Dengue virus type 2 NS3 protease and NS2B-NS3 protease precursor induce apoptosis

Norazizah Shafee and Sazaly AbuBakar

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia

Apoptosis was detected in Vero cell cultures expressing transfected dengue virus type 2 (DENV-2) genes. Approximately 17.5 and 51.5% of cells expressing NS3 serine protease and NS2B-NS3\textsubscript{185} serine protease precursor protein [NS2B-NS3\textsubscript{185(pro)}] genes, respectively, were apoptotic. The percentage of apoptotic cells was significantly higher in cell cultures expressing NS2B-NS3\textsubscript{185(pro)}. NS2B-NS3\textsubscript{185(pro)} was detected as NS2B-NS3\textsubscript{185(pro)}-EGFP fusion protein in cytoplasmic vesicular structures in the apoptotic cells. Site-directed mutagenesis which replaced His\textsuperscript{51} with Ala within the protease catalytic triad significantly reduced the ability of the expressed NS3 and NS2B-NS3\textsubscript{185(pro)} to induce apoptosis. Results from the present study showed that DENV-2-encoded NS3 serine protease induces apoptosis, which is enhanced in cells expressing its precursor, NS2B-NS3\textsubscript{185(pro)}. These findings suggest the importance of NS2B as a cofactor to NS3 protease-induced apoptosis.

Dengue is a mosquito-borne disease endemic in many Southeast Asian countries. The disease is caused by Dengue virus (DENV), a positive single-stranded RNA virus belonging to the Flaviviridae family. There are four antigenically related but distinct DENV serotypes; DENV-1 to -4, all of which can cause dengue fever and the more severe forms of the disease, dengue haemorrhagic fever and dengue shock syndrome (Henchal & Putnak, 1990). The mechanism leading to the severe manifestation of dengue is still unknown. In recent years, however, a number of studies have shown that infection with DENV induces apoptosis in vitro and also in vivo (Despres et al., 1996; Marianneau et al., 1997; Shafee & AbuBakar, 1997; Couvelard et al., 1999; Lei et al., 2001; Huerre et al., 2001). These studies raised the possibility that apoptosis is important in the pathogenesis of dengue. The mechanisms whereby DENV triggers the apoptotic cellular responses, however, have not been thoroughly investigated, though results from a recent study demonstrated the requirement for live virus for induction of apoptosis to occur (Shafee & AbuBakar, 2002). In various other systems, the involvement of serine proteases in the induction of apoptosis has been reported (Verhagen et al., 2002; Martins et al., 2002; Hegde et al., 2002). Serine proteases such as Omi/HtrA2 and Smac/DIABLO have been shown to antagonize the inhibitors of apoptosis proteins (IAP), and thus to promote apoptosis. Among RNA viruses, it was shown that the NS3 protease of Langat virus (Prikhod’ko et al., 2002), and the 3C protease of Enterovirus 71 (Li et al., 2002) and Poliovirus (Barco et al., 2000), induced apoptosis in infected cells. In DENV infection, however, the potential involvement of DENV-2-encoded serine protease in the induction of apoptosis has not been investigated. In the present study, the effects of DENV-2 NS3 protease and NS2B-NS3\textsubscript{185(pro)} overexpression in transiently transfected Vero cells were investigated and compared with results obtained with other dengue virus genes.

All DENV genes used in the present study were cloned into pEGFP-N1 expression vector (Clontech) as EGFP fusion protein, as previously described (Shafee & AbuBakar, 1999). The NS3 protease gene was cloned as a full-length NS3-EGFP, whereas the NS2B-NS3 construct was recloned from the original pQE-30 into pEGFP-N1 and designated NS2B-NS3\textsubscript{185(pro)}. The construct contained 45 amino acids of the hydrophilic domain of NS2B and 185 amino acids from the amino terminus of NS3 (Yusof et al., 2000; Clum et al., 1997). Site-directed mutagenesis of NS3 and NS2B-NS3\textsubscript{185(pro)} was performed based on the earlier report of Clum et al. (1997). Histidine (His\textsuperscript{51}), found within the catalytic triad of NS3 in both NS3 and NS2B-NS3\textsubscript{185(pro)}, was replaced with Ala, hence inactivating the protein’s proteolytic activities (Falgout et al., 1993; Clum et al., 1997; Yusof et al., 2000). For transfection studies, cells were transfected with purified recombinant pEGFP-N1 using Tfx-20 transfection reagent, following the protocol provided by the manufacturer (Promega). Successful expression of the cloned DENV-2 genes was detected by examining the cell cultures under an inverted UV-microscope (Zeiss Axiovert 25). At 48 h after transfection, cells were fixed, counterstained with propidium iodide, and viewed using a confocal microscope (Leica TCS SP2). The presence of apoptotic cells in the cell cultures was detected using the Dead-End Colorimetric Apoptosis Detection System.
following the protocol described by the manufacturer (Promega). The percentages of apoptotic cells and cells expressing EGFP were determined as previously detailed (Shafee & AbuBakar, 2002).

Fluorescence and confocal microscopic analyses of the transfected cells showed that cells expressing prM-EGFP, truncated E[E<sub>34-253</sub>]-EGFP, NB-E-EGFP and E<sub>281-423</sub>-EGFP, B-E-EGFP], NS1-EGFP, NS2A-EGFP (data not shown), NS3-EGFP, and cells expressing mNS3-EGFP and mNS2B-NS3<sub>185(pro)</sub>-EGFP] had similar morphology to cells expressing EGFP alone (Fig. 1). In general, the transfected cells showed diffuse fluorescence with some speckling in the cytoplasm; but intense fluorescing clusters around the nuclear periphery were noted in some cells expressing NS3-EGFP (Fig. 1). Cells expressing NS2B-NS3<sub>185(pro)</sub>-EGFP, on the other hand, had highly vacuolated cytoplasm with NS2B-NS3<sub>185(pro)</sub>-EGFP noted within the vacuoles (Fig. 1, thin arrow, arrowhead). Fluorescent membrane-bound vesicles were also noted in the culture medium of cells expressing NS2B-NS3<sub>185(pro)</sub>-EGFP (Fig. 1, asterisk, thick arrow). Similar vesicular structures, however, were not noted in cells expressing mNS2B-NS3<sub>185(pro)</sub>-EGFP. Immunoblot analysis of the culture medium of NS2B-NS3<sub>185(pro)</sub>-EGFP-expressing cells sedimented at 20 000 g was performed using polyclonal antibodies raised against NS2B-NS3<sub>185(pro)</sub>-EGFP. A polypeptide band of ~65 kDa was detected (Fig. 2a, arrow). The size of this polypeptide band corresponded to the theoretical mass of NS2B-NS3<sub>185(pro)</sub>-EGFP. In addition, a protein band of ~55 kDa (Fig. 2a, asterisk) which corresponded to the expected size of NS3<sub>185(pro)</sub>-EGFP, was also detected, which suggested that the expressed NS2B-NS3<sub>185(pro)</sub>-EGFP was cleaved in vivo to release NS2B. The origin of a thin band below the suspected NS3<sub>185(pro)</sub>-EGFP band is unknown.

At 48h post-transfection, apoptotic cells were detected by colorimetric apoptotic staining in cell cultures expressing the various transfected genes (data not shown). Only 4.2% (±2) of cells expressing the EGFP control plasmid were apoptotic (Fig. 2b). In contrast, cells expressing prM-EGFP, NB-E-EGFP, B-E-EGFP, NS1-EGFP and NS2A-EGFP showed 12.4% (±3), 10.2% (±2), 11.3% (±4), 11.2% (±4) and 7.4% (±3) apoptotic cells, respectively (Fig. 2b). Statistical analyses (ANOVA and Student’s t-test) performed using these mean values suggested that all the DENV-2-EGFP-expressing cells showed means significantly higher (P<0.001) than those of EGFP-expressing cells. However, no significant differences in the means of apoptotic cells between the different DENV-2-EGFP-expressing cells were noted. Cells expressing NS3-EGFP or NS2B-NS3<sub>185(pro)</sub>-EGFP, on the other hand, showed 17.5% (±6) and 51.5% (±13) apoptotic cells, respectively. A significant difference (P<0.001) in the means of apoptotic cells for cells expressing the two genes were obtained when these means were compared to the average mean (10.5%) of all the other DENV-2-EGFP-expressing cells. This suggests that both the NS3-EGFP- and NS2B-NS3<sub>185(pro)</sub>-EGFP-expressing cells had a higher number of apoptotic cells in comparison to cells expressing all other DENV-2 genes. Cells expressing mNS3-EGFP and mNS2B-NS3<sub>185(pro)</sub>-EGFP, however, showed the presence of 8.0% (±6) and 10.0% (±4) apoptotic cells, respectively. These means were significantly lower (P<0.001) from those of cells expressing NS3-EGFP or NS2B-NS3<sub>185(pro)</sub>-EGFP, suggesting that mutation of the protease catalytic site reduced the capacity of the proteins to cause apoptosis. The significant difference (P<0.001) in the means of apoptotic cells observed between the NS3-EGFP- and NS2B-NS3<sub>185(pro)</sub>-EGFP-expressing cells further suggests the potential importance of NS2B as a co-factor for NS3-induced apoptosis. In addition, it is unlikely that DENV-2 NS3 helicase activity is involved in the induction of apoptosis, as the NS2B-NS3<sub>185(pro)</sub> used in the present study lacked the NS3 carboxy end, which contains the viral RNA helicase.

In the present study, it was also noted that the percentage apoptosis for the NS2B-NS3<sub>185(pro)</sub>-EGFP-expressing cells was directly correlated (r=0.72, P<0.001, Pearson) with the number of cells showing the presence of the cytoplasmic vesicular structures. These vesicular structures were absent in cells expressing mNS2B-NS3<sub>185(pro)</sub>-EGFP (Fig. 1), implying that an active protease catalytic site of NS2B-NS3<sub>185(pro)</sub>-EGFP somehow contributed towards the formation of the vesicular structures. Since spontaneous cytoplasmic vacuolation by itself has never been reported to induce apoptosis, it is likely then the DENV-2 NS3 protease activity is responsible for it. It is proposed here that NS2B interacts directly with NS3 within the vesicular complexes to enhance the NS3 proteolytic activity in a manner similar to that previously reported in other systems (Brinkworth et al., 1999; Chambers et al., 1993; Arias et al., 1993; Jan et al., 1995). Furthermore, since the vesicular complexes were also absent in cells expressing NS3-EGFP, it raised the possibility that the interaction between NS3 and its cofactor NS2B directly contributed towards the formation of the vesicular structures and accumulation of NS2B-NS3<sub>185(pro)</sub>-EGFP within these structures leads to the induction of apoptosis.

The importance of DENV NS3 protease in the induction of apoptosis is in line with results for a number of other virus-encoded proteases (Prikhod’ko et al., 2002; Li et al., 2002; Goldstaud et al., 2000; Barco et al., 2000). In Langat virus infection, apoptosis was suggested to be mediated by direct binding of the viral NS3 to caspase-8 (Prikhod’ko et al., 2002). Caspase-mediated pathways have also been suggested for the 3C protease-induced apoptosis of Entrovirus 71 and Poliovirus (Li et al., 2002; Barco et al., 2000). Whether the caspase pathways are also activated by DENV-2 infection is not presently known. However, based on our findings, it is suggested that in DENV-2 infection accumulation of the intracellular NS3 resulted in cleavage of the apoptosis initiator molecules that in turn triggers activation of the apoptotic pathways. This is possible since
DENV NS3 has been shown to act proteolytically in cis and also in trans on a number of potential substrates (Kolykhalov et al., 1994; Bartenschlager et al., 1995; Yusof et al., 2000) and, similar to the findings presented here, NS2B also appeared to function as a cofactor to the DENV-2 NS3 protease activity. Furthermore, alignment of the DENV-2 NS3 amino acid sequence with a number of known serine proteases antagonistic to the inhibitor-of-apoptosis
proteins (IAPs) revealed the presence of a motif resembling the IAP-binding motif within the N terminus of NS3 (Fig. 2c). The first Ala residue of the conserved IAP antagonist N-terminal sequence, however, is substituted with Glu in NS3. Whether this substitution affects the function of DENV-2 NS3 as a bona fide IAP binding protein, requires further investigation. Nonetheless, it is envisaged that DENV-2 NS3 may antagonize the inhibitory function of the IAPs, leading to increased activation of the initiator caspases such as caspase-9 or the downstream effector caspases such as caspase-3 and -7, similar to that mediated by serine proteases Smac/DIABLO or Omi/HtrA2 (Du et al., 2000; Verhagen et al., 2000; Srinivasula et al., 2000, 2001; Verhagen et al., 2002).

The induction of apoptosis in cells expressing other DENV-2 genes was probably mediated by a non-specific mechanism; as the percentages of apoptotic cells observed in these cells were similar. A much lower percentage of apoptosis, approximately half that caused by nonspecific DENV-2 gene expression, however, was obtained in cells expressing EGFP alone. This suggests that the percentage of apoptosis noted in cells expressing DENV-2 proteins other than NS3 or NS2B-NS3185(pro) was probably due to the additive effects of DENV-2 proteins and EGFP accumulation. Intracellular accumulation of the DENV-2 proteins including the mutant forms of NS3 and NS2B-NS3185(pro) triggered apoptosis, perhaps by inducing the endoplasmic reticulum stress-induced apoptotic pathways as previously suggested (Despres et al., 1996).

In summary, results obtained from the present study showed that DENV-2 serine protease NS3 and its precursor protein NS2B-NS3185(pro) induced apoptosis in transiently transfected Vero cells. A significantly higher percentage of apoptosis was observed in cells expressing NS2B-NS3185(pro), suggesting the importance of NS2B as a cofactor for NS3-induced apoptosis. We have also shown that mutation of the NS3 proteolytic site reduced the capacity of NS3 to induce apoptosis. These findings support the importance of NS3 protease in DENV-2-induced apoptosis.

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![Image](attachment://fig2.png)

**Fig. 2.** Expression of DENV-2 protease-EGFP induces apoptosis in Vero cells. (a) Immunoblot analysis of protein sample obtained from the extracellular membrane-bound vesicles in culture medium of cells expressing NS2B-NS3185(pro)-EGFP (lane 1). A sample from culture medium of cells expressing only the EGFP was included as control (lane 2). The immunoblot was probed using polyclonal antibodies against NS2B-NS3185(pro)-EGFP and detected with alkaline phosphatase-conjugated anti-mouse IgG. The presence of NS2B-NS3185(pro)-EGFP is indicated by the detection of the ~65 kDa protein band (arrow). NS2B-NS3185(pro)-EGFP is cleaved to release NS3185(pro)-EGFP (*) and NS2B (not detectable). M, molecular marker. (b) Percentages of apoptotic cells in Vero cell cultures expressing the various DENV-2-EGFP recombinant proteins. Cells expressing NS3-EGFP or NS2B-NS3185(pro)-EGFP showed a significant increase in the percentage of apoptotic cells above those cells expressing other DENV-2 proteins, with cells expressing NS2B-NS3185(pro)-EGFP showing a significantly higher percentage of apoptotic cells than those transfected with NS3-EGFP. Control, cell cultures transfected with pEGFP-N1 plasmid alone. Results are presented as means ± SD of the percentages obtained from at least 10 randomly picked microscope fields. (c) Alignment of the N-terminal sequence of DENV-2 NS3 against selected IAP antagonist serine proteases. The IAP antagonists are mammalian HtrA2 (hu, human; mu, mouse), Smac/DIABLO and *Drosophila* Hid, Grim and Reaper, obtained from GenBank. Dark shading indicates residues identical with NS3 and light shading indicates similar residues.
REFERENCES


