Hepatitis C virus core protein induces expression of genes regulating immune evasion and anti-apoptosis in hepatocytes

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Abstract

Hepatitis C virus (HCV) Core protein is implicated in the development of hepatocellular carcinoma (HCC). We utilized a HepG2 human hepatocyte cell line with inducible expression of HCV Core protein (HCV-1b) to investigate the early effects of Core protein on hepatocyte gene expression and to identify molecular processes modulated by the Core protein. A significant change was observed in the expression of 407 genes, which included genes regulating apoptosis, immune response, and cell cycle. Some of these genes were previously known to be tumor markers. The decreased expression of chemo-attractants such as TNFSF10, CCL20, and osteopontin was observed, which suggested that HCV Core expression could lead to suppression of inflammatory response as well as trafficking of macrophages and neutrophils to the site of HCV infection. An increased expression of anti-apoptosis factors including PAK2, API5, BH1, Tax1BP1, DAXX, and TNFAIP3/A20 was observed. Some of these genes were also linked to the regulation of NFKB activation and that the alteration of their expression levels, by HCV Core, might lead to the suppression NFKB activation of inflammatory responses. Our data suggested that Core expression may contribute to the viral persistence by protecting infected hepatocytes from cell death by the suppressing apoptosis and inflammatory reaction to HCV viral infection.

Keywords: HCV Core; Immune regulation; Apoptosis; Microarray

Introduction

Hepatitis C virus (HCV) is the etiologic agent of acute and chronic hepatitis affecting more than 100 million people worldwide (Uchida, 1994). Chronic hepatitis is one of the leading causes of liver cirrhosis and hepatocellular carcinoma (HCC) (Saito et al., 1990), yet the mechanisms of viral persistence and HCV associated tumorigenesis have not been fully elucidated. HCV, a member of the flavivirus family, has a 9.5-kb positive single-stranded RNA genome, which encodes a polyprotein that is processed into at least 10 different structural and nonstructural proteins (Choo et al., 1989; Ray and Ray, 2001). The HCV Core protein is derived from the N-terminus of the polyprotein and has a highly basic N-terminal region and a highly hydrophobic C-terminus (Kunkel and Watowich, 2002). In addition to being the major component of the viral nucleocapsid, this multifunctional protein has also been implicated in hepatocyte proliferation and cellular transformation, although molecular mechanisms mediating these processes are not fully determined.

Several human and murine studies have investigated mechanisms of HCV Core protein in the development of HCC which included the effects on the cell cycle and growth. We reported that the expression of HCV Core protein modulated cell cycle of hepatocytes as indicated by an increased level of the cell-cycle-dependent kinase inhibitor (CdkI) p21, a target of the p53 tumor suppressor, and found that the increased level of p21 corresponded to a decreased level of cdk2 kinase activity and arrested HepG2 hepatocytes in the G0/G1 phase of the cell cycle (Nguyen et al., 2003). The CdkI p21 has a dominant role in arresting cells in the G0/G1 phase of the cell cycle by inhibiting the kinase activity of Cdk2-cyclin E complex. Other studies have also demonstrated that HCV Core protein modulates the expression of p21 (Oka et al., 2003; Yamanaka et al., 2002). It has been reported that the p53 tumor
suppressor is activated in the presence of HCV Core protein expression (Lu et al., 1999) which could lead to an increased expression of p21. Cyclin E, a protein instrumental in the transversal of the G1/S checkpoint, was shown to be elevated in cells expressing HCV Core protein (Cho et al., 2001). In addition, Core protein activates the c-myc promoter but suppresses the fos promoter, indicating that HCV Core is a potential regulator of oncogenes (Ray et al., 1995). In our previous studies, we observed that HepG2 cells with HCV Core expression accumulated in the G0/G1 phase of the cell cycle (Nguyen et al., 2003). Other studies of HCV Core protein expression using the Huh-7 cell line reported either enhanced or no change in cell proliferation (Fukutomi et al., 2005; Li et al., 2002). The Huh7 cell line contains a point mutation of the p53 gene, while the HepG2 cell line has the wt p53 gene copies, which could lead to different effects of Core protein on cell proliferation. However, these studies together suggest that Core protein may have an impact on HCV pathogenesis through modulation of cell cycle regulatory mechanisms.

HCV Core protein is also linked to the formation of steatosis both in in vitro cell cultures and in vivo. Core protein expression increased the formation of cellular lipid droplets, and Core co-localized with apolipoprotein AII on the surface of cytoplasmic lipid droplets (Barba et al., 1997; Sabile et al., 1999). Expression of Core protein in the liver of transgenic mice induced hepatic steatosis (Moriya et al., 1997) and promoted the development of hepatocellular carcinoma (Moriya et al., 1998). The ability of Core protein to mimic HCV disease in the mouse model suggests that the expression of this protein is integral to HCV pathogenesis.

To investigate the molecular mechanisms of the HCV Core protein induced changes in hepatocytes, we examined the gene expression profiles of HepG2 hepatocytes in the presence of HCV Core protein by DNA microarray analysis. Our data showed that HCV Core protein expression led to modulation of genes regulating immune defense response, anti-apoptosis, and genes previously known as markers of hepatic tumorigenesis. The analysis of the interconnecting network of gene functions suggests that HCV Core may interfere with the immune defense response primarily through regulation and quenching of NFKB activation. The alteration of NFKB function could lead to the suppression of inflammatory responses and ensure the survival of HCV infected hepatocytes from immune surveillance. In addition, Core expression also altered the gene expression that could confer protection of cells from apoptosis. Our data suggest that HCV Core may play an important role in the mechanism of HCV viral persistence in addition to acting as the building blocks of the viral capsid.

Results

HCV Core protein expression led to decreased hepatocyte cell proliferation

We investigated the effect of HCV Core protein on hepatocyte proliferation following the induction of Core expression in HepG2-Core hepatocyte cells. We used an ecdysone-inducible expression system (Materials and methods section) to achieve inducible expression of HCV Core protein in HepG2 hepatocytes. This system allowed us to examine alterations in hepatocyte proliferation and gene expression immediately following the induction of Core protein expression. The advantage of the inducible expression systems is that it prevents any modification of the normal cell phenotype that is associated with exposure to high constitutive expression of Core protein in the cells. We also appended to the N-terminus of HCV Core protein with a sensitive 3XFLAG peptide tag to enhance the sensitivity of detection of HCV Core expression. As shown by Western blot analysis using anti-FLAG antibodies (Fig. 1A), HCV Core protein was expressed in hepatocytes induced by ponasterone A (PonA), a synthetic analog of insect ecdysone, but not in uninduced HepG2 cells. The numbers in the parentheses indicate the subsets of the triplicate experiment. The exaggerated intensity of Core protein expression shown in the figure is due to the sensitivity of the 3XFLAG tag. We have previously shown that the level of Core protein in our expression system was significantly less pronounced when we used anti-Core antibody to directly detect Core protein expression (Nguyen et al., 2003). There was no visual indication of significant cytotoxicity or cell death in HepG2 cells following the induction of Core protein expression, as indicated by the normal morphology of the cells in the cultures and the lack of cellular uptake of the Trypan Blue dye, an

Fig. 1. (A) Expression of HCV Core protein in hepatocytes. Western blot analysis demonstrates the inducible expression of HCV Core protein in HepG2-Core cells. Induced (I) and uninduced (UI) in triplicates. The numbers in the parentheses indicate the individual experiment subsets of the triplicate experiment. (B) Expression of HCV Core protein in HepG2-Core cells reduced cell proliferation. The Core expression in HepG2-Core cells was induced with PonA for 48 h and compared to uninduced HepG2-Core cells. Live cells were counted with Trypan blue dye. Nontransfected HepG2 cells with and without PonA induction served as negative control for cell proliferation assay. Each cell growth assay was performed in triplicates.
indicator for cell death. There was also no visual indication of cytotoxicity in mock-induced control HepG2 cells following the addition of PonA. The expression of Core protein for 48 h in induced HepG2-Core cells resulted in a lower number of cell counts as compared to uninduced HepG2-Core cell cultures (Fig. 1B). The counted cell number was 25% less for cells expressing Core protein compared to cells without Core protein expression. There was no significant difference in the number of cells counted in untreated and PonA treated HepG2 controls (NT I vs. NT U), indicating that the lower cell number was dependent on Core protein expression and was independent of PonA treatment. This observation is consistent with our previous studies, which suggested that HCV Core blocked cell proliferation at the G1-S phase of the cell cycle (Nguyen et al., 2003).

**HCV Core protein induced changes in hepatocyte gene expression**

In order to identify genes and molecular processes associated with Core induced changes in hepatocyte cell proliferation, we evaluated gene expression profiles of Core-expressing HepG2-Core cells in comparison to PonA induced control nontransfected HepG2 cells. The Core expression led to changes (1.3-fold or more and P value of less than or equal to 0.05) in mRNA expression of 534 genes; 453 upregulated genes, 81 down-regulated genes. The initial screenings were conducted with more stringent fold change standards to screen for most prominent changes. However, the high rigid fold change thresholds produced spotty data that omitted many smaller changes of genes that have significant statistical confidential P values. We included in this report genes that have small but consistent fold changes that also accompanied with significant confident P values. Hierarchical clustering graph of the significant gene list showed a distinct expression pattern associated with HCV Core protein expression (Fig. 2). GO Ontological classification of this list of genes showed that Core protein expression led to a modulation of genes involved in the regulation of immune response as well as apoptosis (Tables 1, 2). These genes and their respective biological functions and processes were further analyzed to determine the possible effects from their altered expression.

**HCV Core expression modulates immune response genes**

The expression of Core protein in HepG2 cells resulted in the modulation of genes regulating host immune responses to pathogens (Table 1). The altered expression pattern of immune response genes indicated that the Core protein may dampen the effectiveness of the immune response to HCV infection. HCV Core led to an increased expression of some genes involved in innate immunity. Genes with increased expression included MHC class I polypeptide-related sequence B (MICB), NK cell transcript 4 (NK4/IL-32), defensin beta 1 (DEFB1), beta-2-microglobulin (B2M) and nuclear factor of kappa inhibitor light alpha (NFKBIA/IKBo). MICB is a ligand of NKG2D type II receptor that can activate the cytolytic response of natural killer (NK) cells, CD8 αβ T cells, and γδ T cells (Groh et al., 1998). NK4 was initially discovered as a product of IL-2 activated NK cells (Dahl et al., 1992), and it is recently suggested to induce inflammatory factors, and it is also an antagonist to HGF induced cell growth and an inhibitor of angiogenesis (Matsumoto and Nakamura, 2003). Defensin B1 is a member of the microbactericidal peptide family that plays an important role in innate immune response at mucosal surfaces. Defensin B1 has been shown to be expressed and readily detectable in hepatocytes, and the presence of Defensin B1 in hepatic tissues was proposed to act as defense mechanism against pathogenic infection of the hepatic biliary system (Harada et al., 2004). B2M has a close homology to the immunoglobulins, and its expression is essential for the expression and assembly of HLA class I (Arce-Gomez et al., 1978; D’Urso et al., 1991). The NFKBIA (IKBo) protein has a crucial role in regulating immune response, inflammation and apoptosis since it is the main regulator of NFKB activation (Viatour et al., 2005). The increased expression of IKBo could result in a widespread dysregulation of NFKB transactivated genes. The pro-inflammatory genes downstream of NFKB activation could be suppressed due to the enhanced expression of IKBo by HCV Core.

The expression of HCV Core protein also resulted in the downregulation of tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10/TRAIL), liver activation regulated chemokine (LARC/CCL20/MIP3A), secreted phosphoprotein

![Fig. 2. Graphical hierarchical clustering pattern demonstrated gene expression induced by HCV Core protein in HepG2 cells. The 3 columns on the left represent the mRNA expression profiles of HepG2-Core-expressing cells, and the 3 right columns represent expression profiles of HepG2 control cells (blue = low expression, red = high expression). The graphical cluster was generated using the dChip array analysis program.](image-url)

CELLULAR PROCESSES THAT INCLUDE ACTING AS A SUBSTRATE FOR CASPASE 3 PHOSPHORYLATED BY THE P21 CdKI. PAK2 PARTICIPATES IN MULTIPLE PROTEINS THAT FUNCTION AS STRONG MEDIATORS OF IMMUNE REGULATION (BH1), TAX1 (HUMAN T-CELL LEUKEMIA VIRUS TYPE I) BINDING PROTEIN 1 (TAX1BP1, 200976_s_AT) AND THE INFLAMMATORY RESPONSE. THE PRESENCE OF TAX1BP1/T6BP AND A20 FORM A PROTEIN COMPLEX THAT EFFECTIVELY BLOCKS TNF-α INDUCED APOPTOSIS OF 3T3 CELLS (DE VALCK ET AL., 1999). AN INCREASED EXPRESSION OF DAXX WAS OBSERVED IN CORE-EXPRESSING HEPG2 CELLS. THE EFFECT OF DAXX EXPRESSION ON APOPTOSIS HAS NOT BEEN WELL DEFINED. DAXX WAS SHOWN TO ENHANCE FAS MEDIATED APOPTOSIS (PLUTA ET AL., 1998; YANG ET AL., 1997), HOWEVER, RNAI SILENCING OF DAXX WAS SHOWN TOSENSITIZE CELLS TO APOPTOSIS (CHEN AND CHEN, 2003; MICHAELSON AND LEDER, 2003).

The data showed that the expression of Tax1BP1/T6BP and TNFAIP3/A20 genes was also increased in the presence of HCV Core. Together, Tax1BP1 and A20 form a protein complex that effectively blocks TNF-α induced apoptosis of 3T3 cells (De Valck et al., 1999). An increased expression of DAXX was also observed in Core-expressing HepG2 cells. The effect of DAXX expression on apoptosis has not been well defined. DAXX was shown to enhance Fas mediated apoptosis (Pluta et al., 1998; Yang et al., 1997), however, RNAi silencing of DAXX was shown to sensitize cells to apoptosis (Chen and Chen, 2003; Michaelson and Leder, 2003).

Expression of known tumor markers

The HCV Core protein induced expression of genes previously known to be the markers of various human tumors. Increased levels of Ep-CAM, S100A6, IGF-2, Kras2, and Cyclin D1 were found in Core-expressing hepatocytes (Table 3). The transmembrane glycoprotein Ep-CAM is expressed in normal epithelium of different tissues. Overexpression of this gene has been documented in a variety of human carcinomas (Went et al., 2004) and is considered as a marker associated with the development of HCC (Ruck et al., 2000). DNA microarray analysis showed that the expression of Ep-CAM increased in tissues of individuals identified to be at high risk to develop

### Table 1

<table>
<thead>
<tr>
<th>Gene name and probe set</th>
<th>Fold change</th>
<th>P value</th>
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<tbody>
<tr>
<td>LARC/MIP3A/CCL20 (liver activation regulated)</td>
<td>−3.15</td>
<td>0.00100</td>
</tr>
<tr>
<td>SSP1/Osteopontin (secreted phosphoprotein 1)</td>
<td>−3.04</td>
<td>0.00374</td>
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<tr>
<td>TNFSF10 (tumor necrosis factor ligand)</td>
<td>−1.89</td>
<td>0.01311</td>
</tr>
<tr>
<td>IL1RL1/IL-27L (interleukin 1 receptor-like 1 ligand)</td>
<td>−1.54</td>
<td>0.03166</td>
</tr>
<tr>
<td>FN1 (fibronectin 1)</td>
<td>−1.45</td>
<td>0.01966</td>
</tr>
<tr>
<td>MAP2K3 (mitogen-activated protein kinase 3)</td>
<td>1.36</td>
<td>0.03018</td>
</tr>
<tr>
<td>B2M (Beta-2-microglobulin)</td>
<td>1.42</td>
<td>0.01596</td>
</tr>
<tr>
<td>CTSC (cathepsin C)</td>
<td>1.42</td>
<td>0.00750</td>
</tr>
<tr>
<td>MICB (MHC class I polypeptide-related sequence B)</td>
<td>1.56</td>
<td>0.01557</td>
</tr>
<tr>
<td>NK4 (NK cell transcript 4)</td>
<td>1.63</td>
<td>0.01613</td>
</tr>
<tr>
<td>PLA2G2A (phospholipase A2, group IIA)</td>
<td>1.76</td>
<td>0.00070</td>
</tr>
<tr>
<td>Nuclear factor of kappa inhibitor light alpha (NFKBIA)</td>
<td>1.90</td>
<td>0.00040</td>
</tr>
<tr>
<td>DEFB1 (defensin, beta 1)</td>
<td>3.04</td>
<td>0.00449</td>
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Table 2

<table>
<thead>
<tr>
<th>Gene name and probe set</th>
<th>Fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tax1 (human T cell leukemia virus type I) binding protein 1</td>
<td>1.34</td>
<td>0.032</td>
</tr>
<tr>
<td>BAX inhibitor 1</td>
<td>1.36</td>
<td>0.0016</td>
</tr>
<tr>
<td>Apoptosis inhibitor 5</td>
<td>1.49</td>
<td>0.0358</td>
</tr>
<tr>
<td>Death-associated protein 6</td>
<td>1.55</td>
<td>0.0387</td>
</tr>
<tr>
<td>Tumor necrosis factor, alpha-induced protein 3</td>
<td>1.65</td>
<td>0.0115</td>
</tr>
<tr>
<td>p21 (CDKN1A)-activated kinase 2</td>
<td>1.79</td>
<td>0.0119</td>
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</table>
expression levels were also found to be elevated in Wilms tumor tissues (Scott et al., 1985). The loss of paternal imprinting of IGF2 is a potential correlate of colorectal cancer development (Cui et al., 2003). Western blot analysis of p21 levels could cause the initiation of PCNA transcription to activate the DNA damage and repair surveillance mechanisms. Other studies utilizing DNA microarrays also reported an increased expression of PCNA in HCV infected liver samples (Smith et al., 2003; Zindy et al., 2005). Western blot analysis of PCNA expression was performed to correlate changes observed at the mRNA level to the changes at the protein level. The levels of PCNA protein in induced (I) cells expressing HCV Core protein (Fig. 3, lanes 3 and 6) were found to be higher than in nontransfected (NT, lanes 1 and 4) and uninduced (UI, lanes 2 and 5) cells without HCV Core expression. The addition of PonA to HepG2 cells did not alter PCNA protein levels (lanes 1 vs. 2 and 4 vs. 5). Each lane was loaded with equal amounts of proteins as shown by the GAPDH levels. The change of PCNA protein level is small but consistent with the change of PCNA mRNA expression in HCV Core-expressing cells. Additional Western blots were conducted to verify the protein levels for IKBa and PAK2. The direction of changes of protein levels of IKBa and PAK2 also reflected the direction of changes of their mRNA.

Discussion

In this study, the DNA microarray analysis of HCV Core protein expressing HepG2 cells provided additional information to changes that have been detected in the HepG2 cell line gene expression profile induced by HCV-1b Core protein. The expression data suggested that HCV Core expression led to the decrease of cell proliferation, repression of pro-inflammatory signals, and enhanced expression of anti-apoptosis factors. This could lead to prevention of host cell death from apoptosis and cultures to compensate for differences due to the presence of PonA. A minimal change in gene expression levels between the PonA induced and noninduced cells was observed. This slightly affected the numerical fold change values between induced HepG2-Core and induced nontransfected HepG2 samples, but the compensated values did not alter the direction of change (data not shown).

The DNA microarray data showed that proliferating cell nuclear antigen (PCNA) mRNA was upregulated in HepG2 cells with HCV Core expression. PCNA is an important component of the DNA polymerase complex that is essential for the DNA synthesis process that occurs during cell division and proliferation (Bowman et al., 2004). PCNA is also known to interact with p21 and act as a component of the RAD6-dependent DNA repair mechanism (Hoege et al., 2002). It is possible that the arrest of cells at the G0/G1 and the increase of p21 levels could cause the initiation of PCNA transcription to activate the DNA damage and repair surveillance mechanisms.

<table>
<thead>
<tr>
<th>Gene name and probe set</th>
<th>Fold change</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>IGF2 (insulin-like growth factor 2/Somatomedin A, 202410.X_AT)</td>
<td>3.49</td>
<td>0.00006</td>
</tr>
<tr>
<td>CCND1 (cyclin D1, 208711.S_AT)</td>
<td>2.71</td>
<td>0.00483</td>
</tr>
<tr>
<td>Ep-CAM/TACSTD1 (tumor-associated calcium signal transducer 1, 201839.S_AT)</td>
<td>1.47</td>
<td>0.04533</td>
</tr>
<tr>
<td>S100A6 (S100 calcium binding protein A6, 217728.AT)</td>
<td>1.46</td>
<td>0.00848</td>
</tr>
<tr>
<td>KRAS2 (v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog, 214352.S_AT)</td>
<td>7.5</td>
<td>0.00414</td>
</tr>
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Table 4

RT-PCR validation of DNA microarray data

<table>
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<tr>
<th>Gene name</th>
<th>Fold change (HepG2-Core induced vs. HepG2 induced)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GAPDH normalized</td>
</tr>
<tr>
<td>CCL20/MIP3A</td>
<td>−2.56</td>
</tr>
<tr>
<td>IGF2</td>
<td>1.62</td>
</tr>
<tr>
<td>KRAS2</td>
<td>2.93</td>
</tr>
<tr>
<td>PCNA</td>
<td>2.17</td>
</tr>
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</table>

Table 3

Tumor marker genes were modulated by HCV Core expression

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF2</td>
<td>1.62</td>
<td>2.58</td>
</tr>
<tr>
<td>CCND1</td>
<td>2.93</td>
<td>4.66</td>
</tr>
<tr>
<td>KRAS2</td>
<td>2.17</td>
<td>3.46</td>
</tr>
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</table>
suppression of the immune surveillance system. The analysis of gene functions and regulators suggested that the regulation of NFKB might be a central target for HCV Core in its attempts to modulate apoptosis and immune suppression in hepatocytes. In addition, HCV Core affected the expression of other cellular factors that may result in the alteration of cell growth characteristics. It is unlikely that inhibitions in downstream translational processes would nullify the interpretation of the transcriptional data since, in the HepG2 cell expression system, it was shown that HCV Core expression did not affect cap-dependent translation (Li et al., 2003; Shimoike et al., 2006). Furthermore, cap-dependent translation was not affected by varying the amount of HCV Core in the in vitro translation system (Boni et al., 2005).

The gene expression data from this study provide support to the hypothesis that HCV Core expression decreases cell proliferation. We observed an increased of Geminin and KHDRBS1 (1.5-fold and 1.57-fold increase). These genes were of special interest due to their anti-proliferative functions that could potentially contribute to the observed slower proliferation of Core-expressing cells. The increased expression of Geminin has been shown to inhibit DNA replication during S, G2, and M phases by preventing complete assembly of the DNA pre-replication complex (McGarry and Kirschner, 1998). KHDRBS1 is a tyrosine-phosphorylated, Src-associated protein in mitotic cells. KHDRBS1 expression was demonstrated to inhibit serum-induced DNA synthesis, suggesting that it may play a role in controlling the G1/S cell cycle transition (Barlat et al., 1997). In addition, the decreased expression of LARC, hepatocyte growth factor receptor (HGRF/met, 1.34-fold decrease), and alpha fetal protein (AFP, 2.64-fold decrease) was observed. LARC functions primarily as a chemotaxis factor, it can act as an autocrine/paracrine factor that enhances hepatic cell growth (Fujii et al., 2004). The suppression of LARC expression correlated with decreased proliferation of HCV Core-expressing cells. The decreased expression of two hepatic pro-growth factors, HGF and AFP in HepG2 cells expressing Core protein also are supportive of the observation that HCV Core protein expression suppresses cell proliferation. Decreased expression of HGF could reduce the number of receptors that are available to receive and transmit growth signals. It is thought that an increased serum AFP level is prognostic of active liver regeneration (Kavountzis and Redeker, 1974), and conversely a decrease in AFP transcription could be interpreted as an indicator of lower hepatocyte regeneration and proliferation.

Overall, the change (increased or decreased) in the expression of immune response-associated genes in the Core-expressing hepatocytes seemed to have the potential to negatively impact the anti-viral immune response. The gene expression data suggested that HCV Core may impair inflammatory responses and enhance cell survival of infected cells by interfering with trafficking and infiltration of immune cells to the site of HCV infection and by preventing cell death from necrosis and apoptosis. Downmodulation of the expression of chemotactic factors such as LARC, and osteopontin could suppress immune cell trafficking. LARC is the known chemotactic ligand for the CCR6 receptor on B cells, T cells, dendritic cells, and macrophages. The presence of LARC expression is correlated to the increased necrosis levels of inflamed liver tissue (Shimizu et al., 2001). Previous studies showed the levels of LARC increased in HCV patients that responded to INF treatment as compared to no change in the levels of LARC in patients that were classified as nonresponders (Yamauchi et al., 2002). These findings suggested that LARC plays an important role of recruiting immune cells for the effective mediation of inflammatory response and clearance of viral infection. In this context, HCV Core could impair an effective inflammatory response and prevent viral clearance by repressing the expression of LARC. Our data also showed that HCV Core suppressed osteopontin expression which is consistent with a previous report (Ohkawa et al., 2003). The secretion of the extracellular matrix protein osteopontin by NK cells recruits and activates neutrophils and macrophages (Diao et al., 2004; Weber et al., 2002). The correlation between high osteopontin levels and hepatic tissue injury due to the increase of neutrophil infiltration was demonstrated in the rat model (Apte et al., 2005). The inverse relationship between LARC, osteopontin levels, and HCV Core expression suggests a possible mechanism of immune evasion employed by the Hepatitis C virus.

HCV Core could contribute to viral persistence by regulating anti-apoptosis factors that could prevents apoptosis and enhance the survival of HCV infected host cells. The upregulation of predominantly anti-apoptosis genes such as PAK2, API5, BH1, Tax1BP1, TNFAIP3/A20, and DAXX demonstrated the potential anti-apoptotic property of HCV Core. An enhanced
expression of such anti-apoptosis genes has been shown to inhibit stress-induced apoptosis in various cell lines (De Valek et al., 1999; Kim et al., 2000; Tewari et al., 1997; Xu and Reed, 1998). The functions of some of these anti-apoptosis genes are conserved across a wide spectrum of organisms. For example, the plant homologous of BI1 was shown to suppress Bax induced apoptosis in yeast (Kawai et al., 1999; Sanchez et al., 2000; Xu and Reed, 1998). The normal expression of BI1 and DAXX is required for different cell lines to avoid spontaneous apoptosis (Grzmil et al., 2003; Kawai et al., 1999; Sanchez et al., 2000; Xu and Reed, 1998). The modulation of TNFSF10, AP15, A20/TNFAIP3 by HCV Core was also recently shown by 2-color DNA microarrays (Liu et al., 2005). It was proposed that the TNFSF10 ligand/receptor system is a hepatic paracrine mechanism to eliminate virally infected hepatocytes (Mundt et al., 2003). Suppression of TNFSF10 expression by HCV Core may minimize apoptosis of infected host cells and maintain HCV persistence which suggests an additional anti-apoptosis mechanism employed by HCV Core to prevent viral clearance. These data suggest that HCV Core expression could contribute to the prevention of apoptosis by altering the expression of important the anti-apoptosis factors.

The NFκB is a major transcription factor that is involved in the activation of inflammatory and cell activation factors. The suppression of NFκB activation by HCV Core protein may include multiple mechanisms. Our gene expression analysis has identified a number of genes that could contribute to the mechanisms of inflammatory repression at the molecular level. Recent reports linked HCV Core protein to the inactivation of NFκB (Joo et al., 2005; Ray et al., 2002). Anti-apoptosis genes (DAXX, Tax1BP1, A20) and immune response genes (PLA2G2A, NFKBIA/IκBa) have been previously demonstrated to regulate the activity of NFκB. Using NFκB reporter constructs, it was shown that increased expression of DAXX caused the repression of the NFκB promoter (Michaelson and Leder, 2003). Similarly, overexpression of TaxBP1 led to suppression of the NFκB promoter (Iha et al., 2000). The A20 protein directly interacts with Tax1BP1, and exerts anti-apoptosis effects (De Valek et al., 1999). The A20 protein was a crucial anti-apoptotic factor in the mouse model and that A20-deficient mice were susceptible to sub-lethal doses of TNF (Lee et al., 2000). The A20 protein was shown to block IL-1 and TNF activation of NFκB via its interaction with TNF receptor associated factor 2 (TRAF2) and TRAF6 (Heynicken and Beyaert, 1999; Heynicken et al., 1999) and consequently ensure the cytoplasmic sequestration and inactivation of NFκB by the IkB complex. The function of PLA2G2A protein in inflammation is through its enzymatic activity to release arachidonic acid from cellular phospholipids. Arachidonic acid is the precursor of prostaglandins and leukotrienes. Prostaglandins are vasodilators and chemotaxis factors that promote neutrophil migration and accumulation at the sites of inflammation. PLA2G2A induced production of the monocyte chemotactic protein-1 (MCP-1) chemoattractant (Fuentes et al., 2002) in macrophages. The injection of PLA2G2A has been shown to exacerbate the inflammation of arthritic joints (Bomalaski et al., 1991). Following the stimulation of a RAW264.7 macrophage cell line with PLA2G2A, an enhanced binding of NFκB to its promoter sequences and degradation of the IkBa subunit was reported (Baek et al., 2001). Lastly, HCV Core expression led to increased expression of IkBa subunit of the IkB complex. The increased presence of IkB complexes would enhance sequestration and inactivation of the NFκB transcription complex. Our gene expression data provided novel findings that suggest that expression of genes regulating NFκB was altered to suppress NFκB activation and subsequently would decrease inflammation. Furthermore, the direction of changes for anti-apoptosis, chemotaxis, and inflammation genes support the hypothesis that the Core protein could contribute to the immunosuppressive nature of the HCV.

HCV Core protein was suggested to have an immunosuppressive effect through the downregulation of Cox-2 gene expression (Jhaveri et al., 2005), which might be a result of NFκB inactivation (Joo et al., 2005). Most recently, HCV Core was shown to inhibit T cell function and chemokine production through the interaction with complement receptor gC1qR (Yao et al., 2005). The HCV Core protein expression also led to downmodulation of a number of immunity and inflammatory response related genes in Huh-7 cell line (Fukutomi et al., 2005). The DNA microarray analysis in the chimpanzee model of HCV infection showed that the control and clearance of HCV infection was related to the intrahepatic infiltration of immune cells where as chronic infections correlated with impaired gene expression for T cell recruitment (Su et al., 2002; Thimme et al., 2002). Expression of immune response related genes was downmodulated in liver during chronic HCV infection in humans and chimpanzees (Bigger et al., 2004; Honda et al., 2001; Shao et al., 2005). The mechanisms could include the block in the maturation process of T cells (Bigger et al., 2004) and an enhanced expression of anti-inflammatory genes (Honda et al., 2001). HCV has also been shown to infect and replicate in lymph nodes, PBMC, monocyte/macrophages (Cauussen-Schwemling et al., 2001; Laskus et al., 1998), which could also affect immune functions.

In summary, we investigated the effect of HCV Core on hepatocyte cell proliferation and gene expression. Our results showed that the Core protein expression led to the suppression of chemotaxis and inflammatory factors and enhanced the expression of anti-apoptosis factors that could lead to the protection of infected hepatocytes from immune clearance and apoptosis. Thus, HCV Core protein may play an important role in the survival and persistence of Hepatitis C virus in infected hepatocytes.

Materials and methods

Cell culture

HepG2 cells were obtained from American Type Culture Collection (Manassas, VA.). Cells were maintained in DMEM growth media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U of penicillin-streptomycin/ml ( GibCO-BRL, Rockville, Md.).
Transfection and establishment of a stable HepG2 cell line with inducible Core expression

The establishment of a HepG2 cell line with the ecdsyne inducible expression of HCV Core (HCV-1b) expression was previously described (Nguyen et al., 2003). Briefly, the ecdsyne inducible system consists of an inducible pIND expression plasmid and an inducer pVgRxR. The inducible plasmid pIND-Core was generated by cloning the sequence of the FLAG-tagged Core into ecdsyne-inducible mammalian expression vector pIND (Invitrogen, Carlsbad, CA) containing a neomycin (G418) selection marker. The expression of Core is under the control of Drosophila melanogaster minimal heat shock promoter and the ecdsyne and glucocorticoid response element (E/GRE) hybrid promoter. The E/GRE promoter is driven the RxR and VgEcR ecdsyne receptor subunits that are encoded and constitutively expressed by the pVgRxR plasmid (Invitrogen). The pVgRxR plasmid also contains a Zeocin selection marker for the generation of stable Zeocin-resistant cell clones. The binding of the RxR and VgEcR subunits to the E/GRE promoter is facilitated by the addition of ponasterone A (PonA).

HepG2 cells were co-transfected with linearized pIND-3XFLAG-Core and pVgRxR plasmids by electroporation of 5 × 10^6 cells in 0.5 ml of growth media without serum at 250 V and 950 μF using a Gene Pulser (Bio-Rad, Hercules, CA) and incubated overnight. The transfected cells were expanded in selection media containing 1000 μg/ml of G418 and 200 μg/ml of Zeocin for 2 weeks. The cell colonies were transferred into 24-well plates and maintained in the G418/Zeocein selection media for 2 to 3 weeks. Cell clones that survived the second round of selection were expanded and evaluated for HCV Core expression by Western blots.

Hepatocyte growth conditions and collection

HepG2-Core cells and nontransfected (NT) control cell line were grown in tissue culture plates for mRNA and protein extraction. PonA was used to induce expression of HCV Core protein in the cells (10 μM). PonA was also added to NT cells to serve as parallel controls. Four cell growth conditions were as following: (i) HepG2-Core cells with induction using PonA, (ii) HepG2-Core cells without induction, (iii) untransfected control HepG2 cells with PonA addition, (iv) untransfected control HepG2 cells without PonA addition. Assay was performed in triplicates. Cells were collected and were immediately frozen. Total RNA was isolated from HepG2-Core cells with induction of Core expression (Core I), uninduced HepG2-Core cells (Core U), induced NT control HepG2 cells (NT I), uninduced NT control HepG2 (NT U) and used in the DNA microarray analysis and Real time RT-PCR (TaqMan) assay.

Cell viability and enumeration with Trypan blue

HepG2-Core (Core) and HepG2 control (NT) cells were trypsinized to single cell suspensions and were counted to enumerate cell density. Equal number of cells (5 × 10^5) were seeded in each well of the 6-well plates. Cells were grown overnight and subjected to PonA plates. Cells were harvested 48 h following induction and suspended in 1 ml of media after trypsinization with 0.5 ml of trypsin. Each cell suspension was mixed with equal volume of Trypan Blue dye (25 μl/25 μl) to detect viable cells. The Trypan Blue suspensions were spotted on a hemacytometer and live cell numbers (without Trypan blue uptake) were counted per each sample using a light microscope. For the measurement of cell growth for each set of experiments performed in triplicates, cell numbers of Core uninduced and NT uninduced cell samples were considered to be 100% and were used as controls. The numbers of cells from each Core I and NT I cell samples were compared with the controls and growth percentage values for Core I and NT I cells were derived.

DNA microarray analysis

The human HG-U133A GeneChips were used for the gene expression profiling (Affymetrix, Santa Clara, CA, USA). RNA extraction, amplification, labeling, hybridization, staining, and scanning were performed as per recommendations (Affymetrix Technical Manual). Fluorescence intensity values (.CEL files) generated from hybridized, stained GeneChips were subjected to comparative and hierarchical clustering analyses using the dChip (Version 1.3) software program (Li and Wong, Harvard University). The detection P value of 0.05 or better was used as a statistical criterion for including a given gene in subsequent comparative or clustering analyses. A P value of 0.05 or less was used as a cutoff for identification of significant gene clusters. The initial hierarchical clustering was generated from the list of gene transcripts that were either upregulated or downregulated at least 1.5-fold from comparisons between experimental and control samples. We used D-chip’s Gene Ontology (GO) classification feature to group genes into functional categories. We also re-examined the data to include genes with 1.3-fold change (P value < 0.05) that could provide additional pertinent information.

Immunoblot analysis

HepG2 cells were lysed in NP-40 lysis buffer and 30 μg of total protein was separated on SDS-PAGE gels and transferred to BA-85 membrane (Schleicher and Schuell, Keene, NH). The membrane was stained with Ponceau S to detect protein. It was reacted with 5% nonfat dry milk in phosphate buffered saline (PBS) and 0.1% Tween for 1 hour before the addition of specific primary antibodies to eliminate nonspecific binding. FLAG-tagged HCV Core, PCNA, GAPDH, IKBα, and PAK2 proteins were detected using anti FLAG antibody (Stratagene. La Jolla, CA), anti-PCNA and anti-GAPDH antibodies (Santa Cruz Biotech. Santa Cruz, CA), anti- IKBα and anti-PAK2 antibodies (Cell Signaling Technology. Beverly, MA). After washing and incubation with secondary mouse monoclonal or rabbit polyclonal horseradish-peroxidase linked antibodies (Amer sham Pharmacia, Piscataway, NJ), proteins were detected using ECL (Amersham Pharmacia). The same membrane blot was
used to probe for each protein, the membrane was sectioned into particular molecular weight ranges and probed (or re-probed) with different antibodies to ensure consistency.

**Real-time PCR**

Total RNA was used to synthesize cDNA by reverse transcription (Alluwaimi et al., 2003). Complementary DNA was synthesized using random hexamer primers, and reactions were carried out using PCR master mix (Applied Biosystems, Foster City, CA). The primer probe sets for chemokine (C-C motif) ligand 20 (CCL20), insulin-like growth factor 2 (IGF-2), Kirsten-ras2 (Kras2), proliferating cell nuclear antigen (PCNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosome were used for quantitation by real-time RT-PCR assay (Beekton Dickinson). The housekeeping genes GAPDH and 18S ribosome were used as internal controls for normalization. Each PCR reaction was set up according to manufacturer specificity (TaqMan Universal PCR Mastermix; Applied Biosystems). The reactions were placed in 96-well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System; Applied Biosystems). Amplification conditions included 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. For each gene, a cycle threshold (Ct) value was obtained at the cycle that produced a detectable fluorescence signal. The relative Ct value of each of the candidate gene (in reference to the control gene, i.e., CCL20 to GAPDH) was obtained by subtracting the Ct value of the GAPDH or 18S ribosome gene from the Ct value of each candidate gene. The average relative Ct value for each candidate gene was calculated from the triplicates of RNA samples. The fold change in expression levels was calculated from the triplicates of RNA samples. The fold change in expression levels was calculated from the triplicates of RNA samples.

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