported.⁵⁴ Treatment with ribozyme and antisense oligonucleotides seems to be effective in replicons, however, because of issues with stability and drug delivery, they have not yet been sufficiently developed for clinical use as anti-HCV agents.

Table 1 summarized several treatment targets and ongoing preclinical and clinical trials.

Regulation of Host Genes by HCV Protein

To date, it has been reported that viral proteins, such as E1A⁵⁵ and E1B⁵⁶ of adenovirus, human immunodeficiency virus type 1 Tat protein,⁵⁷ HTLV-1 Tax protein,⁵⁸ human papilloma virus E6⁵⁹ and E7⁶⁰ protein, regulate transcriptional activity of host cells by influencing transcription regulating factors such as CBP/p300, PCAF, histone deacetylase complex, and SRC-1. On the other hand, it has been reported that HCV core protein upregulates transcription of retinoic acid receptor- α (RAR- α) by isolating Sp110b, a transcriptional inhibitor of RAR- α , from intranuclear space to the surface of endoplasmic reticulum.⁶¹ RAR- α is known as a receptor for retinoic acid-induced apoptosis. Similarly, it is possible that HCV protein regulates various other host genes.

HCV and Carcinogenesis (Rb gene and HCV)

The precise mechanism underlying the development from HCV infection to carcinogenesis has not been elucidated, but a relationship with several oncogenes has been proposed. Unlike hepatitis B virus (HBV), integration of the HCV gene into host cell DNA has not been recognized. Therefore, there are possibilities that HCV protein produced by HCV infection affects functions of host cells and heightens carcinogenicity.

Recent studies have revealed that HCV infection affects the cell cycle regulating protein Rb. Rb protein affects cells in the G1-S phase⁶² and inhibits transcription factor E2F.⁶³ In addition, the protein is well known as a tumor suppressor gene, and mutation in the Rb gene has been reported in lung and ovarian cancer cells.^{64,65} We reported that the Rb gene bound to a specific site in the NS5B-encoding polymerase required for HCV replication, which suppressed the expression of Rb protein.⁶⁶ The HCV sequence for binding was the same as the sequence previously reported in adenovirus⁶⁷ and papilloma virus⁶⁸ that bound the Rb gene.⁶⁹ These oncogenes are thought to inhibit Rb gene function and the LxCxE motif by directly binding with them (Fig. 4 and 5), which suggests the involvement of HCV in carcinogenesis.

Table 1 Targets of therapy and emerging therapies in HCV

Target at viral factors	Virus entry	anti E2 monoclonal antibody HCV-AB(XTL)68
	Post translation processing	NS3/4A serine protease inhibitors VX950 (Telaprevir; phase 2), SCH503034 (Boceprevir; phase 2), ITMN-191 (phase 1)
	HCV replication	NS5B RNA dependent RNA polymerase inhibitors (a) nucleoside analogues NM283 (Valopicitabine; phase 2), R1626 (prodrug of R1479; phase 2), MK-608 (preclinical), R1656, R7128 (preclinical) (b) non-nucleoside analogues HCV-796 (phase 2), BILB1941 (phase 1), A-837093 (preclinical), GS-9190 (phase 1)
	HCV RNA translation	NS5A inhibitors A-831 (phase 1), A-689 (preclinical) ribozyme antisense oligonucleotide siRNA
Target at host factors	Virus entry	hepatitis C immunoglobulin Civacir
	Replication	Cyclosporin inhibitors DEBIO-25 (phase 1), NIM811 (preclinical) Myoricin, HMG-CoA reductase inhibitors
	Production of IFN	TLR9 agonist
	Virus particle release	ER glucosidase inhibitors Celgosivir (preclinical)

In addition of these therapies, several interferons have been on going as clinical studies (Albinterferon; phase 3, Pegamax; phase 1, Locteron; phase 1/2, Omega interferon; phase 2).

...LXCXE...

Fig. 4 Various viruses which regulate Rb gene and E2F gene. Rb protein binds to a transcriptional factor E2F and inhibits its transcriptional activity. There are 6 kinds of E2F proteins in mammal genome, and they are divided into 3 categories depending to their transcriptional activity. Rb protein inhibits high active E2F1, E2F2 and E2F3. This E2F-dependent transcriptional inhibition of Rb protein is regulated by chromatin remodeling complex such as SWI/SNF complex, histone deacetylase polycomb group and methylase. Papilloma virus, adenovirus and SV40 T antigen also attenuate Rb protein-induced E2F inhibition. HDACs: histone deacetylases

Fig. 5 Molecular mimicry of amino acids between papilloma virus and HCV. LxCxE domain of human papilloma virus E7 protein and 314–318 amino acids alignment of NS5B region of HCV (Con1 strain and N strain). This region of HCV contains GDD sequence necessary for viral polymerase activity.

Conclusion

The aim of HCV eradication is to eventually reduce the occurrence of HCC. It is therefore important to elucidate the mechanisms by which HCV infection precipitates the onset of HCC. Clinical research in the past years has focused on the improvement of IFN-based treatment regimens. HCV-specific antiviral compounds, some of which have been developed by investigation using the replicon system, are a new perspective in the treatment of chronic hepatitis C. Since the viral RNA-polymerase possesses a high error rate, HCV variants are continuously produced during replication, and selection of drug-resistant HCV strains may occur when viral replication continues. On the other hand, innate and adaptive immune response play an important role in the control of HCV infection, but these responses are hampered by several mechanisms in HCV infection, resulting a weak immunologic response for complete elimination of HCV infected hepatocytes. The replicon system is not a sufficient tool for investigating such an immunological mechanism. A potentially safe and stronger treatment for chronic hepatitis C by combination of several drugs with different mechanisms is expected especially in Japan, where the patients is becoming older, and *in vitro* replicon system will be still expected to produce a useful information for making a new drug design.

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