DEVELOPMENT OF A CELL-BASED ASSAY FOR MONITORING HEPATITIS C VIRUS NS3/4A PROTEASE ACTIVITY

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Summary. – Hepatitis C virus (HCV) NS3/4A (non-structural 3 and 4 B) protease plays a key role in the processing of polyprotein precursor and it becomes an attractive target for antiviral drug discovery. We developed a cell-based assay for monitoring of the NS3/4A protease activity in mammalian cells that is an important step in screening of specific drugs against the protease. The recombinant caspase 3 (rCasp3) was used as the specific substrate for NS3/4A protease. The endogenous cleavage sites in the procaspase 3 molecule were substituted by decapeptides specific for NS3/4A protease. The activation of rCasp3 depended on its specific cleavage by NS3/4A protease and resulted in an apoptosis of stable cells expressing the protease. The difference in cell viability between the cells expressing NS3/4A protease transfected with rCasp3 and the counterparts pretreated with NS3/4A protease inhibitors could be estimated by a spectrophotometry based on 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) staining of cells in microplates. Thus, we developed a simple and cost-effective colorimetric assay for evaluating NS3/4A protease activity enabling the screening of candidate NS3/4A protease inhibitors.

Key words: apoptosis; hepatitis C virus; recombinant Casp3; MTT assay; NS3/4A protease

Introduction

HCV is the causative agent of chronic hepatitis (Choo *et al*., 1989). Over 170 million people worldwide are infected by HCV and this infection is a major health problem (Foy *et al*., 2005; Memon and Memon, 2002; Abrignani *et al*., 1999; Purcell, 1997). The current therapy comprising pegylated-interferon α in combination with ribavirin causes many side-effects (Farrell, 2007; Everson *et al*., 2005; Cornberg *et al*., 2002; McHutchison, 2002). Currently, there is an urgent need for novel effective drugs that targets key HCV enzymes.

HCV is a small enveloped virus that belongs to the family *Flaviviridae* and contains a single-stranded, positive-sense RNA (Clarke, 1997). The 9.6-kb RNA genome has a single ORF that encodes a large polyprotein precursor of 3010–3033 amino acids (Chen *et al*., 1997). Four structural and at least six non-structural proteins are produced by both host and viral proteases in the following order: NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A- NS5B-COOH (Failla *et al*., 1994).

The NS3/4A serine protease directs posttranslational cleavage of the polyprotein and is the key enzyme involved in the polyprotein maturation (Failla *et al*., 1995; Hahm *et al*., 1995). Moreover, it blocks the virus-induced activation of IFN regulatory factor 3, a transcription factor that plays

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Abbreviations: Casp3 = caspase 3; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HCV = hepatitis C virus; IFA = immunofluorescence assay; OD = optical density; LS = large subunit domain; MTT = 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NS3/4A protease = non-structural 3 and 4A protease; ΔNS3/4A protease = non-structural 3 and 4A mutant protease; scNS3/4A = single-chain NS3/4A protease; rCasp3 = recombinant Casp3; SS = small subunit domain

a critical role in the induction of the type I IFN response (Breiman *et al*., 2005; Li *et al*., 2005; Foy *et al*., 2003). Therefore, the NS3/4A protease is regarded as the most promising target for anti-HCV drug development (Reiser *et al*., 2005).

Due to the lack of efficient cell culture propagating HCV, cell-based assays have been developed for evaluating NS3/4A serine protease activity that complements the conventional enzyme assays. Several cell-based assays that monitor NS3/4A protease activity have been described (Chung *et al*., 2005). Some of them involve the use of a chimeric virus such as Sindbis virus containing the gene for HCV NS3/4A protease. Consequently, the production of infectious virions is accompanied by NS3/4A protease activity (Cho *et al*., 1997). Other systems utilize the presence of reporter genes like secreted alkaline phosphatase (SEAP). Release of SEAP into the culture medium depended on the cleavage of NS4A/B junction by the NS3/4A protease (Lee *et al*., 2004, 2003). In particular, the development of subgenomic replicons facilitates research on anti-HCV drugs (Lohmann *et al*., 1999). The HCV replicon system can be used to identify NS3/4A inhibitors with regard to the entire replication complex. However, a replicon-based assay has limitations with respect to the identification of potential inhibitors because replication is dependent on many factors including host factors.

In this report we described the development of colorimetric assay for detection of the intracellular proteolytic activity of NS3/4A protease. The rCasp3 with endogenous cleavage sites substituted by HCV proteolytic cleavage sites functioned as the specific substrate for NS3/4A protease. The specific activation of rCasp3 in stable cell lines expressing the NS3/4A protease could result in apoptosis, while rCasp3 remained inactive in the cells pretreated with NS3/4A protease inhibitors or in cells expressing a mutant inactive NS3/4A protease. Thus, MTT-based assay was developed for evaluating NS3/4A protease activity enabling the screening of potential NS3/4A protease inhibitors in 96-well microplates.

Materials and Methods

Plasmid constructs. T4 DNA ligase and restriction enzymes were purchased from TaKaRa. All plasmids were extracted with a Miniprep plasmid extraction kit (Qiagen) and DNA fragments were purified with a PCR product purification kit (Qiagen). Recombinant DNA techniques were performed according to the standard protocols.

The primers NS3/4A Forward and Reverse were designed according to the nucleotide sequence of the NS3 protease gene Acc. No. AF356827 (Table 1). The gene encoding NS3/4A was obtained by PCR amplification from pRSETA-NS3/4A, a prokaryotic expression plasmid constructed in our laboratory by the method of Dimasi *et al*. (1998). The PCR conditions were as follows: 94°C for 30 secs, 57°C for 30 secs, and 72°C for 60 secs for 30 cycles. The PCR product was digested with *Bam*HI and *Hin*dIII and ligated into pcDNA3.1(–) giving the pcDNA3.1(–)-NS3/4A construct. The mutant protease expression plasmid pcDNA3.1(–)-ΔNS3/4A was used as a negative control. The NS3 protease mutant H1083E was constructed by performing an overlapping PCR with the primer ΔNS3/4A Forward, where the codon GAC that encodes H was substituted by the codon GAG that encodes E.

Total RNA was extracted from Jurkat cells by using TRIzol reagent (Gibco) and cDNA was produced from the total RNA by using SuperScript II RNAse H^- reverse transcriptase (Invitrogen). Next, Casp3 gene (Acc. No. NM004346) was amplified from the cDNA template with the primers Casp3 Forward and Reverse. Finally, the rCasp3 gene was constructed by independent PCR amplifications of large subunit (LS) and small subunit (SS) domains by using of the corresponding primers (Table 1). The LS and SS domains contained the engineered NS4A/B and NS5A/B cleavage sites, respectively. After PCR amplification, the products were mixed with the primers LS-Forward and SS-Reverse. The resultant PCR fragment was cloned into the pMD18-T vector and subcloned into the eukaryotic expression plasmid pcDNA3.1(–). The inserted sequences of these construct pcDNA3.1(–)-rCasp3 were confirmed by nucleotide sequencing (AuGCT Biotech).

Cells and transfection. HepG2 cells (hepatoma cell line) were obtained from the China Typical Culture Collection (Wuhan) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies). The

Table 1. PCR primers

Gene	Primer sequence*	
NS3/4A	NS3/4A Forward	5'-CCGGATCCATGGCGCCTATCGGCTCAG-3'
	NS3/4A Reverse	5'-CGAAGCTTTTACCGCATAGTGGTTTCCATAGA-3'
ΔNS3/4A	NS3/4A Forward	5'-CGCTTGCCAGCCGACGAGTTCCTGGTCCACATTGGTG-3'
	NS3/4 Reverse	5'-CACCAATGTGGACCAGGAACTCGTCGGCTGGCAAGCG-3'
Casp3	Casp3 Forward	5'-TTTGAATTCATGGAGAACACTGAAAACTCAGTGG-3'
	Casp3 Reverse	5'-TTTGGATCCTTAGTGATAAAAATAGAGTTCTTTTGTG-3'
rCasp3	LS-Forward	5'-CGCCTCGAGGGCGGCGAGGACGTCGTCTGCTGCTCGATGTCCTACGGCGGAATATCCCTGGACAACA GTTATAAAATG-3'
	LS-Reverse	5'-GCCGAGGTGTGAGGCGCACTCTTCCATCTCATCGCCGCCTGTCTCAATGCCACAGTCCAG-3'
	SS-Forward	5'-GGCGGCGATGAGATGGAAGAGTGCGCCTCACACCTCGGCGGTGTTGATGATGACATG GCG-3'
	SS-Reverse	5'-GCGAATTCTTAGTGATAAAAATAGAGTTC-3'

*The restriction enzyme digestion site is underlined.

cells were transfected using lipofectamine 2000 (Invitrogen) according to the standard protocol. Briefly, the recombinant plasmids pcDNA3.1(–)-NS3/4A and pcDNA3.1(–)-ΔNS3/4A were transfected into the HepG2 cells. After 6 hrs of incubation, the cultivation medium was refreshed and transfected cells were incubated at 37°C for 48 hrs. The cells were passaged at a dilution of 1:10 in 6-well plates containing DMEM supplemented with 400 μg/ml of antibiotic G418 for inhibition of polypeptide synthesis (Gibco). Stable cell transformants were selected based on the resistance to G418. After 2 weeks, the single-cell clones were selected and seeded in 24-well plates. Finally, the cell lines that were stably transfected with pcDNA3.1(–)-NS3/4A and pcDNA3.1(–)-ΔNS3/4A were identified by RT-PCR, immunofluorescence assay (IFA), Western blot analysis, and were termed scpHepG2 and ΔscpHepG2, respectively. HepG2 cells transiently transfected with pcDNA3.1(–) were used as a negative control.

RT-PCR. The stably transfected cells were grown in a single well of a 6-well plate and lysed with 1 ml TRIzol reagent. Next, total RNA was separated, precipitated, and 1 μg of RNA was primed with 5 pmol of oligo(dT) (TaKaRa), incubated at 42°C for 60 mins in a 20 μl reaction medium containing 50 mmol/l Tris-HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol, 0.5 mmol/l dNTPs, and 20 units of SuperScript II H^- reverse transcriptase. Thereafter, 1 μl of each cDNA preparation was amplified with the primers NS3/4A Forward and NS3/4A Reverse. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified with the Forward 5'-CAGCCTCAAGATCATCAG CA-3' and Reverse 5'-TGTGGTCATGAGTCCTTCCA-3' primers as the internal control. The PCR conditions were as follows: 95°C for 30 secs, 55°C for 30 sesc, and 72°C for 30 secs.

IFA. The stably transfected cells were washed 3 times in PBS, treated with 0.5% Triton X-100 for 2 mins, and fixed for 1 hr in 4% paraformaldehyde. Then, the cells were washed and blocked with 5% skimmed milk in PBS for 30 mins and rewashed. Next, the cells were incubated with monoclonal antibodies 2G11(prepared in our lab, it was against HCV NS3 protease) overnight at 4°C. After washing the cells were incubated with the goat anti-mouse IgG labeled with FITC (Sigma) at room temperature for 1 hr. Finally, the cells were observed under the fluorescence microscope (Olympus BX60).

Western blot analysis. The lysate of the stably transfected cells was loaded on a SDS-PAGE mini gel (25 μg of protein per lane) and run at 120 V. The proteins were transferred for 1 hr to a nitrocellulose membrane (Gibco) at 100 V. The blot was incubated with a blocking buffer (5% non-fat dry milk in Tris-buffered saline [TBS]) for 2 hrs at room temperature and stained with the antibodies against NS3/4A protease (prepared in our laboratory), Casp3 (Cell Signaling Technology), and β-actin (Sigma) in dilution 1:500–1:1000 overnight at 4°C. After washing in TBS containing 0.1% Tween 20, the blot was incubated with goat anti-mouse IgG-IRDye800 conjugate diluted 1:5,000 (Invitrogen) for 1 hr in blocking buffer. Finally, the blot was washed, scanned with Odyssey scanner (LI-COR) and the target band was detected at 800 nm.

Electron microscopy. Ultrathin sections of the cells were stained with uranyl acetate and lead citrate and analyzed under a transmission electron microscope (JEM 2000-EX) at 80 kV.

Flow cytometry. Annexin V-phycoerythrin (5 μl) and propidium iodide (5 μl) were added to the cell suspension and mixed gently. The cells were stained at 4°C in the dark for 15 mins and analyzed with an Epics Elite ESP flow cytometer (Beckman).

MTT assay. The pcDNA3.1(–)-rCasp3 was transfected into scpHepG2, ΔscpHepG2, and HepG2 cells lines. Cell viability was assayed using dye-exclusion staining at 48, 72, and 96 hrs after transfection. The scpHepG2 cells were seeded in 96-well plates. Some wells were pretreated with commercially available NS3/4A protease inhibitors (AnaSpec) for 12 hrs (Ingallinella *et al*., 1998). Next, all cells were transfected with 0.2 μg pcDNA3.1(–)-rCasp3. At 0, 48, 72, and 96 hrs posttransfection, optical density (OD) of the cells was measured by the method described below. MTT (5 mg; Sigma) was dissolved in 1 ml PBS and filtered under sterile conditions. The culture medium was removed and 20 μl of MTT solution was added to each well. After the cells were incubated at 37°C for 4 hrs, the medium was removed and 150 μl of dimethyl sulfoxide was added to each well in order to dissolve the crystals. Finally, the plate was incubated at 37°C for 10 mins and the OD of the cells was measured with a plate reader at 490 nm (Sunrise). Each sample was tested in triplicate.

Results

Construction of plasmid expressing rCasp3 and preparation of HepG2 cell lines expressing NS3/4A and ΔNS3/4A proteases

To modify the gene for Casp3 and to construct a gene for modified rCasp3, the endogenous cleavage site of procaspase 3 was replaced with the NS3/4A protease recognition consensus sequences through overlapping PCR amplification. After 2-round PCR amplification, the cleavage sites DEMEEC-ASHL and EDVVCC-SMSY at the HCV NS4A/B and NS5A/B junctions, respectively, were introduced into procaspase 3 at the positions aa 25 and aa 172, respectively (Fig. 1). Thus, the gene for rCasp3 was constructed and inserted into eukaryotic expression plasmid pcDNA3.1(–) and termed pc3.1(–)-rCasp3.

To construct the single-chain NS3/4A protease (scNS3/4A), the core of the NS4A protein (aa 21–34) was linked to the N-terminus of the NS3 protein. Mutant single-chain NS3/4A protease (ΔscNS3/4A) was prepared by the substitution of

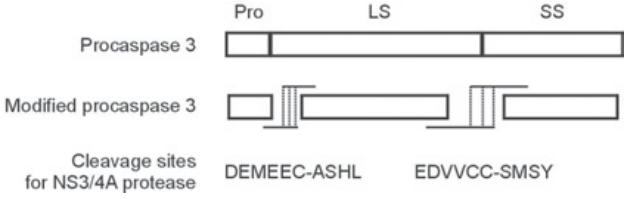

Fig. 1

Scheme of procaspase 3 modification

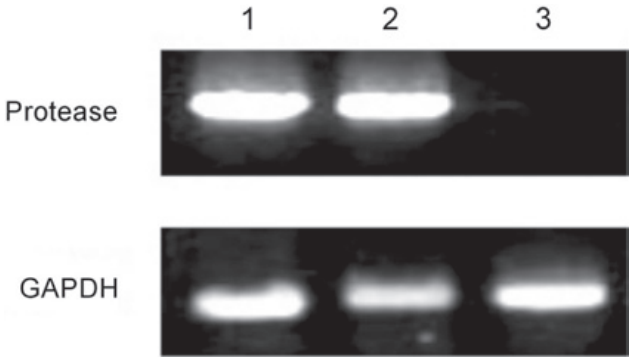

Fig. 2

RT-PCR detection of genes encoding the scNS3/4A protease in scpHepG2 cells (lane 1) and ΔscNS3/4A protease in ΔscpHepG2 cells (lane 2)

GAPDH = internal control gene; non-transfected HepG2 cells (lane 3).

H1083E. The gene encoding ΔscNS3/4A was used as the negative control. Thereafter, the genes encoding scNS3/4A and ΔscNS3/4A proteases were inserted into the eukaryotic expression plasmid pcDNA3.1(–). The resultant recombinant plasmids pcDNA3.1(–)-scNS3/4A and pcDNA3.1(–)-ΔscNS3/4A were transfected into the HepG2 cells by using lipofectamine 2000. The stable cell lines scpHepG2 and ΔscpHepG2 expressing the proteases scNS3/4A and ΔscNS3/4A, respectively, were developed under G418 pressure. In order to identify the expression of the target gene in the stable cell lines, RT-PCR and IFA were used to detect the genes encoding proteases scNS3/4A and ΔscNS3/4A. The specific fragments were amplified from the cDNA template obtained from the corresponding cell lines (Fig. 2). IFA revealed that the scNS3/4A and ΔscNS3/4A proteins were expressed in the cytoplasm of transfected HepG2 cells (Fig. 3). These proteins were visualized using Western blot analysis as well (Fig. 4).

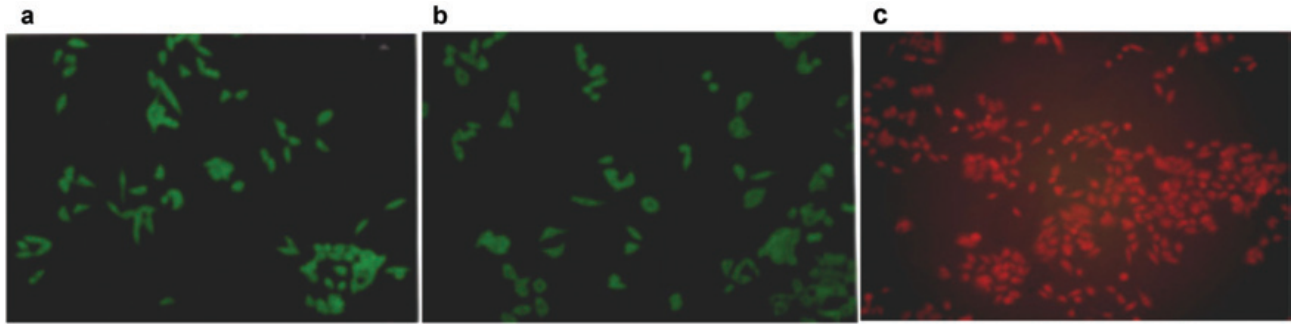

Fig. 3

IFA detection of expressed scNS3/4A protease in scpHepG2 cells (a) and ΔscNS3/4A protease in ΔscpHepG2 cells (b). Non-transfected HepG2 cells stained with Evan's dyestuff (c)

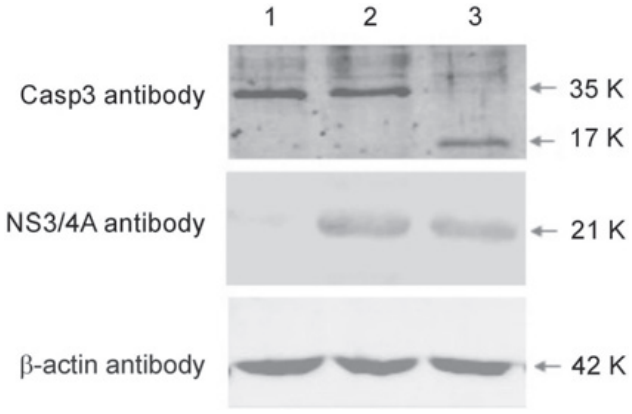

Fig. 4

Apoptosis of rCasp3-transfected HepG2, ΔscpHepG2, and scpHepG2 cells detected by Western blot analysis

HepG2 (lane 1), ΔscpHepG2 (lane 2), and scpHepG2 (lane 3) cells. rCasp3 and proteases detected by the corresponding antibodies. Molecular weights on the right.

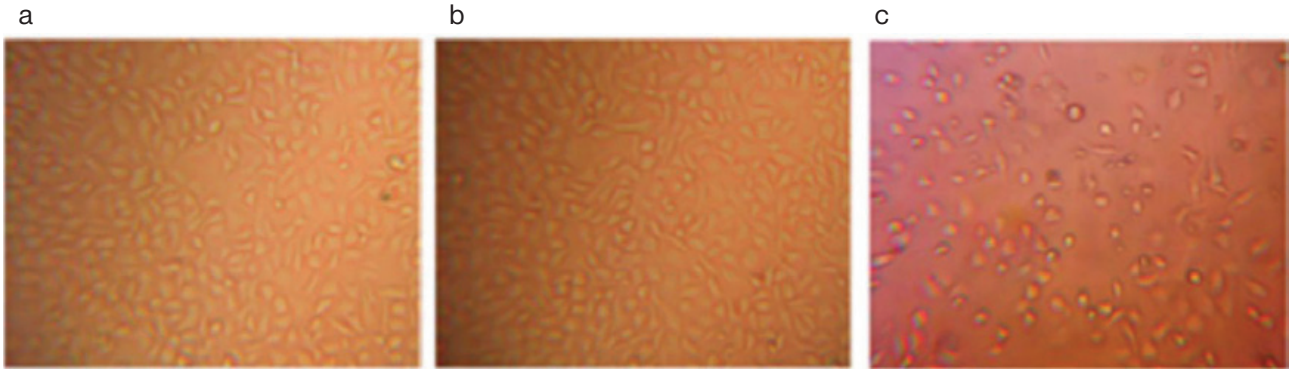

Fig. 5

Apoptosis of rCasp3-transfected HepG2, ΔscpHepG2, and scpHepG2 cells detected by light microscopy

HepG2 (a), ΔscpHepG2 (b), and scpHepG2 (c) cells.

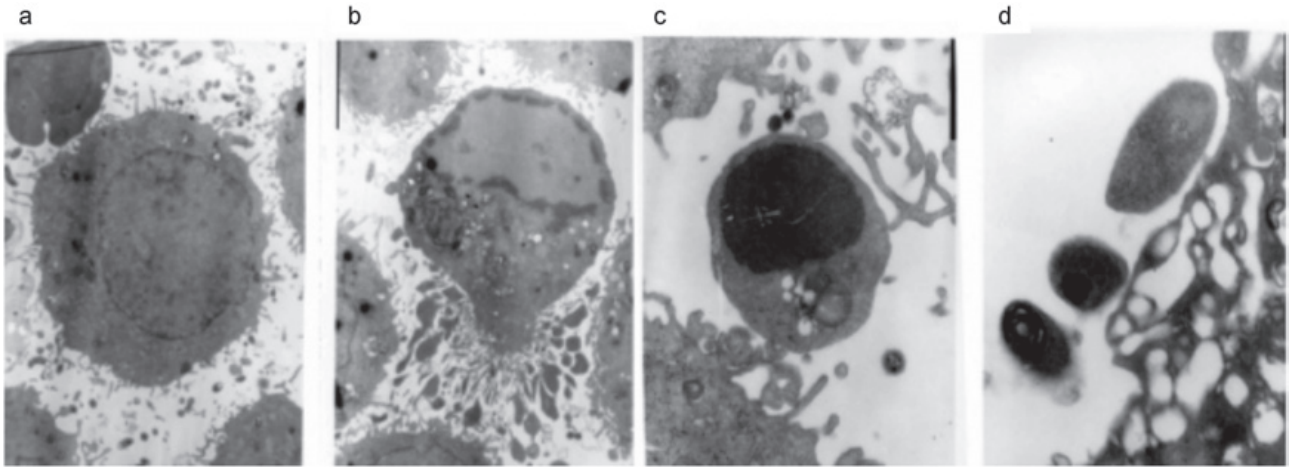

Fig. 6

Apoptosis of rCasp3-transfected HepG2, ΔscpHepG2, and scpHepG2 cells detected by electron microscopy

Control cells (a), apoptotic cells (b, c, d).

Apoptosis of rCasp3-transfected cells is dependent on NS3/4A protease expression

To detect the ability of rCasp3 to induce apoptosis in the cells producing the NS3/4A protease, the recombinant eukaryotic expression plasmid pc3.1(–)-rCasp3 was transfected into the scpHepG2, ΔscpHepG2, and HepG2 cells. The rCasp3-transfected scpHepG2 cells specifically cleaved rCasp3 and the presence of cleaved 17-K subunit was detected by Western blot analysis (Fig. 4). At 48 hrs posttransfection, distinct morphological changes were observed in rCasp3-transfected cells containing NS3/4A protease. The cell size decreased and the intercellular spaces increased (Fig. 5). Electron microscopy revealed that the activation of rCasp3 produced typical apoptotic changes as chromatin shrinkage and formation of apoptotic bodies (Fig. 6).

At 48 hrs posttransfection, the cells were double-stained with annexin V and propidium iodide to facilitate quantification of the apoptotic cells by flow cytometry. The results showed that approximately 45% of the cells underwent apoptosis. Obtained results indicated that the NS3/4A protease could recognize the NS4A/B and NS5A/B cleavage sites of rCasp3. When the NS3/4A protease activity was abrogated through the mutation in the active site of the enzyme as in ΔscpHepG2 cells, rCasp3 could not be cleaved and activated (Fig. 7). Thus, the activation of rCasp3 and

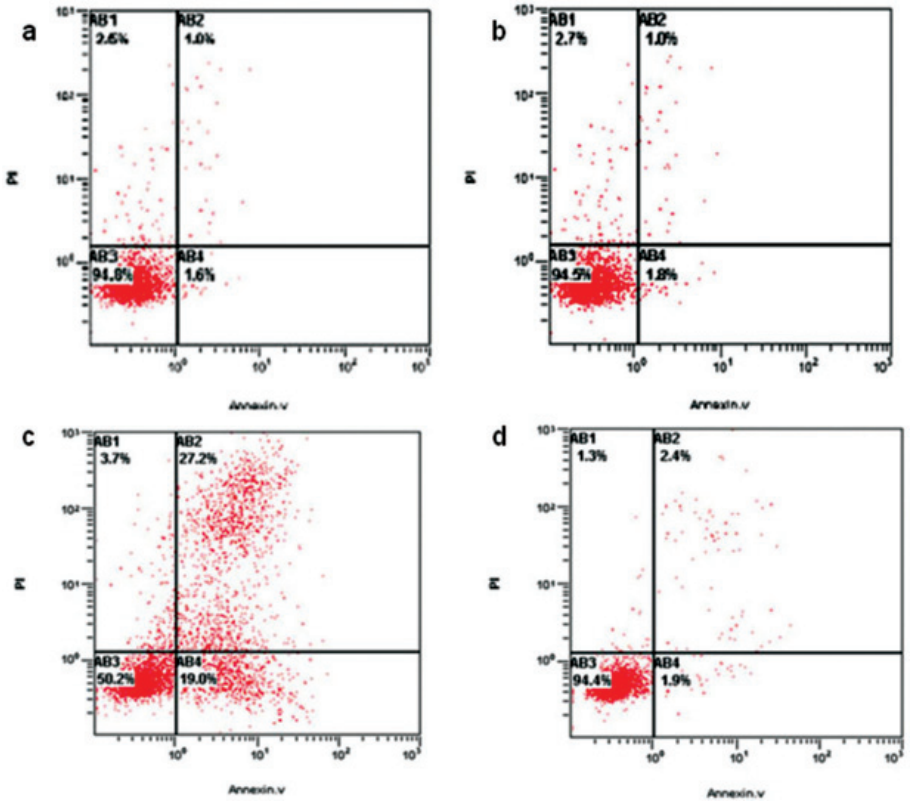

Fig. 7

Apoptosis of rCasp3-transfected cells in the presence and absence of NS3/4A protease inhibitor detected by flow cytometry

HepG2 (a), ΔscpHepG2 (b), and scpHepG2 cells transfected with rCasp3 (c). scpHepG2 cells transfected with rCasp3 in the presence of protease inhibitor Ac-DEMEEC (d). PI = propidium iodide.

consequent apoptosis in the cells was dependent on the NS3/4A protease activity.

Development of an MTT assay for monitoring NS3/4A protease activity

To evaluate the viability of the pc3.1(–)-rCasp3-transfected stable cell lines expressing the NS3/4A protease, a trypan blue exclusion test was performed at 72 hrs after transfection with rCasp-3. Cytotoxicity of the NS3/4A protease inhibitors (Ac-DEMEEC) at a concentration of 0.1–10 μg/ml was tested. No cytopathological changes in the cells incubated with the inhibitor at these concentrations were observed. When the stable cells were transfected with pc3.1(–)-rCasp3, the cells expressing the NS3/4A protease progressed to apoptosis and ultimately died after 72 hrs. When the cells expressing the NS3/4A protease were pretreated with NS3/4A protease inhibitors, the apoptosis was sporadic (Fig. 8a). Based on the specific apoptosis of pc3.1(–)-rCasp3-transfected cells expressing NS3/4A protease, an MTT-based assay was developed that could be performed in 96-well plates. The OD value of the pc3.1(–)-rCasp3-transfected scpHepG2 cells was lower than that of the non-transfected cells or scpHepG2 cells pretreated with NS3/4A protease inhibitors at the same time point. At 72 hrs posttransfection, maximum disparity was observed between the rCasp3-transfected cells and the inhibitor-treated rCasp3-transfected cells (Fig. 8b).

Discussion

The HCV NS3/4A serine protease is essential for viral replication and is undoubtedly the most thoroughly characterized enzyme of HCV (Li *et al.*, 2005). In the recent clinical trials, it has been observed that NS3/4A protease inhibitors such as BILN 2061 and VX-950 considerably reduced the HCV RNA plasma levels. This finding highlighted NS3/4A protease inhibitors as prime targets for anti-HCV drug development (Summa, 2005; Lamarre *et al.*,

2003). The use of BILN 2061 was discontinued in clinical trials, since a cardiac damage was reported in treated laboratory animals (Reiser *et al.*, 2005). Now, many pharmaceutical companies are competing to develop more specific, less toxic, and more active NS3/4A protease inhibitors.

Ideally, a potential NS3/4A protease inhibitor can be screened in cultured cells infected with HCV. However, the testing of the anti-viral molecules has been limited due to the lack of the efficient cells lines suitable for propagation of HCV. The cell-based assays can complement the enzyme assays used for the evaluation of potential inhibitors of NS3/4A protease. There are reports of several assays that are capable of screening NS3/4A protease inhibitors, but still there is an urgent need for a novel cell-based assay suitable for monitoring NS3/4A protease activity.

The HCV NS3/4A protease is a heterodimeric enzyme comprising the NS4A protein and the N-terminal domain of the NS3 protein (De Francesco and Steinkuhler, 2000). The junctions of the polyprotein precursor that are cleaved by the NS3/4A protease have the following consensus sequence that is identical to the junctions of the HCV polyprotein: D/E-(Xaa)4-C/T↓S/A-(Xaa)2-L/W/Y (Kim *et al.*, 2000; Zhang *et al.*, 1997). The role and necessity of NS4A as a cofactor, especially the core region of the NS4A protein became more certain, when the three-dimensional structure of NS3/4A protease was resolved by X-ray crystallography (Kim *et al.*, 1996). It has been shown that the function of NS4A as a cofactor can be efficiently mimicked by the peptides comprising residues 21–34 of the central region of NS4A (Koch *et al.*, 1996; Tanji *et al.*, 1995). Recently, the single-chain protease (scNS3/4A) comprising the core region of NS4A and the N-terminal of NS3 joined by the RGGP linker representing the proteolytic activity has been described (Pasquo *et al.*, 1998). The construction mode of scNS3/4A can mimic the structure of the NS3/4A protease and has been widely used to develop assays for monitoring NS3/4A serine protease activity. In our experiment, the single-chain NS3/4A protease was used and the results showed that it exhibited a good enzymatic activity.

The enzyme Casp3 belongs to a family of cysteine proteases and is involved in the execution step of apoptosis. Activation of Casp3 as a key step in apoptosis is induced by the cleavage of cellular proteins such as poly (ADP-ribose) polymerase, lamin, and fodrin ultimately leading to the cell death (Enari *et al.*, 1998; Woo *et al.*, 1998; Cohen, 1997). Procaspase 3 contains an N-terminal pro-domain followed by the caspase-cleavage recognition site, p17 domain containing the catalytic C residue, second caspase cleavage site, and p12 domain. During apoptotic signaling, the zymogen form of Casp3 is cleaved by upstream caspases such as granzyme B resulting in the formation of active p17:p12 heterotetramer and self-cleavage of the pro-domain. By the modification of the apoptosis-promoting Casp3 protein was engineered HIV protease-activated Casp3 protein, where the endogenous cleavage sites of Casp3 were substituted by HIV proteolytic cleavage sites. The modified protein was activated by HIV protease resulting in the apoptosis of the infected cells (Vocero-Akbani *et al.*, 1999). Later, Hsu *et al.* (2003) introduced an HCV NS3/4A protease cleavage site into BID (modified apoptotic molecule) by using adenovirus expression vectors that were injected into the jugular vein and

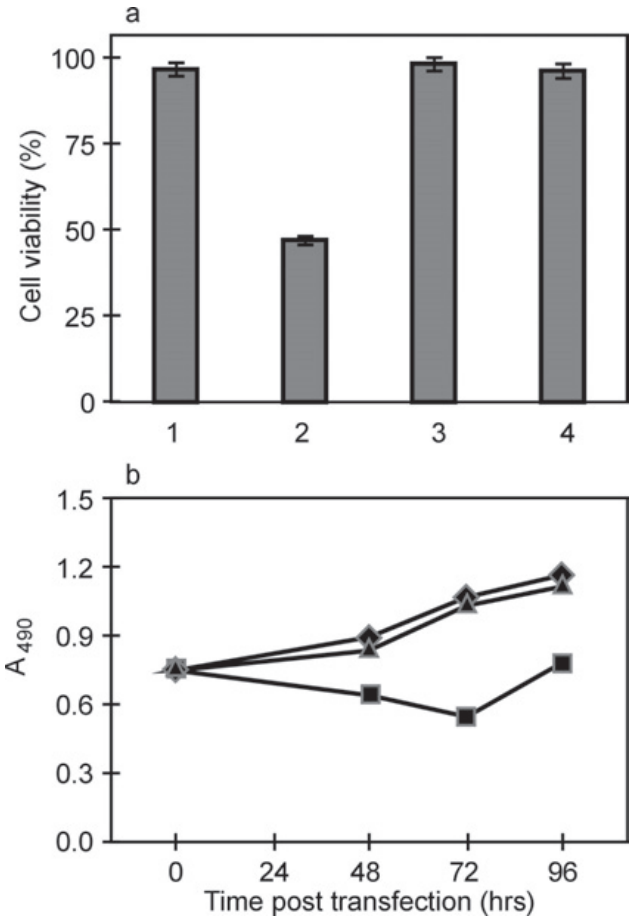

Fig. 8

Apoptosis of rCasp3-transfected cells in the presence and absence of NS3/4A protease inhibitor detected by trypan blue assay (a), and MTT assay (b)

(a): non-transfected scpHepG2 cells (1), scpHepG2 cells transfected with rCasp3 (2), ΔscpHepG2 cells transfected with rCasp3 (3), scpHepG2 cells transfected with rCasp3 in the presence of protease inhibitor Ac-DEMEEC (4). (b): non-transfected scpHepG2 cells (-♦-), scpHepG2 cells transfected with rCasp3 (-■-), scpHepG2 cells transfected with rCasp3 in the presence of protease inhibitor Ac-DEMEEC (-▲-).

efficiently targeted the liver. The non-replicating adenoviruses caused apoptosis of the liver cells containing HCV NS3/4A protease.

In our experiments we used the modified Casp3 to establish a cell-based system for evaluating HCV NS3/4A protease activity. We utilized the characteristics of the NS3/4A protease and the activation of Casp3. In order to establish an MTT-based assay for monitoring NS3/4A protease activity, we developed cell lines containing gene for NS3/4A and ΔNS3/4A proteases that were transfected by rCasp3. To show the dependence of activation of rCasp3 on NS3/4A protease activity, the stable cell lines expressing the ΔNS3/4A protease were used as a negative control. Both NS3/4A protease cleavage sites (NS4A/B and NS5A/B) were inserted between the pro-domain and the large and small subunits of Casp3. Overlapping PCR was used to construct rCasp3 as the substrate of the NS3/4A protease. After transfection of cells with plasmid expressing rCasp3, NS3/4A protease cleaved and activated rCasp3 that finally induced apoptosis. The OD value of the cells following apoptosis could be quantified using the MTT assay. In this experiment, the OD value of the pc3.1(–)-rCasp3-transfected scpHepG2 cells was lower than that of the non-transfected cells and the scpHepG2 cells pretreated with NS3/4A protease inhibitors at the same time point. When the scpHepG2 cells were pretreated with NS3/4A serine protease inhibitors, the OD value of the cells was comparable with that of the negative control. At 72 hrs post transfection, maximum disparity was observed between the pc3.1(–)-rCasp3-transfected cells and the pretreated pc3.1(–)-rCasp3-transfected cells. The cell-based assay described in this report can be used to evaluate intracellular NS3/4A protease activity and to screen for candidate inhibitors by using the apoptosis as an indicator. This system has the following unique advantage that the screened inhibitors would possess a low toxicity and exert a high inhibition effect.

We developed scpHepG2 cell line expressing the NS3/4A protease, whose activity can be indicated by the quantification of the apoptosis of rCasp3-transfected cells. Further, MTT-based assay was established for evaluating NS3/4A protease activity in a microtiter plate, what offered the benefit of assaying numerous samples by using the laboratory equipment. This assay is expected to be helpful in developing and screening of anti-HCV drugs that are important for treatment of the disease caused by HCV.

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