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SCIENCE DIRECT

Virology 336 (2005) 198 – 207

VIROLOGY

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Inhibition of hepatitis C virus core protein expression in immortalized human hepatocytes induces cytochrome *c*-independent increase in Apaf-1 and caspase-9 activation for cell death

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Received 12 March 2005; accepted 15 March 2005
Available online 18 April 2005

Abstract

Hepatitis C virus (HCV) core protein has multifunctional activities. We have previously reported that the core protein of HCV immortalizes primary human hepatocytes, which may relate to multistage hepatocarcinogenic events. These immortalized human hepatocytes (IHH) served as a model to study the mechanism of HCV core protein-mediated cell growth regulation. Inhibition of core protein expression in earlier stages after hepatocyte immortalization leads to the induction of apoptosis. Here, we have observed that introduction of antisense core (AS-Core) sequences for inhibition of core protein expression enhanced the expression of E2F1 and p53 levels in early passage IHH. Inhibition of core protein expression also altered the expression level of Bcl-2 family proteins, displaying an increase of the proapoptotic Bax and a decrease in the level of the anti-apoptotic Bcl-xL proteins. These alterations, however, did not result in the release of cytochrome *c* from the mitochondria. Apaf-1 is frequently deregulated under various pathologic conditions, and examination of AS-Core-expressing apoptotic cells indicated a significant increase in the level of Apaf-1, which coincided with caspase-9 activation. Knockdown of Apaf-1 or the transcriptional regulatory proteins, E2F1 or p53, by small interfering RNA (siRNA) duplexes inhibited the activation of caspase-9 and enhanced cell viability in AS-Core-expressing cells. These findings may contribute to the understanding of the pathophysiology of HCV core protein-mediated hepatocyte growth regulation and disease progression.
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Keywords: Hepatitis C virus core protein; Human hepatocytes; Cytochrome *c*; Apaf-1

Introduction

HCV is a major public health problem, with more than 170 million people chronically infected worldwide. The most important feature of HCV infection is the development of chronic hepatitis in ~80% of the infected individuals and the potential for disease progression to cirrhosis and hepatocellular carcinoma (Jeffers, 2000; Saito et al., 1990). Studies on HCV are challenging due to the lack of a suitable cell culture system and the absence of a small animal model.

Overproduction and release of core protein have been observed in the blood stream of HCV infected hosts (Maillard et al., 2001). HCV core protein immortalizes primary human hepatocytes, the natural host for virus replication (Ray et al., 2000). These immortalized human hepatocytes (IHHs) exhibit activation of telomerase function and continuous growth in cell culture. Hepatocarcinogenesis involves alterations in the concerted action of proto-oncogenes, growth factors, and tumor suppressor genes. HCV core protein regulates p53, p21, c-*myc* promoter activities, and cyclin E (Cho et al., 2001; Kwun and Jang, 2003; Ray et al., 1997, 1998; Siavoshian et al., 2004).

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doi:10.1016/j.virol.2005.03.016

Moreover, HCV core protein constitutively activates AP-1, which correlates with the activation of JNK and MAPKK (Marusawa et al., 1999; Shrivastava et al., 1998). Premature senescence involving p53 is activated in response to constitutive MEK/MAPK mitogenic signaling (Lin et al., 1998). We observed a weak level of core protein expression in IHH, which is sufficient for the maintenance of cell growth, and below which early passage immortalized hepatocytes fail to survive (Basu et al., 2002).

A fundamental aspect of p53 is its ability to participate in cell-cycle checkpoint and apoptosis functions that regulate homeostatic tissue renewal. Exogenous p53 expression can suppress the transformed phenotype of many cell types by inducing growth arrest and apoptosis (Soddu and Sacchi, 1998). The modulation of p53 by adenovirus E4 or E6 proteins, HTLV-1 Tax protein, SV40 T antigen, and the E6/E7 gene products of HPV-16 and 18 plays a role in cell growth regulation (Herzig et al., 1999; Nevels et al., 1999a, 1999b; Steegenga et al., 1999; Uittenbogaard et al., 1995). A constant expression of SV40 T-antigen is necessary for the maintenance of immortalized human fibroblasts (Radna et al., 1989), and antisense E7 RNA of HPV-18 causes death of an oral cancer cell line–1483–after a lag period (Steele et al., 1992). Results from our previous studies have suggested that HCV core protein can modulate p53 gene expression, which may contribute to the immortalization of primary human hepatocytes and growth (Basu et al., 2002; Ray et al., 2000).

Activation of p53 may occur due to an overexpression of E2F1. The p14ARF protein, identified as an E2F target gene, stabilizes p53 and may provide a link between E2F1 and an elevation in p53 levels (Bates et al., 1998). E2F1 is a member of a family of transcription factors and acts to control the G1/S transition in embryotic cells (Harbour and Dean, 2000). In response to mitogenic stimuli, E2F1 is induced in quiescent cells and promotes both cell cycle progression and apoptosis (Nevins, 1998). Apaf-1 is transcriptionally activated by p53 and E2F1 (Moroni et al., 2001), and is a mediator of apoptosis. An increase in Apaf-1 levels has been associated with the direct activation of caspase-9 without mitochondrial damage and leads to the initiation of a caspase cascade (Furukawa et al., 2002).

Immortalization of primary human hepatocytes by HCV core protein may be a result of a regulatory role on cellular gene(s) leading to an alteration in hepatocyte turnover, although the mechanism of core protein-mediated cellular gene regulation is not well understood (Ray and Ray, 2001). HCV core protein has multifunctional activities similar to that seen in a number of other viral proteins with oncogenic potential. The immortalization of a primary human epithelial cell occurs through a multistep process, and many of these steps are not fully understood. Interestingly, inhibition of core protein expression did not alter the growth property of late passage IHH (passage 50) when cells were exhibiting anchorage-independent growth, in contrast to early passage IHH (passage 15). This suggested that HCV core protein is required for the maintenance of this phenotypic change in the early stages after hepatocyte immortalization (Basu et al., 2002; Ray et al., 2000).

In the present study, we have utilized IHH as a model to study the role of HCV core protein-mediated cellular functions, particularly with regard to the mechanism of immortalization. We have investigated the signaling pathway by which IHHs undergo apoptosis upon inhibition of HCV core protein expression. Our results demonstrate a novel mechanism of apoptosis in which hepatocyte death correlates with an increase in Apaf-1. The subsequent activation of caspase-9, leading to the initiation of the intrinsic cell death pathway, occurs in the absence of cytochrome *c* translocation to the cytosol.

Results

Differentially expressed E2F1 and p53 genes in IHH

Atlas cDNA expression membrane (Human Oncogene/Tumor Suppressor Array) was used to identify genes induced in response HCV core protein expression in IHH. Total RNAs isolated from HCV core-transfected IHH and from paired donor hepatocytes were used to generate ^{32}P-labeled cDNA probes for the hybridization of Atlas cDNA expression membrane. Modulated genes were selected as described under Materials and methods and a detailed description of the gene expression analyses will be reported separately. Among the genes of interest from our previous studies (Basu et al., 2002; Ray et al., 2000), both E2F1 and p53 were reduced early after immortalization of the primary human hepatocytes by HCV core (passage number15) as compared to primary hepatocytes from matching donors. Each passage number represented cells from ~4 days of culture. Real-time PCR verified an ~35% decrease in E2F1 and ~50% decrease in p53.

p53 is known to play a key role in growth arrest, DNA repair, and apoptosis after cell stress, primarily through its ability to regulate the transcription of downstream cellular target genes. AS-Core gene expression in early passage IHH (passage 15) induces an apoptotic response (Basu et al., 2002). Inhibition of core gene expression led to an ~5-fold increase in protein expression of p53 as compared to vector control in early passage IHH which preceded apoptosis (Fig. 1, panel A). Inhibition of core gene expression also increased E2F1 protein expression by ~2-fold in early passage IHH (Fig. 1, panel B). These results corroborated the data derived from Atlas cDNA expression and real-time PCR, suggesting that the regulation of p53 and E2F1 genes occurred at the transcriptional level.

To examine whether p53 or E2F1 is responsible for AS-Core-mediated apoptosis, we knocked down p53 or E2F1

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