Cell death induced by cytopathic bovine viral diarrhoea virus is mediated by apoptosis

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Cells infected with two closely related isolates of bovine viral diarrhoea virus (BVDV), one cytopathic (CP) and one non-cytopathic (NCP), were examined for signs of apoptosis. The results from labelling DNA using terminal transferase and biotinylated dUTP and by observing oligonucleosomal-sized DNA fragmentation indicated that the CP strain of BVDV induced apoptosis in cell culture but the NCP strain did not. Induction of apoptosis correlated with infected cells undergoing apoptosis rather than interactions between infected and uninfected cells and the induction of apoptosis by CP BVDV was a dominant trait in cells co-infected with both types of virus.

Bovine viral diarrhoea virus (BVDV) is a pestivirus within the family Flaviviridae and is associated with a variety of disorders of cattle. Infection is common but the severity of the outcome ranges from subclinical or very mild in the majority of cases to fatal mucosal disease. Strains of BVDV fall into two biotypes which differ according to their pathogenicity in certain cultured cells: one biotype causes no visible cytopathology whilst the other causes cell death. Infection of cattle with either virus usually results in a mild transient infection. However, infection of a cow with a virus of the non-cytopathic (NCP) biotype during pregnancy can result in more striking disease. Abortion or teratogenic effects are common and infection during the first 120 days of pregnancy can result in the birth of persistently infected calves. Field observations together with experimental reproduction of mucosal disease in cattle have shown that cattle persistently infected with NCP virus develop mucosal disease on superinfection by an antigenically homologous cytopathic (CP) virus (for review see Brownlie, 1991).

The genome of BVDV is positive ssRNA and encodes a single polypeptide which is subject to post-translational cleavage by both virus and cellular proteinases to produce the mature virus polypeptides. The length of the genome of NCP viruses is approximately 12.5 kb (Deng & Brock, 1992; Meyers et al., 1992). In contrast, the genome of CP viruses varies from 8 kb, in a defective virus (Tautz et al., 1994), to 16.5 kb (Meyers et al., 1992). A striking difference between the two biotypes of BVDV is that CP viruses express a non-structural protein of molecular mass 80 kDa (p80) in infected cells, whereas NCP viruses do not (Donis & Dubovi, 1987; Pocock et al., 1987). The homologue of p80 protein in cells infected by NCP BVDV is a virus protein, p125, and the difference in size is due to distinct changes in genomic organization in the region coding for p125. Protein p80 represents the C-terminal two-thirds of p125 (Collett et al., 1988; Deng & Brock, 1992; De Moerlooze et al., 1993; Meyers et al., 1992) and it has been shown that p80 may be generated by insertion of non-viral sequences into the genome (Collett et al., 1988; De Moerlooze et al., 1993), duplication of regions of the virus genome (Meyers et al., 1992) or deletion within the virus genome (Tautz et al., 1994). All these arrangements affect p125 or p80 and it is therefore likely that the expression of p80 determines the cytopathogenicity of viruses.

Viruses from several genera and families are able to induce apoptosis (programmed cell death) in infected cells. Cells undergoing apoptosis show certain morphological changes (cell shrinkage, pronounced cell surface swelling and chromatin condensation) and DNA fragmentation to oligonucleosomal size, which is taken as one of the biochemical hallmarks of apoptosis. Some viruses encode proteins that block apoptosis, e.g. Epstein–Barr virus (Gregory et al., 1991), adenovirus (Rao et al., 1992) and baculoviruses (Clem et al., 1991), whereas human immunodeficiency virus (Laurent-Crawford et al., 1991), influenza viruses (Takizawa et al., 1993) and Sindbis virus (Levine et al., 1993) are examples of viruses that induce apoptosis. In this report, we examined DNA fragmentation in cells infected with BVDV by two methods and show that the CPE seen in cells infected by CP virus is accompanied by the DNA fragmentation typical of apoptosis.

Cells [calf testis (CTe) or MDBK] grown on cover slips were infected with two BVDV isolates: Pe515 CP and Pe515 NCP (Pocock et al., 1987). Pe515 CP virus was used at an m.o.i. of 10 p.f.u. per cell and Pe515 NCP virus at 10 TCID₅₀ per cell. At 48 h post-infection, the cells were fixed with 4% formaldehyde.
Virus antigen was detected by immunofluorescence using bovine anti-BVDV and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-bovine IgG antibodies. DNA degradation, a marker for apoptosis, was detected using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) method as described by Gavrieli et al. (1992). In this procedure TdT was used to transfer biotin-labelled dUTP to the 3' termini of fragmented DNA which was detected by fluorescence after reaction with rhodamine-labelled avidin. The cells infected with NCP virus showed high levels of FITC fluorescence in the cytoplasm (Fig. 1b, h) in comparison with the uninfected cells (Fig. 1a, g). No significant fluorescence of rhodamine was detected in the nuclei of either uninfected or NCP virus-infected cells (Fig. 1d, e, j, k). In contrast, a strong fluorescence of fluorescein in the cytoplasm and rhodamine in the nuclei was observed in the cells infected by CP virus (Fig. 1c, f, i, l). Moreover, the strong fluorescence of rhodamine correlated with morphological changes in the cells (e.g. shrinkage) caused by BVDV CP virus. These results indicate that CP BVDV causes DNA damage in infected cells but that NCP virus does not.

As the characteristic oligonucleosomal DNA ladder of apoptotic cells is considered a hallmark of apoptosis, experiments were carried out in an attempt to identify oligonucleosomal-sized products in cells infected with BVDV. CTe cells (10⁶) were infected with CP or NCP BVDV at the m.o.i. ratio described above. Cells were lysed at 48 h post-infection in a buffer containing 1% SDS and 100 μg/ml of proteinase K.
Fig. 2. Apoptotic DNA fragmentation caused by infection of CP BVDV. (a) CTE cells (10^6) were infected with CP or NCP 
Pe515 virus at the m.o.i. ratio described in Fig. 1; persistently infected CTE cells (10^6) were prepared by infection with Pe515 
NCP at 1 TCID_{50} per cell and subsequently were superinfected with CP virus at an m.o.i. of 10 p.f.u. per cell. DNA (2 \mu g) 
prepared from these cells at 48 h post-infection, as described by Gavrieli et al. (1992), was subjected to electrophoresis 
through a 1:6% agarose gel and then stained with ethidium bromide. Lane 2, DNA from cells treated with dexamethasone 
preparing the oligonucleosomal banding of apoptotic cells; lane 3, DNA from CP virus-infected cells; lane 4, DNA from CP 
virus-infected cells which were persistently infected with NCP virus; lane 5, DNA from NCP virus-infected cells; lane 6, DNA 
from uninfected cells; lane 1, Haelll-digested \Phi X 174 DNA as molecular size marker. (b) CTE cells (10^6) were infected with CP 
or NCP Pe515 virus at the m.o.i. ratio described in Fig. 1. DNA samples from cells infected with CP virus were isolated at 0, 2, 
4, 8, 12, 24, 36 and 48 h post-infection (lanes 3–10) and DNA from uninfected and NCP virus-infected cells was purified at 
48 h post-infection (lanes 1 and 2), as described by Gavrieli et al. (1992). Two \mu g of each sample were subjected to 
electrophoresis through a 1:6% agarose gel and stained with ethidium bromide. Haelll-digested \Phi X 174 DNA was used as 
molecular size marker (lane 11).

(Boehringer Mannheim) and incubated at 37 °C for 6 h as 
described by Gavrieli et al. (1992). DNA was extracted with 
phenol–chloroform, precipitated with ethanol, resuspended in 
water and treated with ribonuclease at a final concentration of 
100 \mu g/ml at 37 °C for 1 h. To serve as a positive control 
for apoptosis, CTE cells (10^6) were treated with dexamethasone in 
5% ethanol at a final concentration of 10 mm for 18 h at 37 °C 
and DNA was prepared using the method described above. 
DNA (2 \mu g) was subjected to electrophoresis through a 1:6% 
agarose gel and stained with ethidium bromide. A DNA 
staining pattern typical of the oligonucleosomal banding of 
apoptotic cells was obtained from cells treated with dexam-
ethasone (Fig. 2a, lane 2) and an identical DNA pattern was 
observed from cells infected with CP virus (Fig. 2a, lane 3). 
DNA isolated from cells infected with NCP virus showed no 
difference from that of uninfected cells and no oligo-
nucleosomal DNA bands were seen. DNA fragmentation was 
visible from 12 h after infection of cells with CP virus and 
reached maximum levels between 24 and 48 h post-infection 
(Fig. 2b), the time at which CPE becomes apparent. These 
results reinforce the conclusion drawn from the previous 
experiments and show that the DNA damage caused by 
infection of CP BVDV is typical of that shown during 
apoptosis.

As described above, certain DNA viruses produce poly-
peptides that function to block cellular apoptosis. Therefore 
the possibility that the NCP virus might obstruct apoptosis 
was examined by infecting cells with NCP BVDV and 
superinfecting with CP virus. Persistently infected CTE cells 
were obtained by infecting virus-free cells with Pe515 NCP 
virus at m.o.i. of 1 TCID_{50} per cell and incubating the culture 
at 37 °C for 72 h. The final virus titre in the culture fluid was 
10^6 TCID_{50} per ml and 100% of cells were infected as shown 
by immunofluorescence (data not shown). Cells were then 
infected with Pe515 CP virus at an m.o.i. of 10 p.f.u. per cell. 
DNA was extracted at 48 h post-infection and analysed as 
described above. The same characteristic DNA ladder of apo-
ptotic cells as seen in the single infection with CP virus was 
observed (Fig. 2a, lane 4). These observations correlate with 
the ability of Pe515 CP virus to form plaques on cells 
persistently infected with Pe515 NCP (data not shown). These 
results show that CP BVDV is able to induce the DNA 
fragmentation indicative of apoptosis in cells that are persis-
tently infected by NCP virus and that this biotype does not, 
therefore, prevent cell death caused by CP virus infection.

The viruses used in this study are a 'pair' isolated from an 
animal suffering from mucosal disease. The CP virus contains 
a duplication in the genome to produce a copy of p125 and a
copy of p80 (Meyers et al., 1992). The two viruses replicate to similar levels in CTe cells and produce a similar titre of virus in frozen and thawed lysates of infected cells. They produce a similar level of p125 in infected cells, estimated by quantitative Western blotting of cell extracts made at 24 h post-infection and, allowing for the presence of two copies of the p80 epitope in cells infected with CP virus, they produce a similar signal in an ELISA on infected cell lysate using a MAb to p80/p125 (data not shown).

Because CP and NCP viruses can replicate equally well in cells, apoptosis associated with CP virus infection is likely to be induced through a biochemical pathway, in which interaction between virus protein and host factors plays an important role. As p80 protein only exists in cells infected by CP viruses, it is a likely candidate for induction of apoptosis. Collett and colleagues have demonstrated that p80 protein has RNA-stimulated NTPase, RNA helicase and proteinase activities (Wiskerchen & Collett, 1991; Tamura et al., 1993; Warrener & Collett, 1995). There is evidence that certain proteinases are able to induce apoptosis. Two striking examples are the mammalian interleukin-1β-converting enzyme (ICE) (Miura et al., 1993) and a protease resembling ICE (prICE), which has a similar profile of sensitivity to inhibitors as ICE but has a distinct substrate specificity (Lazebnik et al., 1995). However, no homology has been identified between the p80 protein and ICE. The other possibility for the role of p80 protein in cytopathogenicity of BVDV could be that p125 protein interacts with cellular factors to block apoptosis in cells infected by NCP virus and p80 protein inhibits the function of p125 protein. Following this hypothesis, in cells CP virus inhibits an activity of NCP virus which inhibits apoptosis, so the cells undergo apoptosis.

We have examined tissue from animals infected with BVDV either as an asymptomatic persistent infection or as mucosal disease for signs of apoptosis. Our results have been inconclusive because of high levels of background cell death. We cannot therefore conclude, at present, whether cell death in mucosal disease is due to apoptosis of infected cells, as in cell culture. However, such a model is consistent with the pathology of the disease, characterized by destruction of lymphoid follicles in the Peyer’s patches and erosion of the overlying epithelium, which are thought to be a direct consequence of infection with CP virus (Brownlie, 1991).

In conclusion, we have shown that a CP strain of BVDV induced apoptosis in cell culture but a NCP strain did not. Induction of apoptosis correlated with infection of the cells undergoing apoptosis rather than by interactions between infected and uninfected cells. The induction of apoptosis by CP BVDV was a dominant trait in cells co-infected with CP and NCP viruses.

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