Productive replication of caprine arthritis–encephalitis virus is associated with induction of apoptosis

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Caprine arthritis–encephalitis virus (CAEV), an ungulate lentivirus, causes a natural infection in goats, which occurs mainly as a subacute paralysis that begins a few weeks after birth and encephalitis–arthritis that affects adult animals (Narayan et al., 1993). Laboratory strains of CAEV replicate efficiently in goat synovial membrane (GSM) or tahr lung (Yaniv et al., 1985) cell lines. In both cell types, virus replication is associated with a specific cytopathogenic effect (CPE) which precedes cell death. The CAEV specific CPE is characterized by syncytia generated through fusion of infected cells with surrounding susceptible cells, presumably due to the interaction of the viral envelope glycoproteins with cell receptors (Moore et al., 1993).

Recently, it has become evident that virus infection might trigger programmed cell death, and it has been suggested that virus induced apoptosis contributes to the cytopathogenic effects of various viruses, including several lentiviruses (Shen & Shenk, 1995). Moreover, in human immunodeficiency virus (HIV) infection, in addition to the direct killing of infected CD4+ T cells by apoptosis (Laurent-Crawford et al., 1991; Terai et al., 1991), it has been suggested that apoptosis of uninfected T cells can be responsible for T cell depletion and the eventual collapse of the immune system (Meyaard et al., 1992). Also, in human T lymphotropic virus type I infection, lymphocyte apoptosis was recently suggested to be implicated in the development of fulminate disease (Leno et al., 1995). It is thus conceivable that major features of virus pathogenesis might originate from processes associated with apoptosis. The present study provides data suggesting that the major mechanism of cell death in infection with CAEV in vitro is associated with the induction of apoptosis.

To investigate whether CAEV infection is associated with apoptosis, tahr lung or GSM cells were infected at an m.o.i. of 10–20, and at various times post-infection cell monolayers were stained with DAPI to visualize cells with aberrant chromatin organization. As shown in Fig. 1, panel (I) CAEV infection was associated with the appearance of cells with morphological changes characteristic of apoptosis, such as chromatin condensation and nuclear fragmentation. Enumeration of apoptotic CAEV-infected tahr cells as revealed by DAPI staining established the maximal level of apoptosis as 24%. A similar level of apoptosis was detected in CAEV-infected GSM cells also. It is noteworthy that similar relatively low levels of apoptosis were detected in H9 cells following infection with HIV-1 virions (Maldarelli et al., 1995). Several reports have suggested that cell killing by HIV might be due to damaged cell membranes that result from syncytium formation and massive budding of viral particles (Garry, 1989; Stevenson et al., 1990). However, our results show that during the first days of virus infection, prior to syncytia formation, individual cells manifested an apoptotic nucleus (Fig. 1, panel I). This suggests that although the progression of cell apoptosis correlated with the development of CPE, syncytia formation was not a prerequisite for CAEV-induced apoptosis.

To further substantiate the hypothesis that the observed morphological changes represent apoptotic cell death, tahr cell monolayers, on the third day of infection, were analysed by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) method, which identifies cells containing DNA strand breaks (Gavrieli et al., 1992). Fig. 1, panel (II) shows parallel TUNEL (b, d) and DAPI (a, c) staining. The intense fluorescence of the apoptotic cells suggested that the chromatin inside had been fragmented. There was complete correlation between TUNEL positivity and apoptotic nuclei as revealed by DAPI staining, as each one of the TUNEL-positive cells exhibited chromatin morphology.

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Fig. 1. Apoptotic features of CAEV-infected cells. Panel (I), detection of apoptosis by cell morphology. Tahr lung (Yaniv et al., 1985) and GSM cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS). Tahr (a, b, c) or GSM (d) cells were infected with CAEV at an m.o.i. of 10–20. Two hours after adsorption, cells were re-fed with medium containing 10% FCS. Infection was followed by the development of a specific CPE, manifested as multinuclear vacuolated cells, and the detection of reverse transcriptase activity in cell supernatants (not shown). At 6 days after infection, cells were fixed with 4% paraformaldehyde (PFA), air-dried and stained with DAPI (0–1 µg/ml). Cellular morphology was assessed by fluorescence microscopy at 380–420 nm, original magnification ×200. The apoptotic cells detected are either single (a, b) or multinucleated syncytial (c, d) cells. The percentage of CAEV-infected tahr cells with clearly condensed and/or fragmented nuclei was determined as 24%. Panel (II), detection of apoptosis by the TUNEL assay. The assay was done as described by Gavrieli et al. (1992). At 3 days after infection, CAEV-infected tahr cells were fixed in 4% PFA, washed with PBS and stored at −20 °C until use. Cells were then treated with 0–2% Triton X-100, intensively washed with PBS and incubated at room temperature for 10 min in TdT buffer (30 mM Tris pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). After the addition of 50 U TdT (Boehringer) and 1 nM biotin-16-dUTP (Boehringer), cells were incubated at 37 °C for 1 h, washed with PBS and incubated at 37 °C for 30 min in Extra-Avidin-FITC (BioMarker) diluted 1:50 in PBS. After washing with PBS, cells were stained with DAPI. Cells were viewed by fluorescence microscopy for DAPI staining (a, c) as described above, and at 510–530 nm for the TUNEL assay (b, d). (a) and (c), and (b) and (d), depict the same field of cells, respectively. The TUNEL-positive cells in B and D exhibit apoptotic features as detected in A and C respectively. Original magnification ×200. Panel (III), electrophoretic detection of DNA fragmentation. Six days after CAEV infection, 1±5×10⁶-infected (lane B) or mock-infected (lane A) tahr cell monolayers were trypsinized and then digested with 100 µg/ml of proteinase K at 37 °C for 12 h in TNE buffer (10 mM Tris–HCl, pH 7-4, 100 mM NaCl, 1 mM EDTA). After phenol extraction, RNase A (100 µg/ml of TNE) was added for 1 h at 37 °C and following further phenol extraction and ethanol precipitation,
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Fig. 2. Quantification of CAEV-infected tahr cells by infectious centre assay. Tahr cell monolayers were infected with CAEV at an m.o.i. of 50. Following virus adsorption for 1 h at 37 °C, cells were trypsinized, treated with proteinase K (50 µg/ml) for 15 min at 4 °C to eliminate residual unadsorbed virus particles, and then washed with PBS. Equal numbers (10, 50 or 100) of infected or mock-infected cells were then seeded onto 5 cm Petri dishes containing 24 h uninfected tahr cell monolayers. After 2 h incubation at 37 °C, cell monolayers were overlaid with DMEM containing 10% FCS and 1.5% carboxymethylcellulose (CMC). Six days later, cell monolayers seeded with uninfected (c) or infected (a, b) cells were Giemsa-stained and photographed (original magnification: a, × 10; b, c, × 20). Staining with anti-Rev serum (d) was done as follows: after fixing with 4% PFA, cells were washed with PBS and incubated with normal donkey serum for 1 h at room temperature; after washing in PBS, cells monolayers were incubated for 1 h at 37 °C with anti-Rev serum (1:50) that was induced in rabbits against a bacterially synthesized N terminus Rev peptide (Gazit et al., 1996). Cells were then incubated with donkey anti-rabbit IgG–FITC (Jackson; 1:100) for 1 h at 37 °C and visualized by using a confocal microscope (25 mW Ar and He–Ne lasers with 488, 514 and 543 nm maximum lines) coupled to an inverted microscope (LSM 410, Zeiss); magnification, ×10. DAPI staining (e) was done as described above, and cell monolayers were photographed by using an inverted fluorescence microscope at 380–420 nm (magnification, ×10).

DNA samples were electrophoresed on 1% agarose containing 0.5 µg/ml ethidium bromide and photographed under UV illumination. Lane M, HindIII-digested λ DNA size markers. Panel (IV), flow cytometric patterns of nuclear DNA of CAEV-infected tahr cells. Nuclear DNA was fluorescently labelled by the method described by Vindelov et al. (1983). Mock-infected or CAEV-infected tahr cells (10⁶ per sample) were harvested at 3, 6, 8 and 10 days after infection using 5 mM EDTA and centrifuged at 1430 g for 10 min. The cells were resuspended in 200 µl of citrate buffer, pH 7.6, containing sucrose (250 mM), trisodium citrate (40 mM) and dimethylsulfoxide (DMSO) (5%); 1.6 ml of trypsin (30 µg/ml; Sigma) was added for 10 min, then 1.5 ml of a solution containing trypsin inhibitor (2.5 mg/ml; Sigma) and ribonuclease A (0.1 mg/ml; Sigma) for 10 min, followed by the addition of 1.5 ml of a solution containing propidium iodide (PI) (2.08 mg/ml; Calbiochem) and spermine tetrahydrochloride (5.8 mg/ml) for 15 min. PI-stained cells (1 × 10⁶) were then analysed for fluorescence intensity on a FACS IV flow cytometer and data were processed using Consort 40 software (Becton Dickinson). Apoptotic cell nuclei (marked by arrows) containing hypodiploid DNA emitting fluorescence in channels 10–200 were enumerated as a percentage of the total population: 3 days – 4.7%; 6 days – 11%; 8 days – 17%; 10 days – 17.4%.
the activation of an endogenous Ca/Mg-dependent endonuclease activity (Bortner et al., 1995). Gel electrophoresis analysis of DNA extracted from infected versus mock-infected tahr cell monolayers showed the DNA ‘ladder’ commonly observed in apoptotic cells (Fig. 1, panel III).

Evaluation of the level of apoptosis by flow cytometry is based on a reduction of DNA content and cell shrinkage and is associated with the appearance of a hypodiploid peak in the DNA histogram (Darzynkiewicz et al., 1992). Our results showed a progressive increase in the ‘Sub-G1’ cell population of CAEV-infected tahr cells, which occurred with the advance of virus infection, up to a plateau level of 17% that was reached after 8 days of infection (Fig. 1, panel IV).

The fact that the number of apoptotic cells within the CAEV-infected cell population did not appear to exceed 17–24%, although cells were infected with CAEV at an m.o.i. of 10–20, might result from two possibilities: (i) only part of the tahr cell population became infected, in spite of the high virus burden, or (ii) all cells became infected, but just a few underwent apoptosis following infection. To discriminate between these two possibilities, the number of infected cells was determined by the infectious centre technique. Tahr cells were infected with CAEV at an m.o.i. of 50, and following virus adsorption, various numbers of infected cells were seeded onto uninfected tahr cell monolayers and plaque appearance was monitored (Fig. 2). To confirm that each plaque originated from a single CAEV-infected cell, cell monolayers were treated with anti-Rev antibodies, which were previously shown to react with the CAEV Rev protein and to efficiently detect CAEV-infected cells (Gazit et al., 1996). Results indicated that the cells surrounding each plaque contained CAEV-specific Rev protein. Furthermore, DAPI staining confirmed that these CAEV-infected cells exhibited apoptotic features. Enumeration of CAEV-induced plaques established that the initial number of CAEV-infected tahr cells reached 40%. This suggests that tahr cells are in fact a mixed population comprising CAEV-permissive and nonpermissive cells. Further cell cloning studies are required to establish this.

However, since the level of apoptosis, as evaluated by DAPI staining or by FACS analysis, did not exceed 17–24%, experiments were performed to investigate whether all CAEV-infected cells underwent apoptosis. Tahr cells were infected with CAEV at an m.o.i. of 20 and, 1, 2, 4, 6, 8 and 10 days after infection, cell monolayers were reacted with anti-Rev antibodies, and then stained with DAPI. The level of infected cells rose from 3% at day 2, up to 20% at day 6, after which the percentage declined because most of the infected cells detached from the substratum and were washed away during slide processing. All Rev-positive cells exhibited apoptotic features, and the majority of cells that contained condensed chromatin or apoptotic bodies which were still confined within cell membranes were Rev-positive (Fig. 3a, b). Late apoptotic cells, in which apoptotic bodies were already dispersed, were Rev-negative, due presumably to distortion of the cell membrane. Interestingly, in the few cases in which a Rev-negative cell with condensed chromatin was observed, it appeared in close contact with a Rev-positive apoptotic cell. This is reminiscent of the recent data suggesting the induction of apoptosis by cell-to-cell transmission of HIV-1, as a result of CD4–Env interactions (Maldarelli et al., 1995). However, establishment of a similar mechanism for CAEV-induced apoptosis must await further studies.

Our data suggest that the major mechanism of cell death in CAEV infection is associated with apoptosis. The observation
that the level of apoptosis is lower (17–24%) than the initial number of infected cells (40%) can be explained by our observations (unpublished) that the surrounding uninfected cells, in contrast to the infected ones, continue to replicate until reaching confluency. Our data showing that the level of the G2 population did not decrease with the advance of virus infection (Fig. 1, panel III) is consistent with this hypothesis. In addition, this discrepancy might result from the easy detachment of late apoptotic cells during staining.

In HIV, it was recently shown that apoptosis occurs in vivo predominantly in bystander cells, rather than in productively infected cells (Finkel et al., 1995; Li et al., 1995). It thus remains to be determined whether, in CAEV infection, apoptosis also occurs in vivo, and whether it occurs in neighbouring uninfected cells. Understanding these processes might help in revealing the mechanisms associated with CAEV pathogenesis.

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References


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