Dengue virus-induced apoptosis in hepatic cells is partly mediated by Apo2 ligand/tumour necrosis factor-related apoptosis-inducing ligand

Takehiro Matsuda,1,2 Alex Almasan,3 Mariko Tomita,1 Kazumi Tamaki,1 Mika Saito,1 Masayuki Tadano,1 Hideo Yagita,4 Takao Ohta2 and Naoki Mori1

Correspondence
Naoki Mori
n-mori@med.u-ryukyu.ac.jp

1,2Division of Molecular Virology and Oncology, Graduate School of Medicine1 and Division of Child Health and Welfare, Faculty of Medicine2, University of the Ryukyus, Uehara 207, Nishihara, Okinawa 903-0215, Japan

3Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

4Department of Immunology, Juntendo University School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan

Received 17 August 2004
Accepted 7 January 2005

Although hepatic injury is reported in cases with dengue haemorrhagic fever and dengue shock syndrome, its mechanism remains poorly understood. Several findings suggest that dengue virus (DEN) induces apoptosis of hepatocytes in vivo. In this work, DEN type 2 (DEN-2) strain NGC was shown to induce apoptosis in the hepatic cell line HepG2, and infection of HepG2 cells was found to induce Apo2 ligand (Apo2L, also known as tumour necrosis factor-related apoptosis-inducing ligand or TRAIL) expression. Furthermore, Apo2L/TRAIL induced apoptosis in HepG2 cells, which expressed the Apo2L/TRAIL receptor DR5/TRAIL-R2 on their surface. Analysis of the Apo2L/TRAIL promoter revealed that this gene was activated by DEN-2 infection, whose responsive element was overlapping NF-κB- and Sp1-binding sites located at nt −75 to −65. The proteasome inhibitor N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (LLnL) inhibited Apo2L/TRAIL mRNA expression, and LLnL and anti-Apo2L/TRAIL antibody inhibited DEN-2-induced apoptosis. It was proposed that DEN infection promotes apoptosis partly through the induction of Apo2L/TRAIL expression.

INTRODUCTION

Dengue viruses (DENs), mosquito-borne flaviviruses, are major human pathogens affecting about 100 million individuals in tropical and subtropical regions of the world annually, and are classified into four serotypes (dengue virus types 1 to 4, designated here DEN-1, -2, -3 and -4) (Gubler, 1998). All four serotypes of DEN are capable of causing human disease with varying degrees of severity, ranging from asymptomatic infection or dengue fever to the devastating dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Cardinal signs of DHF and DSS include haemorrhage, abrupt onset of vascular leakage and shock, accompanied by severe thrombocytopenia and massive complement activation (Bokisch et al., 1973; Nimmannitya, 1987). In DHF and DSS, liver involvement is a characteristic disease sign (Bhamarapravati et al., 1967). Hepatic injury is similar to that of the early stages of yellow fever, with an increase in plasma transaminase levels, fatty changes in hepatocytes, Kupffer cell hyperplasia, and centrolobular and midzonal necrosis (Innis, 1995). The most characteristic sign is the presence of acidophilic, or Councilman, bodies, which are apoptotic bodies (Feldmann, 1997) and correspond to those seen in the liver of yellow fever patients. DEN antigens have been detected in both hepatocytes and Kupffer cells (Bhamarapravati et al., 1967; Couvelard et al., 1999; Hall et al., 1991; Kangwanpong et al., 1995; Rosen et al., 1989).

Although the pathogenesis of DEN-related disease remains poorly understood, virus-induced cell death may be a crucial pathogenic event. Apoptotic cell death has been implicated as a cytopathological mechanism in response to DEN infection both in vitro and in vivo (Despres et al., 1996, 1998). During the last stage of apoptosis, cells break up into apoptotic bodies, which are then eliminated by phagocytosis. It has been suggested that apoptosis is an innate defence mechanism, which allows the organism to control virus infection by elimination of infected cells (Despres et al., 1996); however, several viruses have been shown to induce apoptosis, which can be detrimental to the host (Koga et al., 1994; Lewis et al., 1996; Shen & Shenk, 1995).
These observations suggest that virus-induced apoptosis may contribute, at least in part, to the pathogenesis of DEN-induced hepatic injury.

Cellular death receptors transmit apoptosis-inducing signals initiated by specific death ligands, most of which are primarily expressed as biologically active type II membrane proteins that are cleaved into soluble forms. Fas ligand (FasL) activates Fas, tumour necrosis factor (TNF) activates TNF receptor 1 (TNFR-1) and Apo2 ligand (Apo2L, also known as TNF-related apoptosis-inducing ligand or TRAIL) activates DR4 (TRAIL-R1) (Pan et al., 1997b) and DR5 (TRAIL-R2) (Walczak et al., 1997). Ligand-mediated activation triggers a cascade of events that begins with death-receptor oligomerization and the close association of their cytoplasmic death domains. This is followed by death domain-associated interaction with adaptor molecules and cellular proteases critical to death receptor-induced apoptosis (Almasan & Ashkenazi, 2003). Ligands of the TNF family and their cognate receptors play a key role in liver pathogenesis (Faubion & Gores, 1999). In this paper, we describe a novel mechanism for DEN-induced hepatic cell death involving the induction of Apo2L/TRAIL expression and subsequent Apo2L/TRAIL-mediated apoptosis.

**METHODS**

**Cell lines, viruses and reagents.** The human hepatoma cell line HepG2 was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS; JRH Biosciences), 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Jurkat is a T-cell line originating from human acute lymphocytic leukaemia (Schneider et al., 1977). C5/MJ (Popovic et al., 1983) and HUT-102 (Poiesz et al., 1983) are human T-cell leukaemia virus type I-infected T-cell lines. These cell lines were grown in RPMI 1640 supplemented with 10% FBS and antibiotics. DEN-2 prototype New Guinea C (NGC) strain was propagated in C6/36 mosquito cells in minimum essential medium containing 10% FBS and antibiotics and stored at −80°C until use. Control C6/36 supernatant was obtained from uninfected cultures of C6/36 cells treated in the same way as those in which virus was propagated. These supernatants were used to mock-infect HepG2 cells. To ascertain the number of HepG2 cells infected with DEN-2, virus titres were determined by a focus-forming assay on HepG2 cells as previously described (Ishimine et al., 1987). Recombinant human soluble Apo2L/TRAIL (Super Killer TRAIL; Alexis Biochemicals) is constituted from the extracellular domain of human Apo2L/TRAIL fused N-terminal to a His-tag and a linker peptide and does not require a cross-linker for biological activities. Human TNF-α and an agonistic anti-human Fas monoclonal antibody (mAb) (7C11) were purchased from PeproTech EC and Beckman Coulter, respectively. A neutralizing anti-human Apo2L/TRAIL mAb (RIK-2) was prepared as described previously (Kayagaki et al., 1997). N-Acetyl-l-leucinyl-l-leucinyl-l-norleucinal (LNL) was purchased from Sigma-Aldrich.

**Virus infection.** Monolayers of HepG2 cells were trypsinized and resuspended in growth medium. Cells were seeded at a density of 1 × 10⁵ cells ml⁻¹ into each plate. After overnight incubation, virus culture fluid or heat-inactivated virus suspension (56°C, 30 min) was added to monolayers of cells at 37°C for 2 h. Virus supernatants were then removed and fresh growth medium was added to each plate for further incubation.

**Detection of DEN-2 antigen.** A mouse mAb (3H5) that reacts with the DEN-2 envelope protein was used to detect DEN viral antigen. Immunostaining was performed as described previously (Tadano et al., 1989).

**Viability and apoptosis assays.** The viability of mock- and DEN-2-infected HepG2 cells in culture plates was examined using the cell proliferation reagent WST-8, a tetrazolium salt (Wako Chemicals). WST-8 (10 μl) was added for the last 4 h of incubation and absorbance at 450 nm was measured using an automated microplate reader. Measuring the mitochondrial dehydrogenase-mediated cleavage of WST-8 to formazan dye indicates the level of proliferation. The early apoptotic event in cells was examined by staining with phycoerythrin-conjugated Apo2.7 mAb (Beckman Coulter) and analysed by flow cytometry (FACSCalibur; Becton Dickinson). The 7A6 antigen, defined by this antibody, is a 38 kDa protein localized to the outer membrane of mitochondria and is involved in the molecular cascade of apoptosis (Seth et al., 1997; Zhang et al., 1996). Expression of 7A6 antigen is preferentially detected on apoptotic cells, but not on the surface of non-apoptotic cells.

**Western blot analysis.** Twenty-four and 48 h after infection with DEN-2, cells were lysed in buffer containing 65 mM Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Samples were cleared by microcentrifugation and assessed for protein concentration. Twenty micrograms of protein per sample was analysed by SDS-PAGE and electroblotted on to PVDF membranes (Millipore). After blocking with 5% non-fat dried milk, membranes were exposed overnight at 4°C to the primary antibody. After washing in TBST buffer (0·1% Tween 20 in Tris-buffered saline), a mouse secondary horseradish peroxidase-conjugated antibody (Amersham Biosciences) was applied for 1 h at room temperature. Proteins were visualized with the enhanced chemiluminescence kit (Amersham Biosciences). mAb against actin (ACTN05; NeoMarkers) was used as a protein loading control.

**RT-PCR.** Total cellular RNA was extracted with Trizol (Invitrogen) from the protocol provided by the manufacturer and the amount of total RNA was determined by measuring absorbance at 260 nm. First-strand cDNA was synthesized from 1 μg total cellular RNA in a 20 μl reaction volume using an RNA PCR kit (Takara Shuzo) with random primers. Thereafter, cDNA was amplified for 30 cycles for Apo2L/TRAIL, 35 cycles for TNF-α and FasL and 28 cycles for β-actin. The oligonucleotide primers used were as follows: for Apo2L/TRAIL, sense, 5'-CAATGACGACAGAGATGATA3'-α, and antisense, 5'-CCCTCTGTAGATGGAAATA3'-α (Satoh et al., 2001); for TNF-α, sense, 5'-ATGAGCGCTAGAAGATGACT3'-α, and antisense, 5'-TCACAGCCGGAATGCCTCAAGAATGACTCGTCCC3'-α; for FasL, sense, 5'-GGATTGGGCCTGGGGATGTTTCA-3; and antisense, 5'-TGTGCGCTAGGGGCAATTGTTG-3' (Chen et al., 1997); and for β-actin, sense, 5'-GTTGGGCCTGGCCACATTG3'-α, and antisense, 5'-CTCCTATGTCGAGGCTTTC-3'-α. Product sizes were 536 bp for Apo2L/TRAIL, 702 bp for TNF-α, 343 bp for FasL and 548 bp for β-actin. Cycling conditions were as follows: denaturation at 94°C for 30 s (TNF-α, FasL and β-actin) or for 40 s (Apo2L/TRAIL), annealing at 57°C for 60 s (Apo2L/TRAIL) or 60°C for 30 s (TNF-α, FasL and β-actin) and extension at 72°C for 60 s (Apo2L/TRAIL) or for 90 s (TNF-α, FasL and β-actin). PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

**Cell-surface expression of Apo2L/TRAIL receptors.** Cells were analysed for the surface expression of DR4 (TRAIL-R1), DR5 (TRAIL-R2), DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) by indirect staining with primary mouse anti-human DR4, DR5, DcR1 and DcR2 mAbs. Briefly, 10⁵ cells were incubated with 1 μg biotinylated control mouse IgG₃, or mAbs specific for DR4 (D1R1), DR5 (D1R2), DcR1 (D1R3) or DcR2 (D1R4) for 30 min. After washing, cells were
incubated with phycoerythrin-conjugated streptavidin (Beckman Coulter) for 30 min on ice and analysed by flow cytometry. For surface staining of Apo2L/TRAIL receptors, cells were detached by incubation with PBS, followed by brief trypsinization. Trypsinization did not affect the Apo2L/TRAIL receptors.

**Plasmids and transfections.** A series of Apo2L/TRAIL promoter pGL3-luciferase reporter constructs described previously (Gong & Almasan, 2000) was used to map the DEN-2-responsive regions. An internal deletion of the NF-κB site (ApoP/1056ΔκB) was also created. Transient transfection of HepG2 cells was achieved with Lipofectamine (Invitrogen) according to the manufacturer’s protocol. Approximately 3 × 10^5 cells were seeded per plate and transfected 16 h later with 0-1 μg of appropriate reporter plasmids. To normalize transfection efficiencies, a thymidine kinase promoter-driven Renilla luciferase plasmid (phRL-TK, 0-5 μg; Promega) was co-transfected as an internal control plasmid. Cells were washed 16-24 h later and infected with virus at an m.o.i. of 8 as described above. After incubation for 24 h, cells were washed in PBS and lysed in reporter lysis buffer (Promega). Cell extracts were prepared by freezing and thawing the cells once. Unbroken cells and debris were pelleted by centrifugation at 20 000 g for 15 min at 4°C. Lysates were assayed for reporter gene activity with the Dual Luciferase Reporter Assay system (Promega). Luciferase activities were normalized based on the Renilla luciferase activity from phRL-TK.

**RESULTS**

**Apoptosis of HepG2 cells induced by DEN-2 infection**

In this study, we employed DEN-2 NGC, a prototype strain, to infect human hepatic HepG2 cells. DEN-infected cells expressed viral antigens, which were confirmed to be DEN-2 by immunostaining using a mAb against the DEN-2 envelope (Fig. 1a).

To investigate whether DEN-2 induced apoptosis, HepG2 cells infected with different doses of DEN-2 [m.o.i. of 0.03, 0.1, 0.3, 1, 3, 8 or 25 focus-forming units (f.f.u.) per cell] or mock treated were analysed using the WST-8 assay at 72 h post-infection (p.i.). Measuring the mitochondrial dehydrogenase-mediated cleavage of WST-8 to formazan dye indicates the level of proliferation. As shown in Fig. 1(b), reduced cell viability of HepG2 cells correlated with increasing m.o.i. We confirmed the results of the WST-8 assay by analysis of the 7A6 antigen, which is expressed on the mitochondrial outer membrane during apoptosis.
To determine the time course of apoptotic cell death, DEN-2-infected HepG2 cells were examined at different times p.i. by analysis of the 7A6 antigen. As shown in Fig. 1(c), more apoptotic cells were observed at 48–72 h p.i. in DEN-2-infected cells (71–87 %) than in mock-infected cells (6–25 %). Heat-inactivated DEN-2 was not effective in inducing apoptosis in HepG2 cells (Fig. 1d).

Expression of Apo2L/TRAIL and TNF-α is induced in DEN-2-infected cells

We next investigated the expression of TNF family members in HepG2 cells after DEN-2 infection. HepG2 cells infected with DEN-2 were harvested at 2, 4, 8, 12 and 24 h p.i. and total RNA was extracted. mRNA expression levels of the TNF family members were examined by RT-PCR. As shown in Fig. 2(a), Apo2L/TRAIL and TNF-α mRNA was upregulated following DEN-2 infection. Supernatant from uninfected C6/36 cells and heat-inactivated DEN-2 did not upregulate Apo2L/TRAIL or TNF-α mRNA expression in HepG2 cells (Fig. 2b). In contrast, FasL mRNA was not expressed in uninfected HepG2 cells and not induced by DEN-2 infection. Apo2L/TRAIL protein expression was also studied by Western blotting. As shown in Fig. 2(c), Apo2L/TRAIL protein expression was also upregulated by DEN-2 infection.

Apo2L/TRAIL induces apoptosis in HepG2 cells

To elucidate the involvement of TNF family members in DEN-2-induced apoptosis, HepG2 cells were incubated with various concentrations of Apo2L/TRAIL, TNF-α or an agonistic anti-human Fas mAb (7C11) for 72 h (the same time at which viability and apoptosis were determined in cells infected with DEN-2), followed by addition of the cell proliferation assay reagent WST-8. As shown in Fig. 3(a–c), Apo2L/TRAIL reduced cell viability of HepG2 cells in a dose-dependent manner, while TNF-α and Fas antibody did not. Apo2L/TRAIL reduced cell viability at 24 h to the same level as at 72 h (data not shown). To examine whether Apo2L/TRAIL-induced apoptosis in HepG2 cells, we analysed expression of 7A6 antigen in these cells. Consistent with the WST-8 assay, Apo2L/TRAIL induced apoptosis of HepG2 cells (Fig. 3d).

Apo2L/TRAIL is released from cells following infection with DEN-2

We sought to determine whether soluble Apo2L/TRAIL was released from DEN-2-infected cells. Following infection of HepG2 cells with DEN-2, supernatants were collected and transferred into Jurkat cells, which are sensitive to Apo2L/TRAIL-induced apoptosis (Fig. 4a). Apo2L/TRAIL-induced cytotoxicity in Jurkat cells was inhibited by an anti-Apo2L/TRAIL antibody, indicating that the reduction in cell viability seen in Jurkat cells was Apo2L/TRAIL-specific. In contrast, TNF-α did not reduce the viability of Jurkat cells. Supernatants collected from virus-infected HepG2 cells at 72 h p.i. reduced cell viability when transferred into Jurkat cells. In contrast, supernatants collected from mock-treated HepG2 cells did not affect cell viability of Jurkat cells. The cytotoxic effects of infected HepG2 cell supernatants were not due to the presence of infectious virus in the transferred supernatant, since DEN-2 virus cannot infect Jurkat cells (data not shown). Furthermore, DEN-2 virus

Fig. 2. Determination of Apo2L/TRAIL and TNF-α expression in DEN-2-infected HepG2 cells. HepG2 cells were infected with DEN-2 at an m.o.i. of 8 and cultured for the indicated time periods. (a) Apo2L/TRAIL, TNF-α and FasL mRNA expression in DEN-2-infected HepG2 cells analysed by RT-PCR. β-Actin mRNA was used as a control. C5/ MJ and HUT-102 cells were used as positive controls (CON+) for Apo2L/TRAIL and TNF-α, respectively, and human T-cell leukaemia virus type I Tax-transfected Jurkat cells were used for FasL. (b) Supernatant from uninfected C6/36 cells and heat-inactivated DEN-2 does not upregulate Apo2L/TRAIL and TNF-α mRNA expression in HepG2 cells. (c) Apo2L/TRAIL expression in DEN-2-infected HepG2 cells cultured for 24 and 48 h, analysed by Western blotting. Protein extracts were separated by 10 % SDS-PAGE, transferred to a membrane and blotted with either a specific anti-Apo2L/TRAIL mAb or an anti-actin antibody (as a protein loading control).
Fig. 3. Apo2L/TRAIl-induced cytotoxicity and apoptosis. (a)–(c) HepG2 cells were incubated with various concentrations of TNF-α (a), agonistic anti-Fas mAb (b) or Apo2L/TRAIl (c) for 72 h. The WST-8 assay was used to measure cell viability. Results are shown as percentage survival compared with the control. (d) The proportion of Apo2L/TRAIl (500 ng ml⁻¹)-treated HepG2 cells in an apoptotic state, as determined by Apo2.7 staining. Values represent the mean ± SD of triplicate measurements.

Fig. 4. Apo2L/TRAIl is released from DEN-2-infected HepG2 cells. (a) HepG2 cells were either mock-treated or infected with DEN-2 (m.o.i. of 8) and then cultured for 72 h. Supernatant from infected or mock-treated HepG2 cells or C6/36 cell supernatant was added to Apo2L/TRAIl-sensitive Jurkat cells as a 25 or 50% mixture with basal medium. Alternatively, Apo2L/TRAIl (100 ng ml⁻¹), TNF-α (100 ng ml⁻¹) or DEN-2 (1 or 2 × 10⁶ f.f.u. ml⁻¹) was added as indicated. Cell viability was assayed 24 h after supernatant transfer and results are shown as percentage survival of Jurkat cells following treatment compared with treatment with basal medium only. Anti-Apo2L/TRAIl mAb (10 μg ml⁻¹) was used as an Apo2L/TRAIl specificity control. Values represent the mean ± SD of triplicate measurements. (b) Supernatant from infected HepG2 cells (as a 50% mixture) and DEN-2 (2 × 10⁶ f.f.u. ml⁻¹) were added with basal medium to Jurkat cells as indicated. Anti-Apo2L/TRAIl mAb (10 μg ml⁻¹) or control IgG (10 μg ml⁻¹) was also added. Cell viability was assayed in Jurkat cells 24 h following supernatant transfer. Results are shown as percentage survival of Jurkat cells following treatment compared with treatment with basal medium only.
appeared to cause no apparent reduction in cell viability in Jurkat cells (Fig. 4a). Anti-Apo2L/TRAIL mAb inhibited cytotoxicity induced by supernatant from virus-infected HepG2 cells, but did not affect cell viability of Jurkat cells cultured with DEN-2 supernatants (Fig. 4b). Together, these results suggested that Apo2L/TRAIL is released from DEN-2-infected HepG2 cells and induces apoptosis in Jurkat cells.

**DEN-2-induced cytotoxicity is mediated in part by Apo2L/TRAIL**

We investigated the role of Apo2L/TRAIL-mediated apoptosis in DEN-2-induced cell death using a mAb directed against Apo2L/TRAIL to block ligand binding during DEN-2 infection. HepG2 cells were pretreated with antibody or control IgG1 for 2 h before viral infection (m.o.i. of 8) and were maintained in antibody- or control IgG1-containing medium following infection with DEN-2. Antibody or control IgG1 was not present during viral infection. Cell viability was determined at 72 h p.i. Anti-Apo2L/TRAIL mAb reduced DEN-2-induced cytotoxicity (71% survival in the presence of antibody versus 31% survival in the presence of control IgG1; Fig. 5a). Using a focus-forming assay, we found that antibody and control IgG1 had no effect on virus infection (data not shown).

**Expression of Apo2L/TRAIL receptors in HepG2 cells**

Restricted expression of Apo2L/TRAIL death receptors DR4 (Pan et al., 1997b) and DR5 (Walczak et al., 1997) and ‘decoy’ receptors DcR1 (TRAIL-R3) (Degli-Esposti et al., 1997; Pan et al., 1997a; Sheridan et al., 1997) and DcR2 (TRAIL-R4) (Pan et al., 1998) is known to regulate the sensitivity of cells to Apo2L/TRAIL. We assessed the expression of Apo2L/TRAIL receptors on the cell membrane of HepG2 by flow cytometry. As shown in Fig. 5(b), surface expression of DR5, but not DR4, DcR1 or DcR2, was observed on HepG2 cells. These results indicated that death receptors, but not ‘decoy’ receptors, are expressed on HepG2 cells.

**Transcriptional control of Apo2L/TRAIL in HepG2 cells**

We investigated whether the DEN-2-induced increase in Apo2L/TRAIL gene expression was a result of enhancement of its promoter activity. HepG2 cells were transiently transfected with a reporter gene construct containing the −1056 nt of the Apo2L/TRAIL upstream regulatory sequences (ApoP/1056). Mock-infection with C6/36 supernatant was used as a control. DEN-2 infection caused an elevation in the activity of this Apo2L/TRAIL-driven reporter construct, suggesting that the virus activated the Apo2L/TRAIL gene at the transcriptional level (Fig. 6). The transcription factor NF-κB has been found to be activated after DEN infection (Avirun et al., 1998; Bosch et al., 2002; Jan et al., 2000; Marianneau et al., 1997). Previous experiments have revealed that two potential NF-κB-binding sites, κB1 (located between nt −264 and −255) and κB2 (located between nt −384 and −375), contained within the Apo2L/TRAIL promoter, are important in upregulation following T-cell activation (Baetu et al., 2001). To assess the importance of the NF-κB-binding sites within the Apo2L/TRAIL promoter, luciferase constructs containing sequential deletions of the Apo2L/TRAIL promoter were transfected into HepG2 cells. As shown in Fig. 6, deletion of sequences down to nt −126 did not diminish promoter activation, while further deletion to nt −33 significantly decreased activation in response to DEN-2. These data suggested that the Apo2L/TRAIL promoter region between nt −126 and −33 is required for DEN-2-induced Apo2L/TRAIL activation. Since the −126/−33 region did not contain the previously characterized NF-κB-binding sites κB1 and κB2, the DNA sequence of this region was analysed. Sequence analysis revealed the presence of overlapping NF-κB- and Sp1-binding sites in the sequence between nt −75 and −65 in the Apo2L/TRAIL promoter (Fig. 7a). To test the role of this site in DEN-2-mediated induction of Apo2L/TRAIL gene transactivation, we generated a −1056/+86 Apo2L/TRAIL promoter/reporter construct bearing an internal deletion of this site (ApoP/1056ΔκB). The effect of DEN-2 on activation of wild-type and ΔκB Apo2L/TRAIL promoter was analysed in HepG2 cells infected with DEN-2. Consistent with previous findings, our experiments...
showed that DEN-2 infection resulted in an increase in Apo2L/TRAIL promoter activation (Fig. 7b). However, deletion of the −74/−65 region resulted in abrogation of promoter activation, confirming the importance of this site in Apo2L/TRAIL gene expression. These observations indicated that the −74/−65 region is involved in DEN-2-mediated activation of Apo2L/TRAIL.

**Transcriptional activity of NF-κB is required for DEN-2-induced apoptosis**

To assess the involvement of Apo2L/TRAIL in DEN-2-induced apoptosis, HepG2 cells were treated with a nontoxic concentration (6–25 μM) of the proteasome inhibitor LLnL (Jeremias et al., 1998), which is known to inhibit the activation of NF-κB by blocking degradation of the IkB protein, for 2 h before DEN-2 infection and for 72 h after infection, and the cell viability was determined. Consistent with the results of Apo2L/TRAIL promoter analysis, treatment of cells with LLnL significantly inhibited DEN-2-induced Apo2L/TRAIL expression (Fig. 8a). Furthermore, LLnL treatment blocked the cytotoxic effect of DEN-2 as measured by WST-8 assay (Fig. 8b). Although proteasome inhibition affects many cellular processes, including activation of transcription factors, cell-cycle progression and apoptosis, these results indicated that DEN-2-induced apoptosis might be mediated, at least in part, by Apo2L/TRAIL.

**DISCUSSION**

Although DEN can infect a variety of cells, including endothelial and neuronal cells, in vitro (Avirutnan et al., 1998; Jan et al., 2000), only a few cell types have been identified as being infected in vivo: the most recognized target cells for DEN in humans are mononuclear phagocytes (Halstead et al., 1977). In recent years, DEN antigens have been found by immunofluorescence staining and in situ hybridization in the liver of patients with fatal DEN infection (Couvelard et al., 1999; Hall et al., 1991; Kangwanpong et al., 1995; Rosen et al., 1989). We found that DEN-2 is able to infect the human hepatic cell line HepG2. HepG2
cells have also been reported to be infected with DEN-1 (Marianneau et al., 1997). In addition, although there is no ideal animal model available for DEN studies, severe combined immunodeficient mice transplanted with HepG2 cells show some similarities to human DEN infection following intraperitoneal DEN infection (An et al., 1999). DEN-2 appeared to cause apoptosis in HepG2 cells, in which the infection was efficient. Although direct damage of HepG2 cells occurs with DEN infection, it has been suggested that apoptosis is induced by the detrimental effects of soluble cytotoxic cytokines, such as Apo2L/TRAIL, TNF-α and FasL released from virus-infected HepG2 cells.

We determined that DEN-2 infection induced the synthesis of functional Apo2L/TRAIL in HepG2 cells. Exogenous Apo2L/TRAIL induced apoptosis in HepG2 cells, which expressed cell-surface DR5. Furthermore, we showed that Apo2L/TRAIL contributed to DEN-2-induced apoptosis. In comparison with the cell death induced by Apo2L/TRAIL, more apoptotic cells were observed in DEN-2-infected cells. Thus, DEN-2 infection might sensitize cells to Apo2L/TRAIL-induced apoptosis. As previously reported by Shigeno et al. (2003), Apo2L/TRAIL reduced cell viability of the human hepatoma cell line HepG2 to 60% in their study. Apo2L/TRAIL preparations similar to that used in this study are also reported to induce increased apoptosis in normal human hepatocytes (Jo et al., 2000). Therefore, our in vitro studies suggest that Apo2L/TRAIL release occurs during DEN infection and may be essential for the induction of apoptosis in human hepatic cells in vivo.

Although Apo2L/TRAIL mRNA expression is detected in various cells and tissues (Pitti et al., 1996; Wiley et al., 1995), regulation of its expression remains largely unknown. Apo2L/TRAIL promoter studies have indicated induction of promoter activity after interferon stimulation (Chen et al., 2001). Two important sites for Apo2L/TRAIL promoter regulation are reported to lie between nt −1371 and −819 and between nt −165 and −35 (Gong & Almasan, 2000; Wang et al., 2000). A recent study demonstrated that the induced expression of Apo2L/TRAIL in Jurkat cells following treatment with a variety of stimuli such as phorbol myristate acetate is linked to two NF-κB binding sites, κB1 (located between nt −264 and −255) and κB2 (located between nt −384 and −375), within the Apo2L/TRAIL promoter (Baetu et al., 2001). We demonstrated that the induction of transcription of Apo2L/TRAIL was triggered by viral infection. Using a reporter gene to assess transcription from the Apo2L/TRAIL promoter, we found for the first time that overlapping NF-κB- and Sp1-binding sites (located between nt −75 and −65) contribute to the control of luciferase reporter gene activity.

NF-κB has been widely proposed to be involved in either protecting or promoting cell death in response to different stimuli in various cell types (Baueurle & Baltimore, 1996; Grimm et al., 1996; Lin et al., 1995). The involvement of NF-κB in DEN-induced apoptosis was first reported by Marianneau et al. (1997). In their studies, NF-κB activation was detected in HepG2 cells during DEN infection. Inhibition of apoptosis was observed when DEN-infected cells were treated with NF-κB decoys. More recently, Jan et al. (2000) reported that DEN infection caused NF-κB activation in a human neuroblastoma cell line. They also reported that pretreatment of cells with NF-κB decoys prevented DEN-induced apoptosis. The requirement for NF-κB activation in DEN-induced apoptosis suggests that NF-κB functions to increase the expression of pro-apoptotic genes. However, NF-κB-responsive pro-apoptotic proteins involved in mediating apoptosis induced by DEN have not been conclusively determined. Although FasL is regulated by NF-κB and contains NF-κB response elements in its

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**Fig. 8.** Proteasome activity is necessary to induce apoptosis in infected cells. HepG2 cells were pretreated for 2 h with LLnL (0.25 μM) before being infected with DEN-2. [(a)](image) Inhibitory effect of treatment with proteasome inhibitor on Apo2L/TRAIL mRNA expression in DEN-2-infected HepG2 cells. After infection (m.o.i. of 8), cells were incubated in medium containing LLnL for 12 h before total cellular RNA was extracted. Human T-cell leukaemia virus type I-infected T-cell line C5/MJ cells were used as a positive control for Apo2L/TRAIL. [(b)](image) Effect of treatment with proteasome inhibitor on cell death in DEN-2-infected HepG2 cells. After infection (m.o.i. of 3 or 8), cells were incubated in medium containing LLnL for 72 h before the percentage of surviving cells was determined. Values represent the mean±SD of triplicate measurements.
promoter (Takahashi et al., 1994). DEN-2 infection failed to induce the expression of FasL. To demonstrate the role of NF-κB in DEN-2-induced apoptosis, the proteasome inhibitor LLnL was used to block cell death. Despite some degree of toxicity being observed in mock-infected cells at the concentration used, LLnL significantly stimulated HepG2 proliferation in the DEN-2-infected cell population. Although the reason for this is currently unclear, any proteasome-dependent activity may be involved. Our present study is the first to demonstrate the involvement of a signalling pathway consisting of sequential activation of NF-κB and Sp1 that controls induction of Apo2L/TRAIL expression during DEN-2-induced apoptosis of human hepatic cells.

The involvement of Apo2L/TRAIL in apoptosis has been implicated in previous studies involving infection with various viruses, including human immunodeficiency virus (Katsikis et al., 1997), reovirus (Clarke et al., 2000), measles virus (Vidalain et al., 2000), cytomegalovirus (Sedger et al., 1999), Theiler’s murine encephalomyelitis virus (Rubio et al., 2003), respiratory syncytial virus (Kotelkin et al., 2003), Newcastle disease virus (Washburn et al., 2003) and Ebola virus (Hensley et al., 2002). Apo2L/TRAIL also plays a key role in viral hepatitis (Mundt et al., 2003). It has been reported that reovirus-induced apoptosis requires NF-κB (Connolly et al., 2000). Similar to DEN-2-induced apoptosis pathways, apoptosis induced by these viruses may require the participation of Apo2L/TRAIL promoter activation regulated by NF-κB. Understanding the signalling pathways used by DEN to induce cellular gene expression and apoptosis will contribute important new information about the mechanisms by which viruses induce cell death and disease.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

REFERENCES


