論文の内容の要旨

論文題目　In Vivo Adaptation of Hepatitis C Virus for Efficient Virus Production and Evasion of Cytokine-Induced Cell Death

和訳　C 型肝炎ウイルスの生体内適応変異によるウイルス生成能の増強とサイトカインにより誘導される細胞死の抑制

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ABSTRACT

Hepatitis C virus (HCV) causes persistent infection in approximately 75 % of infected individuals leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma; however the mechanism of viral persistence remains to be determined. Efforts to elucidate this mechanism were hampered by the lack of HCV strain having the ability to establish infection in *in vivo* as well as *in vitro* settings. This problem was solved recently with the identification of an HCV clone, JFH-1, from a fulminant hepatitis patient in Japan. This clone was found to establish infection in chimpanzees as well as in cell culture. In the previous study, the original patient serum from which the HCV JFH-1 strain was isolated and the cell culture generated JFH-1 virus (JFH-1cc) were inoculated into two different chimpanzees. JFH-1cc infected chimpanzee cleared the infecting virus at 8 week post inoculation, but the virus persisted for 34 weeks in the JFH-1 patient serum-infected chimpanzee. Sequence analysis of the infecting viruses isolated from JFH-1cc infected chimpanzee at week 2 and 7 while from serum-infected chimpanzee at week 2, 19 and 23 post-inoculation showed increasing

number of mutations with the progression of infection. In JFH-1cc infected chimpanzee, no mutation could be detected in infecting virus at week 2, however the virus accumulated 7 non-synonymous mutations at week 7 post-inoculation. On the other hand, in the serum-infected animal, 6 non-synonymous mutations were detected as early as two week after infection and this number kept on increasing with the passage of time eventually reaching to 17 at week 23. I hypothesized that these mutations might have played role in the survival of the virus for longer time in the body of chimpanzee. The present study was designed to investigate the *in vitro* and *in vivo* characteristics of the JFH-1 variants isolated from infected chimpanzees at early and late stages of infection. I generated full-genome JFH-1 constructs with the mutations detected in patient serum-infected chimpanzee at week 2 and 23 post-inoculation and named them JFH-1/S1 and S2 respectively. Similarly, JFH-1 construct with the mutations identified in JFH1cc-infected chimpanzee at week 7 was generated and named JFH-1/C. HuH-7 cells were transfected with *in vitro* transcribed RNA of these constructs and intra- and extracellular HCV RNA and core antigen (Ag) levels were measured at different time points after electroporation. JFH-1/C and JFH-1/S2 displayed lower intracellular RNA and core protein levels as compared to JFH-1/wt and JFH-1/S1, respectively, demonstrating the lower replication kinetics of these strains. On the other hand extracellular RNA and core protein levels of these constructs were significantly higher than JFH-1/wt and JFH-1/S1 respectively, indicating that mutations emerged in these strains might have influenced the later steps of virus life cycle i.e. assembly and/or release. To clarify the effect of these mutations on specific steps of virus life cycle, I performed single cycle virus production assay with a CD81 negative cell line called Huh7-25. Upon electroporation of HCV RNA, these cells assemble and release infectious virus particles into the cultured medium but this progeny of viruses is unable to re-infect the surrounding cells due to the lack of entry receptor. I electroporated *in vitro* transcribed RNA of JFH-1/wt, S1, S2 and C into these cells and measured intra- and extracellular HCV RNA, core Ag and virus infectivity titers at day 3 post-electroporation. The intracellular RNA and core Ag levels of JFH-1/C and S2 were significantly lower than that of JFH-1/wt and S1 respectively, suggesting lower replication efficiency of these strains. However the intracellular infectivity titers of JFH-1/C and S2 were higher than those of JFH-1/wt and

C respectively. Intracellular specific infectivities of JFH-1/C and S2, denoting the number of viruses assembled by these strains for each copy of RNA, were significantly higher than those of JFH-1/wt and S1 respectively, indicating the higher capacity of these strains to assemble infectious virus particles. Subgenomic replicons of JFH-1/C and JFH-1/S2 also showed lower replication efficiency as compared to JFH-1/wt, thus confirming the attenuated replication potential of the variants isolated from chimpanzees at later stages of infection. As JFH-1/S2 has shown more pronounced divergence from JFH-1/wt in terms of ability to replicate and assemble infectious virus particles, I decided to further dissect the mutations responsible for lower replication and enhanced assembly of this strain. For this purpose, I generated the chimeric constructs JFH-1/S2-wt and JFH-1/wt-S2 by replacing core-NS2 region or NS3-NS5B region of JFH-1/wt with the corresponding regions of JFH-1/S2 respectively. I transfected their RNA into Huh7-25 cells and measured intra- and extracellular HCV RNA, core protein and virus infectivity titers on day 3 post electroporation. Analysis of the results showed that the mutations present in the core-NS2 region were responsible for increasing the assembly of the JFH-1/S2 while mutations in NS3-NS5B region decreased replication potential of this virus.

I hypothesized that phenotype of lower replication and higher spread, acquired by JFH-1/S2, might have given this virus advantage to survive against the host defense system. In the liver, HCV-infected hepatocytes are eliminated by targeted apoptosis induced by the different cells of the immune system with ligand- and receptor-mediated signals such as TNF-alpha, FasL, and TNF-Related Apoptosis Induction Ligand (TRAIL). In the previous study, HCV specific T-lymphocytes were detected in the blood of both chimpanzees and their activity correlated with the profiles of viremia. Thus I decided to examine the susceptibility of JFH-1 variants to TNF-alpha or FasL mediated apoptosis. I further confirmed my results by using UV irradiation to induce apoptosis. TUNEL assay of TNF-alpha treated cells showed that JFH-1/S1 and JFH-1/C were almost as equally susceptible to apoptosis as JFH-1/wt. On the other hand, cells transfected with JFH-1/S2 had significantly lower susceptibility to apoptosis as compared with JFH-1/wt. Apoptosis induction index, calculated by dividing the percentage of apoptotic cells in HCV-positive population by the percentage of apoptotic

cells in HCV-negative population, was found to be 1.91, 1.30, 1.28 and 0.94 in JFH-1/wt, JFH-1/S1, JFH-1/C and JFH-1/S2 transfected cells, respectively. Similar results were obtained when susceptibility of cells transfected with JFH-1/wt and JFH-1/S2 to apoptosis was compared after exposing them to FasL or UV irradiation. To further consolidate my results, I detected the extent of apoptosis in cells transfected with JFH-1/wt and JFH-1/S2 by staining with anti-cleaved PARP and obtained very similar results. These results show that JFH-1/S2 clone, which was selected after passage in the patient serum-infected chimpanzee, acquired less susceptibility to the TNF-alpha- and FasL-induced apoptosis.

To examine the effect of mutations in the non-structural region, I compared the susceptibility of subgenomic replicons of JFH-1/wt and JFH-1/S2. In close agreement with full-genome constructs, SGR-JFH1/Luc/S2 showed lower susceptibility than SGR-JFH1/Luc/wt, although the difference was not as pronounced as in case of full-length genomes, which indicates that mutations in structural as well as non-structural region might have contributed to the lower susceptibility of JFH-1/S2 to apoptosis. To confirm it, I induced apoptosis in the cells transfected with JFH-1/S2-wt and JFH-1/wt-S2 chimeric constructs and examined the extent of apoptosis in these cells. Both chimeric viruses showed lower susceptibility to apoptosis as compared to JFH-1/wt, but none of them could reach to the level of JFH-1/S2. These findings confirm that mutations in structural as well as non-structural region of JFH-1/S2 contribute to the lower susceptibility of this virus to apoptosis.

In conclusion, this study has shown that JFH-1/S2 strain, which was isolated from patient serum infected chimpanzee at later stage of infection, acquired mutations which led to the phenotype of lower replication, higher virus production and less susceptibility to apoptosis. Such control of viral functions by specific mutations may be a key viral strategy to establish persistent infection.