Increased Prooxidant Production and Enhanced Susceptibility to Glutathione Depletion in HepG2 Cells Co-expressing HCV Core Protein and CYP2E1

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Hepatitis C virus (HCV) and HCV core protein are hypothesized to induce hepatic oxidative stress and exacerbate injury caused by other toxins such as ethanol that induce the cytochrome P450 enzyme, CYP2E1. In the current study, the effects of HCV core protein [sequence genotype 1b, (nt 342–915)] on parameters indicative of oxidative stress were evaluated in HepG2 cells stably over expressing CYP2E1 (E47), or vector controls (C34). Stable (>10 passages) expression of HCV core protein and CYP2E1 was confirmed in clonal cell lines at the level of mRNA and immunoreactive protein. Prooxidant production, as determined by cellular oxidation of dichlorodihydrofluorescin and dihydroethidium (HE), was increased by expression of HCV core protein in the presence or absence of CYP2E1. Depletion of glutathione (GSH) with buthionine sulfoximine (BSO) enhanced prooxidant production in both C34 and E47 cells. In addition, prooxidant production was greater in BSO-treated cells expressing HCV core protein, and this effect was further enhanced in cells expressing both HCV core and CYP2E1. The CYP2E1 inhibitor, 4-methylpyrazole, could suppress increased prooxidant production in E47 cells. Finally, cells co-expressing both CYP2E1 and HCV core protein showed significantly decreased viability following GSH depletion. These studies show simultaneous expression of HCV core protein and CYP2E1 increases parameters indicative of oxidative stress as well as sensitization to cell injury induced by GSH depletion. These results support the hypothesis that enhanced injury in hepatocytes over expressing both HCV core protein and CYP2E1 is mediated by increases in oxidative stress. J. Med. Virol. 72:230–240, 2004.

KEY WORDS: hepatitis C core protein; CYP2E1; oxidative stress; prooxidant production; glutathione; buthionine sulfoximine

INTRODUCTION

Hepatitis C (HCV) and alcoholic liver disease (ALD) are the two most important causes of liver disease worldwide. In the United States, 1–2% of the population is infected with the virus (Cuthbert, 1994; Hoofnagle, 1997; Tsukomoto and Lu, 2001) and concomitant alcohol consumption is a major risk factor for progressive liver disease [Poynard et al., 1997]. Although the molecular events involved in the synergism of alcohol and HCV are probably multi-factorial, liver injury from either process

Abbreviations: HCV, hepatitis C virus; BSO, buthionine sulfoximine; 4-MP, 4-methylpyrazole; HE, dihydroethidium; DCF, dichlorofluorescein; GSH, glutathione; GSSG, glutathione disulfide; RT-PCR, reverse transcriptase polymerase chain reaction.

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has been hypothesized to depend on the generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide [Nordmann et al., 1992; Farinati et al., 1995; De Maria et al., 1996; Farinati et al., 1996; Ishii et al., 1997].

ROS are considered important for HCV-induced liver disease and oxidative damage is probably generated from cell-mediated target cell cytolyis of HCV infected hepatocytes. Excessive oxidative stress generates lipid peroxidation products such as malondialdehyde (MDA) and other reactive aldehyde metabolites that have been detected in the serum and liver of patients with chronic HCV [Farinati et al., 1995, 1996] and localized to areas of stellate cell collagen synthesis [Paradis et al., 1997]. These patients also show reduced levels of plasma and hepatic glutathione (GSH) [De Maria et al., 1996] and thioredoxin [Sumida et al., 2000] suggesting that chronic depletion of cellular reduced thiol reserves is important for fibrogenesis.

The importance of ROS for chronic ALD has been shown in patients and animal models of acute and chronic ethanol injury [Nordmann et al., 1992; Ishii et al., 1997]. The hepatotoxic effects of ethanol and ROS production are closely tied to ethanol metabolism. An important ethanol oxidizing system in chronic ALD is the cytochrome P450 enzyme CYP2E1 that is found in the cellular microsomes [Lieber, 1993]. CYP2E1 is inducible by ethanol and other drugs and oxidizes ethanol to acetaldehyde [Tesckhe et al., 1974; Tsutsumi et al., 1989]. This metabolic pathway has been linked to increased generation of ROS, lipid peroxidation, heightened cytokine responses, and increased fibrosis of chronic alcoholic liver disease [Lieber, 1997]. Immunohistochemical localization of CYP2E1 is primarily centrilobular [Forker et al., 1991], the characteristic area of fibrosis in ALD.

Because both alcohol and HCV, individually, are thought to promote liver injury through oxidative stress, it is possible that greater than additive disturbances in metabolic oxidation/reduction reactions may be a primary mechanism for the synergistic interaction of these agents. Recent in vitro studies have suggested that hepatitis C viral proteins such as HCV core and NS5A can induce oxidative stress in transfected cells [Gong et al., 2001; Okuda et al., 2002]. Furthermore, a variety of reports demonstrate that HCV core protein can exert transcriptional control over selected cellular functions by interference with cytokine responses, cellular signaling, and apoptosis [Matsumoto et al., 1997; Ray et al., 1997, 1998; Faubion and Gores, 1999; Kato et al., 1999; Marusawa et al., 1999]. The transcriptional regulation of cellular activities by other viral proteins has also been suggested to depend on the modulation of intracellular oxidation/reduction reactions and redox sensitive signaling [Pahl, 1999]. These pathways are also important for the pathogenesis of ALD, consequently, further study is needed to evaluate the effects of viral proteins, in particular HCV core, on enzymes associated with ethanol metabolism such as CYP2E1 and the relationship of these interactions to oxidative stress.

Hepatocyte cell lines that over express CYP2E1 have been well-characterized [Dai et al., 1993; Chen and Cederbaum, 1998]. In the current study, the effects of HCV core protein [sequence genotype 1b, (nt 342–915)] on parameters indicative of oxidative stress was evaluated in HepG2 cells stably over expressing, the P450 enzyme CYP2E1 (E47), or vector controls (C34). Stable (>10 passages) over expression of HCV core protein and CYP2E1 was confirmed at the level of mRNA and immunoreactive protein. Prooxidant production, as determined by cellular oxidation of dichlorodihydrofluorescin and dihydroethidium (HE), was increased by expression of HCV core protein in the presence or absence of CYP2E1. Depletion of GSH with buthionine sulfoximine (BSO) enhanced prooxidant production in both C34 and E47 cells, prooxidant production was greater in BSO-treated cells expressing HCV core protein, and this effect was further enhanced in cells expressing both HCV core and CYP2E1. Finally, cells over expressing CYP2E1 and HCV core protein showed significantly decreased viability following GSH depletion. These studies show that simultaneous expression of HCV core protein and CYP2E1 increases parameters indicative of oxidative stress as well as sensitization to cell injury induced by GSH depletion. These results support the hypothesis that enhanced injury in hepatocytes expressing HCV core protein and over expressing CYP2E1 is mediated by increases in oxidative stress.

MATERIALS AND METHODS

Materials

Molecular biology grade phenol (Aramesco, Inc., Solon, OH), Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), moloney murine leukemia virus reverse transcriptase (Gibco/BRL Lift Technologies, Gaithersburg, MD) were used in these studies. Oligonucleotide primers were prepared by the University of Iowa College of Medicine DNA Core Facility. BSO and 4-methylpyrazole (4-MP), DNAase I (RNAase free) were obtained from Sigma (St. Louis, MO). 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 5-carboxyl-2′,7′-dichlorofluorescein diacetate (carboxy-DCF-DA), and HE were obtained from Molecular Probes (Eugene, OR).

For reverse transcriptase polymerase chain reaction (RT-PCR) detection of HCV core and CYP2E1 mRNAs, specific primers were prepared from the known nucleotide sequences. The genotype 1b primers were: sense 5′-aaatctcaacctcacaaga-3′ and anti-sense 5′-ggaagc- 

tggagttgccaaaca-3′. Nucleotide sequence for CYP2E1 was obtained from GenBank. CYP2E1 primers were sense 5′-ctctcttgtctgtctgctatg and anti-sense 5′-ctttggtcttggtctggct-3′ which produced a fragment of 439 bp which was verified by direct sequencing.

Cell Lines and Culture Conditions

Cell lines stably transfected with CYP2E1 and capable of ethanol metabolism, were a kind gift of Dr. A. Cederbaum, (Mount Sinai, NY). The E47 cell line is a stable clone of HepG2 cells transfected with CYP2E1.
[Cederbaum et al., 2001]. HepG2 cells do not normally make CYP2E1, however, over expression of the enzyme [Dai et al., 1993; Chen and Cederbaum, 1998] makes the cells susceptible to oxidative stress from ethanol and other toxins [Wu and Cederbaum, 1996; Chen et al., 1997; Cederbaum, 1998]. The appropriate control for E47 is the stable C34 cell line that was transfected with the vector only. The lines were routinely passed in Dulbecco’s Modified Minimal Essential Medium (DMEM) with 10% fetal bovine serum containing high glucose, 2mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml), together with selection antibiotic G418 (0.6 mg/ml).

**Plasmids**

PC-DNA3.1 plasmid was obtained from Invitrogen (Carlsbad, CA) with zecon resistance cassette. Plasmid CMV core [Marusawa et al., 1999] containing HCV core sequences, (HCV-J, genotype 1b, nucleotides 341–914), was a kind gift of Dr. M. Hijicata, (Kyoto University, Kyoto, Japan). The genomic sequences of core were amplified by PCR and a 5’ Hind III site was inserted followed by Kozak sequences and an in frame start codon just before the HCV core sequences. A stop codon and terminal 3’ EcoRI were also inserted by PCR. The complete fragment was then cloned into pcDNA3.1 using 5’ HindIII and 3’ EcoRI restriction enzymes. HCV sequences were confirmed by sequence analysis (ABI automated sequencer) as previously described [Stapleton et al., 1999].

**Transfections**

Cells were transfected with pcDNA 3.1 plasmid with or without full-length core sequences using Lipofectamine (Invitrogen, Co.). Transfections were performed in 35 mm plates using a ratio of 5 µg of plasmid DNA per 2 × 10⁶ cells. Forty-eight hours after transfection, cells were washed with fresh medium and then placed into antibiotic selection medium containing zeocin (100 µg/ml, for core selection) and G418 (0.4 mg/ml for CYP2E1 selection). Transfected cells were cloned twice by limiting dilution and then routinely passed in DMEM with 10% FBS containing selection antibiotics. Table I shows the four clonal lines developed from these transfections.

**RNA Isolation and Reverse Transcriptase PCR**

RNA was prepared from 1 × 10⁶ cells using a modification of the phenol-GITC solubilization procedure as we described previously [Schmidt et al., 1995]. After ethanol precipitation the RNA was digested twice with RNAase free DNAase I (50 µ/ml) (Sigma), 1 hr at room temperature, then re-extracted with phenol, precipitated with ethanol, and used for RT-PCR as described below.

RT-PCR was performed as described previously [Schmidt et al., 1995, 1997; Stapleton et al., 1999]. Reverse transcription was conducted at 41°C for 1 hr and the reverse transcriptase inactivated by heating 5 min at 95°C. The cDNA was then reacted with Taq polymerase using 34 cycles of PCR (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min each cycle). DNA products were then analyzed on 1.6% agarose gels using a 1 kb ladder as standard and staining with ethidium bromide.

**MTT Assay and Cellular Viability**

The Cell Titer 96 Non-radioactive Precipitation Assay Kit (Promega, Madison, WI), that utilizes MTT dye conversion, was used for growth and cellular viability experiments. The assay was performed as described by the manufacturer with reference absorption at 630 nm and absorbance of each reaction at 570 nm. The difference in A₅₇₀−₆₃₀ was taken as the index of cell viability. Cells incubated with control medium were used as the 100% viability value. The percent viability was determined by the formula [A₅₇₀−₆₃₀sample/₆₃₀control × 100]. In all cases, direct cell counting and routine trypan blue dye exclusion verified increases in cell number and viability determined by MTT assay.

**Cellular Protein Assay**

Total cellular protein was determined using the Bio-Rad DC protein assay. Cells grown in 96-well plates were washed with three changes of PBS and dissolved in 0.5 N NaOH. Aliquots of the suspension were assayed [Lowry et al., 1951] as instructed by the manufacturer using bovine serum albumin as protein standard.

**Measurements of Prooxidant Production**

Detection of the dichlorofluorescein (DCF) fluorescence (the oxidized product of dichlorodihydrofluorescein) was used as a probe for prooxidant production. Following treatments, cells were washed twice with fresh PBS, (pH 7.4), then incubated with 10 µM DCFH-DA (2′-7′ DCFH-DA) (Molecular Probes, Catalogue no. C-399) for 1 hr in PBS with 1 mg/ml glucose. DCFH-DA is membrane permeable, the ester groups are cleaved by intracellular esterases, and oxidation yields the fluorescent product DCF, which is detectable using 488 nm excitation and 525 nm emission [Hempel et al., 1999]. To ensure that increased DCF fluorescence was measuring changes in oxidation and not increased uptake, ester cleavage, or efflux, the oxidation insensitive analog, carboxy-DCFH-DA (Molecular Probes, Catalogue no. C-369), was used in control experiments. Measurements with the oxidation insensitive probe failed to detect any differences in the amount of DCF fluorescence among the cell lines expressing CYP2E1 and/or HCV core protein, indicating that when changes were seen using the oxidation sensitive probe they were truly representative of altered probe oxidation.

For measurements of prooxidant production using in situ oxidation of HE to the fluorescent product, ethidium, cell lines were grown attached to coverslips for
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48 hr, washed three times in PBS, then incubated with HE (5 µm) for 40 min in the dark. Cells were then washed twice with PBS, fixed 5 min in 3% buffered formalin, and the coverslips mounted. Cells were imaged and photomicrographs taken using confocal laser scanning microscopy with 488 nm excitation and 610 nm emission.

Recombinant Protein Expression and Immunoblot Analysis

Cloned cell lines transfected with pcDNA3.1 plasmid only, or pcDNA3.1 with core sequences were grown to near confluence then washed with PBS and collected by scraping and centrifugation. Cells were lysed by sonication in 10 mM Tris HCl (pH 7.5) containing 0.1 mM PMSF and treated with DNAase I (50 µ/ml) for 60 min on ice. The cellular protein was then mixed with one volume of 2× sample buffer containing 6% SDS and 10% mercaptoethanol, denatured by heating to 95°C for 5 min, and finally separated on 3% acrylamide stacking and 12% Laemmlini running gels for SDS–polyacrylamide gel electrophoresis (SDS–PAGE). After separation, the proteins were electrophoretically transferred to nitrocellulose membranes (Bio Rad, Hercules, CA). Immunoreactive hepatitis C core protein was identified on the blots using mouse monoclonal antibodies (a kind gift of Drs. S. Desai and G. Dawson, Abbott Labs, Abbott Park, IL) to HCV core protein while CYP2E1 enzyme was identified with specific monoclonal antibody to CYP2E1 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive proteins were detected using an appropriate secondary antibody and alkaline phosphatase substrate (AP substrate, Bio-Rad), [Walewski et al., 2001] or 125I labeled staphylococcus protein A followed by autoradiography as previously described [Xiang et al., 2002].

GSH Measurements

Cell monolayers were lysed, then scrape harvested directly from the culture plates using 5% (w/v) sulfosalicylic acid, and the soluble fraction saved for thiol analysis. Total GSH (GSH + GSSG), and GSH disulfide (GSSG) were determined using a spectrophotometric recycling assay as previously described [Lee et al., 1998; Blackburn et al., 1999]. All GSH determinations were normalized for the amount of precipitated cellular protein of the acid-treated samples. Protein pellets were resuspended in 5% (w/v) SDS in 0.1 M NaOH, and the concentrations were measured using the method of bicinchoninic acid protein assay with the Micro BCA protein assay reagent Kit (Pierce, Rockford, IL), with bovine serum albumin as standard.

Statistics

All statistical tests were performed with Sigma Stat (Jandel Scientific software). Statistical significance was assumed for P values < 0.05. Differences in means were assessed using t-test or ANOVA.

RESULTS

Transfection and Characterization of E-47 and C-34 Cells Expressing HCV Core Protein

E47 (HepG2 cells that over express CYP2E1) and C34 (vector control) cells were transfected with plasmids containing full-length HCV core protein sequences or empty vector and cloned in antibiotic selection medium containing G418 and zeocin as described in the “Materials and Methods” section. Initially, the transfected lines grew slowly. However, after several passages, the cells adapted well to the antibiotic selection medium and were passed every 10–14 days at 1:5 dilutions.

It has been suggested that cell lines over expressing CYP2E1 are sensitive to oxidative stress, even under normal culture conditions [Chen and Cederbaum, 1998], but in our experiments the transfected E47 cells were stable in selection medium even after extended passage. Figure 1 shows growth curves for the four clonal cell lines as determined by MTT dye conversion (Fig. 1A,B) or manual cell counts with trypan blue dye exclusion (Fig. 1C,D). Growth rates for the C34 line expressing core (C34/core) were higher than the plasmid only control line whether assayed by MTT dye conversion (Fig. 1A) or cell counting (Fig. 1C). While the rate of MTT dye conversion for the E47 cell line expressing core (E47/core) was comparable to E47 plasmid only control cell line (Fig. 1B), core appeared to stimulate growth when assayed by manual cell counts (Fig. 1D). These findings suggest a growth promoting effect for HCV core protein in the present system similar to the results of others with core transfected HepG2 cells [Erhardt et al., 2002], but in contrast to results for Huh-7 cells expressing core [Okuda et al., 2002].

Detection of mRNA for CYP2E1 and core in the clonal lines was achieved with RT-PCR using specific nucleotide primer sequences for the respective mRNAs. Figure 2A shows that HCV core mRNA was present in E47 and C34 lines transfected with plasmids containing core sequences, but not in cells transfected with plasmid only. RT-PCR assays of mRNA isolated from the respective lines did not show product if the assays were conducted without reverse transcriptase, thus demonstrating the product was amplified from authentic mRNA coding for HCV core protein. Core expression was stable in both E47 and C34 cell lines even after 22 passages (Fig. 2B).

HCV core protein expression was also confirmed on immunoblots using anti-core monoclonal antibodies (Fig. 2C). Only clonal lines transfected with HCV core sequences expressed an immunoreactive band with mobility about 19,000. This band was identical in size to the HCV core protein band identified in extracts of cells containing full-length HCV replicon [Blight et al., 2003].

CYP2E1 expression was also confirmed in the E47 cells with RT-PCR using specific sequence primer sets (Fig. 3A) and on immunoblots using an antibody to CYP2E1 (Fig. 3B). The absence of product from RT-PCR analysis and no specific immunostaining for CYP2E1 in

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the C34 lines (with or without core) confirmed the findings described previously [Cederbaum, 1998]. It was important to establish the stability of CYP2E1 expression under zeocin/G418 antibiotic selection conditions. Consequently, expression of CYP2E1 immunoreactive protein in the parental E47 line was compared to E47 cells transfected with core or plasmid only constructs at various passages after cloning (Fig. 4). Extended passage (25 passages) of transfected E47 cells did not lead to noticeable decreases in CYP2E1 expression (on a per mg protein basis), as compared to parental E47 cells. In fact, expression of CYP2E1 appeared to be mildly enhanced in E47 lines transfected with either HCV core or vector control (Fig. 4).

**Evidence for Oxidative Stress in Cells Over Expressing HCV Core Protein**

Recent data have shown that core protein expression can lead to increased endogenous levels of oxidative species in cells [Okuda et al., 2002]. We compared the endogenous levels of prooxidants generated in each clone by DCF fluorescence assay after 48 hr in cell culture. The relative amount of DCF fluorescence at the end of the 1 hr labeling period in each cell sample was then normalized for the amount of cells as determined by MTT reduction in each sample to correct for the number of metabolically active cells (Fig. 5). No decreases in cellular viability over the incubation period were noted using MTT or trypan blue assays. Cells expressing HCV core protein showed increased DCF fluorescence as compared to vector control cells (Fig. 5, C34 core vs. C34 control plasmid only, P < 0.02, and E47 core vs. E47 control plasmid only, P < 0.01). No differences in DCF fluorescence were noted among cells not expressing core, including wild type HepG2 cells. These results coupled with the fact that no changes in DCF fluorescence were noted between the groups using the oxidation insensitive probe (data not shown) suggest that expression of HCV core protein enhanced prooxidant production in these cells.

Evidence for increased superoxide production \( \left( \text{O}_2^- \right) \) in cells expressing HCV core protein was obtained using in situ labeling with the superoxide sensitive fluorescent probe HE [Carter et al., 1995]. The probe is oxidized to ethidium by intracellular \( \text{O}_2^- \) and intercalates into DNA to produce bright red fluorescence [Carter et al., 1995; Li et al., 2001]. Cell lines expressing HCV core protein showed increased fluorescence as compared to vector controls, consistent with the hypothesis that HCV core protein increases intracellular production of superoxide (Fig. 6, identical exposure times were used for the photomicrographs).

The effects of CYP2E1 enzyme inhibitor, 4-methylpyrazole (4-MP [Feierman and Cederbaum, 1996]), and GSH depleting agent, BSO, on DCF fluorescence were determined in the experiment shown in Figure 7. E47 cells incubated for 48 hr in medium containing BSO showed a significant increase in DCF fluorescence and this effect was further increased in E47 cells that also expressed HCV core protein. While 4-MP blocked the rise of DCF fluorescence in E47 cells co-expressing HCV...
core protein, it had only a slight effect on DCF fluorescence in E47 cells not expressing HCV core protein (Fig. 7). In cellular incubations containing only 4-MP, a small, but significant effect of the inhibitor on DCF fluorescence was noted in E47 cells regardless of core expression, which may reflect the ability of this inhibitor to stabilize CYP2E1 against degradation [Yang and Cederbaum, 1997].

GSH Analysis and Cellular Sensitivity to GSH Depletion

Measurement of GSH in the cell lines revealed that E47 cells had significantly higher cellular levels of total GSH than C34 cells (Table I) thus, confirming the findings of others [Mari and Cederbaum, 2000]. However, lines expressing HCV core protein contained significantly less GSH than parental lines, suggesting that core either promotes increased consumption of GSH, decreased synthesis of GSH, or enhanced efflux of GSH or GSSG from the cells. Intracellular oxidized GSH levels (GSSG) were low for all cells lines, without significant differences between lines, also confirming previous results for C34 and E47 cells [Mari and Cederbaum, 2000].

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Fig. 3. Confirmation of CYP2E1 expression in cell lines. Cell lines were assayed for CYP2E1 mRNA using RT-PCR and specific primers (A), or by immunodetection of CYP2E1 protein on western blots (B) using monoclonal antibody to CYP2E1 as described in “Materials and Methods.” The RT-PCR expected product size is 439 bp. The CY2E1 protein expected size is 54 Kd.

Fig. 4. Stability of CYP2E1 expression in transfected cell lines. Stable cellular clones were maintained for the indicated passages in antibiotic selection medium and assayed on immunoblots. For comparison, non-transfected E47 cells were assayed similarly at various passages after recovery from liquid nitrogen. Equivalent amounts of cellular protein were electrophoresed on 12% Laemmli SDS running gels and antibodies to CYP2E1 or actin were used for immunodetection.
Incubation of cells with medium containing BSO severely depleted GSH in all cell lines and after 48 hr less than 15% of control total GSH levels remained (Fig. 8A). Assessment of cellular viability by MTT dye conversion during GSH depletion showed that C34 cells, which do not contain CYP2E1, were more resistant to GSH depletion than E47 cells (Fig. 8B). Moreover, E47 cells expressing HCV core protein and CYP2E1 were the most sensitive to decreases in viability mediated by GSH depletion of all cell lines tested, showing less than 20% viability after 48 hr of incubation with BSO. The decreased viability of the E47 lines noted with MTT dye conversion was also confirmed with trypan blue staining. E47 cells showed greater than 40% viability while E47 cells expressing core showed only 10% viability after 48 hr of BSO incubation (data not shown). These findings strongly suggest that the increases in prooxidant production seen in BSO treated cells co-expressing HCV core protein and CYP2E1 (Fig. 7) sensitized the cells to the toxic consequences of GSH depletion.

DISCUSSION

Liver injury from ethanol ingestion or HCV infection is thought to involve a metabolic imbalance between prooxidant production and antioxidant capacity leading to oxidative stress, lipid peroxidation, cellular damage, cytokine activation, and subsequent hepatic fibrosis [Nordmann et al., 1992; Farinati et al., 1995; De Maria et al., 1996; Farinati et al., 1996; Ishii et al., 1997]. While synergism of HCV infection and excessive alcohol consumption is likely to be multifactorial, one or more viral proteins, such as core, may exacerbate oxidative stress and thereby promote fibrotic liver disease. The goal of the present work was to test the hypothesis that simultaneous over expression of CYP2E1 and HCV core protein enhanced indices indicative of oxidative stress as well as susceptibility to the toxicity associated with GSH depletion in cells of hepatocyte origin.

The parental HepG2 and E47 cell lines (with C34 vector controls) were chosen for study because over expression of the cytochrome P450 enzyme, CYP2E1, has been well established in this model system [Dai et al., 1993; Wu and Cederbaum, 1996; Chen et al., 1997; Cederbaum, 1998; Chen and Cederbaum, 1998; Cederbaum et al., 2001]. In addition to increased sensitivity to ethanol, agents such as arachidonic acid, isonizid, and acetaminophen, which depend on CYP2E1 oxidation, are toxic to cells over expressing CYP2E1 [Dai and Cederbaum, 1995; Wu and Cederbaum, 1996; Chen et al., 1997; Cederbaum et al., 2001]. Since both CYP2E1 and core have been shown to promote oxidative stress, we felt that extensive characterization of our clonal lines was necessary to ensure their long term stability. Our data revealed persistent, stable expression of both CYP2E1 and HCV core protein in the clonal cell lines for over 30 passages. These cell lines will be a valuable tool to study the transcriptional regulatory effects of HCV core protein on CYP2E1 mediated oxidative reactions and their importance for oxidative injury in the hepatocyte. Clarification of the prooxidant actions of HCV core and its participation in oxidative cellular injury appears important to fully understand the pathology of the virus.

Our experiments supported the hypothesis that over expression of HCV core protein stimulates prooxidant production as determined by increased oxidation of DCFH₂ and HE to their fluorescent products, presumably by ROS or reactive nitrogen species (RNS). The stimulation of prooxidant production seen in cells expressing HCV core was also accompanied by depletion of GSH suggesting that HCV core protein promotes increased GSH utilization, interferes with de novo synthesis of GSH, and/or increases the efflux of GSSG or GS-conjugates to the extra-cellular environment. Further studies are necessary to differentiate between these possibilities, but it is clear that co-expression of HCV core and CYP2E1 cause significant disruptions in GSH metabolism that are presumably the result of increases in prooxidant production.

The hypothesis that HCV core protein stimulates prooxidant production has also been supported by the recent studies of Okuda et al. [2002], who demonstrated increased reactive oxidative species in Huh-7 or HeLa cells after induction of HCV core protein with a tetracycline inducible expression system. Acute core expression was accompanied by increased lipid peroxidation and induction of some antioxidant enzymes [Okuda et al., 2002]. Although the latter data and ours were collected from cultured cells which probably over express HCV core as compared to infected human liver, the amount of viral replication and viral proteins produced during HCV infection in vivo is currently unclear [Lau et al., 1996; Shimizu et al., 1996; Agnello et al., 1998]. On the other hand, available evidence suggests that core protein promotes oxidative stress in vivo and the consequences of this stress cause hepatic...
injury. Transgenic mice that constitutively express HCV core protein showed increased hepatic lipid peroxide species, steatosis, and hepatocellular carcinoma (HCC) [Mori et al., 2001]. Hepatic steatosis and development of hepatocellular carcinoma are highly associated with HCV infection in humans [Lefkowitch et al., 1993; Hoofnagle, 1997]. Moreover, Lerat et al. [2002] reported that transgenic mice that express either HCV structural genes or full-length HCV gene sequences also develop steatosis and HCC. The transgenic mice studies suggest that individual HCV proteins, such as core, manifest extra-viral activities in vivo and that the activities also occur when all the viral genes are present. Collectively, the in vitro and in vivo data imply that the prooxidant actions of core may have a mechanistic role in the pathology of the virus and should be studied further.

While our transfected cells expressed immunoreactive core protein of identical mobility to that seen for cells infected with full-length HCV replicons, further studies are necessary to determine whether alternate reading frame transcripts participate in the prooxidant actions of core. As first shown by Walewski et al. [1998] and later confirmed, [Xu et al., 2001; Gosert and Moradpour, 2002], a second alternate reading frame (ARF) overlaps the core gene. This ARF is expressed and stimulates immune responses in patients. Plasmids containing this region of the HCV genome have the potential to express epitopes encoded in an alternate

Fig. 6. Dihydroethidium (HE) staining of cell lines. Cells grown attached to coverslips were stained for 40 min with 5 μm HE, fixed, mounted, and examined by confocal laser scanning microscopy. Exposure times for the photomicrographs were identical.
reading frame [Gosert and Moradpour, 2002] and additional studies are necessary to evaluate whether these ARF proteins have prooxidant stimulatory properties.

In addition to core, HCV NS5A has also been shown to influence acute oxidative stress in vitro [Gong et al., 2001]. Mechanistically, NS5A appears to evoke calcium efflux from the endoplasmic reticulum (ER) and affect excess oxidant production through an ER overload response (EOR) [Gong et al., 2001]. A number of viral proteins from different systems are also known to elicit an EOR that can occur after viral infection [Pahl, 1999]. In the EOR, cells become programmed to produce large amounts of viral proteins that are processed through the ER. This is followed by efflux of calcium from the organelles and generation of ROS that ultimately act as second messengers for activation of NF-κB, a well-characterized transcription factor and regulator of cytokine signaling. In vitro, HCV core protein interferes with hepatocyte cytokine responses and cellular signaling, ultimately leading to a loss of normal regulation of apoptosis [Matsumoto et al., 1997; Ray et al., 1997, 1998; Kato et al., 1999; Marusawa et al., 1999]. It is possible that the action of core is to increase cellular ROS and these species act as key signaling factors for core activities. Release of calcium may impact the production of prooxidants from flavin containing oxidase enzymes in the mitochondria or cytosol because a non-specific inhibitor of flavin containing oxidase enzymes, diphenyliodonium, appeared to prevent the generation of prooxidants [Okuda et al., 2002]. Because NF-κB activation and cytokine signaling are crucial events in the pathogenesis of alcoholic liver disease [Faubion and Gores, 1999], potential regulation of these processes by core induced ROS may play a role in the accelerated disease process in co-affected patients.

In addition to increasing parameters indicative of increased prooxidant production, HCV core protein also appears to significantly sensitize cells expressing CYP2E1 to the toxic consequences of GSH depletion. E47 cells have been shown to be sensitive to GSH depletion [Mari and Cederbaum, 2000], probably as a consequence of over expression of CYP2E1, an enzyme that promotes the production of prooxidants. To compensate for chronic excess oxidant production, these cells up-regulate antioxidant enzymes involved in GSH metabolism (i.e., gamma-glutamyl cysteine synthetase and GSH transferases) as well as H₂O₂ metabolism (catalase) [Mari and Cederbaum, 2000; Mari and Cederbaum, 2001]. Our studies confirmed the sensitivity of E47 cells to BSO incubation and showed that simultaneous over expression of HCV core protein and CYP2E1 further decreased cellular viability during GSH depletion. While the mechanisms by which HCV core protein sensitizes cells to injury during GSH depletion are unclear, our results demonstrate that simultaneous expression of the HCV core protein and CYP2E1 significantly impact metabolic pathways involved in prooxidant production and antioxidant capacity such that the ability of cells to compensate for GSH depletion is significantly compromised leading to cell injury. These findings are important for eventually understanding the mechanism of liver injury in patients when both HCV and alcoholic liver disease processes are present.

**TABLE I. Glutathione Levels in Clonal Cell Lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>GSH (nm/mg)</th>
<th>SEM</th>
<th>P-value</th>
<th>GSSG (nm/mg)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C34</td>
<td>187</td>
<td>4.5</td>
<td>&lt;0.001</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>C34 core</td>
<td>95</td>
<td>5.1</td>
<td></td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>E47</td>
<td>221</td>
<td>10.8</td>
<td>&lt;0.001</td>
<td>3</td>
<td>0.2</td>
</tr>
<tr>
<td>E47 core</td>
<td>170</td>
<td>13.1</td>
<td></td>
<td>0.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Results are the mean of three separate experiments with three determinations per group. GSH and GSSG were assayed as described in the “Materials and Methods.” Significance between means assessed by Student’s t-test. For comparison of the GSH levels of C34 versus E47 cells, C34 contained significantly less GSH than E47 (P 0.01).
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REFERENCES


