Infectious synthetic HCV transcripts

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Hepatitis C is a common infectious disease. The global prevalence of chronic hepatitis C is estimated ranging from 0.1% to 5% in different countries (average 3%). Although hepatitis C virus infection is oftenly asymptomatic or clinically mild, progression to chronicity occurred at about 85% of the patients (Hoofnagle, 1997). One of the most important reasons for the incapability of host's immunologic defences to clear the virus which resulted in the persistent infection is the continuous changes of sequence of the viral genome leading to sequence heterogeneity (Weiner *et al.*, 1992). The sequence heterogeneity is not distributed evenly throughout the genome. The most variable region is the gene coding for the viral envelope 2 protein where the neutralizing epitope is believed to be located. Therefore, patients can not be protected from (re)-infection of other mutated HCV strains even certain neutralizing antibodies have been developed. This makes it extremely difficult to develop a vaccine for prevention of HCV infection. Whether the severity of the disease and outcome of therapy are also related to the genetic heterogeneity of virus remains unclear.

HCV infection is also regarded as one of the high risk factor for the development of hepatocellular carcinoma (HCC). In some areas, 50-75% of HCC is associated with HCV infection, while in other regions, less than 20% of HCC patients were found to be anti-HCV positive (Hadziyannis *et al.*, 1995). Generally, HCC is considered as the result of cirrhosis, the major long- term serious complication of chronic HCV infection. However, the possible direct carcinogenic effects of HCV have been reported (Ray *et al.*, 1996).

Currently, the treatment for HCV infection is limited to the use of interferon-alpha or beta or interferon-alpha combined with ribavirin. The response rate of the therapy is still relatively poor. In order to develop more efficient new therapeutic agents for HCV infection, efforts are being made to establish a drug screening system efficiently supporting HCV replication. During the last few years, in vitro cell culture systems that permit HCV replication have been reported by infecting human T- and B-cell lines (Shimizu *et al.*, 1992 ; Bertolini *et al.*, 1993), human fetal hepatocytes (Iacovacci *et al.*, 1993), chimpanzee primary hepatocytes (Landford *et al.*, 1994), human primary hepatocytes (Ito *et al.*, 1996), non-neoplastic human hepatocyte line (Kato *et al.*, 1996) and human hepatoma cell line (Seipp *et al.*, 1997) with plasma or serum of infected chimpanzees or patients as infectious inoculum. In these systems, however, HCV replication appeared low, fluctuated and transient. In this regard, it is believed that the presence of relatively low titer of infectious particles and the presence of neutralizing antibodies in the inoculum used may contribute to the low level of infection in vitro.

HCV is an enveloped positive single-stranded virus that belongs to Flaviridae family (Takamizuwa *et al.*, 1991). Its genomic RNA is putatively the template for viral protein translation. Introduction of in vitro transcribed RNA into the host cells should be able to initiate the viral replication and the production of infectious viruses. This approach has been proved to be successful in other RNA viruses (Lad *et al.*, 1991 ; Vassilev *et al.*, 1997). Because of the quasispecies nature of HCV, it is important, however, to identify the infectious sequence for such a purpose.

During the last two years, four infectious HCV cDNA clones have been reported. Two of them were derived from genotype 1a (H77). In these two studies (Kolykhalov *et al.*, 1997 ; Yanagi *et al.* 1997), transcripts whose sequences representing the consensus amino acid sequences were infectious for the chimpanzees, while the others were not. Yanagi *et al.* (1998) have described an infectious chimeric cDNA clone whose genome encodes a polyprotein of genotype lb (HC-J4) of HCV and replicates via 5'- and 3'- UTR of a genotype la isolate (H77). Three transcripts from different clones were injected into the chimpanzee. Only one sequence of these clones could be recovered from the chimpanzee serum at week 2 and 4 p.i., suggesting this is the infectious clone. This infectious clone contains the amino acid sequence that was different from the consensus sequence at three sites ; while other two noninfectious clones had seven and nine mutations respectively compared to the consensus sequence. The noninfectivity of those two clones may be due to the mutations that occurred at the highly conserved residues which could be lethal to the virus, but not as the result of the presence of more mutations. In the last report, an HCV genotype 1b cDNA clone was constructed (Beard *et al.*, 1999). The cloned sequence had 152 substitutions compared to the consensus sequence derived from 20 published com-

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plete HCV genotype 1b genomes. Fifteen of them which were unique to other HCV isolates and graded as non-conserved substitutions were repaired to the consensus amino acid by site-directed mutagenesis. Additional four amino acids insertion in the NS5A region was considered acceptable. The transcript from this clone was infectious as the chimpanzee inoculated by this clone exhibited intermittent viremia over 14 weeks and developed anti-HCV antibodies after injection.

The infectious HCV cDNA clone is an excellent tool for the study of viral gene structure, gene function, replication, host tropism and pathogenesis of HCV. More infectious cDNA clones will be required for detailed molecular characterization of the virus and then obtaining the information of interesting genes for design new antiviral agents and broad protective vaccines. The specific infectivities of the synthetic RNAs from the 4 reported HCV cDNA clones were all tested in chimpanzees. However, this animal model is very expensive and has limited availability. More convenient model is required for identification of infectious RNA transcripts and for comparing the infectivity of RNAs with authentic and mutated sequence.

The strategy of infectious synthetic transcripts was also used for establishing a long-term in vitro cell culture system for HCV propagation. There are several advantages for using infectious synthetic RNA as "inoculum". First, the infectious synthetic RNA can be provided at a much higher titer than those of HCV RNA presented in the patient serum. Secondly, the transcripts only contain one single infectious sequence while in the patient serum many lethal sequences co-exist with the infectious one. Furthermore, there are no antibodies and other factors in the transcripts that may block the infection. It has been reported that transfection of synthetic HCV RNA into human hepatoma cells lead to the production of progeny viruses (Yoo *et al.*, 1995 ; Dash *et al.*, 1997). Viral antigens and replicative intermediate could be detected. The culture supernatant could successfully infect the fresh naive cells.

A full-length HCV cDNA clone has been constructed in our laboratory. The viral genome was isolated from serum of a patient infected with HCV (genotype 1b). Three overlapping fragments covering the entire HCV sequence were combined by subcloning to make the fulllength HCV cDNA clone. The cloned sequence contains HCV genome of 9553 nucleotides. The 5'-UTR region showed a highly identity to those of other HCV sequences (genotype 1b). The 3'-UTR region consisted of 39-nt conventional sequence, 57-nt poly (U-UC) stretch and a 98-nt X sequence whose sequence identity was 97% against other published genotype 1b clones. The ORF encoded a polyprotein which is approximately 96% identity to the consensus HCV infectious sequences. The recognition sites of both host signalase and viral proteinase were quite conserved. The important motifs and cofactors of the viral enzymes (RNA-dependent-RNA polymerase, helicase and proteinase) were also highly conserved. The infectivity of this clone is still the subject of on going experiments. This cDNA clone after proving of infectivity would provide a tool for establishment of long-term in vitro cell culture system for HCV propagation and better understanding of molecular biology and pathogenesis of HCV.

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