Egyptian J. Virol. 5(1): 20-35 (2008)

HepG2 Cell Culture Infection by Hepatitis C Virus Genotype 4: Viral Proteins and RNA

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Genotype 4 hepatitis C virus (HCV) in viremic serum was used for experimental infection of a human hepatoma cell line (HepG2) at a multiplicity of infection (MOI) of one genome copy per 10^3 cells. Stock HepG2 cell cultures were grown in Dulbecco modified minimum essential medium (DMMM) as growth medium with 10% fetal calf serum and antibiotic mixture (100U penicillin and 100 mgm streptomycin/ml) and subcultured at 3-4 days. HepG2 cells were maintained throughout the experiment in Ham F-12 medium maintenance medium with 2% fetal calf serum; 1000 IU penicillin and 1000 mgm streptomycin antibiotic mixture; with 4% (w/v) polyethylene glycol (PEG), 2% dimethyl sulfoxide (DMSO); and 10µl lovastatin for the experimental infection with HCV.

Three markers of HCV virus replication were used. Morphological changes HCV-RNA by reverse transcription polymerase chain reaction (RT PCR) and viral proteins by polyacrylamide gel electrophoresis (PAGE). HCV infected and control HepG2 cells supernatant fluids (SF) were sampled before complete change with fresh MM at weekly intervals for periods extended to one month after infection.

Three SF samples taken 5 days apart after HCV infection showed that detection of HCV-RNA in SF was intermittent but detection of new native protein as well as glycopeptides was consistent.

Key words: HepG2 cells, HCV from patient serum, HCV experimental infection, HCV-RNA, RT-PCR, HCV-protein, PAGE.

Introduction

In 1989 **Choo *et al.*** unraveled the etiology of non-A non-B hepatitis by isolating viral RNA from experimentally infected monkeys' blood. Recombinant-DNA clones were developed and viral proteins were expressed **(Kuo *et al.*, 1989)** to be used in enzyme-linked immunoassays (ELISA) and recombinant immunoblots (RIBA) for the identification of anti-HCV antibodies **(Houghton *et al.*, 1991 a & b).**

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(Received March 2008)
(Accepted May 2008)

The sensitivity of ELISA kits varies and in choosing a kit the predominant HCV genotype should be considered (**Abdel Wahab *et al.*, 1996**). Eventually molecular diagnosis of hepatitis-C virus (HCV) infection was confirmed by detection of HCV-RNA in serum by polymerase chain reaction (PCR) (**Choo *et al.*, 1989; Scott and Gretch, 2007**). Expenses and limited supplies of monkey, the in-vivo model of HCV infection, spurred research for an in-vitro cell culture models.

Natural infection of man with HCV proved that the virus is present in both hepatocytes and lymphoid cells and circulates the blood in association with lipoproteins. Primary human hepatocyte in-vitro cell cultures could support HCV replication (**Ito *et al.*, 1996**). Continuous cell lines of malignant human hepatocytes such as HepG2 and Huh7 were used by **Seipp *et al.*, (1997)**; HepG2 by **El-Awady *et al.*, (2006)**. Experimentally immortalized human hepatocytes were used by **Kanda *et al.*, (2006)** for HCV culture. Parameters of virus replication as replicative gene intermediates, virus protein production, infectious virus yield that can infect monkeys in-vivo, or infect naïve hepatocyte cell cultures in-vitro were assessed. All systems of in-vitro cell culture of HCV used genotypes 1 or 2. Further advances followed the development of full-length HCV genome replicons from genotypes 1α and 1β in Huh7 cells and its derivatives (**Bukh and Purcell, 2007**).

Since **Saed *et al.*, (1991)** report of high incidence of anti-HCV in volunteer Egyptian blood donors, a flow of publications confirmed HCV high prevalence (20 – 37%) that rises to 70-90% in high risk groups (**Darwish *et al.*, 1992; Jeffers *et al.*, 1992; Kamel *et al.*, 1992; Bassily *et al.*, 1992; Hassan and Kotkat 1993; Hibbs *et al.*, 1993; El-Ahmady *et al.*, 1994; Hassaballah and Hegazy, 1994; Bassily *et al.*, 1995; El-Gohary *et al.*, 1995; Kabil *et al.*, 1995; El-Medany *et al.*, 1999; Frank *et al.*, 2000**).

Inclusion of nucleic acid tests (NAT) became a must in Egyptian national blood banking for safe transfusions and for the production of safe medicinal blood fractionation components. Also RT-PCR using commercial expensive kits became a must for diagnosis and assessment of HCV infection, and for post-anti viral therapy viral load response. Further costs of patients' management were added when HCV genotyping is requested.

Genotype 4 HCV is prevalent in Egypt and is problematic (**Zekry *et al.*, 2005**). It is a poor responder to

combined antiviral therapy requiring several RT-PCR assays. With an estimated 16 million HCV patients in Egypt (**Frank *et al.*, 2000**) it is over-taxing the health services as well as resources and patients' budget. Cell culture of viruses reduces the cost of diagnosis of preparation of antigens for serological tests and of development of vaccine. There is a pressing demand to utilize cell culture of HCV. Unlike the successful development of HCV genotypes 1 and 2 molecular infectious clones there is not a similar HCV-genotype 4 infectious clone of in-vitro cell culture based assays for further understanding of the biology of HCV genotype 4 and for the development of molecular diagnosis.

We describe here a pilot study of protein and glycopeptides expression by HCV-genotype 4 infected HepG2 cells which can be of high yield and low cost source of HCV genotype-4 antigen for an in-house serological assay.

MATERIALS AND METHODS

Viremic Sera:

Blood samples with HCV load of $10^4 - 10^6$ genome copies/liter by commercial RT-PCR kits were obtained from 20 chronic HCV infected patients. Sera were pooled in 5 batches, filtered, aliquoted and stored at -80°C until use.

HepG2 Cells

The hepatocellular carcinoma cell line (HepG2) ATCC® no HB-8065™) (**Knowles *et al.*, 1980**) was generously made available from Virology Laboratory in NAMRU # 3 and from the tissue culture laboratory in VACSERA.

HepG2 Cell Culture

The growth medium (GM) was Dulbecco's modified minimum essential medium (DMMEM) supplemented with 10% fetal calf serum and antibiotic mixture (100 U penicillin, 100 mgm streptomycin/ml GM). Stock HepG2 cells were trypsinized twice weekly for subculture at 10^5 cell/ml GM at 37°C.

Experiments were done in freshly trypsinized HepG2 cells suspended at $2x10^5$ cells/ml for tube monolayer cell cultures in Ham's F12 as a maintenance medium (MM) supplemented with 2% fetal calf serum, and antibiotic mixture (1000 U penicillin and 1000 mgm streptomycin/ml) with 4% (w/v) polyethylene glycol (PEG 6000/7500 from Sigma), 2% dimethyl sulfoxide (DMSO from Sigma), 10μM Lovastatin according to **Seipp *et al.* (1997)**. Complete change of medium of both control uninfected or HCV infected cells was done at 5 days

intervals. Our modification was the use of Ham's F12 supplemental MM at the time of HCV inoculation and throughout the experimental infection time.

In-vitro infection of HepG2 cells with hepatitis C virus

Serum (10^4 – 10^6 copies/L of HCV RNA) was used as inoculum to infect HepG2 grown in tubes (10^5 cells/tube) at an approximate multiplicity of infection (MOI) of 1 HCV copy/1000 cells. Adsorption of inoculated virus to cells was for 2 hours at 37°C followed by three washes and the addition of 2 ml Ham's MM/tube. After 24 hours from infection cell washing was done three times to remove any unadsorbed HCV/serum proteins – Fresh Hams F12 MM was added to maintain cell viability while incubated at 37°C. Cells were examined daily by phase contrast light microscopy for morphological changes. The SF were harvested separately for testing for virus replication parameters after 5, 10, 15 days of experimental HCV infection and stored in aliquotes at -20°C. HepG2 cell cultures were replenished with fresh MM to uplift cell viability. Control (uninfected) cells were treated similarly.

RNA extraction

RNA extraction from SF was done by guanidinium isothiocyanate (Sigma) as described by **(Chomozynski and Sacchi (1987)** with silicon dioxide according to **Boom *et al.* (1990).**

HCV RNA detection by reverse transcription polymerase chain reaction (RT-PCR)

Extracted RNA was used as template to be reverse transcribed by Avian myeloblastosis virus (AMV) reverse transcriptase (Stratagene kit). The
c-DNA primer 1 (nucleotide 148 – 167) was '5CTGCGC AACC GGTGAGTACA3' (Research Genetics, UK). For PCR amplification of HCV-cDNA primer 1 was used as reverse primer for the positive sense strand plus the forward RB6B primer 2 (nucleotide 709-729) 5'AGCCGCACG CAAGGG TA TCG3' for the anti-sense strand. PCR cycles were first cycle of denaturation at 94°C for 4 minutes then a cycle of primer annealing at 50°C for 1 minute, extension at 72°C for 2 minutes. This cycle was repeated for 35 times and in the last extension cycle at 72°C for 10 minutes.

Polyacrylamide gel electrophoresis (PAGE) HCV genotype-4 of proteins

Control and HCV infected SF were used for native protein separation **(Davis *et al.*, 1964)** and SDS dissociated protein separation

(Laemmli, 1970) by PAGE. Several PAGE runs were done. Protein bands in gels were stained by Coomassie brilliant blue, by amidoblack, by Sudan black, by fuchsin sulphite (Schiff's reagent). Molecular weight (M.Wt) of protein bands was determined with reference M.Wt marker protein-mixture (Sigma C.NO M4038). Band migration distance from the start point was recorded by GS365W electrophoresis DATA system Microsoft window 3.01version

RESULT

Morphology of experimentally HCV Genotype 4 infected HepG2

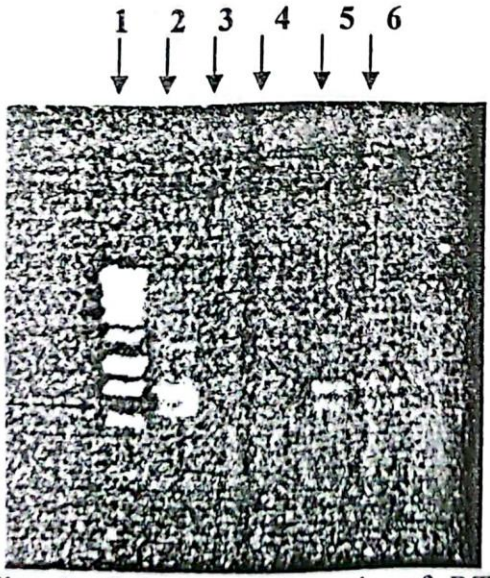


Fig. 1. Gel electrophoresis of RT-PCR of HCV- RNA amplicons in 1.5% agarose gel in TAE buffer.

HCV infected HepG2 tended to form round cell aggregates with increased refractile cytoplasmic inclusions. It did not proceed to a continuous epithelial like monolayer formation as the control HepG2.

Detection of HCV Genotype 4 RNA by RT-PCR

HCV-RNA was not detected in SF harvests at 5 or 10 days post infection. The 15th day post infection harvest of SF contained HCV-RNA detected by RT-PCR (Fig. 1). These results are a measure of extracellular virus release following HCV genotype 4 infection of HepG2 and virus replication with a virus yield that exceeded dilution introduced by SF changes with fresh MM every 5th day post infection.

Lane 1: 100bp DNA ladder
Lane 2: positive control HCV amplicon
Lane 3,4 : negative controls , lanes 5, 6 positive HCV-4 580bp amplicons from Hep G2-CV-4 supernatant fluid from infected cell cultures

Production of proteins by experimentally HCV Genotype 4-infected HepG2 cells

There were variations of native proteins expression by control versus experimentally HCV infected HepG2 cell. New

protein, peptides, and glycopeptides appeared in SF from HCV genotype-4 infected HepG2 cells. Some of these new proteins were consistently present in the 5, 10, and 15 days post infection samples (Fig. 2 to 4).

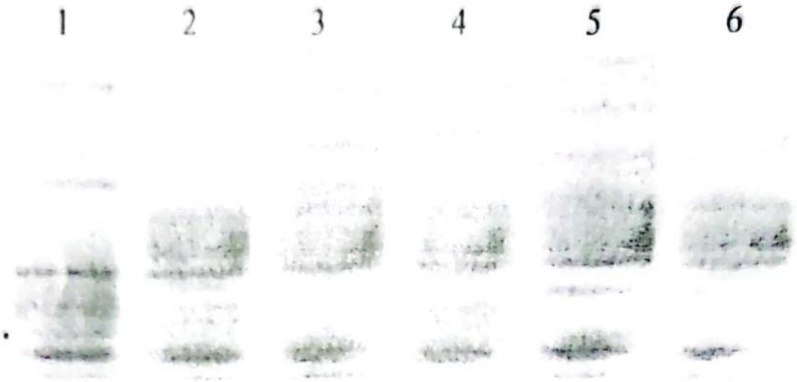


Fig. 2: Scanning of native protein PAGE.
lane 1: control HepG2 cell culture
lane 2-6: HCV-4 virus infected HepG2 cell culture supernatant fluid from five experiments after the 5th day of incubation

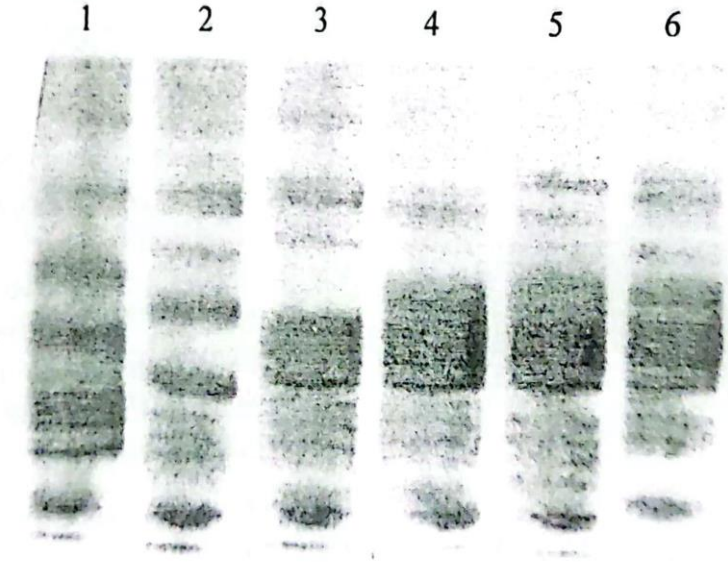


Fig. 3. Scanning of native protein PAGE.
lane 1: control HepG2 cell culture
lane 2-6: HCV-4 virus infected HepG2 cell culture supernatant fluid from five experiments after the 10th day of incubation

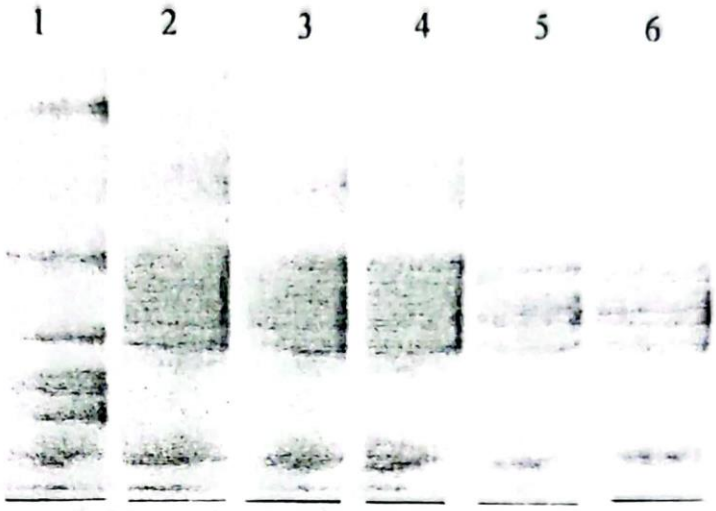


Fig. 4: Scanning of native protein PAGE.
lane 1: control HepG2 cell culture
lane 2-6: HCV-4 virus infected HepG2 cell culture supernatant fluid from five experiments after the 15th day of incubation

SDS-PAGE of proteins and glycoproteins revealed several bands of lower MOL-Wt some shared with control HepG2 but some specific to HepG2 cells infected with HCV (Fig. 5 to 7).

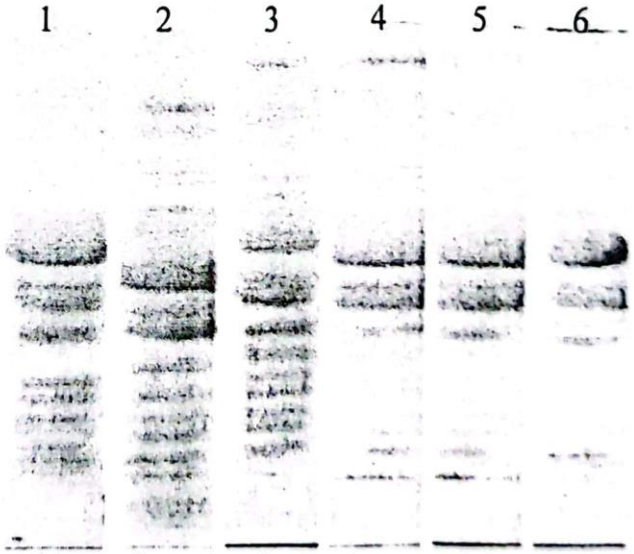


Fig. 5. Scanning of SDS dissociated protein PAGE.
lane 1: control HepG2 cell culture
lane 2-6: HCV-4 virus infected HepG2 cell culture supernatant fluid from five experiments after the 5th day of incubation

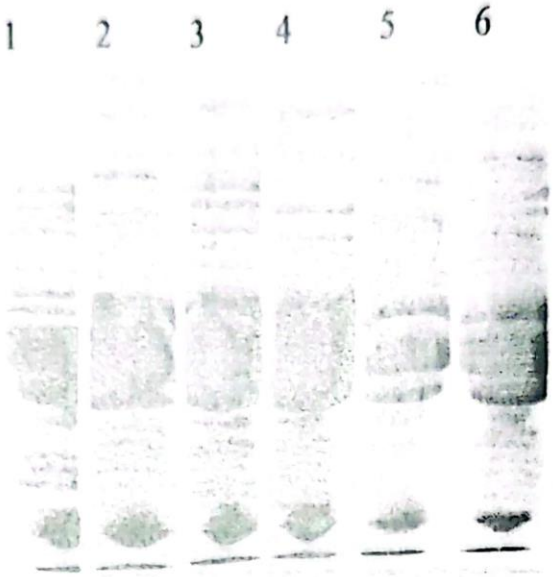


Fig. 6. Scanning of SDS dissociated protein PAGE.
lane 1: control HepG2 cell culture
lane 2-6: HCV-4 virus infected HepG2 cell culture supernatant fluid from five experiments after the 10th day of incubation

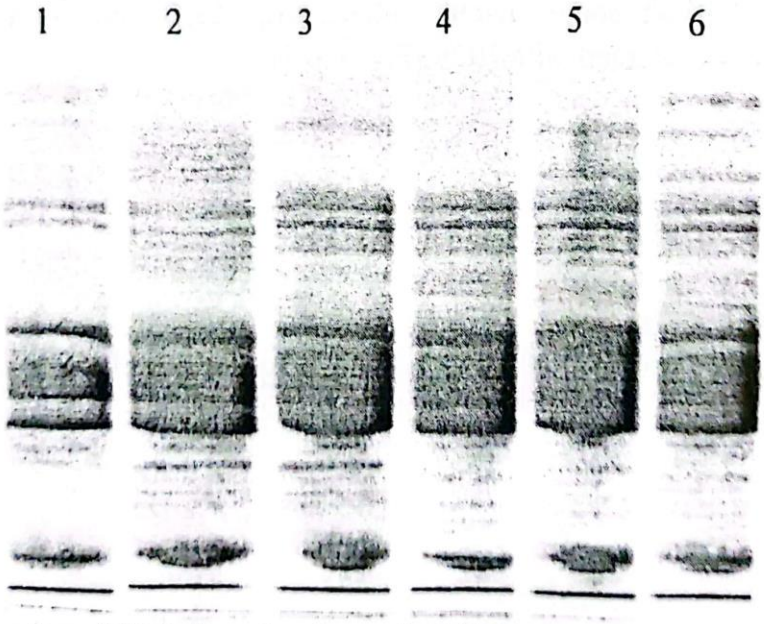


Fig. 7. Scanning of SDS dissociated protein PAGE.
lane 1: control HepG2 cell culture
lane 2-6: HCV-4 virus infected HepG2 cell culture supernatant fluid from five experiments after the 15th day of incubation

Production of glycoprotein by HCV-experimentally infected HepG2 cells

Variation in the mol. Wt. of bands of native glycopeptides from control versus HCV-infected HepG2 was less dramatic (Fig. 8 to 10). There were two new glycopeptide in bands of mol. wt. range from 18-20 kDa and the appearance of 170-189 kDa glycopeptide band in the 15 day post infection SF harvest from 2/5 HCV-infected HepG2 cells (Fig. 10). In the SDS dissociated glycoproteins glycopeptide bands of mol. wt 15 kDa (Fig. 11) were present in the 15 days post infection SF harvest from HCV infected HepG2.

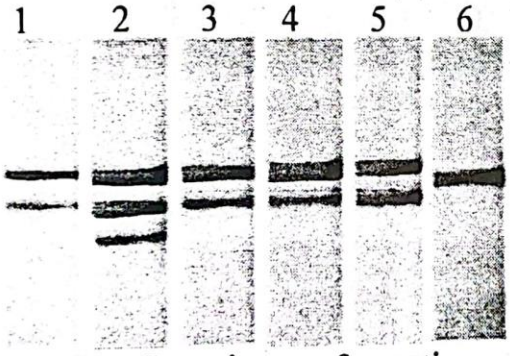


Fig. 8. Scanning of native glycoprotein PAGE.
lane 1: control HepG2 cell culture
lane 2-6: HCV-4 virus infected HepG2 cell culture supernatant fluid from five experiments after the 5th day of incubation

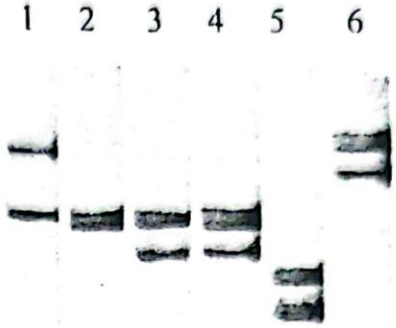


Fig. 9. Scanning of native glycoprotein PAGE.
lane 1: control HepG2 cell culture
lane 2-6: HCV-4 virus infected HepG2 cell culture supernatant fluid from five experiments after the 10th day of incubation

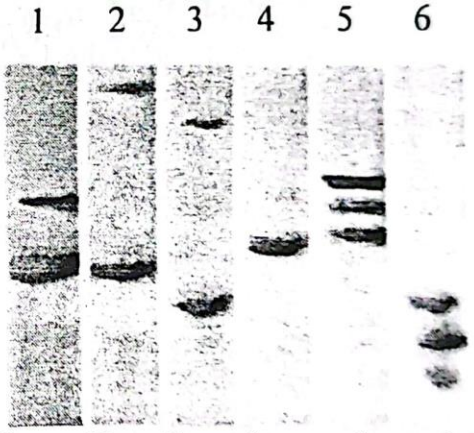
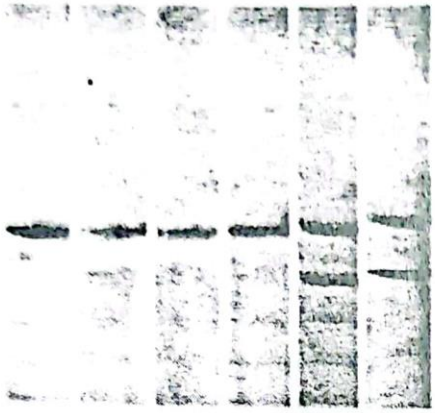


Fig. 10. Scanning of native glycoprotein PAGE.
lane 1: control HepG2 cell culture
lane 2-6: HCV-4 virus infected HepG2 cell culture supernatant fluid from five experiments after the 15th day of incubation

1 2 3 4 5 6

Fig. 11. Scanning of SDS dissociated glycoprotein PAGE.



lane 1: control HepG2 cell culture

lane 2-6: HCV-4 virus infected HepG2 cell culture supernatant fluid from five experiments after the 15th day of incubation

DISCUSSION

Hepatocyte coreceptors for HCV **(Reynolds *et al.*, 2008)** include CD81 tetraspanin protein, SR-B1 scavenger receptor protein, and cludin-1 a tight junction protein. HCV entry into hepatocytes is dependent on the apical expression of the three coreceptors on the polarized hepatocyte. HepG2 cells is a hepatoma cell line that maintain several markers of differentiated hepatocyte but lacks CD81 protein expression. Induced expression of CD81 protein on HepG2 cells conferred increased sensitivity to HCV glycoprotein mediated viral infection **(Zhang *et al.*, 2004)**. Upregulated expression of betalipoprotein receptors of HepG2 cells by lovastatin as well as the improved cell permeability by PEG and DMSO in Ham F-12 medium were recognized by Seipp et al. (1997) to improve HCV genotypes 1a and 1b infection of HepG2 cells.

Our modification of **Seipp *et al.*, (1997)** protocol was the use of Ham F-12 maintenance medium with the supplements at the time of HCV genotype 4 infection of HepG2 cell culture and onwards as medium replenish as long as the experiment was extended and the control HepG2 grow to complete healthy monolayer. Our data showed that cell rounding, clumping, reduced cell density and arrested monolayer formation occurred in HepG2 cells infected with HCV genotype 4 D.

Dash *et al.* (1997) reported that HepG2 cells transfected with infectious HCV genome, showed significant cytotoxicity, and also the cell density decreased over a period of 2 months observation. **Dash *et al.* (2001)** and **Kalkeri *et al.* (2001 a-b)** reported that genotype 1 HCV protein expression induced apoptosis of HepG2 cells. Thus HCV cytotoxicity to HepG2 cells is not genotype specific.

Ahkong *et al.* (1987) reported that PEG and DMSO enhance mechanisms necessary for cell membrane entry and fusion. **Seipp *et al.* (1997)** reported that inclusion of PEG, DMSO and lovastatin in Ham F12 medium at the time of infection increased the efficiency of HCV uptake in HepG2 cells but not the replication. In our experiments the MM contained PEG, DMSO and lovastatin all through the experimental infection of HepG2 with HCV-genotype 4. Our results proved the beneficial effect of these additives on HCV uptake and extend it to HCV replication in HepG2 cells. With our modification of conditions throughout the HCV infection we detected HCV-RNA in the SF up to fifteen days after infection. Thermal degradation at 37°C and repeated complete change of MM excludes carry over of virus from the infecting virus inoculum.

Several precautions were taken in this study to exclude mere survival of HCV in the SF or carry over of HCV from the viremic serum inoculum into the SF of experimentally infected HepG2 cell cultures. Besides only the SF from 15th day past the experimental infection of HepG2 was used for the detection of HCV-RNA by RT-PCR. It should be a sure marker of extracellular virions released after active virus replication. The concentration of virions or viral genome in SF cells infected in-vitro is expected to be too low. In our study we used a commercial PCR kit with detection level of ≈ 400 genome copy/L. Although the extracellular HCV-RNA level in SF of replicas of HCV-experimental infection of HepG2 cells fluctuated; it was present in the SF collected 15 days post infection.

El-Awady *et al.* (2006) using HepG2 cells with a different experimental protocol showed that HCV-genotype 4 replicated in HepG2 cells. In the present study, new (111-236 K.Da) native peptide bands were expressed after the 5th day of HCV infection in 4/5 of SF. While 70-106 K.Da native protein was expressed in all the infected cell culture media and did not appear in the control media. After the 10th day of incubation new bands ranged (141-199 K.Da) native protein were expressed in 4/5 of SF from infected cell culture and (108-123 K.Da) bands appeared in 5/5 of the infected cell culture media. These bands did not appear in the control cell

After 15th day of incubation (198-223 K.Da) bands were present in 3/5 of infected cell culture SF and of the control cell culture. The (119-173 K.Da) bands observed in SF from infected cell culture did not appear in the control media. SDS dissociated protein PAGE

electrophoretic pattern, showed high molecular weight bands (207-245 K.Da) after the 5th day of incubation in all the infected cell SF which did not appear in control cell culture SF. Afterwards on the 10th and 15t day of HCV infection using SDS PAGE of SF did not discriminate between bands from infected or control cell cultures except that new (177-198 K.Da) peptide bands appeared in 3/5 of infected cell culture SF and did not appear in control ones. After the 5th, 10th and 15th days of HCV infection a new band (19-20 K.Da) native glycoprotein peptide band was expressed consistently in SF of infected cells.

It is clear from the present study that these new native or SDS dissociated glycoprotein peptide bands are related to experimental HCV infection of HepG2 cells. Their origin may be newly formed viral glycoproteins or as virus-induced breakdown of the high molecular weight cellular glycoprotein or as virus induced modification of cellular precursor glycoprotein. HCV E proteins interact with host cell membranes and lipids (**Dubuisson *et al.*, 2002**)

HepG2 cell culture experimental infection with HCV genotype 4 is an economic cell substrate for the production of viral peptides/proteins/ glycopeptides for wide scope use in preparation of in-house serology assays. We recommend further experiments to identify released and cell bound HCV proteins after HepG2 cell culture infection with the use of monoclonal antibodies and western blot technique.

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