Apoptosis Precedes Necrosis of Fish Cell Line with Infectious Pancreatic Necrosis Virus Infection

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The current view of infectious pancreatic necrosis virus (IPNV) infection includes a necrotic process that relies primarily on the histological appearance of tissue after the degenerative process. We tested this view by examining the possibility that apoptosis is a component of double-stranded RNA virus (IPNV) that induces fish embryonic cell death. Four kinds of assays for apoptosis were used in analyzing IPNV-infected CHSE-214 cells: (1) assay with terminal deoxynucleotidyl transferase (TdT)-mediated end-labeling of DNA in nuclei of intact cells during virus infection, (2) assay for procoagulant activity, (3) assay for DNA ladders, and (4) electron microscopic assays for the ultrastructural changes in characteristic apoptotic cells. In all p.i. samples, both low and high m.o.i. groups contained apoptotic nuclei, according to TdT-mediated dUTP labeling of intact cells, but in control CHSE-214 cells, apoptotic nuclei were rare at all levels of incubation sampled by TdT-mediated dUTP labeling. Preneoteric or postnecrotic cells were found to express phosphatidylserine on the surface by annexin V–FITC labeling, but normal cells did not. DNAs from both 4 h p.i. of high m.o.i. and 8 h p.i. of low m.o.i. were found to be cleaved into fragments indicative of preferential cleavage at internucleosomal sites. The IPNV-infected CHSE-214 cells were analyzed with an electron microscope and showed a pattern of ultrastructural change, indicating that apoptosis appears before pathological changes of necrosis, including condensed chromatin, fragmented nuclei, nuclei with chromatin margination, and secondary necrosis from preneoteric cells in IPNV-infected CHSE-214 cells. Together, these findings show that apoptosis precedes any detectable necrotic change in CHSE-214 cells that is currently viewed as necrosis. Thus, apoptosis characterizes the onset of pathology in host cells and is followed by necrotic processes.

Key Words: infectious pancreatic necrosis virus, apoptosis, fish cell, secondary necrosis.

INTRODUCTION

There are two major morphologically and biochemically distinct modes of death in eukaryotic cells: apoptosis and necrosis (Duvall and Wyllie, 1986; Kerr and Harmon, 1991; Wyllie et al., 1980). Apoptosis is characterized morphologically by cell shrinkage and hyperchromatic nuclear fragments and biochemically by chromatin cleavage into nucleosomal oligomers (Wyllie et al., 1980). Apoptosis is considered to be a physiological process involved in normal tissue turnover that occurs during embryogenesis, aging, and tumor regression (Wyllie et al., 1980), but pathological stimuli, such as viral infections (Gougeon and Montagnier, 1993; Inoue et al., 1997; Jacotot et al., 1997; Ohno et al., 1993; Noteborn et al., 1994; Rojko et al., 1992; Vasconcelos and Lam, 1994), can also be triggering factors. Necrosis is considered to be a pathological reaction that occurs in response to perturbations in the cellular environment such as complement attack, severe hypoxia, or hyperthermia. These stimuli increase the permeability of the plasma membrane, resulting in irreversible swelling of the cells (Wyllie et al., 1980).

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Infectious pancreatic necrosis virus (IPNV) is the prototype of the group of viruses known as Birnaviridae (Dobos et al., 1979). IPNV was discovered to be associated with a highly contagious disease of susceptible hatchery-reared trout. As the name indicates, the infection among trout produces marked pancreatic necrosis, but histopathological changes sometimes also occur in adjacent adipose tissue, in renal hematopoietic tissue, in the gut, and in the liver (Wolf et al., 1960). Histopathological changes can also occur in renal excretory and hematopoietic tissues, as first reported by Yasutake et al. (1965). Although renal damage is consistent with the high titer of virus typically found in kidneys, at least in carriers, fish, confirmation was first reported by Wolf and Quimby (1969). Sano et al. (1971, 1973) also described, in addition, illustrated renal damage from IPNV in rainbow trout and in amago (Oncorhynchus rhodurus). They found congestion or hemorrhage in glomeruli, edema, and destruction or desquamation of tubule epithelium. Swanson and Gillespie (1979) similarly noted focal degeneration of liver parenchymal cells in yearling Atlantic salmon that had been previously inoculated with IPNV. Liver damage is consistent with virological findings, and Kudo et al. (1973) showed that virions were present in hepatocytes.

An alternative hypothesis, which is proposed here, is
that the death of IPNV-infected host cells is initiated by apoptosis because the criteria used to characterize the death of IPNV-infected cells as an exclusively necrotic process are not sufficient to eliminate the possibility that apoptosis also occurs.

In the present investigation, to examine the possibility that apoptosis may contribute to the death of IPNV-infected cells, we assayed for DNA fragmentation and changes in cell structure that indicate apoptotic cell death. These findings indicated that IPNV induces cell apoptosis and then induces postapoptotic necrosis (secondary necrosis) in fish embryonic cells in vitro.

RESULTS

Light microscopic identification of apoptotic nuclei in prenecrotic CHSE-214 cells

Terminal deoxynucleotidyl transferase (TdT)-dUTP labeling of infected CHSE-214 cells without or with IPNV showed that apoptosis, as indicated by the presence of double-stranded DNA fragments in situ, occurred in virus-infected cells at all times sampled (Figs. 1 and 2). Rarely, the apoptotic nuclei were observed in 1% FCS serum CHSE-214 cells (1%; P < 0.05). This small concentration of apoptotic nuclei in control CHSE-214 cells may reflect an apoptotic, developmental process under low serum conditions. The concentration of apoptotic nuclei observed in the m.o.i. 20 group at 4, 8, and 12 h p.i. was 30%, 62%, and 57% (all P < 0.05), respectively. The apoptotic nuclear population in the m.o.i. 1 group at 4, 8, and 12 h p.i. was 4%, 39%, and 51% (all P < 0.05), respectively.

Visualization of the morphological changes of prenecrotic and postnecrotic cells in CHSE-214 cells

We used the CHSE-214 cell line to test the feasibility of using TdT biotin-dUTP nick end labeling (TUNEL) assay and annexin V-fluorescein labeling for the detection of apoptotic cells. First, we used the in situ TUNEL assay to identify prenecrotic and postnecrotic cells. The results, as shown in Figs. 3A and 3B, can be clearly seen in the prenecrotic cells (Fig. 3A) and postnecrotic cells (Fig. 3B). In a comparison of Fig. 3A with Fig. 3B, we found cell volume increased ~3-fold, and the cell morphology changed more dramatically in prenecrotic than in postnecrotic cells. We then confirmed the cell morphology of the prenecrotic and postnecrotic cells by annexin V in CHSE-214 cells during IPNV infection under the conditions described above. The results are shown in Figs. 3C through 3F. Figures 3C and 3E show the infected m.o.i. CHSE-214 cells double-stained with annexin V and propidium iodide at 8 and 12 h p.i., respectively. The double-stained cells were observed under phase-contrast microscopy. The samples in Figs. 3D and 3F were examined by fluorescence microscopy. The prenecrotic cells were annexin positive (Fig. 3D). At this same time, the membrane integrity remained intact, and the propidium iodide staining was negative. The postnecrotic cells were double positive (Fig. 3F). Postnecrotic cells were only weakly stained with annexin V, but because membrane integrity was lost, they showed positive staining with propidium iodide.

Induction of internucleosomal cleavage by IPNV

Because DNA fragmentation is a well-defined biochemical marker of apoptosis (Schwartzman and Cidlowski, 1993), we first examined the abilities of IPNV (E1-S) to induce DNA fragmentation in the fish embryonic cell line. DNA extracted from CHSE-214 cells with virus and CHSE-214 cells without virus as controls was examined for evidence of internucleosomal fragmentation, which occurs in apoptosis. Intense internucleosomal fragmentation of DNA, a pattern highly specific to apoptosis, was observed in CHSE-214 cells treated with IPNV (Fig. 4). DNA from high and low m.o.i. infected cells were found to be cleaved into fragments both at 4 h p.i. and at 8 h.p.i. On the other hand, the gel of the negative control at 0 and 12 h of incubation showed no DNA fragment formation.

Electron microscopy shows that apoptosis precedes necrosis in CHSE-214 cell

Ultrastructural analysis showed sequentially morphological patterns of apoptosis from the prenecrotic to postnecrotic cells under IPNV (m.o.i. 1) infection. Figures 5A and 5B show uninfected CHSE-214 cells that were incubated for 12 h and for 0 h in 1% serum as a negative control. Condensated nuclei and nuclei with chromatin margination in the early apoptotic cells (at 4 h p.i.) are shown in Figs. 5C and 5D, respectively. At 8 h p.i., cells showed uniformly electron dense micronuclei and small micronuclei protruding from the nuclear periphery (Fig. 5E). Finally, at 12 h of p.i., cells also showed a swollen morphology and exhibited small micronuclei but contained the condensed chromatin and enclosed nuclear membranes (Fig. 5F). At this time, other organelles, such as mitochondria, in the cell appeared seriously swollen and lost their morphologies.

Apoptotic cells phagocytosed by neighbor cells

Figure 6 shows the apoptotic body phagocytosed by a neighbor cell. This apoptotic phagocytosed cell had the morphology typical of apoptotic nuclei, which is characterized by nuclear condensation and multimicronuclei. The phagocytosed fish embryonic cell appeared intact, suggesting that the fish embryonic cell recognized some surface change (as in Figs. 3D and 3F) that promoted their phagocytosis of apoptotic fish cells before lysis (Fadok et al., 1992).
DISCUSSION

The results of the present investigation indicate that the earliest fish cell line (CHSE-214) changes by IPNV are apoptotic rather than necrotic. Specifically, the presence of nuclei containing double-stranded DNA fragments and abnormal chromatin-condensations before evidence of cell necrosis is a strong indicator of apoptosis. Morphological assays for apoptosis are essential in this complex at the intact cell instance of cell death because apoptosis is rapid and cell death in the degenerating CHSE-214 cell is not synchronized. Thus only a small fraction (1%) of nuclei would be expected to be apoptotic at the same time in control cells. The present findings indicate that both m.o.i. 1 (4%) and m.o.i. 20 (30%) are recognizably apoptotic cells at 4 h p.i. when assayed by TdT-mediated dUTP labeling of virus-infected cells. However, nuclei are detectable apoptotic using this technique 2–4 h after the onset of apoptosis, according to time course studies on dexamethasone-stimulated thymocytes (Gavriel et al., 1992).

A recently discovered family of proteins, the annexins, has been found to have a high affinity for aminophospholipids in the presence of Ca\(^{2+}\) (Andree et al., 1990; Raynal and Pollard, 1994; Tait et al., 1989). A member of this family, annexin V, has been shown by several groups to preferentially bind phosphatidylserine (PS) (Andree et al., 1990; Tait et al., 1989; Thiagarajan and Tait, 1990). Thus annexin V provides a convenient tool with which to determine directly whether changes in membrane PS distribution occur as a general feature of apoptosis and to measure the kinetics of these changes on a single-cell basis. In this regard, a recent report demonstrated increased annexin V binding during serum withdrawal-induced apoptosis of murine germinal center B cells as well as a B cell line (Koopman et al., 1994). Here we found that prenecrotic and postnecrotic cells (Figs. 3D and 3F) induced by an m.o.i. 1 of IPNV were accompanied by dramatic changes in PS distribution on the plasma membrane (PM), as assessed by the increased annexin V-binding properties of these cells. The observations of Fadok et al. (1992) suggest that apoptotic lymphocytes lose membrane phospholipid asymmetry.
and expose PS on the outer leaflet of the plasma membrane. Macrophages, then phagocytose apoptotic lymphocytes, specifically recognize exposed PS. CHSE-214 cells that had ingested IPNV-infected CHSE-214 cells were examined by electron microscopy (Fig. 6). Phagocytosed virus-infected cells had the morphology typical of apoptosis, which is characterized by the multimicro- nuclei and cytoplasmic condensation. The phagocytosed IPNV-infected CHSE-214 cells appeared intact, suggesting that the CHSE-214 cells recognized some surface change (as PS) that promoted their phagocytosis of apoptotic IPNV-infected cells before lysis. These data

FIG. 5. Electron micrographs of CHSE-214 cells. (A) CHSE-214 from 12 h of incubation as a negative control. (B) CHSE-214 from 0 h incubation as the negative control. (C and D) Pronecrotic IPNV-infected CHSE-214 cells with m.o.i. 1 at 4 h p.i. showing the chromatin condensed (C) and chromatin margination (D), indicated by arrows. (E) Middle necrotic CHSE-214 cell at 8 h p.i. that formed uniformly electron dense micronuclei indicated by arrows. (F) Postnecrotic cell at 12 h p.i. (Arrow) The condensed chromatin enclosing the nuclear membrane. (Bar) 1 μm.
support the view that IPNV can induce CHSE-214 apoptotic cell death.

Apoptosis is a morphologically distinct cell death that spontaneously occurs in many different tissues under various conditions (Falcieri et al., 1994). It occurs in distinctly separated cells and progresses very rapidly, never causing exudative inflammation in tissues. No cell hydration takes place, but nuclear and cytoplasmic condensation can appear (see Fig. 5C), followed by the formation of numerous membrane-bound cell fragments termed apoptotic bodies. In contrast to necrosis, the nuclear organization is completely lost. Profound chromatin rearrangements take place, followed by the formation of a variable number of compact, electron-dense micronuclei (see Fig. 5E). Surprisingly, despite the extensive nuclear changes, both cytoplasm and organelar components remain intact for some time unless the cell undergoes secondary necrosis (see Fig. 5F). Only in the final apoptotic stage is the whole cell strongly involved and undergoes a secondary necrosis (Falcieri et al., 1994).
In the biochemical features of apoptosis when CHSE-214 cells were infected with different m.o.i. of IPNV E1-S, in the m.o.i. 20 treatment group the nucleosomal degradation quickly happened at 30 min p.i., but in the m.o.i. 1 treatment group nucleosomal degradation did not occur until 6 h p.i. (data not shown). We tested whether the protein synthesis inhibitor cycloheximide (CHX) could prevent cell death by IPNV infection (Ghibelli et al., 1992; Martin, 1993). When treating the 10 µg/ml CHX with CHSE-214 cells before m.o.i. 1 infection, we found that cell death could be prevented, but in the m.o.i. 20 treatment group it could not (data not shown). We proposed that there might be some different mechanism involved in inducing cell death by infection with different m.o.i. in CHSE-214 cells. However, further experiments are required to evaluate the significance of our findings of how different m.o.i. infections relate to the induction of host cell death.

IPNV was discovered to be associated with a highly contagious disease of susceptible hatchery-reared salmonids (Hedrick et al., 1983; Wolf, 1960; Wu et al., 1983) and other nonsalmonid fish (Adair and Ferguson, 1981; Chen et al., 1984, 1985; Hedrick et al., 1983; Ueno et al., 1984). As the name indicates, the infection among trout produces marked pancreatic necrosis, but histopathological changes sometimes also occur in adipose tissue, in renal hematopoietic tissue, in the gut, and in the liver (Sano, 1973). Pancreatic necrosis is typically evident in acinar cells (Sano, 1973, 1971), and extensive areas can be involved. Some affected acinar inclusions; the inclusions, however, as in previous reports, were products of cellular breakdown and not true viral inclusions. We have shown that the exposure of a fish embryonic cell line to IPNV can induce apoptosis with all its associated characteristics: DNA fragmentation, nuclear and cellular segmentation, micronuclei formation, and, finally, post-apoptotic necrosis from early-stage apoptotic cells. Necrotic cell death may occur during natural infections, but these features support the hypothesis that IPNV causes CHSE-214 cells to undergo apoptosis and then post-apoptotic necrosis in vitro.

MATERIALS AND METHODS

Cell culture and virus infection

Chinook salmon embryo cells (CHSE-214) were obtained from the American Type Culture Collection (ATCC). Embryo cells grown at 18°C as monolayers in plastic tissue culture flasks (Nunc) using Eagle’s minimum essential medium (MEM) supplemented with 10% (vol/vol) fetal calf serum (FCS) and 25 µg/ml gentamicin.

E1-S of IPNV Ab strain was isolated from Japanese eel in Taiwan (Wu, 1987). E1-S was propagated in CHSE-214 cell monolayers at an m.o.i. of 0.01 cell. Infected cultures were incubated at 18°C until an extensive cytopathogenic effect (CPE) was observed. Virus plaque assay was performed as previously described by Dobos et al. (1977).

TdT-dUTP labeling

Cells were counted, and 10^3 cells/0.1 ml were seeded onto the two wells of a chamber slide (Nunc) at 18°C for 20 h. Before infection, the 10% FCS MEM was changed to 1% FCS MEM. The virus-infected group received m.o.i. 1 and m.o.i. 20 of IPNV serotype Ab (strain E1-S), which had been propagated and titrated in CHSE-214 cells and had a TCID_{50} of 10^9/0.1 ml. The negative control group received 1.5 ml of 1% FCS MEM. The two treatment groups were then incubated at 18°C for 8 h in an incubator.

At the end of various incubation times (4, 8, and 12 h), each sample was removed from the medium and washed with PBS, and then cell samples were fixed with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at room temperature. Slides were washed with PBS and incubated with blocking solution (0.3% H_2O_2 in methanol) for 30 min at room temperature. Slides were rinsed with PBS and then incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 5 min on ice. Slides were rinsed with PBS. Next, 50 µl TUNEL reaction mixture (as an in situ cell death detection kit; Boehringer-Mannheim) was added to the sample, and the slide was incubated in a humidified chamber for 60 min at 37°C. Samples were either analyzed under a fluorescence microscope in this state or 50 µl of anti-fluorescein antibody conjugated with horseradish peroxidase (POD) was added to the sample to signal conversion that was analyzed under a phase-contrast microscope. The slide was incubated in a humidified chamber for 60 min at 37°C and rinsed with PBS. Finally, 50±100 µl of DAB substrate solution (Boehringer-Mannheim) was added for 10 min at room temperature. The slide was then rinsed with PBS, mounted under a glass coverslip, and analyzed under light microscope.

Cell counts

The slides of both virus-infected CHSE-214 and uninfected control cells were labeled with TdT-dUTP and examined by light microscopy using phase-contrast optics. The number of TdT-dUTP-labeled nuclei in each sample was counted per 200 cells.

Results were expressed as mean ± SEM. Data were analyzed using either paired or unpaired Student’s t tests, as appropriate. A value of P < 0.05 was taken to represent a statistically significant difference between group mean values.

Annexin V-FITC labeling

An analysis of PS on the outer leaflet of apoptotic cell membranes was performed using annexin V-fluorescein and propidium iodide (PI) to determine differentiation of
apo-optic from necrotic cells. The cell preparation and virus infection conditions are described above. At the end of the various incubation times (0, 4, 8, and 12 h), each sample was removed from the medium and washed with PBS, and then cells were incubated with 100 μl of staining solution (annexin V–fluorescein in a HEPES buffer containing PI; Boehringer-Mannheim) for 10±15 min. Evaluation was by fluorescence microscopy using 488-nm excitation and a 515-nm long-pass filter for detection.

DNA preparation and gel electrophoresis

About 10^5 cells/ml were seeded onto a 60-mm petri dish for growth above 20 h. Monolayer cell growths were rinsed twice with PBS. Then all control groups or infection groups were cultured in 1% FCS MEM. The high m.o.i. group received m.o.i. 20 infected CHSE-214 and was incubated over the same time periods. The control and high m.o.i. and low m.o.i. groups were also used for DNA fragmentation studies. At the end of incubation, the cells were lysed with lysis buffer (10 mM Tris–HCl, 0.25% Triton X-100, 1 mM EDTA, pH 7.4). After treatment with phenol–chloroform–isoamyl alcohol (25:24:1), the DNA was precipitated in the presence of 0.3 M sodium acetate and cold 20 mM EDTA, pH 7.4). After treatment with phenol–chloroform–isoamyl alcohol (25:24:1), the DNA was precipitated in the presence of 0.3 M sodium acetate and cold absolute ethanol at –70°C for 2 h and then resuspended in 10 mM Tris–HCl (pH 7.4) and 1 mM EDTA. Aliquots of 20 μl containing ~5±10 μg of DNA were then electrophoresed in 12% agarose gels for 3 h at 40 V. Gels were stained with ethidium bromide and photographed under UV transillumination.

Electron microscopy

The trypsinized cells were collected by centrifugation, washed twice with PBS, and then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h. After a wash with the sodium cacodylate buffer, the cells were postfixed in 1% aqueous osmium tetroxide for 2 h, followed by a wash in the same buffer. They were then dehydrated in a series of ethanol solutions of decreasing dilution and embedded in a Spurr’s mixture. Semithin sections were stained with toluidine blue to count morphological features of apoptosis. Electron microscopy thin sections were stained with lead citrate and uranyl acetate and then observed using an electron microscope (Hitachi H-7000).

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REFERENCES


