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Stable Huh-7 cell lines expressing non-structural proteins of genotype 1a of hepatitis C virus

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A B S T R A C T

Hepatitis C virus (HCV) infection has infected approximately 3% of the world population. HCV genotype 1a is distributed throughout the world, and along with genotype 1b, is relatively resistant to current standards of therapy compared to other HCV genotypes. The present study was designed to produce stable Huh-7 cell lines expressing non-structural proteins of HCV genotype la, representing an in vitro system to facilitate the development of new antiviral drugs against chronic HCV infection. The non-structural genes of HCV genotype 1a were amplified and cloned in a mammalian expression vector pCR 3.1/FlagTag. Huh-7 cells were transfected with one of two expression plasmids, the first containing the NS2, NS3, and NS4a cassette, and second containing the NS5a and NS5b genes. Stable cell lines were produced under the selection of gentamycin (G418). mRNA and protein expression analysis was performed by RT-PCR and Western blotting. The RT-PCR and Western blot results confirmed the stable expression of each of the gene products. Stable Huh-7 cell lines with HCV la non-structural proteins may be helpful for evaluating the role of HCV proteins in molecular pathogenesis, and could facilitate the development of new therapeutic drugs.

1. Introduction

Approximately 300 million people worldwide (Giannini and Brechot, 2003) have been infected with hepatitis C virus, is a major cause of the cirrhosis of liver, liver fibrosis, and hepatocellular carcinoma (HCC), and a primary indication for liver transplantation in developed nations (Feld and Hoofnagle, 2005). A vaccine is not available to prevent HCV infection, although approved therapy with pegylated interferon-alpha in combination with ribavirin and a direct antiviral protease inhibitor is effective for treatment of infection in many but not all patients (Foote et al., 2011). Many patients are not eligible for treatment due to intolerance of side effects, while 20% or more of genotype 1a infections remain resistant to the best current therapies (Nelson, 2011).

HCV is the sole member of the genus *Hepacivirus* within the family *Flaviviridae*. Due to high levels of genetic heterogeneity, it has been classified into six major genotypes and subdivided further into numerous subtypes (Robertson et al., 1998). HCV is a small-enveloped virus with a single-stranded positive-sense RNA molecule of approximately 9.6 kb long encoding polyprotein of approximately 3000–3010 amino acids. The polyprotein is cleaved by viral as well as host enzymes into structural proteins, core, El, E2, P7 and non-structural proteins, NS2, NS3, NS4a, NS4b, NS5a, NS5b (Varaklioti et al., 2002; Walewski et al., 2001; Xu et al., 2001). The non-structural proteins of HCV are important for viral replication via the formation of a membrane bound replication complex. The NS2 protein together with the N-terminal portion of the NS3 protein form the NS2-3 cysteine protease which catalyses the cleavage of the polyprotein NS2/NS3 junction (Grakoui et al., 1993; Santolini et al., 1994). The NS3 protein is multifunctional, with serine protease, RNA helicase, and NTPase activities (Kim et al., 1996). The NS3 serine protease, together with the NS4a cofactor, is responsible for the proteolytic cleavage of the HCV downstream non-structural proteins that are essential for forming the replicative complex, from which viral synthesis occurs (Kim et al., 1998). NS3/4A also plays an important role in HCV persistence by inhibiting innate immune mechanisms by blocking of the RIG-I

Abbreviations: KB, Kilo base; KD, Kilo Dalton; RIG-1, Retinoic acid inducible gene I; ER, Endoplasmic reticulum; GAPDH, Glyceraldehyde phosphate dehydrogenase; RT-PCR, Reverse transcriptase polymerase chain reaction; EDTA, Ethylene diamine tetra acetic acid; SDS-PAGE, sodium dodecyl sulfate poly Acryl amide gel electrophoresis; PMSF, Phenyl methyl sulfonyl fluoride.

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and the toll-like interferon receptor (TRIF, Cardif) and afterwards the interferon-signaling pathways (Meylan et al., 2005). NS4a is involved in targeting NS3 to the ER membrane (Wolk et al., 2000). The NS5a protein is a membrane-associated phosphoprotein (Asabe et al., 1997). It is phosphorylated by different cellular protein kinases that appear to have multiple functions in viral replication. One of the important properties of NS5a is that it contains a domain of 40 amino acids, the so-called IFN-α sensitivity determining region (ISDR), which plays a significant role in the response to IFN- α based therapy (Enomoto et al., 1990,1995). The non-structural protein NS5b represents the RNA-dependent RNA polymerase of HCV. Using the genomic HCV RNA as a template, the NS5b polymerase catylizes the synthesis of minus strand RNA, which then serves as a template for the synthesis of genomic positive strand RNA (Behrens et al., 1998). The viral NS5b lacks a proofreading mechanism leading to the conservation of misincorporated ribonucleotides (Schmidt-Mende et al., 2001). Lack of proof reading mechanisms, and high rate of viral replication, promote a pronounced intra-patient heterogeneity termed viral quasispecies, and significant intra-patient viral evolution (Li et al., 2011).

Researchers have established a number of cell culture based systems, including subgenomic replicons, pseudo particles, and an infection based system, to evaluate novel, innovative, and therapeutically effective antiviral drugs against HCV. In addition, Butt et al. (2011) established stable cell lines expressing successfully different structural and non structural genes of HCV genotype 3. Stable cell lines help to study the role of individual genes in the pathogenesis of the disease. In the present study, a stable Huh-7 cell culture line expressing the non-structural proteins of HCV genotype la was established. Two large cassettes of the non-structural genes, one containing the NS2, NS3, and NS4a genes, and the second containing the NS5a and NS5b genes, were cloned in the mammalian expression vector pCR 3.1/FlagTag and expressed stably in the Huh-7 cell line. Stable gene expression allows long term, defined and reproducible expression of the gene of interest. Stable expression of genes at mRNA and protein levels were confirmed by RT-PCR and Western blot analysis, respectively. These stable cell lines may be useful for in vitro study of HCV induced pathogenesis, and to test new drugs/phytochemicals for their therapeutic effect against HCV genotype 1a.

2. Materials and methods

2.1. PCR amplification

The HFL plasmid containing the complete genome of HCV genotype 1a was kindly provided by Dr. Zafar Nawaz (Biochemistry and Molecular Biology Department, University of Miami, USA). The sequence specific primers were designed for PCR amplification of non-structural genes of HCV using Primer3 software with restriction sites (Forward primers with *EcoRV* and reverse primers with *Xba1* restriction sites). The primer sequences are given in Table 1 with restriction sites. The amplification was performed with 100ng of plasmid (HFL) using forward and reverse primers in a thermal cycler along with long PCR enzyme mix (Fermentas, Maryland USA). The PCR conditions for each amplicon were optimized carefully while adjusting the primer annealing temperatures and %age GC content.

2.2. Construction of expression plasmids

The mammalian expression plasmid pCR3.1/FlagTAG, with the human cytomegalovirus (HCMV) immediate early promoter, was constructed by inserting FlagTAG into pCR3.1 using standard cloning methods. Next, the individual HCV non-structural genes were cloned into the pCR3.1/FlagTAG vector. The cloning of the plasmid was confirmed by PCR and restriction digestion with *EcoRV* and *Xba1* restriction enzymes. All the reaction mixtures were incubated at 37 °C for 2 h.

2.3. Cell culture

The human hepatoma cell line (Huh-7, a well differentiated hepatocellular carcinoma cell line) was grown routinely in high glucose Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Sigma–Aldrich, St. Louis, USA) and 100 μg/ml penicillin plus 100 μg/ml streptomycin, in tissue culture flasks under a humidifying atmosphere containing 5% CO_2 and 95% air at 37 °C. The medium was renewed every 3rd day, and the cells were subcultured every 4–5th day interval. The cells were seeded at a density of 1×10^5 cells/well in 24-well plates, or 3×10^5 cells/well in six well plates, and passages were conducted by washing the mono-layers with phosphate-buffered saline (PBS) followed by a brief incubation with trypsin/EDTA (Invitrogen Life Technologies, Carisbad CA, USA). The washed and proteolyzed cells were collected by centrifugation and resuspended in culture medium for counting and plating. The viable cells were counted by using 0.5% trypan blue dye (Sigma–Aldrich, St. Louis, USA). For 6-well plates, briefly, 3×10^5 cells/well were plated and cultured in complete medium until 60–80% confluent. Cells growing within the exponential phase were used for all experiments.

2.4. Transfection of expression plasmids

A day before transfection, 3×10^5 Huh-7 cells were seeded into 6-well plates. When the cells were 60–80% confluent, they were transfected with 3–4 μg constructed plasmids and Lipofectamine™ 2000 (Invitrogen Life Technologies, Carisbad CA, USA), according to the manufacturer's protocol. The pCR3.1/FlagTAG (empty vector) was used for mock transfection (negative control). After 6–8 h, media with lipofectamine and plasmids was exchanged with simple growing media (DMEM + 10% FBS + 1% antibiotic).

2.5. Total RNA isolation and cDNA synthesis

The total RNA was extracted from transfected Huh-7 cells using the Trizol reagent (Invitrogen Life Sciences, CA, USA) according to the manufacturer's protocol, and RNA samples were stored at −70 °C. The extracted RNA was reverse transcribed into complementary DNA (cDNA) using Moloney murine leukemia virus reverse transcriptase (mMLV-RTase) (Fermentas, Maryland, USA) following the manufacturer's protocol, and amplified with specific RT primers for mRNA confirmation.

2.6. Protein extraction and Western blot analysis

The transfected Huh-7 cells were lysed with cell lysis buffer (NaCl 400 Mm, Tris–HCl 50 mM, EDTA 10 mM, protease inhibitor 2 μl/ml, PMSF 10 mg/ml), and total protein was extracted at 48–72 h following transfection. Alternatively, cells were harvested after three weeks of transfection for stable clones (stable cell lines). After removing media, the cells were washed with 1X PBS. Cells were harvested by adding trypsin and centrifuged at 13,000 × *g* at 4 °C for 2 min. The cell pellet was washed with 1X PBS, and 100 μl of lysis buffer was added to the pellet of the cells, incubated on ice for 10 min, and centrifuged for 25 min at 13,000 × *g* at 4 °C. The supernatant containing protein was transferred to a new eppendorf tube and stored at −20 °C. Based on the protein concentration, equal amounts of total protein samples were loaded in each well of 12%