Induction of apoptosis in vitro and in vivo by H-1 parvovirus infection

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Rodent parvoviruses belonging to the autonomous parvovirus group are small, non-enveloped, nuclear-replicating viruses with a linear single-stranded DNA genome of about 5 kb. Early studies on their pathogenicity showed that these viruses cause foetal and neonatal abnormalities by destroying specific cell populations that proliferate rapidly during normal development (Kilham & Margolis, 1975; Margolis & Kilham, 1975), and that they inhibit tumorigenesis by oncogenic viruses to study the prevention and treatment of human cancer (Rommelaere & Cornelis, 1991).

Increasingly, virus infection is considered to trigger programmed cell death, and virus-induced apoptosis contributes to the cytopathogenic effects of various viruses, such as Sindbis virus (Levine et al., 1993), influenza virus (Takizawa et al., 1993), chicken anaemia virus (Noteborn et al., 1994), human immunodeficiency virus (Meyaard et al., 1992) and herpes simplex virus (Koyama & Adachi, 1997). Whether H-1 parvovirus induces apoptosis in infected cells is not yet known, however. In this communication, we show evidence of apoptosis induced in vitro and in vivo by H-1 virus infection.

H-1 virus, obtained from the ATCC, was passaged in secondary rat embryonic cell cultures and stocks were prepared for subsequent experiments. C6 rat glioblastoma cells, obtained from the Riken Cell Bank (Tsukuba, Japan), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS). The confluent cultured cells were trypsinized, washed with PBS, and suspended in DMEM with 5% FCS, and plated at 4 x 10^4 cells per well in a multi¬well tissue culture plate, and cell viability was measured daily for up to 6 days by Trypan blue staining. Infected C6 cells showed no detectable change in cell number over the first 2 days, and then viability decreased rapidly (data not shown).

To make a preliminary assessment of virus cytotoxicity, infected cells were plated at 2 x 10^4 cells per well in a multi¬well tissue culture plate, and cell viability was measured daily for up to 6 days by Trypan blue staining. Infected C6 cells showed no detectable change in cell number over the first 2 days, and then viability decreased rapidly (data not shown). Most infected cells were dead by 6 days post-infection, while mock-infected cells proliferated exponentially from 2 to 5 days post-infection.

To determine whether cell death in H-1 virus-infected cells was due to apoptosis, we analysed the cells for chromatin condensation and DNA fragmentation. H-1 virus-infected and mock-infected cells were harvested with trypsin at 4 days post-infection, washed with PBS, fixed in 2% buffered paraformaldehyde at 4 °C for 24 h and subsequently stained with Hoechst...
Fig. 1. Chromatin condensation and apoptotic bodies revealed by Hoechst staining (a, b), and DNA fragmentation (c, d), in H-1 virus-infected (b, d) and mock-infected (a, c) C6 cells. Bar, 25 &mu;m. Hardly any DNA was recovered from cells where the DNA was not fragmented, since DNA extraction was carried out by a selective extraction method to obtain only low molecular mass DNA (Hirt, 1967).

Fragmented DNA was extracted from cells by the method of Hirt (1967), with minor modifications, and analysed for the presence of oligonucleosomal DNA ladders by electrophoresis on a 1-5% agarose gel. Morphological changes associated with apoptosis, such as chromatin condensation and the appearance of apoptotic bodies, were seen in H-1 virus-infected C6 cells (Fig. 1b). Oligonucleosomal DNA ladders were seen in infected C6 cells at 2 days post-infection (Fig. 1d). These apoptotic changes were not, however, seen in mock-infected cells (Fig. 1a, c). These findings indicate that H-1 virus infection induces apoptotic changes in C6 cells.

The apoptosis induction mechanism has been extensively studied. Several members of a new cysteine protease family, the caspases, including caspase-1 (ICE/CED-3) (Miura et al., 1993) and caspase-3 (CPP32) (Nicholson et al., 1995), are important components of the mammalian apoptosis pathway. We studied the participation of caspases in H-1 virus-induced apoptosis in C6 cells by an inhibitor assay. Two types of inhibitor were used: acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) as a caspase-1-like protease inhibitor, and acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO; Bio-mol) as a caspase-3-like protease inhibitor. Infected cells were cultured for 12 h in DMEM plus 10% FCS, and various concentrations of tetrapeptides were added. At 72 h post-infection, infected cells were harvested with trypsin, and the percentage of apoptotic cells was determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL), as described below. The per-
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Fig. 2. Effect of caspase inhibitors on H-1 virus-induced apoptosis. C6 cells were mock-infected or infected with H-1 virus and cultured for 72 h with various concentrations of inhibitors of caspase-1 (Ac–YVAD–CHO, hatched bars) or caspase-3 (Ac–DEVD–CHO, filled bars). The graph shows the percentage of apoptotic cells with each treatment, as determined by the TUNEL assay. The percentage of apoptotic cells was calculated by counting 200 cells on triplicate slides. Virus-induced apoptosis was inhibited in a dose-dependent manner by the caspase-3-specific inhibitor, but not by the caspase-1-specific inhibitor (Fig. 2). Our results suggest that the caspase-3-dependent apoptosis activation pathway is induced by H-1 virus infection in C6 cells.

To test whether apoptosis can be induced by H-1 virus infection in vivo, newborn rats (Sprague–Dawley rats, purchased from CLEA Japan) were inoculated intraperitoneally with 10⁵ TCID₅₀ H-1 virus. Infected newborns suffered emaciation, jaundice and ataxia. They were sacrificed by bleeding under ethylether anaesthesia at 7 days post-infection. Brain tissue was collected and fixed in 4% buffered paraformaldehyde at 4 °C for 24 h, embedded in paraffin wax, and 5 µm sections were cut. Serial sections were examined for the distribution of infected and apoptotic cells by in situ hybridization and in situ end-labelling, respectively. For in situ hybridization, a 370 bp digoxigenin (DIG)-labelled oligonucleotide probe from the H-1 virus NS region was prepared as described elsewhere (Lo et al., 1988). Hybridization and visualization procedures were performed essentially according to Gaertner et al. (1993). Briefly, deparaffinized sections were rehydrated, digested with 40 µg/ml proteinase K (Sigma) for 10 min at 37 °C, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, and denatured at 65 °C for 10 min in hybridization buffer (0.3 M NaCl, 20 mM Tris–HCl pH 7.5, 1% SDS, 20 µg/ml salmon sperm DNA, 50% formamide) at 42 °C for 60 min. The slides were then hybridized with the DIG-labeled probe for 18 h at 42 °C. Following hybridization, the slides were washed and visualized with a fluorescein anti-DIG antibody conjugated with alkaline phosphatase (Roche Diagnostics) at 37 °C for 1 h. The slides were then incubated with NBT/ST reagents (SIGMA) for 5 min at room temperature.

Fig. 3. Detection of virus DNA and apoptosis in cerebellum sections from newborn rats. In situ hybridization to virus DNA in sections from newborn rat cerebellum showed high concentrations of virus DNA in the external granular cells of the cerebellum of H-1 virus-infected rats (a) but not in mock-infected rats (b). H-1 virus-infected rats also showed a significant increase in apoptosis in the cerebellum, as detected by the TUNEL assay (c), compared with mock-infected rats (d). Bar, 50 µm.
formamide and 1 x Denhardt’s solution). Sections were covered with 100 μl hybridization solution supplemented with 200 ng/ml DIG-labelled probe, incubated overnight at 50 °C, and visualized with a DIG DNA detection kit (Boehringer Mannheim) and alkaline phosphatase-labelled anti-DIG antibody. TUNEL was performed as described elsewhere (Gavrieli et al., 1992), with minor modifications. Tissue sections, digested with 40 μg/ml proteinase K for 15 min at room temperature, were treated with 2% H2O2 in PBS for 5 min to inactivate endogenous peroxidase activity. Sections were rinsed with PBS, and TdT (0·3 U/μl, BRL) and biotin-16-dUTP (10 μM, Boehringer Mannheim) in TdT buffer (30 mM Tris–HCl pH 7·5, 50 mM MgCl2, 0·6 mM 2-mercaptopethanesulfonic acid and 0·5 mg/ml BSA) were added. Sections were incubated in a humid atmosphere at 37 °C for 60 min. After rinsing, sections were reacted with peroxidase-conjugated streptavidin (Vectastain, Vector Laboratories), and stained with 3,3′-diaminobenzidine tetrachloride. Slides were counterstained with methyl green.

Virus DNA was easily detected in the granular layer cells of the cerebellum. This observation is consistent with previous descriptions of virus distribution in infected rats (Gaertner et al., 1993). The apoptosis-specific signal was present at a significantly higher frequency in infected tissue than in mock-infected controls, and was observed in the same areas as the virus-infected cells in the cerebellum (Fig. 3). Interestingly, parvovirus pathogenesis following transplacental infection in newborn rats or hamsters includes cerebellar hypoplasia, hepatitis, and craniofacial and periodontal lesions (Toolan, 1979). These lesions may be related to apoptosis induced by H-1 virus.

Our study shows that H-1 virus infection induces caspase-3-dependent apoptosis in C6 cells. Apoptosis induced by H-1 virus may also play an important role in parvovirus pathogenicity in vivo.

Several other agents, including TNF-α (Yin et al., 1995), s-Myc (Asai et al., 1994a) and IGF (Resnicoff et al., 1995), were recently shown to induce apoptosis in C6 cells, but the pathway mediating apoptosis in response to these signals is only poorly understood. An increased severity of the parvovirus cytopathic effect correlates with the effect of transformation by various virus and cellular oncogenes, including simian virus 40 large T and polyomavirus middle T antigens and Ha-ras, and by physical and chemical carcinogens (Legrand et al., 1992). Telerman et al. (1993) showed that p53 gene mutations are associated with sensitivity to H-1 virus-induced cell death in a human leukaemia cell line. Asai et al. (1994b) showed that C6 cells expressed wild-type but not mutant p53, and that apoptosis was induced by overexpression of s-Myc. s-Myc might mediate apoptosis in H-1 virus-infected C6 cells, since caspase-3-like proteases play a critical role in s-Myc-mediated apoptosis (Kagaya et al., 1997). It is well known that the apoptotic response is different depending on the cell type. Doering et al. (1986) demonstrated a nucleosomal repeat pattern, indicating apoptosis, in MVM(i)-infected lymphoma cells. Op De Beeck & Caillet-Fauquet (1997), however, detected no sign of apoptosis in fibroblasts expressing NS-1 of MVM(p), even though NS-1 expression induced the formation of nicks in the chromatin. Several researchers have reported that the intracellular accumulation of autonomous parvovirus NS proteins is cytotoxic, especially in neoplastic cells (Mouset et al., 1994), and that the cytotoxicity of parvovirus NS protein is modulated by cellular factors (Caillet-Fauquet et al., 1990).

Further research clarifying the molecular links between cytotoxic NS proteins and the apoptotic signalling pathway should provide interesting insights into parvovirus pathogenicity and oncosuppression.

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References


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