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Pseudorabies virus induces apoptosis in tissue culture cells

Brief Report

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Summary. The process of cell death as a result of exposure to pseudorabies virus (PRV) in cultured cells was examined and specific features characteristic of apoptosis were observed. At early times of infection, externalization of membrane phospholipid phophatidylserine was detected by flow cytometry analysis. During the infection process, caspase 3-like protease activity was induced and the activity increased in a time dependent manner. Cellular DNA degradation was demonstrated by agarose gel electrophoresis. Morphologic changes of the nucleus that included chromatin condensation and margination to the periphery of the nucleus were evident in electron microscopy analysis. These biochemical and morphologic changes demonstrated that, during PRV replication, the host cell was induced to undergo apoptosis.

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Apoptosis, also known as programmed cell death, is a process of cellular suicide that occurs under normal physiological conditions [3]. It is a mode of cell death commonly associated with cell turnover, tissue homeostasis, aging and embryogenesis. There are specific morphologic and biochemical changes characteristic of apoptosis [3, 14]. They include cell shrinkage, loss of cell-cell contact, chromatin aggregation, nuclear and cytoplasmic condensation, partition of nucleus and cytoplasm into membrane-bound vesicles. Specific nucleases are activated and they cause degradation of chromosomal DNA. The degraded DNA may be present as high molecular weight fragments or cleaved into internucleosomal oligomers [2, 6, 18, 29, 31]. Although some of the nucleases that are active during apoptosis have been identified [1, 9, 10, 20, 25], the factors and mechanisms involved in DNA fragmentation are still unclear.

A wide variety of viruses have been reported to induce apoptosis [23, 27] and an increasing number of viral components have been shown to induce or inhibit apoptosis [13, 19]. The anti-apoptotic activities enable the virus to block premature death of the host cell for the propagation of progeny viruses or establishment of persistence, and the pro-apoptotic activities ensure efficient dissemination of progeny viruses to neighboring cells. Pseudorabies virus (PRV) belongs to the alphaherpes virus subfamily and the association of PRV infection with apoptosis has not been established. In this work, we showed that PRV induces programmed cell death in Madin-Darby bovine kidney (MDBK) cells.

The Indiana-Funkhauser strain of PRV was used to infect MDBK cells cultivated in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. The experiments were carried out at a multiplicity of infection (m.o.i.) of 10 and the virus titer was quantitated by a plaque assay previously described [5]. One of the earliest morphologic changes during apoptosis is the translocation of membrane phospholipid phophatidylserine (PS) from the inner to the outer leaflet of the plasma membrane [17]. Simultaneous staining of cells with annexin V, which binds PS, and propidium iodide (PI), which is excluded by viable cells, allows differentiation of cells undergoing apoptosis (annexin V positive, PI negative) or necrosis (annexin V positive, PI positive) [28]. Previously, we have demonstrated that progeny PRV start to appear 8 h after infection of MDBK cells [4]. Therefore, we analysed the externalization of PS of MDBK cells during the early stages of PRV infection. Infected cultures at different times postinfection (p.i.) were analysed by flow cytometry after staining with FITC-annexin V and/or PI. Dexamethasone, which has been widely used to induce apoptosis in many cell types, was included as a positive control [26]. In comparison to untreated cells, the dexamethasone-treated and PRV-infected cultures showed an increased number of annexin V positive and PI negative cells soon after exposure to dexamethasone or PRV (Fig. 1), indicating an increased externalization of PS in viable cells.

During apoptosis, one or more cascades of proteolytic enzymes are activated. Caspase 3, which is a key component of the apoptotic pathway, is responsible for proteolytic cleavage of a large number of substrates [7, 22]. A commercially available kit was used to monitor caspase 3-like activity in mock-infected and PRV-infected MDBK cells. Two independent experiments were conducted and similar results were obtained. One of the experiments is presented in Fig. 2 and the results demonstrated that as PRV infection progressed, caspase 3-like activity increased, as shown by cleavage of the substrate, DEVD-pNA. At all time points tested, caspase 3-like activity was higher in PRV-infected cell lysates than in mock-infected cell lysates. The caspase 3-like activity was significantly reduced in the presence of the caspase 3 inhibitor, DEVD-fmk.

Degradation of cellular DNA inside a cell is a hallmark of apoptosis. Experiments were carried out to visualize cellular DNA fragmentation triggered by PRV. Total cell DNA was isolated from PRV-infected MDBK cells at different times p.i. and subjected to agarose-gel electrophoresis. The uninfected cell DNA appeared as high molecular weight form and the PRV-infected cell contained a significant proportion of lower molecular weight DNA, which appeared as a

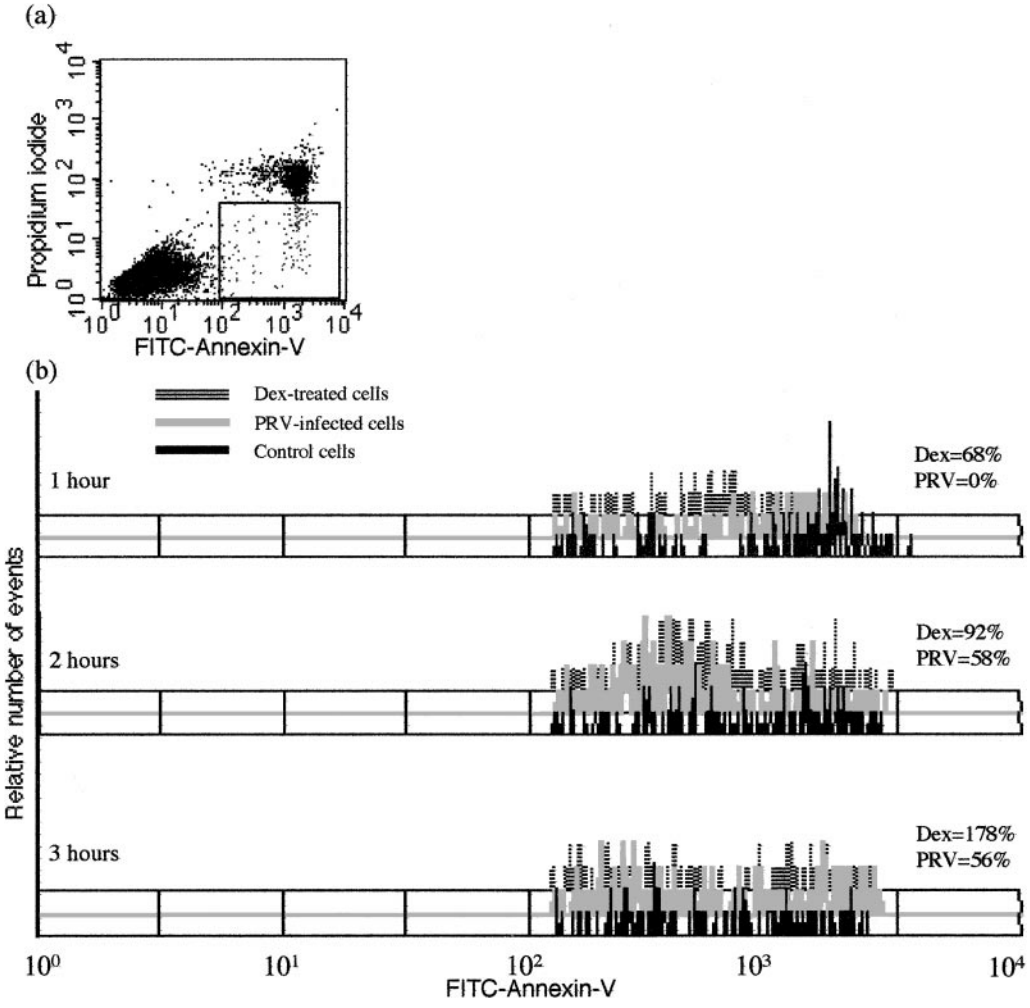

Fig. 1. Flow cytometry analysis. The cells were prepared and stained with FITC-annexin V and/or PI according to the manufacturer's instructions (PharMingen, San Diego, CA) and then analysed by flow cytometry with a FACSan instrument (Becton Dickenson, Franklin Lakes, NJ). **a** A typical FITC-annexin V and PI profile with the apoptotic cell population (annexin V positive and PI negative) enclosed in a box. **b** The cytometric profiles of apoptotic cells at the indicated time after mock-treatment, PRV-infection or dexamethasone-treatment are presented. The increase of annexin V positive and PI negative cells (in percentage) above the control sample is also indicated

smear after separation by agarose gel electrophoresis (Fig. 3a). The presence of internucleosomal ladder of 180 to 200 base-pairs was not readily apparent. This observation was reproduced in many subsequent experiments. To ensure that this observation was not specific to MDBK cells, we conducted the same experiment in swine testicular (ST) cells. Previously, we demonstrated that the kinetics of PRV replication in ST and MDBK cells were identical (unpubl. data) and that ST cells were capable of exhibiting internucleosomal ladder formation upon

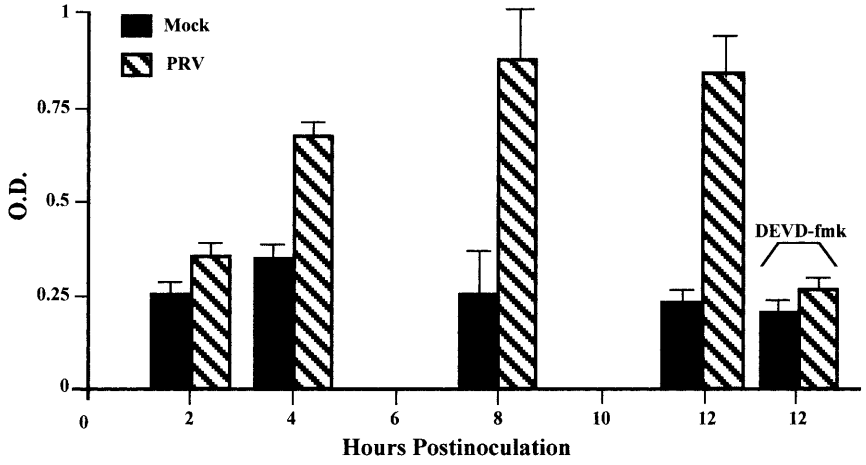

Fig. 2. Comparison of caspase 3-like activity in PRV-infected and mock-infected MDBK cells. The ApoAlert CPP32/Caspase-3 Colorimetric Assay Kit was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Preparation of cell lysates and measurement of caspase-3-like activity was carried out according to the manufacturer's instructions. In this assay, a colorimetric tetra-peptide labeled with p-nitroanilide (DEAD-pNA) is cleaved by proteases with caspase-3-like activity and samples are read in a spectrophotometer at 405 nm [11]. The experiment was carried out in duplicate and DEVD-fmk was included in one of the assays

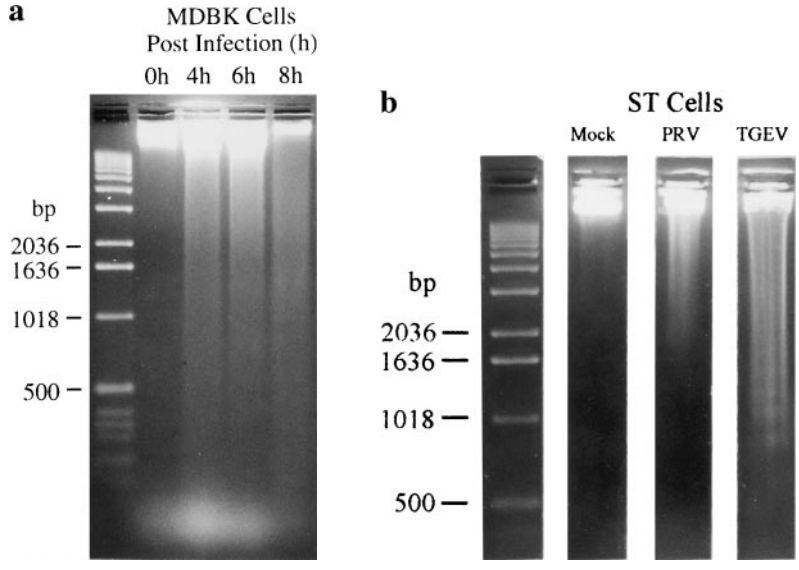

Fig. 3. Analysis of DNA fragmentation by agarose gel electrophoresis. Total cell DNA was isolated using the STAT-60 DNA extraction kit purchased from TEL-TEXT "B", Inc. (Friendswood, TX). Briefly, virus infected cells were lysed and extracted with chloroform according to the manufacturer's instructions. The samples were then incubated with RNase, ethanol precipitated and electrophoresed in 1.3% agarose gel. The DNA patterns were photographed after staining with 0.05 μg/ml of ethidium bromide. **a** MDBK cells were infected with PRV and **b** ST cells were infected at m.o.i. 10 with PRV or TGEV for 18 h. Total cell DNA was harvested at the indicated times p.i.

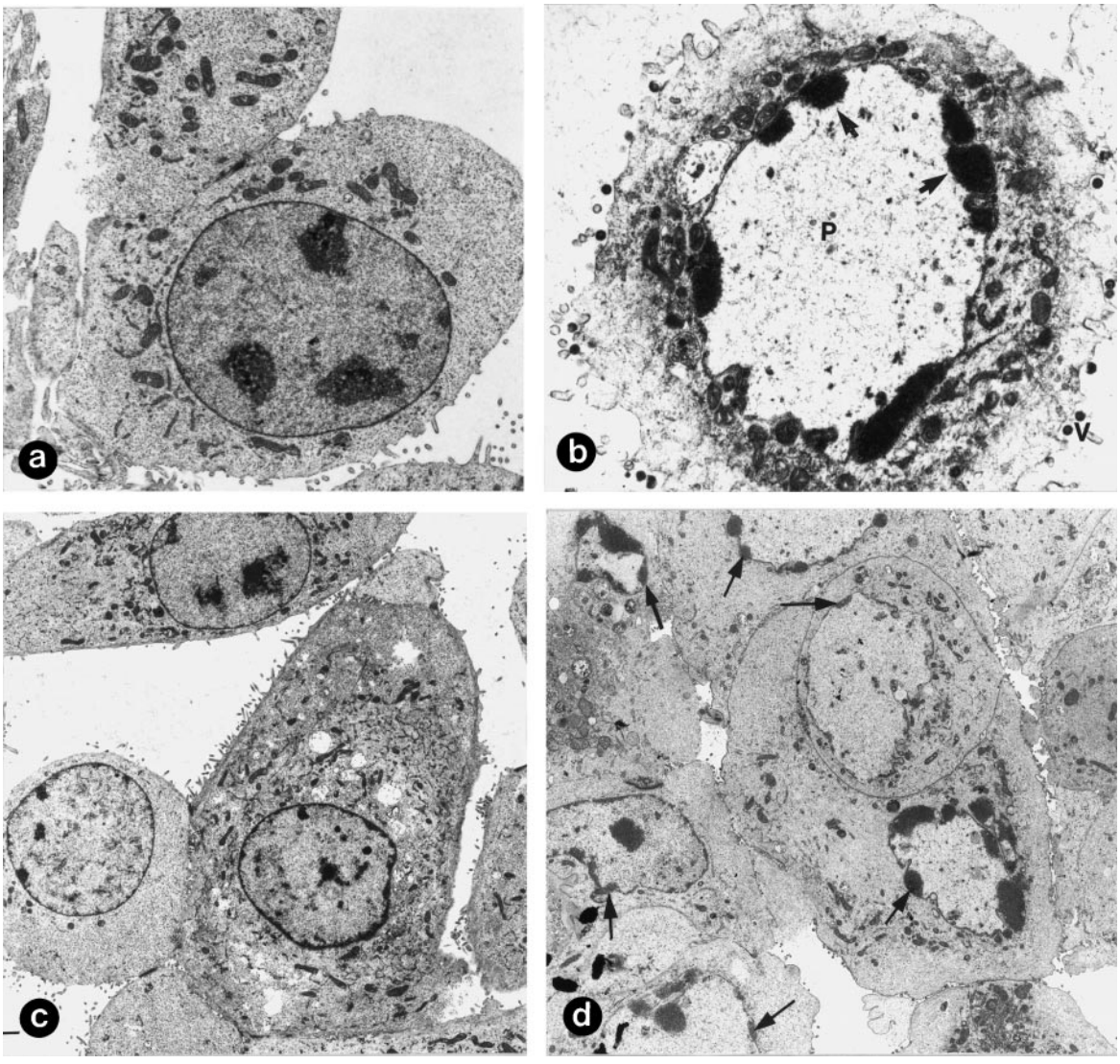

Fig. 4. Electron micrographs. PRV-infected cells were scraped from cell culture plates, collected by centrifugation, washed twice in PBS, fixed in 2.5% glutaraldehyde (pH 7.4) for 4 h and stored overnight at 4 °C in 0.1 M cacodylate buffer (pH 7.4). The samples were post-fixed in osmium tetroxide (pH 7.4), followed by dehydration through a series of ethanol solutions. After clearing with propylene oxide, the samples were passed through a series of propylene/Eponate 12 resin (3:1, 1:1 and 1:3), and then allowed to polymerise in the Eponate 12 resin. Thin sections were stained with lead citrate and uranyl acetate and examined on a Philips EM 410 electron microscope (Philips Electronic Instruments Co. Mohwah, NJ). **a**, **c** Uninfected cells of magnifications 5800× and 2800×, respectively. **b**, **d** 12 h and 48 h PRV-infected MDBK cells (magnifications of 6900× and 2800×, respectively) with condensed and marginalized chromatin (arrows), viral particle (*P*) and complete virion (*V*)

infection with transmissible gastroenteritis virus (TGEV) [8]. The results showed that both TGEV and PRV induced DNA degradation in ST cells. However, internucleosomal DNA laddering that was prominent with the TGEV-infected ST cells was not readily detected with the PRV-infected cells (Fig. 3b).

Morphologic changes associated with apoptosis can also be visualized by transmission electron microscopy (EM). At different times p.i., MDBK cells exposed to PRV were prepared for EM examination. Nuclear chromatin condensation and margination, commonly associated with apoptosis, were observed in the infected cells. The EM images of uninfected and PRV-infected MDBK cells were presented for comparison (Fig. 4). The condensed chromatin appeared as fragmented electron dense masses that were marginalized to the periphery of the nucleus. Intra-cellular and extra-cellular viruses associated with PRV-infected cells were readily observed at 12 h p.i. At 48 h p.i., this chromatin condensation pattern was evident in all cells where intracellular viral particles were observed.

In this report, we examined whether PRV can induce apoptosis in MDBK cells during virus replication. Several features characteristic of apoptosis were detected. The morphologic changes detected by flow cytometry and electron microscopy showed externalization of PS and chromatin condensation and margination, respectively. Cellular DNA fragmentation, considered a hallmark of programmed cell death as the result of endonucleolytic activity, can exist in several forms, including large fragments of 30–500 kilo-bp, 180–200 bp oligomeric internucleosomal fragments and single-stranded DNA breaks [3, 21, 29, 30]. The relationships between these types of DNA degradation are not clear. Agarose gel electrophoresis analysis demonstrated that PRV could induce DNA fragmentation in MDBK as well as in ST cells. However, PRV did not induce a distinct oligonucleosomal DNA cleavage pattern as exhibited by other alphaherpes viruses, e.g. herpes simplex virus type 1 [15, 16], varicella-zoster virus [24] and bovine herpes virus type 1 [12]. Activation of caspase 3-like activity, commonly associated with programmed cell death, was detected. Our experiments showed that caspase 3-like activity increased in a time dependent manner after PRV infection of MDBK cells. Thus, the morphologic and biochemical results from the above experiments demonstrated that PRV can induce apoptosis in cultured cells.

PRV can undergo a lytic or a latent infection when exposed to a susceptible host. As shown in this study, progeny viruses were produced during a lytic infection which resulted in death of the host cell. During a latent infection, the host cell survives and harbors the PRV genome. As such, the apoptotic process inducible by PRV must be inhibited during the establishment of a latent infection.

Therefore, understanding how PRV induces apoptosis will provide insights into the events involved in PRV latency.

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