HCV core protein promotes liver fibrogenesis *via* up-regulation of CTGF with TGF-β1

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Abstract

Liver cirrhosis is one of the major complications of hepatitis C virus (HCV) infection, but the mechanisms underlying HCV-related fibrogenesis are still not clear. Although the roles of HCV core protein remain poorly understood, it is supposed to play an important role in the regulation of cellular growth and hepatocarcinogenesis. The aim of this study was to examine the role of HCV core protein on the hepatic fibrogenesis. We established an *in vitro* co-culture system with primary hepatic stellate cell (HSC) isolated from rats, and a stable HepG2-HCV core cell line which had been transfected with HCV core gene. The expressions of fibrosis-related molecules trans- forming growth factor β1 (TGF-β1), transforming growth factor β receptor II (TGFβRII), α-smooth muscle actin (α-SMA) and connective tissue growth factor (CTGF) were analyzed *via* histo- logical or molecular methods. In addition, the ex- pression levels of matrix metaloprotinase-2 (MMP- 2) and collagen type I (Col I) from the co-cultured media were measured by zymogram and ELISA, respectively. The expressions of α-SMA, TGF-β1, Col I, TGFβRII and MMP-2 were significantly in- creased in the co-culture of stable HepG2-HCV core with HSC. Moreover, the significant increases of CTGF and TGF-β1 in the HCV core- expressing cells were observed by either Northern or Wes- tern blot analysis. These results suggest that HCV core protein may contribute to the hepatic fibro- genesis *via* up-regulation of CTGF and TGF-β1.

Keywords: connective tissue growth factor; fibroge- nesis; hepatitis C virus; liver cirrhosis; stellate cell

Introduction

Hepatitis C virus (HCV) infection, one of the major public health problems worldwide, causes progressive liver disease that may end in liver cirrhosis and, eventually, in hepatocellular carcinoma. HCV is a positive-stranded RNA virus whose genome contains a single open reading frame (ORF) encoding poly-peptides of 3,010 - 3,011 amino acids (Choo *et al.,* 1991; Ogata *et al.,* 1991).

Earlier studies have shown that HCV core protein regulate cellular events and induce hepatocarcino-genesis (Moriya *et al.,* 1998; Okuda *et al.,* 2002). We previously established a stable HepG2-core cell line which was permanently transfected with HCV core gene into HepG2 cells, and we observed that the HCV core protein play a key role in the signal trans-duction of apoptosis (Yang *et al.,* 2002; Hosui *et al.,* 2003). However, hepatic fibrogenesis relation to HCV core protein is not well understood to date due to the lack of an efficient cell culture system for HCV (Brass *et al.,* 2002).

When the liver is injured by any cause, the liver cells including proper hepatocytes, Kuffer cells, sinu-soidal endothelial cells and stellate cells secret va-rious cytokines, hormones or oxygen free radicals, leading to hepatic damage, apoptosis or regeneration (Neuman *et al.,* 2001). Among them, transforming growth factor β1 (TGF-β1) is known to play a key role in the fibrogenic process. Liver injury induces stellate cell activation leading to up-regulation of TGF-β1,

which stimulates the production and deposition of extracellular matrix (ECM), resulting in liver fibrosis (Bissell, 2001). Connective tissue growth factor (CTGF), multi-functional matricellular protein, is shown to act as an essential mediator in the hepatic fibrogenesis by stimulating ECM production (Rachfal and Brigstock, 2003). In addition, matrix metalloproteinase 2 (MMP-2), which degrades the normal subendothelial matrix, is up-regulated by activated hepatic stellate cell (HSC), which results in the destruction of collagen type IV in the basement membrane, and is then replaced with fibril collagen (Karelina *et al.,* 2000).

Because the mechanisms of hepatic fibrogenesis by HCV have been mainly demonstrated *via in vivo* study (Schuppan *et al.,* 2003), it is not clear which HCV protein directly influences the process of fibrosis. For this reason, in the present study, we investigated whether HCV core protein plays a direct role in fibrogenesis or not, using an *in vitro* co-culture system which is composed of a stable HepG2-core cell line and primary HSC. To more clarify the mechanism of fibrogenesis *in vitro*, we analyzed the expression of various proteins, growth factors and receptors including α-smooth muscle actin (α-SMA), MMP-2, collagen type I (Col I), TGF-β1, CTGF, and transforming growth factor β receptor II (TGFβRII) (Nelson *et al.,* 1997; Reinehr *et al.,* 1998; Giannelli *et al.,* 2002; Leask *et al.,* 2002; Qin *et al.,* 2002).

Materials and Methods

Isolation of primary HSC and cell culture

HSC were isolated from the liver of six-week old Sprague-Dawley male rats by the collagenase perfusion two-step method described previously (Friedman, 1993). Liver isolates were centrifuged for purification by 15% nycodenz[R] (Sigma, St. Louis, MO) density gradient separation. Extracted HSC was plated on an uncoated 100-mm culture dish and maintained with conditioned media composed of Williams' E and F-12 media supplemented with 10% fetal calf serum (FCS, Invitrogen, Carlsbad), standard antibiotics, 1 mM non-essential amino acid, 1 mM sodium pyruvate and 0.5 µM hydrocortisone hemisuccinate (Sigma) (Ferrini *et al.,* 1997; Castet *et al.,* 2002). After confirming a purity level of more than 95% by endogenous vitamin A fluorescence, HSC was characterized by immunocytochemistry to analyse myofibroblasts. A HepG2 cell line purchased from ATCC (American Type Culture Collection, Manassas, VA) was cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37°C in a humidified chamber of 5% CO_2. A stable HepG2-core cell line, such as C18, which contained a transfected HCV core gene in HepG2 cell line (gifted by Dr. Sung, Pohang University, Korea) was cultured in DMEM containing 200 µg/ml G-418 (Invitrogen).

In vitro co-culture system of a stable HepG2-core cell line with HSC

To elucidate the relationship between HCV core protein and hepatic fibrosis, we established two-way *in vitro* co-culture systems of a stable HepG2-core cell line with HSC. One is cell-to-cell contacted co-culture system composed of a mixture of two different cell lines designed for morphological analysis, and the other is cell-to-cell non-contacted co-culture system for molecular evaluation (Harimoto *et al.,* 2002; Uyama *et al.,* 2002). In the cell-to-cell non-contacted co-culture system, a stable HepG2-core cell line was separated from HSC by a 0.2 - µm Anopore semi-permeable membrane (Nalge Nunc. Naperville, IL). In the mixed co-culture, plating of HSC at 1.5×10^5 cells per Lab Tek II eight-chamber slide is followed by stable HepG2-core cells or HepG2 cells as a negative control at the same number of cells in different chamber slide for immunocytochemistry. Following 24 h culture in DMEM supplemented with 10% FBS, the cells were washed with phosphate buffer saline (PBS) and cultured for another 24 h in serum-free DMEM. After culture, the media was collected and stored at -20°C until use.

Enzyme linked-immunosorbent assay (ELISA)

To quantify the expression levels of TGF-β1 and Col I in single culture and co-culture, ELISA from culture media was performed over time. In brief, each sample from culture media was dispensed into 96-well microtiter plates (Nalge Nunc) with coating buffer (50 mM carbonate, 1.59 g/L Na_2CO_3 and 2.93 g/L $NaHCO_3$, pH 9.6) and incubated at 4°C overnight. The plates were then rinsed with PBS with 0.05% Tween-20 (PBST), reacted firstly with specific antibodies against TGF-β1 and Col I at 37°C for 1 h and secondly with supplied horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Zymed, San Francisco; diluted at 1:3,000).

After being washed three times with PBST, the plates were developed by incubation for 30 min at room temperature with 3,3', 5,5-tetramethyl benzidine (TMB) as the substrate. Reaction was stopped by 1 M H_2SO_4 and the absorbance at 450 nm was determined on a microplate reader. The experiment was independently performed three times.

Immunocytochemistry

For the immunocytochemical staining, the mixed cells of HSC with stable HepG2-core or HepG2 were fixed in pre-cold methanol at -20°C for 15 min and dried at room temperature. The fixed cells were rehydrated in PBS for 10 min and blocking was performed with 2% bovine serum albumin (BSA) in PBS for 1 h (Peehl *et al.,* 1997). The primary monoclonal antibodies against α-SMA (Santa Cruz, CA; diluted at 1:1,000), TGF-β1 (R&D system, MN; diluted at 1: 500) and CTGF (abcam, MA; diluted at 1:1,000) were applied to the cells. After incubation overnight

at 4°C, the cells were treated with DAKO Chemmate™ (DAKO, Glostrup, Denmark) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) as substrates following the manufacturer's specifications. Finally the cells were counterstained with Mayer's haematoxylin (Sigma).

Northern blot analysis

After total RNA extraction from cultured cells by Trizol (Invitrogen), each RNA sample of 10 μg per lane was separated on 1.2% agarose-formaldehyde gels, electrotransferred onto nylon membranes, and cross-linked by UV irradiation. To detect CTGF mRNA, a cDNA probe containing the human CTGF ORF, of size 850 bp, was radiolabeled with [^{32}P]-dCTP (Amersham Pharmacia Biotech, Aylesburym, UK) by random priming and then hybridized overnight at 65°C in 1% BSA, 7% sodium dodecyl sulfate (SDS), 0.5 M NaH$_2$PO$_4$ and 1 mM ethylenediaminetetraacetic acid (EDTA). The membranes were washed twice in 2 X SSC, 0.1% SDS for 5 min at 37°C, twice more times in 1 X SSC, 0.1% SDS for 30 min at 65°C and finally once more in 0.2 X SSC for 5 min at 37°C. They were then exposed to AGFA X-ray blue film (AGFA Gevaert, N.V.) with intensifying screens. To assess equivalent RNA loading in each lane, the membranes were rehybridized with β-actin cDNA probe (Clontech, CA).

Western blot analysis

All of the cultivated stable HepG2-core, HepG2 and cell-to-cell non-contacted co-cultured HSC were lysated in RIPA lysis buffer (50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM Na$_3$VO$_4$, 1 mM NaF). Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). After boiling for 5 min lysates were separated under 10% SDS-PAGE polyacrylamide gel. Proteins were transferred to nitrocellulose membranes which were blocked in Tris-buffered saline with Tween-20 (TTBS) containing 5% non-fat dried milk. The membranes were incubated with specific antibodies against α-SMA (diluted at 1 : 500), TGFβRII (Santa Cruz, CA; diluted at 1 : 1,000), Col I (Sigma; diluted at 1 : 1,000) and CTGF (diluted at 1 : 2,500) at 4°C overnight. After rinsing three times in TTBS, the membranes were incubated HRP conjugated anti-mouse and -goat IgG antibodies (diluted at 1 : 2,000 and 1 : 3,000, respectively, Amersham Bioscience UK) at room temperature for 2 h and then developed using a chemiluminescence detection kit (Amersham Bioscience), according to the manufacturer's guide, and exposed to AGFA X-ray blue film (AGFA). The band density was measured using TINA image software (Raytest, Straubenhardt, Germany).

Zymograpy

The culture media from cell-to-cell non-contacted co-culture was mixed with non-denatured 5 X sample buffer (1 M Tris-Cl pH 6.8, 1% Bromophenol Blue, 20% SDS). Media mixture was electrophoresed in 8% polyacrylamide gel containing 1 mg/mL gelatin (Sigma) (Heussen *et al.,* 1980), and the gel was firstly rinsed twice in distilled water with 2.5% Triton X-100 for 10 minutes and then secondly incubated in the activation buffer (50 mM Tris buffer, pH 7.4 containing 5 mM CaCl$_2$ and 1 μM ZnCl$_2$) at 4°C for 12 h. In the gel stained with Coomassie brilliant blue R-250, MMP-2, appeared as a clear area and the band density was measured using TINA image software (Raytest).

Statistical analysis

Data are shown as mean ± SEM. Statistical comparisons were performed with unpaired Student's *t*-test. The degree of significance of the differences was considered when *P*-value below 0.05.

Results

Up-regulation of TGF-β1 expression in stable HepG2-core cells

To verify the presence of HCV core protein in the stable HepG2-core cell, we performed Western blot and then confirmed the expression of HCV core protein with 19 kDa molecular weight in the stable cell line (data not shown).

To investigate whether HCV core protein stimulates proinflammatory cytokine, we measured TGF-β1 level in the supernatant between HepG2-core cells and HepG2 cells at 6, 9, 12 and 24 h by ELISA. The level of TGF-β1 in stable HepG2-core cells over time was significantly higher than that in HepG2 cells (*P* < 0.05), suggesting that HCV core protein enhances TGF-β1 expression which promotes liver fibrogenesis (Figure 1).
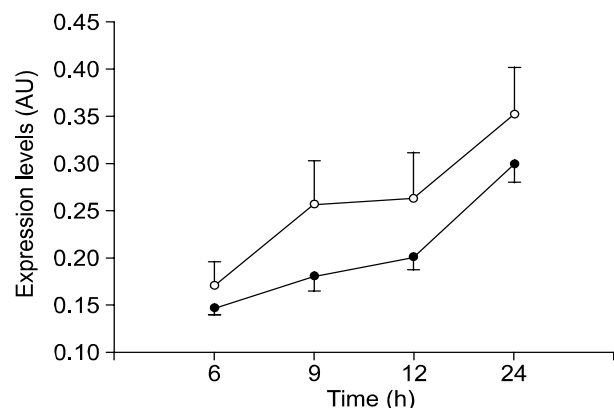

Figure 1. TGF-β1 expression in stable HepG2 core cells (○) and HepG2 cells (●). The levels of TGF-β1 secreted from serum free DMEM were determined at 6, 9, 12 and 24 h using a specific ELISA. The levels of TGF-β1 in stable HepG2-HCV core cells over time were significantly higher than that in HepG2 cells (*P* < 0.05). Data from three independent experiments are shown as means ± SD.
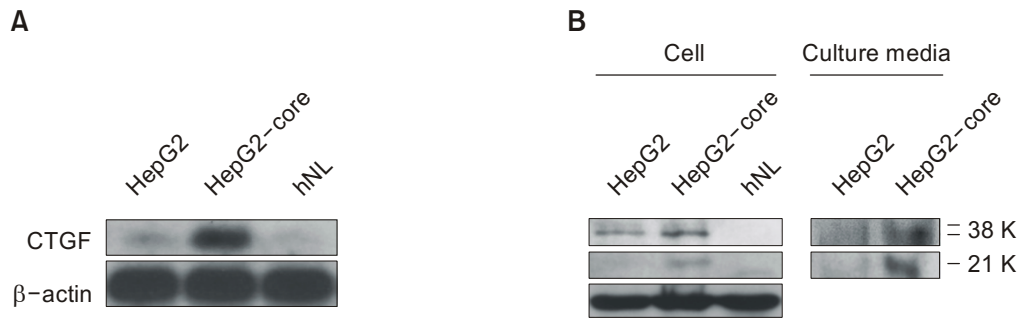
A

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Figure 2. CTGF mRNA and protein among stable HepG2-HCV core cells, HepG2 cells and human normal liver tissue (hNL) by Northern blot and Western blot, respectively. They were cultured for 24 h in serum-free DMEM. (A) The expression of CTGF mRNA was significantly higher in HepG2-HCV core cells than that HepG2 cells. (B) The expressions of both the 38 kDa doublet and 21 kDa CTGF forms in stable HepG2-HCV core cells and HepG2 cells but not hNL. Proteolytically processed form, of 21 kDa, was shown in stable HepG2-HCV core cells and cultured media but HepG2 cells weakly.

Effect of HCV core protein on CTGF expression

A previous report suggested that CTGF is closely related in fibrogenic processes which involve proliferation, collagen synthesis and chemotaxis in mesenchymal cells (Chen *et al.,* 2001).

We performed Northern blot analysis to evaluate the expression of CTGF mRNA in HepG2-core cells which express HCV core protein. The expression of CTGF mRNA was more pronounced in HepG2-core cells than in other HepG2 cells or normal human liver tissue as negative controls (Figure 2A). In contrast, the levels of human β-actin mRNA were similar in the three tested samples.

The CTGF protein appeared as a proteolytically processed form, of 21 kDa, in addition to the 38 kDa doublet pattern as described in a previous report (Paradis *et al.,* 1999). In this study, the expressions of both the 38 kDa doublet and 21 kDa CTGF forms were higher in stable HepG2-core cells than in HepG2 cells. Nevertheless, no target band was observed in human normal liver tissue (Figure 2B). Taken together, our results demonstrated that the levels of CTGF expression are up-regulated by HCV core protein.

Comparison of the expression levels of α-SMA, procollagen, TGF-β1 and TGFβRII in HSC co-cultured with stable HepG2-core cells and with HepG2 cells

In general, α-SMA and desmin are known as indicating markers of activated HSC. In this study, we observed the expression of α-SMA and desmin through immunocytochemistry and Western blot (data not shown).

In the morphological and molecular analyses, the expressions of TGF-β1 and CTGF were predominantly observed in HSC co-cultured with stable HepG2-core cells compared to with HepG2 cells (Figure 3A).

In addition, we quantitated the expression levels of Col I from media of HSC co-cultured with stable HepG2-core cells and HepG2 cells using ELISA. The
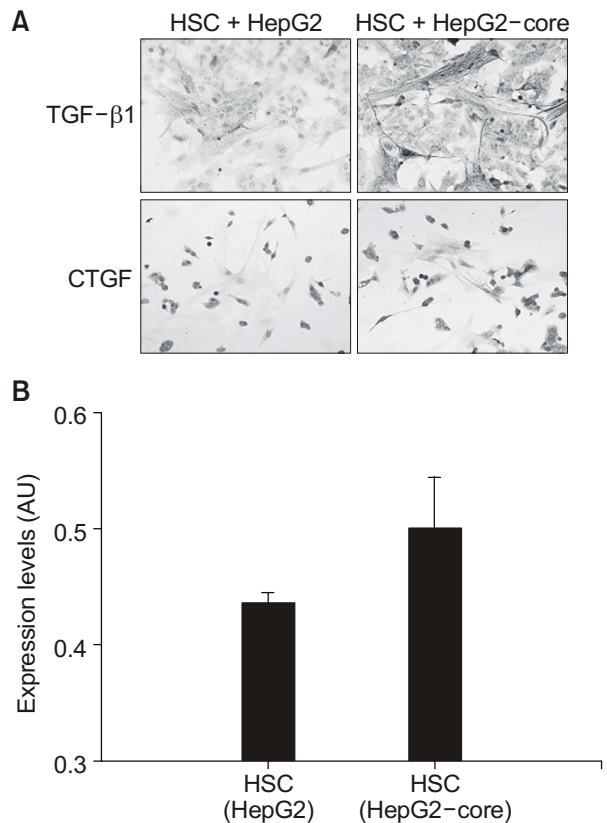
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Figure 3. The expression levels of fibrosis-related proteins after HSC was co-cultured with either stable HepG2-HCV core cells or with HepG2 cells. (A) Immunocytochemical staining of TGF-β1 and CTGF in mixed co-culture system. The characteristic HSC with long cytoplasmic process was differentiated from round hepatocytes. (B) Collagen type (Col I) expression in cell-to-cell non-contacted co-culture system. The expression level of Col I was determined in serum free DMEM using a specific ELISA. The expression level of Col I in stable HepG2 core was higher 27% than that of HepG2 with HSC. The level of Col I in HSC with stable HepG2-HCV core cells was significantly higher than that with HepG2 cells (*P* < 0.05). Data from three independent experiments are shown as means ± SD.
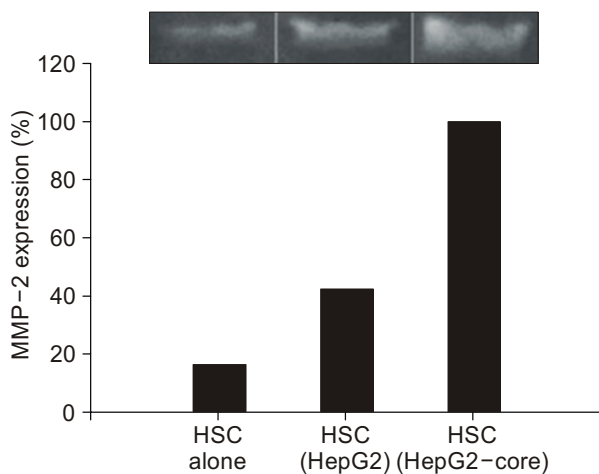
Figure 4. MMP-2 expression in HSC alone and co-cultured HSC. Each cell was cultured in serum free DMEM for 24 h and then the level of MMP-2 from cultured media was measured by zymogram. The expression level in HSC co-cultured with stable HepG2-core cells was 2.5 times higher than in HSC co-cultured with HepG2, and 6 times higher than in that with HSC alone
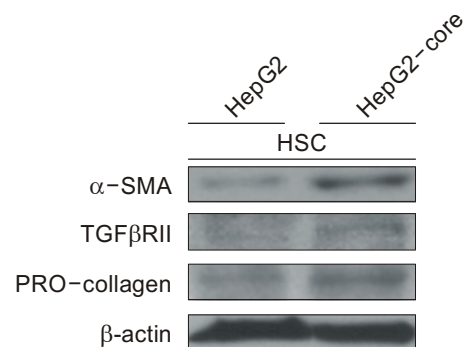

Figure 5. Western blotting for α-SMA, procollagen and TGFβRII in co-cultured HSC. HSC from co-culture with stable HepG2-core cells showed higher expression of α-SMA, procollagen and TGFβRII than those from co-culture with HepG2 cells, suggesting that HCV core protein up-regulated fibrogenesis-related proteins.

expression levels of Col I in HSC co-cultured with stable HepG2-core cells were 27% higher than in those with HepG2, respectively (Figure 3B). These results suggest that HCV core protein enhances the fibrogenesis-related proteins leading to fibrosis and then cirrhosis in the setting of chronic liver diseases by HCV.

As described by a previous report, MMP-2, degrades basement membrane collagen and denatures collagens by up-regulation during fibrogenic process (Friedman, 1993). In the present study, MMP-2 was quantified in culture media from HSC alone, and from HSC co-cultured with stable HepG2-core cells and with HepG2 cells by zymogram. The expression level in HSC co-cultured with stable HepG2-core cells was 2.5 times higher than in HSC co-cultured with HepG2 and 6 times higher than in that with HSC alone (Figure 4). From these results, fibrogenesis in chronic HCV liver diseases is stimulated and advanced by HCV core protein.

The previous data demonstrated that TGFβRII is up-regulated in addition to other fibrogenesis related proteins such as α-SMA and procollagen during fibrogenesis (Qin *et al.,* 2002). In this regard, we analyzed the expressions of fibrosis-related proteins such as α-SMA, procollagen and TGFβRII in the separate-cultured HSC using Western blot. In our result, HSC from co-culture with stable HepG2-core cells showed higher expression of α-SMA, procollagen and TGFβRII than that from co-culture with HepG2 cells (Figure 5), suggesting that HCV core protein stimulates HSC and makes it produce α-SMA and pro-collagen, and up-regulates TGFβRII which enhances TGF-β1 expression.

Discussion

Although HCV infection induces a high rate of chronic liver diseases including chronic hepatitis and liver cirrhosis, the mechanism of this induction has not been elucidated yet. Moreover, chronic hepatitis C finally progresses into liver cirrhosis *via* fibrogenesis at a high rate despite anti-viral or immunomodulating therapy.

As already mentioned in numerous previous studies, HCV core protein represents multi-functional activity that plays a key role in the modulation of cell proliferation and viability, the activation of signal transduction and induction of steatosis and lipid peroxidation (Giannini and Brechot, 2003). However, the studies of HCV core-related liver fibrosis have been limited until now and they have been mainly accomplished by animal models.

The aim of this work was to investigate the role of HCV core protein related to hepatic fibrogenesis *in vitro* culture system. Recently, co-culture systems have been employed for specific purposes like research or therapeutic approach in tissue engineering (Rojkind *et al.,* 1995; Bhatia *et al.,* 1998). In this study, we established two different types of co-culture systems, which we named cell-to-cell contacted and non-contacted co-culture of HSC with stable HepG2-core constitutively expressing HCV core protein. In particular, no study of the co-cultivation of HSC and hepatocyte expressing HCV core protein has been reported to date.

Hepatic fibrosis induced by variably harmful stimuli such as virus, hypoxia, chemicals, bile stasis, drugs and herbals, is characterized by abnormally excessive accumulation of ECM accompanied by exaggerated cytokine releasing. HCV core protein is thought to produce reactive oxygen species (ROS) through con-

sequent derangement of lipid metabolism, leading to the induction of proinflammatory cytokine TGF-β1 (Okuda *et al.,* 2002). However, the direct mechanism by which HCV core protein induces fibrogenesis has not been clarified. In this study, we observed that HCV core protein significantly produced fibrosis-related proteins in an *in vitro* co-culture system. TGF-β1, recognized as the strongest inducers of fibrogenesis, underwent pronounced expression in media of HSC co-cultured with stable HepG2-core cells compared to that of HSC co-cultured with HepG2 cells. These results suggested that HCV core protein might regulate TGF-β1 expression. In addition, Col I, which was produced during fibrogenesis, was increased from media of HSC co-cultured with stable HepG2-core cells, strongly suggesting HCV core protein is closely correlated to fibrogenesis.

CTGF is known to be a multifunctional matricellular protein, which has been implicated in wound healing, and several fibrotic diseases including atherosclerosis, pulmonary fibrosis, renal fibrosis and scleroderma (Igarashi *et al.,* 1993; Igarashi *et al.,* 1996; Oemar *et al.,* 1997; Ito *et al.,* 1998; Lasky *et al.,* 1998). Although some data showed that CTGF was mainly derived from HSC during liver fibrogenesis (Paradis *et al.,* 1999; Williams *et al.,* 2000), it has been shown that all major cell types in the liver have the potential to produce CTGF according to initial hepatic injury and type of damage (Rachfal *et al.,* 2003). Based on such previous results, we have immunocytochemically examined CTGF in HSC co-cultured with either stable HepG2-core cells or with HepG2 cells. In our results, CTGF was predominantly expressed in co-culture of HSC with stable HepG2-core cells than in HSC with HepG2 cells. It is interesting to note that CTGF was expressed in both HSC and HepG2-core cells. We also observed that CTGF mRNA and proteins were significantly expressed in the stable HepG2-core cells compared to in the HepG2 cells alone and in normal human liver. Furthermore, CTGF bands of both the 21 kDa proteolytically processed form and the 38 kDa doublet were detected in stable HepG2 core cells but not in normal human liver. However, a 38 kDa doublet, but not the 21 kDa form, was detected in HepG2 cells alone, suggesting that HCV core protein up-regulates CTGF in the liver with HCV infection.

In general, fibrogenesis is progressed by an up-regulation of ECM synthesis and a down-regulation of MMP activity by an increase of TIMP, a physiological inhibitor of MMP. These MMPs, TIMPs and collagens which are major components of ECM, are mainly produced in myofibroblastic cells derived from activated HSC (Knittel *et al.,* 1999; Friedman, 2000). However, it has been reported that MMP-2, among several identified MMPs, degrades basement membrane collagen so that denatured fibril collagens replace normal collagen as ECM during fibrogenesis (Karelina *et al.,* 2000; Schuppan *et al.,* 2001). Our study demonstrated that MMP-2 and fibril collagen (Col I) are significantly increased in HSC co-cultured with stable HepG2-core cell compared to in HSC co-cultured with HepG2 cell. This observation suggests

that HCV core protein stimulates HSC to induce fibrogenesis.

During hepatic fibrogenesis, TGF-β1 is mainly produced from myofibroblasts and its corresponding receptor (TGFRII) is activated in an autocrine fashion (Tahashi *et al.,* 2002). In this study, TGFβRII expression was higher in lysates of activated HSC co-cultured with stable HepG2-core cells than in those of HSC co-cultured with HepG2 cell. Taken together with the above findings, it is evident that HCV core protein regulates TGF-β1 and TGFβRII expression to direction of progressive fibrosis. The results from this co-culture system therefore support the previous findings during hepatic fibrogenesis in human and animal models (Qi Z *et al.,* 1999).

It is not well known whether HCV directly affects HSC to induce liver fibrosis in chronic HCV carriers, because an efficient *in vitro* HCV culture system is not available and *in vivo* animal models such as HCV core-transgenic mice don't appear fibrosis. Therefore, our co-culture system promises to be useful for studying the dynamic signal transduction of fibrogenesis as well as for verifying new anti-fibrotic agents for liver fibrosis.

In summary, our data suggest that HCV core protein plays an important role in liver fibrogenesis by up-regulation of fibrogeneis-related proteins. Nevertheless, further studies of the role in fibrogenesis of other HCV proteins, in addition to that of HCV core protein, are required.

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