Temporal Changes in Chromatin, Intracellular Calcium, and Poly(ADP-Ribose) Polymerase during Sindbis Virus-Induced Apoptosis of Neuroblastoma Cells

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Sindbis virus (SV) induces apoptosis in many vertebrate cells, but the mechanism is unknown. To gain insight into this mechanism, the nature and time course of intracellular changes related to programmed cell death were studied in SV-infected mouse neuroblastoma cells. New virus production began at 5 h after infection and reached a peak at 12 h. Hoechst 33342 staining of DNA analyzed by flow cytometry demonstrated changes in chromatin beginning 6 h after infection. These chromatin changes were cell cycle dependent, affecting cells in G₀/G₁ but not S phase. Apoptosis was not dependent on increases in intracellular Ca²⁺ and occurred more rapidly in the absence of extracellular Ca²⁺. Nuclear changes were accompanied by activation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), resulting in increased consumption of NAD which was apparent by 10 h after infection. SV-induced apoptosis also involved the proteolytic cleavage of PARP. This cleavage was detectable at 16 h after infection approximately the same time that DNA fragmentation was apparent by agarose gel electrophoresis. We conclude that SV-induced apoptosis of neuroblastoma cells is dependent on viral replication, is not dependent on a rise in intracellular Ca²⁺, and is accompanied by activation of PARP and of a protease that cleaves PARP.

Virus infection may result in death, persistent infection, or oncogenic transformation of the infected cell. The specific outcome is dependent on characteristics of both the virus and the host cell. Some viruses are typically lytic in cell culture, while others usually cause a persistent infection. However, there are many examples of viruses that replicate lytically in one cell type but replicate persistently without causing cell death in another cell type. Identifying the determinants of these differences is important to understanding the pathogenesis of viral infections.

Death of virus-infected cells has traditionally been thought to be caused primarily by virus-induced alterations in transcription and translation of essential cellular proteins or by damage to cell membranes resulting from virus morphogenesis and release from the cell. However, it has recently been demonstrated that for a number of apparently lytic viruses, cell death results from viral induction of apoptosis (21, 26, 32, 43, 45, 46, 65), a cellular program leading to cell shrinkage, cytoplasmic blebbing, and endonucleolytic cleavage of chromosomal DNA prior to altered permeability of the plasma membrane (12, 27).

Apoptosis is a cellular response to many different insults, both physiological and pathological, including irradiation (58), lysis by cytotoxic T cells (15), withdrawal of growth factors (30,

42, 52), and drug treatment of tumor cells (4, 24). Intracellular changes often associated with the induction of apoptosis include increases in intracellular free calcium (40, 41) and increased expression of a wide variety of genes, including those encoding cellular transcription factors (57, 60, 71, 73). Activation of cellular proteases has been implicated in cell death by the observation that protease inhibitors block cell lysis by cytotoxic T cells (56, 59); by the identification of the *Caenorhabditis elegans* death gene *ced-3* as a homolog of interleukin-1 β -converting enzyme (ICE) gene (22, 44, 77); by the ability of the poxvirus ICE inhibitor gene *crm-A* (53) to protect neurons from apoptosis induced by growth factor withdrawal (17); and by the ability of protease inhibitors to inhibit chemotherapy-induced apoptosis in cells (8, 25) as well as in a cell-free system that reproduces the events of apoptosis in vitro (33). Whether these changes are the cause of programmed cell death or the late effects of activation of a cellular program leading to apoptosis is unclear. Many of these changes are unique for specific cellular systems, and so it is likely that there are a variety of programs associated with induction of apoptosis. The intracellular events in virus-infected cells that lead to cell death have received limited study. Studies of virus-induced apoptosis offer the opportunity to provide insights into cellular responses to viral infection and to identify common elements essential to multiple mechanisms for induction of apoptosis.

Alphaviruses plaque readily in a variety of cell lines and are generally considered to be lytic. However, alphaviruses can also cause persistent infection, and this has been most clearly described for mosquito cells (51, 64, 68) and differentiated mammalian neurons (35). We have used Sindbis virus (SV), the prototype alphavirus, to study virus-induced death of mammalian cells. SV induces apoptosis in most vertebrate cell lines,

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and this can be blocked in some cells by expression of cellular inhibitors of apoptosis such as *bcl-2* (35). In this study, we have used N18 mouse neuroblastoma cells infected with SV to identify the time course of changes in nuclear DNA by flow cytometry, to assess changes in intracellular free Ca^{2+} , and to identify changes in poly(ADP-ribose) polymerase (PARP) that accompany virus-induced apoptosis.

MATERIALS AND METHODS

Virus. The TE strain of SV (39) was grown and assayed in BHK-21 cells.

Cells. N18, a mouse neuroblastoma cell line (1), was cultured in Dulbecco's minimal essential medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Biocell Laboratories, Rancho Dominguez, Calif.) and 50 μg of gentamicin (GIBCO) per ml.

Flow cytometry analysis. N18 cells were infected with SV at a multiplicity of 5 PFU per cell. At various time points, infected and uninfected cultures were trypsinized and harvested. The cells were washed twice with Dulbecco's minimal essential medium without fetal bovine serum, incubated with the DNA-staining dye Hoechst 33342 (10 $\mu\text{g}/\text{ml}$; Molecular Probes, Junction City, Ore.) for 30 min at 37°C, and analyzed by flow cytometry. Analysis was performed on a Becton Dickinson FACStar Plus flow cytometer running the FACStar Plus research software (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) and equipped with an Inova 90 argon laser (Coherent Laser Products Division, Palo Alto, Calif.).

Detection of DNA fragmentation by gel electrophoresis. The in situ extraction protocol of Sorenson et al. was used (63). A gel with 2% agarose in Tris-borate-EDTA buffer was cast. After solidification, the agar between the comb and the buffer reservoir above the wells was removed and replaced with 1% agarose containing 1.2 mg of proteinase K per ml and 2% sodium dodecyl sulfate (SDS). One million N18 cells, previously infected with SV at a multiplicity of 5 PFU per cell, were suspended in sample buffer (Tris-borate-EDTA) containing 10 mg of RNase per ml, 15% Ficoll, and 0.01% Coomassie blue. The cells were lysed in the well as SDS contacted the cells with the initiation of electrophoresis, which was carried out at 20 V for 1.5 h and at 100 V for 3 h. After electrophoresis, the agarose gel was incubated overnight at room temperature in Tris-EDTA buffer containing 2 mg of RNase per 100 ml of Tris-EDTA. DNA was detected by staining with ethidium bromide.

Intracellular Ca^{2+} measurement. N18 cells were grown on glass coverslips in six-well plates. These cultures were infected for 1 h at 37°C with SV (5 PFU per cell), loaded for 1 h at room temperature with 2.5 μM Fura-2 acetoxymethyl ester (Molecular Probes), and then washed three times to remove extracellular Fura-2. Coverslips were rigidly mounted in a machined cuvette (28) and placed in an SLM-Aminco fluorometer (SPF-500C). Fluorescent emission at 510 nm (20-nm bandpass) was measured in response to alternating excitation wavelengths of 340 and 380 nm (1-nm bandpass). The 340/380-nm fluorescence ratio was calculated after subtraction of cellular autofluorescence values. Free calcium concentrations were calculated from 340/380-nm ratios by using the equation of Grynkiewicz et al. (19), assuming $K_d(\text{Ca}^{2+}) = 224$ nm. Other parameters in the equation ($R_{\text{max}} = 23.1$, $R_{\text{min}} = 0.9$, and $\beta = 9$) were estimated from the calcium-sensitive dye released from cells by 50 μM digitonin and were identical to those for pure de-esterified Fura-2.

Detection of PARP activation and proteolysis. To estimate NAD content, cells were extracted with ice-cold 0.5 M perchloric acid. The extracts were neutralized with 1 M KOH containing 0.2 M potassium phosphate (pH 7.5 after neutralization) and stored at -70°C until analysis was performed by using the enzyme cycling assay of Bernofsky and Swan (7). To detect PARP proteolysis, samples containing 50 μg of protein from SV-infected N18 cells were prepared for electrophoresis, separated on SDS-5 to 15% polyacrylamide gels, transferred to nitrocellulose, and reacted with monoclonal antibody C-2-10 (31) as previously described (25).

RESULTS

SV replication in N18 cells. The time course of SV replication in N18 mouse neuroblastoma cells after infection at a multiplicity of 5 PFU per cell was determined (Fig. 1). New virus was detected in supernatant fluids 5 h after infection and indicated the length of time for one cycle of replication. Peak virus production was evident by 12 h, and cytopathic effect was apparent between 16 and 18 h, although cellular membrane integrity was maintained as indicated by trypan blue exclusion.

Flow cytometry analysis of DNA changes. Flow cytometry has recently been used to study cells undergoing apoptosis induced by drugs and by growth factor withdrawal (48). Hoechst 33342 diffuses through the cell membrane, recognizes

FIG. 1. Growth curve of the TE strain of SV in N18 mouse neuroblastoma cells. Infection was at a multiplicity of 5.

a native chromatin structure, and binds the minor groove. During apoptosis, the structure of chromatin is altered and Hoechst 33342 binds less well. Therefore, apoptotic cells appear to contain less DNA and are visualized as a sub- G_0/G_1 peak (13, 67). This is not observed in necrotic cell death (48).

SV-infected N18 neuroblastoma cells stained with Hoechst 33342 were analyzed at various times after infection (Fig. 2). An increase in the sub- G_0/G_1 peak could be detected as early as 6.5 h postinfection (arrows in Fig. 2) and increased significantly by 18 h. The change in the histogram was correlated with 90° light scatter (Fig. 3). By side scatter, the apoptotic cells are seen to be largely intact but shrunken. The appearance of a DNA peak below the G_0/G_1 region was in proportion to the increase in the shrunken cell population detected by side scatter.

Susceptibility to chemically induced apoptosis in many well-studied systems is related to the cell cycle (76). To identify the relationship between cell cycle and susceptibility to SV-induced apoptosis, the histograms of DNA from uninfected cells

FIG. 2. Flow cytometry histogram analysis of uninfected and SV-infected N18 neuroblastoma cells at 4, 6.5, and 18 h after infection. Fluorescence intensity represents amount of DNA staining by Hoechst 33342. Arrows indicate apoptotic cell populations.

FIG. 3. Side scatter analysis of apoptotic N18 mouse neuroblastoma cells by flow cytometry. Infected cells were assessed 6.5 h after SV infection. The arrow indicates the apoptotic cell population.

and infected cells were analyzed and the percentages of cells in each region at various times after infection were determined (Table 1). The number and proportion of cells in the G_0/G_1 phase gradually decreased, while the apoptotic cells continuously increased postinfection. In contrast, the proportion of cells in S phase remained fairly constant until the very late stages of infection. Cells in G_2/M constituted only 2 to 6% of cells at all times, and so the importance of changes in this population was difficult to discern.

Role of Ca^{2+} and Mg^{2+} in virus-induced DNA fragmentation. To determine the potential role of divalent cations in SV-induced apoptosis, changes in intracellular Ca^{2+} in neuroblastoma cells after SV infection were determined by using Fura-2 (Table 2). Levels of free intracellular Ca^{2+} were measured after virus had penetrated the N18 cells (1 h postinfection) and at the time of virus release, 1 h before chromatin changes were detected (5 h postinfection). No increase in intracellular Ca^{2+} was detected at either time after infection. At 5 h postinfection, Ca^{2+} levels were lower than in uninfected cells. To confirm that SV-induced apoptosis was not dependent on extracellular Ca^{2+} or Mg^{2+} , N18 cells were infected with SV and incubated with and without Ca^{2+} and Mg^{2+} and then analyzed by flow cytometry at various times postinfection (Fig. 4). In both culture conditions, alteration of chromatin structure was first present 6 h postinfection but progressed more rapidly in the absence of divalent cations.

Detection of PARP enzymatic activity and proteolysis during SV infection. PARP is a nuclear, chromatin-associated enzyme that is activated by DNA single-strand and double-strand breaks (47). It converts NAD to nicotinamide and protein-linked ADP-ribose polymer, leading to the consumption of intracellular NAD (74), unwinding of nucleosomes, and enhanced DNA repair (6). We examined the possible activation of PARP by measuring the level of intracellular NAD at various times after infection (Fig. 5). The level of NAD de-

TABLE 1. Cell cycle distribution during SV-induced apoptosis

h postinfection	No. of expts	Avg % of cells in each cell cycle region			
		Sub- G_0/G_1	G_0/G_1	S	G_2/M
4	2	17	53	24	6
6–6.5	2	35	29	29	6
7.5–8.5	3	44	30	23	2
10.5–14	3	47	27	20	5
16–18	2	67	15	16	2

TABLE 2. Ca^{2+} concentration in N18 cells after SV infection

h postinfection	Expt	Ca^{2+} (nM) in N18 cells	
		SV infected	Uninfected
1	1	165	200
	2	130	145
	3	150	ND ^a
5	1	71	120
	2	65	92

^a ND, not done.

creased by 10 h after infection and continued to decrease throughout the course of the infection.

To determine whether SV infection is associated with proteolysis of PARP, N18 cellular proteins were examined by immunoblotting at various times (Fig. 6A). Degradation of the 116-kDa PARP molecule to a 85-kDa fragment was detectable at 14 h postinfection. The time course of PARP cleavage paralleled the appearance of DNA fragmentation (Fig. 6B).

DISCUSSION

Studies of virus-induced cell death provide insights into the mechanisms by which virus infections induce disease. SV causes cell death by inducing apoptosis in a virus- and cell-dependent manner (35, 69). Neurons develop resistance to SV-induced apoptosis with maturation (35), which contributes to the age-dependent resistance to fatal encephalitis that is characteristic of SV infection in mice (23, 36). In these studies, we have further defined the intracellular changes associated with SV-induced apoptosis in murine neuroblastoma cells and have shown that nuclear DNA changes are detectable within 6 h after infection, that infection does not cause an increase in

FIG. 4. Role of extracellular Ca^{2+} in apoptosis induced by SV. SV-infected N18 mouse neuroblastoma cells were cultured with and without Ca^{2+} and Mg^{2+} . Progression of apoptosis was assessed by staining with Hoechst 33342 and flow cytometry analysis at 4, 8.5, and 16 h after infection.

FIG. 5. Consumption of NAD at various times after SV infection of N18 mouse neuroblastoma cells.

basal intracellular levels of free Ca^{2+} , and that apoptosis is accompanied by activation and subsequent proteolysis of the DNA repair enzyme PARP.

A number of viruses are now known to cause cell death by inducing apoptosis (26, 37, 43, 45, 46), and some viruses encode proteins that inhibit this process (11, 20, 66, 72). However, few studies have examined the mechanism by which virus replication initiates this cellular program. Replication of SV in N18 cells is rapid. A number of studies have implicated proteins that are involved in cell cycle progression (e.g., p53) in apoptosis (37, 38, 76). The number of cells in S phase was maintained until late in infection, while those in G_0/G_1 decreased steadily as the number of apoptotic cells increased. This finding suggests that cells in G_0/G_1 are the primary cells undergoing apoptosis, although a contribution from the small number of cells in G_2/M cannot be excluded.

The mechanisms by which a virus activates a cellular program leading to apoptosis is unknown. Intracellular synthesis of viral proteins is generally required, but expression of a single

viral protein may be sufficient. It has been proposed that human immunodeficiency virus induces apoptosis in CD4^+ lymphocytes through the interaction between the gp120-gp41 complex and CD4 in the cell membrane (32). Apoptosis has also been linked to adenovirus E1A stabilization of p53 (37) and to expression of parvovirus nonstructural proteins (9, 34), human T-cell leukemia virus type 1 Tax (75), and chicken anemia virus VP3 (46). The mechanism of SV-induced apoptosis is not yet clear. In order to induce apoptosis in neuroblastoma cells, some component of viral replication is necessary. Binding of UV-inactivated SV at a concentration of 100 PFU per cell did not induce apoptosis in neuroblastoma cells (data not shown). Flow cytometry indicates no evidence of apoptosis until viral replication has reached the stage of new virion formation. Expression of nonstructural proteins alone can cause cell death that may or may not be associated with apoptosis, but death occurs much more rapidly when structural proteins are expressed (16). These data suggest that synthesis of structural proteins is associated with induction of apoptosis.

Although there are many methods to induce apoptosis, it is believed that they all involve a common final pathway involving DNA fragmentation. Activation of a cellular endonuclease is likely to have a major role in DNA fragmentation and morphologic changes in the nucleus (2). Several candidate endonucleases have been characterized (49). NUC-18 and DNase I, prime candidates for this role, require Ca^{2+} for activation (49, 50). Sustained increases in levels of intracellular Ca^{2+} induce apoptosis and DNA fragmentation in some cells (40). SV infection did not increase, and appeared to lower, intracellular Ca^{2+} levels in mouse neuroblastoma cells, and apoptosis progressed more rapidly in the absence of extracellular Ca^{2+} . The resting level of Ca^{2+} in neuroblastoma cells is high compared with the levels of intracellular Ca^{2+} in other cell lines (40), and this may be sufficient to support activation of endonuclease activity in the presence of another signal.

Programmed cell death does not always require increased intracellular Ca^{2+} and, in fact, has been associated with decreased intracellular Ca^{2+} (3). For example, *fas* cross-linking and etoposide treatment can induce Ca^{2+} -independent apoptosis (5, 55). Therefore, DNase II has been suggested to play a role in internucleosomal digestion of genomic DNA during apoptosis of these cells (5). Mammalian cells contain other Ca^{2+} -independent endonucleases which could be involved in apoptosis but are less well studied (49). Which of these endonucleases plays a role in neuronal cell death due to SV infection needs further investigation.

PARP, a highly conserved, abundant nuclear enzyme (14), may also play a role in apoptosis. In the presence of DNA strand breaks, this enzyme converts NAD to nicotinamide and protein-linked ADP-ribose polymers (10). The enzyme is composed of three major domains: a DNA-binding domain, an automodification domain, and a catalytic (NAD-binding) domain (18, 61). Previous results indicate that PARP is activated in cells undergoing apoptosis (25, 29, 70), that PARP is proteolytically cleaved when cells undergo apoptosis (24, 25), and that inhibition of PARP activity induces apoptosis (54). In etoposide-treated HL-60 cells, the experimental system in which these events have been most extensively studied, the rapidity of these events has made it difficult to determine their precise order. The slower time course in SV-induced N18 cells has permitted a more precise ordering of these events. The decrease in NAD levels suggested that PARP was activated within 10 h of SV infection, whereas PARP cleavage was not detectable until 16 h postinfection. These data suggest the following course of events. Alteration in chromatin structure and initial breakage of DNA appear to occur by 6 h postinfection.

FIG. 6. Cleavage of PARP and chromosomal DNA. (A) Immunoblot analysis of proteolytic cleavage of PARP at various times after SV infection. Lanes: 1, uninfected cells; 2 to 7, cells 4, 7, 10, 16, 18, and 20 h after infection. (B) Detection of DNA fragmentation by gel electrophoresis in the same cells. Lanes: 1, molecular weight markers; 2, uninfected cells; 3 to 7, cells 6, 10, 16, 18, and 20 h after infection.

tion, i.e., shortly after the first cycle of viral replication is completed. Attempts to repair early DNA strand breaks take place, as evidenced by activation of PARP (consumption of NAD). Several hours later, but still well in advance of membrane permeability changes, PARP is proteolytically cleaved. It has recently been shown that this cleavage occurs through activation of prICE, a protease related to ICE (33). How SV infection leads to the DNA strand breaks that activate PARP and the prICE activation that cleaves PARP remains to be elucidated. Because cleavage of PARP was detected only at late times following SV infection, it seems unlikely that PARP cleavage is an initiator of apoptosis. However, the 85-kDa C-terminal domain of PARP can continue to consume NAD at a low rate in the absence of DNA binding (61), and it has been postulated that consumption of NAD by PARP and consequent depletion of ATP can lead to cell death (6, 62, 78).

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