Non-structural protein 4A of *Hepatitis C virus* accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis

Yuki Nomura-Takigawa,† Motoko Nagano-Fujii,† Lin Deng,† Sohei Kitazawa, Satoshi Ishido,† Kiyonao Sada† and Hak Hotta†

Correspondence
Hak Hotta
hotta@kobe-u.ac.jp

Divisions of Microbiology† and Molecular Pathology†, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

Non-structural protein 4A (NS4A) of *Hepatitis C virus* (HCV) functions as a cofactor for NS3 by forming a complex with it to augment its enzymic activities. NS4A also forms a complex with other HCV proteins, such as NS4B/NS5A, to facilitate the formation of the viral RNA replication complex on the endoplasmic reticulum (ER) membrane. In addition to its essential role in HCV replication, NS4A is thought to be involved in viral pathogenesis by affecting cellular functions. In this study, it was demonstrated that NS4A was localized not only on the ER, but also on mitochondria when expressed either alone or together with NS3 in the form of the NS3/4A polyprotein and in the context of HCV RNA replication in Huh7 cells harbouring an HCV RNA replicon. Moreover, NS4A expression altered the intracellular distribution of mitochondria significantly and caused mitochondrial damage, as evidenced by the collapsed mitochondrial transmembrane potential and release of cytochrome c into the cytoplasm, which led ultimately to induction of apoptosis through activation of caspase-3, but not caspase-8. Consistently, Huh7 cells expressing NS3/4A and those harbouring an HCV RNA replicon were shown to be more prone to undergoing actinomycin D-induced, mitochondria-mediated apoptosis, compared with the control Huh7 cells. Taken together, these results suggest the possibility that HCV exerts cytopathic effect (CPE) on the infected cells under certain conditions and that NS4A is responsible, at least in part, for the conditional CPE in HCV-infected cells.

INTRODUCTION

*Hepatitis C virus* (HCV), a member of the family *Flaviviridae*, has a single-stranded, positive-sense RNA of about 9.6 kb in length. The virus genome encodes a precursor polyprotein of about 3000 aa, which is cleaved into at least 10 mature viral proteins, such as Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Reed & Rice, 2000). HCV is known to evade the host-defence mechanisms to establish persistent infection, causing chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Kiyosawa et al., 1990; Tong et al., 1995). It has been suggested that liver-cell injuries, either apoptotic or necrotic changes, are mediated principally by antiviral immune responses, such as HCV-specific cytotoxic T lymphocytes. However, the possible involvement of viral cytopathic effect (CPE) should also be taken into consideration.

Apoptosis involves two major pathways: the Fas-mediated pathway and the mitochondria-mediated pathway (Ashkenazi & Dixit, 1998; Gewies et al., 2000). Fas-mediated apoptosis is conducted through facilitation of caspase-8 activation. Regarding HCV infection, Core was shown to induce Fas-mediated and tumour necrosis factor (TNF) receptor-mediated apoptosis (Ruggieri et al., 1997). On the other hand, a number of apoptosis-inducing signals are concentrated at mitochondria to facilitate the release of cytochrome c from mitochondria, which induces formation of an apoptosis complex that includes apoptosis protease-activating factor-1 (Apaf-1) and procaspase-9 (Deveraux et al., 1998; Fearnhead et al., 1998; Gross et al., 1999; Skulachev, 1998). This results in the activation of caspases and finally the cleavage of chromosomal DNA. Thus, mitochondria are intensive and central organelles regulating apoptotic signals.

A wide variety of viral proteins have been shown to localize specifically on mitochondria, such as cytomegalovirus vMIA (Goldmacher et al., 1999), myxoma virus M11L (Everett et al., 2002), Kaposi’s sarcoma-associated herpesvirus K7 (Feng et al., 2002; Wang et al., 2002), human
immunodeficiency virus type 1 Vpr (Jacot et al., 2000, 2001), human T-cell leukemia virus type 1 p13\textsuperscript{11} (D’Agostino et al., 2002), influenza virus PB1-F2 (Chen et al., 2001), hepatitis B virus X protein (Rahmani et al., 2000) and HCV Core (Schwer et al., 2004). Some of them exert anti-apoptotic effects, and the others pro-apoptotic ones, by binding to apoptosis-regulating host-cell factors.

HCV NS4A is a non-structural protein of about 7 kDa that consists of 54 aa with a hydrophobic N-terminal region and a hydrophilic C terminus. NS4A is known to function as a cofactor for NS3 to augment its enzymic activities, such as serine protease (Failla et al., 1995; Reed & Rice, 2000; Satoh et al., 1995) and RNA and DNA helicases (Kuang et al., 2004; Pang et al., 2002; Reed & Rice, 2000). NS3 and NS4A, together with the other non-structural proteins, are incorporated into the HCV RNA replication complex, which is localized primarily on the endoplasmic reticulum (ER) and related membrane structures (Aizaki et al., 2004; Egger et al., 2002; Gosert et al., 2003; Kim et al., 1999; Wölk et al., 2000). Little is known, however, about the possible effect(s) of NS4A on cellular functions, except for a few studies, including ours, showing that NS4A markedly inhibits the translation of the host cell (Florese et al., 2002; Kato et al., 2002).

In this study, we report that NS4A is localized not only on the ER, but also on mitochondria, and that NS4A induces apoptosis through a mitochondria-mediated pathway, as demonstrated by the decreased mitochondrial transmembrane potential, the release of cytochrome c from mitochondria and the activation of caspase-3, followed by the morphological changes characteristic of apoptotic cell death. We have also observed that HuH7 cells harbouring an HCV subgenomic RNA replicon are more prone to apoptosis than control cells when treated with mitochondria-mediated apoptosis through a mitochondria-mediated pathway, as demonstrated by the decreased mitochondrial transmembrane potential, the release of cytochrome c from mitochondria and the activation of caspase-3, followed by the morphological changes characteristic of apoptotic cell death.

In this study, we report that NS4A is localized not only on the ER, but also on mitochondria, and that NS4A induces apoptosis through a mitochondria-mediated pathway, as demonstrated by the decreased mitochondrial transmembrane potential, the release of cytochrome c from mitochondria and the activation of caspase-3, followed by the morphological changes characteristic of apoptotic cell death. We have also observed that HuH7 cells harbouring an HCV subgenomic RNA replicon are more prone to apoptosis than control cells when treated with mitochondria-mediated apoptosis through a mitochondria-mediated pathway, as demonstrated by the decreased mitochondrial transmembrane potential, the release of cytochrome c from mitochondria and the activation of caspase-3, followed by the morphological changes characteristic of apoptotic cell death.

**METHODS**

**Construction of expression plasmids.** A cDNA fragment encoding the full-length NS4A of HCV subtype 1b (Con1 strain) was amplified by PCR from pFKSB284Gly (a kind gift from Dr R. Bartenschlager, University of Heidelberg, Heidelberg, Germany) (Lohmann et al., 2001). The amplified fragment was digested with EcoRI and subcloned into the unique EcoRI site of pSG5 (Strategene) to generate pSG5-NS4A. Plasmids to express FLAG-tagged full-length NS4A and a C-terminally deleted mutant were constructed as reported previously with minor modifications (Florese et al., 2002; Taguchi et al., 2004). Other pSG5-based expression plasmids, such as pSG5-Core, -NS3, -NS4B, -NS3/4A, -NS5A and -NS5B, were described elsewhere (Deng et al., 2006; Florese et al., 2002; Ishido et al., 2000; Song et al., 1999; Wang et al., 2000).

**Cell culture and transfection.** HuH7 cell lines harbouring an HCV subgenomic RNA replicon (HuH7-FK2884Gly-1 cells) that expresses NS3 to NS5B were reported previously (Lohmann et al., 2001; Taguchi et al., 2004; Takigawa et al., 2004). The parental HuH7 cells served as a control. For transient expression of each HCV protein, HuH7 cells were transfected with an expression plasmid by using FuGENE 6 transfection reagent (Roche Diagnostics).

**Cell-viability assay.** Cells were seeded in 96-well plastic plates. Cell viability was determined based on mitochondrial NADH-dependent dehydrogenase activity by WST-1 assay using a sulfonated tetrazolium salt, 2-(4-iophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo- phenyl)-2H-tetrazolium monosodium salt, as reported previously (Fujita et al., 1996; Ishido et al., 2000). A\textsubscript{490} was read with a microplate photometer (Bio-Rad). Octuplicate cultures were prepared for each sample and the results were presented as a percentage of the value for untreated controls.

**Subcellular fractionation.** Cells (1 × 10\textsuperscript{7}) were harvested with a cell scraper and the cell suspension was centrifuged at 200 g for 5 min at 4 °C. The cells were resuspended in an ice-cold homogenization buffer containing 100 mM Tris/HCl (pH 8-0), 250 mM sucrose, 2 mM EDTA and protease inhibitors (Complete; Roche Molecular Biochemicals) and homogenized by using a homogenizer by 30 strokes at speed 4-4 of a motor-driven pestle (Wheaton over stirrer). The homogenate was centrifuged at 900 g for 10 min at 4 °C twice. Subsequently, the supernatant, deprived of the nuclei and unbroken cells, was centrifuged at 10,000 g for 10 min at 4 °C to collect mitochondria. The supernatant was further centrifuged at 100,000 g for 1 h and the pellet containing the ER was obtained. Each subcellular fraction was determined by immunoblotting with antibodies against protein disulfide isomerase (PDI) (Becton Dickinson) and mHSP70 (Affinity BioReagents, Inc.) as markers for ER and mitochondria, respectively.

**Immunoblotting.** Cell lysates in a buffer containing 50 mM Tris/HCl (pH 6-8), 2 % SDS, 10 % glycerol and bromophenol blue were electrophoresed on 14–16 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes as described previously (Muramatsu et al., 1997). After being blocked with skimmed milk for 1 h at room temperature followed by washing with PBS containing 0.05 % Tween 20 (PBS-T), the membrane was incubated with an appropriate first antibody for 1 h, washed three times with PBS-T and incubated with peroxidase-labelled second antibody at room temperature for 30 min. After being washed three times with PBS-T, the positive bands were visualized by using the ECL detection system (Amersham Biosciences) according to the manufacturer’s instructions.

**Immunofluorescence microscopy.** Cells were fixed with 3-7 % formaldehyde for 10 min at room temperature and permeabilized with 0-1 % Triton X-100 for 10 min at room temperature. After being washed with PBS, the cells were incubated with a first antibody for 1 h, followed by washing three times with PBS-T and staining with a fluorescein isothiocyanate (FITC)-, Cy3- or Alexa Fluor 546-labelled secondary antibody. The first antibodies used were mouse mAbs against Core, NS3, NS4A and NS5A (kind gifts from Dr I. Yoshida, Research Institute for Microbial Diseases, Osaka University, Kan-Onji branch, Kagawa, Japan). The cells were washed again with PBS-T, mounted with 80 % glycerol and observed under a fluorescence microscope (Olympus) or a confocal immunofluorescence microscope (Carl Zeiss). MitoTracker (Molecular Probes) and pEYFP-Golgi (Clontech) were used for staining mitochondria and the Golgi apparatus, respectively.

**Immunoelectron microscopy.** Immunoelectron microscopy was performed as described previously with some modifications (Hidajat et al., 2005). In brief, cells were fixed with 4 % paraformaldehyde and 1 % glutaraldehyde in 150 mM HEPEs-KOH (pH 7-4) for 10 min at room temperature. The cells were collected by a cell scraper, centrifuged and dehydrated through a series of 50, 70, 80, 90 and 100 % ethanol. The sample was embedded in LR White resin (London Resin Co. Ltd) and kept at −20 °C for 2 days. After ultrathin...
sectioning, sections were blocked with 0.5% BSA solution and incubated with anti-NS4A mouse mAb for 1 h at room temperature. After being washed with PBS, the sections were incubated with goat anti-mouse IgG conjugated to 10 nm gold (Sigma) for 30 min at room temperature. After being washed with PBS and extra-pure water, the sections were dried, stained with lead citrate and observed under an electron microscope (JEM-1200EX; JOEL).

Mitochondrial transmembrane potential. Changes in the mitochondrial transmembrane potential were examined by using rhodamine 123 (Rho123; Sigma). Rho123, a fluorescent, lipophilic, cationic dye, accumulates in mitochondria of living cells and has been used for evaluating changes in the mitochondrial transmembrane potential (Davis et al., 1985; Leprat et al., 1990; Li et al., 1999; Lin et al., 2004). Cells (5 × 10^{5}) were washed with PBS and stained with a staining solution containing Rho123 (0.5 μg ml⁻¹) for 15 min at 37°C. The fluorescence emitted from Rho123 was analysed by a flow cytometer (Becton Dickinson).

Caspase enzymic activity. Caspase-3 activity was measured by using Caspase-GloTM 3/7 reagent (Promega) according to the manufacturer’s instructions. In brief, a luminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, was added to cells cultured on a microplate. The cells were incubated for 30 min at room temperature and the luminescence of each sample was measured by a microplate luminometer (Luminescencer-JNP AB-2100; ATTO). Caspase-8 activity was measured by using a FLICE/Caspase-8 colorimetric protease assay kit (Medical Biological Laboratories Co. Ltd) according to the manufacturer’s instructions. Cells were detached from the dishes with trypsin and lysed in an ice-cold lysis buffer supplied with the kit for 10 min. The cytosolic extracts obtained were mixed with IETD-pNA, the substrate of caspase-8, and incubated for 2 h at 37°C. A_{405} was read with a microplate photometer (Bio-Rad).

Cytological markers for apoptosis: morphological changes of the nuclei. Cells were fixed with 100% methanol at −20°C for 20 min, washed twice with PBS and stained with 10 μM Hoechst 33342 at room temperature for 10 min, as described previously (Fuiita et al., 1996; Ishido et al., 2000). The morphology of the nuclei of the cells was examined under a light microscope.

RESULTS

NS4A is localized on mitochondria

We first examined the intracellular localization of NS4A. Immunofluorescence analysis revealed that NS4A colocalized with MitoTracker, a marker for mitochondria, in Huh7 cells using a transient-expression system (Fig. 1a, top). It should be noted that, in NS4A-expressing cells, mitochondria accumulated in the perinuclear region, exhibiting a doughnut-like appearance. NS4A was also localized at the ER to a considerable degree (Fig. 1a, middle) and, to a much lesser degree, at the Golgi apparatus (bottom). Mitochondrial localization of NS4A was observed when NS4A was co-expressed with NS3 in cis as well (Fig. 1b, top). We also tested the possible mitochondrial localization of the other HCV proteins, such as Core, NS3, NS4B and NS5A, and found that Core and NS5A were partially localized on mitochondria. Unlike NS4A, however, none of these HCV proteins altered the intracellular-distribution pattern of mitochondria. The mitochondrial localization of NS4A in Huh7 cells was confirmed by further experiments. Subcellular-fractionation analysis revealed that NS4A was detected abundantly in a mitochondrial fraction of Huh7 cells in a transient-expression system (Fig. 1c). Moreover, NS4A was detected in a mitochondrial fraction obtained from Huh7 cells harbouring an HCV subgenomic RNA replicon. PDI, an ER marker, was barely detected, if at all, in the mitochondrial fraction, with the result excluding the possible contamination of the mitochondrial fraction with the ER and thereby verifying the specificity of the mitochondrial localization of NS4A in those cells. Confocal immunofluorescence microscopic analysis revealed that NS4A was partially colocalized on mitochondria in Huh7 cells harbouring the HCV subgenomic RNA replicon, although to a lesser extent than that observed in cells expressing NS4A or NS3/4A transiently (Fig. 1d). Also, immunoelectron microscopic analysis demonstrated clearly that NS4A was localized on both ER and mitochondria of Huh7 cells expressing NS4A transiently (Fig. 1e) and those harbouring the HCV RNA replicon (Fig. 1f). The immunogold staining was not observed in the control cells without NS4A expression, ensuring the specificity of the staining (data not shown). From these results, we concluded that NS4A was localized preferentially on mitochondria. We also speculated that NS4A could potentially alter the intracellular distribution and functions of mitochondria.

NS4A induces cell death

While analysing the intracellular localization of NS4A, we noticed that NS4A-expressing cells exhibited severe cell damage after prolonged cultivation. In line with this observation, we could not generate any Huh7 cell line stably expressing NS4A (data not shown). We therefore examined the possible effect of NS4A on cell viability. The result demonstrated that NS4A caused cell death (Fig. 2). When NS4A was co-expressed with NS3 in cis (NS3/4A), cell death was not observed, although a comparable level of NS4A expression was achieved in these cells. These results suggest that, under these experimental conditions, the cell death-inducing effect of NS4A was abolished after forming a complex with NS3. This result, however, does not necessarily exclude the possibility that NS3/4A could affect cell death under certain conditions (see below).

NS4A induces the collapse of the mitochondrial transmembrane potential

To assess the molecular mechanism of NS4A-induced cell death, we first measured the mitochondrial transmembrane potential by using Rho123. In this analysis, Huh7 cells transiently transfected with the pSG5 vector and those treated with staurosporine served as a negative and a positive control, respectively. As shown in Fig. 3, a larger number of NS4A-expressing cells showed decreased mitochondrial transmembrane potential (24.0%) compared with the non-expressing control (7.6%) and cells expressing NS3 alone (10.5%). Again, the mitochondria-damaging effect of NS4A was counteracted by NS3 (NS3/4A; 9.5%).
Fig. 1. Localization of NS4A on mitochondria. (a) Immunofluorescence analysis. Huh7 cells expressing NS4A transiently were double-stained with anti-NS4A mouse mAb (left panels) and MitoTracker Red (upper middle panel), anti-calregulin rabbit antiserum as an ER marker (centre) or pEYFP-Golgi (lower middle panel). Second antibodies used were either FITC-conjugated anti-mouse IgG (green), Cy3-conjugated anti-rabbit IgG (red) or Alexa Fluor 546-conjugated anti-mouse IgG (red). Overlaid pictures are shown on the right. Note a doughnut-like, perinuclear staining of mitochondria in NS4A-expressing cells (upper middle panel, arrowhead). (b) Huh7 cells transiently expressing NS3/4A were stained with anti-NS4A antibody plus FITC-conjugated anti-mouse IgG (left panel) and MitoTracker Red as a mitochondrial marker (centre). Also, cells expressing Core, NS3, NS4B and NS5A transiently were double-stained with specific antibodies (Core, NS3 and NS5A) or HCV-infected patient serum (NS4B) and MitoTracker Red. Second antibodies used were FITC-conjugated anti-mouse IgG or anti-human IgG. Overlaid pictures are shown on the right. (c) Subcellular fractionation. Huh7 cells expressing NS4A transiently, those harbouring an HCV subgenomic RNA replicon and the parental control were fractionated. The ER and mitochondrial fractions were probed with anti-NS4A antibody. The absence of ER in the mitochondrial fraction was confirmed by staining with antibodies against PDI. (d) Huh7 cells harbouring an HCV subgenomic RNA replicon were stained with anti-NS4A antibody plus FITC-conjugated anti-mouse IgG (left panel) and MitoTracker Red (centre). An overlaid picture is shown on the right. (e) Immunoelectron microscopic analysis of Huh7 cells expressing NS4A transiently. Arrows and arrowheads indicate NS4A localized at mitochondria and ER, respectively. (f) Immunoelectron microscopic analysis of Huh7 cells harbouring an HCV subgenomic RNA replicon. Arrows and arrowheads indicate NS4A localized at mitochondria and ER, respectively. Mit, Mitochondrion; rER, rough ER.
NS4A induces the release of cytochrome c from mitochondria

The collapse of the mitochondrial transmembrane potential would impair the intrinsic functions of mitochondria, triggering apoptosis and/or necrosis. Cytochrome c is normally present in the mitochondrial intermembrane space and is released to the cytosol when cells undergo apoptosis. We therefore examined whether expression of NS4A could trigger the release of cytochrome c from mitochondria. As had been expected, nearly 70% of NS4A-expressing Huh7 cells, but not the empty vector-transfected control, showed the release of cytochrome c into the cytoplasm 48 h after transfection, as evidenced by diffuse staining of cytochrome c throughout the cell (Fig. 4a, b). Expression of Core (Fig. 4b), NS4B and NS5A (data not shown), but not NS3, each induced cytochrome c release only slightly (~10%). Cells expressing NS3/4A did not induce cytochrome c release, but rather exhibited doughnut-like, perinuclear staining of cytochrome c (Fig. 4a, b). The absence of cytochrome c release in NS3/4A-expressing cells was confirmed even 96 h after transfection (data not shown). The doughnut-like staining pattern of NS4A closely resembled that of mitochondrial localization in cells expressing either NS4A alone or NS3/4A (Fig. 1a, b). These results collectively suggest the possibility that both NS4A by itself and the NS3/4A complex accumulate on mitochondria, but that they exert differential effects on the cellular conditions depending upon its molecular status.

Cytochrome c release was observed in cells expressing NS4AAC14 (Fig. 4b). A comparable expression level of full-length NS4A and NS4AAC14 was verified by immunoblotting. These results suggested that the C-terminal 14 residues of NS4A were not involved in the induction of cytochrome c release.

NS4A activates caspase-3, but not caspase-8, and induces apoptosis

Cytochrome c, once released to the cytosol, plays an important role in the activation of caspase-3, which is a principal effector for induction of apoptosis (Liu et al., 1996). Consistent with this idea, we observed that caspase-3 activity was enhanced in NS4A-expressing Huh7 cells compared with the non-expressing control (Fig. 5a, left panel). On the other hand, caspase-8 was not activated by NS4A expression (Fig. 5a, right panel). Caspase-8 is known to be involved in the Fas-mediated apoptotic pathway (Muzio et al., 1996; Shu et al., 1997). NS4A-mediated cell death was inhibited almost completely by treatment with Z-VAD-fmk, a general inhibitor of caspases (Fig. 5b). These results collectively suggested that NS4A induced apoptosis through the mitochondria-mediated, but not the Fas-mediated, pathway.

To confirm that the NS4A-mediated cell death was due to apoptosis, the nuclei of the cells were stained with Hoechst 33342 and their morphology was examined. Chromatin condensation and nuclear fragmentation, typical cytological markers for apoptosis, were observed in NS4A-expressing cells as well as in staurosporine-treated, positive-control cells (Fig. 6a). A similar morphological change of the nucleus was also observed in NS3/4A-expressing cells more frequently than in the vector-transfected control (Fig. 6b). This result implies the possibility that the NS3/4A complex exerts certain effects on cell function, but that the possible effect on cell survival may not become evident in the absence of additional factor(s).

NS3/4A-expressing Huh7 cells are prone to undergoing mitochondria-mediated apoptosis

We hypothesized that the NS3/4A complex might direct the cells to a pre-apoptotic status that, upon exposure to an otherwise ineffective low dose of apoptotic stimuli, leads the cells to apoptosis. To examine this possibility, cells expressing NS3/4A or NS4A alone and the non-expressing control were treated with a suboptimal dose of actinomycin D (100 ng ml⁻¹) and cell viability was determined. The results obtained revealed that NS3/4A-expressing cells, as well as those expressing NS4A alone, were more prone to undergoing actinomycin D-induced (mitochondria-mediated)
apoptosis than the control cells (Fig. 6c). Similar results were obtained when the cells were treated with 50 ng actinomycin D ml⁻¹ (data not shown).

**Huh7 cells harbouring an HCV subgenomic RNA replicon are prone to undergoing mitochondria-mediated, but not Fas-mediated, apoptosis**

In cells infected with HCV or those harbouring an HCV RNA replicon, NS4A is expressed in the context of virus replication, where NS4A is principally incorporated into the viral RNA replication complex together with other

**Fig. 3.** Collapse of the mitochondrial transmembrane potential by NS4A. Huh7 cells expressing NS4A, NS3 and NS3/4A for 24 h transiently, as well as a vector-transfected, non-expressing control, were measured for the mitochondrial transmembrane potential by staining the cells with Rho123 followed by flow-cytometric analysis. Cells treated with staurosporine (STS, 1 μM) for 6 h served as a positive control. Reduced Rho123 staining indicates the mitochondrial transmembrane potential reduction.

**Fig. 4.** Cytochrome c release from mitochondria into the cytoplasm by NS4A. (a) Huh7 cells expressing NS4A, NS3 and NS3/4A transiently for 48 h, as well as the vector-transfected, non-expressing control, were stained with anti-cytochrome c mouse mAb and Alexa Fluor 546-conjugated anti-mouse IgG. Expression of NS4A and NS3 was confirmed by staining the cells with HCV-infected patient serum that reacted strongly to NS4A and NS3, followed by FITC-conjugated anti-human IgG (right upper corner of the three panels). Cells treated with actinomycin D (ActD; 300 ng ml⁻¹) for 24 h served as a positive control. (b) Percentage of cells showing cytochrome c release among plasmid-harbouring cells. Huh7 cells expressing Core, NS3, NS3/4A, FLAG-tagged NS4A and FLAG-tagged NS4AΔC14 transiently, as well as the vector-transfected, non-expressing control, were tested. *P<0·01 compared with the control (Student’s t-test). †All of the NS3/4A-expressing cells showed perinuclear accumulation of cytochrome c staining (see Fig. 4a), which coincided with the mitochondrial-localization pattern, as evidenced by staining with a mitochondrial marker (MitoTracker). An equivalent expression level of FLAG-tagged NS4A and FLAG-tagged NS4AΔC14 was verified by immunoblotting (bottom).
non-structural proteins. We therefore examined whether Huh7 cells harbouring an HCV subgenomic RNA replicon are prone to undergoing apoptosis under some circumstances. As shown in Fig. 7(a), the replicon-harbouring cells underwent actinomycin D-induced (mitochondria-mediated) apoptosis to a significantly larger extent than that observed with the non-expressing control. On the other hand, no difference in the degree of TNF-α-induced apoptosis was observed between the replicon-harbouring cells and the control Huh7 cells. Similar results were obtained reproducibly with two other independent clones harbouring the same HCV RNA replicon (data not shown).

The collapse of the mitochondrial transmembrane potential was significantly more evident in the replicon-harbouring cells (35.7%) than in the control (10.0%) when treated with actinomycin D (Fig. 7b).

**DISCUSSION**

HCV infection in the liver causes apoptotic and/or necrotic cell death of hepatocytes. The cell death is thought to be mediated principally by HCV-specific cytotoxic T cells; however, direct CPE by the virus itself should not be overlooked. Many viruses, including HCV, possess viral proteins that either promote or inhibit cell death. Among HCV proteins, Core (Sacco et al., 2003), NS2 (Erdtmann et al., 2003), NS3 (Fujita et al., 1996) and NS5A (He et al., 2002; Lan et al., 2002) have been reported to possess anti-apoptotic functions. Also, there are reports showing that Core (Hahn et al., 2000; Moorman et al., 2003; Ruggieri et al., 1997, 2003; Soguero et al., 2002; Zhu et al., 2001), E1 (Ciccarello et al., 2003, 2004), NS3/4A (Hsu et al., 2003), NS5A and NS5B (Siavoshian et al., 2005) function as pro-apoptotic proteins.

NS4A is known to localize in the ER (Mottola et al., 2002; Reed & Rice, 2000). On the other hand, we demonstrate in the present study that NS4A was localized not only in the ER, but also on mitochondria (Fig. 1). Normally, mitochondria take a filamentous form, with a minor fraction exhibiting a micropunctate appearance, and are distributed evenly in the cell. In NS4A-expressing cells, however, mitochondria took...
a dumpy form and were aggregated in the perinuclear region, exhibiting a doughnut-like appearance (Fig. 1a). Similar perinuclear accumulation of mitochondria was observed also in NS3/4A-expressing cells (Fig. 4a; data not shown). These results suggest the possibility that NS4A, either expressed alone or co-expressed with NS3 so as to form a complex with it, accumulates on mitochondria. The next question that we raised was what would be the consequence of NS4A accumulation on mitochondria. In this regard, we found that NS4A induced mitochondrial transmembrane potential reduction (Fig. 3) and the release of cytochrome c into the cytoplasm (Fig. 4). Expression levels of Bax and Bcl-2 were not affected by NS4A or NS3/4A (data not shown), suggesting that a Bax/Bcl-2 imbalance was unlikely to be the cause of the observed mitochondrial damage. The molecular event(s) triggering the mitochondrial damage is/are currently unknown. In any case, cytochrome c is usually bound to the inner mitochondrial membrane through an association with the anionic phospholipid cardiolipin. The release of cytochrome c from mitochondria triggers the formation of an apoptosome complex that includes Apaf-1, procaspase-9 and ATP, leading to the activation of caspases and eventually apoptosis of the cell. Consistent with this scenario, we

Fig. 6. Evidence for NS4A-induced apoptosis. (a) Huh7 cells expressing NS4A and NS3/4A transiently for 48 h and the vector-transfected, non-expressing control were stained with Hoechst 33342 (10 μM) for 10 min and observed under a fluorescent microscope. Cells treated with staurosporine (STS, 1 μM) for 6 h served as a positive control. (b) Percentage of cells showing nuclear fragmentation was calculated. *P<0.01 (Student’s t-test). (c) Huh7 cells expressing NS4A or NS3/4A transiently and the vector-transfected, non-expressing control were treated with a suboptimal dose of actinomycin D (100 ng ml⁻¹) for 24 h and cell viability was determined.

Fig. 7. Increased sensitivity of HCV RNA replicon-harbouring cells to mitochondria-mediated, but not TNF-α-receptor-mediated, apoptotic stimuli. (a) Huh7 cells harbouring an HCV subgenomic RNA replicon (empty bars) and the parental control (filled bars) were left untreated or treated with either actinomycin D (ActD, 100 ng ml⁻¹) for 24 h or TNF-α (50 ng ml⁻¹) and cycloheximide (CHX, 5 μg ml⁻¹) for 9 h. Cell viability was measured by WST-1 assay. *P<0.01 (Student’s t-test). (b) The cells in (a) were analysed for mitochondrial transmembrane potential by using Rho123. Reduced Rho123 staining indicates the mitochondrial transmembrane potential reduction.
observed NS4A-induced caspase-3 activation (Fig. 5a) and cell death (Fig. 2), the latter of which was blocked by a broad-spectrum caspase inhibitor Z-VAD (Fig. 5b). NS4A-expressing cells exhibited nuclear fragmentation (Fig. 6), which is considered as an apoptosis marker. On the other hand, NS4A did not induce caspase-8 activation (Fig. 5a). It is well-known that caspase-8 is activated upon Fas- and TNF-α-receptor-mediated apoptosis (Muzio et al., 1996; Shu et al., 1997). These results collectively suggest that NS4A induces mitochondria-mediated, but not Fas- or TNF-α-mediated, apoptosis.

NS4A consists of 54 aa, with its N-terminal and central regions being hydrophobic (Failla et al., 1995). Deletion mutational analysis revealed that a C-terminally deleted mutant (NS4AΔC14) also induced the release of cytochrome c (Fig. 4b). In this regard, we previously observed that NS4AΔC14 (aa 1–40), but not NS4AΔN17 (aa 18–54) or NS4AΔN17ΔC14 (aa 18–40), inhibited the translation in the cell (Florese et al., 2002). These results imply an important role for the N-terminal hydrophobic region of NS4A in affecting host cellular functions. On the other hand, NS4A is known to bind to NS3 and NS4B/NS5A through its hydrophobic central region. Our results showed that the NS4A-induced apoptosis and reduction in the mitochondrial transmembrane potential were alleviated by NS3 (Figs 2–4). It should be emphasized, however, that the mitochondrial morphology and intracellular localization (Fig. 4a) and the nuclear morphology (Fig. 6a, b) were altered in NS3/4A-expressing cells. This result implies the possibility that the NS3/4A complex, after being transported to mitochondria by virtue of NS4A, exerts a significant effect on mitochondrial function, and possibly even other cellular functions, without affecting mitochondrial transmembrane potential. In fact, we observed that NS3/4A-expressing cells were more sensitive to actinomycin D-induced, mitochondria-mediated apoptosis than the non-expressing control (Fig. 6c). It is not surprising that a virus can mediate mitochondrial dysfunction through a number of different mechanisms. It has recently been reported that NS3/4A cleaves the mitochondrial antiviral signalling protein, MAVS, thereby inhibiting the retinoic acid-inducible gene I-mediated induction of beta interferon production (Li et al., 2005). It is possible that NS3/4A cleaves another mitochondrial protein(s) to mediate mitochondria-mediated cellular activities.

The possible mitochondria-damaging effect of NS4A alone might be weakened in HCV-infected cells, where NS4A is incorporated into the RNA replication complex with NS3, NS4B, NS5A and NS5B. It may explain why HCV RNA replicon-harbouring cells grew well under normal conditions, despite the apoptosis-inducing function of NS4A. Upon receiving a suboptimal degree of apoptotic stimuli, however, Huh7 cells harbouring an HCV subgenomic RNA replicon underwent apoptosis to a larger extent than HCV replicon-free control cells (Fig. 7). This result suggests the possibility that HCV infection renders host cells more sensitive to mitochondria-mediated apoptosis. This notion is in line with previous observations that, in hepatocytes of HCV-infected patients, mitochondria exhibited irregular and dumpy appearances with thin and fragmented cristae (Barbaro et al., 1999) and that hepatocytes of HCV-infected patients underwent apoptosis in vivo (Bantel et al., 2001; Bantel & Schulze-Osthoff, 2003; Hayashi et al., 1997; Hiramatsu et al., 1994). HCV-induced apoptosis was also observed in B-cell lymphoma cells in vitro (Sung et al., 2003). It should be mentioned that, after receiving a pro-apoptotic stimulus, such as calcium ionophore treatment, cells undergo either apoptosis or necrosis depending upon the amount of ATP available in the microenvironment (Eguchi et al., 1997). Therefore, HCV infection may induce necrosis as well under some conditions. In conclusion, our present data imply the possibility that NS4A is responsible, at least partly, for conditional cell death (CPE) of hepatocytes in HCV-infected patients.

ACKNOWLEDGEMENTS

The authors are grateful to Dr R. Bartenschlager (University of Heidelberg, Heidelberg, Germany) for providing an HCV subgenomic RNA replicon (pFK5B2884Gly). Thanks are also due to Dr I. Yoshida (Research Institute for Microbial Diseases, Osaka University, Kan-Onji Branch, Kagawa, Japan) for providing mouse mAbs against HCV NS3, NS4A and NS5A. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, the Japan Society for the Promotion of Science and the Ministry of Health, Labour and Welfare, Japan. This study was also carried out as part of the 21COE Program at Kobe University Graduate School of Medicine.

REFERENCES


HCV NS4A induces mitochondria-mediated apoptosis


Muzio, M., Chinnaiyan, A. M., Kischkel, F. C. & 11 other authors (1996). FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 85, 817–827.


