Tatsuo Miyamura · Stanley M. Lemon
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Hepatitis C Virus I

Cellular and Molecular Virology

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Part I
Hepatitis C Virus

Towards the Control of Hepatitis C

Michael Houghton

Abstract The discovery and characterisation of the hepatitis C virus (HCV) genome using a bacteriophage expression screening approach in 1989, quickly led to the development of blood tests to protect the blood supply and to diagnose and facilitate management of HCV patients. The viral-encoded serine protease and replicase then became major drug targets that in combination with viral NS5a-targeting drugs, that were facilitated by the use of in vitro genome replicon systems, has now led to most HCV patients being curable after just short treatment regimens. Natural immunity has been demonstrated in multiply-exposed individuals along with the identification of cellular immune correlates of protection. A growing role for neutralising antibodies in protection has also been indicated following the ability to grow HCV and viral pseudoparticles in cell culture. This knowledge has led to the pre-clinical and clinical testing of various promising vaccine candidates. Approval of HCV vaccines along with the development of much cheaper antiviral drugs will eventually lead to the effective global control of this virus which currently infects an estimated 150 million carriers around the world.

Keywords Non-A, non-B hepatitis • HCV virus discovery • HCV replication • Blood screening • Therapeutic approaches • Daclatasvir • HCV vaccine

1 From Non-A, Non-B to C

Following the discovery of the hepatitis B virus (HBV) in 1968 (Bayer et al. 1968) and the hepatitis A virus in 1973 (Feinstone et al. 1973), it became clear in 1974 (Prince et al. 1974) and 1975 (Feinstone et al. 1975) that most transfusion cases of hepatitis were due to neither virus from which the term Non-A, Non-B hepatitis (NANBH) was born. Studies from Harvey Alter at the NIH (Alter 1980) and separately from an independent consortium (TTTV) of collaborators (Hollinger et al. 1980) indicated that the risk of transfusion-associated NANBH could be as

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high as 10 % and that the resulting liver disease persisted frequently. Over the course of many years, this initial mild disease could progress into severe clinical liver diseases such as liver cirrhosis (Dienstag and Alter 1986) and hepatocellular carcinoma (Kiyosawa et al. 1984). Unfortunately, methods used to identify HAV and HBV were unsuccessful at isolating and identifying the etiological agent of NANBH (Shih et al. 1986) due essentially to the much lower titer of NANBH as compared with the known hepatitis viruses (Prince 1983). This problem was solved eventually by the application of sensitive molecular cloning and screening methods. Working with my colleagues Qui-Lim Choo and George Kuo at the Chiron Corporation in the USA, prior to the routine use of PCR amplification technology, we cloned all nucleic acids from an ultracentifuged pellet of infectious chimpanzee plasma into the bacteriophage expression vector lambda gt11 resulting in large proteomic libraries. This plasma, of relatively high titer for NANBH, was obtained from my collaborator Daniel Bradley at the Centers for Disease Control (CDC). While antibodies to NANBH had not then been identified, we assumed their existence and screened the proteomic libraries with NANBH patient sera and identified antibody-tagged clones using a radioactive second antibody. Fortunately, after many failed attempts, this inherently risky approach finally succeeded in identifying one small clone (5-1-1) that we were able to show was not derived from the chimpanzee genome, hybridised to a large RNA molecule present only in NANBH-infected chimpanzee and human samples and which encoded a protein reactive with antibodies found only in NANBH-infected blood. Furthermore, the sequence of the RNA-derived cDNA clones indicated that that it was novel and very distantly related to flaviviruses. This novel method for identifying an infectious agent resulted in the identification of the hepatitis C virus (HCV; Choo et al. 1989; Kuo et al. 1989). This work was a result of a team effort from Qui-Lim Choo, a most thorough molecular biologist working in my own laboratory and George Kuo, who had his own protein chemistry and immunochemistry laboratory adjacent to mine. George provided me with an analysis of the likely limiting concentrations of NANBH antigen in vivo which was largely responsible for persuading me to attempt the precarious recombinant DNA expression screening approach using patient sera as a presumptive, albeit unproven source of NANBH antibodies. Daniel Bradley at the CDC was my long-term external collaborator who provided numerous characterised chimpanzee-derived NANBH samples throughout the course of this HCV virus discovery project in my laboratory from 1982 to 1989.

2 The Hepatitis C Virus (HCV)

A very distant relative of the flaviviruses and pestiviruses, HCV contains a positive-stranded RNA genome of around 10,000 nucleotides which encodes a large polyprotein of over 3000 amino acids which is cleaved co-and post-translationally into virion structural proteins (nucleocapsid (C) and envelope glycoproteins gpE1

and gpE2) and a plethora of non-structural proteins involved in viral replication and assembly (Lindenbach and Rice 2013; Paul et al. 2014). Notable features of HCV replication include it's ability to induce a membranous web within the e.r (Moradpour et al. 2003) in which virus is replicated (Romero-Brey et al. 2015), assembled on lipid droplets (Barba et al. 1997; Miyanari et al. 2007) and secreted using the low-density lipoprotein secretory pathway resulting in the production of apolipoprotein-associated viral particles of very light density (Huang et al. 2007). HCV induces the recruitment of nuclear pore proteins into the membranous web possibly to facilitate a protective subcellular environment from the cell's innate immune response and to create a custom-made virus factory (Neufeldt et al. 2013). Meanwhile, the innate immune response is down-regulated via cleavage of MAVS and TRIF by the viral serine protease (Foy et al. 2005; Li et al. 2005).

Being a RNA virus replicated via it's own RNA-dependent RNA polymerase that lacks proof-reading activity, mutations are common in every replication cycle leading to a highly fluid and heterogenous viral genome currently comprising at least seven basic genotypes, whose distribution varies around the world with numerous subtypes (Smith et al. 2014). As such, each HCV strain comprises a highly adaptable quasi-species of RNA genomes, a feature that quickly emerged from the laboratory of Tatsuo Miyamura who using our original strain, went on to identify the world's most common HCV genotype, the 1b subtype (Kubo et al. 1989).

Despite intense efforts for many years, HCV could not be grown efficiently in tissue culture until 2005 when Takaji Wakita and collaborators identified a Japanese 2a strain that could complete the entire replication cycle in vitro with significant yields of progeny virus (Wakita et al. 2005). Prior to this, infectious HCV pseudoparticles (HCVpp) could be produced in cell cultures co-expressing defective HIV and lentiviral genomes along with HCV envelope glycoproteins (Flint et al. 2004; Sandrin et al. 2005). Earlier, replicons of HCV were produced in Ralf Bartenschlager's (Lohmann et al. 1999) and Charles Rice's (Blight et al. 2000) laboratories in human hepatoma cell-lines. These have proven to be of great value in HCV drug discovery programs and in basic research. An immunodeficient SCID mouse model for HCV infection involving transplantation of human hepatocytes has also proven valuable in research, drug development and virus neutralisation studies (Mercer et al. 2001) and an immunocompetent mouse model shows much promise in future vaccine studies (Dorner et al. 2013). Visualisation of the virion proved difficult and became possible only recently, many years after it's molecular isolation (Wakita et al. 2005; Fig. 1).

3 Prevention and Therapy

Once we identified the viral genome, it was possible to quickly develop blood tests to capture and detect circulating HCV-specific antibody in infected individuals (Kuo et al. 1989). In addition, with the advent of PCR- and TMA-nucleic acid

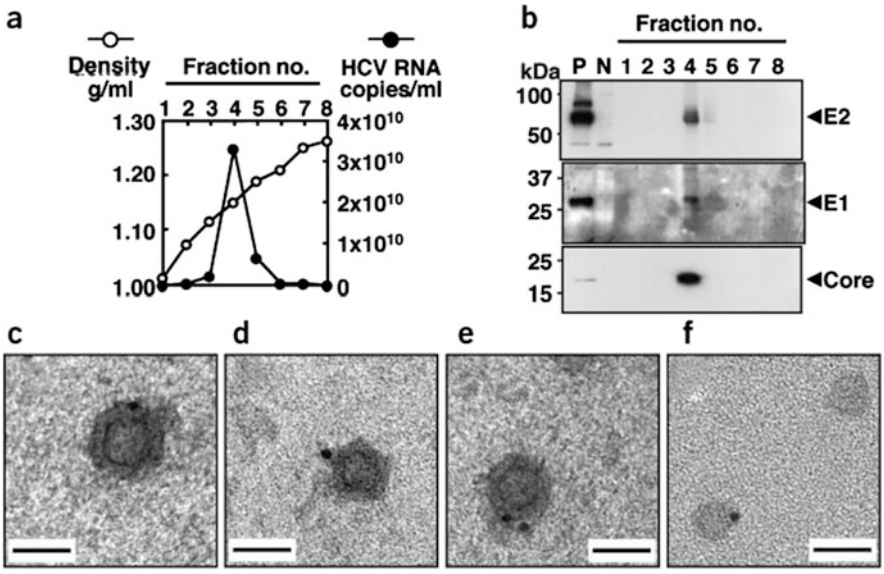

Fig. 1 Density gradient and electron microscope analysis of recombinant HCV particles. (**a**, **b**) Co-sedimentation of viral RNA and structural proteins. (**a**) Concentrated culture medium collected from JFH1/E2HA RNA–transfected cells was fractionated using a 10–60 % sucrose density gradient. HCV RNA titer in each fraction was determined. (**b**) Density gradient fractions were further concentrated and analyzed by western blotting for core, E1 or E2-hemagglutinin. P, cell lysate prepared from JFH1/E2HA RNA–transfected Huh7 cells; N, cell lysate from untransfected Huh7 cells. *Arrowheads* indicate positions of HCV proteins. (**c**–**f**) Electron micrograph of spherical structures shown by immunogold labeling. Grids were incubated with a concentrated JFH1 virus stock and then with the E2 monoclonal antibody CBH5 (Hadlock et al. 2000). Bound antibodies were detected with Protein A coupled to gold particles 10 nm in diameter. (**c**–**e**) Three representative examples showing the same structure. (**f**) Control grid coated with concentrated cell-free supernatant derived from mock-transfected cells. In rare cases, we observed gold particles attached to unstructured protein aggregates. *Scale bar*, 50 nm (Reprinted from Wakita et al. (2005))

amplification technologies, these tests were quickly approved around the world and have effectively eliminated transfusion-associated HCV transmission where blood screening is performed.

Successful therapeutic approaches were initiated prior to the identification of HCV with the seminal discovery of alpha interferon able to cure a small minority of NANBH patients (Hoofnagle et al. 1986). Stabilising the interferon by conjugation with polyethylene glycol led to better potency as did the addition of ribavirin, a guanosine analogue that works to prevent relapse using an unknown mechanism (Manns et al. 2001; Feld 2012). This combination was the mainstay of therapy for many years although it was limited by high toxicity and by only partial potency (McHutchison and Fried 2003).

Surprisingly, it took more than 20 years to develop approvable drugs targeting the HCV protease and polymerase, despite these targets being evident from the