

West Nile virus-induced bax-dependent apoptosis

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Abstract The mechanism of cell death induced by West Nile virus (WNV), a causative agent of human febrile syndrome and encephalitis, was investigated. WNV-infected K562 and Neuro-2a cells manifested the typical features of apoptosis, including cell shrinkage, chromatin condensation and subdiploid DNA content by flow cytometry. DNA fragmentation into nucleosomal size and changes in outer cell membrane phospholipid composition were also observed in K562 cells. UV-inactivated virus failed to induce the above-mentioned characteristics, suggesting that viral replication may be required for the induction of apoptosis by WNV. Additionally, signals involved in WNV-induced apoptosis are associated with the up-regulation of bax gene expression. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Flavivirus; West Nile virus; Apoptosis; Cell death; bcl-2; Bax

1. Introduction

West Nile virus (WNV), an arthropod-borne, positive-stranded and enveloped RNA virus, is a member of the genus flavivirus of the family Flaviviridae. It belongs to the Japanese encephalitis antigenic complex which includes Kunjin, Murray Valley encephalitis, Saint Louis encephalitis and Japanese encephalitis viruses [1]. Birds are natural hosts for WNV and serve as a reservoir from which vector mosquitoes may infect humans and other mammals [1–10]. A possible airborne route of infection has also been proposed for many of the laboratory infections occurring in persons working with arboviruses [11]. The *Culex* species appear to be the primary vector for WNV [4,7,8] although recently this virus was also detected in some *Aedes* and one *Anopheles* species [12]. The virus is prevalent in Africa and the Middle East region and is the causa-

tive agent of the disease syndrome known as West Nile fever. Encephalitis is a common complication of West Nile fever, resulting in fatal neurologic disease [1–3,8,13–15]. Recently, an outbreak of WNV occurred in the northeastern USA focused in the New York city area. The outbreak resulted in cases of fatal encephalitis in humans, birds and equines [4–8,10,12,15,16].

WNV can multiply in the cytoplasm of several cultured cells showing a cytopathic effect [1,17,18]. Apoptosis is an active and highly conserved process of cellular self-destruction with distinctive morphological and biochemical features. It is important for normal development, host defense and the suppression of oncogenesis [19–21]. Apoptosis is characterized by a reduction in cell volume, membrane blebbing, chromatin condensation and endonuclease cleavage of DNA into oligonucleosomal length fragments. Another feature of apoptosis is the change in the lipid composition of the outer cell membrane, which appears to be important in eliciting a phagocytic response [20–22]. A number of stimuli can provoke programmed cell death including viral gene products [20,21,23–25], and there is a large amount of evidence indicating that apoptosis is an efficient mechanism by which viruses can cause death of host cells [26–29]. Conversely, it has been proposed that apoptosis may support viral progeny dissemination while limiting induction of inflammatory and immune responses [25].

The pathways for the induction of apoptosis are very complex and still unclear in many aspects. The bcl-2 gene family plays an important role in both positive and negative regulation of programmed cell death [20,30–32]. Bcl-2 was the first gene shown to be involved in the regulation of apoptosis [33], it has proved to be unique among proto-oncogenes in blocking programmed cell death rather than promoting cell proliferation [34]. The human bax gene encodes a protein that binds the Bcl-2 protein and acts in opposition to it [35]. In vivo, bax is expressed more widely than bcl-2 in cell types that show a high rate of apoptosis [36]. The ratio of bcl-2/bax determines the death or survival response to an apoptotic stimulus [34]. The bcl-x gene was identified due to its similarity to the bcl-2 gene. Bcl-x_L, the larger protein product of this gene, renders cells resistant to apoptosis upon growth factor deprivation, while Bcl-x_s, a shorter alternatively spliced protein, can inhibit the ability of Bcl-2 to prevent apoptotic cell death [30].

In the present study, we investigated whether apoptosis contributes to the death of cultured cells infected by WNV. We demonstrated several characteristics of apoptosis in WNV-infected cells and showed that viral replication may be required for the induction of apoptosis. We also provide

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Abbreviations: WNV, West Nile virus; E-MEM, Eagle's minimum essential medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; pi, post-infection; m.o.i., multiplicity of infection; PS, phosphatidylserine; TAE, Tris-acetate-EDTA; PI, propidium iodide; RT-PCR, reverse transcription-polymerase chain reaction; E, mean square error

results suggesting that bax gene expression is involved in WNV-induced cell death. These findings are important because to our knowledge this is the first report suggesting that WNV induces cell death by apoptosis.

2. Materials and methods

2.1. Cell culture and viruses

WNV strain Eg101 was propagated in the C6/36 *Aedes albopictus* cell line [37] at 28°C in Eagle's minimum essential medium (E-MEM) supplemented with 2% fetal calf serum (FCS) and non-essential amino acids (0.1–0.2 mM). Virus titration was done by focus formation assay using the BHK-21 cell line. Briefly, cells grown in a monolayer were infected with the appropriate dilution of virus stock and overlaid with 0.5% methyl cellulose in maintenance medium (2×E-MEM). After incubation for 72 h, cells were fixed with 5% formaldehyde, permeabilized with 1% Nonidet-P40 in phosphate-buffered saline (PBS), and foci of virus-infected cells were visualized by immunoperoxidase staining.

Inactivated WNV was produced by heating at 56°C for 40 min or by UV exposure (254 nm) for 20 min at a distance of 5 cm as previously described [38]. The loss of infectivity was confirmed by the loss of infectivity to C6/36 cells as well as by attempts to rescue infectious virus.

The K562 cell line (ATCC number CCL-243) [39], a human mononuclear cell line derived from a patient with chronic myeloid leukemia, supports flavivirus growth very well [40]. These cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% FCS at 37°C in a 5% CO₂ atmosphere. The Neuro 2a (N2a) cell line (ATCC number CCL-131) is a mouse neuroblastoma cell line that also supports flavivirus growth [41]. This cell line was grown under the same conditions as K562 but using E-MEM medium.

For virus infection, 5×10^5 or 1×10^6 cells were adsorbed with virus, at a multiplicity of infection (m.o.i.) of 1 for 2 h. Then, non-adsorbed virus was removed and RPMI 1640 or E-MEM medium containing 2% FCS was added. The cells were then incubated at 37°C for various time periods.

2.2. Cell viability assay

Cell viability was determined by the trypan blue exclusion test. Cells were harvested at different intervals post-infection (pi), resuspended in PBS and mixed with an equal volume of 0.1% trypan blue. The cells that excluded the dye were counted under a microscope using a hemocytometer chamber.

2.3. Viral antigen detection

Virus- and mock-infected cells were harvested at 24, 48 or 72 hpi, fixed with 2% formaldehyde in PBS and permeabilized with IC-Perm[®] (cell permeabilization buffer, Biosource International, Tokyo, Japan). Viral antigen was detected by flow cytometry using a FITC-labeled anti-flavivirus group-specific monoclonal antibody (FITC-6b6c-1) [42]. The green fluorescence of individual cells was measured using a FacScan flow cytometer (Becton Dickinson). The analysis of green fluorescence-positive cells was performed with 10 000 events from each sample.

2.4. Nuclear Hoechst staining

Chromatin condensation, a characteristic of cells undergoing apoptosis [20,21], was visualized by Hoechst staining [43]. The Hoechst dye is membrane-permeant and thus labels the nuclei of both living and dead cells. WNV- and mock-infected cells were harvested at 24 or 72 hpi. The cells were washed once with PBS and treated with 10 µg/ml Hoechst 33258 (Sigma) in PBS at 37°C for 5 min. The cells were then washed with PBS and examined under a fluorescence microscope (Zeiss, Axiovert 135M).

2.5. Annexin V staining

The redistribution of phosphatidylserine (PS) from the inner to the outer layer of the cell membrane of apoptotic cells can be detected using the phospholipid binding protein annexin V [44]. In this study, the Annexin V-cy 3 Apoptosis Detection Kit (Medical and Biological Laboratories Co., Nagoya City, Japan) was used and the proportion of positive cells was counted using a fluorescence microscope (Zeiss, Axiovert 135M).

2.6. DNA fragmentation by ladder analysis

Fragmentation of cellular DNA into characteristic apoptotic ladders was assessed using the method previously published by Park and Patek [45].

2.7. Propidium iodide (PI) staining

The staining of the isolated nuclei was done as previously published [46]. Virus-inoculated cells were harvested at 24, 48 or 72 hpi. The PI fluorescence of individual nuclei was measured using a FacScan flow cytometer (Becton Dickinson) and 10 000 events were collected for each sample. A reduced cellular DNA content due to fractioning of the apoptotic cells and formation of apoptotic bodies is a characteristic feature of cells undergoing apoptosis [21]. This effect leads to the occurrence of a sub-G1 cell population containing cells with lower DNA content compared to cells in the G1 phase of the cell cycle.

2.8. RNA extraction and cDNA synthesis

Total RNA preparations from 3×10^5 or 6×10^5 uninfected cells and cells infected with WNV were obtained by the guanidine isothiocyanate method using TRIzol (Gibco BRL) [47] according to the manufacturer's instructions. Possible contamination with genomic DNA was eliminated by treating the extract of RNA with DNase I enzyme. To assess mRNA expression, a reverse transcription-polymerase chain reaction (RT-PCR) method was used. For the RT reaction, total RNA was primed with hexanucleotide random primers (TaKaRa) and reverse-transcribed with SuperScript[®] II reverse transcriptase (Gibco BRL) in a 20 µl reaction.

2.9. Real-time PCR quantification

2.9.1. Amplification. The newly synthesized cDNA was subjected to amplification using the Light cycler-DNA Master SYBR Green I Kit (Roche Molecular Biochemicals) for real-time PCR analysis, following the manufacturer's instructions. SYBR Green I is a DNA double-strand-specific dye. As DNA is synthesized, SYBR Green I binds to the amplified PCR product and the increase in amplicon concentration can be quantified by its fluorescence. Amplification was performed in glass capillaries in a 20 µl reaction mixture containing 1×SYBR Green I Master Mix, 0.5 pmol of forward and reverse primers (5'-CGGGCATTTCAGTGACCTGAC-3' and 5'-TCAGGAACGACGGTTGAAG-3' [48] for the human bcl-x gene; 5'-CTGACGGCAACTCAACTGG-3' and 5'-TCTTGGATCCAGCCCAACAG-3' [49] for the bax gene; 5'-GCTGCACCTGACGCCCTTCA-3' and 5'-CTTCAGAGACAGCCAGGAGA-3' for the bcl-2 gene; 5'-CATGAAGTGTGACGTGGACATCC-3' and 5'-GCTGATCCACATCTGCTGGAAGG-3' for the β-actin gene), 4 mM of MgCl₂, and target cDNA (2 µl for the detection of the apoptosis-regulating genes and 1 µl for the detection of the β-actin gene). The β-actin gene was used as an internal control for the expression of the three former genes. The generated PCR products were 340, 160, 301 and 229 bp long for the bcl-x_L, bax, bcl-2 and β-actin genes, respectively. Conditions for cycling were 95°C for 30 s, followed by 40 cycles of 0 s at 95°C (short denaturation), 5 s at 58°C and 16 s at 72°C. Amplification and detection were performed with the Light Cycler instrument (Roche Diagnostics). A negative control was included in each run and specificity of amplification reactions was checked by melting curve analysis [50].

2.9.2. Data analysis. PCR data were obtained by considering the log-linear portion of the fluorescence curve. Analysis of raw data was done with the Light Cycler software version 3.0 (Roche Diagnostics). A standard curve for each PCR product was calculated automatically by plotting the threshold cycle values against each standard of known concentration in picogram quantities and calculation of the linear regression line of this curve. All standard curves had a correlation coefficient of 1 and mean square error (*E*) < 0.2. The experimentally determined standard curve was, in turn, used to estimate the concentration of each sample.

3. Results

3.1. Infection of K562 and N2a cells by WNV

WNV-infected cells express viral antigens in their cytoplasm [1]. FITC-labeled anti-flavivirus group-specific monoclonal antibody was used to detect viral protein, and the number of infected cells was quantitated by flow cytometry.

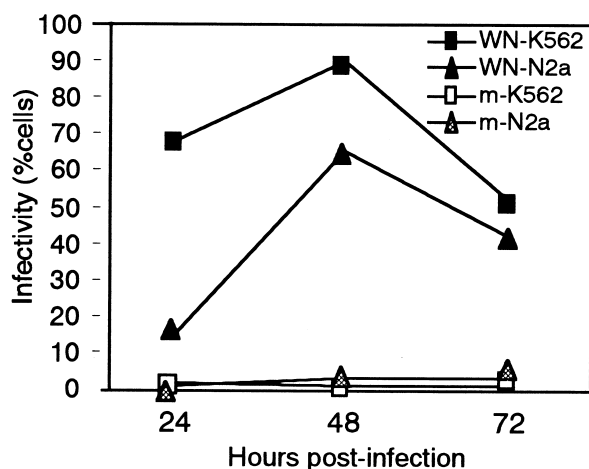


Fig. 1. Kinetics of WNV infection in K562 and N2a cells. WNV-infected K562 and N2a cells were harvested at different times pi. The fixed and permeabilized cells were treated with FITC-labeled anti-flavivirus group-specific monoclonal antibodies [41] and analyzed by flow cytometry. A data set representative of three experiments is shown. WN and m indicate WNV- and mock-infected cells, respectively.

About 68% of the K562 cells were infected at 24 h after inoculation at a m.o.i. of 1, and almost all cells were infected after 48 h of inoculation (90%) (Fig. 1). Fifteen percent of the N2a cells were infected with WNV at 24 hpi and the peak (65%) was observed at 48 hpi (Fig. 1). No fluorescence was detected for either cell line at any of the times pi when inoculated with heat or UV-inactivated WNV. Similarly, controls with FITC-labeled normal IgG2a antibody yielded negative results with both WNV-infected and uninfected cells (data not shown).

3.2. Cell viability

At 24 hpi, K562 cells infected with WNV started to exhibit some cytopathic changes such as membrane blebbing and cell shrinkage. Such changes were much increased at 72 hpi (Fig. 2A). N2a cells exhibited similar cytopathic changes, rounding of the cells, detachment from the monolayer and cell shrinkage.

The trypan blue exclusion test revealed that the decrease in viable cells was time-dependent. Ninety-three percent, 79% and 24% of K562 cells remained viable after infection with WNV for 24, 48 and 72 hpi, respectively, whereas less than 4% of mock-infected cells were permeable to the dye at 72 hpi (Fig. 2B). WNV-infected N2a cells showed similar results with the percentage of viable cells decreasing from 94% to 48% at 24 and 72 hpi, respectively. However, for mock-infected cells a much lower degree of cell mortality was observed (less than 4% throughout the experiment).

3.3. Apoptosis of WNV-infected cells

In searching for the characteristic features of apoptotic cell death three methodologies were used in this study. Mock- and virus-infected K562 cells were harvested at different times pi. In the first method, cellular DNA was isolated, run on 2% agarose gels and tested for the presence of characteristic DNA fragmentation. DNA laddering with increments of 180–200 bp in size was barely noticeable at 24 hpi but became evident at 72 hpi (Fig. 3). No such DNA ladders were observed for

mock-infected cells. To support this finding, nuclear staining was carried out to check for cellular damage using the Hoechst staining method. At 24 hpi almost 13% of the nuclei of the WNV-inoculated K562 cells were irregularly shaped,

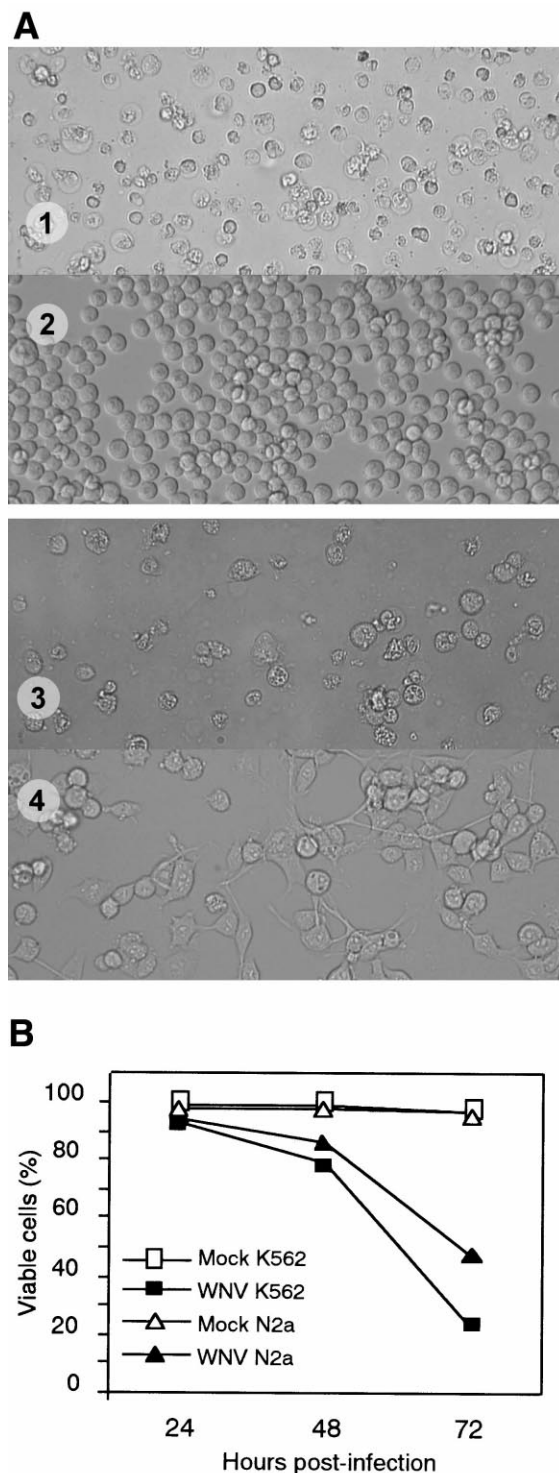


Fig. 2. Cell death by WNV infection. K562 and N2a cells were infected by WNV (m.o.i. = 1). A: Photographs of WNV-infected K562 (1) and N2a (3) cells, and mock-infected K562 (2) and N2a (4) cells at 72 h after infection (18 \times magnification). WNV-infected cells exhibited cytopathic changes such as rounding of the cells, detachment from the monolayer and cell shrinkage. B: The number of viable cells was counted by the trypan blue exclusion test at the indicated times pi. A data set representative of three experiments is shown.

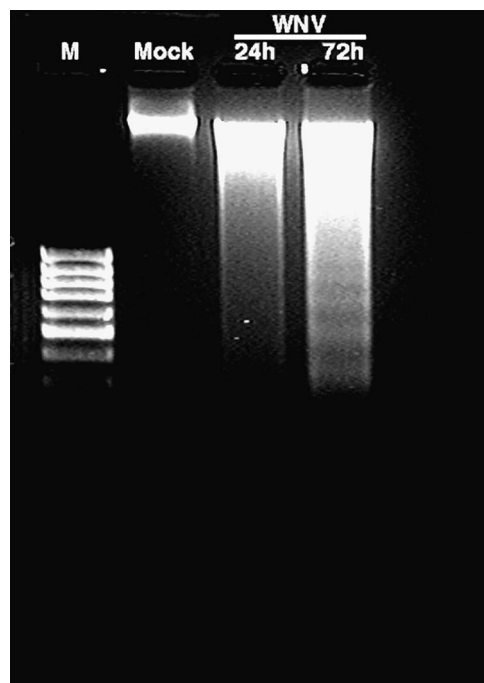


Fig. 3. Oligonucleosomal DNA fragmentation induced by WNV infection. DNA was extracted from mock-infected and WNV-infected K562 cells (m.o.i. = 1) at 24 and 72 hpi using the method proposed by Park and Patek [44]. The samples were analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Lane M contains a 100-bp DNA ladder used as a marker. Internucleosomal DNA cleavage in infected cells was barely noticeable at 24 hpi but became evident at 72 hpi. No such DNA ladders were seen in mock-infected cells.

and the percentage rose markedly to 87% by 72 hpi (Fig. 4). Internally segmented nuclei were evident in some cells, as a typical feature of cells undergoing apoptosis on viral infection. On the other hand, under the fluorescence microscope the vast majority of mock-infected cells showed normal round nuclei with evenly distributed chromatin even at 72 hpi. Only a little over 2% of these cells showed irregularly shaped nuclei.

The relocation of PS to the outer layer of the cell membrane was detected by the use of annexin V [22,44] (Fig. 5). The

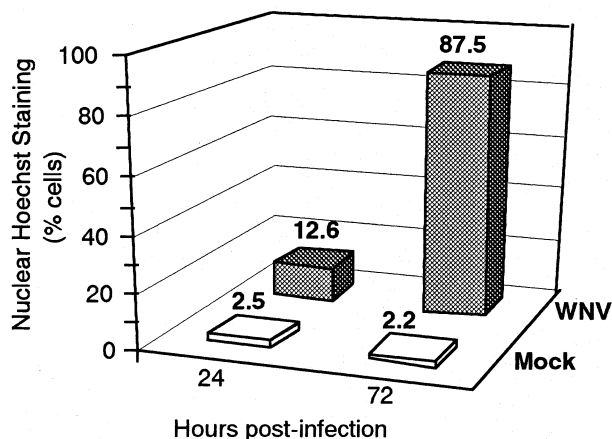


Fig. 4. Nuclear staining of apoptotic cells. K562 cells were infected by WNV (m.o.i. = 1) and harvested at different times pi and treated with Hoechst 33258 in PBS. A data set representative of two experiments is shown.

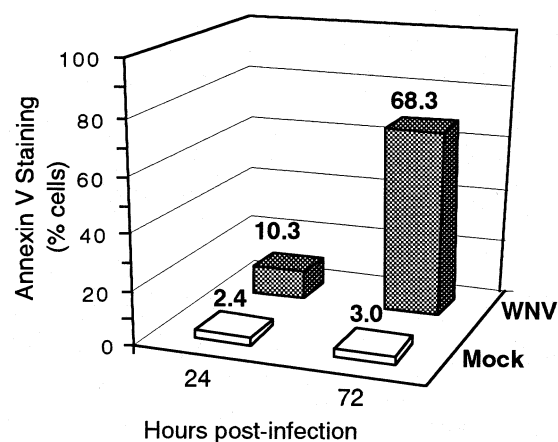


Fig. 5. Visualization of the change in the lipid composition of the outer cell membrane of apoptotic cells. K562 cells were infected by WNV (m.o.i. = 1), harvested at different times after infection and treated with Annexin V-cy 3. A data set representative of two experiments is shown.

number of annexin V-positive cells was significantly elevated in infected cells. The values at 24 and 72 hpi were 10% and 68% for WNV-infected cells, and 2% and 3% for mock-infected cells, respectively.

To quantify apoptotic cells, K562 and N2a cells were inoculated with either WNV or inactivated WNV or were mock-infected. The cells were then analyzed by flow cytometry using DNA staining with PI [46] (Fig. 6a). The proportion of cells showing subdiploid DNA content increased with time after infection. WNV-inoculated K562 cells manifested a significant reduction in the percentage of nuclei with diploid DNA content, while the hypodiploid DNA peak was greatly enhanced from 4.8% at 24 hpi to 52% at 72 hpi (Fig. 6b). Conversely, the proportion of mock-infected cells with subgenomic DNA content was 3% at 72 hpi. Likewise, infected N2a cells showed an increase in the percentage of cells with subdiploid DNA content with the progression of time. At 48 and 72 hpi, 7% and 37% of cells were apoptotic, respectively. On the other hand, only a very small proportion of mock-infected N2a cells showed signs of apoptosis even at 72 hpi (Fig. 6c). Cells infected with heat-inactivated WNV, used as a second negative control, did not show a significant level of apoptosis (less than 5% at 72 hpi, data not shown).

In order to determine whether WNV-induced apoptosis is triggered by the attachment of virions to the cell membrane or by signals originating from within the infected cells, cultured K562 and N2a cells were inoculated with UV-inactivated WNV. The inoculated cells were not stained when specific antiserum was used in immunofluorescence and no infectious focus was detected by focus-forming assay (data not shown). Accordingly, UV-inactivated virus failed to induce apoptosis in inoculated cells judging by the low percentage of cells with subdiploid DNA content at 72 hpi (Fig. 6b,c).

3.4. WNV-induced apoptosis is associated with upregulation of bax mRNA

To investigate the mechanism by which WNV induced apoptosis in K562 and N2a cells, the levels of expression of bcl-2 family members were determined. For N2a cells, bcl-2 and bax gene expression was measured. Otherwise, for K562 cells, the expression of bcl-x_L and bax mRNA was measured in-

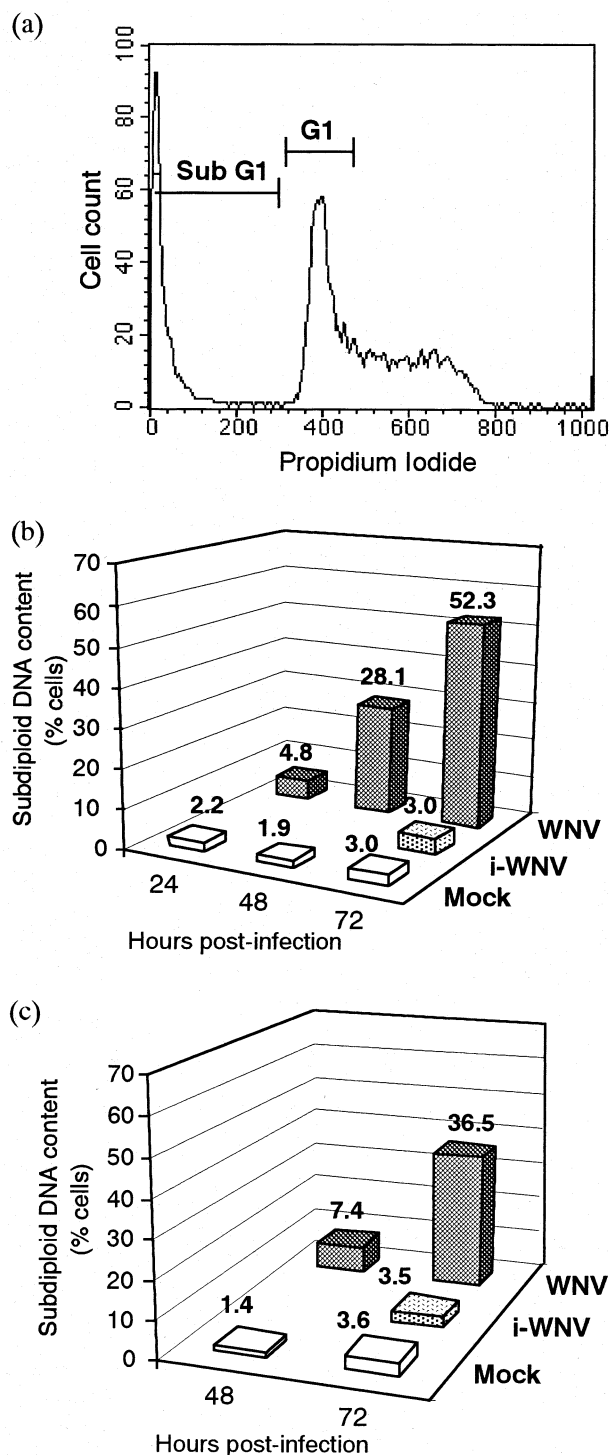


Fig. 6. Subdiploid DNA content determination for the detection of apoptotic cells. Cells were infected with WNV (m.o.i.=1) and i-WNV (UV-inactivated WNV), while the control cells were mock-infected. They were harvested at various intervals pi. Cell pellets were resuspended in 1 ml of hypotonic fluorochrome solution as previously described [45]. The PI fluorescence of individual nuclei was measured by flow cytometry. Ten thousand events were collected from each sample. a: Illustration of a typical histogram plot. Cells with lower DNA content (sub-G1) appear on the left side of cells with diploid DNA content, showing a lower PI intake. Data sets for K562 (b) and N2a (c) cells representatives of four and three experiments, respectively, are shown.

stead. This was because K562 cells do not express the *bcl-2* gene [48]. Expression of apoptosis-related genes was tested in lysates of mock-infected and WNV-infected cells harvested at 0 and 6 hpi, using a quantitative RT-PCR (Fig. 7a,b). For K562 cells, the expression of *bcl-x_L* mRNA did not change in WNV-infected cells (7.1×10^{-4} pg), however *bax* mRNA levels were markedly elevated (from 8.9×10^{-5} pg to 1×10^{-3} pg) at 6 h after inoculation. In contrast, the expression of *bcl-x_L* ($4-5 \times 10^{-4}$ pg) and *bax* (3.5×10^{-4} pg) mRNA was not affected in mock-infected cells. The ratios *bcl-x_L*/*bax* were 7.98 and 0.71 for WNV-infected K562 cells at 0 and 6 hpi, respectively. For N2a cells, the levels of *bax* mRNA were also elevated (from 1×10^{-3} pg to 4.5×10^{-3} pg) at 6 h after inoculation when infected with WNV. Otherwise, for mock-infected cells the expression of this gene remained unchanged (6×10^{-4} pg). The *bcl-2* gene expression for both mock- and WNV-

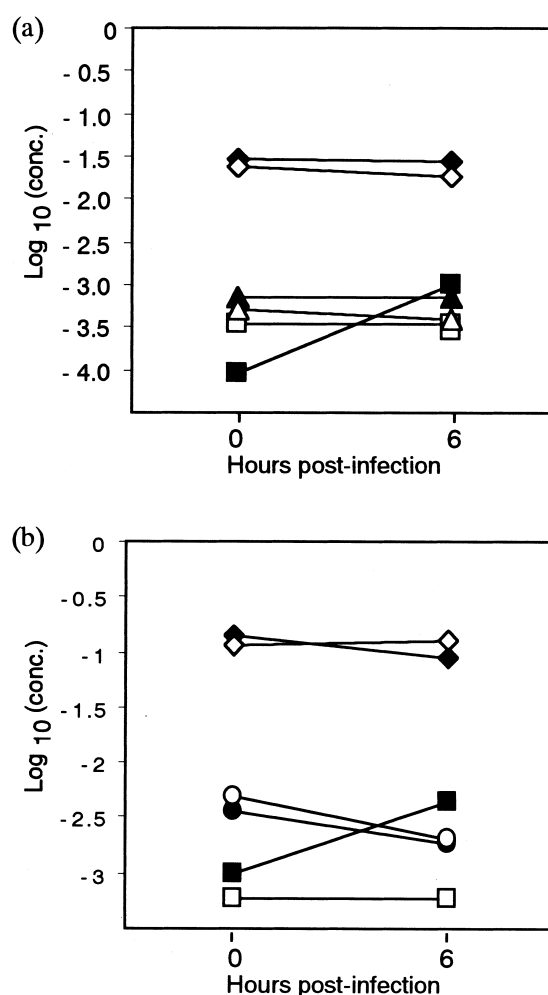


Fig. 7. Gene expression in WNV-infected apoptotic cells. Total RNA was extracted from mock- and WNV-infected cells at 0 and 6 hpi. cDNA was synthesized and real-time PCR was performed to quantify the expression of *bcl-2*, *bcl-x_L* and *bax* genes. As an internal control, the gene expression of β-actin was also measured. The results plotted were estimated using an experimentally determined standard curve and represent one data set of two experiments. a: K562 cells. b: N2a cells. ♦, β-actin; ■, *bax*; ▲, *bcl-x_L*; and ●, *bcl-2* gene expression in WNV-infected cells; ◇, β-actin; □, *bax*; △, *bcl-x_L*; and ○, *bcl-2* gene expression in mock-infected cells. The y-axis shows the log₁₀ of the concentration in picograms and the x-axis shows the time in hours.

infected N2a cells showed a slight decrease at the two times considered (from 3.5×10^{-3} pg to 1.8×10^{-3} pg for the mock-infected and from 5×10^{-3} pg to 2×10^{-3} pg for the WNV-infected). The ratios bcl-2/bax were 3.50 and 0.40 for WNV-infected N2a cells at 0 and 6 hpi, respectively. As a control, the level of β -actin mRNA remained unchanged throughout the course of viral infection.

4. Discussion

Comprehension of the mechanisms by which viruses induce cell death is important in our understanding of the pathogenesis of viral infections. To our knowledge this is the first report demonstrating that the cytopathic effect of WNV infection is mediated by the induction of apoptosis. Cell shrinkage and the appearance of DNA fragmentation were observed in WNV-infected but not in mock-infected cells. FacScan analysis revealed that the number of apoptotic cells increased with time. These data are also consistent with results obtained for Hoechst 33258 staining and annexin V binding of PS in K562 cells. The observation of a combination of typical morphological and biochemical characteristics of apoptosis allows us to conclude that apoptotic cell death is important in establishing a pathogenic role in this model of virus infection. Cardoso et al. [38] reported that UV-inactivated measles virus retains its capacity to bind cells and to fuse with the plasma membrane delivering its nucleoprotein into the cytosol to be processed for antigen presentation. Hence, the same conditions were applied for inactivation of WNV. The observation that UV-inactivated WNV failed to induce apoptosis suggests that active viral replication might be necessary for the induction of apoptosis by WNV. These findings indicate that, in this model, apoptosis might not be induced only by the attachment or penetration of viral particles into the host cell.

The bcl-2 family includes a series of homologous genes, which include bcl-2, bax, bcl-x_L, bcl-x_s and bad, all of which are important intracellular regulators of programmed cell death [20,32,51]. These genes are involved in different pathways of induction or repression of apoptosis. Usually bcl-2 and bcl-x_L inhibit apoptosis [20,34,48,52–54] while bax, bad and bcl-x_s induce apoptotic cell death [20,35,36,55,56]. Enforced expression of Bcl-2 or Bcl-x_L proteins has been reported to block apoptosis induced by various stimuli [57], including viruses such as alphavirus vectors [58], Japanese encephalitis virus [59], La Crosse virus [60], Sindbis virus [31], resulting in prolonged cell survival. Conversely, another cellular protein, Bax, was reported to be widely expressed in various cell types showing high rates of apoptosis [36]. In this study, the mechanism by which WNV induces apoptosis in infected K562 and N2a cells was investigated. K562 cells do not express the bcl-2 gene [48] or they do so at very low levels [56], in spite of showing resistance to many chemotherapeutic agents and radiation [61]. Therefore, the expression of the bcl-x_L and bax genes was investigated in WNV-infected and in mock-infected K562 cells. The expression of the pro-apoptotic bax gene showed an 11-fold increase within 6 h of infection, while the level of the anti-apoptotic bcl-x_L gene was unaffected. For the mock-infected cells, the expression of both genes remained at the same levels. WNV-infected N2a cells also showed an increase in bax mRNA levels (4.5 times) 6 hpi while mock-infected cells did not. The levels of bcl-2 mRNA slightly decreased in both mock- and virus-infected cells. The

lower increment of bax gene expression shown by N2a cells may explain the lower degree of apoptosis noted for this cell line at 72 hpi (37% vs. 52% for K562 cells). These results suggest that the bax gene was specifically upregulated by WNV infection and that it might be involved in the induction of apoptosis in WNV-infected K562 and N2a cells. We can thus hypothesize that the increased expression of bax is one of the mechanisms responsible for WNV-induced apoptosis. Similar findings were reported by Kobayashi et al. [56], who using a gene transfer system to modulate bax gene expression demonstrated that overexpression of this gene can predispose K562 cells to apoptosis induced by a wide range of chemotherapeutic agents.

It has been known that the bcl-2 family of genes forms homodimers and heterodimers [20]. For example, bcl-2 and bcl-x_L form homodimers and heterodimerize with bcl-x_s, bax, bad, Mcl-1, and also with each other [62–65]. It has also been proposed that the bcl-2/bax ratio in a cell acts as a rheostat that regulates susceptibility to apoptosis [34]. This rheostat may be directed towards the development of apoptosis by repressing bcl-2 and promoting bax expression [35,66,67]. Similarly, as bcl-x_L, but not bcl-2, is expressed in K562 cells, we can speculate that the bcl-x_L/bax ratio may act as a rheostat as well. In this report, the level of bax gene expression was upregulated in WNV-infected K562 and N2a cells at 6 hpi while the expression of bcl-x_L and bcl-2 was unchanged or slightly decreased. The ratio bcl-2/bax for WNV-infected N2a cells decreased almost nine times within 6 h of infection while only a two-fold decrease was observed for mock-infected cells. In addition, whereas the bcl-x_L/bax ratio decreased 11-fold for WNV-infected K562 cells it remained unchanged for mock-infected cells. Therefore, an imbalance in the relative proportion of these apoptosis-regulating genes could be responsible for the WNV-induced apoptotic cell death observed in the two cell lines considered. Similarly, recent results published by Gallaher et al. [68] on the expression of apoptosis genes in cells treated with colcemid concluded that, considering the ratio of RT-PCR densities, in either of the cell lines tested, the level of apoptosis correlated better with the bcl-x_L/bax ratio than with the bcl-2/bax ratio.

WNV is the causative agent of West Nile fever and encephalitis is a common complication that may result in fatal neurologic disease mainly involving damage to neurons of the brainstem [8]. Immunostains of tissues from several birds collected during the outbreak of WNV that occurred in the northeastern USA in 1999 revealed that the brains of most birds were infected with this virus and that WNV antigen was also present in the neurons of the brainstem [5]. In addition, WNV was found to infect mainly neurons in cultures of spinal cord slices, a model for studying the pathogenicity of neurotropic viruses [18]. Moreover, it was also recently reported that apoptotic cell death is an important cause of neuronal injury in experimental viral infection in vivo [27,69,70]. Our study shows that WNV infection of the N2a cells resulted in cell death by apoptosis, judging from the increase in the proportion of cells showing subdiploid DNA content (Fig. 6c). Therefore, it is possible that apoptosis may represent a pathogenic mechanism of this virus.

Several viral proteins has been shown to contribute to the induction of apoptosis [71–74]. Determining whether any WNV protein per se is able to induce apoptosis or if the

process is initiated by the release of soluble factors, such as cytokines, from WNV-infected cells requires further investigation.

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