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RAPID COMMUNICATION

Differential Regulation of Bcl-2 and Bax Expression in Cells Infected with Virulent and Nonvirulent Strains of Sindbis Virus

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Sindbis virus is an alphavirus that infects cells in either lytic or persistent infection. In this study we examined the effects of Sindbis virus on cell apoptosis and on the expression of Bcl-2 and Bax. Of the two strains studied, SVA and SVNI, only the neurovirulent strain, SVNI, induced apoptosis of astrocytes and PC-12 cells. SVA, which infects cells in a persistent manner, induced up-regulation of bcl-2 mRNA and Bcl-2 protein, whereas SVNI induced an increase in Bax levels. Our results indicate a differential regulation of Bcl2 and Bax expression by SVA and SVNI, which may be associated with the apoptotic potential of the viruses.

Introduction. Sindbis virus (SV) is a member of the alphaviruses that has been used as a model system for studying the pathogenesis of viral-induced encephalitis (*1*). SV infects cells in either a persistent or a lytic infection depending on the cell type and the viral strain (*2*). Viral persistence has been attributed to the overexpression of anti-apoptotic proteins, such as bcl-2 (*3*). SV-induced apoptosis appeared to involve activation of caspases or death pathways that are similar to those induced by activation of the TNF receptor superfamily (*4–6*).

Members of the Bcl-2 family have been shown to exhibit both anti-apoptotic and proapoptotic activities (*7*). The cellular oncogene bcl-2 has been shown to delay or block apoptosis induced by numerous, often unrelated physiological and pathological stimuli (*8*). Bcl-2 can heterodimerize with a conserved homologue, Bax, that accelerates programmed cell death (*9*). Excess of Bax is associated with apoptosis, whereas increased levels of Bcl-2 lead to cell survival. In addition, alternative pathways in which Bcl-2 and Bax can act independently of each other or can heterodimerize with other members of the Bcl-2 family are also known (*10*).

Bcl-2 and Bax have been implicated as important factors in the apoptosis induced by SV. Overexpression of bcl-2 oncogene was suggested as playing a role in the establishment of persistent viral infection by blocking virus-induced programmed cell death in rat prostatic adenocarcinoma (AT-3) cells (*3*). In addition, it has been reported that mature neurons are more resistant to SV infection than are neuronal precursors, due to their higher endogenous levels of Bcl-2 protein (*3*). In a recent study, it was reported that overexpression of Bax played a role in the inhibition of SV-induced apoptosis in some neuronal populations (*11*).

In this study we report that SVA, which leads to persistent infection, increases the expression of Bcl-2, whereas SVNI, which induces cell apoptosis, increases the expression of Bax.

Results. We first examined the effects of SVA and SVNI on apoptosis of astrocytes and PC-12 cells, which serve as a model for neuronal cells. Using TUNEL, we found that SVA and SVNI induced differential effects on cell apoptosis. Thus, infection of astrocytes with SVNI induced a small degree of apoptosis after 24 h (data not shown). By 48 h, nearly 70% of the astrocytes had undergone apoptosis (Fig. 1A). In contrast, SVA did not induce any visible changes in glial cell nuclei up to 6 days postinfection. Similar results were obtained for PC-12 cells. Using a DNA-laddering assay we found that SVNI induced apoptosis in PC-12 cells, whereas SVA had no significant effect (Fig. 1B). These results were further confirmed using additional techniques such as FACS analysis and ELISA using anti-histone 1 antibody (data not shown).

The differences in the apoptotic effects of SVA and SVNI were probably not related to their degrees of infection or replication in the different cells. In astrocytes, SVA

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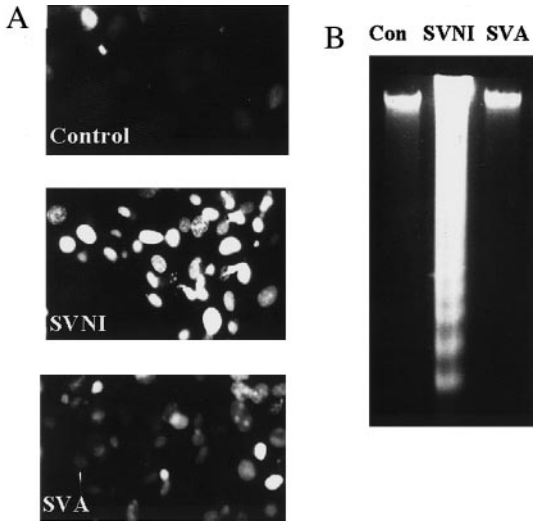

FIG. 1. Apoptosis of astrocytes and PC-12 cells infected with SVA and SVNI. Astrocytes (A) and PC-12 cells (B) were infected with either SVA or SVNI for 48 h. Apoptosis of astrocytes was measured using a TUNEL assay 48 h postinfection as described under Methods (A). DNA fragmentation analysis was performed for PC-12 cells (B). Low-molecular-weight DNA was isolated from control and virus-infected cells. Samples were electrophoresed in a 2% agarose gel and visualized with ethidium bromide. Results of a representative experiments out of four similar experiments are presented.

and SVNI infected the cells and replicated to similar degrees, as determined by immunofluorescence staining using anti-SV antibodies and by PFU assay, respectively (data not shown). In PC-12 cells, SVNI exhibited higher infection and replication rates than SVA. However, infection of PC-12 with 100 m.o.i. SVA resulted in a rate of infection similar to that of 1.0 m.o.i. SVNI, but still did not induce cell apoptosis.

It had been reported previously that apoptosis induced by SV is inhibited by overexpression of Bcl-2 (*3*). We therefore wanted to examine whether the differential effects of SVA and SVNI on cell apoptosis could be associated with changes in the expression of apoptosis-related genes. We first examined the effects of SVA and SVNI on the levels of endogenous Bcl-2. Astrocytes and PC-12 cells were infected with SVA and SVNI for different periods of time, and total RNA was extracted, reverse transcribed into cDNA, and then amplified with bcl-2-specific primers. We found that infection of the cells with the nonneurovirulent strain, SVA, increased bcl-2 mRNA levels in cultured cortical astrocytes (Fig. 2A) and in PC-12 cells (Fig. 2B). The increase in bcl-2 mRNA levels was observed following 4 h of infection (data not shown) and reached plateau levels after 6 h. In contrast, the neurovirulent strain, SVNI, which induced apoptosis in both glial and neuronal cells, did not increase the level of bcl-2 mRNA in either of the cells examined (Figs. 2A, 2B).

Similar results were observed in the levels of Bcl-2 protein. Thus, PC-12 cells infected with SVA for 24 h showed a strong increase in expression of Bcl-2 protein, whereas SVNI infection induced a small decrease in Bcl-2 expression (Fig. 2C).

Bax is a bcl-2-like protein that forms heterodimers with bcl-2 and has been reported as acting as a proapoptotic protein (*7*). We found that, unlike its effects on bcl-2, SVA did not induce significant changes in the expression of bax mRNA or Bax protein in either astrocytes or PC-12 cells (Figs. 3A, 3B). In contrast, astrocytes (Fig. 3A) and PC-12 cells (Fig. 3B) infected with SVNI exhibited an increase in bax mRNA expression. The increase in bax mRNA reached plateau levels after 6–8 h postinfection (data not shown). Similarly, we found that SVNI increased Bax protein levels in infected PC-12 cells (Fig. 3C) and astrocytes (data not shown). The effects were maximal 24 h postinfection. Similar changes in the effects of SVA and SVNI on the expression of Bax and Bcl-2 were observed also in cortical neurons (data not shown).

The bax/bcl-2 ratio in cells infected with SVNI was 1.83 $\pm$ 0.15 in astrocytes and 2.3 $\pm$ 0.1 in PC-12 cells, whereas infection with SVA resulted in a dramatic de-

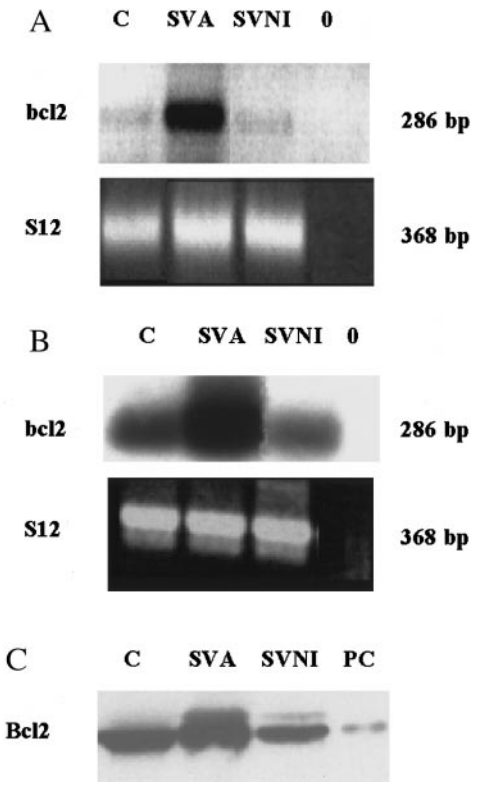

FIG. 2. Bcl-2 expression in cells infected with SVA and SVNI. Astrocytes (A) and PC-12 cells (B) were infected with either SVA or SVNI. RNA was extracted after 6 h and the samples were reverse transcribed. As a control, we used reaction mixture with 1 μl RNA instead of cDNA to exclude any contamination as a source of amplified fragments (0 control). The S-12 RT–PCR products were visualized by ethidium bromide staining. bcl-2 mRNA was detected using Southern blot followed by hybridization with ^{32}P-labeled internal primer. Bcl-2 protein was measured in PC-12 cells by immunoprecipitation followed by Western blot analysis (C). Results of a representative experiment out of four similar experiments are presented. PC, positive control.

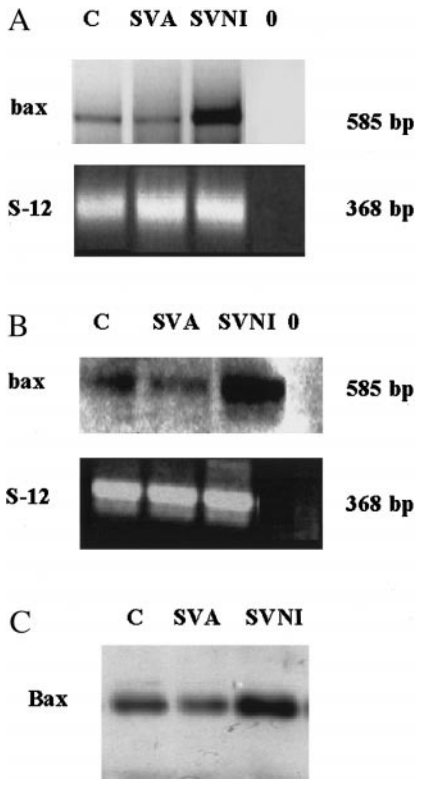

FIG. 3. Bax expression in cells infected with SVA and SVNI. Astrocytes (A) and PC-12 cells (B) were infected with either SVA or SVNI. RNA was extracted after 6 h and the samples were reverse transcribed. As a control, we used reaction mixture with 1 μl RNA instead of cDNA to exclude any contamination as a source of amplified fragments (0 control). The S-12 RT–PCR products were visualized by ethidium bromide staining and the bax mRNA was detected using Southern blot followed by hybridization with ^{32}P-labeled internal primer. Bax protein was measured in PC-12 cells using Western blot analysis (C). Results of a representative experiment out of four similar experiments are presented.

crease in the bax/bcl-2 ratio: 0.18 $\pm$ 0.02 in astrocytes and 0.15 $\pm$ 0.03 in PC-12 cells.

Discussion. In this study we compared the effects of two strains of SV, SVA and SVNI, which differ in their neurovirulence, on cell apoptosis and on expression of apoptosis-related genes Bcl-2 and Bax. SVA and SVNI differ in their neurovirulence in adult mice and rats (*12, 13*). SVA is a non-neurovirulent strain that can infect neurons following intracranial infection and is similar to the described AR339 strain and its derivative, SV1A (*14, 15*). SVA, similarly to SV1A, induces encephalitis and death in suckling mice, but an asymptomatic infection in weanling mice. In contrast, SVNI induces encephalitis and death in both suckling and weanling mice. SVA and SVNI also exert different effects on the activation of astrocytes (*16*). We found that in addition to their differences in neurovirulence in weanling mice, SVA and SVNI also exerted different effects on the apoptosis of neuronal and glial cells in culture. Thus, SVNI induced cell apoptosis of both astrocytes and neuronal cells, whereas SVA infected cells in a persistent manner.

We demonstrated for the first time differential regulation of endogenous Bcl-2 and Bax expression in response to infection with different strains of Sindbis virus. Infection of both glial and neural cells with SVNI, but not SVA, induced increases in bax mRNA and protein. In contrast, infection of astrocytes with SVA, but not SVNI, increased the level of bcl-2 mRNA and protein. Thus, induction of Bcl-2 by SVA and that of Bax by SVNI may play roles in determining the type of infection induced by each of the two viruses, persistent or lytic, respectively.

There have been no reports regarding changes in the endogenous levels of Bcl2 and Bax in response to SV infection. On the other hand, overexpression of exogenous Bcl-2 has been reported as playing a role in the establishment of persistent viral infection by blocking virus-induced programmed cell death in rat AT-3 cells (*3*). Similarly, mature neurons were reported to express higher levels of Bcl-2 protein than neuronal precursors and were also more resistant to apoptosis in response to SV infection. Recently, it was reported that Bax can protect suckling mice and some neuronal cells from apoptosis induced by SV and that the ability of Bax to act in an anti-apoptotic or proapoptotic manner depends on its interactions with cell-specific factors (*11*).

The mechanism by which Sindbis virus regulates the expression of Bcl-2 and Bax is currently not known. There have been a number of studies showing regulation of endogenous levels of Bax and Bcl-2 in response to apoptotic or anti-apoptotic stimuli. For example, TGFβ1 has been reported to down-regulate endogenous Bcl-2 expression (*17, 18*). It has been also reported that, in several hematopoietic cell types, the expression of HIV-1 tat protein resulted in down-regulation of Bcl-2 at both transcriptional and translational levels and in increased expression of Bax (*19*) and that the HTLV-1 Tax protein repressed transcription of the human *bax* gene (*20*). Since SVA and SVNI induced changes in the mRNA of both proteins, it appears that the viral protein(s) may affect directly or indirectly the cell transcription machinery that is involved in the regulation of these genes. Our data suggest that this regulation is differentially controlled by viruses that induce lytic and persistent SV infection. The mechanisms involved in the differential inductions of Bcl-2 and Bax by SVA and SVNI and the roles of these proteins in apoptosis induced by SVNI are currently being studied.

Methods. Primary glial cultures were prepared from cerebral cortices of 2-day-old mice as previously described (*16*). The culture consisted of 95% astroglia cells as determined by GFAP staining. For virus infection, growth medium was removed and was replaced with 10% volume of medium containing virus (10^6 PFU/ml, m.o.i. approx. 1). Viruses were absorbed in the cells for

1 h at 37°C, after which they were removed, the cultures were washed twice, and fresh medium was added.

Relative levels of bcl-2 and bax mRNA were estimated by a semiquantitative polymerase chain reaction (PCR) in comparison to the mRNA of the ribosomal protein S-12 as previously described (*23*). Total RNA was extracted from primary cultures of mouse cortex astrocytes and PC-12 cells with TRI Reagent (MRC) according to the manufacturer's manual. A Bcl-2 286-bp cDNA fragment corresponding to nucleotides 3352 to 3638 of the mouse bcl-2 locus was amplified by semiquantitative PCR using forward primer, 5′-TGCACCTGAGCGCCTTCAC-3′, and bcl-2 reverse primer, 5′-TAGCTGATTCGACCATTTGC-CTGA-3′ (*21*). A Bax 585-bp cDNA fragment corresponding to nucleotides −6 to 579 of mouse bax cDNA (*9*) was obtained by semiquantitative PCR using the following primers: forward, 5′-GGAATTCGCGGTGATGGACG-GGTCCGG-3′; bax reverse, 5′-GGAATTCTCAGCCCATCT-TCTTCCAGA-3′. Bax primers contain an *Eco*RI linker to facilitate subcloning. Optimal conditions for reverse transcription and amplification of bcl-2 (40 cycles), bax (30 cycles), and S-12 (25 cycles) were such that the rate of reaction did not plateau. PCR products were size-fractionated by electrophoresis in 1.5% agarose gels and ethidium bromide stained in the case of S-12 and bax or, in the case of bcl-2, downward transferred to Gene-Screen PlusTM nylon membranes (Dupont) using 0.4 N NaOH, 0.6 N NaCl transfer solution according to the method of Chomczynski (*22*). Hybridization was done as previously described (*23*) using a ^{32}P-end-labeled internal probe (bcl-2,5′-CCAGGAGAAATCAAACAAAGG-3′). Some gels of bax–PCR were also Southern-blotted, but this time with random primer ^{32}P-labeled cDNA obtained by PCR and cloned into Blue Script. After hybridization the filters were washed in 2× SSC, 0.1% SDS for 30 min at room temperature and in 0.1× SSC, 0.1% SDS for 1 h at 60°C and exposed to Kodak XAR films at −70°C with an intensifying screen for 5–6 h.

For Western blot analysis, cells were lysed in 100 μl of lysis buffer (25 mM Tris–HCl, pH 7.4; 50 mM NaCl; 0.5% Na deoxycholate; 2% NP-40; 0.2% SDS; 1 mM PMSF; 50 μg/ml aprotinin; 50 μM leupeptin; 0.5 mM Na_3VO_4) on ice for 15 min. The cell lysates were centrifuged for 15 min at 14,000 rpm in an Eppendorf microcentrifuge, supernatants were removed, and 2× sample buffer was added. Lysates (20 μg protein) were resolved by SDS–PAGE (12%) and were transferred to nitrocellulose membranes. Similar protein contents of individual samples were verified by staining the membranes with 0.1% Ponceau S solution in 5% acetic acid (Sigma). The protein staining was found to be linear to up to 30 μg of protein/lane. The Ponceau S staining was removed by several washes with phosphate-buffered saline (pH 7.4). The membranes were then blocked with 5% dry milk in phosphate-buffered saline and subsequently stained with anti-Bax or anti-Bcl-2 antibody (Transduction Laboratories, Lexington, KY). Specific reactive bands were detected using a goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) and the immunoreactive bands were visualized by the ECL Western blotting detection kit (Amersham, Arlington Heights, IL).

For immunoprecipitation, samples were preabsorbed with 25 μl protein A/G–Sepharose (50%) for 10 min and immunoprecipitation was performed using 4 μg/ml anti-Bcl-2 antibody and 30 μl of A/G—Sepharose at 4°C. Following the washes, the pellets were resuspended in 25 μl SDS sample buffer and boiled for 5 min. The entire supernatants were subjected to Western blotting. Membranes were probed with anti-Bcl-2 antibody.

TUNEL assay was performed on PC-12 cells grown in chamber slides. Cells were washed with wash solution (PBS +1 % BSA) and fixed with a solution of methanol/acetic acid (75/2.5%). Following fixation, cells were washed three times with wash solution and incubated for 2 h in a moist chamber with TUNEL reaction solution containing terminal deoxynucleotidyl transferase and FITC-conjugated dUTP. Cells were visualized under a fluorescence microscope.

Low-molecular-weight DNA was extracted from 10^6 cells/ml. Detached and adherent cells were collected and washed once with PBS. Cells were then incubated for 1 h in 400 μl of ice-cold lysis buffer containing 10 mM Tris (pH 7.5), 10 mM EDTA, and 0.2% Triton X-100. Lysates were centrifuged at 10,000*g* at 4°C for 20 min and DNA was extracted from the supernatant using buffered phenol followed by extraction with buffered phenol–chloroform and by extraction with chloroform–isoamyl alcohol. DNA was precipitated with ethanol, and samples were resuspended in 20 μl water and treated with RNase A (1 μg/ml) for 20 min. Samples were run on a 2% agarose gel in Tris–borate EDTA buffer and the gel was stained with ethidium bromide.

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