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Hepatitis C Virus Core Protein Sensitizes Cells to Apoptosis Induced by Anti-Cancer Drug

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The core protein of the hepatitis C virus (HCV) is a multifunctional protein. The HCV core protein was reported to regulate cellular gene expression and transform primary rat embryo fibroblast cells. However, the role of the core protein in the pathogenesis of HCV-associated liver diseases is not well understood. To investigate the functional role of the core protein in cytopathogenicity, we have constructed stable expression systems of full length or truncated HCV core protein lacking the C-terminal hydrophobic domains and established HepG2 cell clones constitutively expressing the core protein. The full length core protein was localized in the cytoplasm and the C-terminal truncated core protein was localized in the nucleus. HepG2 cells expressing nuclear, truncated core protein showed elevated cell death during cultivation compared to untransfected cells and full length core-expressing cells. In the treatment with bleomycin, both cell clones expressing full length or truncated core protein appeared to be more sensitive to bleomycin than the parental HepG2 cells. These results suggest that the core protein may play a role in HCV pathogenesis promoting apoptotic cell death of infected cells.

Key words: Apoptosis, hepatitis C virus, core protein, anti-cancer drug

Hepatitis C virus (HCV), belonging to the *Flaviviridae* family, is well known as the major cause of post transfusional and sporadic non-A, non-B hepatitis (1,18,20). HCV contains a positive-stranded RNA genome of about 9.5 kb that encodes a large polyprotein precursor of 3000 amino acids (5, 10, 31). The precursor polypeptide is cleaved into its functional units by viral and host proteases, yielding three structural and six nonstructural proteins. The structural proteins core, E1, and E2, are produced from the N-terminal portion of the precursor protein by host signalases and the nonstructural proteins are processed by virally encoded proteases (6, 7, 9, 28).

HCV infection frequently persists for a long time and eventually leads to hepatic cirrhosis and hepatocellular carcinoma (27). However, the molecular mechanism of HCV pathogenesis is not well understood. It has been suggested that the cytotoxic T lymphocytes play an important role in the clearance of viral infection and are associated with the immunopathogenesis of type C hepatitis (2,16). Recently, the TNF- or Fas-mediated apoptosis of the hepatocyte was shown to be involved in the pathogenicity of HCV (8, 26, 34). Apoptotic cell death has been observed in many cases of cytopathic effects induced by infection of viruses such as the Epstein-Barr virus and human immunodeficiency virus (11,19). In the case of type C hepatitis, the core protein was considered to be involved in apoptotic cell death induced by HCV infection. However, the effects of the core protein on the cellular apoptosis have been reported differently depending on the cell lines (24, 26, 34).

The core protein of HCV, which is a nonglycosyated protein of 22 kd, is well conserved among all identified strains (3, 32). Antibodies to the core protein are most frequently detected in patients and carriers of HCV and thus, utilized for the diagnosis of HCV infection(4). The HCV core protein is known to have multifunctions in infected cells. It forms the nucleocapsids of HCV and regulates the cellular gene expression. It suppresses the promoters of c-*fos*, the retinoblastoma susceptibility gene, β-interferon gene, β-actin gene and the human immunodeficiency virus type 1 long termi- nal repeat (14, 22). In addition, it was recently reported that the HCV core protein may cooperate with the *ras* oncogene and transform rat embryo fibroblast cells (23).

In this study, to investigate the pathogenicity of

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HCV and the involvement of the HCV core protein in the regulation of cellular growth, we have expressed the core protein as full length or truncated forms in the human hepatoblastoma, HepG2 cells, and attempted to establish stable cell clones which express the core protein permanently. In the human liver cell line it is extremely difficult to express foreign genes permanently. By using a versatile expression vector, pEF321, we could obtain several HepG2 cell clones which stably express the core protein. We have also established the HepG2 cell clone which stably express the nuclear core protein by expressing the truncated core gene lacking the C-terminal hydrophobic domain. By testing the obtained stable cell clones, we demonstrated a possible role of the core protein in the pathogenesis of HCV.

Materials and Methods

Cell culture and transfection

The human hepatoblastoma cell line, HepG2, was grown in DMEM (Gibco) supplemented with 10% FCS. Subconfluent culture of the cells was transfected with plasmid DNA (20 μg/10 cm plate) by the calcium phosphate-DNA coprecipitation method (33). The DNA precipitates were formed at room temperature by mixing DNA and calcium chloride with Hepes buffer (pH 7.0). The DNA precipitates were applied to the cells for 6 h and the cells were then treated with growth medium containing 20% dimethyl sulfoxide for 3 min. After washing with fresh medium, the cells were maintained in 5% CO_2 at 37°C.

Indirect immunofluorescence assay

Subconfluent cells grown on 15 mm coverslips were washed twice in PBS and fixed in ethanol : acetone (1:1) for 18 min at –20°C. The fixed cells were incubated with the chronic hepatitis C patient serum containing antibody against the hepatitis C virus core protein in a 37°C moisture chamber for 2 h. After washing wash with PBS, the cells were incubated again with the fluorescein isothiocyanate-labeled rabbit anti-human immunoglobulin G antibody (Tago) at 37°C for 2 h. The coverslips were then embedded on glass slides with glycerol and the fluorescence was visualized by a UV microscope (Axoskop, Zeiss)(15).

DNA fragmentation assay

For DNA fragmentation analysis, the cells on a 10 cm plate were harvested and washed twice in PBS. Total DNA was extracted by incubating cells in 10 mM Tris-HCl (pH 8.0) containing proteinase K (200 μg/ml, Boehringer Mannheim) and 10% SDS at 50°C for 2 h. After centrifugation at 10,000 rpm at 20°C for 5 min, the DNA in supernatant was precipitated with ethanol. The obtained DNA was treated with RNase A (50 μg/ml, Sigma), extracted with phenol/chloroform, and precipitated with ethanol. The DNA fragmentation was analysed by electrophoresis of total DNA using a 1.5% agarose gel in TAE buffer.

Results and Discussion

Construction of plasmids and expression of the core protein

The full length core protein of HCV bears two hydrophobic domains at its C-terminus. It was previously reported that these hydrophobic domains allow the core protein localized in the cytoplasm attaching at the membranes of the cytoplasmic organelles (14, 30). It was also proposed that the truncated core protein lacking the C-terminal hydrophobic domains translocates into the nucleus by three probable nuclear localization signals found at the N-terminus of the core protein (14, 30)

The HCV core protein has a regulatory function in the cellular gene expression of the infected cells (14, 20). The activities of the viral or cellular promoters appeared to be suppressed by the core protein. This suppressive effect on gene expression has been shown only by the full length core protein which is localized in the cytoplasm. The truncated, nuclear core protein which is lacking C-terminal hydrophobic domains did not show regulatory function indicating that the intracellular localization is one of the important factors needed for the core protein to display its functions in the infected cells. Therefore, we have expressed both the full length and truncated core proteins to investigate the pathogenic effects of the HCV core protein in human liver cells. The HCV cDNA used in this study was derived from a healthy Japanese HCV carrier (17, 32). As shown in Fig. 1A, the region of 39 has the full coding sequences of the core protein, and the region of 37 has truncated coding sequences lacking two hydrophobic domains of the C-terminus. Each region of 39 and 37 was inserted between the EF-1α promoter and SV40 early poly(A) addition signal of the pEF321 expression vector (Fig. 1B). The EF-1α promoter in the pEF321 vector contains the promoter region and the first intron of the human polypeptide chain elongation factor 1α (EF-1α) gene (12). This promoter including the first intron is very

strong and transcribes a large amount of mRNA of the cloned DNA. In particular, the pEF321 vector is known to be very efficient in the stable expression of foreign genes in human liver cells (13).

The bacterial neomycin resistance gene directed by the LTR of the Rous sarcoma virus was included in the expression plasmids as a selection marker for the isolation of the cell clones (Fig. 1B).

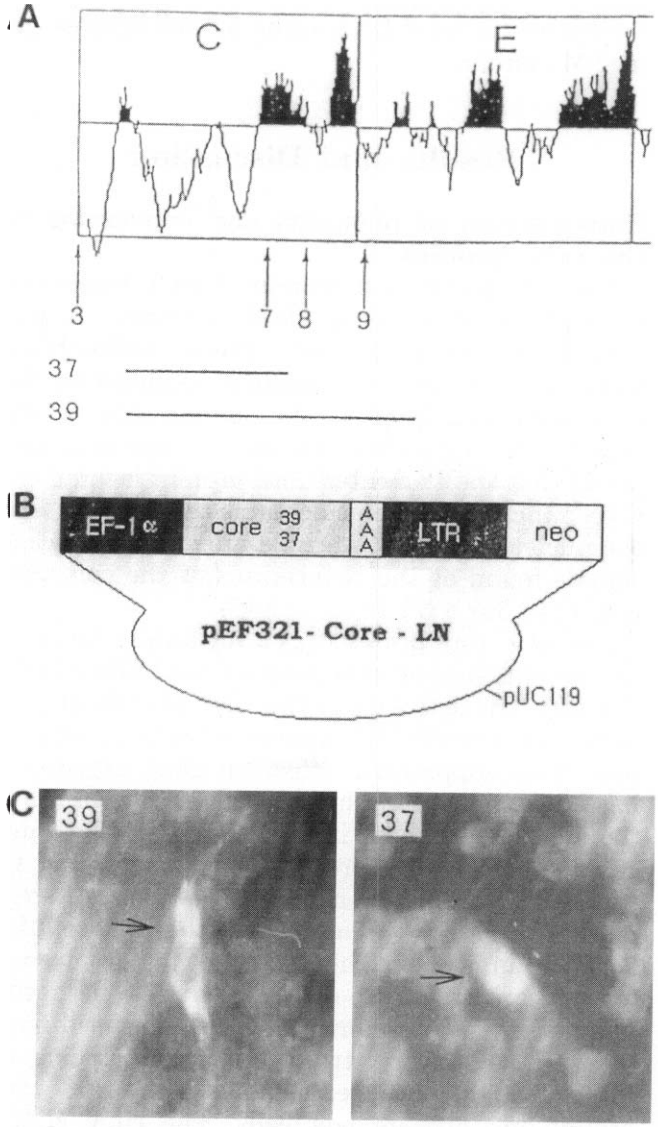

Fig. 1. Expression system of intact and truncated HCV core protein. (A) Hydropathic plots of amino acids of the HCV core protein. The hydrophobic domains were indicated by black areas above the midline. The numbers of 3, 7, 8, 9 indicate the nucleotide numbers 300, 700, 800, 900 of HCV cDNA, respectively. The HCV cDNA fragments used for expression were indicated by lines. (B) Structure of the expression plasmids containing full length or truncated cDNA fragment of the HCV core protein. (C) Immunofluorescence photomicrographs of transiently expressed HCV core protein. After 48h of transfection with each of the expression plasmids, HepG2 cells were fixed and subjected to a indirect immunofluorescence assay as described in Materials and Methods. Arrowheads indicate positive fluorescence in the cytoplasm (39) and in the nucleus (37).

In order to examine the expression and intracellular localization of the full length and truncated core proteins in the transient expression system, we transfected the plasmids, pEF321-core (39)-LN and pEF321-core(37)-LN, into HepG2 cells. As shown in Fig. 1C, both the full length and truncated core proteins were expressed in large quantities and showed similar strong fluorescence signal in the indirect immunofluorescence assay. As expected, the full length core protein was localized in the cytoplasm and the truncated core protein was localized in the nucleus of HepG2 cells.

Establishment of HepG2 cell clones which stably express the HCV core protein

In order to investigate the effects of the HCV core protein on human liver cell growth, we attempted to establish HepG2 cell clones which express the core protein stably. We transfected the expression plasmids, pEF321-core (39)-LN and pEF321-core (37)-LN, into the HepG2 cells as linear forms digested at the unique *ScaI* site on the ampicillin resistance gene of the plasmids and then selected with G418 (0.8 mg/ml) for 30 days. Many G418 resistant colonies were obtained from both of the plates transfected with each of the two expression plasmids. Among the colonies, HepG2 cell clones which stably expressed the core protein were screened by indirect immunofluorescence assay using the serum of a type C hepatitis patient containing the antibody to the HCV core protein.

The full length core protein was expressed in almost all of the G418 resistant colonies in large amount showing a strong fluorescence signal. But the truncated core protein was expressed only in about 30% of the G418 resistant colonies. Unexpectedly, the fluorescence signal of truncated core protein in all of the stable cell clones was strongly weaker than that of the full length core protein indicating that the expression level of the full length core protein is higher than the truncated core protein in stable expression (Fig. 2).

As in the transient expression system, the full length core protein was localized in the cytoplasm and the truncated core protein was localized in the nucleus of the stably expressing cell clones (Fig. 2). We selected each one cell clone showing relatively strong fluorescence from the stable cell clones of full length and truncated core protein for further study.

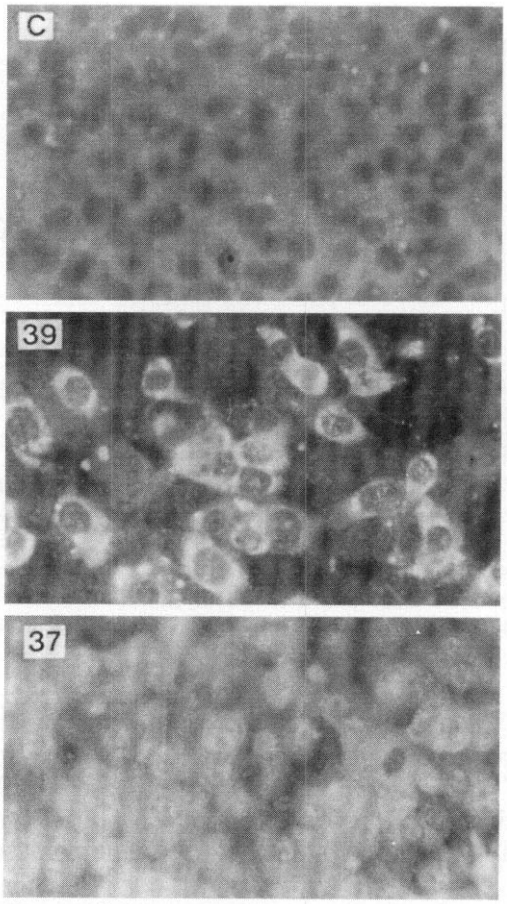

Fig. 2. Immunofluorescence photomicrographs of HepG2 cells stably expressing the HCV core protein. After selection with G418 (0.8 mg/ml) for one month, the cells were subjected to indirect immunofluorescence assay. The core protein was detected in the cytoplasm (39) and nucleus (37), but the core protein was not detected in the untransfected HepG2 cell (C).

Growth characteristics of HepG2 cell clones which express the core protein stably

We have examined the growth characteristics of the stable cell clones which constitutively express the full length or truncated core protein.The HepG2 cell clone stably expressing the full length core protein did not show any distinctive features in the growth pattern or morphology compared to the untransfected cells. However, a large number of the cells in the clone which stably express the nuclear, C-terminal truncated core protein displayed a round shape in morphology and were detached from the plate. During cultivation of the cell clones which express the truncated core protein, large quantities of dead cells floating in the medium were observed (data not shown). Similar result, indicating that the core protein localized in the nucleus inhibits the cell growth and consequently may induce cell death, was also observed in the DNA fragmentation assay. As shown in Fig. 3, the

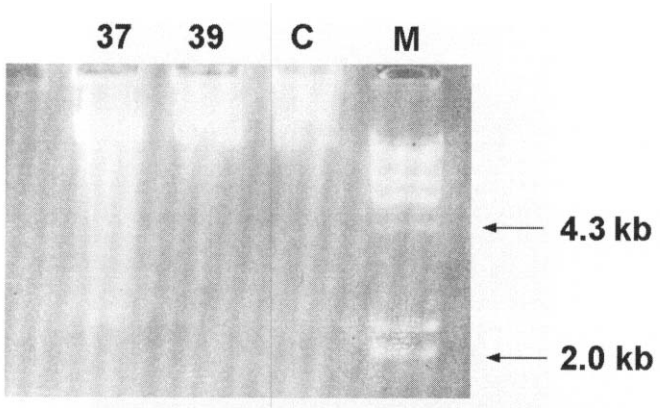

Fig. 3. Degradation of chromosomal DNA of HepG2 cells stably expressing the full length or truncated core protein. Total chromosomal DNAs isolated from the untransfected HepG2 cells (C) and the HepG2 cell clones expressing full length (39) or truncated (37) core protein were analyzed by 1.5% agarose gel electrophoresis. The DNA size markers of the λ-*Hind*III digest are shown (M).

degradation of the chromosomal DNA of the cells constitutively expressing the truncated core protein was more apparent than the full length core-expressing cells or untransfected cells. The fact that we could not obtain the stable cell clone which strongly express the nuclear core protein seems to suggest that the core protein in the nucleus may be toxic and induce cell death.

Effect of core protein on apoptosis induced by anti-cancer drug

To examine the susceptibility of HepG2 cells expressing the HCV core protein to anti-cancer drugs, the cells were treated with bleomycin and 5-fluorouracil which are apoptosis-inducing agents. Subconfluent cultures of the HepG2 cells stably expressing the full length or truncated core protein were first treated with 12 μg/ml of bleomycin for 40 h in a culture medium containing 2.5% FCS. Apoptosis was evaluated by the morphological change which was observed under a microscope and by the DNA fragmentation assay. As shown in Fig. 4, the HepG2 cell clones expressing full length or truncated core protein revealed typical morphology of cellular shrinkage undergoing apoptosis whereas untransfected control cells did not show any notable change in treatment with bleomycin. Cellular DNAs isolated from the HepG2 cells expressing the core protein also exhibited an elevated fragmentation pattern compared to the DNA isolated from the untransfected cells, indicating that both of the full length and truncated core protein sensitize the HepG2 cells to apoptosis induced by bleomycin (Fig. 5).

We then treated the same HepG2 cells with 5-fluorouracil in various doses of 100, 300, and 500 μM.

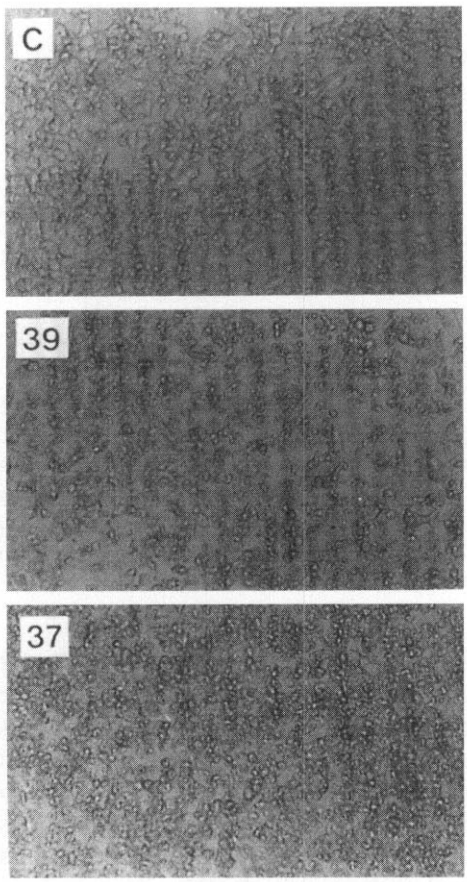

Fig. 4. Morphology of HepG2 cells after treatment with bleomycin. Untransfected HepG2 cells (C) and cell clones which stably express the full length (39) or truncated (37) core protein were treated with 12 μg/ml of bleomycin for 40 h and photographed by microscopy (magnification, × 100).

However, the sensitization to the apoptosis was not observed by the 5-fluorouracil in HepG2 cells expressing the full length or truncated core protein(data not shown). These results suggest that the HCV core protein has a role in causing cells to enter the apoptotic pathway which is activated by the anti-cancer drug. The fact that cell death by 5-fluorouracil was not stimulated in core-expressing cells suggests that the apoptosis related to the core protein is restricted to a specific pathway.

Previous reports indicated that the HCV core protein has some regulatory functions in the process of cellular apoptosis although both inductive and suppressive effects are observed in different conditions and in different cell lines (21, 24, 25, 26, 34). Interestingly, the Fas- and TNF-mediated apoptosis was clearly activated by the core protein and it was demonstrated that the core protein is associated with the liver cell injury by HCV infection (26, 34). Our results also suggest that the HCV core protein, regardless of its intracellular localization, plays an important role in the induction of cell death during

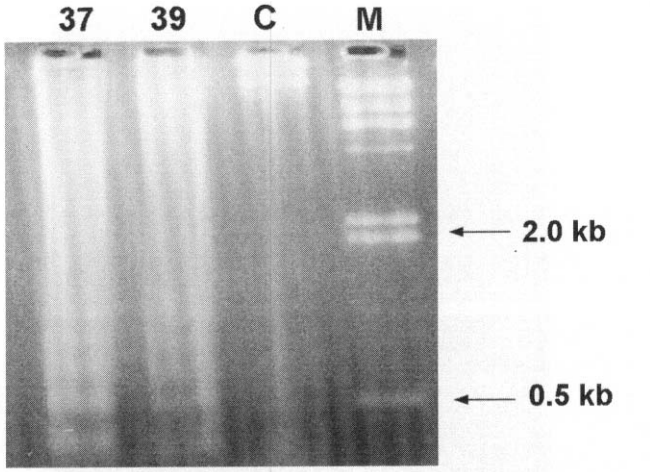

Fig. 5. DNA fragmentation of HepG2 cells stably expressing the HCV core protein. Total chromosomal DNAs of untransfected HepG2 cells (C) and HepG2 cell clones which stably express the full length (39) or truncated (37) core protein were isolated after treatment with belomycin and analyzed by 1.5% agarose gel electrophoresis. The DNA size markers of λ-*Hind*III digest are shown (M).

HCV infection, although the molecular mechanism is not known. Furthermore, the result that the HCV core protein causes cells to be highly susceptible to bleomycin suggests a potential target for therapy against hepatocellular carcinoma associated with chronic HCV infection.

In conclusion, by establishing HepG2 cell clones which stably express the full length or truncated core protein, we demonstrated that the HCV core protein is implicated in the pathogenicity of HCV infection enhancing apoptotic cell death.

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