Hepatitis C Virus Impairs p53 via Persistent Overexpression of 3β-Hydroxysterol Δ24-Reductase

Received for publication, July 10, 2009, and in revised form, October 24, 2009. Published, JBC Papers in Press, October 27, 2009, DOI 10.1074/jbc.M109.043223

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Persistent infection with hepatitis C virus (HCV) induces tumorigenicity in hepatocytes. To gain insight into the mechanisms underlying this process, we generated monoclonal antibodies for their ability to bind antigens overexpressed in hepatoblastoma-derived cell line, RzM6-LC, showing augmented tumorigenicity. We identified 3β-hydroxysterol Δ24-reductase (DHCR24) from this screen and showed that its expression reflected tumorigenicity. HCV induced the DHCR24 overexpression in human hepatocytes. Ectopic or HCV-induced DHCR24 overexpression resulted in resistance to oxidative stress-induced apoptosis and suppressed p53 activity. DHCR24 overexpression in these cells paralleled the increased interaction between p53 and MDM2 (also known as HDM2), a p53-specific E3 ubiquitin ligase, in the cytoplasm. Persistent DHCR24 overexpression did not alter the phosphorylation status of p53 but resulted in decreased acetylation of p53 at lysine residues 373 and 382 in the nucleus after treatment with hydrogen peroxide. Taken together, these results suggest that DHCR24 is elevated in response to HCV infection and inhibits the p53 stress response by stimulating the accumulation of the MDM2-p53 complex in the cytoplasm and by inhibiting the acetylation of p53 in the nucleus.

Hepatitis C virus (HCV) is composed of a single-stranded RNA genome of positive polarity (1). Translation of viral proteins is initiated from an internal ribosome entry site (2) and results in a single polypeptide that is subsequently cleaved by host and viral proteases to yield viable proteins (3). The HCV genome does not rely on canonical translation factors and can readily establish chronic infection without integrating into the host genome, resulting in hepatic steatosis and hepatocellular carcinoma (HCC) (4). More than 170 million people worldwide are infected with HCV (5); chronic HCV infection and aging are the major risk factors for HCC (6–8). Liver cancer is the fifth most common cause of cancer mortality worldwide (9). The frequent inactivation of p53 in human HCC suggests that the loss of p53-dependent apoptosis may promote hepatocarcinogenesis (10). Chronic HCV infection results in chronic liver inflammation and induces endoplasmic reticulum stress and oxidative stress, which are thought to induce hepatocarcinogenesis (11, 12). The mechanistic details underlying HCC development are not fully understood. To gain insight into the molecular mechanisms underlying HCV-induced pathogenesis, we previously established RzM6 cells (13), a human hepatoblastoma (HepG2)-derived cell line in which expression of the full-length HCV genome is controlled by a Cre/loxP system. Expression of the HCV genome promoted anchorage-independent growth of RzM6 cells after 44 days of culture from the onset of HCV expression (RzM6-44d cells) but not in RzM6 cells after 0 days (RzM6-0d cells) (13). In the present study, we generated monoclonal antibodies against RzM6 cells cultured for longer than 44 days (RzM6-LC cells) and then screened the antibodies for their ability to bind antigens overexpressed in these cells. We identified 3β-hydroxysterol Δ24-reductase (DHCR24) from this screen and characterized its role in the HCV-induced cell growth deregulation.

EXPERIMENTAL PROCEDURES

Cells, Growth Assay, and Plasmids—HepG2 human hepatoblastoma cells, HuH-7 human hepatoma cells, WRL68 human embryonic hepatic cells, HEK293 human embryonic kidney cells, and human WI-38 fibroblast cells were purchased from the American Type Culture Collection. NIH3T3 mouse fibroblast cells were from Japanese Collection of Research Bioresoruce. Cells were cultured under the growth conditions described in the supplemental material. RzM6 cells were established by
transfection of HepG2 cells with the plasmid HCR6-Rz, which contains the full-length HCV cDNA (nucleotides 1–9611; GenBank™ accession number AY045702), and stably transformed cell lines were selected in media containing G418 (800 μg/ml bioactive; Invitrogen). These cell lines, termed 2–18, were then transfected with pCAG-Mer-Cre-Mer (Cre/loxP system) and were selected in media containing puromycin (Sigma), as described previously (13), to generate the RZm6 cell line. HCV expression was induced by treatment with 4-hydroxy-tamoxifen (100 nM). Cells expressing HCV for 44 days (RZm6-44d) displayed augmented anchorage-independent cell growth. Cells expressing HCV for more than 44 days are referred to as RZm6-LC cells.

The tumor formation assay was performed by injecting RZm6-0d, RZm6-44d, or RZm6-LC cells in the exponential growth phase into nude mice. Cells in culture were harvested with trypsin, and 2 × 10^6 or 1 × 10^7 cells were subcutaneously injected into the backs of athymic nude mice (iCR strain, Charles River). HepG2 and WRL68 cells with plasmid DNA or small interfering RNA (siRNA) were transiently transfected using Lipofectamine 2000. Amplified DHCR24 cDNA was also subcloned into the pcDNA3.1 vector containing an HA or FLAG tag (kindly supplied by Dr. N. Takahashi, Tokyo University, Japan), was modified by replacing the green fluorescent protein gene with the hygromycin phosphotransferase gene to construct pCSII-EF-MCS-EMCV IRES-Hygro. DHCR24 fused to the 5′-HA or 5′-FLAG tag-encoding sequence were cloned into the pGEM-T easy vector (Promega). In vitro translation was performed using TNT(R) reticulocyte lysate (Promega) and the Express Protein Labeling Mix (New England Nuclear) in the presence of either [35S]Met/Cys or non-radioactive methionine. Amplified DHCR24 cDNA was digested with Xhol and subcloned into the pcAG-PURO vector (16) for transfection into WRL68 cells using Lipofectamine 2000. Amplified DHCR24 cDNA was also subcloned into the pcDNA3.1 vector containing an HA or FLAG tag (kindly supplied by Dr. N. Takahashi, Tokyo University of Agriculture and Technology) for transfection into RZm6 or HepG2 cells using Lipofectamine LTX (Invitrogen). Transfected cells were selected in media containing G418.

The lentiviral vector, pCSII-EF-MCS-EMCV IRES-GFP (generous gift from Hiroyuki Miyoshi, RIKEN, Tsukuba, Japan), was modified by replacing the green fluorescent protein gene with the hygromycin phosphotransferase gene to construct pCSII-EF-MCS-EMCV IRES-Hygro. DHCR24 fused to the 5′-HA or 5′-FLAG tag-encoding sequence were cloned under the EF promoter. The resulting plasmid was cotransfected with packaging plasmid (pCAG-HIVgvp and pCMV-VSVG-RSV-Rev) in 293FT cells (Invitrogen) to produce recombinant lentivirus. Following infection, cells were selected with hygromycin B (600 μg/ml; Sigma).
Impairment of p53 by HCV through DHCR24 Overexpression

Silencing of DHCR24 and HCV by siRNA—The DHCR24 stealth siRNA was designed to target the human DHCR24 mRNA sequence 5′-GCAAGCUGAUAGCUGGCUAU-3′ (nucleotides 970–993) using the BLOCK-IT RNAi designer (Invitrogen). A mutated siRNA (5′-GAGUCUACAGAUACGGAAGUUA-3′) was synthesized as a control. An alternative siRNA, siDHCR24-1024, was designed as 5′-GAGAACUAUCUGAAAGCAATT-3′. The HCV siRNA was synthesized as previously described (17). Cells were transfected with a 1 nM concentration of the chemically synthesized siRNAs using Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen) and then incubated for 4–6 h at 37 °C. Cells were characterized 48 h after transfection.

Caspase and Reporter Assays—Cells (1 × 10⁶ cells/well) were seeded into white 96-well plates (Sumitomo Bakelite), treated with 1 mM H₂O₂, and then lysed with caspase-Glo 3/7 luminescent substrate containing the sequence DEVD (Promega). Caspase 3/7 activity was determined by measuring the absorbance resulting from cleavage of a polyclonal substrate containing the sequence DEVD (Promega) using a multilabel counter (PerkinElmer Life Sciences). The p21(−)/p21+ promoter activity was assayed in HepG2, RzM6-0d, or RzM6-LC cells transfected with pWWP-Luc (kindly supplied by Dr. Bert Vogelstein (The Johns Hopkins University)). Cells were cotransfected with phRL-TK(Int-) (Promega) for normalization of promoter activity. Cells were incubated for 2 days after transfection and were then treated with 1 mM H₂O₂ for 4 h. Promoter activity was measured using the Dual-Luciferase reporter assay system (Promega).

HCV Infection of Humanized Chimeric Mouse Liver and mRNA Quantification by Quantitative Reverse Transcription-PCR—Detailed procedures are described in the supplemental material.

Statistical Analysis—Student’s t test was used to test the statistical significance of the results. p values less than 0.05 were considered statistically significant.

RESULTS

Expression of DHCR24 Parallels Hepatocarcinogenesis—RzM6 cells expressing full-length HCV were established using the Cre/loxP expression-switching system (13). RzM6 cells cultured for longer than 44 days (termed RzM6-LC) had a greater ability to form colonies (13) and to induce tumors in nude mice (Fig. 1A). We produced monoclonal antibodies against RzM6-LC cells (see supplemental materials) and screened them for their ability to bind RzM6 antigens overexpressed upon the onset of hepatocarcinogenesis. Antibody clone 2-152a bound to a ~60-kDa protein (p60) that was expressed at higher levels in RzM6-LC cells than in RzM6 cells before the onset of HCV expression (termed RzM6-0d) (13) (Fig. 1B). p60 was strongly expressed in hepatoma-derived HuH-7 cells but was less abundant in the less aggressive cancer cell line, HepG2, or in the normal embryonic cell lines WRL68, HEK293, or NIH3T3 (supplemental Fig. 1A). To identify p60, the protein was purified from RzM6-LC cells using immunoaffinity chromatography and subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (supplemental Fig. 1B). Through this process, p60 was identified as DHCR24 (also known as seladin-1) (18–20), an enzyme that catalyzes the reduction of the Δ-24 double bond of sterol intermediates during cholesterol biosynthesis and is up-regulated by oxidative stress (19, 21). DHCR24 cDNA was cloned and translated in vitro (Fig. 1C) and also expressed in human embryonic hepatic WRL68 cells (Fig. 1D), and the monoclonal antibody 2-152a (green) or anti-protein-disulfide isomerase antibody (PDI; red). Scale bars, 25 nm.

![Figure 1](image1)

**FIGURE 1.** Analysis of RzM6 cell tumorigenicity and identification of DHCR24 overexpression in RzM6-LC cells. A, summary of tumor formation in nude mice injected with RzM6-0d or RzM6-LC cells. B, detection of a ~60-kDa protein (arrow, upper panel) in whole-cell lysates (30 μg/lane) from RzM6-0d and RzM6-LC cells by Western blotting with monoclonal antibody 2-152a. Anti-actin antibody was used for normalization (arrow, lower panel). Data are representative of two independent experiments. C, empty vector (pGEM3, left lane) or pGEM-DHCR24 (right lane) was subjected to in vitro transcription/translation using rabbit reticulocyte lysates in the presence of [³⁵S]methionine. Samples were subjected to SDS-PAGE followed by autoradiography. D, cell lysates from untransfected WRL68 cells (mon), WRL68 cells transfected with empty pCAG vector (vector), and WRL68 cells transfected with pCAG-DHCR24 vector were examined by Western blotting with monoclonal antibody 2-152a (upper panel) or monoclonal anti-actin antibody (lower panel). E, WRL68 cells were transfected with pCAG-DHCR24 or pCAG vector alone and subjected to immunocytochemistry with monoclonal antibody 2-152a (green) or anti-protein-disulfide isomerase antibody (PDI; red). Scale bars, 25 nm.
protein and mRNA in a panel of hepatic and embryonic cell lines (Fig. 2A). Northern blotting revealed that DHCR24 mRNA expression was notably higher in RzM6-LC cells than in RzM6-0d cells, indicating that induction of DHCR24 occurs at the transcriptional level. DHCR24 protein levels were also higher in HuH-7 and RzM6-LC cells relative to RzM6-0d cells (Fig. 2A). To examine whether persistent up-regulation of DHCR24 in RzM6-LC cells resulted from HCV expression, we utilized an siRNA to knockdown HCV expression (17) (Fig. 2B). Silencing HCV by greater than 99% with siRNA reduced the expression of DHCR24 and p53 in Rzm6-LC cells. When we induced HCV expression with tamoxifen, the induction of DHCR24 was observed after 2, 4, and 6 days (Fig. 2C). These results indicate that expression of the full-length HCV genome induced DHCR24 overexpression. DHCR24 was not induced in HuH-7 cells infected with the HCV strain JFH-1 (22) (data not shown). This result might be explained by the substantial endogenous expression of DHCR24 in HuH-7 cells (Fig. 2A and supplemental Fig. 1A). To examine whether HCV infection can induce DHCR24, human hepatocytes in chimeric mice were infected with hepatitis B virus (HBV) or HCV (Fig. 2, D and E). Notable up-regulation of DHCR24 mRNA was detected in HCV-infected human hepatocytes but not in HBV-infected human hepatocytes (Fig. 2F).

Persistent Overexpression of DHCR24 Induces Apoptotic Resistance to Oxidative Stress—As HCV infection increased the expression of DHCR24, we further examined the effect of DHCR24 on hepatocytes. Because DHCR24 regulates oxidative stress-induced apoptosis (19, 21, 23, 24), the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay was performed with RzM6 cells to examine the effect of DHCR24 overexpression on H2O2-induced apoptosis (Fig. 3A). Fragmentation of genomic DNA was less pronounced in DHCR24-overexpressing cells (RzM6-LC cells and RzM6-0d cells transduced with DHCR24 lentivirus) than in RzM6-0d cells or RzM6-0d cells transduced with empty lentiviral vector. To quantify the apoptotic response, we examined the effect of DHCR24 overexpression on caspase activity (Fig. 3, B–D). Caspase activation by H2O2 was suppressed in Rzm6-LC cells compared with Rzm6-0d cells (Fig. 3B). Transfection with HCV siRNA recovered the caspase response in Rzm6-LC cells. Caspase 3/7 activity was also examined following the transfection of HepG2 cells with pCA-Rz (Fig. 3C). Induction of caspase activation by H2O2 was inhibited by expression of the HCV gene; the inhibition was partially recovered by transfection with DHCR24
Impairment of p53 by HCV through DHCR24 Overexpression

A

RzM6-0d

H₂O₂ -  H₂O₂ +

Phase

RzM6-0d lenti-empty

H₂O₂ -  H₂O₂ +

Phase

TUNEL

RzM6-0d DHCR24-lenti

H₂O₂ -  H₂O₂ +

TUNEL

B

RzM6-0d  RzM6-LC

C

D

RzM6-0d

H₂O₂ -  H₂O₂ +

HCV siRNA  control siRNA

vector  pCA-Rz  pcDNA-DHCR24  DHCR24 siRNA  control siRNA

Caspase 3/7 activity

Caspase 3/7 activity

Caspase 3/7 activity
Persistent Overexpression of DHCR24 Inhibits p53 Activity—H$_2$O$_2$ induces p53-dependent apoptosis (26). Expression of the p53-induced apoptotic response mediators, Bax (27) and Puma (28), did not increase after H$_2$O$_2$ treatment of RzM6-LC cells (Fig. 4A). Therefore, we examined p53 expression and function in DHCR24-overexpressing cells. H$_2$O$_2$ increased the expression of DHCR24 and p53 in RzM6-0d cells. By comparison, the expression of these proteins was already elevated prior to H$_2$O$_2$ treatment in RzM6-LC cells, and we found no further H$_2$O$_2$-induced increase in expression (Fig. 4A). Consistent with these findings, H$_2$O$_2$ activated transcription from the p21$^{WAF1/CIP}$ promoter in RzM6-0d cells but not in RzM6-LC cells (Fig. 4B). This response of the p21$^{WAF1/CIP}$ promoter is impaired in cells overexpressing DHCR24 via the lentivirus vector (Fig. 4, C and D) or via the expression vector (Fig. 4E). Induction of Bax and Puma expression after H$_2$O$_2$ treatment was decreased in DHCR24-overexpressing cells, the function of p53 in the oxidative stress pathway is impaired.

Overexpression of DHCR24 Enhances the Interaction between p53 and MDM2—Since DHCR24 is a regulator of the p53-MDM2 interaction (21), we examined the interaction between p53 and its specific ubiquitin ligase, MDM2. Unexpectedly, the interaction between p53 and MDM2 was stronger in RzM6-LC cells than in RzM6-0d cells (Fig. 5, A and B). Lentiviral vector overexpression of DHCR24 in RzM6-0d cells increased the binding of p53 to MDM2 (data not shown). Furthermore, cell fractionation analysis revealed that the interaction between MDM2 and p53 mostly occurred in the cytoplasmic fraction, even after H$_2$O$_2$ treatment of RzM6-LC cells (Fig. 5, C and D). Consistent with these findings, H$_2$O$_2$ activated transcription from the p21$^{WAF1/CIP}$ promoter in RzM6-0d cells but not in RzM6-LC cells (Fig. 4B). This response of the p21$^{WAF1/CIP}$ promoter is impaired in cells overexpressing DHCR24 via the lentivirus vector (Fig. 4, C and D) or via the expression vector (Fig. 4E). Induction of Bax and Puma expression after H$_2$O$_2$ treatment was decreased in DHCR24-overexpressing cells, the function of p53 in the oxidative stress pathway is impaired.

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FIGURE 5. DHCR24 overexpression enhances the interaction between p53 and MDM2 in the cytoplasm. A, p53 was immunoprecipitated (IP) from RzM6-0d and RzM6-LC cells with polyclonal anti-p53 antibody (FL393) followed by Western blotting with monoclonal antibodies against MDM2 (top) and p53 or DHCR24 (bottom). B, MDM2 was immunoprecipitated from RzM6-0d and RzM6-LC cells using polyclonal anti-MDM2 antibody (H221) followed by Western blotting (WB) with monoclonal antibodies against p53 (top) and MDM2 or DHCR24 (bottom). C, p53 was immunoprecipitated from cytoplasmic (C) or nuclear (N) fractions of RzM6-0d and LC cells using anti-p53 antibody (FL393) followed by Western blotting with anti-MDM2 antibody (top) and anti-p53 antibody (bottom). Cell fractionation was confirmed by Western blotting with anti-histone H1 and tubulin (WB). D, p53 was immunoprecipitated from cytoplasmic or nuclear fractions of HepG2 cells transfected with control pcDNA (vec) or pcDNA-DHCR24 expression vector using polyclonal anti-p53 (FL393) followed by Western blotting with antibodies against the proteins indicated at the right. Reaction with secondary antibodies (anti-rabbit or mouse IgG conjugated with horseradish peroxidase) alone did not show any signal (data not shown), and data representative of three independent experiments are shown (A–D and G). E, RzM6-0d or RzM6-LC cells with H2O2 treatment were fractionated into total (T), cytoplasmic, and nuclear fractions and subjected to Western blotting with the antibodies indicated on the left. F, Western blotting of WI38 cells with or without H2O2 treatment (0.5 mM, 4 h) with antibodies against p53, DHCR24, and actin. G, immunoprecipitation of p53 from cells transfected with pcDNA vector and pcDNA-DHCR24 (DHCR) with or without H2O2 treatment (0.5 mM, 4 h) followed by Western blotting with antibodies against MDM2 (first column) and p53 (second column). Cells were examined with Western blotting with anti-DHCR24 (third column) and anti-actin (fourth column). H, the average ratio of the quantified results of immunoprecipitation of p53 and Western blotting of MDM2 in RzM6-0d, RzM6-LC, and WI38 cells with transfection of pcDNA vector or pcDNA-DHCR24 with or without H2O2 treatment are indicated. Vertical bars, S.D. *, p < 0.05 (two-tailed Student’s t test). I, a caspase 3/7 assay was performed in WI38 cells with pcDNA vector (vector) and pcDNA-DHCR24 overexpression vector. Vertical bars, S.D. *, p < 0.05 (two-tailed Student’s t test).
### Impairment of p53 by HCV through DHCR24 Overexpression

#### A

|        | RzM6-0d | RzM6-LC |
|--------|---------|---------|
| H\textsubscript{2}O\textsubscript{2} | 0 \textsuperscript{1} 0.5 1.0 | 0 \textsuperscript{1} 0.5 1.0 |
| Ser\textsuperscript{15} | | |
| p53 | | |
| actin | | |
| ratio p.-p53/p53 | 1.0 5.0 3.3 | 0.9 5.0 3.4 |

#### B

|        | RzM6-0d | RzM6-LC |
|--------|---------|---------|
| H\textsubscript{2}O\textsubscript{2} | - + | - + |
| Ser\textsuperscript{46} | | |
| Ser\textsuperscript{392} | | |
| p53 | | |
| actin | | |

#### C

|        | RzM6-0d | RzM6-LC |
|--------|---------|---------|
| Ser\textsuperscript{15} | C N | C N |
| p53 | | |
| Histone H1 | | |
| Tubulin | | |
| H\textsubscript{2}O\textsubscript{2} | - - + + - - + + |

#### D

|        | RzM6-0d | RzM6-LC | HepG2 vec. | DHCR24 |
|--------|---------|---------|------------|---------|
| H\textsubscript{2}O\textsubscript{2} | 0 d | LC | - | + |
| IP:p53 | | | | |
| WB:Ac-Lys\textsuperscript{373, 382} | | | | |
| IP: p53 | | | | |
| WB:p53 | | | | |
| WB:core | | | | |
| WB:Histone H1 | | | | |
| WB:Tubulin | | | | |
| Ratio | 1.0 12.4 2.2 2.5 | 1.2 7.3 0.4 2.3 |

#### E

|        | RzM6-0d | RzM6-LC |
|--------|---------|---------|
| IP:p53 | C N | C N |
| WB:Ac-Lys\textsuperscript{373, 382} | | |
| IP: p53 | | |
| WB:p53 | | |
| WB:core | | |
| WB:Histone H1 | | |
| WB:Tubulin | | |
| H\textsubscript{2}O\textsubscript{2} | - - + + - - + + |

#### F

|        | HepG2 vec. | DHCR24 |
|--------|------------|---------|
| IP:p53 | C N | C N |
| WB:Ac-Lys\textsuperscript{373, 382} | | |
| IP: p53 | | |
| WB:p53 | | |
| WB:Histone H1 | | |
| WB:Tubulin | | |
| H\textsubscript{2}O\textsubscript{2} | - - + + - - + + | - - + + - - + + |
Impairment of p53 by HCV through DHCR24 Overexpression

A

|          | RzM6-0d | RzM6-LC |
|----------|---------|---------|
| IP:p53   | non     | cont.   |
| WB:Ac-Lys<sup>373</sup>, 382 | siRNA    | siRNA    |
| WB:p53   |         |         |
| WB:DHCR24 |         |         |
| WB:actin |         |         |

B

|          | RzM6-0d | RzM6-LC |
|----------|---------|---------|
| IP:p53   | non     | cont.   |
| WB:Ac-Lys<sup>373</sup>, 382 | siRNA    | siRNA    |
| WB:p53   |         |         |
| WB:DHCR24 |         |         |
| WB:actin |         |         |

FIGURE 7. Silencing of DHCR24 increases p53 acetylation. A, RzM6-0d and RzM6-LC cells were untreated (non) or treated with control siRNA (cont. siRNA) or DHCR24 siRNA. Acetylated p53 Lys<sup>373</sup> and Lys<sup>382</sup> (top) and total p53 (bottom) were immunoprecipitated (IP) with anti-p53 antibody (DO-1) followed by Western blotting (WB), and the expression of actin and DHCR24 by Western blotting (bottom) was examined. The average ratio of acetylated p53 to total p53 in RzM6-0d cells without treatment is indicated at the bottom. B, RzM6-0d cells were transfected with control siRNA (cont. siRNA), DHCR24 siRNA (DH1024 or DH283), or untreated (non). Acetylated p53 Lys<sup>373</sup> and Lys<sup>382</sup> (top) and total p53 (bottom) were immunoprecipitated with anti-p53 antibody followed by Western blotting, and the expression of actin and DHCR24 by Western blotting (bottom) was examined. Reaction with secondary antibodies (anti-rabbit or mouse IgG conjugated with horseradish peroxidase) alone did not yield any signals (data not shown), and data representative of three independent experiments are shown (A and B).

A previous in vitro study that used bacterially expressed and purified protein (21), the interaction between p53 and MDM2 may be due to the different regulatory systems of p53 and MDM2. The expression of DHCR24 in HepG2 cells decreased the interaction between p53 and MDM2 (supplemental Fig. 2A). Overexpression of DHCR24 in HepG2 cells increased the interaction between p53 and MDM2 in the cytoplasm (HepG2-DHCR24; Fig. 5D and supplemental Fig. 2, B and C). Level of p53 in the nucleus after treatment with H<sub>2</sub>O<sub>2</sub> was low in RzM6-LC cells (Fig. 5E).

In a previous in vitro study that used bacterially expressed and purified protein (21), the interaction between p53 and MDM2 was decreased when the amount of DHCR24 was increased. These discrepancies with our results may be due to the different experimental systems. p53 and MDM2 were induced by H<sub>2</sub>O<sub>2</sub> in WI38TERT cells (21), as observed in our system (Fig. 5F). However, the interaction between MDM2 and p53 was much lower in WI38 cells than in RzM6 cells (Fig. 5G and H). Moreover, ectopic expression of DHCR24 did not inhibit apoptotic stress to H<sub>2</sub>O<sub>2</sub> in WI38 cells (Fig. 5I) but suppressed apoptosis in HepG2 cells (Fig. 3). This different response may be due to the different regulatory systems of p53 and MDM2 in the liver and lung; MDM2 phosphorylation at Ser<sup>166</sup> is regulated by the MEK-ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) pathway in hepatocytes and Akt in lung cells (29). The response of MDM2 at Ser<sup>166</sup> to H<sub>2</sub>O<sub>2</sub> was significantly high in liver (HepG2) cells but was low in WI38 cells (supplemental Fig. 2, D and E), as previously observed in A549 cells (29). The up-regulation of MDM2 phosphorylation at Ser<sup>166</sup> accelerates its E3 ligase activity (30).

We also found that overexpression of DHCR24 did not up-regulate the transcription of p53 or MDM2 genes (supplemental Fig. 3, A and B). However, DHCR24 overexpression inhibited polyubiquitination in RzM6-LC cells and H358 (p53 null) cells (supplemental Fig. 3, C–E). This inhibition of polyubiquitination would inhibit p53 degradation, resulting in an increased amount of p53 available to interact with MDM2.

Posttranslational Modification of p53 in DHCR24-overexpressing Cells—We did not detect any p53 nucleotide substitutions in RzM6-LC cells compared with the p53 in RzM6-0d cells (data not shown). Thus, we examined the posttranslational modification of p53, which is thought to be tightly connected to the regulation of its function and localization (31). We examined the phosphorylation of p53 at Ser<sup>15</sup> (Fig. 6A); at Ser<sup>46</sup>, Ser<sup>9</sup>, Ser<sup>20</sup> and Ser<sup>37</sup> (supplemental Fig. 4A); and at Ser<sup>146</sup> and Ser<sup>392</sup> (Fig. 6B) by Western blotting and found no marked differences in phosphorylation after H<sub>2</sub>O<sub>2</sub> treatment between RzM6-0d and RzM6-LC cells. The phosphorylation of p53 at Ser<sup>15</sup>, Ser<sup>46</sup>, Ser<sup>9</sup>, Ser<sup>20</sup> and Ser<sup>37</sup> (supplemental Fig. 4B) was examined. The average ratio of acetylated p53 to total p53 at Lys<sup>373</sup> and Lys<sup>382</sup> by immunoprecipitation followed by Western blotting and found that acetylation of p53 was significantly low in RzM6-LC cells compared with RzM6-0d cells following H<sub>2</sub>O<sub>2</sub> treatment (Fig. 6C). We assessed the acetylation of p53 at Lys<sup>373</sup> and Lys<sup>382</sup> by immunoprecipitation followed by Western blotting and found that acetylation of p53 was significantly low in RzM6-LC cells compared with RzM6-0d cells following H<sub>2</sub>O<sub>2</sub> treatment (Fig. 6D). H<sub>2</sub>O<sub>2</sub> induced the acetylation of p53 at Lys<sup>373</sup> and Lys<sup>382</sup> in RzM6-0d cells (Fig. 6E). This acetylation

FIGURE 6. Posttranslational modification of p53 in cells overexpressing DHCR24. A, phosphorylation of p53 at Ser<sup>15</sup> was examined in RzM6-0d and RzM6-LC cells after treatment with 0, 0.5, or 1.0 mM H<sub>2</sub>O<sub>2</sub> by Western blotting with specific rabbit polyclonal antibodies. p53 was detected with anti-p53 monoclonal antibody (DO-1). Actin was analyzed as a control. B, phosphorylation of p53 at Ser<sup>46</sup> and Ser<sup>392</sup> in RzM6-0d and RzM6-LC cells after treatment with 0 or 1.0 mM H<sub>2</sub>O<sub>2</sub> was analyzed by Western blotting with specific rabbit polyclonal antibodies. p53 was detected with anti-p53 monoclonal antibody. Actin was analyzed as a control. C, RzM6-0d or RzM6-LC cells with or without H<sub>2</sub>O<sub>2</sub> treatment were fractionated into cytoplasmic (C) and nuclear (N) fractions that were subjected to Western blotting with the antibodies indicated on the left. D, acetylation of p53 Lys<sup>373</sup> and Lys<sup>382</sup> (top) and total p53 (bottom) was characterized by immunoprecipitation (IP) with anti-p53 antibody followed by Western blotting (WB) with the rabbit polyclonal antibodies indicated on the left in RzM6-0d and RzM6-LC cells or HepG2 cells transfected with pcDNA vector (vec.) or pcDNA-DHCR24 expression vector (DHCR24). The average ratio of acetylated p53 to total p53 in RzM6-0d cells without H<sub>2</sub>O<sub>2</sub> treatment is indicated at the bottom. E, acetylation of p53 Lys<sup>373</sup> and Lys<sup>382</sup> (top) and total p53 (second panel) was characterized by immunoprecipitation with anti-p53 antibody followed by Western blotting with the rabbit polyclonal antibodies indicated on the left in RzM6-0d and RzM6-LC cells. HCV core protein was detected by Western blotting with monoclonal antibody (31, 32). Cell fractionation was confirmed with antibodies against histone H1 and tubulin, p53 and acetylated p53 were quantitated, and the average ratio of acetylated p53 to total p53 in the cytoplasmic (C) fraction of RzM6-0d cells is indicated. Vertical bars, S.D. *, p < 0.05 (two-tailed Student’s t test), F, acetylation of p53 Lys<sup>373</sup> and Lys<sup>382</sup> (top) and total p53 (second panel) was characterized by IP with anti-p53 antibody followed by Western blotting with the rabbit polyclonal antibodies indicated on the left in HepG2 cells transfected with pcDNA vector or pcDNA-DHCR24 vector with or without H<sub>2</sub>O<sub>2</sub> treatment. Cell fractionation was confirmed with antibodies against histone H1 and tubulin, p53 and acetylated p53 were quantitated, and the average ratio of acetylated p53 to total p53 in the cytoplasmic fraction of Hep-vec cells is indicated. Vertical bars, S.D. *, p < 0.05 (two-tailed Student’s t test). Reaction with secondary antibodies (anti-rabbit or mouse IgG conjugated with horseradish peroxidase) alone did not show any signals (data not shown), and data representative of three independent experiments are indicated (A–F).
was significantly inhibited in RzM6-LC cells (Fig. 6E). Persistent ectopic overexpression of DHCR24 also repressed H$_2$O$_2$-induced p53 acetylation in the nuclei of HepG2 cells (Fig. 6F). In addition, silencing of DHCR24 by siRNA caused up-regulation of p53 acetylation at Lys$^{373}$ and Lys$^{382}$ in RzM6-0d and RzM6-LC cells (Fig. 7A). When the alternative siRNA for DHCR24 (DH1024) was transfected into RzM6-0d cells, the acetylation of p53 was accelerated (Fig. 7B). The mutant DHCR24 vector suppressed the effect of DHCR24 siRNA (supplemental Fig. 4B). These results indicate that overexpression of DHCR24 down-regulates p53 acetylation.

DISCUSSION

This study demonstrates that DHCR24 expression parallels hepatocarcinogenesis and that HCV induces overexpression of DHCR24 at both the mRNA and protein levels. Moreover, persistent DHCR24 overexpression suppresses the p53 response to H$_2$O$_2$. These findings are consistent with the previous report that inactivation and mutation of p53 plays a role in the development of HCC (32). Hepatocytes with p53 abnormalities are likely to escape from cell cycle check points and acquire resistance to apoptosis, thereby increasing their tumorigenic potential. Likewise, genetic inactivation of p53 is associated with late stage disease (32). HCV RNA levels are notably lower in cancerous tissues (33). Thus, impairment of p53 function by overexpression of DHCR24 overexpression might play a crucial role in early stage disease progression.

In DHCR24-overexpressing cells, p53 was mostly distributed in the cytoplasm (supplemental Fig. 2, A and B). This change in the distribution pattern of p53 might be caused by an increased interaction between p53 and MDM2, which might be induced by increased phosphorylation of MDM2 and suppression of polyubiquitination by overexpression of DHCR24. The up-regulation of the p53-MDM2 interaction negatively regulates p53 and shuttles p53 from the nucleus to the cytoplasm (34, 35). MDM2 inhibits both p53 transcriptional activity and p300-mediated p53 acetylation upon ternary complex formation with p300 and p53 (36–38). Acetylation of p53 by CBP/p300 mostly occurs in the nucleus (36). Therefore, the increase in cytoplasmic p53-MDM2 complexes in DHCR24-overexpressing cells may account for the observed suppression of p53 acetylation in the nucleus, even after treatment with H$_2$O$_2$ (supplemental Fig. 5). Impaired acetylation of C-terminal lysine residues decreases the sequence-specific DNA-binding activity (39) and the stability of p53 (37, 40). Taken together, our data suggest that DHCR24 overexpression may down-regulate p53 function by inhibiting degradation, increasing the formation of the p53-MDM2 complex in the cytoplasm, and suppressing acetylation of p53 in the nucleus.

In conclusion, we propose that HCV infection impairs the function of p53 through DHCR24 overexpression, which up-regulates the interaction between p53 and MDM2 in the cytoplasm and suppresses p53 acetylation in the nucleus. Because overexpression of DHCR24 is observed in other cancers, including melanoma (24) and prostate cancer (41), the findings from this study might provide a foundation for investigations into the mechanisms underlying the formation of these cancers.

We plan to examine the liver-specific regulatory network of the p53-MDM2 interaction by DHCR24 in a future study.

ACKNOWLEDGMENTS—We thank S. Imajoh-Ohmi, H. Fukuda, T. Watanabe, S. Nakagawa, K. Tanaka, and R. Takehara for technical support and N. Sonenberg, Y. Furutachi, T. Tsukiyama, S. Tone, S. Sekiguchi, and F. Yasui for valuable comments. We thank S. Harada (Kumamoto University) for kind encouragement.

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