Hepatitis C Virus Protein Expression Induces Apoptosis in HepG2 Cells

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The mechanisms of hepatocyte death and the events that lead to a high rate of chronic liver disease in patients infected with hepatitis C virus are not known. We established a HCV replication system in HepG2 cell culture and utilized this model to address the effect of HCV proteins on HepG2 cell growth and viability. After transfection of HepG2 cells with full-length RNA, a truncated RNA, or an antisense RNA, cell proliferation and cell viability were analyzed by tryptophan blue uptake and the trypan blue exclusion method, respectively. Full-length RNA transfected HepG2 cells showed a decrease in cell proliferation and viability compared to cells transfected with HCV truncated RNA and antisense RNA control. A subset of cells expressing HCV proteins underwent apoptosis as documented by morphological studies, ultrastructural analysis, cell cycle analysis by flow cytometry, terminal transferase enzyme mediated end labeling of DNA, and DNA laddering. This study suggests that expression of HCV proteins can lead to cell death by apoptosis, which may be an important event in the pathogenesis of chronic hepatitis C virus infection in humans. © 2001 Academic Press

Key Words: hepatitis C virus; immunohistochemistry; RNA transfection; cell viability; apoptosis; TUNEL assay; electron microscopy

INTRODUCTION

Histopathological analysis of liver biopsies from patients with chronic hepatitis C virus infection suggests the presence of apoptotic cell death (Hiramatsu et al., 1994). Several other RNA viruses are known to induce apoptotic cell death, which plays an important role in disease pathogenesis. Both measles virus (a paramyxovirus) and human immunodeficiency virus (a retrovirus) cause T-cell death through apoptosis that contributes to immunosuppression (Pignata et al., 1998; Plymale et al., 1999). The alphaviruses Sindbis virus and Semliki Forest virus induce apoptosis in neurons of mouse brains and oligodendrocytes, respectively, causing encephalitis (Nava et al., 1998; Glasgow et al., 1997). Influenza virus (myxovirus) causes apoptosis of cultured MDCK and U937 cells (Price et al., 1997). Dengue virus and rabies virus induce apoptosis in a hepatoma cell line (Marinneau et al., 1997) and in cultured lymphocytes (Thouloze et al., 1997) respectively.

Hepatitis C virus (HCV) is a member of the Flaviviridae family. Infection with HCV leads to chronic hepatitis followed by liver fibrosis and hepatocellular carcinoma. The HCV genome consists of a linear positive-stranded RNA molecule of approximately 9600 nucleotides (nt) encoding a single polyprotein of approximately 3010 amino acids (Choo et al., 1989). The polyprotein is cleaved by both host and viral proteases to generate three structural proteins (core, E1, and E2) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). HCV replication occurs via synthesis of a genomic length minus-strand RNA that serves as a replicative intermediate. At present, not much is understood regarding the overall effect of all the HCV proteins on cells due to the lack of a suitable in vitro cell culture model for hepatitis C virus. Recently, our laboratory has established a HCV cell culture system based upon HCV genome length RNA transfection of a hepatoma cell line (Dash et al., 1997). In this model, we observed toxicity in the cells transfected with full-length HCV RNA and a decrease in cell density over time. By using different approaches, we report here that expression of HCV proteins from transfected HCV RNA in a hepatic cell line leads to decreased viability of the cells by apoptotic cell death.

RESULTS

Demonstration of HCV proteins in the HCV RNA transfected HepG2 cells

The constructs used for the in vitro transcription reaction are shown in Fig. 1. To confirm the expression of the structural and nonstructural proteins of HCV in the RNA transfected HepG2 cells and controls, immunohistochemistry was performed. HepG2 cells transfected with full-length HCV RNA expressed HCV proteins (Fig. 2). This staining was found to be specific as demonstrated by various controls employed in the experiment (Fig. 2). HepG2 cells transfected with truncated HCV RNA 6.7 also expressed HCV proteins (data not shown). Immu-
staining results revealed that staining for HCV proteins was mostly in the cytoplasm. This cellular distribution of HCV proteins is consistent with previous reports that investigated the expression of subsets of HCV proteins (Santolini et al., 1994; Selby et al., 1993). Approximately 30–35% of HCV RNA transfected cells were positive by immunohistochemistry for the HCV envelope (E2) protein. Counterstaining with hematoxylin revealed that some of the cells with cytoplasmic staining for HCV proteins had condensed nuclei (Fig. 2).

HCV RNA transfected HepG2 cells showed reduced cell proliferation

The effect of HCV protein expression on cell proliferation was assayed by [3H]thymidine uptake. Dividing cells are known to incorporate [3H]-labeled thymidine in DNA, which can be estimated as an indicator of cell proliferation. Proliferation of HepG2 cells transfected with full-length RNA or truncated RNA or mock transfected in the presence of [3H]thymidine was positive by immunohistochemistry for the HCV envelope (E2) protein. Counterstaining with hematoxylin revealed that some of the cells with cytoplasmic staining for HCV proteins had condensed nuclei (Fig. 2).

Transfection of HepG2 cells with HCV RNA leads to reduced viability

There was a decrease in the viability of HepG2 cells transfected with full-length HCV RNA compared to antisense RNA or mutant RNA transfected cells (Fig. 5A). Relative to mock-transfected cells, full-length HCV RNA transfected cells showed a 30% reduction in cell viability. Cells transfected with mutant RNA lacking the HCV internal ribosomal entry site (508–9565 mutant) did not express HCV proteins (Fig. 4) and failed to show a reduction in cell viability (Fig. 5A). To determine whether similar results could be obtained with other HCV clones including clones shown to be infectious in vivo, HepG2 cells were transfected with RNA transcribed from a chimpanzee infectious clone, pCVJ4L6S (Yanagi et al., 1998), and cell viability was determined. The viability of HepG2 cells transfected with RNA derived by in vitro transcription of the infectious clone was also reduced by 37%

FIG. 1. Construct used to prepare HCV full-length and antisense RNA by in vitro transcription. Full-length HCV RNA 9565 nt in length (1) was prepared by in vitro transcription using T7 RNA polymerase from pGEM 9.6-T7 after restriction digestion with HindIII. Antisense HCV RNA (9100AS) (3) was prepared from the same plasmid using SP6 polymerase after digestion with NotI. Similarly truncated HCV RNA 6700 nt in length (2) was prepared by in vitro transcription using T7 polymerase from pGEM 6.7-T7 after restriction digestion with EcoRI enzyme. All the structural and nonstructural HCV proteins are expressed from full-length HCV RNA (1), whereas truncated HCV RNA (2) encodes all the HCV proteins except part of NS5A and NS5B. Antisense RNA (3) does not express any HCV proteins. The positions of the restriction sites are shown in the figure.
compared to the mock transfected control. To ascertain whether this effect was due to expression of HCV proteins from transfected RNA or replication of RNA, HepG2 cells were transfected with HCV cDNA from a mammalian expression plasmid (pcDNA3/333-9425). Interestingly, we observed a similar reduction (30%) in cell viability of HepG2 cells transfected with this construct.

To correlate reduction in viability of transfected cells with the amount of HCV RNA, increasing amounts of HCV RNA were used for transfection. As shown in Fig. 5B, a progressive decrease in cell viability was observed, from 75.4% viable cells for 1 µg of HCV RNA to 63.22% viable cells for 6 µg of HCV RNA. The overall levels of HCV protein production were proportional to the amount of

FIG. 2. Demonstration of HCV envelope (E2) protein in the transfected HepG2 cells and controls. (A) Expression of HCV envelope (E2) protein (arrows) in full-length HCV RNA transfected HepG2 cells (X400). Inset shows a positive HepG2 cell with condensed nucleus (X1000). (B) Full-length HCV RNA transfected HepG2 cells stained with preadsorbed anti-E2 antibody (X400). (C) Mock-transfected HepG2 cells stained with anti-E2 antibody (X400). (D) Full-length HCV RNA transfected HepG2 cells stained with normal mouse IgG (X400).

FIG. 4. Expression of HCV proteins in HepG2 cells transfected with HCV full-length RNA and controls. HepG2 cells were transfected with HCV mutant RNA lacking the internal ribosomal entry site and translational start codon (HCV 508-9565 mutant) (A), or HCV truncated RNA (HCV 6.7) (B), or HCV full-length RNA (HCV 9.6) (C), at different time points posttransfection followed by immunohistochemistry using anti-E2 antibody as described under Materials and Methods (I, 7 days; II, 14 days; III, 21 days). At 21 days, fewer cells were positive in the truncated RNA transfected cells than in the full-length RNA transfected cells.
HCV RNA transfected (data not shown). These results indicated that HCV protein expression resulted in decreased viability of HepG2 cells and that this effect was dose dependent. To confirm the finding that altered cell viability is due to the expression of HCV proteins, an additional experiment was performed with three different constructs, HCV full-length RNA, HCV truncated RNA, and mutant RNA lacking the HCV internal ribosomal entry site (mutant RNA). The results shown in Fig. 5C indicate that RNA that does not express HCV proteins did not affect cell viability compared to mock control at 3, 7, 14, and 21 days posttransfection, whereas cells transfected with the other two constructs expressed proteins and cell viability was affected.

TUNEL (terminal deoxynucleotidyltransferase mediated dUTP–biotin nick end-labeling) assay

An important feature of apoptotic cell death is the presence of fragmented DNA in the chromatin. There-
Therefore, we examined HCV RNA in the transfected HepG2 cells, for DNA fragmentation by terminal transferase enzyme (TdT) labeling (Fig. 6). Antisense RNA transfected HepG2 cells served as negative control. As shown in Fig. 6, about 10% of cells were TUNEL positive. Cells transfected with antisense RNA and mock controls were negative. This was further confirmed using an alternative approach to detect DNA fragmentation: cells were stained with propidium iodide. Typical chromatin condensation and fragmentation characterizing apoptotic cells were evident in HCV full-length RNA transfected HepG2 cells (Fig. 7).

Morphological and ultrastructural studies

HCV RNA transfected cells were processed for transmission electron microscopy to confirm morphological changes during apoptosis such as nuclear condensa-
tion, nuclear fragmentation, and cell shrinkage. One to 2-μm-thick sections of cell pellets were stained with toluidine blue and paraphenylediamine and then examined by light microscopy for differentiation of nuclear morphology and cytoplasmic compartments. Ten to 15% of cells transfected with full-length HCV RNA showed nuclear condensation (Fig. 8B), as evidenced by darker staining nuclei when compared with controls (Fig. 8A) by random unbiased counting. These pellets were further subjected to transmission electron microscopy to study the ultrastructural changes. Cells with nuclear condensation and fragmentation of nuclei but with intact cytoplasmic organelles and plasma membranes (shown in Fig. 9A) were seen, compared to control cells (Fig. 9B). These morphological changes were suggestive of apoptosis. Morphological changes characteristic of apoptosis were not observed in HepG2 cells transfected with antisense RNA or mock-transfected controls.

Cell cycle analysis

The presence of apoptotic cells in the HCV RNA transfected cell cultures was further confirmed by flow cytometry. Hypodiploid DNA peaks in the sub-G0/G1 region were detected in propidium iodide stained HepG2 cells transfected with HCV RNA. Antisense RNA transfected HepG2 cells did not show a sub-G0/G1 peak, indicating the absence of apoptotic cells (Figs. 10A and 10B). Approximately 10% of the HCV transfected cells were found to be apoptotic.

DNA fragmentation

Apoptotic cells are characterized by the presence of fragmented DNA in their nucleus. This fragmented DNA can be extracted and analyzed on 2% agarose gels. Necrotic cells are characterized by the presence of a DNA smear, whereas apoptotic cells have fragmented DNA. To confirm the apoptotic changes of HCV RNA transfected cells, we evaluated DNA laddering of full-length, truncated, and mock transfected cells (Fig. 11). However, a moderate DNA ladder was present in HCV 6.7 RNA transfected cells, thus confirming our earlier observation that reduced cell viability in HCV 6.7 RNA transfected cells was due to the expression of HCV proteins.

DISCUSSION

Histochemical examinations of biopsy samples from patients with hepatitis C have been reported to show apoptosis of hepatocytes (Kerr et al., 1979; Calabrese et al., 2000). It is not clear whether these apoptotic bodies are due to direct the cytopathic effect of HCV or a consequence of immunological reactions. To investigate the former hypothesis, we determined the effects of HCV protein expression on hepatic cells. Since HCV is a hepatotropic virus, we used a hepatoblast cell line to study the effects of HCV expression on cells. Previously we had reported that transfection of full-length HCV RNA to HepG2 cells leads to replication and formation of infectious viral particles in cell culture. We observed that cells transfected with HCV RNA showed significant cytotoxicity and a decrease in cell density over time. In this report, we extended these observations and determined the effect of HCV expression on cell viability. To exclude toxicity associated with RNA transfection, an antisense RNA and a mutant RNA lacking the internal ribosomal entry site were used as controls. We demonstrated that HepG2 cells transfected with full-length as well as truncated HCV 6.7 RNA produced HCV proteins after transfection. A small proportion (30–35%) of these cells showed nuclear condensation and fragmentation.
showed distinct cytoplasmic positive staining as detected by immunohistochemistry using specific anti-HCV protein antibodies. Some of the cells expressing HCV proteins had condensed nuclei, a characteristic feature of apoptotic cell death (Garen et al., 1999; Price et al., 1997; Hoff and Donis, 1997). We have also confirmed the expression of all the structural and nonstructural proteins of HCV from the cDNA construct used in the present study (Myung et al., in press) manuscript submitted for publication). We showed that cells transfected with full-length HCV RNA affected cell viability and cell proliferation at 7, 14, and 21 days after transfection. HepG2 cells transfected with mutant RNA had a similar effect at the earlier time point possibly because this RNA may still translate protein. To confirm these observations, expression of HCV protein in the transfected cells was examined at 7, 14, and 21 days. As expected, cells transfected with full-length as well as mutant HCV RNA 6.7 express HCV protein at 7, 14, and 21 days. However, there are many fewer HCV-positive cells present in the 6.7 RNA transfected cells compared to full-length RNA (Fig. 4, B3). The decrease in proliferation of cells transfected with truncated RNA at earlier time points (7 and 14 days) might be due to the expression of HCV proteins, but these cells recovered at 21 days probably because of the absence of HCV protein expression (Fig. 4). It is of interest to note that there is a reduction of cell viability at 14 and 21 days. This is probably due to the fact that not all HepG2 cells in the culture were transfected uniformly with HCV RNA. Cells transfected with many copies of HCV RNA translated more HCV protein and became positive by immunocytochemistry and exhibited reduced cell viability at earlier time points. However, cells transfected with fewer copies of HCV RNA may not have been detected at earlier time points by immunocytochemistry possibly due to lower levels of HCV protein expression. We feel that the replication and translation of HCV RNA in these cells over time led to the accumulation of HCV proteins that affected cell viability on days 14 and 21.

Reduction in cell viability was not exclusively due to

FIG. 11. Demonstration of DNA laddering in HCV RNA transfected HepG2 cells. Mock transfected cells (lane 2), and cells transfected with HCV 9.6 RNA (lane 3), 6.7 RNA (lane 4), and antisense (9100 AS) RNA (lane 5) were subjected to DNA laddering. Lane 1, 100-bp molecular weight marker. The brighter band in the molecular weight marker lane is 600 bp. Fragmented nuclear DNA in HCV full-length RNA transfected cells can be seen as a brighter DNA ladder, compared to a faint ladder in truncated RNA transfected cells.
replication of transfected RNA since similar toxicity was observed in cells that were transfected either with a truncated HCV RNA control (Fig. 5) or with a pcDNA3/333-9425 construct lacking the 5’UTR and parts of the 3’UTR at 3 days posttransfection, both presumably replication defective. This effect might be due to translation of HCV proteins by this construct. To support this assumption, we performed additional experiments and determined that cells transfected with HCV RNA with a deletion of internal ribosome entry site sequences did not produce proteins and had no effect on cell viability (Fig. 5). To exclude the possibility that the reduction in cell viability in HCV RNA transfected cells might be due to the nonspecific effect of transfected RNA, cells transfected with antisense RNA and mutant RNA with a deletion in the internal ribosomal entry site were used as controls. Cells transfected with these RNAs did not show reduced cell viability. These results suggest that expression of HCV proteins is toxic to cells.

To determine whether HepG2 cells expressing HCV proteins had morphological features of apoptosis, DNA fragmentation, cell cycle analysis, TUNEL assays, and electron microscopy studies were performed. Cells showing evidence of apoptosis were observed in a HCV RNA transfected HepG2 cell culture by different assays and compared to control cultures. To further characterize the ultrastructural changes in these cells, high-magnification transmission electron microscopy was performed and cells with fragmented and crescent-shaped nuclei, but with intact cytoplasmic organelles and membranes characteristic of apoptotic cell death were observed (Fig. 8). TUNEL assays and cell cycle analysis provided further evidence of apoptotic cell death, because of the appearance of a brown staining nucleus (Fig. 6) and a sub-G0/G1 peak (Fig. 10), respectively. There is a difference in DNA fragmentation in cells transfected with full-length versus truncated HCV RNA. This difference could be due to a combination of RNA replication, RNA stability, translation, and persistence of full-length RNA compared to truncated HCV 6.7 RNA. It is interesting to note that though about 30–35% of transfected cells expressed HCV proteins, only 10–15% of the cells underwent apoptosis. This discrepancy demonstrates a difference in various assays employed to assay cell death. [3H]Thymidine uptake measures cell proliferation and trypan blue dye exclusion measures membrane permeability changes, whereas cell cycle analysis and DNA laddering measure hypodiploid DNA and fragmented DNA, respectively.

The apoptotic form of cell death is reported in many other virus infections in vitro as well as in vivo. This occurs either due to toxic effects of viral proteins or due to host–virus interactions. At present there are several reports representing both pro- and anti-apoptotic effects of HCV proteins. Most of these studies have relied on systems that express either individual proteins of HCV (core, NS3) or systems in which only a few HCV proteins were expressed in cells. Some of these reports indicate that core protein expression induced apoptosis in response to anti-fas monoclonal antibody (Ruggieri et al., 1997) and enhanced the susceptibility of hepatocytes to TNFα mediated apoptosis (Zhu et al., 1998). Some reports indicate that expression of HCV core protein suppresses c-myc overexpression induced apoptosis in Chinese hamster ovary cells, cisplatin mediated apoptosis in human cervical epithelial cells (Ray et al., 1996), and TNFα induced apoptosis in a human breast carcinoma cell line (Ray et al., 1998). Marusawa et al. (1999) reported inhibition of TNFα and fas mediated apoptosis of HepG2 cells via NF-κB activation by HCV core protein. Activation of NF-κB is shown to be responsible for the resistance of hepatic cells expressing HCV structural proteins to TNFα (Tai et al., 2000). Fujita and co-workers (1998) suggested that NS3 protein inhibits actinomycin D induced apoptosis in NIH 3T3 cells. Expression of NS5A protein in HepG2 cells is also shown to protect against TNFα induced apoptosis (Ghosh et al., 2000). In contrast, Dumoulin and co-workers (1999) have shown that HCV core protein does not inhibit apoptosis in HepG2 cells induced by either TNFα or fas. However, some other reports have suggested proapoptotic effects of HCV protein expression. An osteosarcoma cell line indubitably expressing both structural and nonstructural proteins of HCV exhibited cytotoxicity (Moradpour et al., 1998). Chinese hamster ovary cells stably expressing core protein were shown to undergo apoptosis in response to serum starvation (Honda et al., 2000). Thus, both anti-apoptotic and pro-apoptotic effects of HCV proteins are evident. It is possible that the effects of HCV proteins on the cells might be different depending upon whether HCV proteins are expressed alone or in the presence of other HCV proteins. Differences in these contradicting reports about the pro- and anti-apoptotic effects of HCV proteins might also be due to the different cell lines and HCV proteins used. To mimic the in vivo HCV infection, in which all the HCV proteins are present, we used full-length HCV RNA and cDNA transfection. Though it was recently reported that replication of HCV might be responsible for liver injury (Chang et al., 2000), toxicity associated with HCV truncated 6.7 RNA and HCV cDNA (pcDNA3/333-9425) expression in our experiments indicates that reduction in cell viability is due to HCV protein expression. The circumstances in which these events are relevant and their role in virus infection in vivo and in cellular transformation need to be studied in detail.

Several unsuccessful attempts by different laboratories including ours have been made to establish stable cell lines for full-length HCV genome. This may be partly related to the cytotoxicity associated with the expression of HCV proteins. We have observed apoptosis induced by the expression of HCV proteins in a hepatic cell culture model. Currently we are investigating which
structural or nonstructural HCV proteins induce apoptotic cell death in HepG2 cells. This will allow us to understand the mechanism of host–virus interactions in HCV induced apoptotic cell death. Further studies of the relevance of apoptosis in HCV infections in humans are needed.

MATERIALS AND METHODS

Plasmid constructs

The full-length HCV cDNA clone used in this study has been described previously (Dash et al., 1997). Full-length HCV RNA was prepared from this construct using T7 RNA polymerase. It was not possible to prepare an antisense RNA from the plasmid construct pMO9.6-T7 since this plasmid construct does not have an SP6 promoter or a T7 promoter in the opposite orientation. Therefore, another construct, pGEM 9.6-T7, was prepared. For this purpose, we first removed the full-length HCV cDNA from pMO9.6-T7 by HindIII digestion and cloned it into the pGEM 3Zf(+)-vector. This allowed us to generate an antisense RNA from HCV cDNA using SP6 RNA polymerase. To generate run-off transcripts, this plasmid was linearized with HindIII and antisense RNA was prepared using SP6 RNA polymerase. To generate truncated 6.7 RNA, pGEM6.7-T7 was prepared. For this purpose, we first removed the full-length HCV cDNA from pMO9.6-T7 by HindIII digestion and cloned it into the mammalian expression vector pcDNA3. This construct does not contain the UTR and has only 50 nucleotides of the 3’UTR. The resultant plasmid, pcDNA3/333-9425, was used for transfection of HepG2 cells using FuGene6 (Roche, Indianapolis, IN) transfection reagent, according to the manufacturer’s protocol. cDNA for HCV mutant RNA was prepared by restriction digestion of pMO9.6-T7 with NotI and cloned into pMO9.4-T7 plasmid. The HindIII fragment from this construct containing HCV nt 333–9425 sequence was again recloned into the mammalian expression vector pcDNA3. This construct does not contain the 5’UTR and has only 50 nucleotides of the 3’UTR. The resultant plasmid, pcDNA3/333-9425 was used for transfection of HepG2 cells using FuGene6 (Roche, Indianapolis, IN) transfection reagent, according to the manufacturer’s protocol. cDNA for HCV mutant RNA was prepared by restriction digestion of pMO9.6-T7 with NotI and HindIII enzymes followed by cloning into pGEMEX 2.0 (Promega Inc., Madison, WI). This clone lacks the first 508 bp of HCV cDNA encoding the 5’UTR and internal ribosomal entry site. Mutant RNA was prepared by using HindIII linearized pGEMEX 508-9565 as a template in the in vitro transcription reaction.

Preparation of HCV RNA transcripts

Sense and antisense HCV RNA was prepared from plasmid construct pGEM 9.6-T7 using T7 and SP6 RNA polymerase, respectively (Promega). Mutant HCV RNA was prepared using T7 RNA polymerase from HindIII linearized pGEMEX 508-9565. HCV RNA was in vitro transcribed using 40 mmol/L Tris/ HCl (pH 7.5), 6 mmol/L MgCl₂, 10 mmol/L dithiothreitol, 2.5 mmol/L ribonucleotides, and 5 µg of DNA templates. The reaction mixture was incubated for 1 h at 37°C. Next the reaction mix was treated with DNase I (RNase, Promega) for 1 h and a final phenol-chloroform extraction was performed. A second DNase digestion was performed to ensure no carryover of DNA in the in vitro transcripts. The RNA was extracted with phenol-chloroform and then with chloroform-isooamyl alcohol. Finally the RNA transcripts were precipitated with 2.5 vol of absolute ethanol after 1 vol of 7.5 mol/L ammonium acetate was added. The integrity of the RNA transcripts was evaluated after electrophoresis through a 1% agarose gel.

Cell culture and RNA transfection

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in minimal essential medium (MEM; Gibco BRL) with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, and antibiotics. HepG2 cells are well-differentiated cells with biosynthetic capabilities similar to those of human hepatocytes. Separate 60-mm dishes of HepG2 cells (2×10⁶ cells) seeded 1 day before transfection were transfected with 20 µg of HCV RNA, antisense RNA, HCV 6.7 RNA or mock transfected using the diethylamino-ethyl (DEAE)–dextran method. For this purpose we complex of DEAE–dextran (400 µg) and 20 µg of HCV RNA in 0.5 ml of 10 mmol/L HEPES buffer, pH 7.3, was incubated. After 30 min 0.5 ml of complete medium was added to the complex and layered on HepG2 cells in 60-mm dishes for 2 h. Finally the complex was removed, and the cells were washed three times with 5 ml of PBS and treated for 2 min with 2 ml of PBS containing 10% dimethyl sulfoxide. The cells were then washed and maintained in 3 ml of complete medium.

Immunohistochemistry of HCV proteins

We determined whether HCV RNA transfected HepG2 cells produced HCV proteins by immunohistochemistry using anti-E2 antibody (gift from Michael Houghton, Chiron Corp., Emeryville, CA) and the ABC method (Vectorstain, Vector Laboratories, Burlingame, CA). Since detection of HCV proteins is very critical in this system, we first determined the specificity of anti-E2 antibody based upon following evidence: (i) Anti-E2 antibody reacted in both Western blotting and immunofluorescence assays using a vaccinia–T7 binary expression system that ex-
presses HCV proteins; (ii) aeadsorption of anti-E2 antibody with HCV positive antibody followed by immunostaining of HCV RNA transfected cells revealed a negative reaction; and (iii) there was a linear correlation between the amount of HCV RNA used for transfection and the number of positive cells detected by immunohistochemistry. At 3 days after transfection, immunohistochemistry for HCV was performed. After the cells were washed twice with phosphate-buffered saline (PBS), pH 7.4, cells were air-dried and fixed with cold acetone (Fisher Scientific, Pittsburgh, PA) for 10 min. The cells were permeabilized with 0.06% saponin after being rinsed with PBS thrice for 5 min each. Blocking of these cells was performed with 1.5% normal goat serum (Sigma Chemical Co., St. Louis, MO) and 2% BSA for 30 min at room temperature. The cells were incubated with primary antibodies (1:500) overnight at 4°C. Negative controls with 1:500 normal mouse IgG (Biotechnology Inc., Santa Cruz, CA) were used instead of primary antibody. After being rinsed with PBS, the slides were incubated with biotinylated goat anti-mouse antibody (Biotechnology Inc., Santa Cruz, CA). Blocking for endogenous biotin and peroxidase was done with 0.9% H₂O₂ for 30 min followed by Elite avidin–biotin peroxidase complex (Vector Laboratories, Burlingame, CA). The slides were developed by incubation with diaminobenzidine (Dako, Carpinteria, CA) for 1 min. Counterstaining was done with hematoxylin (Sigma Chemical Co.) for 1 min. The slides were mounted with permount after dehydration and observed under an Olympus light microscope.

Cell proliferation

Cell proliferation was measured by [³H]thymidine uptake by using a standard protocol published previously (Tolleson et al., 1996). HepG2 cells transfected with HCV RNA and controls at different time points were incubated with 0.1 μCi [³H]thymidine for 4 h in MEM with 10% fetal bovine serum. Cells were washed once with PBS and 10% trichloroacetic acid (TCA). Cells were then incubated with 10% TCA for 10 min at 37°C. After TCA was removed from the cell culture plates, cells were lysed with 0.5 ml of lysis buffer containing 0.33 N NaOH and 1% sodium dodecyl sulfate. The cell lysates were then mixed with 6 ml of scintillation fluid and [³H]thymidine incorporation was measured in a liquid scintillation counter (Beckman Instruments, Fullerton, CA).

Cell viability

HepG2 cells transfected with HCV RNA and controls were subjected to cell viability assays using a standard trypan blue dye exclusion assay as indicated. Transfected cells were trypsinized and resuspended in 1 ml of complete minimal essential medium. Twenty microliters of this solution was mixed with 30 µl of PBS and 50 µl of 0.4% trypan blue (Sigma Chemical Co.) and incubated at room temperature for 1 min. Live and dead cells were observed and counted in the neubar’s chamber. The number of dead cells was counted as those stained with trypan blue dye within four microscopic fields of neubar’s slide. The percentage of total cells excluding the dye is expressed as the percentage viability. The percentage viability of mock transfected cells was taken as 100% and other results were normalized.

TUNEL assay

The TUNEL technique detects endonucleolytically cleaved DNA by the addition of labeled dUTP to DNA ends by TdT. HepG2 cells transfected with full-length HCV RNA and controls were used for the TUNEL assay using a TACS in situ apoptosis detection kit (Genzyme Inc., Cambridge, MA) according to the manufacturer’s protocol. The cells were counterstained with 1% methyl green followed by observation under a light microscope. Objective analysis of TUNEL stained cells was performed by the method previously reported (Plymale et al., 1995). Propidium iodide staining was performed by adding propidium iodide to 1 × 10⁴ cells to a final concentration of 10 mg/ml followed by fluorescence microscopy.

Morphological and ultrastructural studies

HepG2 cells transfected with full-length HCV RNA and antisense RNA controls were examined at 3 days post-transfection by electron microscopy. Pellets of the transfected and control HepG2 cells were fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol, infiltrated with propylene oxide, and embedded in Epon mixture. To determine the percentage of apoptotic cells, thick sections of the pellet (1–2 µm) were fixed on a glass slide and stained with toluidine blue (TB) (Fisher Scientific) and paraphenyldiamine (PDA). A mixture of TB and PDA differentially stains cytosolic and nuclear compartments. The slides were then washed with warm water, air-dried, and examined with a light microscope for cellular morphology. Ten high-power fields were randomly examined in the control as well as HCV transfected cells and changes were recorded. For transmission electron microscopy, ultrathin sections were stained with a saturated solution of uranyl acetate and lead citrate. Cell pellets were examined by transmission electron microscopy using a Hitachi H.7100 electron microscope without knowledge of the origin of the cells.

Flow cytometry

For flow cytometry analysis of HepG2 cells transfected with HCV RNA and controls, cells were washed with PBS twice, trypsinized, and resuspended in 200 µl of PBS. These cells were fixed with 70% ethanol. After incubation at 4°C for 30 min, cells were again washed twice with PBS. To remove double-stranded RNA, cells were further
incubated with 3 units of RNase for 30 min at 37°C, followed by staining with 100 μl of propidium iodide (Roche Molecular Biochemicals, Indianapolis, IN) for 10 min and 10,000 cells were analyzed in an EPICS-Elite flow cytometer (Coulter, Hialeah, FL).

DNA fragmentation

Apoptosis of HCV RNA transfected HepG2 cells was confirmed by a DNA laddering assay. A total of 6 x 10^5 HepG2 cells transfected with RNA and controls were mixed with 20 μl lysis buffer [20 mM EDTA, 100 mM Tris, pH 8.0, 0.8% (w/v) SDS] and 10 μl RNase A/T1 cocktail mix (500 units/ml and 20,000 units/ml, respectively from Ambion Inc.) and incubated at 37°C for 60 min. Ten microliters of proteinase K (20 mg/ml, Ambion Inc.) was then added to the cell lysates followed by incubation at 50°C for 90 min. Five microliters of 6× DNA loading buffer was added to the lysates and electrophoresed on 1.5% agarose gel in 1× Tris–acetate–EDTA buffer at 35 V for 4 h.

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