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HCV E2 may induce apoptosis of Huh-7 cells via a mitochondrial-related caspase pathway

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Abstract

Introduction: One unusual characteristic of HCV is to establish chronic infection and the precise mechanisms remain unclear.

Materials and methods: Huh-7 cells were transiently transfected with E2 and subjected to MTT assay, DNA fragmentation assay, and Western blotting to see the impact of E2 protein on apoptosis.

Results and discussion: E2 may inhibit cell proliferation by inducing apoptosis and pro-caspases 3, 8, and 9 were cleaved and activated to result in the presence of active forms in a time-dependent fashion, which suggest that E2-induced apoptosis is caspase-dependent. Furthermore, the cytosolic level of cytochrome *c* was increased together with a gradually down-regulated Bcl-2 and up-regulated Bax protein expression. The continuing reduction of Bid protein and the gradual increase of tBid protein also indicated that a time-dependent increased turn-over of Bid protein into tBid. Taken together, our data suggested that HCV E2 may induce apoptosis through a mitochondrial damage-mediated caspase pathway.

Keywords: Hepatitis C virus; Envelope protein 2; Apoptosis; Cytochrome *c*; Caspase

Since being identified as the major etiological agent of most transfusion-associated non-A, non-B hepatitis in 1989, hepatitis C virus (HCV) infection has been one of major causes of chronic hepatitis and liver failure worldwide. One unusual characteristic of hepatitis C is its capability to establish chronic infection in most infected individuals. Such persistent infection may be mild, or sometimes even asymptomatic, in early phases of the disease, however, after a decade or two, may cause liver cirrhosis and eventually hepatocellular carcinoma [1], which then have a great social and economical impact.

Generally, cellular antiviral responses are effective to clear most viral infections, but various viruses possess mechanisms that counteract these antiviral activities to establish a persistent infection and lead to viral pathogenesis. A variety of mechanisms to maintain viral persistence have been proven or proposed, including immune escape derived from quasispecies diversity or high mutation rates, impaired immune functions resulted from the interference of viral proteins, failure to develop an effective immune response, and viruses escaping from the apoptotic mechanism. As for HCV, the precise related mechanisms of carcinogenesis and mechanisms involved in evading host immune surveillance remain to be elucidated, however, analysis of peripheral blood mononuclear cells and liver biopsies from chronic patients suggested that HCV infection could induce apoptosis, which may help the virus escape the immune surveillance and cause liver injuries [2–7]. In vitro studies with either HCV full-length RNA or cDNA [8] have demonstrated that apoptosis could be induced by viral proteins. Several independent studies

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suggested that in addition to mediating pathological effects of HCV through several of its multifunctional activities [9,10], core protein of HCV could modulate cellular apoptosis [11–14] and is involved in cell growth promotion and immortalization [14–17]. However, others using similar systems obtained inconsistent results [13,15,16]. Furthermore, the involvement of other HCV proteins in the induction of apoptosis has not been clearly identified.

Known as the viral attachment protein responsible for the viral entry, HCV envelope protein 2 (E2), a structural protein, has been interested for its potential to induce apoptosis since this protein shares a similar genetic characteristic as the envelope proteins of certain Flaviviruses, which have been reported to be able to induce apoptosis in cultured mammalian cells to maintain persistent infection [18–22]. Although a study has indicated that expression of HCV E2 protein could induce apoptosis in cultured mammalian cells to contribute the liver injury [23], another study showed that E2 inhibits apoptosis by inhibiting TRAIL-induced cytochrome *c* release from the mitochondria, which may subsequently augment persistent HCV infection [24]. Therefore, further study is required to clarify the impact of HCV E2 protein on apoptosis. In this study, we examined the expression levels of apoptosis-related molecules in an HCV E2-expressing Huh-7 cell line to elucidate the role of HCV E2 protein in establishing chronic infection and liver injury.

Materials and methods

Cell culture. Huh-7, a human hepatocellular carcinoma cell line, was grown in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, 0.1 mM nonessential amino acid, and 1 mM sodium pyruvate and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Construction of a plasmid containing E2 gene and transfection. A DNA fragment containing full-length E2 coding region (aa 498–777 of the HCV polyprotein) was obtained from a PCR amplification with primers 5′-GCGAATTCACCCACACGACGGGGAGGGT-3′ (*Eco*RI site underlined) and 5′-GCGGATCCGGCTGAGTTCTGACCTATCC-3′ (*Bam*HI site underlined) [25] and then cloned into a expression vector pEGFP-N1 (Beckton–Dickinson) to yield an E2 expression vector, pEGFP-E2.

At the day prior to transfection, cells were plated on 6-cm tissue culture dishes at a density of 4×10^5 cells/dish and cultured for 18 h. A lipofectamine reagent (Invitrogen, Life Technologies) was used to carry out transfection according to the manufacturer's instructions. Each plate received a mixture of 6 μg of pEGFP-N1 or pEGFP-E2 and 6 μL Lipofectamine reagent in 2.0 mL DMEM (without serum). After the cells were exposed to the DNAs for 6 h, an additional 4.0 mL of fresh DMEM was added to each plate. A mock transfection with pEGFP-N1 only will be simultaneously performed to act as a mock control and transfection efficiency was monitored by pEGFP-N1 which encodes an enhanced green fluorescent protein. At 24 and 48 h post-transfection, cells were harvested and analyzed for cell proliferation, apoptosis, and Western blot.

Cell viability (MTT assay). Cells were seeded in 24-well plates at a density of 3.0×10^4 cells/well and then transfected with pEGFP-E2 or pEGFP-N1. At 24 or 48 h after transfection, 0.5 mg/mL 3-[4,5]-2,5-diphenyltetrazolium bromide (MTT) in fresh medium was added and incubated for additional 4 h. Afterwards, the blue formazan crystals were dissolved in 1 mL isopropanol and measured spectrophotometrically at 563 nm.

DNA fragmentation. Cells were harvested and incubated in lysis buffer (10 mM/L Tris, 1 mM/L EDTA, 100 mM/L NaCl, 5 g/L SDS, and 1 μg/μL RNase A, pH 8.0) at 37 °C for 30 min. At the end of incubation, proteinase K was added to a final concentration of 0.1 mg/mL and the incubation was continued at 55 °C for 4 h. DNA was extracted with phenol/chloroform and precipitated with ethanol. DNA pellets were dissolved in TE buffer and analyzed on a 1% agarose gel.

Western blot analysis. Cell lysates were separated in a 12.5% polyacrylamide gel and transferred onto a nitrocellulose membrane as previously described [26]. The blot was subsequently incubated with 5% non-fat milk in PBS for 1 h to block non-specific binding, and probed with a corresponding antibody against a specific protein (H1920-19 for E2 was obtained from United States Biological; B-10 for Fas, C-178 for Fas-L, H-181 for FADD, C-2 for Bcl-2, N-20 for Bax, N-19 for Bid, H-104 for cytochrome *c*, H-38 for caspase 9, and L-18 for caspase 3 were purchased from Santa Cruz, California, USA) for 2 h, and then with an appropriate peroxidase-conjugated secondary antibody for 1 h. All incubations were carried out at 37 °C and intensive PBS washing was performed between incubations. After the final PBS washing, signal was developed by ECL detection system and relative photographic density was quantitated by scanning the photographic negatives on a gel documentation and analysis system (Alpha Imager 2000, Alpha Innotech Corporation).

Statistical analysis. Statistical significances of difference throughout this study were calculated by Student's *t*-test (SigmaStat 2.0, Jandel Scientific) with $P < 0.05$ being regarded as statistically significant.

Results

E2 inhibits the proliferation of Huh-7 cells by inducing apoptosis

To test the impact of E2 protein on the proliferation of Huh-7 cells, an E2 gene fragment was cloned into pEGFP-N1, a plasmid containing EGFP gene, to construct an E2-expressing plasmid, pEGFP-E2, and then transiently transfected into cells. The successful transfection was confirmed by microscopic observation and Western blot. After transfection, pEGFP-E2-transfected cells showed significant cell death by microscope observation (data not shown) revealing reduced cell density and cell shrinkage situation. The effect of E2 protein on cell proliferation was further analyzed by MTT analysis. As shown in Fig. 1A, E2 expression inhibited Huh-7 cell proliferation in a time-dependent fashion with cell viability being reduced to 70% at 48 h post-transfection, as compared with that of mock transfection.

Among several possible mechanisms involved in reduced cell proliferation, to determine if apoptosis is the underlying mechanism for the reduced cell proliferation of E2 expressing cells, cells transfected with pEGFP-E2 or control vector pEGFP-N1 were subjected to DNA fragmentation assay. As shown in Fig. 1B, the formation of DNA ladder in pEGFP-E2 transfected cells was observed in a time-dependent manner while cells transfected with pEGFP-N1 showed no evident DNA fragmentation. Therefore, we conclude that E2 protein inhibits the proliferation of Huh-7 cells through the induction of apoptosis.

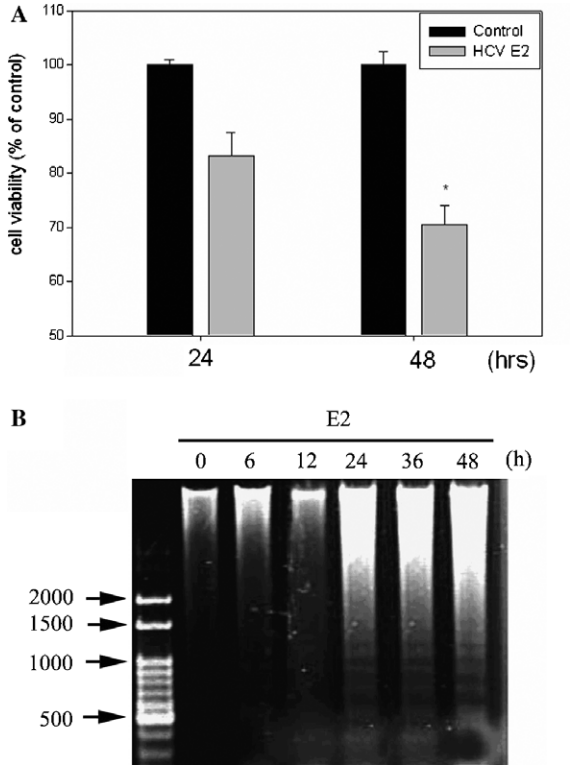

Fig. 1. HCV E2 inhibits the proliferation of Huh-7 cells by inducing apoptosis. (A) At 24 or 48 h after E2 transfection, 0.5 mg/mL MTT in fresh medium was added and incubated for additional 4 h. The survival of cells was detected by measuring the absorbance at a wavelength of 563 nm and expressed as the ratio of control. Data represent means ± SD of three independent experiments. * indicates that this difference reached a statistical significance with a $P < 0.01$. (B) At the indicated time after transfection, cells were harvested and subjected to DNA extraction. The DNA fragments were separated on 1% agarose gel electrophoresis, stained with ethidium bromide, and then visualized under ultraviolet light.

Activation of caspases in E2-transfected Huh-7 cells

To explore the molecular mechanism responsible for E2-induced apoptosis, expression levels of several apoptosis-related genes, including Fas, Fas ligand, FADD, Pro-caspases, and caspases 3, 8, and 9 were analyzed by Western blots. The unchanged expressions of Fas, Fas ligand or FADD before and after the E2-transfection indicated that these molecules were not involved in the E2 protein-induced apoptosis (data not shown). It has been well established that caspases are involved in and indispensable for the final stage of apoptosis. Upon responding to various stimuli, pro-caspases would be classically divided into active forms to carry out their functions. As shown in Fig. 2, pro-caspases 8 and 9 were cleaved and activated in pEGFP-E2 transfected cells to result in the presence of active forms of caspases 8 and 9. Furthermore, caspase 3, a downstream protease of caspases 8 and 9, was also cleaved and activated in cells after being transfected with pEGFP-E2. These cleavage and activation of caspases appeared to be time-dependent and these results suggested that E2-induced apoptosis is caspase-dependent.

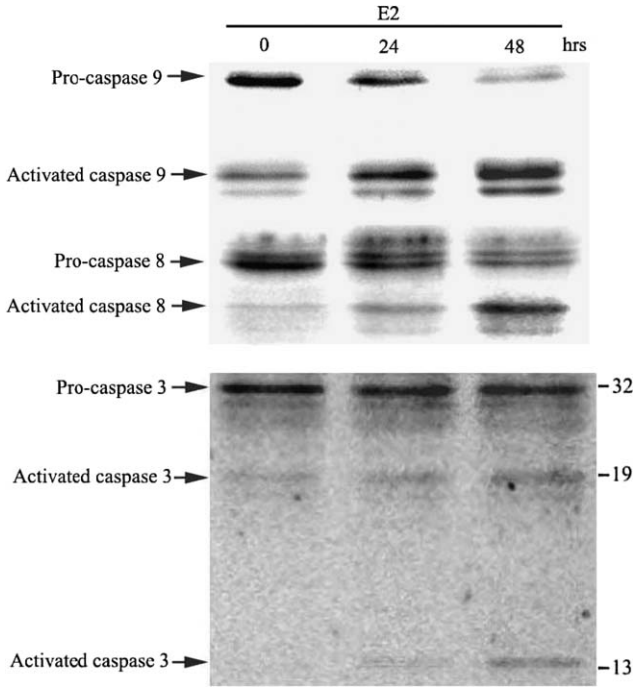

Fig. 2. Activation of caspases in E2-transfected Huh-7 cells. At 24 or 48 h after E2 transfection, cells were harvested and the whole cell lysate was prepared for Western blotting with an antibody against caspase 3, 8 or 9 as described in Materials and methods.

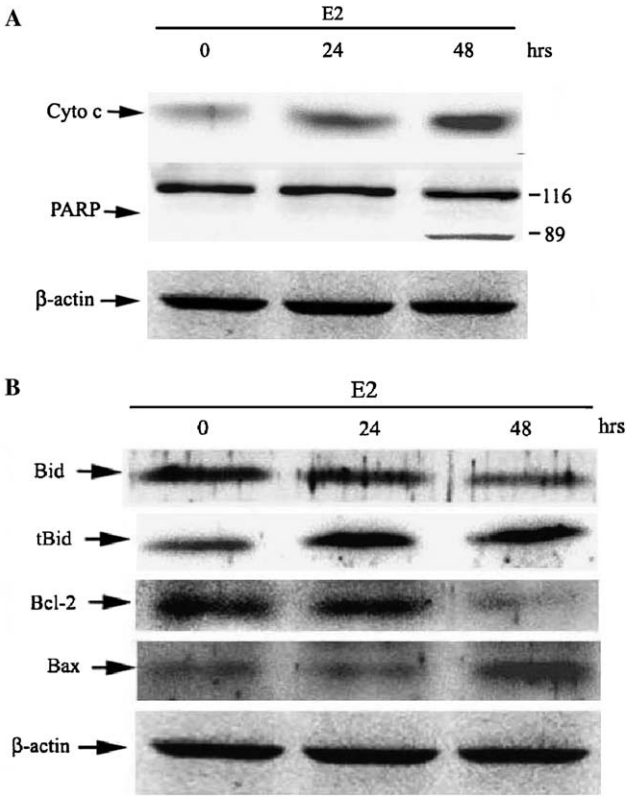

Fig. 3. Involvement of cytochrome *c* release and caspase-dependent Bcl-2 cleavage in E2-transfected Huh-7 cells. At 24 or 48 h after E2 transfection, cells were harvested and the whole cell lysate was prepared for Western blotting with an appropriate antibody against cytochrome *c* or PARP (A), or Bid, tBid, Bcl-2, or Bax (B) as described in Materials and methods.

Involvement of cytochrome c release and caspase-dependent Bcl-2 cleavage in E2-transfected Huh-7 cells

The release of cytochrome *c* from mitochondria to cytosol is an important step in the apoptotic pathway. Such release leads to a caspase-9-dependent activation of caspase-3, as well as a cleaved form of PARP (with a molecular weight of 89 kDa). As shown in Fig. 3A, the cytosolic level of cytochrome *c* increased in a time-dependent manner in E2-transfected cells while the cleaved form of PARP clearly appeared at 48 h post-transfection. Since mitochondrial cytochrome *c* release could be induced through a series of actions involving members of Bcl-2 family, such as Bax, Bcl-2, and Bid, we thus examined the impacts of E2 expression on these proteins. As shown in Fig. 3B, the protein level of Bcl-2 was down-regulated in a time-dependent fashion, accompanied with a gradual increase of Bax protein expression. Furthermore, the continuing reduction of Bid protein and the gradual increase of tBid protein also indicated that a time-dependent increased turn-over of Bid protein into tBid, a truncation form of Bid protein.

Discussion

One of the most irritated and unresolved problems caused by HCV is the establishment of chronic infection in most infected people despite of effective immune response and such chronic HCV infection may lead to cirrhosis and hepatocellular carcinoma. In addition to that the high mutation rate of viral envelope proteins [11,12] and the suppression of host immune system [13–15] are generally believed to be the main causes, apoptosis has been proposed to be one contributor to viral chronic infection. Although such programmed death of cells infected by viruses may appear suicidal, this has been suggested to be a response to virus infection as a mean of facilitating virus dissemination.

Apoptosis is a tightly regulated progress under the control of several signaling pathways, such as caspase and mitochondrial pathways [1–3] and it has been reported that apoptosis is involved in the pathogenesis of hepatitis C. There is evidence that immune response (cytotoxic T lymphocyte) might be involved in the apoptosis of hepatocytes in HCV infected patients [4]. Iken et al. recently reported that HCV structural proteins core, envelope 1 (E1), and envelope 2 (E2) may enhance apoptosis of activated T cells by upregulating FasL [27]. Increased apoptosis of activated T cells induced by HCV structural proteins could amplify the ability of the liver to down-modulate T cell responses, leading to attenuation of anti-viral responses and facilitating viral persistence. However, another study indicated that HCV core-E1-E2 transgenic mice develop significantly larger tumors than transgenic mice expressing core alone or nontransgenic mice. The accelerated tumor phenotype is attributable to suppression of apoptosis rather than enhanced proliferation. These data implicate HCV E1 and/or E2 in conjunction with core as antiapoptotic, tumor accelerator proteins [28]. Furthermore, a recent study suggested that HCV core protein induces the expression of FasL and the activity of FasL promoter in HepG2 cell system to induce apoptosis while other studies have obtained controversial results. Therefore, HCV core protein may not the only viral protein involved in HCV-induced apoptosis and the contribution of other HCV proteins should be clarified.

Among other HCV proteins, E2 protein has been suspected to possess a capability of inducing apoptosis due to its genetic similarity as the envelope proteins of dengue and Langat viruses, which could induce apoptosis during infection. As for the impact of HCV E2 protein on apoptosis, inconsistent results have been obtained, while a study has indicated that HCV E2 protein could induce apoptosis to contribute the liver injury [23], another study showed that E2 inhibits apoptosis by inhibiting TRAIL-induced cytochrome *c* release from the mitochondria, which may subsequently augment persistent HCV infection [24]. Therefore, to clarify the impact and underlying mechanism of HCV E2 protein on apoptosis, as well as to elucidate the role of HCV E2 protein in establishing chronic infection and liver injury, HCV E2 was transiently expressed in Huh-7 cells. Microscopic observation showed that E2-transfected Huh-7 cells displayed a shrunk morphology while cell proliferation of E2-transfected Huh-7 cells was significantly reduced, compared to those vector-transfected cells. Our further assay for DNA fragmentation demonstrated that reduced proliferation of E2-transfected cells was apoptosis-related. Results of subsequent Western blotting indicated that E2 protein caused activations of caspase-3, caspase-8, and caspase-9 to result in increased expressions of activated caspases, which is consistent with a study of Langat virus demonstrating that expression of envelope (E) protein could induce apoptosis via the caspase-3 pathway [7]. Furthermore, the cleavage of PARP into 116 kDa and 89 kDa also proved the occurrence of apoptosis and the activation of caspase pathway. Taken together, we have proven that HCV E2-induced apoptosis is caspase-dependent. Moreover, a time-dependent release of cytochrome *c* was also observed after the E2 transfection indicating a mitochondria-initiated apoptosis. Such apoptosis process is believed to be related to the expression of proteins of Bcl-2 family, which has been reported to regulate the induction of apoptosis at least through the control of mitochondrial function [8,9]. For examples, Bcl-2 could influence the permeability of intracellular membranes and cytochrome *c* release from mitochondria to control apoptosis [10]. Furthermore, during apoptotic process, Bid is cleaved by caspase-8 and the resultant truncated Bid could cause mitochondrial cytochrome *c* releasing into the cytoplasm. In the present study, the reduction of Bcl-2 protein level was observed in E2-transfected Huh-7, accompanied wit a reduced Bid protein level and an increased tBid level, which are concordant and reflect the increased apoptosis. According to current understanding, BH3-only proteins, such as Bid, could activate multidomain members, Bax or

Bak, which have the capability of altering the integrity of outer mitochondrial membrane [13–16], by stimulating its oligomerization in the outer membrane. Our result showing an increased Bax level caused by E2 transfection was consistent with such regulation.

An increased Fas-mediated apoptosis PBMC from chronically infected patients has been reported [6] and core protein has been shown to be able to increase the sensitivity of a T cell line to Fas-mediated apoptosis. However in this study, expression levels of Fas and FasL were not significantly altered by the transfection of HCV E2 (data not shown). Furthermore, other pathways regulating apoptosis, such as JNK and p38 pathways, were not examined in this study yet and therefore the impact of E2 on these pathways could not be ruled out and should be clarified.

Taken our data together, HCV E2 protein may first activate caspase-8 to lead to Bid truncation and Bcl-2 reduction, and then activates mitochondria-mediated apoptotic pathway including cytochrome *c* release and subsequent activation of caspase-9 and caspase-3. These results indicated that HCV E2-induced apoptosis of Huh-7 cells may involve a mitochondrial-related caspase pathway. In summary, our data have revealed that E2 protein may be involved in HCV-induced pathogenesis by inducing apoptosis of Huh-7 cells.

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References

[1] S.E. Bassett, D.L. Thomas, K.M. Brasky, R.E. Lanford, Viral persistence, antibody to E1 and E2, and hypervariable region 1 sequence stability in hepatitis C virus-inoculated chimpanzees, J. Virol. 73 (1999) 1118–1126.

[2] N. Hiramatsu, N. Hayashi, K. Katayama, K. Mochizuki, Y. Kawanishi, A. Kasahara, H. Fusamoto, T. Kamada, Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C, Hepatology 19 (1994) 1354–1359.

[3] F. Calabrese, P. Pontisso, E. Pettenazzo, L. Benvegnu, A. Vario, L. Chemello, A. Alberti, M. Valente, Liver cell apoptosis in chronic hepatitis C correlates with histological but not biochemical activity or serum HCV-RNA levels, Hepatology 31 (2000) 1153–1159.

[4] K. Emi, K. Nakamura, K. Yuh, S. Sugyo, H. Shijo, M. Kuroki, K. Tamura, Magnitude of activity in chronic hepatitis C is influenced by apoptosis of T cells responsible for hepatitis C virus, J. Gastroenterol. Hepatol. 14 (1999) 1018–1024.

[5] E. Toubi, A. Kessel, L. Goldstein, G. Slobodin, E. Sabo, Z. Shmuel, E. Zuckerman, Enhanced peripheral T-cell apoptosis in chronic hepatitis C virus infection: association with liver disease severity, J. Hepatol. 35 (2001) 774–780.

[6] N. Taya, Y. Torimoto, M. Shindo, K. Hirai, C. Hasebe, Y. Kohgo, Fas-mediated apoptosis of peripheral blood mononuclear cells in patients with hepatitis C, Br. J. Haematol. 110 (2000) 89–97.

[7] S. Pianko, S. Patella, G. Ostapowicz, P. Desmond, W. Sievert, Fas-mediated hepatocyte apoptosis is increased by hepatitis C virus infection and alcohol consumption, and may be associated with hepatic fibrosis: mechanisms of liver cell injury in chronic hepatitis C virus infection, J. Viral. Hepat. 8 (2001) 406–413.

[8] G. Kalkeri, N. Khalap, S. Akhter, R.F. Garry, C.D. Fermin, S. Dash, Hepatitis C viral proteins affect cell viability and membrane permeability, Exp. Mol. Pathol. 71 (2001) 194–208.

[9] H.C. Ma, C.H. Ke, T.Y. Hsieh, S.Y. Lo, The first hydrophobic domain of the hepatitis C virus E1 protein is important for interaction with the capsid protein, J. Gen. Virol. 83 (2002) 3085–3092.

[10] J. McLauchlan, Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes, J. Viral. Hepat. 7 (2000) 2–14.

[11] R.B. Ray, K. Meyer, R. Ray, Suppression of apoptotic cell death by hepatitis C virus core protein, Virology 226 (1996) 176–182.

[12] R.B. Ray, K. Meyer, R. Steele, A. Shrivastava, B.B. Aggarwal, R. Ray, Inhibition of tumor necrosis factor (TNF-alpha)-mediated apoptosis by hepatitis C virus core protein, J. Biol. Chem. 273 (1998) 2256–2259.

[13] H. Marusawa, M. Hijikata, T. Chiba, K. Shimotohno, Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation, J. Virol. 73 (1999) 4713–4720.

[14] M. Honda, S. Kaneko, T. Shimazaki, E. Matsushita, K. Kobayashi, L.H. Ping, H.C. Zhang, S.M. Lemon, Hepatitis C virus core protein induces apoptosis and impairs cell-cycle regulation in stably transformed Chinese hamster ovary cells, Hepatology 31 (2000) 1351–1359.

[15] R.B. Ray, R. Steele, K. Meyer, R. Ray, Transcriptional repression of p53 promoter by hepatitis C virus core protein, J. Biol. Chem. 272 (1997) 10983–10986.

[16] R.B. Ray, K. Meyer, R. Ray, Hepatitis C virus core protein promotes immortalization of primary human hepatocytes, Virology 271 (2000) 197–204.

[17] N. Zhu, A. Khoshnan, R. Schneider, M. Matsumoto, G. Dennert, C. Ware, M.M. Lai, Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis, J. Virol. 72 (1998) 3691–3697.

[18] P. Avirutnan, P. Malasit, B. Seliger, S. Bhakdi, M. Husmann, Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis, J. Immunol. 161 (1998) 6338–6346.

[19] C.N. Duarte dos Santos, M.P. Frenkiel, M.P. Courageot, C.F. Rocha, M.C. Vazeille-Falcoz, M.W. Wien, F.A. Rey, V. Deubel, P. Despres, Determinants in the envelope E protein and viral RNA helicase NS3 that influence the induction of apoptosis in response to infection with dengue type 1 virus, Virology 274 (2000) 292–308.

[20] G.G. Prikhod'ko, E.A. Prikhod'ko, A.G. Pletnev, J.I. Cohen, Langat flavivirus protease NS3 binds caspase-8 and induces apoptosis, J. Virol. 76 (2002) 5701–5710.

[21] M. Hijikata, N. Kato, Y. Ootsuyama, M. Nakagawa, K. Shimotohno, Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis, Proc. Natl. Acad. Sci. USA 88 (1991) 5547–5551.

[22] C.L. Wang, L.X. Zhu, J. Liu, Z.C. Zhang, Y. Wang, G.D. Li, Expression and characterization of hepatitis C Virus E2 glycoprotein fused to hepatitis B virus preS1(21-47) fragment in CHO cells, Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai) 34 (2002) 400–404.

[23] L.X. Zhu, J. Liu, Y.H. Xie, Y.Y. Kong, Y. Ye, C.L. Wang, G.D. Li, Y. Wang, Expression of hepatitis C virus envelope protein 2 induces apoptosis in cultured mammalian cells, World J. Gastroenterol. 10 (2004) 2972–2978.

[24] S.H. Lee, Y.K. Kim, C.S. Kim, S.K. Seol, J. Kim, S. Cho, Y.L. Song, R. Bartenschlager, S.K. Jang, E2 of hepatitis C virus inhibits apoptosis, J. Immunol. 175 (2005) 8226–8235.

[25] M.S. Yurkova, A.H. Patel, A.N. Fedorov, Characterisation of bacterially expressed structural protein E2 of hepatitis C virus, Protein Expr. Purif. 37 (2004) 119–125.

[26] J. Liu, L. Zhu, X. Zhang, M. Lu, Y. Kong, Y. Wang, G. Li, Expression, purification, immunological characterization and application of *Escherichia coli*-derived hepatitis C virus E2 proteins, Biotechnol. Appl. Biochem. 34 (2001) 109–119.

[27] K. Iken, L. Huang, H. Bekele, E.V. Schmidt, M.J. Koziel, Apoptosis of activated CD4+ and CD8+ T cells is enhanced by co-culture with hepatocytes expressing hepatitis C virus (HCV) structural proteins through FasL induction, Virology 346 (2006) 363–372.

[28] Y. Kamegaya, Y. Hiasa, L. Zukerberg, N. Fowler, J.T. Blackard, W. Lin, W.H. Choe, E.V. Schmidt, R.T. Chung, Hepatitis C virus acts as a tumor accelerator by blocking apoptosis in a mouse model of hepatocarcinogenesis, Hepatology 41 (2005) 660–667.