HIV and HCV Cooperatively Promote Hepatic Fibrogenesis via Induction of Reactive Oxygen Species and NFκB

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HIV/HCV coinfection leads to accelerated hepatic fibrosis progression, with higher rates of cirrhosis, liver failure, and liver death than does HCV mono-infection. However, the profibrogenic role of HIV on hepatocytes and hepatic stellate cells (HSC) has not been fully clarified. We hypothesized that HIV, HCV independently regulate hepatic fibrosis progression, with higher rates of cirrhosis, liver failure, and death compared with patients with HCV mono-infection, even despite successful control of HIV with antiretroviral therapy (9).

HIV/HCV coinfection induces a significant increase in TGF-β1, a central mediator of liver fibrogenesis, in the liver and serum of patient and in cell culture (4, 8, 10–12). We have demonstrated that HCV increases hepatocytes TGF-β1 expression in HCV replicon cells (13), and that this occurs through the enhanced production of reactive oxygen species (ROS) in HCV JFH1 cell culture (6, 14). Liver fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) components, reduction of ECM-removing matrix metalloproteinases (MMPs), and an up-regulation of tissue inhibitors of MMPs (TIMPs), mainly TIMP-1 (15). However, both HIV and its envelope gp120 protein have been demonstrated to induce cell signaling within hepatocytes, hepatic stellate cells (HSC) and other immune cells through its interaction with CCR5 or CXCR4 chemokine receptors (4, 6–8). Of note, it has been demonstrated that HIV/HCV coinfection leads to accelerated hepatic fibrosis progression, higher rates of liver failure and death compared with patients with HCV mono-infection, even despite successful control of HIV with antiretroviral therapy (9).

Hepatitis C virus (HCV)3 is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. HCV and human immunodeficiency virus (HIV) infect ~180 million and 40 million people, respectively, worldwide. Among these, 5 million persons are coinfected with HIV/HCV(1–4). HCV does not have a major impact on HIV disease progression, whereas HIV accelerates HCV-related liver disease (3, 5). HIV infects human CD4 T lymphocytes, macrophages, and dendritic cells, but does not replicate in human hepatocytes. However, both HIV and its envelope gp120 protein have been demonstrated to induce cell signaling within hepatocytes, hepatic stellate cells (HSC) and other immune cells through its interaction with CCR5 or CXCR4 chemokine receptors (4, 6–8). Of note, it has been demonstrated that HIV/HCV coinfection leads to accelerated hepatic fibrosis progression, higher rates of liver failure and death compared with patients with HCV mono-infection, even despite successful control of HIV with antiretroviral therapy (9).

EXPERIMENTAL PROCEDURES

Cell Cultures—Huh7.5.1 human hepatoma cells (16), HCV JFH1-infected Huh7.5.1 cells (17), and HSC LX-2 (18) cells
Table 1. List of primer sequences for real time PCR

| Gene name          | Forward primer | Reverse primer |
|--------------------|----------------|----------------|
| Human GAPDH        | ACACTTCATCGCCCTCCTGCC | GGCCTGCTGTACACCTCTGTC |
| JFH1-HCV           | TCTTGCTGACACCCTAG | TCCGGCA GTTACCACAAGGC |
| Human TIMP-1       | GTGGTTGCTGCTGTCATGAC | TGCGTTGCTCCCCAGACATT |
| Human procollagen α1(I) | CAGGCCCTCTCACTCALC | TAATCACCTGTTGCCGCC |
| Human MMP-3        | GTCCGGCTGTTCTGAGTGA | GGGGACGTTTCCGGAGTA |

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were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) medium. The JFH1 cells used in this study were harvested at between days 4–20 post-infection. To monitor the effect of inactivated HIV on TIMP1 and MMP3 protein expression in culture supernatant, cells were cultured in UltraCulture Serum-Free Medium (BioWhittaker, Walkersville, MD) supplemental with 2 mM glutamine (Mediatech, Inc. Herndon, VA).

HIV Stocks—Inactivated HIV supernatant was produced as previously described (8). HIV and control supernatant were heat-inactivated at 56 °C for 30 min. Viral stock concentrations were measured for HIV-1 p24 using the Alliance p24 Antigen ELISA kit (PerkinElmer, Waltham, MA). HIV p24 concentrations are 65 ng/ml for NL4–3 CXCR4-tropic HIV (X4 HIV); and 80 ng/ml for BaL CCR5-tropic HIV (R5 HIV). The cells were incubated in UltraCulture serum-free medium with or without HIV stock supernatant (1:10 diluted for final concentration) for 24 h.

JFH1 HCV—JFH1 RNA was generated and transfected into Huh7.5.1 cells by electroporation as previously reported (16, 17). The JFH1-infected cells used in this study were between day 4 and 20 post-infection.

Protein Sample Preparation—Protein samples for ELISA or Western blot were prepared as previously described (19, 20).

Western Blot—Western blot was performed as previously reported (19, 20). The primary antibodies used for the Western blots included mouse anti-actin (Sigma Life Science and Biologicals), and phosphorylated and unphosphorylated NFkB (p65) (Cell Signaling Technology, Inc., Danvers, MA).

ELISA—HCV core concentration was measured by Ortho HCV core antigen ELISA (Ortho Diagnostics Systems, Raritan, NJ) and quantified by qPCR as previously described (19). The primer sequences are listed in Table 1. Each gene mRNA level was normalized to GAPDH to calculate a fibrosis gene/GAPDH arbitrary unit (fold).

ROS Measurements and Cell Viability Assay—Cells (10,000 cell/well) were seeded in 100 μl of 10% FBS DMEM in 96-well clear bottom white assay plates overnight (14 h). Huh7.5.1 or JFH1 cells were incubated with 10 μl of heat-inactivated HIV supernatant for 24 h. LX2 cells were incubated with 10 μl of heat-inactivated HIV supernatant and 10 μl of JFH1 supernatant for 24 h. The negative control supernatant was obtained from Huh7.5.1 cells grown for 3 days in 10% FBS DMEM (DMEM). Diphenyliodonium (DPI) (ROS inhibitor) (20 μM final concentration) (EMD Chemicals, Inc., Gibbstown, NJ) was added to the appropriate wells for 3 h. ROS levels were measured as previously described (23–25). Briefly, the cells were incubated with 10 μM carboxy-derivative of fluorescein (carboxy-H2DCFDA) (ROS dye) (Invitrogen, Carlsbad, CA) in warm PBS for 1 h according to the manufacturer’s protocol. ROS levels were assessed through the measurement of fluorescence at an excitation of 485 nm and an emission of 528
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**TABLE 2**

**JFH1 infects Huh7.5.1 cells, but not HSCs**

To determine the binding or replication activity of JFH1 HCV in HSCs or Huh7.5.1 cells, 200,000 cells/well were seeded in 1 ml of medium in well-plates for 12 h. JFH1 stock virus (100 μl) was added to the correspondent well for 1 min, 10 min, 1 hr, 24 hr, and 96 hrs. The JFH1 supernatants were harvested, and the cells washed twice with PBS, followed by analysis of the cell lysates using the HCV core protein ELISA. HCV core protein content (pg/ml) is expressed as means and STDs from three sample replicates.

| HCV core | HCV cor |
|---------|---------|
| HSC+JFH1 | Ave STD | Ave STD |
| pg/ml   | pg/ml |
| 1 min   | 63.4 5.0 | 1 min | 43.5 3.8 |
| 10 min  | 217.2 18.9 | 10 min | 217.5 10.2 |
| 60 min  | 653.6 24.9 | 60 min | 786.6 41.3 |
| 24 h    | 1088.1 87.4 | 24 h | 4540.7 315.7 |
| 48 h    | 1044.0 71.3 | 48 h | 29144.7 1568.6 |
| 96 h    | 906.1 40.9 | 96 h | 43902.5 3012.5 |

nm. Cell viability was monitored through the Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Fluorescent microscopy was utilized to view ROS production. ROS/Cell Viability Arbitrary Unit (fold) was calculated by normalizing ROS level to cell viability.

**siRNA and Transfection**—To determine the specific molecular pathways of HIV and HCV regulated fibrosis-related gene expression, we performed RNAi to knock down NFkB gene expression. siRNAs were transfected into cells using HiPer-Fect Transfection Reagent (Qiagen, Valencia, CA). The siRNAs used for gene knock-down were as follows: SignalSilence NFkB p65 siRNA kit (Cat. 6536) which including (NFkB p65 siRNA I, product 6261, siRNA duplex sequences: 5'-GCCCUAUCCCUUACGUA-C3', and 5'-UGACGUAAGGGAUAGGC-3'), and (NFkB p65 siRNA II, product 6534, siRNA duplex sequences: 5'-GCU-GAAAGUGCAUCCAAAGG-3', and 5'-CCUUUGGAUG-CACUUCAG-C3') (Cell Signaling Technology, Inc.). SignalSilence Control siRNA (product 6568, siRNA duplex sequences: 5'-CGUAGCGGGAAUACUUCGA-3', and 5'-UCGAAGAUAUCCCGCUAGC-3') (Cell Signaling Technology, Inc.) were used as negative controls for siRNA transfection. Knockdown of NFkB protein expression was confirmed by Western blot.

**Statistics**—Data analysis was carried out using a 2-tailed Student's t test with pooled variance. Data are expressed as mean ± S.D. of at least three sample replicates, unless stated otherwise.

**RESULTS**

**JFH1 Does Not Replicate in Human Stellate Cells**—To test the interaction and infectivity of JFH1 in HSC and Huh7.5.1 cells, we incubated JFH1 supernatant with HSC or Huh7.5.1 cells. We found that HCV core levels in JFH1-incubated HSC cells increased from 63.4 ± 5.0 pg/ml at 1 min to 217.2 ± 18.9 pg/ml, 653.6 ± 24.9 pg/ml, 1088.1 ± 87.4 pg/ml at 10 min, 60 min, and 24 hr, respectively. However, longer periods of incubation (48 h and 96 h) did not further increase HCV core level (1044.0 ± 71.3 pg/ml, and 906.1 ± 40.9 pg/ml, respectively), suggesting that JFH1 did not replicate in HSC cells (Table 2). In contrast, we found that HCV core expression in JFH1-infected Huh7.5.1 cells increased 5.8-, 37-, and 55.9-fold from 786.6 ± 41.3 pg/ml at 60 min of incubation to 4540.7 ± 315.7 pg/ml, 29144.7 ± 1586.6 pg/ml, and 43902.5 ± 3012.5 pg/ml at 24, 48, and 96 h, respectively (Table 2), confirming JFH1 HCV replication in Huh7.5.1 cells.

**HIV Increases Procollagen α1(I) mRNA and Protein Expression in HSC; and mRNA Expression in Huh7.5.1, and JFH1 Cells**—To explore the effects of HIV on procollagen α1(I) (Col1A) gene and protein expression in LX-2 HSC, Huh7.5.1, and JFH1-infected Huh7.5.1 cells, we performed qPCR to measure the level of Col1A gene activity in cell and collagen type I protein level in supernatant in cell incubated with inactivated HIV infection supernatants. We found that heat-inactivated X4 HIV or R5 HIV enhanced Col1A mRNA expression in HSC by 2.03 ± 0.36-fold (p = 0.01), and 1.93 ± 0.22-fold (p = 0.003) respectively, compared with cells treated with medium alone (Fig. 1A). Incubation of HSCs with JFH1 HCV supernatant modestly increased Col1A mRNA expression by 1.32 ± 0.21-fold (p = 0.09) compared with untreated HSC cells. Incubation of HSCs with either X4 HIV or R5 HIV plus JFH1 further significantly enhanced Col1A mRNA expression by 2.24 ± 0.22 fold (p = 0.001), and 2.30 ± 0.23-fold (p = 0.001), respectively, compared with HSCs alone (Fig. 1A). In line with the Col1A mRNA data, we found that X4 HIV or R5 HIV enhanced collagen type I protein levels in HSC from 0.34 ± 0.04 μg/ml to 1.94 ± 0.26 μg/ml, and 1.60 ± 0.17 μg/ml respectively, compared with cells treated with medium alone (Fig. 1B). Incubation of HSCs with JFH1 HCV supernatant increased collagen type I protein levels to 2.02 ± 0.23 μg/ml. Incubation of HSCs with either X4 HIV or R5 HIV plus JFH1 further increased collagen type I protein levels to 7.85 ± 0.58 μg/ml, and 6.67 ± 0.38 μg/ml, respectively, compared with HSCs alone (Fig. 1B). We found that Huh7.5.1 cells express some Col1A mRNA. However, the qPCR threshold cycle (Ct) value in Col1A mRNA is roughly 10-fold higher in Huh7.5.1 cell (Ct range was between 27–30) than which in HSC cell (Ct range was between 17–20). We also found that CXCR4 and CCR5 HIV enhanced Col1A mRNA expression in Huh7.5.1 cells by 1.59 ± 0.14-fold (p = 0.01), and 1.46 ± 0.19-fold (p = 0.004), respectively, compared with untreated Huh7.5.1 cells (Fig. 1C). JFH1 HCV infection increased Col1A mRNA expression by 1.54 ± 0.26 fold (p = 0.04) in Huh7.5.1 cells. Again, X4 or R5 HIV further increased Col1A mRNA expression in JFH1-infected Huh7.5.1 cells by 3.70 ± 0.73-fold (p = 0.003), and 4.68 ± 0.39-fold (p < 0.001), respectively, compared with uninfected Huh7.5.1 cells. In contrast, HIV negative control supernatant had no effect on Col1A gene expression in Huh7.5.1 or JFH1-infected cells (Fig. 1C). Collagen type I protein levels in uninfected or JFH1-infected Huh7.5.1 cells were below the reliable range of ELISA detection (data not shown).

**HIV Increases TIMP1 mRNA and Protein Expression in HSC, Huh7.5.1, and JFH1-infected Huh7.5.1 Cells**—Heat-inactivated X4 HIV or R5 HIV enhanced HSC TIMP-1 mRNA expression by 2.01 ± 0.36-fold (p = 0.01), and 2.07 ± 0.37-fold (p = 0.01), respectively, compared with medium-only treated HSC cells (Fig. 1D). This was accompanied by an increase of secreted TIMP-1 protein from 50.3 ± 5.5 ng/ml in medium-treated HSC cell to 147.3 ± 10 ng/ml (p = 0.001) in X4 HIV and 151.1 ± 17.4 ng/ml (p = 0.001) in R5 HIV (Fig.
HSC cells incubated with JFH1 HCV supernatant exhibited minimal increases in TIMP-1 mRNA expression (1.32 ± 0.21-fold, p = 0.9) compared with medium-treated HSC cells, while HSC cells incubated with X4 HIV or R5 HIV combined with JFH1 HCV supernatant showed further significant up-regulation of TIMP-1 mRNA expression by 2.24 ± 0.22-fold (p = 0.001), and 2.30 ± 0.23-fold (p = 0.001), respectively, compared with medium-treated HSC cells (Fig. 1D). Incubation with JFH1 HCV supernatant increased TIMP-1 protein expression in HSC cells to 64.7 ± 4.7 ng/ml (p = 0.03) compared with medium-treated HSC cells (50.3 ± 5.5 ng/ml). Incubation of HSC cells with X4 HIV or R5 HIV alone or plus JFH1 HCV supernatant further significantly induced TIMP-1 protein expression to 169.8 ± 19.6 ng/ml (p < 0.001), and 170.3 ± 20.2 ng/ml (p < 0.001) respectively, compared with medium-treated HSC cells (Fig. 1E) or JFH1-infected cells. TIMP-1 mRNA expression was up-regulated by X4 HIV or R5 HIV in Huh7.5.1 cell by 1.55 ± 0.17-fold (p = 0.015), and 1.61 ± 0.23-fold (p = 0.021), respectively, compared with medium-treated Huh7.5.1 cells, while JFH1 HCV infection increased TIMP-1 mRNA expression by 2.02 ± 0.16-fold (p = 0.002). X4 and R5 HIV further induced TIMP-1 mRNA expression in JFH1 cell by 4.32 ± 0.76-fold (p = 0.002), and 3.62 ± 0.28-fold (p < 0.001), respectively, compared with medium-treated Huh7.5.1 cells (Fig. 1F). Equally, X4 HIV and R5 HIV significantly increased TIMP-1 protein levels to 2.08 ± 0.25 ng/ml (p = 0.001), and 1.91 ± 0.19 ng/ml (p = 0.001) respectively, compared with medium-treated Huh7.5.1 cells (0.71 ± 0.06 ng/ml). In Huh7.5.1 cells, Neg HIV control had no significant effect on TIMP-1 protein levels in Huh7.5.1 cells.
cells. In line with the mRNA data, JFH1 mono-infection also increased TIMP1 cytokine level to 1.97 ± 0.13 ng/ml ($p = 0.001$) in Huh7.5.1 cells. X4 HIV or R5 HIV further significantly enhanced TIMP-1 protein levels to 3.87 ± 0.17 ng/ml ($p < 0.001$), and 3.68 ± 0.31 ng/ml ($p < 0.001$), respectively, compared with medium or JFH1-treated Huh7.5.1 cells (Fig. 1G).

**HIV Suppresses MMP-3 Gene and Protein Expression in HSC Cells**—X4 HIV or R5 HIV suppressed MMP-3 mRNA expression to 0.70 ± 0.06-fold ($p = 0.008$) and 0.75 ± 0.06-fold ($p = 0.01$), respectively, compared with medium-treated HSC cells, while incubation of HSCs with JFH1 HCV supernatant did not affect MMP-3 mRNA expression. X4 HIV or R5 HIV each combined with JFH1 HCV supernatant did not further suppress MMP-3 mRNA expression compared with HIV alone treated HSCs (Fig. 2A). X4 HIV and R5 HIV significantly down-regulated MMP-3 protein secretion to 14.1 ± 1.37 ng/ml ($p = 0.005$) and 13.99 ± 1.22 ng/ml ($p = 0.005$), respectively, compared with medium treated HSC (26.15 ± 3.56 ng/ml). However, MMP-3 protein secretion was not further suppressed in HSC incubated with JFH1 HCV supernatants (Fig. 2B). MMP-3 protein in uninfected or JFH1-infected Huh7.5.1 cells was below the limits of detection (data not shown).

**HIV/HCV Co-infected and HCV Infected Increase Col1A and TIMP1 mRNA and Decrease MMP3 mRNA Levels in Human Liver Tissue**—To determine whether HIV/HCV co-infected and HCV infected have effects on liver fibrosis in vivo, we measured mRNA levels in liver biopsy from non-infected, HCV infected, and HIV/HCV co-infected patients. HIV alone data were not available due to the lack of availability of HIV mono-infected liver biopsy material. We found the following transcript mRNA levels for procollagen I (CoL1A) and TIMP-1: HIV/HCV co-infected > HCV infected > non-infected; and for MMP3: HIV/HCV co-infected < HCV infected < non-infected (Fig. 3, A–C). These data indicate that the results from the cell culture model are consistent with patient liver tissue samples, confirming that HIV/HCV co-infection and HCV infection produce a milieu that accelerates hepatic fibrosis.

**HIV Increases ROS Production in HSC, Huh7.5.1, and JFH1 Cells**—ROS production has been suggested to induce liver fibrogenesis (13, 15). We have previously shown that HCV replication induces ROS production in both JFH1 and HCV replicon cells. To determine whether HIV has an effect on ROS generation, we monitored ROS production in HSC, Huh7.5.1, and JFH1-infected Huh7.5.1 cells incubated with inactivated HIV supernatants. We found that X4 HIV and R5 HIV significantly induced ROS production by 1.62 ± 0.12
ROS fluorescence was measured by using ROS dye. Cell viability was monitored by the Cell Viability Assay. ROS level was normalized by cell viability to calculate the ROS/Cell Viability Arbitrary Unit (fold). ROS data represent mean ± S.D. (n = 4). ROS fluorescent images in live cells were monitored by fluorescent microscopy. *, p < 0.05; **, p < 0.01; and #, p < 0.001 for comparison of indicated treatment and Neg HIV. A, HIV and HCV enhanced ROS production in HSC cells. Empty bar: HSC cell, Gray bar: HSC incubated with JFH1 HCV. B, ROS fluorescence images in live HSC cells. C, HIV and HCV increased ROS production in Huh7.5.1 cells. Empty bar: Huh7.5.1 cells, gray bar: JFH1-infected Huh7.5.1 cells. D, ROS fluorescence images in live Huh7.5.1 and JFH1 cells.

(p = 0.003) and 1.70 ± 0.24 (p = 0.01)-fold in HSC, respectively, compared with control (Neg HIV) treated cells (Fig. 4A). Although JFH1 HCV did not replicate in HSC cells (Table 2), we found that HSC cells incubated with HCV JFH1 supernatant also increased ROS production by 1.69 ± 0.14 (p = 0.003)-fold compared with HSC cells incubated with uninfected Huh7.5.1 supernatant (Fig. 4A). X4 HIV or R5 HIV combined with JFH1 HCV supernatant further enhanced ROS production by 2.40 ± 0.42 (p = 0.002) and 2.09 ± 0.15 (p = 0.001) fold, respectively, compared with untreated HSC cells (Fig. 4A). Furthermore, we observed that X4 HIV or R5 HIV supernatant added to Huh7.5.1 cells significantly induced ROS production by 1.88 ± 0.17 (p = 0.002) and 1.81 ± 0.25 (p = 0.008)-fold, respectively, compared with Neg HIV supernatant-treated Huh7.5.1 cells (Fig. 4C). We confirmed that JFH1 infection increased ROS production by 2.35 ± 0.29-fold (p = 0.001) in Huh7.5.1 cells compared with uninfected controls (Fig. 4C) (6). X4 HIV or R5 HIV further increased ROS production by 4.15 ± 0.26 (p < 0.001) and 4.2 ± 0.33 (p < 0.001)-fold, respectively, in JFH1-infected Huh7.5.1 cells compared with uninfected Huh7.5.1 cells (Fig. 4C). To visualize intracellular ROS activity, we obtained ROS fluorescent images in live cells that confirmed X4 and R5 HIV induced higher levels of ROS activity in HSC, Huh7.5.1, and JFH1-infected Huh7.5.1 cells than seen in Neg HIV controls (Fig. 4, B and D).

HIV and HCV Induced TIMP1 and CoL1A Gene Expressions are ROS-dependent—To assess whether HIV and HCV induction of TIMP1 and CoL1A gene expression are linked to ROS production, we used DPI, a potent inhibitor of ROS formation. DPI efficiently blocked HIV- and HCV-induced ROS enhancement in HSC, Huh7.5.1, and JFH1 cells (Fig. 5, A and D). DPI also inhibited HIV- and HCV-induced enhancement of TIMP-1 and CoL1A mRNA expressions in HSC, Huh7.5.1, and JFH1-infected Huh7.5.1 cells (Fig. 5, B and C, G and H). We found that DPI had no effect on HSC cell viability (Fig. 5B), and moderately decreased cell viability in Huh7.5.1- and JFH-infected Huh7.5.1 cells (Fig. 5F). These data indicate that HIV and HCV regulation of TIMP1 and CoL1A gene expression occur through ROS production.

HIV- and HCV-induced NFkB Phosphorylation Is ROS-dependent—To explore the effects of HIV and HCV on NFkB activity, we monitored NFkB phosphorylation by Western blot. We found that HIV and HCV each increased NFkB phosphorylation in HSC (Fig. 6A) and Huh7.5.1 cells (Fig. 6B). We also observed that DPI blocked HIV- and HCV-induced NFkB phosphorylation in HSC and Huh7.5.1 cells (Fig. 6, A and B), indicating that HIV and HCV activate NFkB via ROS production.

HIV- and HCV-induced TIMP1 and CoL1A Expressions Enhancement Are Abrogated by NFkB siRNA—To determine whether NFkB participates in HIV- and HCV-mediated enhancement of TIMP1 and CoL1A expression, we performed siRNA-mediated knockdown of NFkB in both HSC and Huh7.5.1 cells. We found that both HIV- and HCV-induced TIMP1 mRNA and protein, and CoL1A mRNA expressions were inhibited by NFkB-specific siRNA in HSC (Fig. 7, A–C) and Huh7.5.1 cells (Fig. 8, A–C). Western blotting confirmed
that NFκB protein expression was knocked down by NFκB siRNA in both cell lines (Figs. 7D and 8D). These data indicate that HIV- and HCV-induced TIMP1 and procollagen α1(I) production is dependent on NFκB activation. However, we found that HIV- and HCV-mediated ROS production was not inhibited by NFκB siRNA in HSC and Huh7.5.1 cells (Fig. 9, A and B), indicating that NF-κB activation lies downstream of ROS production.

DISCUSSION

HIV/HCV-coinfected patients show an accelerated progression to cirrhosis, liver failure, and liver-related death compared with HCV-monoinfected patients (3, 4, 8). However, the putative profibrogenic role of HIV upon (HCV-infected) hepatocytes and HSCs has not been carefully explored. In a previous study, we found that inactivated HIV or its envelope glycoprotein gp120 were capable of up-regulating TGF-β1 expression in Huh7.5.1- and JFH1-infected Huh7.5.1 cells (8). Our previous work had suggested that alterations in the circulating and intrahepatic cytokine environment that accompany HIV infection, particularly the increased levels of the profibrogenic cytokine TGF-β1, may contribute to the accelerated liver fibrosis observed in HIV-HCV-coinfected persons (8). HCV infection increases the generation of ROS (14) (26). HCV replication has been demonstrated to increase TGF-β1 production through the generation of ROS in an HCV subgenomic replicon, as well as in the JFH1 infectious model (14) (6, 13). HCV protein expression has also been shown to induce oxidative stress in a TGF-β1-dependent manner (27). TGF-β1 is a potent inducer of extracellular matrix synthesis. It enhances the production of many ECM proteins and down-regulates their MMP-dependent degradation by increasing TIMP expression, and induces production of
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type I collagen by HSCs (28). TGF-β1 can also stimulate ROS production, which further contributes to the progression of liver fibrosis (28, 29). TGF-β1 has also been shown to induce liver fibrosis through Kupffer cell-mediated HSC activation and induction of collagen I and TIMP1 expression (30). It has been demonstrated that TGF-β1 stimulates vascular endothelial growth factor (VEGF) through the p38 mitogen-activated protein kinase (p38 MAPK)-dependent pathway, and leads to induction of type I collagen synthesis in murine mesangial cells (31, 32). ROS are natural byproducts of normal oxygen metabolism that have important roles in cell signaling. ROS are predominantly produced in the mitochondria via the electron transport chain through endoplasmic reticulum (ER) stress, which is induced by viral infection (33–35). HCV and other viruses can induce ROS, resulting in significant damage to cell structures, as well as induction of apoptosis and fibrosis, as has been shown in both this report and previous studies (14) (6, 25, 36, 37). We found that both HIV and HCV enhance ROS production in HSC and Huh7.5.1 cells. However, the impact of excess ROS on activation of fibrogenic signaling has not been well characterized. Hepatocytes are the predominant cells in the liver, while activated HSC are the main effector cells of fibrogenesis (15, 38, 39). Liver fibrosis is the product of excessive accumulation of ECM components that is not matched by removal of ECM by proteases, such as fibrolytic MMPs, and the overproduction of mainly TIMP-1 (15, 38, 39). In this study, we investigated the mechanism by which HIV and HCV cooperatively induce fibrosis-related gene and protein expression in HSC and Huh7.5.1 cells. We found that JFH1 HCV infection significantly induced Col1A1 and TIMP1 activity in Huh7.5.1 cells. The finding of low level of Col1A1 expression in Huh7.5.1 cells is in line with several studies that demonstrate epithelial-mesenchymal transition phenotypes in both hepatocytic cell lines and cultures hepatocytes in vitro (40), but this phenotype apparently does not exist in vivo (41, 42). Interestingly, we also found that JFH1 HCV induced pro-fibrogenic activity in HSCs, despite the fact that JFH1 did not
replicate in these cells. In support of this finding, it has been previously shown that binding of the HCV envelope protein E2 can up-regulate MMP2 through E2/CD81 interactions in HSC cells (43). X4 HIV and R5 HIV have been demonstrated to enter and infect activated HSC LX2 and primary HSCs, inducing procollagen I expression and secretion of monocyte chemoattractant protein 1 (MCP-1) (7, 44). We have now demonstrated that HIV and HCV can individually and jointly induce the profibrogenic genes procollagen H9251/H9260 I and TIMP1, as well as reduce the potentially antifibrogenic expression of MMP3. We also demonstrate that the induction by HIV and HCV of fibrogenic gene expression in HSC and Huh7.5.1 cells is ROS-dependent. Activation of ROS downstream of the NFkB pathway plays major roles in both regulating the immune response to infection and in profibrogenic activation. This is in line with NFkB being one of the major signal-transducers activated in response to viral infection (6, 45). It has been reported that multiple families of viruses, including HIV, HCV, HBV, HTLV-1, EBV, and influenza virus activate NFkB, which subsequently promotes expression of over 100 target genes (46). In this study, we demonstrate that HIV and HCV regulate fibrosis-related genes through ROS induction and activation of the NFkB pathway. However, in this study and in a previous report (14), we found that siRNA to NFkB did not significantly affect JFH1 replication in Huh7.5.1 cells. Our results provide the first reported mechanistic data on how HIV and HCV may cooperatively drive hepatic fibrogenesis, thereby broadening our understanding of the mechanisms underlying liver disease in patients coinfected with HIV/HCV. It would now appear that strategies to limit HIV/HCV induc-

![Figure 8](image-url)

**FIGURE 8.** NFkB siRNA blocks HIV and HCV activated TIMP1 and Col1A expression in Huh7.5.1 and JFH1 cells. NFkB siRNA was transfected into Huh7.5.1 or JFH1-infected Huh7.5.1 (Day 1 postinfection) cells for 24 h, Neg siRNA was used as transfection control. The siRNA-transfected cells were then incubated with X4 HIV, R5 HIV, or Neg HIV supernatants for another 24 h. *, p < 0.05; **, p < 0.01; and #, p < 0.001 for comparison of indicated treatment with negative control siRNA in presence of Neg HIV. A, NFkB siRNA inhibited HIV- and JFH1 HCV-induced TIMP1 mRNA in Huh7.5.1 cells. B, NFkB siRNA blocked HIV- and JFH1 HCV-induced TIMP-1 protein in Huh7.5.1 cells. C, NFkB siRNA inhibited HIV- and JFH1 HCV-induced Col1a mRNA in Huh7.5.1 cells. D, NFkB siRNA knocked down NFkB protein expression in Huh7.5.1 cells. Lanes 1 and 7, Neg HIV; 2 and 8, X4 HIV; 3 and 9, R5 HIV; 4 and 10, Neg HIV + JFH1; 5 and 11, X4 HIV + JFH1; 6 and 12, R5 HIV + JFH1.

![Figure 9](image-url)

**FIGURE 9.** NFkB siRNA does not affect HIV and HCV-induced ROS production. HSC, Huh7.5.1, or JFH1 cells were transfected with Neg siRNA or NFkB siRNA for 24 h. The siRNA-transfected cells were incubated with Neg HIV, X4 HIV, or R5 HIV with or without JFH1 supernatant for another 24 h. ROS fluorescence was measured by using ROS dye. Cell viability was monitored by Cell Viability Assay. ROS level was normalized by cell viability to calculate the ROS/Cell Viability Arbitrary Unit (fold). ROS data represent mean ± S.D. (n = 4). *, p < 0.05; **, p < 0.01; and #, p < 0.001 for comparison of indicated treatment with negative control siRNA in presence of Neg HIV. A, NFkB knock down has no effect on X4 HIV, R5 HIV and JFH1 HCV-enhanced ROS production in HSC cells. B, NFkB knock down has no effect on X4 HIV or R5 HIV-induced ROS production in Huh7.5.1 and JFH1 cells.
HIV/HCV Promotes Hepatic Fibrogenesis

A new study has been published in the Journal of Biological Chemistry, which provides evidence for the role of oxidative stress and inhibition of NFκB activation in the promotion of hepatic fibrosis. The researchers demonstrated that HIV/HCV infection leads to an increased expression of pro-fibrotic factors, which warrant further investigation into potential therapeutic targets.

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