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Dengue virus disrupts Daxx and NF-κB interaction to induce CD137-mediated apoptosis

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ABSTRACT

Dengue virus (DENV) is a positive-strand RNA virus of the *Flavivirus* family with 4 different serotypes. Clinical manifestations of DENV infection include dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. Following DENV infection, apoptosis of hepatic cells is observed both *in vitro* and *in vivo*. However, the molecular mechanisms revealing how viral components affect cellular apoptosis remain unclear. In the present study, the role of death domain-associated protein 6 (Daxx) in DENV-mediated apoptosis was characterized by RNA interference and overexpression studies, and the anti-apoptotic function of Daxx during DENV infection was identified. Furthermore, the viral component, DENV capsid protein (DENV C), interacted with Daxx to disrupt interaction between Daxx and NF-κB. The liberated NF-κB activated the promoter of CD137, which is a member of the TNF family, and is previously shown to induce apoptosis during DENV infection. In summary, DENV C disrupts Daxx and NF-κB interaction to induce CD137-mediated apoptosis during DENV infection.

1. Introduction

Dengue virus (DENV), a positive-strand RNA virus of the *Flavivirus* family with 4 different serotypes (DENV-1, -2, -3, and -4), is transmitted to man by the mosquito *Aedes aegypti* [1]. All four serotypes of DENV cause disease with a varied degree of severity. Most of the DENV-infected patients develop dengue fever (DF). However, some patients reach severe forms, which are classified into dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. Hepatic injury concerns one of the most clinical symptoms, which leads to severe diseases. Evidence of hepatic injury is also demonstrated by hepatomegaly and an increase in transaminase levels [3]. Hepatic biopsy specimens, obtained from fatal cases of DSS, show cellular apoptosis, which may be related to the pathogenesis of DHF/DSS [4].

Following DENV infection, apoptosis of hepatic cells was observed both *in vitro* and *in vivo* [5–12]. The transcription factor NF*k*B is activated concomitantly with synthesis of DENV proteins before the appearance of apoptotic cells in HepG2 cells [6]. Viral components, including membrane (DENV M) and capsid (DENV C), contribute to DENV-mediated apoptosis [7,8,13]. The molecular mechanisms confirming how viral components affect cellular apoptosis need further investigation. The interplay between DENV C and human death domain-associated protein Daxx in DENV-mediated apoptosis is reported [13]. The loss of nuclear localization of DENV C disrupts the interaction with Daxx in the nucleus, and decreases apoptosis [13]. However, how Daxx is involved directly in DENV-induced apoptosis is not known. Moreover, the mRNA expression of CD137, which is a member of the TNF receptor super family 9 (TNFRSF 9) and is involved in apoptosis [14]was up-regulated in HepG2 cells expressing DENV C [15]. However, how DENV C mediates CD137-induced apoptosis is not known.

This study characterized the role of Daxx during DENV infection by RNA interference and overexpression studies, and the anti-apoptotic function of Daxx during DENV infection is identified. To demonstrate the molecular mechanism how Daxx mediated an anti-apoptotic function when HepG2 cells were infected with DENV, co-immunoprecipitation studies were performed. The results show that DENV C disrupts interaction between anti-apoptotic Daxx and

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the p65 subunit of NF-κB. The liberated NF-κB then activates the CD137 promoter to induce apoptosis.

2. Materials and methods

2.1. Cell culture and preparation of DENV

Up to 6×10^5 HepG2 cells were seeded in a 6-well plate and cultured for 24 h prior to infection. HepG2 cells were grown 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 at 37 °C in a humidified atmosphere, containing 5% CO_2. DENV serotype 2 strain 16681 was propagated as described previously [15,16].

2.2. Construction of plasmids

To construct a plasmid expressing Daxx, RNA was extracted from HepG2 cells and converted to cDNA. The nested-PCR was performed by using a set of outer primers including NTDaxx_F 5′CCATGCGAGGTTCTGAG3′ and NTDaxx_R 5′CACCAAAAGGGGA TTAG3′ and inner primers including DaxxBam_F 5′CTAGGA TCCGACATGGCCACCGCTAAC3′ and DaxxXba_R 5′CTA TAGACTAAT CAGAGTCTAAG3′; the cDNA was sub-cloned into pcDNA 3.1 His C (Invitrogen), and verified by DNA sequencing. The expression of Daxx was confirmed by Western blot analysis using both anti-Daxx (sc-7152; Santa Cruz Biotechnology) and anti-His antibodies (sc-8036; Santa Cruz Biotechnology).

To construct plasmids, containing the CD137 promoter and its three deleted constructs (Fig. 4A and B), genomic DNA was extracted from the monocyte cell line THP-1. The nested PCR was then performed by using outer primers, including CD137 Promoter_F 5′AGGTACCTGCCATGTTGGACGTC3′ and CD137 Promoter_R 5′TAAG CTTGATGAAATCTGGCACAG3′, and these sets of inner primers to construct CD137 full promoter and the three deleted constructs, including Full CD137_F 5′CTAGGTACCAATCCCTCCTAGCTCTCAG3′; Del1_F 5′CTAGGTACCCCATTATCAGGCAGG3′; Del2_F 5′CTAGGTAC CGAGACCCCGCCCCTG3′; Del3_F 5′TAGGTACCG ACCTGAAGTCCTC3′ and Full CD137_R 5′GTTAAGCTTAGATCTCAGGGCTGCCGG3′, respectively. PCR products were separately sub-cloned into the pGL3 basic vector (Promega), and verified by DNA sequencing.

2.3. RNA interference directed against Daxx during DENV infection, and in HepG2 cells either expressing DENV C or NLS-mutated DENV C (R85 DENV C)

HepG2 cells were transfected with either siRNA against Daxx (SiDaxx; 5′CAGCCAAGCUCUAUGUCUACAUCAA3′) (Invitrogen) or siRNA control (SiC; 5′CACGCCTCTTTGTCTTGTTTCGAAA3′) (Invitrogen). At 24 h post transfection, DENV serotype 2 was infected at MOI of 5. DENV-infected HepG2 cells were harvested 24 h after infection. Both life and dead cells were collected, subjected to an apoptosis assay by annexin V/PI staining (BD Biosciences), and analyzed by flow cytometry. The expression level of Daxx was verified by Western blot analysis using anti-Daxx antibody, and compared with β-actin antibody, which was used as a loading control.

HepG2 cells, stably expressing either DENV C or NLS-mutated DENV C (R85 DENV C) were also constructed according to methods, described previously [11]. The culture supernatant, containing infectious viral particles, was collected after 24 h post transfection and added to the HepG2 cell line, which was pre-incubated with 8 μg/ml of polybrene. At 24 h after incubation, the HepG2 cells stably expressing DENV C or R85 DENV C were selected with media containing 0.5 mg/ml G418 (Calbiochem). The G418-resistant cells were grown and maintained in DMEM medium containing 0.5 mg/ml G418, and the expression of DENV C was examined by flow cytometry and Western blot analysis using an antibody to DENV C (D2-C1) [17]. Up to 6×10^5 of stably HepG2 cells expressing DENV C or R85 DENV C were then placed into a 6-well plate for 24 h prior to transfection. The transfections of siRNA directed against Daxx or siRNA control were performed, and treated with 0.5 μg/ml anti-Fas mAb antibody (Sigma) and 1 μg/ml cycloheximide (Sigma) for 24 h in culture media. Both adherent and floating cells and culture supernatant were collected for an apoptosis assay by annexin V/PI staining, and analyzed by flow cytometry. The expression level of Daxx was verified by Western blot analysis using an anti-Daxx antibody compared with β-actin antibody.

2.4. Overexpression of Daxx during DENV infection, and in HepG2 cells expressing either DENV C or R85 DENV C

Up to 6×10^5 of HepG2 cells were plated into a 6-well plate and transfected with either plasmid, coding for Daxx (His/Daxx) or vector control (His). At 24 h post transfection, DENV serotype 2 was added at MOI of 5. The cells were harvested 24 h after infection. Apoptosis was then measured as described previously. The expression level of Daxx was verified by Western blot analysis using anti-Daxx antibody and compared with β-actin antibody.

Up to 6×10^5 of HepG2 cells, stably expressing DENV C or R85 DENV C, were also placed into a 6-well plate for 24 h prior to transfection. The transfection with either plasmid coding for Daxx (His/Daxx) or vector control (His) was performed, and the cells were treated with 0.5 μg/ml anti-Fas mAb antibody (Sigma) and 1 μg/ml cycloheximide (Sigma) for 24 h in culture medium. Apoptosis was measured as described previously. The expression level of Daxx was verified by Western blot analysis using anti-Daxx antibody compared with β-actin antibody.

2.5. Co-immunoprecipitation

Up to 6×10^5 of HepG2 cells were plated for 24 h, followed by DENV infection at MOI of 1. Cells were harvested, and immunoprecipitation was carried out with either 5 μg of purified mouse antibody to DENV C (D2-C1) or 5 μg of rabbit antibody anti-Daxx antibody (sc-7152). The mixture was then incubated with a soft rotation at 4 °C overnight. The incubation was continued for 4 h after addition of protein G Sepharose beads. The bound proteins were eluted and subjected to Western blot analysis either with an antibody to DENV C (D2-C1), or a rabbit antibody anti-Daxx antibody (sc-7152), or a rabbit anti-p65 polyclonal antibody (sc-372; Santa Cruz Biotechnology), or a rabbit anti-AP1 polyclonal antibody (sc-1694; Santa Cruz Biotechnology). Cell lysates from HepG2 cells expressing DENV C or vector control were also immunoprecipitated either with a purified anti-DENV C antibody or an anti-Daxx antibody. The complexes were detected either with an anti-DENV C antibody, or an anti-Daxx antibody or an anti-NF*k*B antibody (p65) or an anti-AP1 antibody. The membranes were washed and incubated with HRP-conjugated secondary antibody. Immune complexes were detected by enhanced chemiluminescence (Pierce).

2.6. Dual-luciferase® reporter assay

Up to 1.2×10^5 of HepG2 cells were placed in a 24-well plate and transfected with either a plasmid, containing the full CD137 promoter or with a deleted CD137 promoter; Del1, Del2 or Del3 CD137. The luciferase empty vector was used as a negative control, and the vector containing the p65 subunit of NF-κB was used as a