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Role of cathepsin B in dengue virus-mediated apoptosis

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ABSTRACT

Dengue virus (DENV) infection is one of the most important mosquito-borne viral diseases, which is endemic in the tropical and sub-tropical regions. Patients with dengue hemorrhagic fever (DHF) generally present hemorrhagic tendencies, plasma leakage, thrombocytopenia, and hemoconcentration. Hepatic dysfunction is also a crucial feature of DENV infection. Hepatic biopsy specimens obtained from fatal cases of DENV infection show cellular apoptosis, which apparently relate to the pathogenesis. Cathepsins, which are cysteine proteases inside the lysosome, were previously reported to be up-regulated in patients with DHF. However, their functions during DENV infection have not been thoroughly investigated. We show for the first time that DENV induces lysosomal membrane permeabilization. The resulting cytosolic cathepsin B and S contributed to apoptosis via caspase activation. The activity of caspase 3 was significantly reduced in DENV-infected HepG2 cells treatedwith cathepsin B or S inhibitors. Treatment with cathepsin B inhibitor also reduced the activity of caspase 9, suggesting that cathepsin B activates both caspase-9 and caspase-3. Reduced cathepsin B expression, effected by RNA interference, mimicked pharmacological inhibition of the enzyme and confirmed the contribution of cathepsin B to apoptotic events induced by DENV in HepG2 cells.

1. Introduction

Dengue virus (DENV) infection is one of the most important mosquito-borne viral diseases and is endemic in several countries. DENV has four serotypes (DENV-1, -2, -3 and -4). Clinical manifestations of infection include febrile disease, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). The last occurs in cases of subsequent infection with a different serotype of DENV [1]. Patients with DHF present with hemorrhagic tendencies, plasma leakage, thrombocytopenia, and hemoconcentration. Evidence of hepatic injury is also demonstrated by hepatomegaly and increases in transaminase levels [2–4]. Hepatic biopsy specimens obtained from fatal cases of DSS show cellular apoptosis, which may relate to the pathogenesis of DSS [5–7].

Following DENV infection, apoptosis of hepatic cells was observed both *in vitro* and *in vivo* [6–17]. DENV infection promotes apoptosis in the hepatoma cell line, HepG2, partly through the induction of TRAIL, a member of the death receptor pathway [18]. TNFR and Fas signaling also contribute to DENV mediated apoptosis [11,15]. Changes in mitochondria typical of the apoptotic process have also been demonstrated in DENV-infected HepG2 cells, indicating a role of the mitochondrial pathway [16]. In Huh-7 cells, another hepatoma cell line, DENV infection also increases mitochondrial membrane potential and p53 expression [19,20]. In a mouse model of DENV infection, intrahepatic infiltrating $CD8^+$ T cells cause liver cell death [21]. A contribution of apoptosis to the pathogenesis of fatal DHF/DSS in humans is suggested by immunolocalization studies [7]. Apoptotic cells were present in the livers offive of six patients from a Cuban dengue epidemic [7].

Lysosomes are membrane-bound organelles filled with hydrolytic enzymes including cysteine proteases and cathepsins [22]. Whereas intra-lysosomal cathepsins can degrade proteins and participate in several cellular processes, such as autophagy, antigen presentation and cytokine maturation, cytosolic cathepsins mediate apoptosis. [23,26–28]. In hepatocytes, cathepsins process Bid or caspase 2, degrade Bcl-2, and trigger the intrinsic apoptosis pathway. Although cathepsin genes have been shown to be up-regulated in DENV-infected HepG2 cells [13] and in patients with DHF [29], the role of cathepsins in DENV-mediatedapoptosis has not been investigated.

This study aims to characterize the role of cathepsins in DENV-mediated apoptosis. We show here that DENV induces lysosomal

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membrane permeabilization thereby releasing cathepsins to induce apoptosis and that cathepsin B contributes to DENV-mediated apoptosis in HepG2 cells.

2. Materials and methods

2.1. Culture and infection of HepG2 cells

HepG2 cells were cultured for 24 h before infection in DMEM medium (Gibco-BRL), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml of penicillin and streptomycin at37 °C in a humidified atmosphere containing 5% CO_2. Cells were infected with DENV serotype 2 (DENV-2) strain 16681 at a multiplicity of infection (MOI) of 5. The fraction of cells infected was measured by flow cytometry following immunofluorescent staining of DENV envelope protein (DENV E) [30,31].

2.2. Apoptosis assay

About 1 to 2 $\times$ 10^5 HepG2 cells were seeded in a 12-well culture plate, infected with DENV-2 for 2 h, washed with PBS, and incubated for 24 h, 36 h and 48 h, respectively. Following incubation, cells were stained with annexin V/FITC and propidium iodide (PI) (BD Biosciences). Apoptotic cells, which were annexin V+/PI−, werequantitated by flow cytometry.

To assess the caspase activity, up to 2 $\times$ 10^4 HepG2 cells were seeded in a 96-well plate and pre-treated with 50 μM cathepsin B inhibitor (CA-074 Me, Calbiochem), 10 μM cathepsin L inhibitor (Z-FY (t-Bu)-DMK, Calbiochem) or 5 μM cathepsin S inhibitor (Z-FL-COCHO, Calbiochem) for 3 h before infection with DENV-2. All concentrations tested in this study were proven to be non-toxic to the HepG2 cells (data not shown). An equal volume of DMSO (Sigma) was used as a vehicle control. The cells were infected with DENV-2 for 48 h. The activity of caspases 3 and 7 was measured using the fluorescence-based assay, Apo-One® Homogeneous Caspase-3/7 assay (Promega). In addition, the activity of caspase 8 and caspase 9 was separately measured using luminescence-based assays, Caspase-Glo™ 8 Assay andCaspase-Glo™ 9 Assay (Promega), respectively.

2.3. Lysosome permeability assay

About 1 to 2 $\times$ 10^5 HepG2 cells were seeded in a 12-well culture plate, infected with DENV-2 for 2 h, washed with PBS, and incubated for 24 h, 36 h and 48 h, respectively. At the indicated time points, lysosomes were labeled by incubation of cells with 0.5 μg/ml of acridine orange (Molecular Probes) for 15 min at 37 °C using the protocol described previously [32,33]. Acridine orange accumulates in intact lysosomes and fluoresces red. Fluorescence was analyzed with flow cytometry. The reduced red fluorescence of DENV-infected cells relative tomock-infected cells was taken as a measure of lysosome permeabilization. The role of reactive oxygen species (ROS) in lysosomal membrane permeabilization was assessed. HepG2 cells were seeded in a 12-well cul-

Table 1
Primers used for quantitative real time PCR.

Gene	Primer sequence (5′–3′)		Product size (bp)
	Forward	Reverse	
CTSB	TGTGTATTCGGACTTCCTGCT	GTGTGCCATTCTCCACTCC	113
CTSS	GGGTACCTCATGTGACAAG	TCACTTCTTCACTGGTCATG	400
CTSL	CAGTGTGGGAGAAGAACATG	TGATTCTTCACAGGAGTCAC	247
CTSD	GTTTCGTGCATTGGAAGACC	GGTTTTGTCCCCTCTCACTC	302
ACTB	AGAAAATCTGGCACCACACC	CTCCTTAATGTCACGCACGA	395

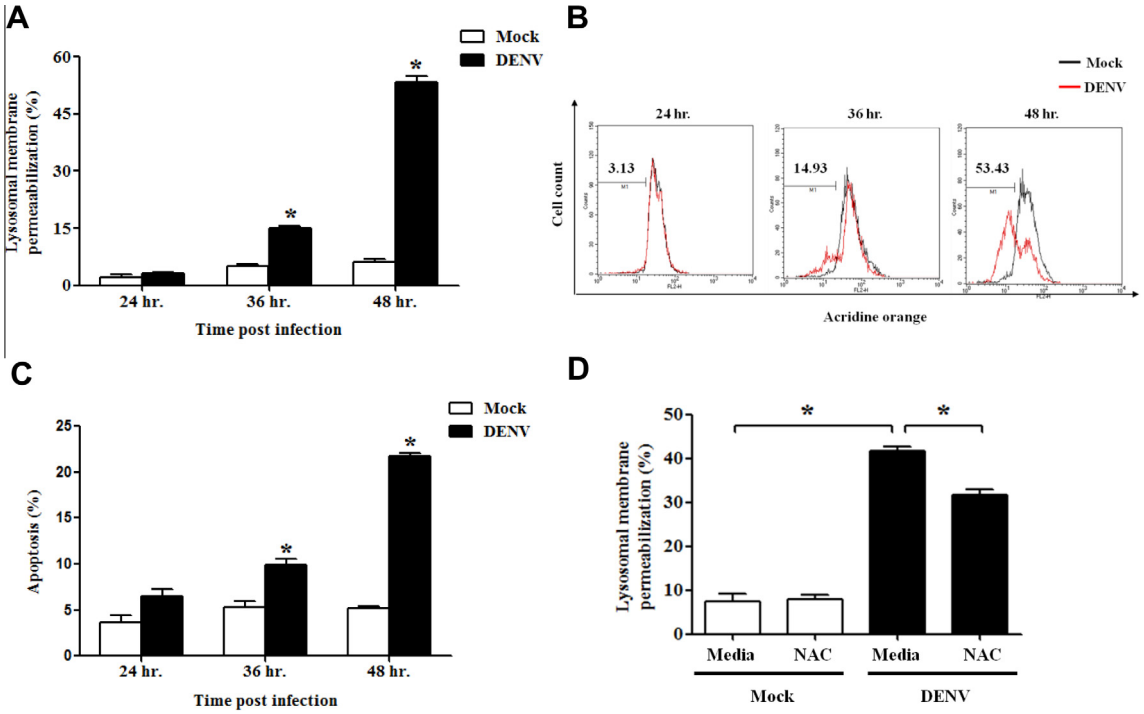

Fig. 1. DENV induced lysosomal membrane permeabilization, partly mediated by ROS in HepG2 cells. HepG2 cells were infected with DENV-2 at a MOI of 5 for 24, 36 and 48 h. Apoptosis was determined by annexin V/PI staining (C). Lysosomal membrane permeabilization was determined by acridine orange staining (A and B). To determine the role of ROS in mediating lysosomal membrane permeabilization, HepG2 cells were infected with DENV-2 at a MOI of 5 and cultured in the presence or the absence of 5 mM of NAC for 48 h (D). The results were obtained from flow cytometry analysis and expressed as the average of three independent experiments ± SEM. The asterisks indicate statistically significant differences between groups ($p < 0.05$).

ture plate, infected with DENV-2 for 2 h, washed with PBS, and incubated in the presence or the absence of 5 mM N-acetyl cysteine (NAC) (Calbiochem) for 24 h, 36 h and 48 h, respectively. At the indicated time points, lysosomes were labeled by incubation of cells with 0.5 μg/ml of acridine orange (Molecular Probes) for 15 min at 37 °C using the protocol described previously [32,33].

2.4. Real-time PCR

Up to 2×10^6 HepG2 cells were seeded in a 60 mm dish and were infected with DENV-2 for 24 h and 48 h, respectively. Total RNA from mock-infected or DENV-infected cells was isolated and reverses transcribed using the High Pure RNA isolation kit (Roche) and SuperScript® III First-Strand Synthesis System (Invitrogen), respectively. *CTSB*, *CTSD*, *CTSL*, *CTSS* or *ACTB* mRNA were PCR amplified using gene-specific primers (Table 1). Amplification was monitored using SYBR Green I Reaction Mix (Roche) in a Roche Light Cycler 480. The *Ct* of each mRNA and ACTB control was measured and the difference between their ΔCt was calculated. The relative expression values ($2^{-\Delta\Delta Ct}$) between mock-infected and DENV-infected HepG2 cells was then determined.

2.5. RNA interference

Up to 1.5×10^5 HepG2 cells were seeded in a 12-well culture plate and transfected either with 200 nM of small interfering RNA (siRNA) directed against *CTSB* (5′ UCU CUU UGA UGG UGG GAC ACU GUG G 3′)(Invitrogen) or with siControl (stealth RNAi negative control Hi GC) (Invitrogen) using lipofectamine 2000 (Invitrogen). At 24 h post transfection, cells were infected with DENV-2 and harvested 48 h later. The cells were lysed in RIPA buffer and subjected to Western blot analysis [34] using primary antibodies against CTSB(Cell Signaling), cleaved caspase 3 (Cell signaling), DENV E [30, 31] or β-actin (Santa Cruz Biotechnology), respectively.

2.6. Statistical analysis

All data were obtained from three independent experiments and reported as the mean ± SEM. Statistical differences between the groups were tested with an unpaired *t*-test using StatView version 5.0 and *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. DENV induced lysosomal membrane permeabilization in HepG2cells

Following DENV infection, apoptosis of hepatic cells was observed both *in vitro* and *in vivo* [6–17] However, the role of cathepsins in DENV-mediated apoptosis has not been investigated. Different stimuli have been shown to cause lysosomal membrane

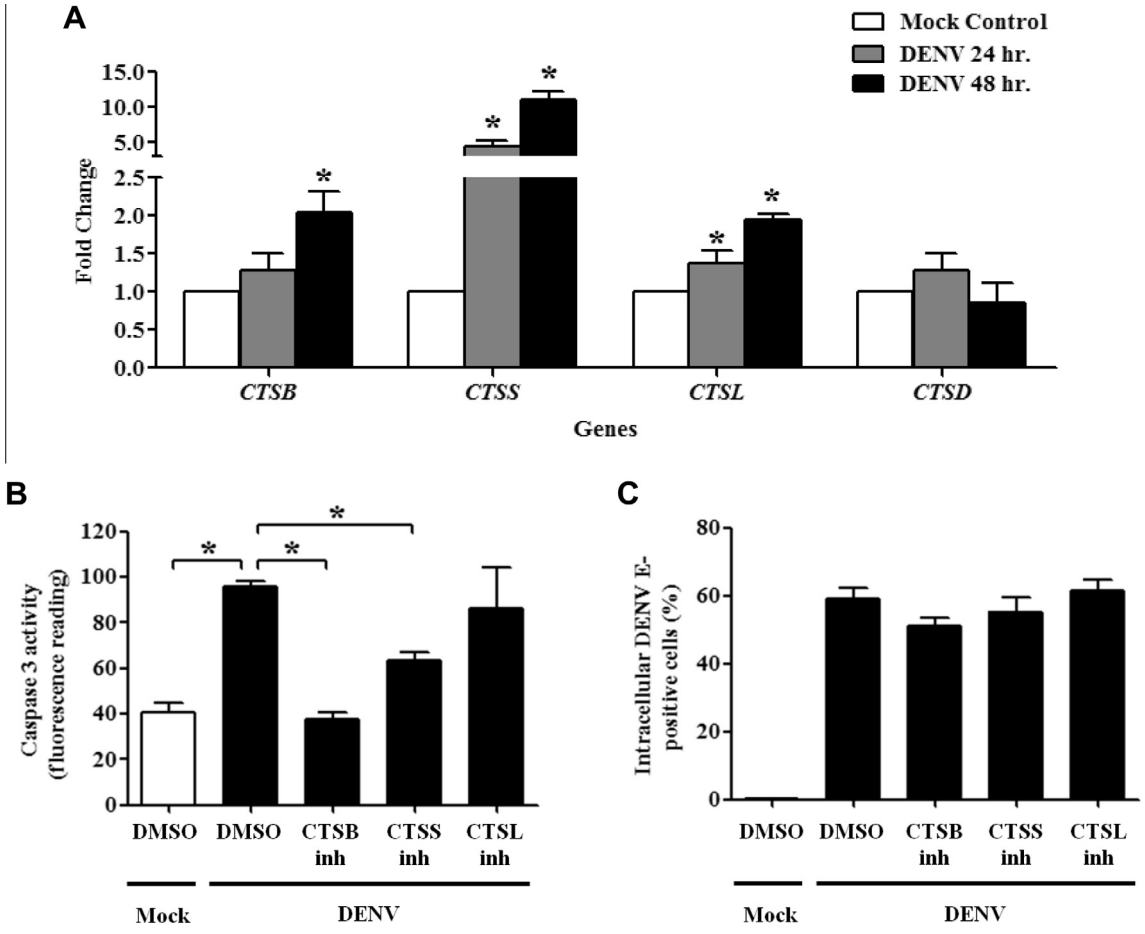

Fig. 2. DENV mediated up-regulation of cathepsin genes, which involved in apoptosis in HepG2 cells. HepG2 cells were infected with DENV-2 at a MOI of 5 for 24 and 48 h. *CTSB*, *CTSS*, *CTSL* and *CTSD* gene expression was determined by real time PCR using specific primers (A). To determine the role of cathepsins in mediating apoptosis, HepG2 cells were pre-treated with the specific inhibitors of CTSB (50 μM), CTSS (5 μM) and CTSL (10 μM) or an equal volume of DMSO vehicle control (0.2%) for 3 h before infection with DENV-2 at a MOI of 5. The cells were cultured in the presence of the inhibitors or DMSO for 48 h post infection before measurement of caspase 3 activity (B) and the percentage of infection (C). The results are expressed as the average of three independentexperiments ± SEM. The asterisks indicate statistically significant differences between groups ($p < 0.05$).

permeabilization and the released cytosolic cathepsin can mediate apoptosis [24–28]. We first asked whether DENV infection induced lysosomal membrane permeabilization. At 24 h post infection, 6% of the cell population exhibited an apoptotic phenotype and this increased to 10% at 36 h and 21% at 48 h (Fig. 1C). Theincrease in apoptotic cells was paralleled by an increase in the number of cells with reduced acridine orange red fluorescence. As show in Fig. 1A and B, reduced lysosomal fluorescence was evident in 3% of cells at 24 h, increasing to 14% and 53% at 36 and 48 h, respectively. The data suggested that DENV-induced apoptosis was accompanied by lysosomal membrane permeabilization.

DENV infection is known to induce ROS accumulation [36] and increased ROS has been shown to mediate lysosome destabilization [35]. Consequently, we next asked whether ROS contributed to lysosomal destabilization during DENV infection. HepG2 cells were infected with DENV in the presence or the absence of 5 mM N-acetyl cysteine (NAC), which confers antioxidant effect and isable to reduce free radicals. As expected, NAC partially reversed the effects of DENV-induced lysosomal destabilization (Fig. 1D) implying that DENV-induced ROS contributes to lysosomal membrane permeabilization. However, NAC reduced permeabilization by only 25%, implying that factors other than ROS make a significant contribution to this effect [37]. In other stimuli, death receptor engagements by their ligands, including TNF-α or TRAIL, result in activation of caspase 8 and lysosomal destabilization in mouse hepatocytes [27,38].

3.2. DENV-induced apoptosis in HepG2 cells is mediated by cathepsin B

Previous gene expression studies from our laboratory and others identified cathepsin B and Sas differentially expressed during DENV infection [13,29,39]. However, a role of cathepsins in DENV-mediated apoptosis has not been investigated. Cathepsin B, D, L and S were selected for further study as they were shown to contribute to apoptosis of hepatocytes [27, 40–42]. HepG2 cells were infected with DENV-2 for 24 h and 48 h and the change in cathepsin mRNAs were evaluated. The average percent of HepG2 cells infected with DENV-2 were 80 and 90, respectively (data not shown). Our results (Fig. 2A) showed that *CTSB*, *CTSL*, *CTSS* but not *CTSD* mRNA expression increased in DENV-infected cells. *CTSS* expressionincreased most dramatically, 5-fold by 24 h post infection and 10-fold by 48 h. *CTSB* and *CTSL* mRNA expression each increased 2-fold by 48 h post infection while *CTSD* was not increased at both time points. We then tested whether cathepsin B, L, and S were involved in DENV-mediated apoptosis. HepG2 cells were pre-treated with cathepsin B, L, and S inhibitors before infection with DENV-2 and incubating for 48 h. The results showed that caspase 3 activity was significantly decreased in DENV-infected cells treated with cathepsin B and S inhibitors but not with cathepsin L inhibitor (Fig. 2B). The decrease was not due to a reduction in DENV infection since similar levels of infection were seen between vehicle- and inhibitor-treated cells (Fig 2C).This data suggest that DENV-induced apoptosis was mediated by cathepsin B and S in HepG2 cells.

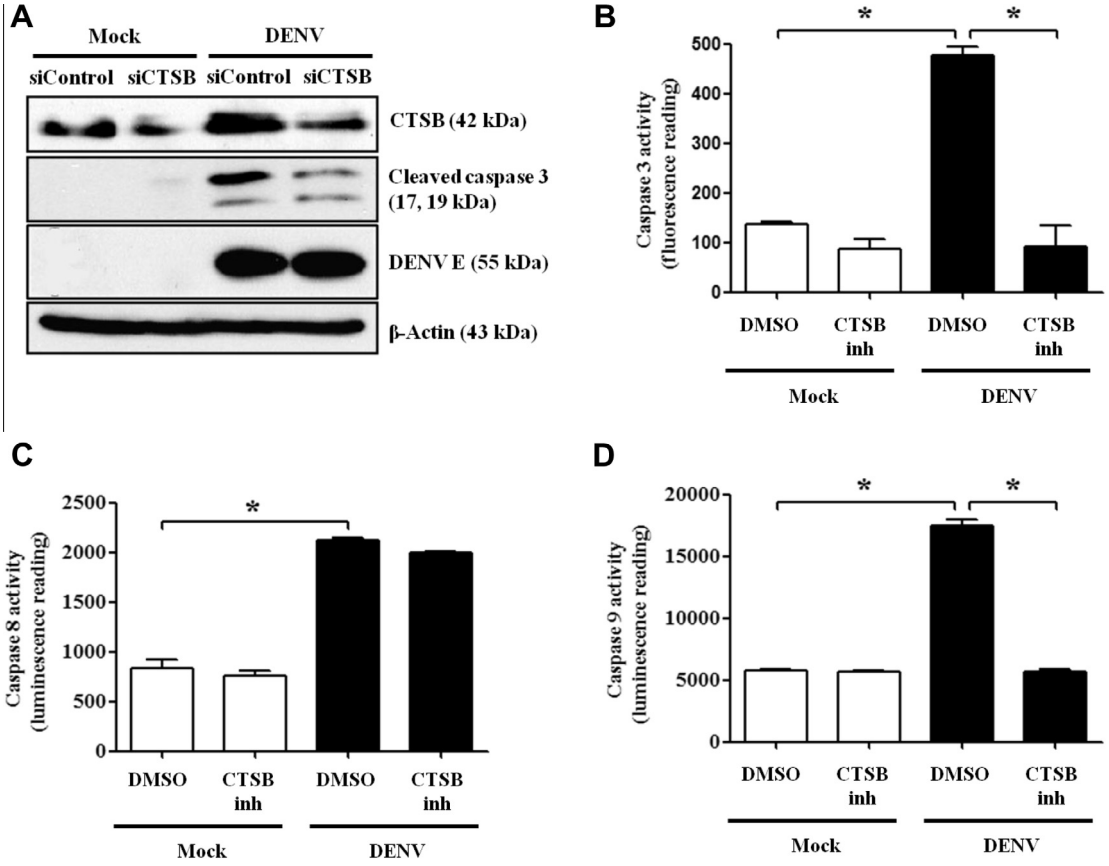

Fig. 3. Cathepsin B mediated caspase 9 and caspase 3 activation in DENV-infected HepG2 cells. HepG2 cells were transfected either with 200 nM of siRNA directed against *CTSB* or siControl Hi GC for 24 h before infection with DENV-2 at a MOI of 5 for 48 h. Cell lysates were subjected to Western blot analysis to determine the knock down efficiency and the effect to apoptosis using CTSB and cleaved caspase 3 antibodies, respectively (A). To determine the apoptosis pathway influenced by cathepsin B, HepG2 cells were pre-treated with the specific inhibitors of CTSB (50 μM) or equal volume of DMSO vehicle control (0.2%) for 3 h before infection with DENV-2 at a MOI of 5. The cells were cultured in the presence of the inhibitors or DMSO for 48 h post infection before measurement of caspase 3 activity (B), caspase 8 activity (C) and caspase 9activity (D). The results are expressed as the average of three independent experiments ± SEM. The asterisks indicate statistically significant differences between groups ($p < 0.05$).

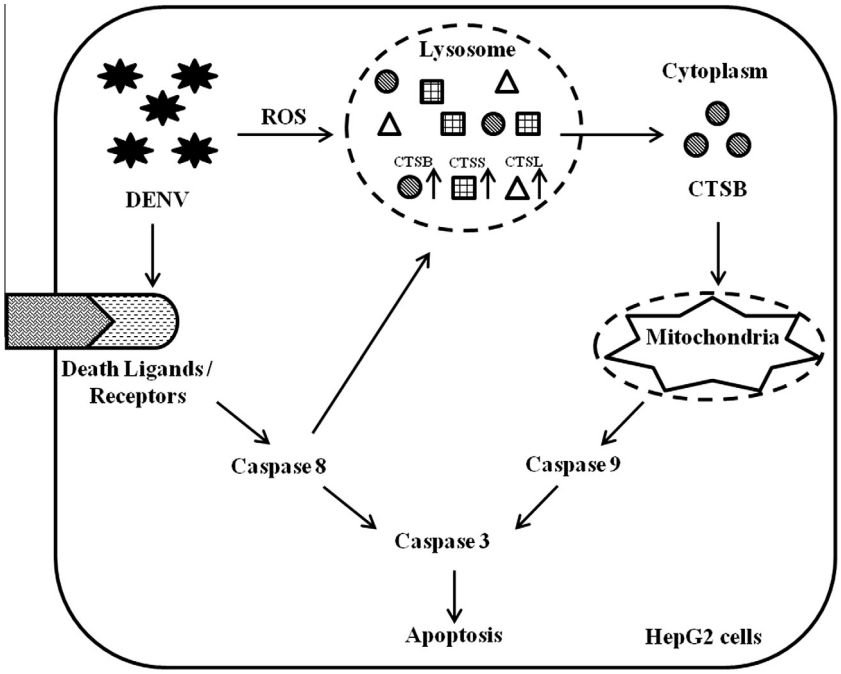

Fig. 4. The proposed model of the lysosomal pathway of apoptosis after DENV infection in HepG2 cells. DENV infection of HepG2 cells induces lysosomal membrane permeabilization, and up-regulation of cathepsin B, which turns on the intrinsic pathway of apoptosis by activation of caspase 9 and caspase 3. Lysosomal membrane permeabilization is partly mediated by ROS and possibly by caspase 8 through ligands/receptors signaling.

Since treatment with cathepsin B inhibitor strongly inhibited activation of caspase 3 activity, we next tested the specificity of this effect by RNAi knockdown of cathepsin B. HepG2 cells were transfected with siRNA directed against *CTSB* before infection with DENV. The efficiency of cathepsin B knockdown by siRNA and the effect on apoptosis were examined by Western blot analysis using primary antibodies against CTSB and cleaved caspase 3, respectively. CTSB protein expression was up-regulated in DENV-infected HepG2 cells (Fig. 3A, siControl, Mock vs. DENV) and siRNA against CTSB prevented this increase (Fig. 3A, DENV, siControl vs. siCTSB). In addition, treatment with siRNA directed against*CTSB* reduced the amount of cleaved caspase 3 in DENV-infected cells (Fig. 3A, DENV, siControl vs. siCTSB) suggesting a role for CTSB in DENV-mediated apoptosis. The result of siRNA directed against *CTSB* in DENV-infected HepG2 cells is in agreement with the result of pharmacological inhibition of CTSB (Fig. 3B). Therefore, genetic inhibition of CTSB expression reproduces pharmacological inhibition of the enzyme and confirms the contribution of CTSB to apoptotic event induced by DENV. Cathepsin B was previously shown to involve in cell death in herpes simplex virus-infected monocytic cells[33]. In addition, cathepsin B secreted from HIV-infected macrophages contributes to neuronal apoptosis [43]. Interestingly, inhibition ofcathepsin B was shown to protect glioma cells from pavovirus-induced cytolysis [44].

3.3. Cathepsin B mediates caspase 9 activation in DENV-infected HepG2 cells

The initiator caspase 8 of the death receptor pathway and the initiator caspase 9 of the mitochondrial pathway activate the effector caspase 3. Activated caspase 3 then cleaves cellular target proteins leading to cell death [45]. We next asked whether caspase 8 or caspase 9 pathways were activated by cathepsin B. HepG2 cells pre-treated with cathepsin B inhibitor were infected with DENV-2 and the activity of caspase 8 and 9 were measured using a luminescence-based assay. In the absence of cathepsin B inhibitor, the activities of both caspase 8 and caspase 9 were drastically increased in DENV-infected cells suggesting the involvement of both extrinsic and intrinsic pathways (Fig. 3C and D). However, treatment with cathepsin B inhibitor significantly prevented the increase of caspase 9, but not caspase 8 (Fig. 3C and D). These results suggested that caspase 8 is not the downstream of cathepsin B and that cathepsin B activation of caspase 3 occurs via caspase 9. The data are in agreement with *in vivo* studies demonstrating that cathepsin B inhibition suppresses LPS/D-GalN-induced caspase-9 and caspase-3 activation and prevents hepatic failure in mice [46]. Furthermore, caspase 8 was previously shown to be the upstream of cathepsin B, which subsequently induced downstream caspase 9 and caspase 3 in TNF-alpha-mediated hepatocyte apoptosis [27].

We propose a lysosomal pathway of apoptosis after DENV infection in HepG2 cells (Fig. 4). DENV induces lysosomal membrane permeabilization partly via ROS thereby releasing cathepsins to induce apoptosis and cathepsin B contributes to DENV-mediated apoptosis in HepG2 cells via caspase-9 and caspase-3 activation. The molecular mechanisms by which cathepsin B mediates apoptosis merit further investigation.

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