Hepatitis C virus (HCV) infection causes chronic hepatitis in a significant number of infected individuals, which gradually progresses to liver cirrhosis and subsequently to hepatocellular carcinoma (HCC) (14, 21). HCV gene expression has been observed in HCC, but the mechanism by which chronic HCV infection results in HCC is unknown. It has been proposed that HCV-induced chronic inflammation and the effects of cytokines in the development of fibrosis and liver cell proliferation could contribute to hepatocarcinogenesis (21). HCV-induced free radical-mediated injury that occurs as part of chronic liver damage might cause DNA damage and also contribute to the development of HCC (21, 23).

HCV is a member of the Flaviviridae family, which has a positive-sense single-stranded RNA genome of about 9.6 kb. The RNA genome encodes a polyprotein of about 3,000 amino acids, which is cleaved by viral and host proteases into structural (core, E1, and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (1, 6, 25, 27). The development of the Flaviviridae virus involves the formation of a lumen leading to the formation of new blood vessels and finally assembly into a tubular structure. Subsequent formation of a lumen leads to the formation of new blood vessels (12, 13, 35).

Angiogenesis is the formation of new blood vessels from preexisting blood vessel, which is the prerequisite for tumor growth and spread (10). Tumors cannot grow beyond a certain size, generally 1 to 2 mm due to lack of oxygen (hypoxia) and other essential nutrients (3). Tumors induce blood vessel growth (neovascularization) by secreting various soluble growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor, which can induce neovascularization into the tumor by inducing endothelial cell proliferation, supplying required nutrients, and allowing tumor to expand (10, 13). Thus, angiogenesis is a necessary and required step for transition from a small harmless cluster of cells to a large malignant tumor. Angiogenesis is also required for the spread of a tumor or metastasis. A single cancer cell can break away from an established solid tumor, enter the blood vessel, and be carried to a distant site, where it can implant and begin the growth of a secondary tumor (10, 13, 33). Angiogenesis in HCC and other solid tumors is based on the same fundamental principles of activation, proliferation, and migration of endothelial cells mediated by secreted angiogenic factors and finally assembly into a tubular structure. Subsequent formation of a lumen leads to the formation of new blood vessel (12, 13, 35).

Hypoxia-inducible factor 1α (HIF-1α) and HIF-1β are constitutively expressed in cells. HIF-1α, but not HIF-1β, is degraded by ubiquitin-proteasome pathways under normoxic conditions (17, 18). In hypoxia, HIF-1α stabilizes and translocates to the nucleus, where it dimerizes with HIF-1β and binds to the hypoxia-responsive element (HRE) of the target gene promoter. The dimeric complex also recruits transcriptional coactivators such as CREB-binding protein (CBP)/p300 and activates the transcription of target genes (8, 18). Direct HIF-1α target genes that are particularly relevant to cancer progression include angiogenic factors such as VEGF, cell proliferation and survival factors such as insulin-like growth factors, glycolytic enzymes, and invasion and metastatic factors such as matrix metalloproteinases (36). In addition to hypoxia, HIF-1α is also stabilized and activated during normoxia by growth factors, cytokines, hormones, nitric oxide, and even cellular and viral oncogenes as well as inactivation of tumor suppressor genes (16, 17, 46, 50). Signaling pathways induced by these factors mediate the activation of HIF-1α (15, 16, 46, 50).
Although the crucial roles of HIF-1α in angiogenesis have been investigated extensively in different cancers, the role of HIF-1α in HCV-associated hepatocarcinogenesis has not yet been reported. We investigated here the role of HCV in the induction of angiogenic factors in the human hepatoma cell line, Huh-7. The results of these studies show that viral infection mediates angiogenesis by stabilizing HIF-1α under normoxic growth conditions. HCV-stabilized HIF-1α subsequently stimulates the synthesis and secretion of VEGF. Both the stabilization of HIF-1α and the stimulation of VEGF synthesis occur via oxidative stress and calcium signaling induced by HCV gene expression. Using the chick chorioallantoic membrane (CAM) assay, we further show that HCV-infected cells secrete angiogenic cytokines that promote neovascularization in vivo. This activity was abrogated in the presence of dominant-negative mutants of NF-κB and STAT-3. Both transcription factors are activated by HCV gene expression (42). Our results support the functional role of HCV gene expression in angiogenesis associated with hepatocarcinogenesis.

MATERIALS AND METHODS

Plasmids and reagents. The HRE-Luc reporter plasmid contained the trimeric units of HRE sequences cloned into the pGL2 basic vector (Promega, Madison, WI; a gift from N. S. Chandel, Northwestern University, Chicago, IL). The human VEGF promoter-luciferase reporter construct in which VEGF promoter (−1176/+54) was cloned into the pGL2 basic vector, was kindly provided by Gilles Pages (University of Nice, Nice, France). Pyrrolidine dithiocarbamate (PDTC), N-acetyl-i-cysteine (NAC), PD98059, SB203580, and NS398 were purchased from Sigma. N398, Celecoxib, BAPTA-AM, and LY294002 were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA).

Cell culture. The human hepatoma cell line, Huh-7, was cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin sulfate/ml. FCA4 and K2040 are the Huh-7 cell lines that stably express the HCV subgenomic replicon. FCA4 contains a deletion of serine (40, 49). After infection, cells were harvested at different time points (days 1, 2, 3, 4, 5, 6, and 7), and cellular lysates were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for HCV protein detection. HCV-infected cells harvested at day 3 were used for further studies.

Luciferase reporter assay. Huh-7 cells were plated at a density of 2 × 10^5 cells per six-well cluster plate. Cells were transfected with firefly luciferase reporter plasmids using Lipofectamine 2000 reagent (Invitrogen). At 36 h posttransfection, cells were treated with PD98059 (50 μM) for 6 h, LY294002 (50 μM) for 12 h, NS398 (50 μM) for 24 h, and Celecoxib (100 μM) for 24 h. The transfection efficiency was normalized by cotransfection of pRL-TK vector (Promega), which expressed Renilla luciferase gene under the control of the thymidine kinase promoter. Luciferase assays were done by using a Dual-Luciferase reporter assay kit (Promega) according to the manufacturer’s protocol.

RESULTS

HCV gene expression stabilizes HIF-1α. In most of the HCC tumors associated with HCV, the presence of viral RNA has been demonstrated, suggesting a possible role of HCV in the maintenance of liver neoplasia (14). Angiogenesis is an essential step in tumor progression and invasion. In the present study, we investigated the potential role of HCV gene expression in inducing angiogenesis with a particular focus on HIF-1α. HIF-1α is constitutively expressed and degraded, but under hypoxia, it is stabilized (17, 18). We analyzed the stabilization of HIF-1α, an inducer of angiogenic factors, in HCV-infected cells by Western blot assays. The results revealed the stabilization of HIF-1α in HCV-infected cell lysates harvested at day 3 (Fig. 1A, lane 2) but not in uninfected lysates (lane 1). This stabilization also occurred in Huh-7 cells expressing HCV subgenomic replicon RNA (FCA4 and K2040) (Fig. 1B, lanes 2 and 3). FCA4 and K2040 represent two stable clones of Huh-7 cells that harbor HCV genotype 1b subgenomic replicon RNA.
expressing all of the nonstructural proteins (from NS3 to NS5B) (12, 38). These results indicated that nonstructural protein expression was sufficient for the HIF-1α stabilization. These results further demonstrated that the stabilization of HIF-1α occurred under normoxia. Thus, HCV mimicked hypoxic conditions, which resulted in the stabilization of HIF-1α. To demonstrate the involvement of proteasomal pathways in the degradation of HIF-1α in Huh-7 cells, these cells were treated with the proteasome inhibitor, MG132. Cellular lysates were prepared and subjected to Western blot analysis. The results show the stabilization of HIF-1α in MG132-treated cells (Fig. 1C, lane 2).

We and others have previously shown that HCV gene expression induces oxidative stress via calcium signaling (5, 11, 39). Elevated levels of reactive oxygen species (ROS) lead to the activation of cellular kinases, which then activate latent transcription factors in the cytoplasm such as NF-κB and STAT-3 (39, 42). To investigate whether HIF-1α stabilization involved oxidative stress, we treated HCV-infected cells with the antioxidant, PDTC. In antioxidant-treated cellular lysates, HIF-1α stabilization was abrogated (Fig. 1D, lane 3). HCV-infected cells treated with a calcium chelator also displayed similar abrogation of HIF-1α stabilization (Fig. 1D, lane 4). We have previously shown that HCV gene expression activates different cellular kinase pathways through oxidative stress and calcium signaling (39, 42). To test whether activation of these kinases affect the stabilization of HIF-1α, we used a series of kinase inhibitors to identify the cellular kinases responsible for HIF-1α stabilization. HCV-infected cells were treated with phosphatidylinositol 3-kinase (PI3-kinase) (LY294002), Src kinase (SU 6656), p38 mitogen-activated protein (MAP) kinase (SB203580), and MEK (PD98059) inhibitors. Both the MEK and PI3-kinase inhibitors abolished HIF-1α stabilization (Fig. 2B, lane 3). Src kinase inhibitor, on the other hand, affected the stabilization partially (Fig. 2A, lane 4), p38 MAP kinase inhibitor did not have any affect (Fig. 2A, lane 5). The effect of kinase inhibitors on viral replication and/or gene expression was previously shown to be modest (43). Together, these results indicated that cellular signaling pathways mediated the HIF-1α stabilization, which is consistent with previous observations (19, 32, 48).

HCV-activated STAT-3 and NF-κB are involved in HIF-1α stabilization. It has been previously reported that both STAT-3 and NF-κB modulate the stability and activity of HIF-1α (9, 10, 44). HCV gene expression constitutively activates STAT-3 and NF-κB via calcium signaling and oxidative stress (39, 42). To investigate whether HCV regulates HIF-1α stabilization via STAT-3 and NF-κB activation, HCV-infected cells were transiently transfected with the dominant-negative mutants of STAT-3 (Y705F and S727A) and NF-κB (IkB-
These mutants were transfected into HCV-infected Huh-7 cells, and cellular lysates were analyzed by Western blot assays. The results were uniformly negative for the stabilization of HIF-1α/HIF-2α (Fig. 2C, lanes 3, 4, 5, 6, and 7), indicating that both STAT-3 and NF-κB signaling pathways are involved in the stabilization of HIF-1α.

HCV infection stimulates VEGF promoter activity. HIF-1α is a transcription factor that binds to the HREs in the promoter region of genes involved in angiogenesis such as VEGF (31). The promoter of the human VEGF gene contains HRE, the binding sites for HIF-1α among others, including AP-1, AP-2, NF-κB, and Sp1. The luciferase reporter constructs under the control of three HRE elements or VEGF promoter sequences were transiently transfected into uninfected and HCV-infected Huh-7 cells in the presence of antioxidants or treated with various kinase inhibitors. Luciferase activity was stimulated in HCV-infected cells (Fig. 3A). PI3-kinase and MEK inhibitors reduced luciferase activity (Fig. 3A). Antioxidant-treated cells, as well as those transiently transfected with Mn-SOD (superoxide dismutase) expression vector, also displayed reduced luciferase activity (Fig. 3A). These cell-based assay results confirmed that HIF-1α stabilization was mediated via oxidative stress and subsequent activation of cellular kinase. The VEGF-promoter luciferase vector transfected into HCV-infected cells showed similar inhibition but to a lesser degree (Fig. 3B). Because VEGF promoter contains binding sites for several other transcription factors, all of which may not respond to oxidative stress, the reduction of luciferase activity was less pronounced in the HCV-infected cells transiently transfected with an Mn-SOD expression vector (Fig. 3B). MEK inhibitor (PD98059) was most effective in reducing the luciferase activity. Two additional inhibitors of COX-2 (Celecoxib and NS398) were also used in this assay. We have previously shown that COX-2-stimulated VEGF synthesis via prostaglandin E2 (PGE2) (22) and HCV gene expression also results in the stimulation of COX-2 synthesis (41). In agreement with these studies, we also observed a downregulation of VEGF promoter activity in the presence of both inhibitors of COX-2 (Fig. 3B).

![FIG. 2. HCV infection stabilizes HIF-1α through cellular kinase pathways.](http://jvi.asm.org/)

(A) Uninfected Huh-7 cells and HCV-infected Huh-7 cells were treated with DMSO (lane 2), PI3-kinase inhibitor LY294002 at 50 μM for 12 h (lane 3), Src kinase inhibitor SU6656 at 10 μM for 2 h (lane 4), or p38 MAP kinase inhibitor SB203580 at 10 μM for 15 h (lane 5). Whole-cell lysates were prepared and subjected to Western blot assays with anti-HIF-1α antibody. Actin is the protein loading control, and core is used as a representative HCV protein expressed in infected cells. (B) Uninfected and HCV-infected Huh-7 cells were treated with DMSO (lane 2) or p42/44 MAP kinase inhibitor PD98059 at 50 μM (lane 3) for 6 h. Whole-cell lysates were prepared and subjected to Western blot assays with anti-HIF-1α antibody. (C) HCV activates HIF-1α through the STAT-3 and NF-κB pathways. Uninfected and HCV-infected Huh-7 cells were transiently transfected with empty vector plasmid (lane 2), STAT-3 dominant-negative mutant Tyr705 to Phe (lane 3), STAT-3 dominant-negative mutant Ser727 to Ala (lane 4), and IκBα dominant-negative mutants Tyr 42 to Phe (lane 5), Tyr 305 to Phe (lane 6), and Ser 32,36 to Ala (lane 7). Whole-cell lysates were prepared and subjected to Western blot analysis with anti-HIF-1α antibody. Actin is the protein loading control, and core is a representative HCV protein expressed in infected cells.
HCV induces the expression and secretion of VEGF. Expression and secretion of VEGF, which acts as a paracrine growth factor of endothelial cells, are increased significantly in many cancer cell lines (7, 20). To investigate whether HCV gene expression can induce the expression and secretion of VEGF, the CM was concentrated from both uninfected and HCV-infected Huh-7 cells and analyzed under reducing conditions. Western blot assay showed spliced forms of VEGF-A in supernatants from HCV-infected cells using anti-VEGF-A monoclonal antibody (Fig. 4A, lane 2). Since Huh-7 cells were derived from an HCC, a basal level of VEGF-A secretion was observed (Fig. 4, lane 1). In the CM from the HCV-infected Huh-7 cells, however, there was a notable increase in the VEGF secretion. The intracellular levels of VEGF protein expression in the HCV-infected cells were also examined. Huh-7 and HCV-infected cellular lysates were analyzed by Western blot analysis. The results demonstrated the induction of VEGF protein (~43 kDa) in HCV-infected cells (Fig. 4B, compare lane 2 with lane 1). To determine whether HCV infection also induces the transcriptional stimulation of VEGF mRNA, total cellular RNA was extracted from Huh-7 cells and HCV-infected cells, and the level of VEGF mRNA was quantified by qRT-PCR. The results showed a modest increase in VEGF mRNA synthesis in HCV-infected cells (Fig. 4C), indicating that HCV infection contributes to the biosynthesis of VEGF mRNA.

HCV induces neovascularization in the chick CAM assay. The CAM assay is a widely used method to demonstrate angiogenesis in vivo (2). We used the CAM assay to investigate the angiogenic potential of HCV. HCV-infected cells secrete various angiogenic factors such as VEGF and other unknown factors in the CM, which can induce neovascularization. CM collected from Huh-7 and HCV-infected cells was used to demonstrate the angiogenic potential of HCV in vivo using the CAM assay. Recombinant VEGF (VEGF165) was used as a positive control, which induced strong neovascularization on the CAM surfaces (Fig. 5B), whereas PBS-treated CAM displayed this activity at negligible levels (Fig. 5A). Stimulation of angiogenesis or neovascularization is measured by the emergence of branch points from the preexisting blood vessels. CM derived from HCV-infected cells produced significantly a higher level of neovascularization than CM from uninfected control cells (Fig. 5, compare panels C and D). Since Huh-7 is a cancer cell line, it secretes basal levels of angiogenic cytokines, which induced some degree of neovascularization (Fig. 5A and C). CM derived from HCV-infected cells transfected with dominant-negative mutants of STAT-3 and NF-κB inhibited neovascularization (Fig. 5E and F). A quantitative analysis of the neovascularization is shown in Fig. 5G. These data provide in vivo evidence for the HCV-induced angiogenesis that is mediated through the activation of STAT-3 and NF-κB.

DISCUSSION

The expression of HIF-1α plays important roles in angiogenesis, tumor growth, invasion, and metastasis (24, 48). Up-regulation of HIF-1α has been observed in a broad range of cancers, including cancers of the brain, breast, cervix, oropharynx, ovary, and uterus (36, 37). HIF-1α is an essential transcription factor for principal angiogenic cytokines, including VEGF, that are required for the continued growth and survival of tumors (24, 48). Although HCV infections are strongly linked with the development of HCC, the exact mechanism(s) by which HCV induces HCC is not clearly established. The
The present study was undertaken to investigate the potential roles of HCV gene expression in inducing angiogenesis. These results demonstrate that HCV induces the stabilization of HIF-1α/HIF-1β in the human hepatoma cell line under normoxic conditions, which is mediated by oxidative stress and calcium signaling. Cellular kinases, including PI3-kinase/Akt and p42/44 MAP kinase, and subsequent activation of transcription factors such as NF-κB and STAT-3 play key roles in the induction of angiogenesis by HCV. Using the CAM assay, we provide in vivo evidence for the release of angiogenic factors by HCV-infected cells leading to neovascularization.

Our studies demonstrated that full-length HCV genome derived from HCV genotype 2a, as well as subgenomic replicon (genotype 1b), induced the stabilization of HIF-1α, suggesting that HCV nonstructural proteins are sufficient for this process. The HCV structural protein core and the nonstructural protein 5A (NS5A) have been implicated in various signal transduction pathways, but these proteins were unable to stabilize HIF-1α individually (unpublished results).

Oxidative stress is associated with nearly all pathological states, especially those involved in inflammatory processes (39, 42). A number of studies support the role of ROS in the initiation and progression of cancer (30, 34). We have previously shown that HCV gene expression elevates the levels of ROS through calcium signaling (11). ROS have been implicated in the signal transduction pathways for the induction of HIF-1α. Hypoxia elevates mitochondrial ROS at complex III, causing the accumulation of HIF-1α protein (4). Since HCV induces HIF-1α under normoxic conditions of growth, the HCV-induced ROS mimicked the hypoxic condition of cell growth. In the present study, we observed the destabilization of HIF-1α in the presence of antioxidants (NAC) or an antioxidant enzyme, Mn-SOD.

The PI3-kinase/Akt signaling pathway has been linked with oncogenesis (29). It is upregulated in diverse human cancers. The PI3-kinase/Akt pathway is also central to the regulation HIF-1α and VEGF activity (47). We have previously shown that HCV gene expression induces PI3-kinase/Akt activity (43). Our results demonstrated the potential role of HCV-induced PI3-kinase in the stabilization of HIF-1α protein and the subsequent stimulation of VEGF promoter. The functional role of p42/44 MAP kinase in the phosphorylation of HIF-1α has been established (32). Hepatitis B virus X protein upregulates HIF-1α in the Chang liver cell line, which is sensitive to the MEK inhibitor, PD98059 (46). In the case of HCV, both PI3-kinase and p42/44 MAP kinase are involved in the stabilization of HIF-1α and stimulation of VEGF synthesis. Although HCV activates the p38 MAP kinase pathway (43), this activation is not involved in HIF-1α stabilization.

Several studies have demonstrated that STAT-3 signaling...
pathways are involved in the activation of HIF-1α and VEGF expression. Previously, we have demonstrated that HCV gene expression activates STAT-3 via oxidative stress (43). In the present study, we demonstrated that HCV infection induces HIF-1α by both serine and tyrosine kinases, and blocking STAT-3 tyrosine phosphorylation reduced angiogenesis in vivo. Phosphorylation of STAT-3 is essential for VEGF induction and blocking STAT-3 completely inhibits the migration of endothelial cells and tube formation (45). Our observation is consistent with these findings. We also observed that NF-κB activation was necessary in HIF-1α stabilization (Fig. 2C) and VEGF synthesis in vitro (unpublished data) and neovascularization in vivo. We have previously shown that HCV induces Cox-2 in an NF-κB-dependent manner, which in turn stimulated the synthesis of PGE2 (41). PGE2 plays a positive role in VEGF synthesis (22). Therefore, VEGF synthesis is induced by HCV via activation of NF-κB, and this involves the stabilization of HIF-1α and the stimulation of PGE2.

In summary, our studies demonstrated the role of HCV gene expression in the induction of cellular angiogenic factors by various signal transduction pathways. HCV-induced ROS plays major roles in the induction of signal transduction pathways, such as the activation of PI3-kinase, p42/44 MAP kinase, STAT-3, and NF-κB. These, in turn, stabilize HIF-1α, which then stimulates VEGF and other important cytokines involved in angiogenesis. The fact that VEGF promoter stimulation was reduced in the presence of COX-2 inhibitors suggests that PGE2, whose secretion is induced by HCV, also plays a role in the secretion of VEGF in the extracellular media. Besides VEGF, HCV induces the secretion of other angiogenic cytokines, such as interleukin-8, and metastatic factors such as MMP-2 and MMP-9 (26, 28, 29, 44, 45). The combined action
of these factors may be involved in the neovascularization. We demonstrate here that HCV induces angiogenesis by signaling pathways that are induced by the virus. These considerations need to be taken into account for effective therapeutic strategies for HCV-induced angiogenesis in liver tumors.

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