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Hepatitis C virus Core protein overcomes stress-induced premature senescence by down-regulating p16 expression via DNA methylation

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ABSTRACT

Hepatitis C virus Core plays a vital role in the development of hepatocellular carcinoma; however, the mechanism is still controversial. Here, we show that Core overcomes premature senescence provoked by a reactive oxygen species inducer, H_2O_2, in human liver cells. For this effect, Core down-regulated levels of p16 via promoter hypermethylation and subsequently induced phosphorylation of Rb in the presence of H_2O_2. Levels of p21 and p27, however, were little affected by Core under the condition. The potentials of Core to inactivate Rb and suppress H_2O_2-mediated cellular senescence were abolished when levels of p16 were recovered by either exogenous complementation or inhibition of DNA methylation. Considering that cellular senescence provoked by oxidative stresses is an important tumor suppression process, our present study provides a new strategy by which HCV promotes development of hepatocellular carcinoma.

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1. Introduction

Hepatitis C virus (HCV) is a major cause of non-A and non-B hepatitis, which frequently leads to liver cirrhosis and hepatocellular carcinoma (HCC) [1]. As a member of *Flaviviridae* family, it contains a positive stranded RNA genome of 9.5 kb, encoding a polyprotein processed into four structural proteins and six non-structural proteins [2]. In addition to its function in regulating viral RNA translation and capsid assembly, Core has been strongly implicated in HCC pathogenesis by virtue of its role in alteration of various signaling pathways, transcriptional activation, modulations of immune responses, apoptosis, and lipid metabolism [3,4]. In addition, Core has been directly implicated in cellular transformation and immortalization [5]. Furthermore, direct induction of HCC by Core in transgenic mice has been reported [6]. Despite of steadily increasing knowledge about Core in HCV-associated oncogenesis, its action mechanism is still controversial.

Normal somatic cells enter a state of irreversible growth arrest after a limited proliferative life span [7]. This process, known as replicative senescence, is normally signaled by shortened telomeres that result from repeated rounds of DNA replication in the absence of telomerase expression [8]. Activated oncogenes also can trigger cell senescence, thereby blocking progression to a transformed cell phenotype [9]. In addition, cells exposed to various types of sublethal oxidative stress display a senescent-like phenotype termed stress-induced premature senescence (SIPS). The stress conditions inducing SIPS include exposure to hydrogen peroxide (H_2O_2) [10], *tert*-butylhydroperoxide [11], hyperoxia [12], UV light [13], and radioactivity [14]. In addition to irreversible cell cycle arrest, cellular senescence is characterized by several molecular and cytological markers, such as a large flat morphology, induction of a senescence-associated β-galactosidase activity (SA β-gal) [15], and formation of several heterochromatin domains, called senescence-associated heterochromatin foci (SAHF) [16]. Numerous studies have elucidated the molecular mechanisms that direct cellular senescence. Among several cell cycle regulators, p16, p21, and p27 act as key regulators of senescence [17]. As cyclin-dependent kinase inhibitors (CKIs), they block the activity of G_1- or G_1/S-Cdks, leading to up-regulation of Rb activity in the cells. Continuous Rb activation then induces cellular senescence by recruiting heterochromatin proteins such as histone deacetylase to the E2F-responsive promoters to form SAHF, resulting in stable repression of E2F target genes [16].

Oxidative stress, imposed either by viral proteins or host immune responses, has been suggested as an important pathologic mechanism in hepatitis C and other chronic liver diseases [18,19]. Free radicals can trigger a cascade of epigenetic, genomic and post-genomic alterations that lead to HCC [19]. However, it is relatively unknown how hepatocytes maintain their growth potential and support virus replication under the oxidative microenvironment causing cellular senescence. It might be possible if the regulators of cellular senescence are dysfunctional in these cells. Indeed, p16 is frequently inactivated via either epigenetic or genetic alterations in a large proportion (>30%) of human tumors including HCC

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[20,21]. In addition, the p16-deficient human fibroblasts are resistant to RAS-induced senescence [22]. Interestingly, epigenetic inactivation of p16 in HCC is strongly associated with infection of HCV [23]. Furthermore, we have recently shown that HCV Core down-regulates p16 expression via promoter hypermethylation [24]. In the present study, we further investigated whether Core overcomes SIPS provoked by H_2O_2, enabling continuous cell proliferation under oxidative stress. We also investigated whether this effect is directly associated with the potential of Core to down-regulate p16 via promoter hypermethylation.

2. Materials and methods

2.1. Plasmids

To construct the Core-expressing plasmid pCMV-3 × HA1-Core, the full-length HCV core sequence (genotype 1b) was cloned into pCMV-3 × HA1, in frame, downstream of three copies of the influenza virus hamagglutinin (HA) epitope [a nonapeptide sequence (YPYDVPDYA)] as described before [25]. For construction of pCMV-3 × HA1-p16, a full-length p16 cDNA prepared from total RNA of HepG2 cells was amplified with the use of INK4aF (5′-GCC TCG AGC CGG CGG CGG GGA-3′) and INK4aR (5′-CTC TAG ATC TTT CAA TCG GGG ATG TC-3′) and subcloned, in frame, into pCMV-3 × HA1 [26].

2.2. Cell culture and transfection

Two human liver cancer cell lines, HepG2 (KCLB No. 88065) and Huh7 (KCLB No. 60104), were obtained from the Korean Cell Line Bank. Another human liver cell line named Chang liver (ATCC No. CCL-13) was kindly provided by Dr. JaeHun Cheong (Pusan National University, Korea). All cells were maintained in DMEM supplemented with 10% FCS (Gibco). For transient expression, 2×10^5 cells per 60-mm-diameter plate were transfected with 2 μg of appropriate plasmid(s) using WelFect-EX PLUS (WelGENE) following the manufacturer's instructions. Stable cell lines, HepG2-vector and HepG2-Core, were established by transfection with either an empty vector or pCMV-3 × HA1-Core, respectively, followed by selection in the presence of 500 μg/ml G418 (Gibco) [25]. To induce oxidative stress, cells were treated with H_2O_2 as described before [10]. Briefly, 5×10^5 cells per 60-mm-diameter plate were treated with 200 μM H_2O_2 for 2 h. After changing with a normal medium, the cells were incubated for additional 72 h. Cells were also treated with 5 μM 5-Aza-2′dC (Sigma) for the indicated samples.

2.3. Determination of intracellular reactive oxygen species (ROS) levels

Chloromethyl dichlorodihydrofluorescein diactate (CM-H_2DCFDA, Invitrogen), which acts as an H_2O_2-sensitive fluorophore, was employed for the detection of H_2O_2-induced reactive oxygen species (ROS) as described before [27]. Briefly, 4×10^4 cells per well in 12-well plate were treated with 200 μM H_2O_2 for 2 h, followed by incubation with 10 μM CM-H_2DCFDA in serum-free media for 1 h. The fluorescent signal was then quantified with a luminometer (VICTOR III, Perkin-Elmer) with excitation and emission wavelengths of 485 and 535 nm, respectively.

2.4. RNA interference

SilenCircle RNAi system (Allele Biotech), a plasmid-based RNA interference system that uses U6 RNA-based polymerase III promoter, was employed to silence specific gene expression. Based on the target sequence of HCV core (5′-AAG GCG ACA ACC TAT CCC CAA-3′) [25], siRNA inserts composed of both sense and antisense sequences separated by a central loop sequence were designed. The siRNA inserts were ligated into pre-cut pSilenCircle vector and positive clones were selected.

2.5. Reverse transcription–polymerase chain reaction (RT–PCR) and quantitative RT–PCR

Total cellular RNA was extracted using a total RNA isolation kit (Qiagen). RNA (3 μg) was reverse transcribed with the corresponding reverse primer. One-quarter of the reverse-transcribed RNA was amplified with *Taq* polymerase (95 °C for 5 min; 30 cycles at 95 °C for 1 min, 56 °C for 1 min; 72 °C for 30 s, 72 °C for 5 min) using forward primers, 5′-CGG GGT GAA AAG ATA AAG-3′ and 5′-TGG CGT GAT TCT GAG CAA-3′ and reverse primers, 5′-TGC GGT CAC CAT TCA TCC A-3′ and 5′-CTG CCA AGC TGC CCA AGG-3′ to detect RNA levels of apolipoprotein J and SM22, respectively [28]. The PCR condition for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was described before [26]. For quantitative RT–PCR, one twentieth of the RT reaction mixture was used for PCR amplification by the StepOne™ Real-Time PCR system (Applied Biosystems) and SYBR Premix Ex Taq TMII (Takara). GAPDH was used for cDNA normalization. Relative expression was calculated using the comparative C_t method [29].

2.6. Western blot analysis

Cells were lysed in buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40] with protease inhibitors. Cell extracts were separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF, Amersham). Membranes were then incubated with antibodies against p16, p21, p27 (Santa Cruz Biotechnology), phosphorylated Rb, γ-tubulin (Sigma), Rb (Calbiochem), and HA (Roche) for 2 h at room temperature and subsequently with the appropriate horse-radish peroxidase-conjugated secondary antibodies: anti-rabbit IgG (H + L)-HRP (Bio-Rad) and anti-mouse IgG (H + L)-HRP (Bio-Rad) for 1 h at room temperature. The chemiluminescent ECL kit (Amersham) was used to visualize protein bands on X-ray films.

2.7. Methylation-specific PCR (MSP) and bisulfite DNA sequencing

Genomic DNA (1 μg) denatured in 50 μL of 0.2 N NaOH was modified by treatment with 30 μL of 10 mM hydroquinone (Sigma) and 520 μL of 3 M sodium bisulfite (pH 5.0; Sigma) at 50 °C for 16 h. Treatment of DNA with bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. For MSP, the modified DNA (100 ng) was amplified with *Taq* polymerase with both methylated and unmethylated primer pairs of p16 as described before [30]. In addition, the region from −145 to +136 of the p16 promoter was amplified by PCR using a bisulfite sequencing primer set, p16-F (5′-GGG TAG GTG GGG AGG AGT TTA GTT T-3′) and p16-R (5′-AAT AAC CAA CCA ACC CCT CCT CTT T-3′) [31]. The PCR products were subcloned into the T-easy vector (Promega) and their nucleotide sequences were determined.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's specifications. The sheared chromatin was immunoprecipitated with anti-Ets1 and anti-Ets2 antibodies (Santa Cruz Biotechnology), and a negative control rabbit IgG (Santa Cruz Biotechnology). DNA released from precipitated complexes was amplified by PCR using p16 primers (sense, 5′-GCC CAG TCC TCC TCC TCC CTT GC-3′; antisense, 5′-CAA GCT TCT CCC CGC CC-3′) [31].

2.9. SA β-gal and SAHF assays

SA β-gal assay was performed as described by Dimri et al. [15]. Briefly, cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10 min. SA β-gal activity was determined by incubation with SA β-gal staining solution (Cell Signaling) at 37 °C for 18 h. The cells were rinsed twice with PBS and washed with methanol followed by microscopic examination. SAHF formation was detected as previously described [16]. Briefly, cells on a coverslip were fixed with 70% ethanol for 1 h, stained with 1 μg/ml DAPI for 5 min, and observed under a fluorescence microscope (Zeiss).

2.10. Cell proliferation assay

For the determination of cell growth, 5×10^4 cells per well in 6-well plates were incubated for 20 h. Cells were either mock-treated or treated with either 200 μM H_2O_2 (Sigma) and/or 5-Aza-2′dC (Sigma) for 2 h and incubated in a normal medium for additional 72 h, followed by counting the total cell number in each well after trypsinization.

2.11. Statistical analysis

The values indicate means ± SD from at least three independent experiments. The difference between the means of the treatment groups and their controls were assessed with the paired two tailed *t* test; A *P* value of <0.05 was considered to be statistically significant.

3. Results

3.1. HCV Core overcomes stress-induced cellular senescence

We first investigated whether HCV Core overcomes stress-induced premature senescence by treating HepG2 cell lines with or without Core expression with an oxidative stress inducer, H_2O_2. Several biomarkers of cellular senescence, including morphological changes (enlarged and flattened), SA β-Gal staining, and SAHF were clearly observed in the control cells (HepG2-vector) at 72 h after exposure to 200 μM H_2O_2 for 30 min (Fig. 1A). Treatment with H_2O_2 also markedly increased RNA levels of senescence-related genes such as apolipoprotein J and SM22 [28] in these cells (Fig. 1B). In addition, the senescence-related effects of H_2O_2 on cell

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