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SUPPRESSION OF SERUM STARVATION-INDUCED APOPTOSIS BY HEPATITIS C VIRUS CORE PROTEIN

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KEY WORDS

Hepatitis C virus; core protein; serum starvation; apoptosis; inhibition; p38 MAP kinase

ABSTRACT

Hepatitis C virus (HCV) core protein either enhances or inhibits apoptosis depending on the apoptosis-inducing stimuli and cell conditions. In this paper we studied possible effect of HCV core protein on apoptosis induced by serum starvation. NIH3T3 cells stably expressing HCV core protein were more resistant to serum starvation-induced apoptosis than were the non-expressing control. Neither p53, p21Wafl nor Bax was detectably induced after serum starvation, irrespective of HCV core protein expression, suggesting that the observed apoptosis is p53-independent. Serum starvation-induced apoptosis was partially inhibited by SB203580, a specific inhibitor of p38 mitogen-activated protein (MAP) kinase, in the non-expressing control, but not in HCV core protein-expressing cells. Moreover, activation of p38 MAP kinase after serum starvation, as measured by the amount of its phosphorylated form, was inhibited in HCV core protein-expressing cells. Our results suggest that HCV core protein inhibits serum starvation-induced apoptosis through inhibition of p38 MAP kinase activation.

INTRODUCTION

Hepatitis C virus (HCV), a member of the *Flavivividae* family, has been known to be a major causative agent of post-transfusion and sporadic non-A, non-B viral hepatitis worldwide, which readily establishes chronic persistent infection that often leads to liver cirrhosis and hepatocellular carcinoma.[11] The viral genome,

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single-stranded, positive sense RNA of approximately 9.6 kb, exhibits a considerable degree of sequence variation, based on which HCV is currently classified into at least 6 clades (previously called major genotypes) and more than 60 subtypes.[6, 20. 29)] Geographic distribution and clinico-pathological features are thought to vary with different HCV clades and subtypes.[2, 6, 20. 35)] The viral genome encodes a polyprotein precursor consisting of about 3,010-3,033 amino acid residues, which is cleaved by the host signal peptidase and two virally encoded proteases into at least 10 viral proteins; core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B.[11)]

HCV core protein exerts versatile effects on host cells. It exhibits transcriptional suppression of certain promoters[28, 32, 33)] while exhibiting transcriptional activation of another promoter through differentially regulating nuclear transcription factors.[5, 19, 34)] HCV core protein was shown to associate with cellular lipid storage droplet[3)] and induce hepatic steatosis in transgenic mice.[22)] Also, cells expressing HCV core protein exhibit oncogenic phenotypes[4, 25, 38)] and, moreover, transgenic mice that express HCV core protein in the liver tends to develop hepatocellular carcinoma.[21)] These results strongly suggest an oncogenic property of HCV core protein. In addition, HCV core protein is likely involved in the regulation of apoptosis, either positively or negatively. For example, it was reported that HCV core protein enhanced Fas- and tumor necrosis factor (TNF)-mediated apoptosis.[31, 41)] In contrast, other research groups demonstrated that HCV core protein inhibited DNA-damaging agent-induced apoptosis[26)] and even TNF-mediated apoptosis.[19, 27)] Thus, HCV core protein appears to exert varying effects on apoptosis depending on the apoptotic stimuli as well as cell types and conditions.

Apoptosis includes cell death caused by trophic factor deficiency, in which p38 mitogen-activated protein (MAP) kinase plays a role in mediating apoptotic signal.[15, 39)] It is interesting to know whether HCV core protein enhances or inhibits apoptosis induced by serum starvation, the deficiency of growth factors. In this report, we describe that HCV core protein inhibits serum starvation-induced apoptosis, possibly through inhibiting p38 MAP kinase signaling pathway.

MATERIALS AND METHODS

Plasmid construction and generation of HCV core protein-expressing stable cell lines

A cDNA fragment encoding the entire region of HCV core protein (amino acids I to 191) was amplified from HCV C980 cDNA9) by PCR using a set of primers (5 '-TAGAGAATTCATCATGAGCACAMAATCCTAA-3'and5'-TGCGGAATTC CTAAGCGGAAGCGGAAGCTGGGATGG-3 '; the E*co*RI recognition sequence is underlined, and the initiation codon and a complimentary sequence of a

stop codon in boldface letters). The amplified fragment was digested with E*co*RI and subcloned into the unique ECORI site of pH8 expression plasmid.[16] The resultant plasmid, pH8-Jcore-F, could mediate constitutive expression of HCV core protein under the control of murine leukemia virus long terminal repeats.

NlH3T3 cells were maintained in Dulbecco's modification of Eagles' medium containing 10% heat-inactivated fetal calf serum and gentamicin (50 µg/ml) at 37 'C in a C02 incubator. The cells were transfected with pH8-Jcore-F by calcium phosphate co-precipitation method36) and cultured in the presence of a neomycin derivative G418 (400 µg/ml). As a control, NlH3T3 cells were transfected with pH8 vector plasmid. After 2 to 3 weeks, each colony of G418-resistant stable transfectants was cloned using a cloning cylinder and grown to bulk.

Analysis of apoptotic cell death

Apoptotic cell death under serum-starved condition was examined by the following methods. (i) Cell survival rate: Cells were seeded into each well (6 mm in diameter) of a 96-well plate at a concentration of 5 x 10s cells/well and allowed to grow overnight. After rinsed once with serum-free medium, the cells were incubated in fresh serum-free medium for 7 days. Cell viability was measured by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] conversion assay as described previously.7) In brief, 10 u1 of MTT stock solution (5 mg/ml) was added to each well of the tissue culture plates, which were incubated at 37'C in a C02 incubator. M~ is known to produce an insoluble purple formazan product in the presence of mitochondrial enzymes in living cells. After 4 h, 100 u1 of HCl/isopropanol solution was added to each well and the plates were incubated at room temperature overnight to dissolve the MTT formazans in the cell. The absorbance of each well was measured at 570 nm using a microplate reader. (ii) Morphological changes of the nuclei: Cells seeded on a plastic coverslip were washed once with phosphate-buffered saline (PBS), fixed with 100% methanol at -20 'C for 20 min, and stained with Hoechst 33342 at room temperature for 10 min. The morphology of the nuclei was observed under a light microscopy. For quantitative analysis, at least 1,000 cells were counted for each clone and percentages of cells with condensed chromatin and/or fragmented nuclei were determined.

Western blot analysis

Cells were lysed in a buffer consisting of 50 mM Tris-HCI (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue and 10% glycerol. The samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically blotted onto a polyvinylidene difluoride filter.

After blocking in PBS containing 3.% skim milk, the filters were incubated with a first antibody, washed five times with PBS containing 0.5.qif.o Tween 20, and then incubated with a peroxidase-labeled second antibody. After washing five times, the protein bands were visualized by enhanced chemical luminescence (ECL-protein detection; Amersham Pharmacia Biotech., Buckinghamshire, U.K.).

Other chemicals and antibodies

A specific inhibitor for p38 MAP kinase, SB203580, was used at a final concentration of I μM. Mouse monoclonal antibody against HCV core protein was a kind gift from Dr. M. Kohara, Tokyo Metropolitan Institute for Medical Science, Tokyo, Japan. Mouse monoclonal antibody against p38 MAP kinase (A-12; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U. S. A.) and rabbit polyclonal antibody against phosphorylated p38 MAP kinase (New England Biolabs, Inc., Bevery, MA, U. S. A.) were purchased.

RESULTS

HCV core protein suppresses serum starvation-induced apoptosis

Five clones expressing HCV core protein (C-3, -6, -7, -12, -14) and five control clones (P-1, -3, -4, -5, -17) were obtained by plasmid transfection, followed by G418 selection and cell cloning. Immunofluorescence analysis revealed a granular staining of HCV core protein predominantly in the cytoplasm, with a minor fraction of the staining in the nucleus (Fig. 1a), the result being consistent with previous report.14) Western blot analysis confirmed a single band of 21 kDa of HCV core protein in the positive clones (Fig. 1b).

Using the clones obtained, possible effects of HCV core protein on serum starvation-induced apoptotic cell death were analyzed. We first examined survival ratios of cells under serum-starved condition. Control cell clones began to die within 1-2 days after serum starvation and the number of dead cells increased with time (Fig. 2a). On the other hand, HCV core protein-expressing clones almost completely survived serum starvation up to 7 days. Fig. 2b shows percent survival of each clone 7days after serum starvation. The difference in the mean survival ratio between HCV core protein-expressing clones and the control was statistically significant ($P < 0.01$). To confirm that the cell death was in fact due to apoptosis, we performed morphological observation of the nuclei. Chromatin condensation and nuclear fragmentation were clearly observed in the control cells, but barely in HCV core protein-expressing cells (Fig. 3). Practically the same results were obtained with the other cell clones (data not shown). These results indicated that cell death caused by serum starvation was principally apoptosis and that HCV core

protein suppressed serum starvation-induced apoptosis.

Anti-apoptotic effect of HCV core protein could be detected by morphological observation of the nuclei as early as 36 h after serum starvation. As shown in the Table, 8.6% of the control clones showed chromatin condensation and/or fragmentation of the nuclei while only 2.5% of HCV core protein-expressing cells showed the same morphological changes. The difference between the two groups was statistically significant ($P < 0.01$).

p38 MAP kinase activation is involved in serum starvation-induced apoptosis and is inhibited by HCV core protein

It has recently been reported that apoptosis induced by withdrawal of trophic factors in a neuronal as well as a non-neuronal cell line is mediated by activated p38 MAP kinase.[15] We tested whether this was the case in our experimental system. A specific inhibitor of p38 MAP kinase, SB203580, was used to assess this issue. Treatment with SB203580 inhibited serum starvation-induced apoptosis in non-expressing control cells, with the difference between the treated and untreated cells being statistically significant (Fig. 4). On the other hand, the same inhibitor treatment did not affect serum starvation-induced apoptosis in HCV core protein-expressing cells.

p38 MAP kinase is activated upon phosphorylation.[8, 24] Therefore, the activation status of p38 MAP kinase can be measured by the amount of its phosphorylated form. Western blot analysis revealed that the amounts of the phosphorylated form of p38 MAP kinase before serum starvation were practically the same between HCV core protein-expressing cells and the control (Fig. 5, a and c). After 16 h of serum starvation, the phosphorylated form of p38 MAP kinase increased markedly in the non-expressing control cells, but barely in HCV core protein-expressing cells (Fig. 5, b and c). It should be noted that total amounts of p38 MAP kinase did not differ significantly between HCV core protein-expressing cells and the control. These results suggested that activation of p38 MAP kinase after serum starvation was inhibited in HCV core protein-expressing cells.

DISCUSSION

Apoptosis is one of the important host defense mechanisms and, therefore, the inhibition of apoptosis is thought to be a major mechanism of viral persistence, which is also an important step toward malignant transformation. Indeed, many viruses are known to possess apoptosis-inhibiting proteins, such as adenovirus EIB, human papillomavirus E6, cowpox virus CrmA, Epstein-Barr virus BHRFI and LMP 1, and hepatitis B virus X protein.37) It is not surprising that HCV possesses an

ability to counteract apoptosis. We previously reported that the NS3 protein of HCV interacts with p53, a key molecule for apoptosis induced by DNA damaging agents, and inhibits actinomycin D-induced apoptosis.[7, 12, 13, 23] We have also observed that HCV core protein-expressing cells are more resistant to actinomycin D-induced apoptosis than are the NS3-expressing cells (data not shown).

In addition to DNA-damaging agents and other apoptosis-inducing stimuli, such as TNF and Fas, trophic factor deficiency is known to induce apoptosis.[15, 39] Therefore, we analyzed the possible effect of HCV core protein on serum starvation-induced apoptosis. Our results have clearly demonstrated that HCV core protein inhibits serum starvation-induced apoptosis (Figs. 2 and 3, Table I). It should be noted that the cell growth in normal medium did not differ between HCV core protein-expressing clones and the control (doubling time, 20.2±2.6h and 21.9±4.1h, respectively; P = 0.34). It was unlikely, therefore, that HCV core protein-expressing cells proliferated more rapidly and hence showed higher survival rate.

We then tried to look into the possible mechanism by which HCV core protein inhibits serum starvation-induced apoptosis. The tumor suppressor protein p53 or its downstream mediators, such as p21Wafl and Bax, was barely induced in serum-starved cells, irrespective of HCV core protein expression (data not shown). This result suggests that serum starvation-induced apoptosis is p53-independent.

It has been reported that apoptosis induced by withdrawal of trophic factors is mediated by increased p38 MAP kinase activity.[15] p38 MAP kinase is a member of the MAP kinase superfamily, which is activated by inflammatory cytokines, such as interleukin-1 and TNF-a, by environmental stresses including UV, heat, hyperosmolarity, and by withdrawal of growth factors.[8, 15, 17, 24, 30] p38 MAP kinase is distinguished from extracellular signal-regulated kinases (ERKS) of the MAP kinase superfamily that are regulated by mitogens and growth factors via Ras/Raf-dependent pathway.[17] By using SB203580, a specific inhibitor of the p38 MAP kinase signaling pathway, serum starvation-induced apoptosis was significantly lowered in the control cells (Fig. 4), suggesting that serum starvation-induced apoptosis was indeed mediated by p38 MAP kinase in our experimental system as well. Inhibition of p38 MAP kinase by SB203580 is thought to reduce caspase 3 activity, resulting in the inhibition of apoptosis.[18] On the other hand, the same inhibitor treatment did not exert any detectable effect on serum starvation-induced apoptosis in HCV core protein-expressing cells (Fig. 4). This result could be interpreted in two ways; (i) the extent of serum starvation-induced apoptosis was so low in HCV core protein-expressing cells that the inhibitory effect of SB203580, if any, was not significantly detected, or (ii) activation of p38 MAP kinase after serum starvation was already impaired by HCV core protein so that the inhibitory effect of SB203580 was masked. In favor of the latter idea, the activated (phosphorylated)

form of p38 MAP kinase after serum starvation was reduced in HCV core protein-expressing cells compared with the non-expressing control cells (Fig. 5). Taken together, our present results suggest the possibility that HCV core protein inhibits serum starvation-induced apoptosis by inhibiting p38 MAP kinase activation. In this connection, it has been reported that HCV core protein interacts with 14-3-3 proteins, [1] the proteins shown to block apoptosis through p38 MAP kinase inhibition and/or ERK activation.[40] Thus, the involvement of HCV core protein in regulating MAP kinase cascades is likely important in determining the cell fate upon various stimuli.

It was recently reported that, upon serum starvation, Chines hamster ovary cells expressing HCV core protein underwent apoptosis to a larger extent, concomitantly exhibiting increased expression of c-Myc, p53, p21Wafl and Bax, compared with the non-expressing control cells.[10] Those results are inconsistent with ours; in our system, neither p53, p21Wafl nor Bax was induced by serum starvation in NIH3T3 cells (data not shown) and, moreover, HCV core protein-expressing NIH3T3 cells (Figs. 2-4, Table I) and L929 cells (data not shown) were more resistant to serum starvation-induced apoptosis than were the non-expressing control. The reason for the discrepancy between their results and ours is currently unknown. In this connection, it should be noted that similar discrepancy was reported, i. e., the apparently opposite effects, either pro-apoptotic or anti-apoptotic, of HCV core protein on TNF-α-mediated apoptosis.[19, 27, 31, 41] It is possible that serum starvation, or an apoptosis-inducing stimulus in general, affects the network of intracellular signal transduction pathways in a different manner depending on the cell types tested, and therefore, the effect of HCV core protein on serum starvation-induced apoptosis might vary with different cell lines. Further study is needed to elucidate the issue.

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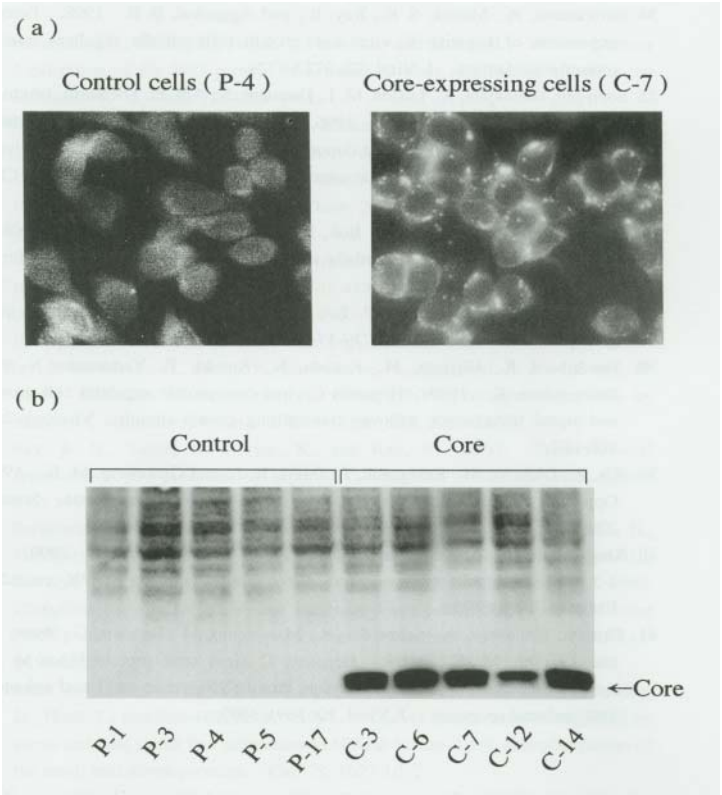


Fig. 1. Expression of HCV core protein in NlH3T3 stable transfectants.
(a) Indirect immunofluorescence analysis of a control (P4) and a HCV core protein-expressing clone (C-7) using monoclonal antibody against HCV core protein. (b) Western blot analysis of the control (P-1, -3, 4, -5 and -17) and HCV core protein-expressing clones (C-3, -6, -7, -12 and -14) using the same monoclonal antibody as in (a).

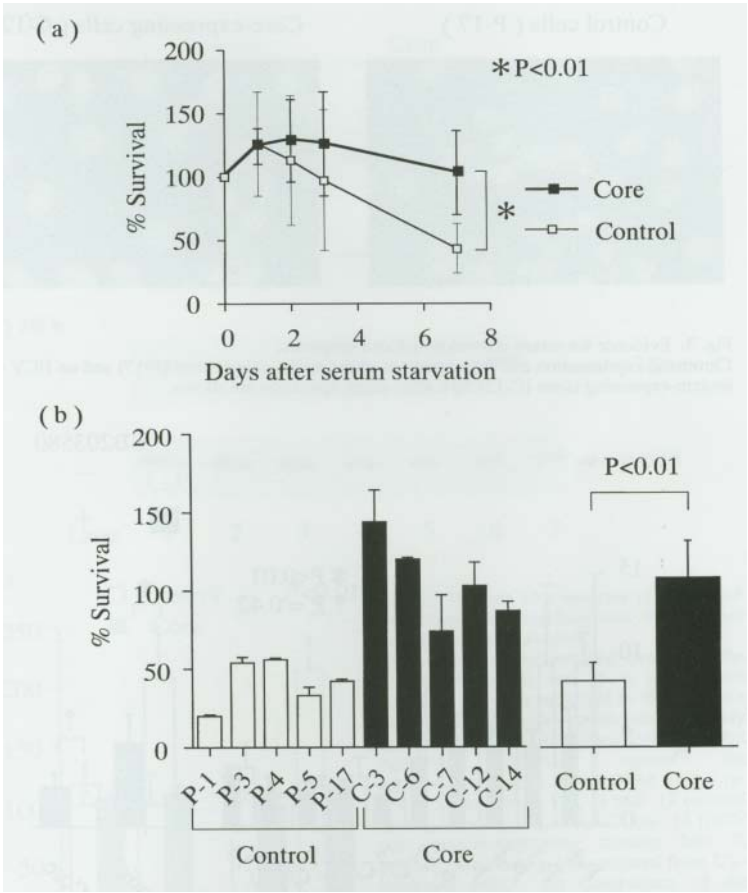


Fig.2. Cell viability of HVC core protein-expressing clones and the control after serum starvation.

(a) Time course of the mean viability, as determined by MTT assay, of five HCV core protein-expressing cloncs (filled squares) and five non-expressing control clones (empty squares). Vertical lines indicate S.E. *, P <0.01. (b) The viability of each clone 7 days after serum starvation. Empty columns, control clones; filled columns, HCV protein-expressing clones. Mean values ± S.E. of the five clones are shown on the right.

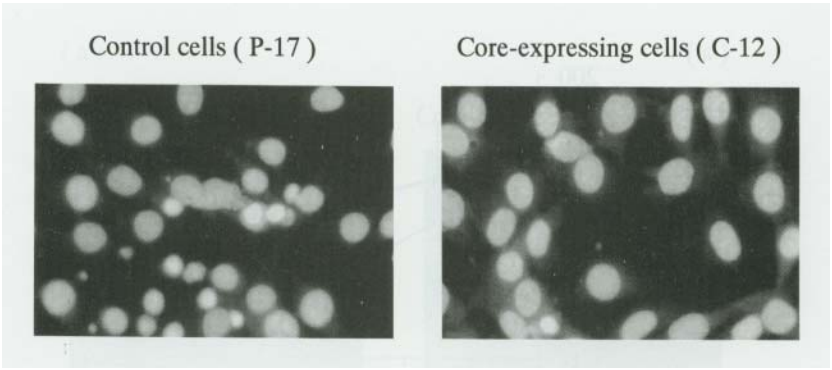


Fig. 3. Evidence for serum starvation-induced apoptosis.
Chromatin condensation and fragmentation of the nuclei of a control (P-17) and an HCV core protein-expressing clone (C- 12) 36 h after serum starvation are shown.

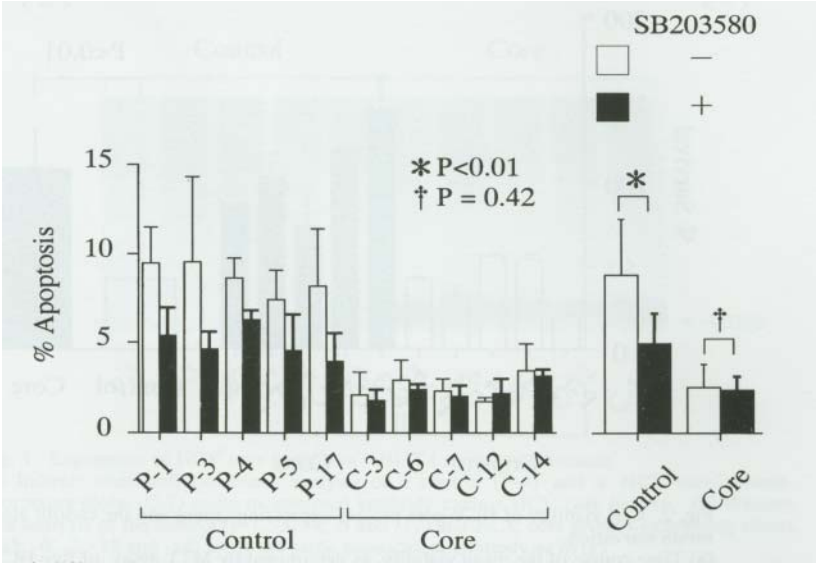
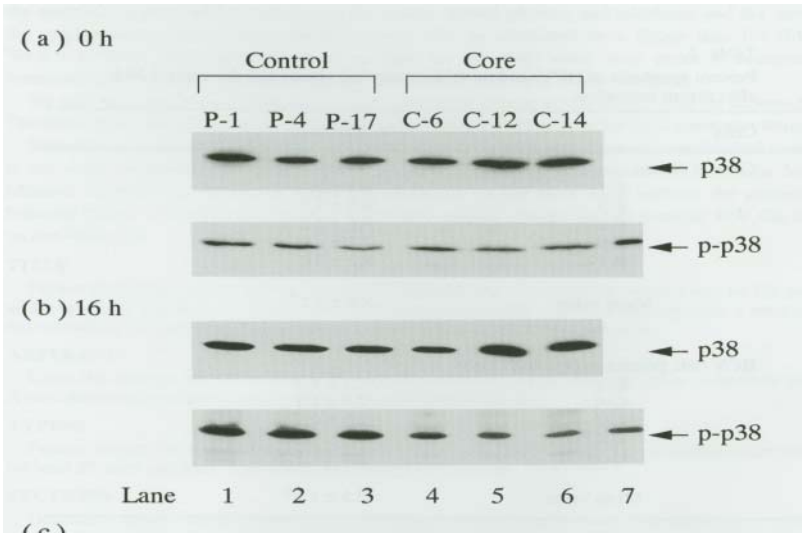


Fig.4.Effect of a specific p38 MAP kinase inhibitor, SB203580, on serum starvation-induced apoptosis in HCV core protein-expressing clones and the control 36 h after serum starvation.
Empty columns indicate cells without inhibitor treatment and filled columns those treated with the inhibitor. Data represent mean values ± S.E. obtained from four independent experiments. *, P <0.01.



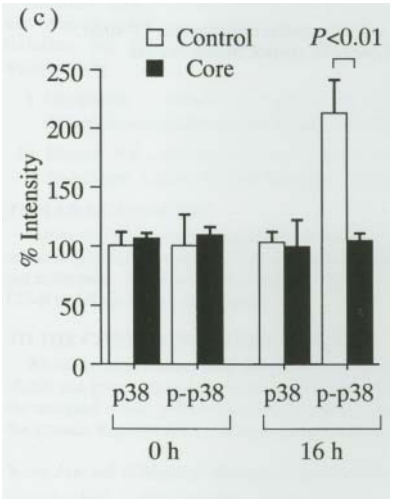


Fig. 5. Western blot analysis of p38 MAP kinase and its phosphorylated form before and after serum starvation. HCV core protein-expressing clones and the control before (a) and 16 h after serum starvation (b) were subjected to Western blot analysis using mouse monoclonal antibody against total p38 MAP kinase (p38) or rabbit polyclonal antibody against the phosphorylated form of p38 MAP kinase (p-p38). Lanes I to 3, P-1, -4 and -17 (control clones); Ianes 4 to 6, C-6, -12 and -14 (HCV core protein-expressing clones); Iane 7, positive control for p-p38 prepared from UV-irradiated cells. (c) Comparison of the amounts of p38 and p-p38 between HCV core protein-expressing clones (filled columns) and the control (empty columns) before (0 h) and 16 h after serum starvation. The mean values of the three control clones before serum starvation were referred to as 100%.

Table I.

Percent apoptosis of HCV core protein-expressing clones and the control 36 h after serum starvation.

Cells	% Apoptosis
Control clones	
P-1	9.4 ± 2.0
P-3	9.5 ± 4.8
P-4	8.6 ± 1.2
P-5	7.4 ± 1.6
P-17	8.2 ± 3.2
Mean value	8.6 ± 3.1*
HCV core protein-expressing clones	
C-3	2.1 ± 1.3
C-6	2.9 ± 1.1
C-7	2.3 ± 0.7
C-12	1.7 ± 0.3
C-14	3.4 ± 1.5
Mean value	2.5 ± 1.2*

Note. Values are means ± S.D. of n = 4 independent experiments. *P <0.01, HCV core protein-expressing clones vs. control, Student's t test.