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New Mechanism of Hepatic Fibrogenesis: Hepatitis C Virus Infection Induces Transforming Growth Factor β1 Production through Glucose-Regulated Protein 94

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ABSTRACT

Hepatitis C virus (HCV) is one of the leading causes of chronic liver inflammatory disease (hepatitis), which often leads to more severe diseases, such as liver fibrosis, cirrhosis, and hepatocellular carcinoma. Liver fibrosis, in particular, is a major pathogenic consequence of HCV infection, and transforming growth factor β1 (TGF-β1) plays a key role in its pathogenesis. Several HCV proteins have been suggested to either augment or suppress the expression of TGF-β1 by HCV-infected cells. Here, we report that TGF-β1 levels are elevated in HCV-infected hepatocytes cultured *in vitro* and in liver tissue of HCV patients. Notably, the level of TGF-β1 in media from *in vitro*-cultured HCV-infected hepatocytes was high enough to activate primary hepatic stellate cells isolated from rats. This indicates that TGF-β1 secreted by HCV-infected hepatocytes is likely to play a key role in the liver fibrosis observed in HCV patients. Moreover, we showed that HCV E2 protein triggers the production of TGF-β1 by HCV-infected cells through overproduction of glucose-regulated protein 94 (GRP94).

IMPORTANCE

Hepatic fibrosis is a critical step in liver cirrhosis caused by hepatitis C virus infection. It is already known that immune cells, including Kupffer cells, mediate liver fibrosis. Recently, several papers have suggested that HCV-infected hepatocytes also significantly produce TGF-β1. Here, we provide the first examination of TGF-β1 levels in the hepatocytes of HCV patients. Using an HCV culture system, we showed that HCV infection increases TGF-β1 production in hepatocytes. Furthermore, we confirmed that the amount of TGF-β1 secreted by HCV-infected hepatocytes was sufficient to activate primary hepatic stellate cells. To understand the molecular basis of TGF-β1 production in HCV-infected hepatocytes, we used HCV replicons and various stable cell lines. Finally, we elucidated that HCV E2 triggered TGF-β1 secretion via GRP94 mediated NF-κB activation. This study contributes to the understanding of liver fibrosis by HCV and suggests a new potential target (GRP94) for blocking liver cirrhosis in HCV patients.

Hepatitis C virus (HCV) is one of the leading causes of chronic inflammatory liver disease (hepatitis) (1). According to World Health Organization reports, about 170 million people currently suffer from HCV infection worldwide (2). Chronic HCV infection often results in the development of hepatic fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (3).

HCV is a positive-sense, single-stranded RNA virus whose 9.6-kb genome encodes at least 10 viral proteins, including four structural and six nonstructural proteins (4). Error-prone RNA-dependent RNA polymerase (RDRP) generates a wide variety of mutations (5), which complicates development of HCV vaccines (6). Several therapeutic agents have recently been developed for HCV-related chronic hepatitis, including direct-acting antivirals (DAAs) that target HCV viral proteins (7, 8).

Liver fibrosis is known to be tightly linked with HCV infection and ultimately leads to liver dysfunction and cirrhosis (9). Liver fibrosis is involved in the process of healing wounds caused by chronic inflammation (one of the prominent features of HCV infection-related pathogenesis) and the death of HCV-infected hepatocytes (10). In fibrotic states, liver tissue shows abnormal accumulation of extracellular matrix (ECM) and changes in ECM composition (11, 12).

Generally, liver fibrosis progression is promoted by activated hepatic stellate cells (HSCs) (13). In the normal liver, HSCs reside in the sinusoidal space in a quiescent state (14), but upon activation by cytokines, they secrete large amounts of ECM (9). Transforming growth factor β1 (TGF-β1), a multifunctional cytokine that controls growth, adhesion, migration, apoptosis, and differentiation of cells (15), is the most important cytokine involved in fibrogenesis (16). During liver fibrosis, TGF-β1 induces *trans*-differentiation of quiescent HSCs to myofibroblasts, which, in turn, produce other cytokines and matrix proteins (17).

It has been suggested that Kupffer cells, activated by the continuous liver injury caused by HCV infection, are the main source of TGF-β1 (18). However, there have been several reports suggesting that HCV-infected hepatocytes themselves secrete TGF-β1 (19–21). Elevation of reactive oxygen species (ROS) in HCV-infected cells was suggested as a mechanism for the TGF-β1 produc-

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tion in HCV-infected cells (21), and HCV core protein was shown to be responsible for TGF-β1 secretion (19, 20). In contrast, several reports have shown that HCV viral protein(s) represses TGF-β1 signaling and/or TGF-β1 gene expression (22, 23). For instance, NS5A protein was shown to inhibit TGF-β1 signaling (22). Moreover, NS5A protein was suggested to inhibit the function of activating protein 1 (AP-1), a transcriptional activator required for TGF-β1 gene expression (24), by perturbing Ras-ERK signaling pathway (23).

In this study, we investigated the molecular basis of the fibrogenesis induced by HCV infection. Particularly, we sought to identify HCV protein(s), if it exists, that augments TGF-β1 production by HCV-infected cells. Using molecular biological and biochemical approaches, we found that HCV-infected hepatocytes secrete TGF-β1 proteins which are sufficiently high to activate primary HSCs. Moreover, high level of TGF-β1 was observed from HCV-infected liver cells of HCV patients. Our investigation of the molecular basis of TGF-β1 production in HCV-infected cells revealed that HCV core protein and envelope protein 2 (E2) participate in elevated TGF-β1 production. We also demonstrated that overproduction of glucose-regulated protein 94 (GRP94) by HCV E2 triggers the signal transduction cascades that result in the elevated TGF-β1 production.

MATERIALS AND METHODS

Cell culture. Huh-7 and Huh-7.5.1 cells were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with antibiotics (100 U/ml of penicillin and 10 μg/ml of streptomycin) and 10% fetal bovine serum (FBS; HyClone) in a humidified 5% CO_2 environment. Huh-7 cells containing HCV replicons were grown under the same conditions, with the additional inclusion of the antibiotic G418 (500 μg/ml; AG Scientific) (25). Huh-7 cells expressing HCV E2 or GRP94 were cultured under the same conditions, with the additional inclusion of the antibiotic hygromycin B (300 μg/ml; AG Scientific) (25, 26).

Western blotting. Cells were lysed in passive lysis buffer (Promega). Proteins in lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose membranes (Whatman). Antibodies against GRP94, NS5B, and α-smooth muscle actin (α-SMA; Santa Cruz), β-actin (ICN), and TGF-β1 (BD Biosciences) were used as primary antibodies. The monoclonal anti-E2 antibody H52 was a kind gift from Jean Dubuisson (University of Lille), and an antibody against core protein was kindly provided by Ralf Bartenschlager (University of Heidelberg). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG, sheep anti-mouse IgG (GE Healthcare), and donkey anti-goat IgG (Santa Cruz) were used as secondary antibodies. Chemiluminescence detection was performed using WEST-ZOL plus (iNtRON).

siRNA transfection. Knockdown of GRP94 was performed by transfection with a specific small interfering RNA (siRNA) duplex targeting GRP94 (5′-UGAUGUGGAUGGUACAGUA-3′; Bioneer) using Oligofectamine (Invitrogen) (26). Cells were incubated for 4 h, after which serum-free medium was replaced with DMEM containing 10% FBS.

qRT-PCR. Total RNA was isolated with TRI-solution (Bioscience Technology). cDNA synthesis was performed by reverse transcription reagent (Promega). Quantitative reverse transcription-PCR (qRT-PCR) was performed with the TaKaRa SYBR Premix EX *Taq* II protocol using an IQ5 multicolor real-time PCR detection system (Bio-Rad). Sequences of primer pairs for qRT-PCR are as follows: TGF-β1, 5′-GGC GAT ACC TCA GCA ACC G-3 and 5′-CTA AGG CGA AAG CCC TCA AT-3′; HCV, 5′-TCT GCG GAA CCG GTG AGT A-3′ and 5′-TCA GGC AGT ACC ACA AGG C-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-AGG GCT GCT TTT AAC TCT GGT-3′ and 5′-CCC CAC TTG ATT TTG GAG GGA-3′.

Quantification of TGF-β1 by ELISA. Huh-7 cells were seeded in a 100-mm dish and cultured for 12 h. After three washings with phosphate-buffered saline (PBS), fresh serum-free medium was added. Cells were then incubated for 24 h, after which culture media were collected and filtered through a 0.2-μm Millipore filter. The amount of secreted TGF-β1 in the culture medium was determined using a human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's protocol.

Immunofluorescence assays. The subcellular localization of TGF-β1 and HCV core protein was monitored using anti-TGF-β1 (BD Biosciences) and anti-core (Affinity Bioreagents) antibodies, respectively. For immunocytochemistry, HCV-inoculated and uninoculated Huh-7.5.1 cells were fixed with 4% paraformaldehyde (10 min) and then incubated for 0.5 h in blocking solution containing 1% bovine serum album (BSA) and 0.1% Tween 20 in PBS to permeabilize cells and block nonspecific binding of antibodies. Cells were then washed with PBS and incubated at room temperature for 1 h with primary antibodies. After a washing with PBS, cells were incubated with secondary antibodies for 1 h.

For immunohistochemistry, paraffin-embedded liver tissue specimens were deparaffinized and rehydrated with xylene and ethanol. Antigenic epitopes of samples were exposed by treatment with 10 mM citrate buffer and heating in a microwave oven. Samples were incubated in blocking solution containing 5% horse serum and 0.02% Triton X-100 in Tris-buffered saline (TBS) at room temperature for 2 h and then incubated overnight at 4°C with primary antibodies. After a washing with TBS containing 0.01% Triton X-100, the samples were incubated with secondary antibodies (Invitrogen; Jackson ImmunoResearch) for 2 h. Immunostained samples were observed under an Olympus FV1000 confocal laser scanning microscope.

Quantification of the imaging data. Images were analyzed using MetaMorph software. The data from immunocytochemical study of 134 cells and the data from immunohistochemical study of 57 cells were analyzed using the software. The fluorescence intensities (TGF-β1, red; HCV core, green; and Hoechst, blue) of cells were measured by the linescan tool in MetaMorph software. To calculate the level of protein expression in cells, the sum of fluorescence intensities (TGF-β1, red, and HCV core, green) was divided by the sum of Hoechst intensities in the corresponding cells. The average value of fluorescence intensity in each group was calculated by dividing the sum of protein level in the group by the number of cells belonging to the group. The version of MetaMorph used is 7.04r4.

Virus infection and production. *In vitro* transcription of HCV RNA (derived from JFH-1) and transfection of RNAs were performed as described previously (27). Infectious HCV particles were collected from the culture media of Huh-7.5.1 cells 3 days after transfection with HCV RNA. The levels of TGF-β1 in the media of HCV-infected Huh-7.5.1 cells were measured 3 weeks after HCV infection using a TGF-β1 ELISA kit.

Isolation of HSCs. Primary HSCs were isolated from the livers of male Sprague-Dawley rats according to an established method (28). Briefly, rat livers were perfused with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) containing 0.025% collagenase B. The resulting liver suspension was incubated at 37°C for 20 min, and HSCs were separated by centrifugation with an 11.9% Histodenz (Sigma) cushion. The purity of HSC isolates was greater than 90%, as assessed by fluorescence of an anti-GFAP (glial fibrillary acidic protein) antibody (Abcam) under UV excitation (29). Rats were maintained under specific-pathogen-free conditions. All animal procedures were approved by the Animal Care Committee of POSTECH Biotech Center.

Detection of HSC activation. HSCs were cultured in DMEM containing 3% FBS. After 3 days of incubation, HSCs were transferred to 6-well plates and cultured for 12 h. The cells were further cultured with serum-free DMEM for 24 h. The culture media were replaced with the incubation media of various cells with or without pretreatment of antibodies for 4 h. After being cultured for an additional 10 h, HSCs were collected and lysed with passive lysis buffer (Promega). A recombinant human TGF-β1 protein (R&D Systems) was used as a positive control for activation of HSCs.

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Liver tissue samples. Liver tissue samples were donated by liver cancer patients who provided informed consent, and the utilization of specimens for this research was authorized by the institutional review boards of the universities which provided the samples. Tissue sample number 06-30414N (a nontumor region of a liver cancer patient who was not infected with HCV), numbers N1 and N2 (nontumor regions of hepatitis B virus [HBV] patients), and numbers 375N, 388N, 02-27024N, 03-10521N, and 06-9008N (nontumor regions of HCV patients) were provided by the Liver Cancer Specimen Bank, supported by National Research Resource Bank Program of the Korea Science and Engineering Foundation in the Ministry of Science and Technology. Tissue sample numbers s10-6410-B and s09-20494 (nontumor regions of HCV patients) were provided by Ewha Womans University.

RESULTS

HCV infection increases TGF-β1 secretion by hepatocytes. To determine whether HCV infection affects the secretion of TGF-β1 by hepatocytes, we measured the amount of TGF-β1 in the culture media of Huh-7.5.1 cells after HCV (JFH-1) infection using a TGF-β1 ELISA. The level of TGF-β1 in the culture media from HCV-infected cells (3 weeks after infection with HCV at a multiplicity of infection [MOI] of 0.1) was increased ~2-fold compared with that in culture media from mock-infected cells (Fig. 1A). HCV infection was monitored by Western blotting of cell extracts with an antibody against HCV core protein (Fig. 1A).

We also investigated the acute effect of HCV infection on TGF-β1 gene expression by inoculation with a high titer of HCV (an MOI of 5, ensuring infection of more than 99% of the cells). Surprisingly, both TGF-β1 mRNA and pro-TGF-β1 protein (a precursor of TGF-β1) were increased ~4-fold in the HCV-infected cells at 2 days after infection (Fig. 1B and C, respectively). The results indicated that HCV infection stimulated the production and secretion of TGF-β1 by hepatocytes.

TGF-β1 protein levels are high in HCV-infected cells and in neighboring cells. TGF-β1 levels in individual hepatocytes infected with JFH-1 were investigated using fluorescence immunocytochemistry. HCV-infected cells were detected using an antibody against HCV core protein, and intracellular TGF-β1 was stained using a specific anti-TGF-β1 antibody. Much higher levels of TGF-β1 (red) were observed in HCV-infected cells (green) than in uninfected cells (compare Fig. 2A-1 and A-2). In order to compare the levels of TGF-β1 in individual cells in the HCV-inoculated sample, we further analyzed the immunocytochemistry data shown in Fig. 2A-2 using MetaMorph software (Fig. 2B). We classified HCV-inoculated cells into three groups: (i) HCV-infected cells, which showed high levels of HCV core protein (Fig. 2B-1); (ii) uninfected neighboring cells, which showed undetectable levels of HCV core protein but were in direct contact with HCV-infected cells (Fig. 2B-2); and (iii) uninfected nonneighboring cells, which did not show HCV core protein and were not in contact with HCV-infected cells (Fig. 2B-3). The intensity of signals emitted by HCV core (green), TGF-β1 (red), and Hoechst (blue) along transects through individual cells, indicated by red lines in the left images in Fig. 2B, are depicted in the traces in the right images in Fig. 2B. These data indicate that high levels of TGF-β1 proteins were present in the cytoplasm of HCV-infected cells and that TGF-β1 protein was not colocalized with HCV core proteins even though both proteins were found in the cytoplasm. Statistical analyses of TGF-β1 protein in individual cells (Tables 1 and 2) showed that the average amount of TGF-β1 protein in HCV-infected cells was more than 20-fold higher than that in uninoculated cells (Table 1) and that the average amounts of TGF-β1 protein in uninfected cells neighboring HCV-infected cells and in uninfected nonneighboring cells were 5.6- and 2.8-fold higher than that in mock-inoculated cells, respectively (Table 1). Many HCV-infected cells showed more than 30-fold-higher relative TGF-β1 levels (RTLs) (Table 2), whereas none of the un-

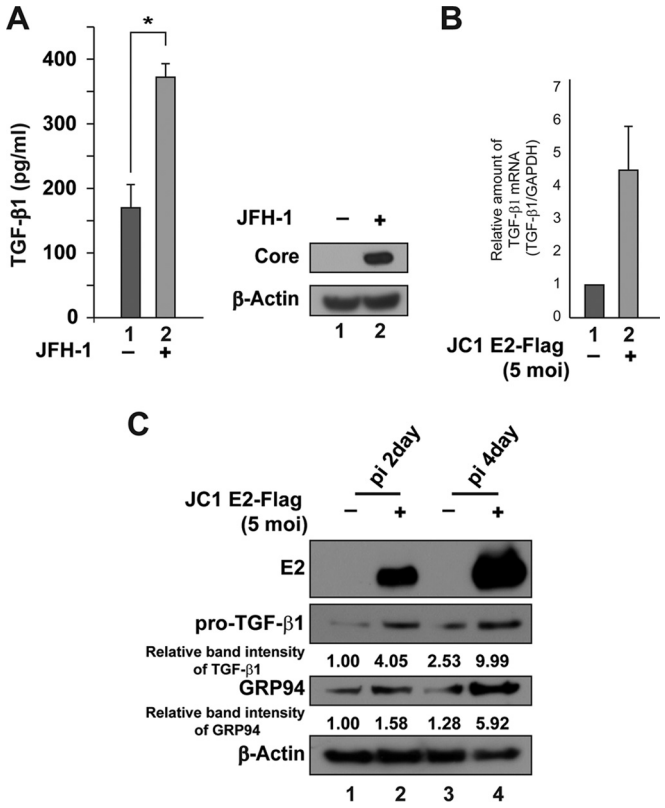

FIG 1 HCV infection induces TGF-β1 secretion. (A) TGF-β1 levels in the culture media of Huh-7.5.1 cells, infected with HCV (JFH-1) or mock infected, were examined by ELISA. Huh-7.5.1 cells were infected with HCV (MOI, 0.1) and maintained for 3 weeks; then the same numbers of HCV-infected cells and mock-infected cells (7×10^5) were seeded on 100-mm dishes and cultured for 12 h. The media were changed to DMEM without FBS, and the cells were cultured for an additional 24 h. Culture media were collected and analyzed by ELISA after being filtered through a 0.2-μm-pore filter. Data are presented as means and standard deviations (bars and error bars, respectively) from three independent experiments. HCV infections were confirmed by Western blotting for HCV core protein. The extracts from mock-infected (lane 1) and HCV-infected (lane 2) Huh-7.5.1 cells were resolved by SDS-PAGE on 12% gels and analyzed by Western blotting with antibodies against HCV core protein and β-actin (used as a loading control). (B) HCV infection induces TGF-β1 mRNA production. Huh-7.5.1 cells were inoculated with HCV (JC1 E2-Flag; MOI, 5) and cultivated for 2 days. The amounts of TGF-β1 mRNA in the HCV-infected (lane 2) and mock-infected cells were measured by qRT-PCR using the total RNAs isolated by TRI-solution method (Bioscience Technology). The amount of TGF-β1 mRNA was normalized by that of GAPDH mRNA. The relative amounts of TGF-β1 mRNA in the HCV-infected cells and the mock-infected cells are depicted. The experiments were performed three times, and the means and standard deviations are depicted by bars and error bars, respectively. (C) HCV infection augments production of pro-TGF-β1 (a precursor of TGF-β1) and GRP94. Huh-7.5.1 cells were inoculated with HCV (JC1 E2-Flag; MOI, 5) and cultivated for 2 days (lane 2) or 4 days (lane 4). Mock-infected cells were cultivated for 2 days (lane 1) or 4 days (lane 3) as negative controls. The amounts of HCV E2, pro-TGF-β1, GRP94, and β-actin proteins were monitored by Western blotting analyses using corresponding antibodies. Asterisks in the figures indicate *P* values: *, $P < 0.05$; **, $P < 0.001$.

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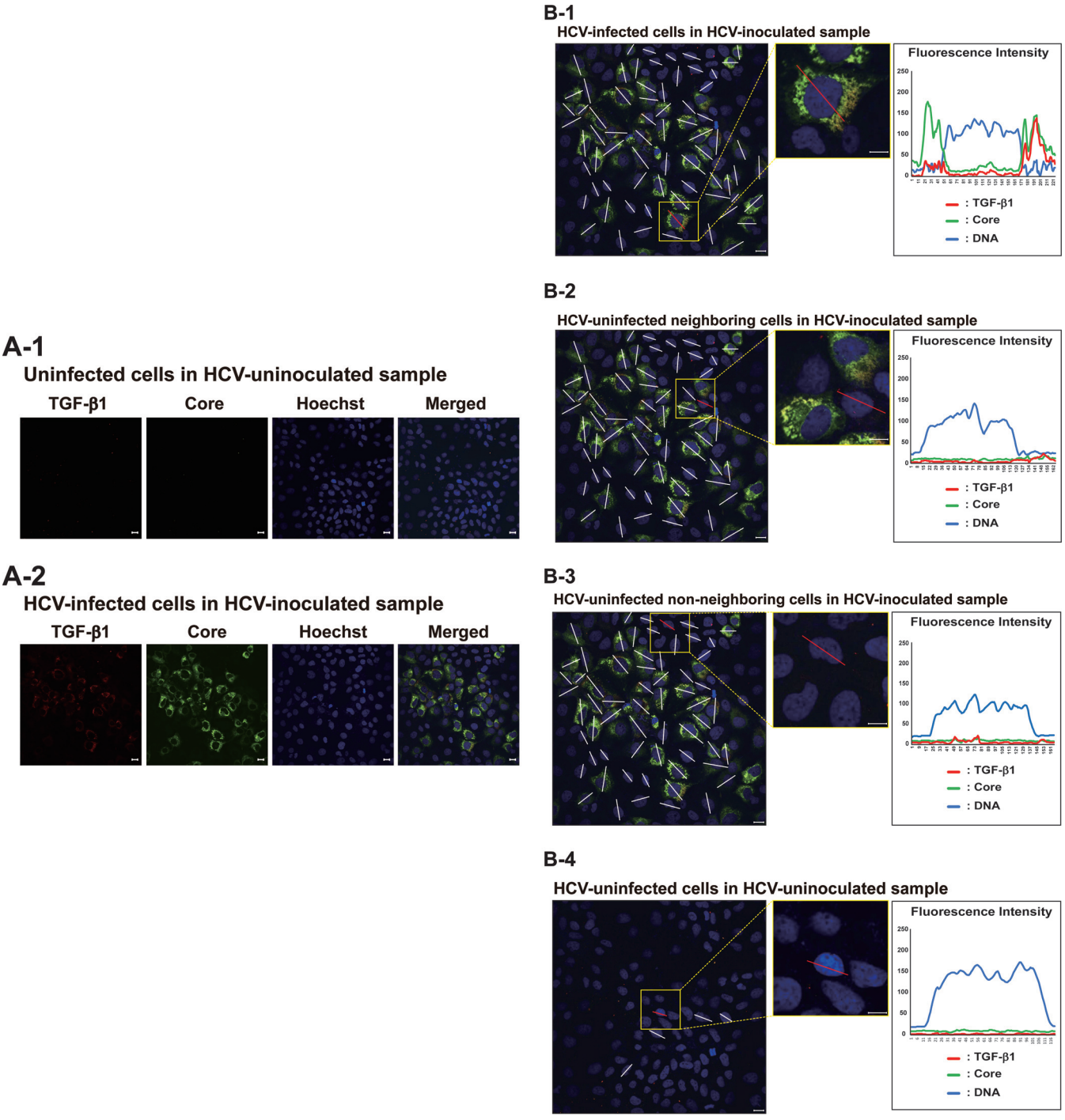

FIG 2 TGF-β1 expression is augmented in HCV-infected cells and their neighboring cells. (A) Huh-7.5.1 cells were inoculated or mock inoculated with HCV (JFH-1; MOI, 0.1) and then cultivated for 3 days. The uninoculated (A-1) and HCV-inoculated (A-2) samples were stained with Hoechst 33342 (blue) and immunostained with antibodies against HCV core (green) and TGF-β1 (red) proteins. Representative fluorescence microscopic images of TGF-β1, HCV core protein (Core), and nuclei (Hoechst) are shown. Merged images are also presented. Scale bar = 10 μm. (B) Quantitative analyses of fluorescence intensities of TGF-β1 and HCV core protein signals in HCV-infected cells (B-1), uninfected neighboring cells (B-2), and nonneighboring cells (B-3) in HCV-inoculated samples and uninfected cells in uninoculated samples (B-4) were performed using MetaMorph software.

infected cells in the HCV-inoculated sample showed RTLs greater than 20-fold (Table 2). The relationship between HCV infection and TGF-β1 production was evaluated by calculating the Pearson correlation coefficient from the MetaMorph analysis results (RTL values) shown in Table 2. The Pearson correlation coefficient value (0.88) indicates that HCV infection and TGF-β1 production are highly correlated. The biochemical and immunocytochemical results indicate that TGF-β1 expression was augmented in HCV-

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TABLE 1 Relative amounts of TGF-β1 protein in various groups of cells[a]

Sample type	Cell category	Relative amt of TGF-β1 (avg)
HCV-inoculated sample	HCV-infected cells	21.8
	Uninfected neighboring cells	5.6
	Uninfected nonneighboring cells	2.8
Uninoculated sample	Uninfected cells	1.0

[a] The amounts of TGF-β1 proteins in the cells were estimated by MetaMorph software using the immunocytochemical data shown in Fig. 2B and immunocytochemical data not shown (total of 134 cells). The amount of TGF-β1 in the uninoculated, uninfected cells was set to 1 in normalization.

TABLE 2 Numbers of cells showing various levels of TGF-β1 in the different groups[a]

Sample type	Cell category	Relative TGF-β1 level (RTL)	Cell no.
HCV-inoculated sample	HCV-infected cells	Subtotal	87
		40 ≤ RTL	5
		30 ≤ RTL < 40	12
		20 ≤ RTL < 30	26
		10 ≤ RTL < 20	29
		5 ≤ RTL < 10	11
		RTL < 5	4
	Uninfected neighboring cells	Subtotal	12
		40 ≤ RTL	0
		30 ≤ RTL < 40	0
		20 ≤ RTL < 30	0
		10 ≤ RTL < 20	1
		5 ≤ RTL < 10	5
		RTL < 5	6
	Uninfected nonneighboring cells	Subtotal	25
		40 ≤ RTL	0
		30 ≤ RTL < 40	0
		20 ≤ RTL < 30	0
		10 ≤ RTL < 20	0
		5 ≤ RTL < 10	1
		RTL < 5	24
Uninoculated	Uninfected cells	Subtotal	10
		40 ≤ RTL	0
		30 ≤ RTL < 40	0
		20 ≤ RTL < 30	0
		10 ≤ RTL < 20	0
		5 ≤ RTL < 10	0
		RTL < 5	10
		Total	134

[a] The amounts of TGF-β1 proteins in the cells were estimated by MetaMorph software using the immunocytochemical data shown in Fig. 2B and data not shown (total of 134 cells). The relative amount of TGF-β1 proteins (RTL) in the cells was calculated by setting the average amount of TGF-β1 proteins in the uninoculated, uninfected cells as 1.

infected cells. Moreover, the elevated RTL of uninfected nonneighboring cells compared with the RTL of uninoculated, uninfected cells suggests that one or more cytokines secreted from HCV-infected cells stimulated expression of TGF-β1 in HCV-uninfected cells in the same culture dish.

HCV-infected liver cells of HCV patients express high levels of TGF-β1. We next investigated whether HCV infection results in overproduction of TGF-β1 protein in the liver cells of HCV patients as it does in the *in vitro* culture system. For this purpose, we performed Western blot analysis with nontumor regions of liver tissues from liver cancer patients with (Fig. 3A, lanes 2 to 6) or without (lane 1 in Fig. 3A) HCV infection. Clinical backgrounds of the specimens are summarized in Materials and Methods. TGF-β1 protein was highly expressed in the nontumor regions of liver tissues of HCV patients. On the other hand, TGF-β1 protein was not detectable from the nontumor region of a liver tissue of a liver cancer patient who was not infected with HCV (Fig. 3A, compare lane 1 with lanes 2 to 6).

In order to investigate TGF-β1 expression in the individual cells of HBV and HCV patients' liver tissues, we performed immunohistochemical studies using nontumor regions of liver tissues of HBV and HCV patients (Fig. 3C). To monitor HCV-infected cells and TGF-β1 levels, we used antibodies against HCV core protein (green) and TGF-β1 (red), respectively. As expected, core-positive signals were detected in HCV patients' tissues (Fig. 3C, panels j, n, and r) but not in HBV patients' tissues (Fig. 3C, panels b and f). Consistent with *in vitro* HCV culture system results, HCV-infected cells in liver tissue from patients expressed high levels of TGF-β1 (Fig. 3C, panels i to t). In contrast, TGF-β1 signals were much weaker in cells negative for HCV core protein (noninfected cells) (Fig. 3C, panels i to t) and were very weak in cells from HBV patients' tissues (Fig. 3C, panels a to h). We estimated the amounts of TGF-β1 and HCV core proteins in the individual cells of HBV and HCV patients using the immunohistochemical data shown in Fig. 3C with MetaMorph software similarly to the cases with Fig. 2B and Tables 1 and 2. In this analysis, we classified the liver cells into three groups: group 1 consisted of HBV-infected cells, which were in the nontumor regions of HBV patients' liver tissues; group 2 consisted of HCV-infected cells, which were in the nontumor regions of HCV patients' liver tissues and showed high levels of HCV core protein; and group 3 consisted of uninfected cells which were in the nontumor regions of HCV patients' liver tissues but did not show HCV core protein. The average amount of TGF-β1 protein in group 2 was ~14-fold higher than that in group 1. We further investigated the relationship between HCV infection and TGF-β1 production by calculating the Pearson correlation coefficient using the estimated amounts of TGF-β1 and HCV core proteins in the immunohistochemical data. The Pearson correlation coefficient (0.94) indicates that HCV infection and TGF-β1 production were very highly correlated. These results suggest that one or more HCV proteins stimulated the production and/or stabilization of TGF-β1 protein in the HCV-infected cells of liver tissue from HCV patients.

HCV core and E2 proteins enhance TGF-β1 secretion by HCV replicon-containing cells. Results from our *in vitro* HCV culture system and our investigation of patients' liver tissues showed that HCV-infected hepatocytes produced more TGF-β1 protein than uninfected cells (Fig. 1 to 3). To understand the molecular basis of the elevated TGF-β1 expression in HCV-infected cells, we sought to identify the HCV protein(s) responsible for the overproduction of TGF-β1. In order to mimic HCV infection, we used various HCV replicons expressing various structural proteins of HCV (core, E1, and E2). The replicons Core(UAA), E1(UAA), and E2(UAA) direct expression of core, core plus E1,

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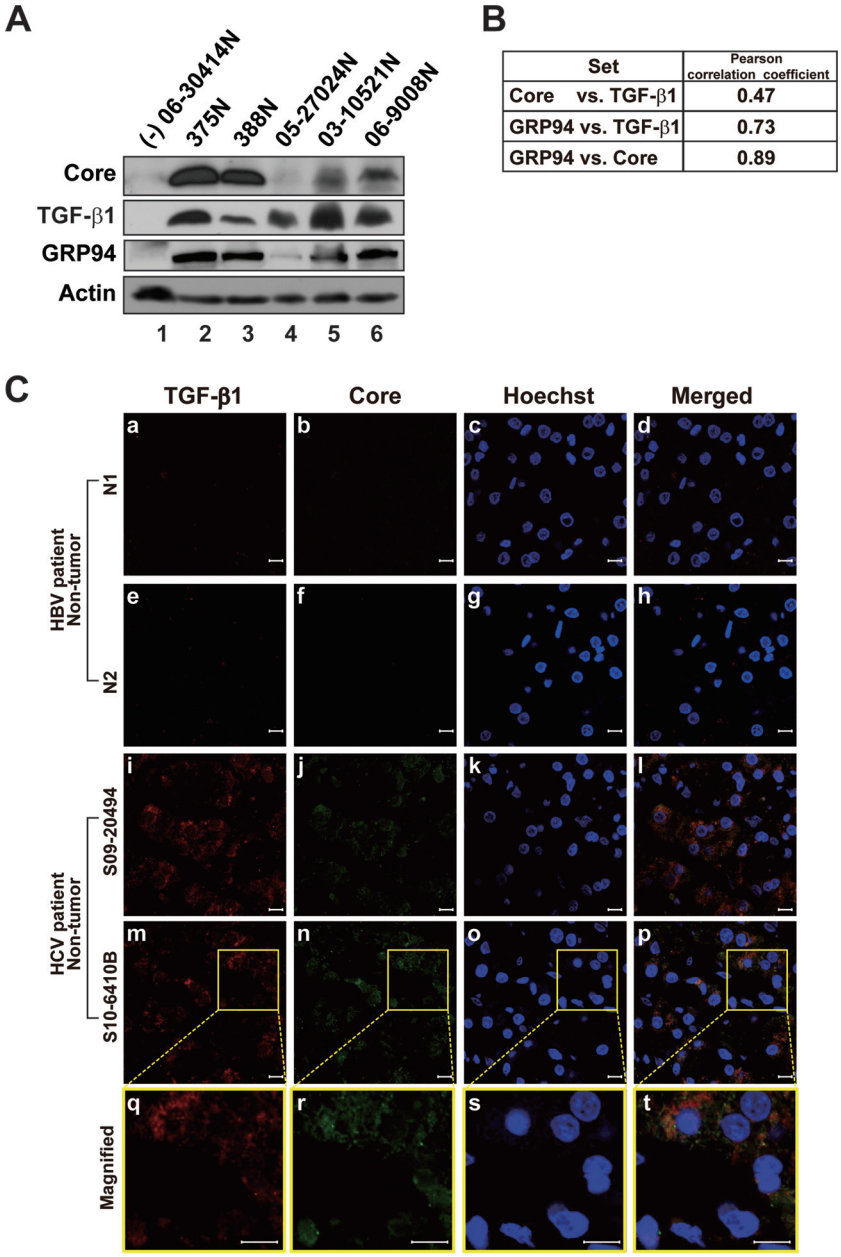

FIG 3 HCV-infected liver cells of HCV patients express high levels of TGF-β1 protein. (A) Protein levels of HCV core, TGF-β1, GRP94, and β-actin were analyzed by Western blotting using nontumor regions of liver tissues from liver cancer patients with (lanes 2 to 6) or without (lane 1) infection of HCV. (B) Pearson correlation coefficients of protein levels of HCV core, TGF-β1, and GRP94 in the liver tissues were calculated by using the protein band intensities shown in panel A. (C) Nontumor regions of liver tissues from HBV patients (N1 and N2) and HCV patients (s09-20494 and s10-6410-B) were stained with Hoechst 33342 (blue) or immunostained with antibodies against HCV core (green) and TGF-β1 (red) proteins. Representative fluorescence microscopic images of TGF-β1, HCV core protein (Core), and nuclei (Hoechst) are shown. Merged images are also presented. Scale bar = 10 μm.

and core plus E1 plus E2, respectively, together with nonstructural proteins NS3 to NS5A, as described by Lee et al. (25) (Fig. 4A); the replicon +2 C399T expresses only nonstructural proteins. The replicon +2 C399T showed reduced levels of TGF-β1 protein in the culture media compared to the culture media of control Huh-7 cells (Fig. 4B, compare bar 2 with bar 1). This indicates that nonstructural protein(s) of HCV inhibits expression of TGF-β1 by host cells (see Discussion). Huh-7 cells containing replicons Core(UAA) and E1(UAA) restored TGF-β1 levels to values comparable to those in control Huh-7 cells (Fig. 4B, compare bars 3 and 4 with bar 1). This result indicates that HCV core contributes to secretion of TGF-β1. This result is consistent with a previous report suggesting that the HCV core augments TGF-β1 through ROS generation (21). Interestingly, TGF-β1 levels in the media were greatly increased by the replicon E2(UAA), which produces HCV E2 (Fig. 4B, lane 5). This suggests that HCV E2 augments TGF-β1 expression via an unknown mechanism. To determine whether HCV E2 is capable of enhancing TGF-β1 expression

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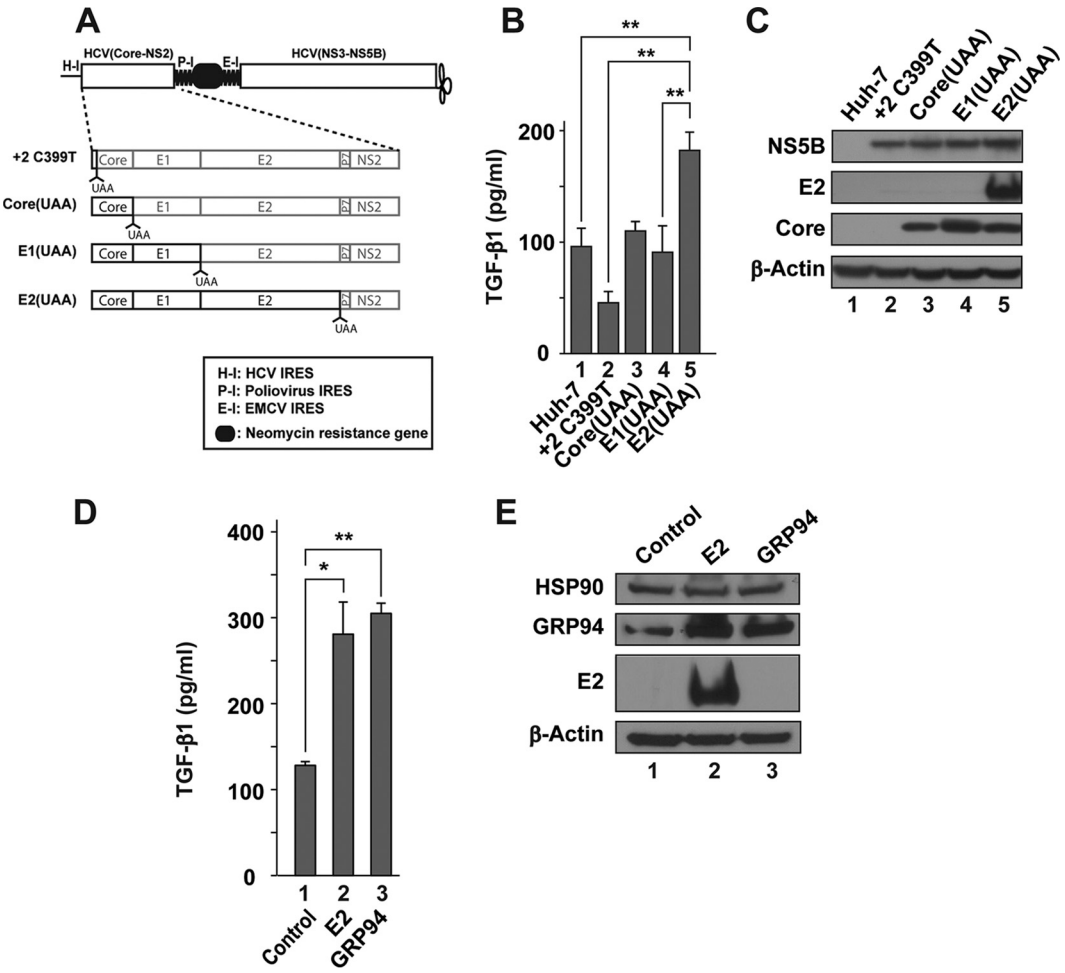

FIG 4 HCV E2 augments secretion of TGF-β1. (A) Schematic diagram of HCV replicons used in this study. Replicon +2 C399T expresses only HCV nonstructural proteins NS3 to NS5B. Replicons Core(UAA), E1(UAA), and E2(UAA) express core, core plus E1, and core plus E1 plus E2, respectively, in addition to the nonstructural proteins NS3 to NS5B (25). (B) TGF-β1 levels in culture media of Huh-7 cells containing various HCV replicons were examined by ELISA. Replicon-containing cells (7×10^5) were seeded on 100-mm dishes and cultured for 12 h. The media were replaced with serum-free DMEM, and the cells were cultured for an additional 24 h. Culture media were collected and analyzed by ELISA after being filtered through a 0.2-μm-pore filter. (C) The levels of HCV NS5B, E2, and core proteins in control Huh-7 cells (lane 1) and Huh-7 cells containing replicon +2 C399U (lane 2), Core(UAA) (lane 3), E1(UAA) (lane 4), or E2(UAA) (lane 5) were determined by Western blotting analyses with anti-NS5B, anti-E2, anti-core, and anti-β-actin antibodies. (D) TGF-β1 levels in culture media of control Huh-7 cells (hygromycin-selected cells) and Huh-7 cells expressing HCV E2 or GRP94 were examined by ELISA, as described for panel B. (E) Levels of HSP90, GRP94, HCV E2, and β-actin proteins in control cells (lane 1), E2-overproducing cells (lane 2), and GRP94-overproducing cells (lane 3) were analyzed by Western blotting with anti-HSP90, anti-GRP94, anti-E2, and anti-β-actin antibodies. All data are presented as means and standard deviations (bars and error bars, respectively) from three independent experiments.

without the help of other HCV proteins, we used a Huh-7 cell line that expresses HCV E2. HCV E2-expressing Huh-7 cells exhibited greatly increased TGF-β1 secretion (Fig. 4D), suggesting that HCV E2 alone is able to mediate increased TGF-β1 production.

GRP94 activity is required for HCV E2-mediated overproduction of TGF-β1. We investigated the mechanism underlying TGF-β1 overproduction by HCV E2. Previously, we reported that HCV E2 activates the nuclear factor κB (NF-κB) signaling pathway through overproduction of GRP94, which is a molecular chaperone that functions in processing and transporting secretory proteins (26). Notably, ectopic expression of HCV E2 in Huh-7 cells strongly augmented the expression of GRP94 but did not affect the expression of HSP90, a cytosolic homologue of GRP94 (Fig. 4E). In addition, overproduction of GRP94 was observed in the Huh-7.5.1 cells infected with HCV (Fig. 1C and 5E) and the liver tissues of HCV patients (Fig. 3A).

We next examined whether GRP94 overproduction triggered by HCV E2 contributed to the augmented TGF-β1 secretion. Indeed, GRP94-overproducing cells exhibited elevated TGF-β1 secretion in the absence of E2 protein (Fig. 4D, bar 3). Moreover, depletion of GRP94 using a small interfering RNA (siRNA) reduced TGF-β1 secretion by Huh-7 cells ectopically expressing HCV E2 (Fig. 5A, bar 4) or GRP94 (Fig. 5B, bar 4). Notably, TGF-β1 secretion by control Huh-7 cells was reduced by depletion of GRP94 (Fig. 5A and B, bars 2), indicating that the rather high basal secretion of TGF-β1 from control Huh-7 cells is attributable, at least in part, to the high level of GRP94 in these cells.

Depletion of GRP94 by an siRNA against GRP94 reduced TGF-β1 secretion by Huh-7 cells containing HCV E2(UAA) replicon (data not shown). Also, depletion of GRP94 reduced production of pro-TGF-β1 by HCV-infected cells (Fig. 5E). Moreover, strong relationships among HCV infection, GRP94, and

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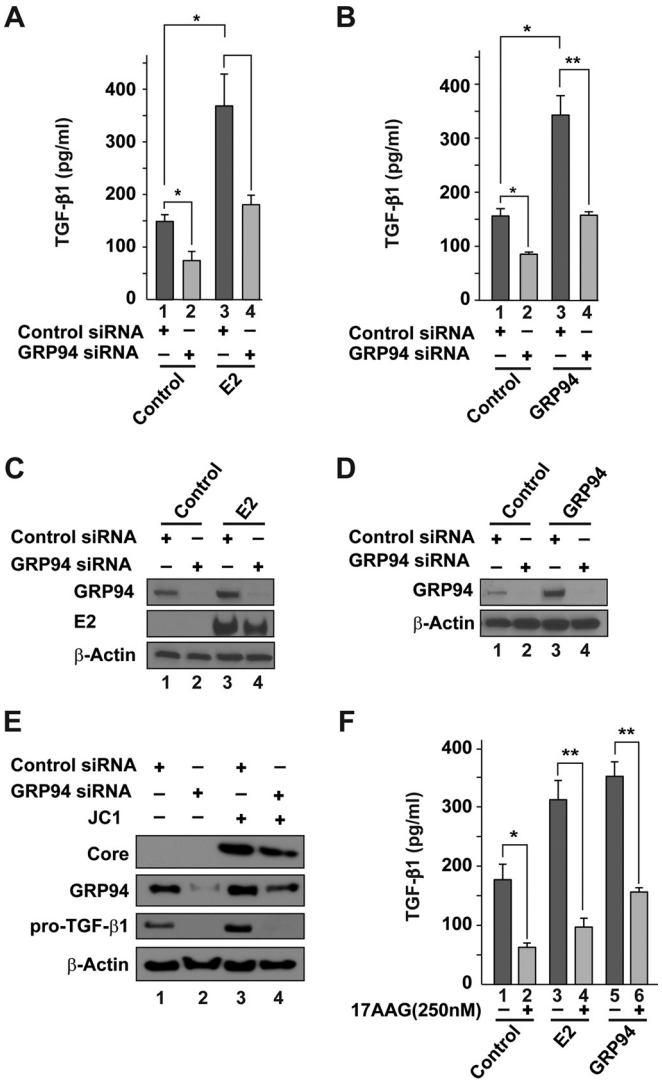

FIG 5 Overproduction of GRP94 is required for E2-dependent TGF-β1 production. (A) Effect of GRP94 knockdown on the secretion of TGF-β1 from control Huh-7 cells (bars 1 and 2) and Huh-7 cells ectopically expressing HCV E2 (bars 3 and 4). Cells were seeded in 12-well plates at 4×10^4 cells/well, cultured for 12 h, and then transfected with an siRNA against GRP94 using Oligofectamine and cultured for 48 h. After culturing of cells in serum-free media for an additional 24 h, TGF-β1 levels in culture media were examined by ELISA. (B) The effect of GRP94 knockdown on the secretion of TGF-β1 from the Huh-7 cells ectopically expressing GRP94 (bars 3 and 4) was determined as described for panel A. (C and D) GRP94 siRNA knockdown efficiencies were monitored by Western blotting analyses. (C) Control (lanes 1 and 2) and HCV E2-overproducing (lanes 3 and 4) Huh-7 cells described for panel A were analyzed by Western blotting with anti-GRP94, anti-HCV E2, and anti-β-actin antibodies. (D) Control (lanes 1 and 2) and GRP94-overproducing (lanes 3 and 4) Huh-7 cells described for panel B were analyzed by Western blotting with anti-GRP94 and anti-β-actin antibodies. (E) Effect of GRP94 knockdown on the production of pro-TGF-β1 by HCV-infected cells. Huh-7.5.1 cells were inoculated with HCV (JC1; MOI, 0.1) and cultivated for 4 weeks. HCV-infected and mock-infected cells were seeded on 12-well plates (5×10^4 cells/well) and cultivated for 12 h, and then the cells were transfected with an siRNA against GRP94 using Oligofectamine. The cells were further cultivated for 72 h, and then the levels of HCV core, GRP94, pro-TGF-β1, and β-actin proteins in the cells were examined by Western blotting using corresponding antibodies. (F) Effect of the GRP94 inhibitor 17-AAG on the production of TGF-β1 by Huh-7 cells ectopically expressing GRP94 or HCV E2. Cells were seeded on 12-well plates at 5×10^4 cells/well and cultured for 12 h. After treatment with 17-AAG (250 nM) in DMEM containing 10% FBS for 12 h, cells were cultured in serum-free DMEM with 17-AAG (250 nM) for an additional 24 h. The culture media were collected, filtered through a 0.2-μm-pore filter, and analyzed by ELISA. All data are presented as means and standard deviations (bars and error bars, respectively) from three independent experiments.

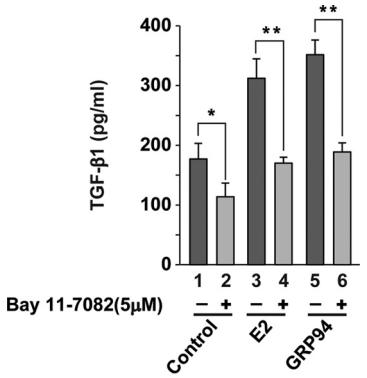

FIG 6 Activation of NF-κB is required for E2-dependent TGF-β1 production. Effects of the NF-κB inhibitor Bay 11-7082 on the production of TGF-β1 by Huh-7 cells ectopically expressing HCV E2 or GRP94 were analyzed. Cells were seeded on 12-well plates at 5×10^4 cells/well and cultured for 12 h. Media were replaced with DMEM containing 10% FBS and Bay 11-7082 (5 μM), and cells were incubated for 12 h. After culturing of cells in serum-free media containing Bay 11-7082 (5 μM) for an additional 24 h, TGF-β1 levels in culture media were examined by ELISA. Data are presented as means and standard deviations (bars and error bars, respectively) from three independent experiments.

TGF-β1 in the human liver are also indicated by the high Pearson correlation coefficients among these items (Fig. 3B). Taken together, these data strongly suggest that HCV E2 triggers expression of TGF-β1 through overproduction of GRP94.

We further investigated the role of GRP94 activity in TGF-β1 secretion using 17-allylamino-demethoxygeldamycin (17-AAG), an inhibitor of GRP94 that blocks the activities of GRP94 and HSP90. Treatment with 17-AAG (250 nM) reduced secretion of TGF-β1 protein by control Huh-7 cells as well as Huh-7 cells ectopically expressing either E2 or GRP94 (Fig. 5F, bars 2, 4, and 6). Moreover, treatment of 17-AAG (250 nM) reduced TGF-β1 secretion by Huh-7 cells containing the HCV E2(UAA) replicon (data not shown). These results indicate that GRP94 activity is required for high levels of TGF-β1 secretion by HCV-infected cells.

Activation of NF-κB is required for the enhanced secretion of TGF-β1 protein by HCV-infected hepatocytes. Previously, we reported that GRP94 overproduction by HCV infection enhances the expression of anti-apoptotic proteins in an NF-κB-dependent manner (26). Moreover, Lin et al. reported that TGF-β1 production is increased by HCV infection via NF-κB activation (21). On the basis of these previous observations, we investigated whether the augmented secretion of TGF-β1 proteins by HCV E2 is mediated by NF-κB activation. To this end, we examined the effect of the IKKα/β inhibitor Bay 11-7082 on TGF-β1 secretion by Huh-7 cells overproducing HCV E2 or GRP94 (Fig. 6). Treatment with Bay 11-7082 decreased TGF-β1 secretion by both E2- and GRP94-overproducing cells (Fig. 6). Moreover, treatment with Bay 11-7082 decreased TGF-β1 secretion by Huh-7 cells containing the HCV

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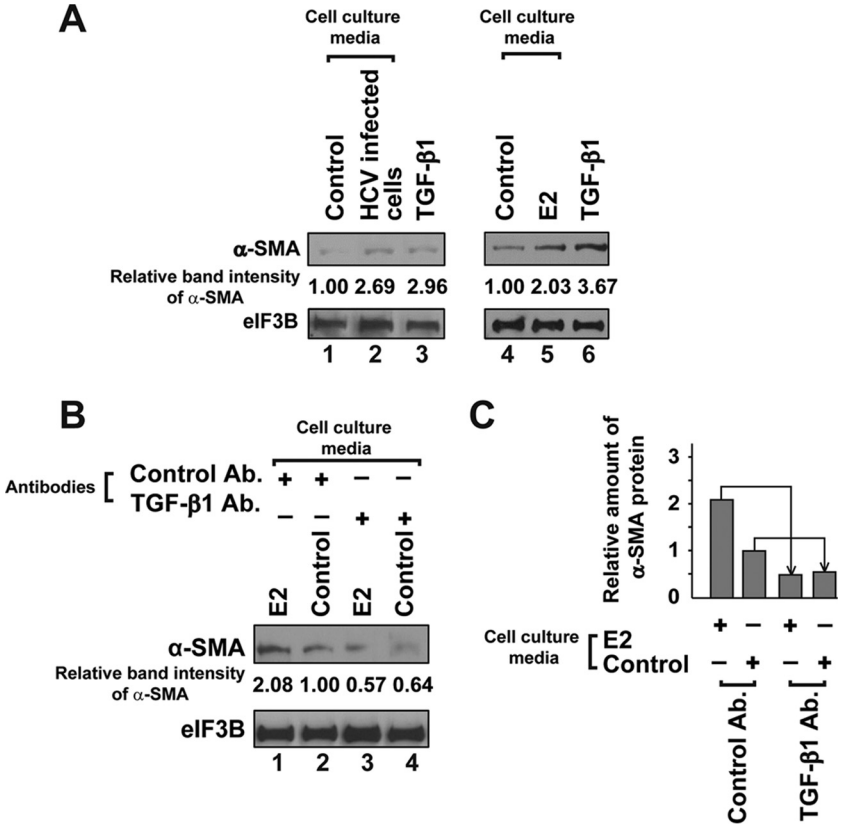

FIG 7 TGF-β1 secreted by HCV E2-overproducing cells activates primary HSCs. (A) HSCs isolated from Sprague-Dawley rats were incubated in DMEM containing 3% FBS for 3 days. HSCs were then seeded on 6-well plates at 8×10^4 cells/well and cultured for 12 h. After culturing of cells for 24 h in serum-free media, media were replaced with culture media from control Huh-7.5.1 cells (lane 1) or HCV-infected Huh-7.5.1 cells (lanes 2) or with serum-free media supplemented with 2 ng/ml of recombinant human TGF-β1 (lane 3), and HSCs were incubated for an additional 10 h. HSCs were then harvested, and levels of α-SMA (a marker of HSC activation) and eukaryotic initiation factor 3B (eIF3B; control) were monitored by Western blotting. Similar experiments were performed using culture media from control Huh-7 cells (lane 4), HCV E2-overproducing cells (lane 5), and serum-free media supplemented with 2 ng/ml of recombinant human TGF-β1 (lane 6). The relative amounts of α-SMA in cells normalized to those of eIF3B are depicted at the top. (B) Isolated HSCs were incubated in DMEM containing 3% FBS for 3 days, after which they were seeded on 6-well plates at 5×10^4 cells/well and cultured for 12 h. After culturing of cells for 24 h in serum-free media, media were replaced with culture media from control Huh-7 cells (lanes 2 and 4) or HCV E2-overproducing cells (lanes 1 and 3), and cells were incubated for an additional 10 h. Culture media were pretreated with either a control mouse antibody (lane 1 and 2) or neutralizing anti-TGF-β1 antibody (lanes 3 and 4) at 4°C for 4 h before culturing of HSCs. The cells were harvested, and levels of α-SMA and eIF3B were monitored by Western blotting. The relative amounts of α-SMA in cells normalized to those of eIF3B are depicted on top of the panel. The same experiment was performed independently two times. (C) Relative amount of α-SMA in HSCs from panel B, depicted graphically.

E2(UAA) replicon (data not shown). These results indicate that NF-κB activation is involved in HCV E2-dependent TGF-β1 secretion, although additional research, currently ongoing in our laboratory, will be necessary to establish the mechanistic details.

TGF-β1 protein secreted by HCV-infected cells activates primary HSCs. Activated HSCs play a key role in liver fibrosis, and TGF-β1 is the major cytokine involved in activating HSCs. Accordingly, we investigated whether TGF-β1 protein secreted by HCV-infected cells and HCV E2-overproducing cells is sufficient to activate HSCs. For this purpose, we isolated rat primary HSCs using a perfusion and collagenase digestion method (28). Using these HSCs, we examined the effects of culture media from HCV-infected cells or HCV E2-overproducing cells on HSC activation by Western blotting for α-smooth muscle actin (α-SMA), an indicator of HSC activation (30). Treatment with culture media (5-fold diluted) from HCV-infected Huh-7.5.1 cells increased α-SMA in primary HSCs about 3-fold compared with that in primary HSCs treated with control uninfected Huh-7.5.1 cell culture media (Fig. 7A, compare lane 2 with lane 1). The level of primary HSC activation was comparable with the activation of the cells by stimulation with TGF-β1 protein at a final concentration of 2 ng/ml (Fig. 7A, lane 3). Treatment with culture media from Huh-7 cells expressing HCV E2 (5-fold diluted) also activated primary HSCs (Fig. 7A, lanes 4 to 6).

In order to test whether TGF-β1 protein secreted by Huh-7 cells overproducing HCV E2 is the major cytokine that activates HSCs, we examined the effect of a TGF-β1-neutralizing antibody. Addition of a TGF-β1-neutralizing antibody to culture media from both control cells and HCV E2-overproducing cells reduced the levels of α-SMA in primary HSCs (Fig. 7B). Importantly, the level of α-SMA was reduced to the same level as in the control cells after treatment of the TGF-β1 antibody (Fig. 7B and C). This clearly demonstrates that TGF-β1 is the key cytokine activating HSCs by the HCV E2. Taken together, these results indicate that

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the amount of TGF-β1 protein produced by HCV infection is sufficient to activate HSCs.

DISCUSSION

HCV infection is closely associated with liver fibrosis, a major risk factor related to fatal liver diseases, such as liver cirrhosis and HCC (31). TGF-β1 is the most important cytokine involved in triggering liver fibrosis. It has been suggested that Kupffer cells and liver-infiltrating lymphocytes are the major sources of TGF-β1 protein (32, 33). However, a number of reports have suggested that HCV-infected hepatocytes could be the original source of TGF-β1 protein (21, 34, 35). In this study, we monitored the effect of HCV infection on the production of TGF-β1 in individual hepatocytes for the first time using an immunocytochemical method. We found that HCV-infected cells expressed the highest levels of TGF-β1 but that cells neighboring HCV-infected cells also produced high levels of TGF-β1 (Fig. 2 and Tables 1 and 2). Moreover, TGF-β1 levels in uninfected, nonneighboring cells on the same culture dish were higher than those in cells on an uninoculated culture dish. These results indicate that one or more HCV protein(s) triggers the production of TGF-β1 in HCV-infected cells and further suggest that protein(s) secreted from HCV-infected cells acts in a paracrine manner to induce TGF-β1 production in uninfected cells on the same dish (see below). Importantly, high levels of TGF-β1 protein were also observed in HCV-infected hepatocytes in liver tissue from HCV patients (Fig. 3), suggesting that the strong expression of TGF-β1 in HCV-infected hepatocytes occurs under physiological conditions.

Elevated production of TGF-β1 mRNA and pro-TGF-β1 protein was observed as early as 2 days after infection with HCV (Fig. 1B and C). On the other hand, a detectable increase of TGF-β1 proteins secreted in the media was observed only at 3 weeks after infection with HCV (Fig. 1A). The delayed secretion of TGF-β1 may be attributable in part to the complex processes of proteolytic cleavage and secretory pathway of TGF-β1 (36, 37). Interestingly, it was reported that HCV infection reduces the expression of proprotein convertase subtilisin/kexin type 9 (PCSK9) (38), which is also known to mediate the proteolytic processing of TGF-β1 (39). Therefore, it is likely that the secretion of TGF-β1 is modulated after the production of pro-TGF-β1 by HCV infection. The regulatory mechanism of TGF-β1 secretion from the HCV-infected cells remains to be elucidated.

In an effort to understand the molecular basis of TGF-β1 production by HCV infection, we analyzed the effects of HCV structural proteins on TGF-β1 production. To minimize artifacts resulting from ectopic production of a heterologous gene in a cell line, we used various HCV replicon-containing cells that mimic the HCV infection state of hepatocytes. These replicons direct expression of nonstructural proteins NS3 to NS5B and a limited number of HCV structural proteins (none, core, core plus E1, or core plus E1 plus E2) (Fig. 4A). To our surprise, TGF-β1 production by Huh-7 cells was reduced in the presence of the subgenomic replicon expressing only the nonstructural proteins NS3 to NS5B (Fig. 4B). This indicates that one or more of the HCV nonstructural proteins inhibit TGF-β1 production. We speculate that NS5A is a potential candidate inhibitory viral protein, since it inhibits expression of the transcription activator AP-1 (23), which is responsible for promoting TGF-β1 expression. NS5A may also contribute to TGF-β1 reduction by inhibiting TGF-β receptor function through protein-protein interactions (22).

The reduced production of TGF-β1 by HCV nonstructural protein(s) was restored to the original level by coexpression of nonstructural proteins with core protein or core plus E1 (Fig. 4B). This indicates that HCV core protein augments TGF-β1 production, consistent with previous reports by Shin et al. (19) and Taniguchi et al. (20). The generation of ROS by core protein could be the underlying mechanism of TGF-β1 production by the core protein (21).

Notably, we found that HCV E2 further enhanced the secretion of TGF-β1 from the cells containing the HCV replicon E2(UAA), which expresses E2 in addition to core and E1 proteins and nonstructural proteins (Fig. 4B). This suggests that HCV E2 mediated the enhanced TGF-β1 production by HCV-infected cells. To test this, we generated a Huh-7 cell line ectopically expressing only E2 and found that expression of HCV E2 is sufficient to trigger overproduction of TGF-β1. To elucidate the mechanism underlying E2-mediated TGF-β1 production, we investigated the role of GRP94, since E2 is known to trigger overproduction of GRP94 (26). Several lines of evidence indicated that GRP94 is responsible for the E2-mediated TGF-β1 production: (i) ectopic expression of GRP94 in Huh-7 cells induced production of TGF-β1 (Fig. 4D), (ii) knockdown of GRP94 reduced production of TGF-β1 by both control Huh-7 cells and E2-expressing Huh-7 cells (Fig. 5A), (iii) TGF-β1 production was reduced by a chemical inhibitor (17-AAG) of GRP94 (Fig. 5F), (iv) depletion of GRP94 reduced production of pro-TGF-β1 by HCV-infected cells (Fig. 5E), and (v) the levels of GRP94 and TGF-β1 were highly correlated in the human liver tissues (Fig. 3A and B). On the basis of these observations, we conclude that HCV E2 augments TGF-β1 production by triggering overproduction of GRP94.

It is not fully understood how the overproduction of GRP94 induces TGF-β1 expression. However, we previously showed that overproduction of GRP94 activates the NF-κB signaling pathway, which results in production of anti-apoptotic proteins (26). In this study, using an inhibitor of NF-κB signal transduction, we demonstrated that NF-κB activation is involved in mediating TGF-β1 expression, providing a clue to the molecular mechanism linking GRP94 to TGF-β1 production (Fig. 6). Consistent with this result, it has been shown that NF-κB activation is involved in TGF-β1 production in hepatocytes (21). Moreover, Chiao and colleagues have shown that NF-κB activation enhances AP-1 activation in murine embryonic fibroblasts (MEF) (40), and Sullivan and coworkers have suggested that increased c-Fos expression and c-Jun phosphorylation triggered by tumor necrosis factor alpha (TNF-α) constitute the mechanism underlying TNF-α–induced TGF-β1 expression in lung fibroblasts (24). c-Jun and c-Fos proteins are components of AP-1 (24), which is a transcription factor that enhances TGF-β1 expression (41). Indeed, we also observed that HCV E2-mediated overproduction of GRP94 triggers TNF-α secretion through the activation of NF-κB (unpublished data) and that treatment of Huh-7 cells with TNF-α induces TGF-β1 production (data not shown). Moreover, we have found that TGF-β1 secretion by Huh-7 cells expressing E2 or GRP94 is decreased by addition of a TNF-α-neutralizing antibody to the culture media (data not shown). Therefore, we speculate that E2 in HCV-infected cells indirectly induces secretion of TNF-α, and this cytokine, in turn, triggers TGF-β1 production by HCV-infected cells and neighboring cells in an autocrine or paracrine manner.

What would be the physiological consequence of TGF-β1 pro-

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duction by HCV-infected hepatocytes apart from its role in the pathogenesis of hepatic fibrosis? Studies have revealed that patients with chronic HCV have higher levels of regulatory T (Treg) cells—which play important roles in immunological tolerance and viral persistence (42, 43)—in the peripheral blood compared with healthy control subjects or patients whose HCV infection spontaneously resolves (44, 45). Moreover, Treg cells have been shown to suppress the proliferation of HCV-specific cytotoxic T lymphocytes during persistent HCV infection (46, 47). There have also been reports that HCV-infected hepatocytes directly induce the development of Treg cells, which contribute to the impaired host T-cell responses (34, 48). Considering that TGF-β plays a pivotal role in the generation of Treg cells from precursor cells (49), we speculate that TGF-β1 produced by HCV core and E2 proteins contributes to the induction of Treg cells and the induction of immune tolerance against the HCV proteins.

In conclusion, as a result of our efforts to elucidate the molecular basis of the pathogenesis of hepatic fibrosis caused by HCV infection, we have uncovered a novel mechanism of TGF-β1 induction. We found that the cellular protein GRP94, which directly interacts with HCV E2, plays an important role in TGF-β1 induction, suggesting that GRP94 is a potential target for the development of drugs that prevent hepatic fibrosis caused by HCV infection. Moreover, such a GRP94-inhibiting drug would also likely boost immunity against HCV infection by blocking the induction of Treg cells, which direct the immune tolerance against HCV.

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