

HCV core protein modulates Rb pathway through pRb down-regulation and E2F-1 up-regulation

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Abstract

It has been recognized that the HCV (hepatitis C virus) core protein plays an important role in hepatocarcinogenesis. The functional inactivation of the Rb pathway appears to be a major event for multi-step cancer carcinogenesis. To elucidate the role of the HCV core protein in hepatocarcinogenesis, we investigated the effect of the HCV core protein on the Rb pathway in both Rat-1 cell lines, stably expressing the HCV core protein and the doxycycline-regulated cell lines. The HCV core stable transfectants showed a dramatic decrease in the pRb levels and E2F-1 up-regulation. In the doxycycline-regulated cell lines, the pRb levels were significantly decreased which are followed by E2F-1 up-regulation. HCV core stable transfectants showed higher cell growth rates and were sensitized to apoptosis. Thus, our results first indicate that the HCV core protein decreases the expression of pRb, thereby allowing E2F-1 to be constitutively active, which is thought to result in rapid cell proliferation or sensitizing to apoptosis. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Hepatitis C virus; Core protein; Hepatocarcinogenesis; Rb pathway

1. Introduction

Hepatitis C virus (HCV) is an important causative agent of the acute and chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma [1]. HCV infection is now recognized to be a major risk factor for hepatocellular carcinoma. However, the precise role of HCV in hepatocarcinogenesis remains unknown.

Many researchers have reported that the core pro-

tein of HCV transforms fibroblasts with or without the cooperation of the ras oncogene [2,3], *trans*-suppresses some cellular promoters [4–6], and modifies the susceptibility of cultured cells to apoptotic signals [7–9]. Although the mechanisms of these regulations are still unclear, it is possible that these properties of the HCV core protein influence host cell growth, survival, and carcinogenesis. Recently, it was also reported that transgenic mice expressing the HCV core gene developed hepatocellular carcinoma [10]. We previously reported that the Rat-1 cell line, stably expressing HCV core protein, exhibited focus formation, anchorage-independent growth, and tumor formation in nude mice [3]. Furthermore, depending on the expression level of the core protein,

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the HCV core protein is able to induce the transformation of Rat-1 cells at various efficiencies.

Recent data have suggested that deregulation of the restriction point control in the cell cycle G1 phase appears to be required for malignant transformation [11,12]. The pRb (retinoblastoma tumor suppressor protein) pathway has been regarded as a functional unit controlling the G1 phase and as an obligatory oncogenic target [13,14]. In addition, pRb is important for suppressing growth, facilitating differentiation, and inhibiting apoptosis [15]. Therefore, the Rb pathway perturbation is directly involved in malignant transformation processes.

To understand the role of the HCV core protein in hepatocarcinogenesis, it is necessary to investigate whether the HCV core protein can modulate the Rb pathway during the transformation processes. In this study, we investigated the effect of the HCV core protein on the Rb pathway in both Rat-1 cell lines, stably expressing the HCV core protein, and doxycycline-regulated cell lines. In this present study, we reported that the HCV core protein decreases the expression of pRb through the down-regulation of Rb mRNA, thereby allowing E2F-1 to be constitutively active, which is thought to result in rapid cell proliferation or sensitizing to apoptosis.

2. Materials and methods

2.1. Plasmids and cells

As previously described [3], pCI-neo-core K was constructed by inserting the PCR-amplified core region (amino acids 1–191) of the HCV-K isolate [16] into the expression vector, pCI-neo (Promega, USA). The PCR-amplified core sequences were also inserted into the expression plasmid, pUHD 10-3, and designated as pUHD core. The pUHD 10-3 plasmid, kindly provided by Dr H. Bujard of the University of California, Berkeley, carries the regulatory region with human cytomegalovirus (hCMV) minimal promoter and heptamerized upstream tet operator, as previously described [17,18].

Rat-1 cells, immortalized Rat embryo fibroblasts, were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Germany) supplied with 10% fetal bovine serum and transfected with pCI-neo

or pCI-neo-core K using the calcium phosphate coprecipitation method and selected by G418 treatment for 2 weeks. Resistant clones were separately propagated and the expression of core gene was analyzed by western blot. pUHD core was cotransfected with a pUHD 172-neo in 5:1 ratio into Rat-1 cells to generate a core-inducible cell line. pUHD 172-neo provided by Dr H. Bujard encodes a neomycin phosphotransferase gene and carries the doxycycline (dox) regulatory region along with the hCMV promoter/enhancer elements and the rtTA-gene [17]. To select clones that express core under the control of dox, G418-resistant clones were incubated with medium containing with or without dox (Sigma, USA). After incubation for 48 h, the cells were harvested and used for Western blot.

2.2. Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was isolated by the guanidinium/cesium chloride centrifugation method [19] and quantitated by spectrophotometry. One microgram of total RNA was reverse transcribed using M-MLV Reverse Transcriptase (Promega). The PCR reaction was carried out under the conditions recommended by the manufacturer's instructions (Perkin Elmer Cetus, USA). Briefly, 50 µl of a reaction mixture including 2.5 units of *Taq* polymerase (Takara, Japan), 5 µl of 10× buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 1 µl of first-strand cDNA, and 25 pmol each of primer, was subjected to 28 PCR cycles (denaturation at 94°C for 1.5 min, annealing at 58°C for 1 min, and polymerization at 72°C for 1 min). The following primer pairs were used for PCR amplification: (1) GAPDH (sense: 5'-ATCACTGCCACTCAGAAGAC-3', antisense: 5'-CTTGCTCTCAGTATCCTTGC-3'); (2) Rb (sense: 5'-GTACCGTCTAGCATATCTCC-3', antisense: 5'-CTCTGTCACTG-TTACACACC-3').

2.3. Western blot analysis

Cells were lysed in lysis buffer (10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, PMSF (10 µg/ml), aprotinin (10 µg/ml), leupeptin (10 µg/ml), 5 mM phenanthroline, and 28 mM benzamidinium-HCl) for 30 min on ice. The lysates were cleared by centrifugation at 12 000 rpm for 30 min.

Lysates were quantitated using the Bradford assay (Bio-Rad, USA) with bovine serum albumin as a reference standard. For the Western blotting experiments, each gel slot was loaded with 100 μ g of cell lysates protein. Electrophoresis was performed in 6.5% or 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and transferred to PVDF (polyvinylidene difluoride membrane, Millipore, USA). After incubation with primary antibodies, proteins were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham, USA), followed by ECL according to the manufacturer's instructions (Amersham). Primary antibodies against Rb (G3-245; PharMingen, USA), E2F-1 (KH95; Santa Cruz, USA), and β -actin (I-19; Santa Cruz) were applied at optimized concentrations. HCV core proteins were detected with using anti-core positive patient's sera and horseradish peroxidase-conjugated anti-human immunoglobulin G as the primary and secondary antibodies, respectively.

2.4. Determination of pRb half-life

Pulse-chase analysis was performed as described previously [20]. Briefly, cells were prestarved for 30 min in methionine-free DMEM media (Gibco BRL, Germany) and then pulse-labeled for 15 min with 250 μ Ci of [35 S]methionine (Amersham) followed by chase for the indicated time periods. At each time point, cells were washed with cold PBS and lysed in EBC lysis buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 0.5% NP-40, 100 mM NaF, 200 μ M sodium orthovanadate). Lysates were pre-cleared, and equal concentration of lysates were immunoprecipitated with anti-Rb antibody and resolved by 6.5% SDS-PAGE. The signals were detected by autoradiography.

2.5. ac-DEVD-pNA enzymatic cleavage activity

Cells were lysed in lysis buffer (50 mM Tris (pH 7.5), 0.03% NP-40, 1.0 mM DTT) for 30 min on ice. After centrifugation at 12000 rpm for 30 min at 4°C, the supernatant was quantitated using the Bradford assay (Bio-Rad) with bovine serum albumin as a reference standard. Lysates (20 μ g) were incubated with 0.2 mM ac-DEVD-pNA (Enzyme System Prod-

uct, USA) in a total volume of 0.1 ml. Assays were performed in duplicate, and the results are presented as the average increase in absorbance at 405 nm.

3. Results

3.1. Establishment of HCV core stable transfectants and doxycycline-regulated cell lines

The expression of the HCV core protein in the stable transfectants (cell clones; cores 1, 5, and 6) and doxycycline-regulated cell lines was examined by Western blotting analysis (Fig. 1). The HCV core protein was expressed in three different stable transfectants, especially prominent in core 1 and 6 (Fig. 1A). In the doxycycline-regulated cell lines, the expression of the HCV core protein was tightly

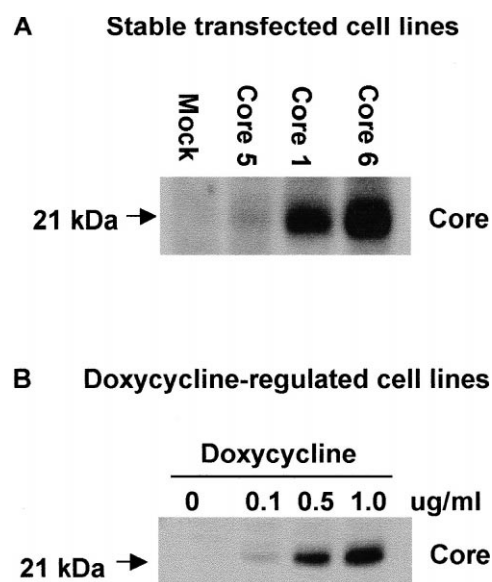


Fig. 1. Expressions of HCV core protein in the stable transfectants (cores 1, 5, and 6) and the doxycycline-regulated cell lines (pUHD core). Detection of the HCV core protein in core 1, core 5, and core 6 cells by Western blot analysis (A). The expression levels of the HCV core protein were different in each clone: core 5 = +, core 1 = +++, and core 6 = +++. Regulation of steady-state HCV core protein expression by doxycycline in the culture medium (B). pUHD core cells were cultured for 48 h in a medium containing the indicated concentrations of doxycycline. Equal amounts of cell lysates (100 μ g) were resolved by 12% SDS-PAGE and subjected to Western blotting using anti-core positive patient's sera and horseradish peroxidase-conjugated anti-human immunoglobulin G as the primary and secondary antibodies, respectively.

regulated over a broad range by the concentration of doxycycline (Fig. 1B).

3.2. Effect of HCV core protein on the levels of pRb and E2F-1

To investigate the effect of the HCV core protein on pRb and E2F-1 levels, we used anti-Rb or anti-E2F-1 immunoblotting of the whole cell lysates in both HCV core stable transfectants and doxycycline-regulated cells, respectively. As shown in Fig. 2, in each case the HCV core-expressing cells, especially cores 1 and 6, showed a dramatic decrease in the levels of pRb compared to the mock cells, whereas E2F-1 were up-regulated in the HCV core stable transfectants. We hypothesized that the HCV core protein down-regulates the levels of the pRb. To confirm this hypothesis, we performed anti-Rb immunoblotting in the doxycycline-regulated cells (Fig. 3). When the cells were cultured for 72 h in a medium containing various concentrations of doxycycline, the pRb levels remarkably decreased (Fig. 3A), and a time-dependent decrease in the pRb levels was seen following the HCV core protein expression (Fig. 3B). This directly supports the role of the HCV core protein in inducing a decrease in the pRb levels. Consistent with stable transfectants, in doxycycline-

Stable transfected cell lines

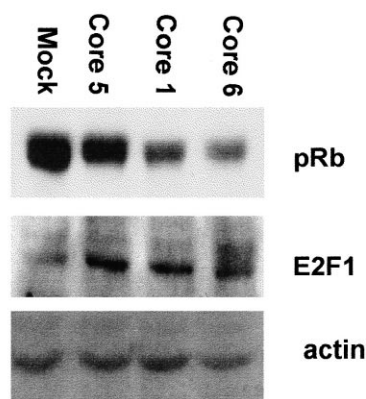


Fig. 2. Effect of HCV core protein on the levels of pRb and E2F-1 in HCV core stable transfectants (cores 1, 5 and 6). Note the dramatic decrease in the pRb levels in the HCV core stable transfectants, especially in cores 1 and 6. Equal amounts of cell lysates (100 μ g) were resolved by 6.5% SDS-PAGE and subjected to Western blotting with monoclonal anti-Rb or anti-E2F-1 antibodies. Actin was used as a loading control. The same results were shown repeatedly by three independent experiments.

Doxycycline-regulated cell lines

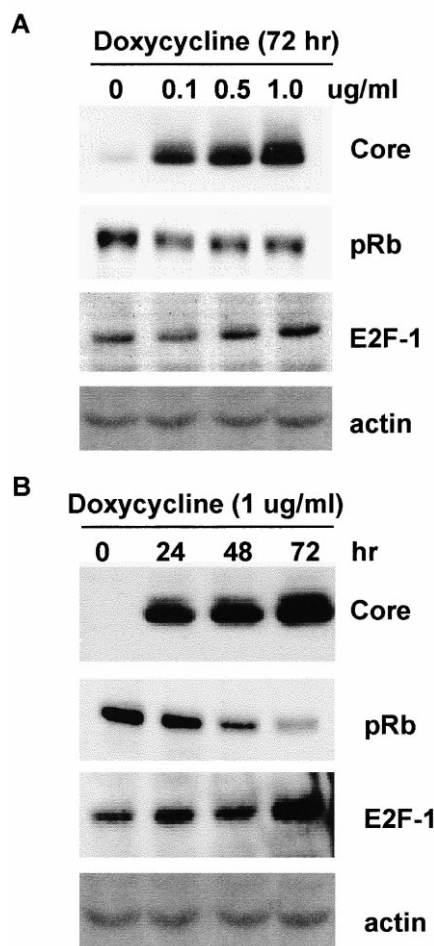


Fig. 3. Effect of HCV core protein on the levels of pRb and E2F-1 in doxycycline-regulated cell lines. The cells were cultured in the absence and presence of various doxycycline concentrations for 72 h (A) and cultured in the presence of doxycycline (1 μ g/ml) for the indicated times (B). Consistent with the HCV core stable transfectants, a marked decrease in the pRb level was shown in the doxycycline-regulated cell line. Equal amounts of cell lysates (100 μ g) were resolved by 6.5% SDS-PAGE and subjected to Western blotting with anti-Rb or anti-E2F-1 antibodies. Actin was used as a loading control. The same results were shown repeatedly by three independent experiments.

regulated cell lines, E2F-1 was also increased in a concentration- and time-dependent manner (Fig. 3A,B).

3.3. Effect of the HCV core protein on Rb mRNA levels

A marked decrease in the levels of pRb suggests

the possibility that the HCV core protein also decreases the Rb mRNA expression. To determine whether the HCV core protein down-regulates the Rb mRNA expression, we investigated the levels of Rb mRNA in both HCV core stable transfectants and doxycycline-regulated cells. As shown in Fig. 4A, the Rb mRNA levels showed a dramatic decrease in the core 6 cells. Consistent with the stable transfectants, a time- and concentration-dependent decrease in the levels of Rb mRNA was also observed following the HCV core protein expression (Fig. 4B,C).

3.4. Effect of HCV core protein on pRb-half life

The levels of pRb were also determined by destruction of the protein itself, by various mechanisms. According to pulse-chase analysis (Fig. 5), the pRb showed a similar half-life in the mock and core 6 cells. Furthermore, consistent with previous data,

mock cells showed a slight increase in basal pRb levels compared to core 6 cells.

3.5. Effect of pRb down-regulation on cell viability

To determine whether pRb down-regulation can modulate susceptibility to cell death following contact inhibition, cells were continuously cultured 5 days after 100% confluency status.

According to the viable cell count, core 6 cells had a five times higher cell death rate than mock cells (Fig. 6A). Consistent with viable cell count, we also observed that core 6 cells showed marked cell detachment in overconfluent conditions, but not in mock cells (Fig. 6B). Fig. 6C showed that cell death was mediated by an apoptotic process through caspase-3 activation. In addition to contact inhibition, we also observed that core 6 cells showed increased apoptotic cell death to various cellular stresses, such as, etoposide and serum starvation (data not shown). These

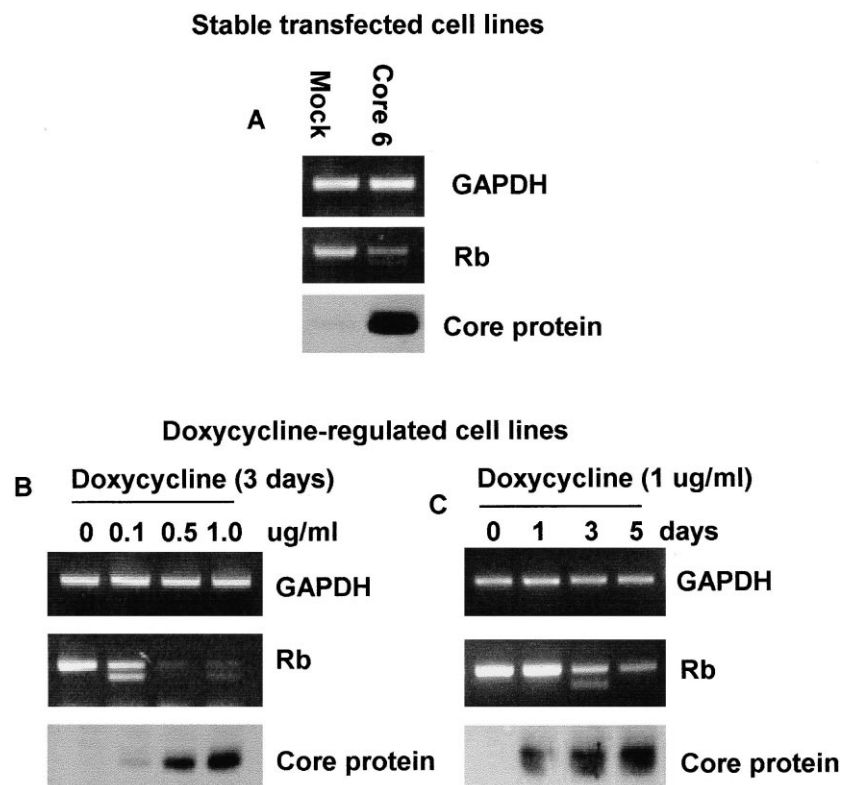


Fig. 4. Effect of HCV core protein on the levels of Rb mRNA in the HCV core stable transfectant (core 6) and doxycycline-regulated cell lines. Note a marked decrease in the Rb mRNA levels of the core 6 cells (A). The cells were cultured in the absence and presence of various doxycycline concentrations for 3 days (B), and cultured in the presence of doxycycline (1 μ g/ml) for the indicated times (C). The Rb mRNA levels were dramatically decreased after HCV core protein expression. The expressions of HCV core protein were confirmed by anti-core immunoblotting. The same results were shown repeatedly by three independent experiments.

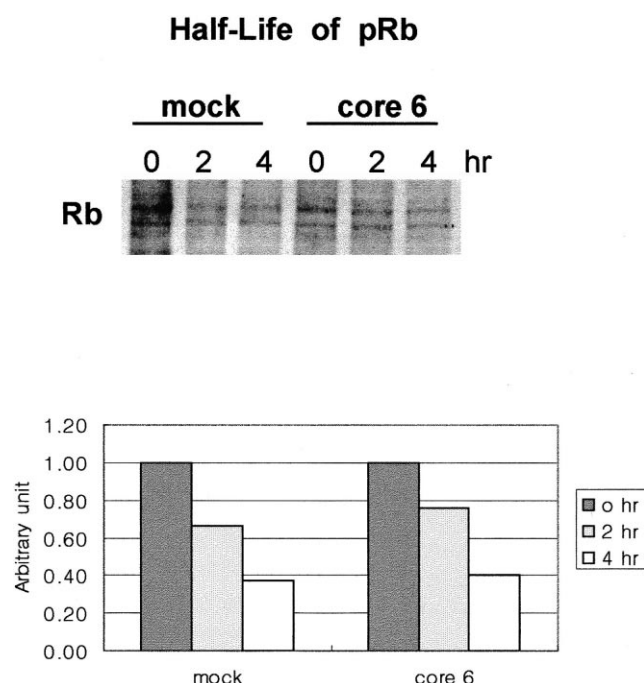


Fig. 5. Determination of the pRb half-life. Pulse-chase analysis of the pRb was performed in mock and core 6 cells. The cells were metabolically pulse-labeled with [35 S]methionine for 15 min and chased for various time periods. Equal amounts of cell lysates (200 μ g) were immunoprecipitated using an anti-Rb antibody, and separated by 6.5% SDS-PAGE. pRb signals decreased with similar kinetics in mock and core 6 cells. Quantification of pRb signals was performed with a densitometer (Scion Corp.).

results suggest that the pRb down-regulation may sensitize core 6 cells to apoptosis.

4. Discussion

Cancer is a multi-step process that requires an accumulative effect altering the key biological pathways, such as the restriction point control, genome integrity control, and cell survival or death pathway [11,12]. The pRb, as a tumor suppressor protein, plays a key role in a late G1 phase checkpoint control [13]. A loss of pRb function by different mechanisms has been reported in many human cancers [12–14]. In addition to the loss of pRb function, recent studies suggest that levels of the pRb are important for determining of cellular fates [21,22]. DNA tumor viruses such as adenovirus, human papilloma virus (HPV), and SV40 virus can induce malignant

transformation by loss of pRb function [23]. In the case of HCV, RNA virus, the correlation between loss of pRb function and HCV core protein remains to be elucidated.

In this study, the HCV core protein decreased the level of pRb by down-regulation the Rb mRNA. Kim et al. [4] also reported that the HCV core protein suppressed the expression of Rb promoters in HepG2 cells using CAT (chloramphenicol acetyl transferase) assay. Thus, the regulation of the Rb expression by the HCV core protein may be modulated at the transcriptional levels. However, it seems like that level of Rb mRNA does not immediately decrease in the presence of HCV core protein. In addition, pRb levels were decreased 48 h after induction of HCV core protein. These results suggest that the down-modulation of the pRb levels could be delayed and indirect effect of the core protein overexpression. Furthermore, according to pulse-chase analysis, HCV core protein cannot induce a rapid pRb destruction observing in the presence of HPV E7 oncoprotein.

The expression levels of E2F-1 were higher in the HCV core stable transfectants. Unexpectedly, core 6 cells showed relatively lower E2F-1 levels than core 5 and core 1 cells, suggesting that core 6 cells with higher E2F-1 levels may undergo apoptosis, due to various reasons, such as, the core itself toxicity or cell cycle perturbation during routine culture. In fact, we usually observed that a number of core 6 cells were detached from the culture dish, whereas, core 1 and 5 cells were not. The delicate balance between pRb and E2F-1 is very important for determining the alternative cellular fates, such as cell proliferation or apoptosis [22,24]. Overexpression of E2F-1 can induce aberrant S-phase entry or apoptosis depending on cell status. The HCV core stable transfectants used in this study grew rapidly compared to the mock cells (data not shown). However, cellular stress such as contact inhibition induced more sensitive cell death in core 6 cells through apoptosis. Even though HCV core protein appears to be capable of both inducing and blocking apoptosis under different conditions [7,8], in this study, it was observed that core 6 cells showed increased apoptotic cell death to various cellular stresses, such as contact inhibition.

Thus, our data suggest that HCV core protein can

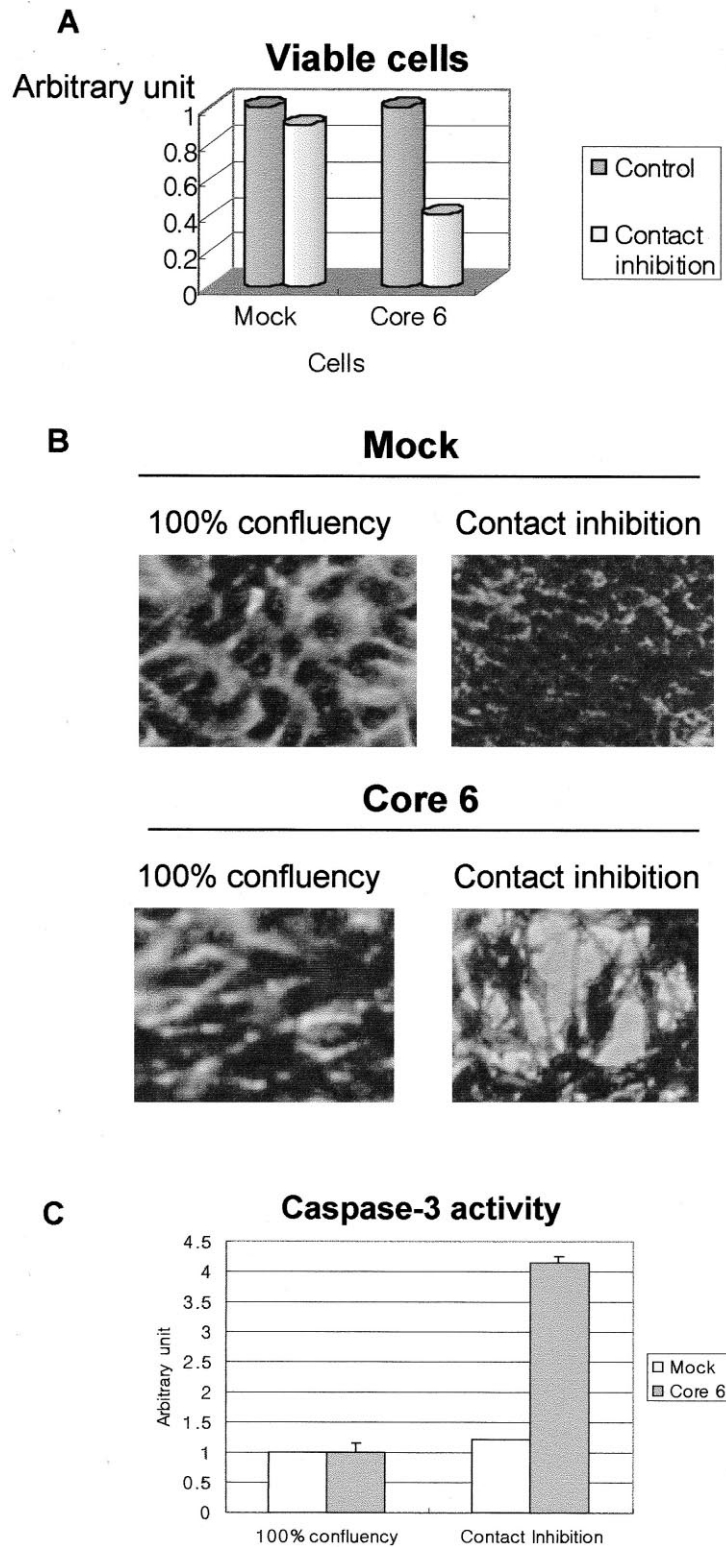


Fig. 6. Effect of HCV core protein on the viabilities of mock and core 6 cells in overconfluent growth. Cells were continuously cultured 5 days after 100% confluency status. Trypan blue exclusion assay (A), cell morphologies (B), and caspase-3 activity showed that core 6 cells were more sensitive to cell death following contact inhibition than mock cells. Photographs were taken after crystal violet staining using an inverted phase contrast microscope ($\times 100$). Enzymatic ac-DEVD-pNA cleavage activity was monitored on a spectrophotometer (Amersham Pharmacia) at an absorbance of 405 nm (C).

induce a down-regulation of pRb levels by a delayed or indirect pathway, and the release of active E2F transcription factors and accumulation of E2F-1 may result on deregulating the Rb pathway by other means. Consequently, disruption of pRb/E2F-1 balance by the HCV core protein may result in increased cell proliferation or making cells prone to apoptosis, depending on the conditions. However, to progress into hepatocellular cell carcinoma, virus infected cells should overcome the apoptotic pathway. To understand hepatocarcinogenesis by HCV infection, it is necessary to investigate how HCV core protein can inhibit the apoptotic process in the presence of Rb pathway perturbation.

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