Hepatitis C virus core protein induces hypoxia-inducible factor 1α-mediated vascular endothelial growth factor expression in Huh7.5.1 cells

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Abstract. Hepatitis C virus (HCV) infection is one of the major causes of hepatocellular carcinoma (HCC). It has been demonstrated that the overexpression of angiogenic factors are associated with the maintenance of liver neoplasia. Hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) are important regulators of angiogenesis and are important in wound healing, the regeneration of new vessels and reproductive functions. The present study investigated the role of the HCV core protein in the induction of HIF-1α and VEGF expression. The HCV core gene and HIF-1α siRNA were transfected into Huh7.5.1 cells. The results demonstrated that the induction of HCV core gene expression in Huh7.5.1 cells leads to the overexpression and stabilization of HIF-1α, and the activation of HIF-1α leads, in turn, to the stimulation of VEGF, which is one of the most important angiogenic factors. These results provide new information to facilitate the understanding of HCC oncogenesis.

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Abbreviations: Flag2B, pCMV-Tag2B; Flag2B-core, pCMV-Tag2B-core; HIF-1α siRNA, siRNAs against HIF-1α; NC siRNA, negative control siRNA

Key words: hepatitis C virus, hepatocellular carcinoma, hypoxia-inducible factor 1α, vascular endothelial growth factor

Introduction

It is estimated that 2-3% of the world's population are chronically infected with the hepatitis C virus (HCV), which is considered to be a major risk factor for the development of hepatocellular carcinoma (HCC) (1,2). HCV belongs to the Flaviviridae family of enveloped RNA viruses and contains a 9.6 kb single-stranded positive-sense RNA genome. This genome is translated into a large polyprotein which is then cleaved by viral and host proteases into structural (core, E1 and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (3-5). In addition to their unique involvement in the life cycle and assembly of the virus, these HCV proteins also participate in processes, including transcriptional activation, cell signaling, apoptosis and transformation by way of interaction with host factors (6-10). In particular, the core gene product has long been proposed as a candidate protein implicated in liver oncogenesis.

Hypoxia-inducible factor 1 (HIF-1) is a heteroduplex that contains a constitutively expressed HIF-1β subunit and an oxygen- and growth factor-regulated HIF-1α subunit (the major determinant for the activity of HIF-1). HIF-1 is the most important regulator of oxygen homeostasis, which is required for cellular metabolism (11). Under physiological and normoxic conditions, HIF-1α is subjected to rapid degradation by ubiquitin-proteasome pathways (12). However, under hypoxic induction or non-hypoxic growth factor induction conditions, HIF-1α is overexpressed and stabilized, resulting in the activation of genes that stimulate angiogenesis, including vascular endothelial growth factor (VEGF) (13). In human cancers, HIF-1 mediated angiogenesis and metabolic adaptation are important in tumor formation, progression and metastasis (14).

Extensive investigations have been conducted to elucidate the inter-relationships between viral products and host cellular factors. Previous studies have demonstrated that HCV infection stabilizes HIF-1α and stimulates the synthesis of VEGF (15). The present study demonstrated that induction of HCV core protein expression in Huh7.5.1 cells enhances the transcriptional level and protein amount of HIF-1α, as well as VEGF, and also confirmed that the HCV core protein increases the expression of

VEGF directly via the activation of HIF-1α. Thus, we propose a novel molecular mechanism of the core protein in modulating gene expression that is associated with HCC.

Materials and methods

Plasmids and siRNAs. The plasmid pCMV-Tag2B (Flag2B; Stratagene, La Jolla, CA, USA) was used to construct the HCV core expression plasmid pCMV-Tag2B-core (Flag2B-core). The selection of siRNAs against HIF-1α (HIF-1α siRNA) and negative control siRNA (NC siRNA) were based on the study by Gillespie *et al* (16).

Cell culture and transfection. The human hepatoma cell line, Huh7.5.1, was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U of penicillin/ml and 100 μg of streptomycin sulfate/ml at 37˚C in a humidified 5% CO_2 incubator. Transient transfections of Huh7.5.1 cells with the plasmids and siRNAs described above were conducted using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

Reverse transcriptase (RT)-PCR. Following 48 h of transfection, total cellular RNAs were extracted using TRIzol (Invitrogen Life Technologies) and the cDNA was reverse transcribed from 1 μg of total RNA using an oligo (dT) primer. The resulting cDNA was PCR amplified with the following gene-specific primers: HIF-1α, forward 5'-TAGTGCCACATCATCACC-3' and reverse 5'-ACATGCTAAATCAGAGGG-3'; VEGF, forward 5'-GGGCAGAATCATCACGAAGT-3' and reverse 5'-GGCTCCAGGGCATTAGACA-3'. PCR amplification was performed under the following conditions: 10 min at 95˚C, followed by 35 cycles of 94˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec and finishing with a dissociation protocol. The PCR products were detected by 2% agarose gel electrophoresis and visualized under UV light with ethidium bromide staining.

Western blot analysis. Following 48 h of transfection, cell samples were lysed with Nonidet P-40 lysis buffer [10 mM of Tris-HCl (pH 7.4), 10 mM of NaCl, 3 mM of $MgCl_2$ and 0.5% Nonidet P-40]. The cell lysates were then centrifuged at 3,000 x g for 10 min and the supernatants were used in the assay. Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. Following the inhibition of non-specific binding sites, western blot analysis was performed using specific antibodies against HIF-1α (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), VEGF (Sigma, St. Louis, MO, USA), HCV core protein (Affinity Bioreagents, Golden, CO, USA) and, as an internal control, a monoclonal antibody against β-actin (Sigma). Following washing, blots were developed with horseradish peroxidase-labelled goat anti-rabbit IgG, using an enhanced chemiluminescence kit (Amersham Life Sciences, Piscataway, NJ, USA).

Enzyme-linked immunosorbent assay (ELISA) analysis. Following 48 h of transfection, the VEGF concentration in cell supernatants was measured by ELISA, which was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical analysis. SPSS 13.0 software was used for statistical analysis. Values are expressed as the means ± SD. The comparison of two means was performed by t-tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HCV core protein enhances the expression levels of HIF-1α mRNA and protein in Huh7.5.1 cells. HIF-1 is a heterodimeric (HIF-1α coupled with HIF-1β) protein that regulates oxygen homeostasis for cellular metabolism and acts as an inducer of angiogenic factors. Under physiological conditions, HIF-1α is constitutively expressed and degraded, however, under hypoxia or other conditions, HIF-1α is overexpressed and stabilized (11,13). In the present study, the expression levels of HIF-1α mRNA and protein were measured by RT-PCR and western blot analysis, respectively, in Huh7.5.1 cells transfected with the HCV core gene eukaryotic expression vector (Flag2B-core) or the empty vector (Flag2B). The results in Fig. 1A demonstrated a moderate increase of HIF-1α mRNA in HCV core induced Huh7.5.1 cells relative to non-induced cells. The western blot assay demonstrated a significant increase of HIF-1α protein in HCV core induced Huh7.5.1 cells compared with the control (Fig. 1B).

HCV core protein induces the expression and secretion of VEGF in Huh7.5.1 cells. VEGF stimulates angiogenesis and vascular permeability in neoplastic tissues, which means the expression and secretion of VEGF are increased significantly in numerous types of cancer (17,18). In order to investigate whether HCV core gene expression alone can induce the expression and secretion of VEGF, VEGF mRNA and protein levels in Huh7.5.1 cells were measured according to the same instructions used to measure HIF-1α expression levels. An increase in VEGF mRNA (Fig. 2A) and protein expression (Fig. 2B) was identified in HCV core induced Huh7.5.1 cells, indicating that the HCV core protein contributes to the biosynthesis of VEGF.

As VEGF can be secreted into the extracellular media, we further examined the concentrations of VEGF in cell supernatants by ELISA. The supernatant was removed from all wells and a human VEGF ELISA (R&D Systems) was performed on the cell supernatants 48 h post-transfection, as described in the Quantikine human VEGF ELISA instructions. Student's t-test was used for statistical analysis. The results in Fig. 2C demonstrated that VEGF concentrations in the supernatants of HCV core induced Huh7.5.1 cells were significantly elevated compared with the controls (654.5±43.7 vs 365.9±26.8 pg/ml).

RNA interference disrupts HIF-1α-induced upregulation of VEGF. The present study utilized HIF-1α siRNA that, when transfected into cells, targets HIF-1α mRNA for degradation, thus reducing the expression of HIF-1α RNA and protein. The VEGF mRNA (Fig. 3A) and protein levels (Fig. 3B) in Huh7.5.1 cells cotransfected with Flag2B-core and HIF-1α siRNA were significantly reduced compared with the Flag2B-core plus NC siRNA-transfected cells. The ELISA

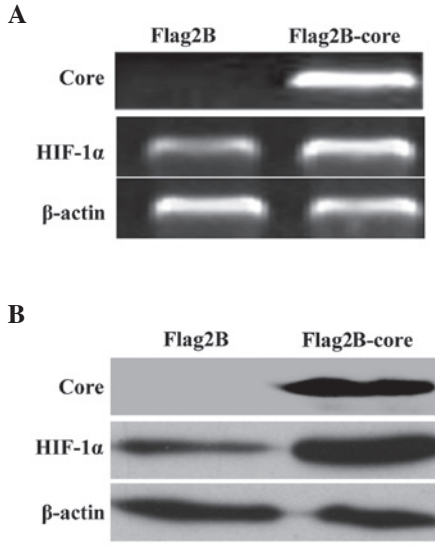

Figure 1. HCV core protein enhances the expression levels of HIF-1α mRNA and protein in Huh7.5.1 cells. (A) RT-PCR analysis was used to compare the relative levels of HIF-1α mRNA in Huh7.5.1 cells transfected with Flag2B (Lane 1) or Flag2B-core (Lane 2). The β-actin gene was amplified as an internal control. PCR products were detected by 2% agarose gel electrophoresis with ethidium bromide stain. (B) Western blot analysis of HIF-1α protein expression in Huh7.5.1 cells. Lane 1, Huh7.5.1 cellular lysates transfected with Flag2B; Lane 2, Huh7.5.1 cellular lysates transfected with Flag2B-core. The middle panel represents the expression of HCV core protein, and the bottom panel represents the expression of β-actin as an internal control. HCV, hepatitis C virus; HIF-1α, hypoxia-inducible factor-1α; RT-PCR, reverse transcriptase-PCR; Flag2B-core, pCMV-Tag2B-core; Flag2B, pCMV-Tag2B.

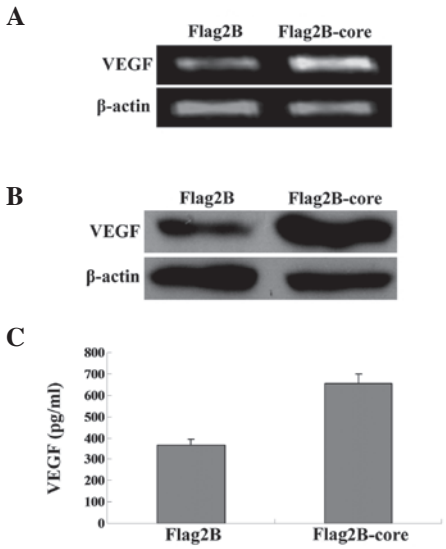

Figure 2. HCV core protein induces the expression and secretion of VEGF in Huh7.5.1 cells. (A) RT-PCR analysis was used to compare the relative levels of VEGF mRNA in Huh7.5.1 cells transfected with Flag2B (Lane 1) or Flag2B-core (Lane 2). The β-actin gene was amplified as an internal control. PCR products were detected by 2% agarose gel electrophoresis with ethidium bromide staining. (B) Western blot analysis of VEGF protein expression in Huh7.5.1 cells (Lane 1), Huh7.5.1 cellular lysates transfected with Flag2B (Lane 2) and Huh7.5.1 cellular lysates transfected with Flag2B-core. The middle panel represents the expression of the HCV core protein and the bottom panel represents the expression of β-actin as an internal control. (C) ELISA analysis of VEGF concentrations in the supernatants of HCV core induced Huh7.5.1 cells and non-induced controls. HCV, hepatitis C virus; RT-PCR, reverse transcriptase-PCR; ELISA, enzyme-linked immunosorbent assay; VEGF, vascular endothelial growth factor; Flag2B-core, pCMV-Tag2B-core; Flag2B, pCMV-Tag2B.

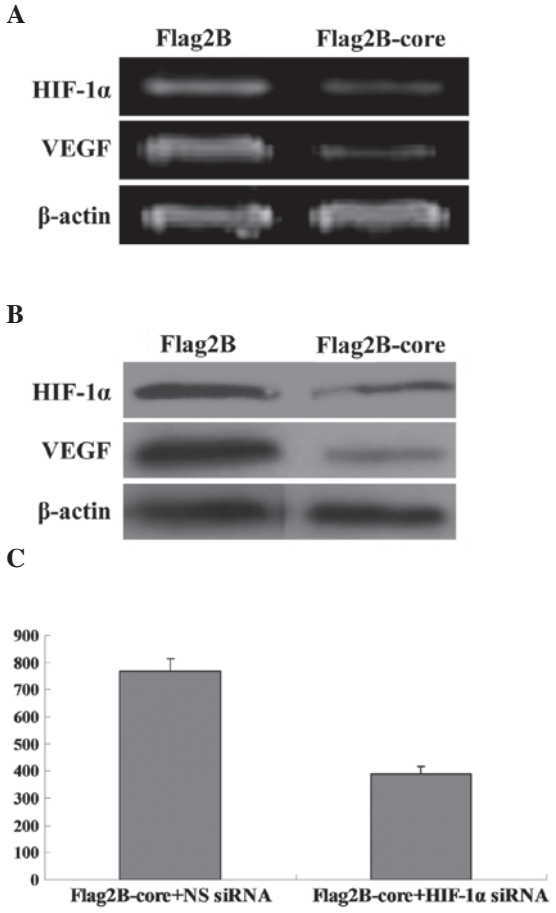

Figure 3. RNA interference disrupts HIF-1α-induced upregulation of VEGF. (A) RT-PCR analysis was used to compare the relative levels of VEGF mRNA in Huh7.5.1 cells transfected with Flag2B-core plus NC siRNA (Lane 1) and Flag2B-core plus HIF-1α siRNA (Lane 2). The β-actin gene was amplified as an internal control. PCR products were detected by 2% agarose gel electrophoresis with ethidium bromide staining. (B) Western blot analysis of VEGF protein expression in Huh7.5.1 cells. Lane 1, Huh7.5.1 cellular lysates transfected with Flag2B-core plus NC siRNA; Lane 2, Huh7.5.1 cellular lysates transfected with Flag2B-core plus HIF-1α siRNA. The middle panel represents the expression of the HCV core protein and the bottom panel represents the expression of β-actin as an internal control. (C) ELISA analysis of VEGF concentrations in the supernatants of Flag2B-core plus NC siRNA transfected Huh7.5.1 cells and Flag2B-core plus HIF-1α siRNA transfected Huh7.5.1 cells. HCV, hepatitis C virus; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; RT-PCR, reverse transcriptase-PCR; Flag2B-core, pCMV-Tag2B-core; Flag2B, pCMV-Tag2B; HIF-1α siRNA, siRNAs against HIF-1α; NC siRNA, negative control siRNA.

results in Fig. 3C (389.2±29.6 vs 768.8±47.3 pg/ml) were in line with the results in Fig. 3A and B.

Discussion

HCV infections are associated with the development of HCC, however, the underlying mechanisms by which HCV induces HCC are not well understood. Indeed, although accumulating studies have implicated the specific roles of HCV proteins in the modulation of cell proliferation and pathogenesis, it is unclear which viral gene products are crucial for the establishment of HCC (19,20). It has been demonstrated that the core protein of HCV can induce HCC in transgenic mice by the modulation of cellular gene products, which has brought the core protein to the attention of researchers (21).

The core protein is located at the N-terminal portion of the HCV polyprotein and is highly conserved among various HCV subtypes. Apart from functioning as the building block of the viral nucleocapsid, which is involved in binding and packaging the viral RNA genome, the core protein exhibits pleiotropic roles in numerous activities, including gene transcription, cell proliferation and cell death through interference with the normal functions of an extensive list of cellular proteins (21). In this regard, the present study was undertaken to investigate whether HCV core gene expression is able to trigger angiogenesis, which is pivotal in tumor formation and maintenance. Our results demonstrated that the induction of HCV core protein expression enhances the transcriptional level and amount of HIF-1α as well as VEGF in Huh7.5.1 cells. HIF-1α and VEGF are regulators of angiogenesis and are important in wound healing, the regeneration of new vessels and reproductive functions. Therefore, these results indicated that the HCV core protein is able to stimulate angiogenesis.

The first study of HIF-1α overexpression in human cancer was ~10 years ago. Since then, a large amount of data has been collected demonstrating that HIF-1α overexpression is associated with tumor angiogenesis and increased mortality in cancer of the brain, breast, oropharynx, esophagus, colon, ovary and uterine cervix (22-25). Notably, proteins encoded by transforming viruses that cause tumors in humans, including EBV latent membrane protein 1, hepatitis B virus X protein, human papillomavirus E6/E7 proteins and human T-cell leukemia virus Tat protein, also induce HIF-1α activity (26-29). Therefore, it is evident that HIF-1α activity represents a fundamental common pathway in cancer pathogenesis.

What are the mechanisms by which the HCV core protein activates HIF-1α? In various types of human cancer, the increased expression of HIF-1α is induced either by intratumoral hypoxia or by genetic alterations affecting key oncogenes and tumor suppressor genes. For example, *ras* signaling has been demonstrated to be instrumental in hypoxia-induced stabilization of HIF-1α and inactivation of *p53* in tumor cells, which contributes to the activation of the angiogenic switch via amplification of normal HIF-1 dependent responses to hypoxia (30). Regarding HCV-induced tumors, the HCV core protein is able to co-operate with the *ras* oncogene in the transformation of rodent fibroblasts under certain conditions and is able to exert transcriptional repression of the *p53* promoter (31). However, we were unable to draw a conclusion regarding the mechanism of activation of HIF-1α by the HCV core protein as there is not enough evidence that HCV can directly activate any oncogene or deactivate any tumor suppressor genes at present. As the complete cell culture systems of HCV are now available, it may be useful to study more aspects of the HCV core protein in order to elucidate the exact mechanisms underlying HCV core protein induction of HIF-1α.

Our results confirmed that the core protein activates HIF-1α, which, in turn, increases the expression of VEGF. At present, VEGF inhibitors are undergoing clinical testing as a strategy for the prevention and treatment of certain malignancies. These findings may prompt worldwide study into the inhibition of HIF-1α, which may be a novel approach to cancer therapy.

In conclusion, the role of HCV in inducing oncogenesis is complicated and awaits further detailed investigation. In the case of the prevention and control of the virus induced tumors, the cellular response factors activated by viral infection warrant further studies. Thus, a mixture of antibodies or inhibitors may be required that target the virus itself and such cellular factors.

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