Mechanism of cell death during infectious salmon anemia virus infection is cell type-specific

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Infectious salmon anemia virus (ISAV) is a very important fish virus in the Northern hemisphere and there is continued interest in understanding the mechanisms of its pathogenesis and persistence in fish. In this study, the permissive fish cell lines SHK-1, CHSE-214 and TO were used to determine whether ISAV-induced cytopathic effect (CPE) is due to apoptosis or necrosis. Characteristic apoptotic DNA fragmentation was observed only in ISAV-infected SHK-1 and CHSE-214 cells. Apoptosis in ISAV-infected SHK-1 cells was confirmed by fragment end-labelling assay, suggesting that CPE in these cells is associated with apoptosis. ISAV-infected TO cells did not undergo apoptosis, but showed leakage of high-mobility group 1 (HMGB1) protein from the nucleus, which is characteristic of cells undergoing necrosis; this suggests that CPE in these cells is associated with necrosis. ISAV-infected SHK-1 cells did not show leakage of HMGB1 protein. Infection with two different strains of ISAV showed that induction of apoptosis was correlated with the appearance of CPE in SHK-1 cells. ISAV-induced apoptosis was inhibited by a pan-caspase inhibitor, Z-VAD-fmk, indicating a caspase-activation pathway. The ISAV putative PB2 protein and proteins encoded by RNA segment 7 bound caspase-8 specifically in vitro, suggesting that these viral proteins may have a role in ISAV-induced apoptosis. These findings demonstrate for the first time that the mechanism of cell death during ISAV infection is dependent on the cell type, which may have implications for ISAV pathogenesis and persistence.

INTRODUCTION

Infectious salmon anemia (ISA) virus (ISAV) is the causative agent of ISA, which is a highly infectious disease of farmed Atlantic salmon in the Northern hemisphere. This virus is a member of the family Orthomyxoviridae, genus Isavirus (Falk et al., 1997; Krossøy et al., 1999; www.ncbi.nlm.nih.gov/ICTVdb/ictv/index.htm). The clinical disease in farmed Atlantic salmon is characterized by high mortality with exophthalmia, pale gills, ascites, haemorrhagic liver necrosis, renal interstitial haemorrhage and tubular nephrosis. ISAV is known to cause overt and fatal systemic infection in farmed Atlantic salmon and asymptomatic infection in feral fish, a situation analogous to that caused by avian influenza viruses in domestic poultry and feral birds (reviewed by Kibenge et al., 2004). ISAV may infect and replicate in sea trout, brown trout, rainbow trout, eels, herring and Arctic char, resulting in asymptomatic, probably lifelong carriers of the virus (reviewed by Kibenge et al., 2004). In Atlantic salmon, ISAV targets leukocytic cells (Falk et al., 1995) and endothelial cells (Falk & Dannevig, 1995), or endothelial-associated cells (Falk et al., 2001) and macrophages (Moneke et al., 2003). Permissive fish cell lines for ISAV include salmon head kidney (SHK-1) cells (Dannevig et al., 1995); TO cells (Wergeland & Jakobsen, 2001); and Atlantic salmon kidney (ASK-2) cells (Rolland et al., 2002), which are macrophage-like cell lines (Dannevig et al., 1997; Wergeland & Jakobsen, 2001; Rolland et al., 2002) in which virus replicates with production of cytopathic effect (CPE). Although all three cell lines are derived from the Atlantic salmon pronephros, they are distinctly different in their growth characteristics (e.g. media requirements, split ratio and cell composition), they display distinctly different virus-induced CPE (Kibenge et al., 2001; Rolland et al., 2002) and they interact differently with virus–antibody mixtures during the virus neutralization test (Joseph et al., 2003). Some strains of ISAV can also replicate and cause CPE in the Chinook salmon embryonic cell line (CHSE-214) (Bouchard et al., 1999; Kibenge et al., 2000; Griffiths et al., 2001). Virus replication also occurs in the Atlantic salmon (AS) cell line (Sanchez et al., 1993) and the rainbow trout gills (RTgill-W1) cell line (Bols et al., 1994), but in these cases the virus is non-cytopathic.

Two general pathways that are known to cause CPE and eukaryotic cell death during virus infection are apoptosis
and necrosis. Morphological characteristics of necrosis include swelling and rapid cell degradation, disruption and loss of plasma membrane integrity, accompanied by extensive cytoplasmic vacuolation (Goudie, 1985). Necrosis is also characterized by the release of high-mobility group 1 (HMGB1) protein, a chromatin-binding factor, from necrotic cells. The release of HMGB1 protein triggers inflammatory reactions that result in extensive tissue damage (Scaffidi et al., 2002). In contrast, apoptosis is an energy-dependent, genetically controlled process of cell death that occurs in response to a wide variety of stimuli (Budihardjo et al., 1999). Apoptosis plays an integral role in many normal physiological processes, including homeostasis, tissue differentiation and elimination of harmful cells such as tumorogenic, mutated or virus-infected cells (Everett & McFadden, 1999; Green, 2000). Apoptosis progresses through a series of morphological and biochemical changes, including cytoplasmic shrinkage, chromatin condensation and intranucleosomal cleavage, phosphatidylserine exposure, plasma membrane blebbing and cell fragmentation into apoptotic bodies that are phagocytosed by macrophages or other surrounding cells without provoking an inflammatory response (White, 1996; Vaux & Strasser, 1996; O’Brien, 1998; Bowen-Pope & Schaub, 2001; Hay & Kannourakis, 2002; Watanabe et al., 2002). Most, if not all, of these changes are effected by members of a family of cysteine proteases called caspases, which consist of an initiator caspase (such as caspase-8 and -9), which cleave and activate other caspases, and an effector caspase (such as caspase-3, -6 and -7), which cleave a variety of cellular substrates, thereby disassembling cellular structures or inactivating enzymes within the nucleus of the affected cell (reviewed by Degterev et al., 2003).

A critical step in viral pathogenesis is the ability of a virus to inhibit host antiviral responses through the inhibition of host gene expression and/or by interfering with programmed cell death. Apoptosis of virus-infected cells can be caused by virus-induced inhibition of host gene expression or by the antiviral response of the host. Viruses have developed various strategies either to promote or inhibit apoptosis, or even to do both at different stages in their replication cycle in the host cell (reviewed by Everett & McFadden, 1999; Roulston et al., 1999; Alcamí & Koszinowski, 2000; Barber, 2001; Boya et al., 2001). Many viruses block the apoptotic responses to ensure efficient virus production; some induce apoptosis, resulting in virus dissemination and protection from an immune response (Hay & Kannourakis, 2002). In some cases, the onset of apoptosis can be beneficial to the virus, where phagocytosis of apoptotic bodies by neighbouring cells leads to dissemination of the virus (Mi et al., 2001). Certain viruses may depend on apoptosis to destroy the integrity of cells near the end of the replication cycle, facilitating the release and spread of the viral progeny (Hay & Kannourakis, 2002). Influenza A virus is known to induce apoptosis both in vivo and in vitro in the latest stages of infection. Influenza virus infection induces the expression of Fas, and Fas-mediated apoptosis has been suggested as an important mechanism of cell death during influenza virus infection. The role of Fas in influenza A virus-induced apoptosis is supported by the activation of caspase-8, but not caspase-9, in virus-infected cells (Balachandran et al., 2000). It has been shown that the induction of Fas expression by influenza virus or viral dsRNA is mediated partly by the dsRNA-dependent protein kinase (PKR), indicating the involvement of the antiviral response of the host cell in the induction of apoptosis during influenza A virus infection (Balachandran et al., 2000). Caspase-mediated cleavage of nucleocapsid protein (NP) of human strains of influenza A virus also supports the antiviral role of apoptosis during influenza virus infection (Zhirnov et al., 1999). In this case, caspase activation may limit virus production, as the truncated form of NP cannot be used for proper virus assembly (Zhirnov et al., 1999).

The role of various viral proteins during influenza virus-induced apoptosis has also been studied. It has been shown that NS1 (non-structural) protein is capable of inducing apoptosis when expressed in cell cultures (Schultz-Cherry et al., 2001). Other studies, however, suggest that NS1 also possesses anti-apoptotic potential (Zhirnov et al., 2002b). Experiments in Madin Darby canine kidney (MDCK) cells demonstrated that neuraminidase (NA) could activate latent transforming growth factor beta to its biologically active form, a broad inducer of apoptosis (Morris et al., 1999). Inhibitors of NA delayed the onset of apoptosis when added shortly after infection. Also, viruses with highly active NA induced apoptosis in host cells more rapidly than did those with less active NA (Morris et al., 1999). Interaction of M1 (matrix) protein of influenza A virus with cellular caspase-8 suggests that M1 protein may have a role in virus-induced apoptosis (Zhirnov et al., 2002a). A new influenza virus gene product, PB-F2, produced by a +1 reading frame in the viral RNA segment that encodes polymerase subunit PB1, has also been shown to play important roles in influenza virus-induced apoptosis (Chen et al., 2001). Thus, influenza viruses may have multiple mechanisms that contribute to the induction of apoptosis in host cells.

Earlier studies also suggested that apoptosis facilitated replication of influenza virus in cell cultures. Overexpression of the anti-apoptotic protein Bcl-2 inhibited influenza A virus-induced apoptosis and reduced virus production in MDCK cells; the blocking effect was attributed to modified glycosylation of the haemagglutinin (HA) protein (Olsen et al., 1996). This argument is supported by a recent study that demonstrated that caspase-3 activation is essential for influenza A virus propagation in cell cultures (Wurzer et al., 2003). Inhibition of caspase-3 activity by caspase-3 inhibitor or small interfering RNAs blocked efficient replication of influenza A virus in cell cultures. Inhibition of virus replication correlated with the retention of viral ribonucleoprotein complexes in the nucleus, which prevented production of progeny virus particles (Wurzer et al., 2003).
The role of apoptosis in ISAV pathogenesis has not been studied. Studies on ISAV-induced apoptosis may provide a clearer picture of the cellular mechanisms of viral persistence and pathogenesis in ISAV infection. In the present study, we investigated whether the death of cultured cells infected by ISAV was associated with apoptosis or necrosis. As caspase-8 is an upstream initiator caspase that cleaves and activates other caspases (Degterev et al., 2003), we also wished to determine whether it interacts with any ISAV protein. Such an interaction could shut down the caspase-activation pathway.

**METHODS**

**Cells and virus infection.** Each well of a six-well tissue-culture plate was seeded with 3 ml growth medium containing 1·5 × 10⁵ cells ml⁻¹. CHSE-214 cells were grown at 16 °C in HMEM (Eagle’s minimum essential medium containing Hanks’ salts; Invitrogen Life Technologies) as described previously (Kibenge et al., 2000). SHK-1 cells were grown at room temperature (24 °C) in Leibovitz L-15 medium (Invitrogen Life Technologies) as described previously (Dannevig et al., 1995). TO cells were grown in HMEM (BioWhittaker) as described by Wergeland & Jacobsen (2001). Cell monolayers (24 h old) were infected with ISAV isolate NBISA01 at an m.o.i. of 10 in maintenance medium; cells were harvested at 6, 12 and 18 h and 1, 2, 3, 4, 5 and 6 days post-infection (p.i.) for TO cells and SHK-1 cells, and at 1, 3, 5, 7, 9, 11 and 13 days p.i. for CHSE-214 cells. In a repeat experiment, SHK-1 and TO cells that were infected with a less virulent strain of ISAV, isolate U5757-1, were harvested at 12 h and at 1, 2, 3, 4, 5, 6 and 7 days p.i. The duration of infection studied in each cell line and virus were based on the development of CPE, determined in previous studies (Kibenge et al., 2000, 2001). All cell monolayers were washed twice with PBS before harvesting.

**Chromosomal DNA-fragmentation assay.** For isolation of chromosomal DNA, harvested cells were lysed with 500 μl lysis buffer [50 mM Tris·HCl (pH 8), 10 mM EDTA, 0·5% Sarkosyl, 0·5 mg proteinase K ml⁻¹] (Hong et al., 1998). Cells from two wells were pooled for each sample and the pooled lysate was incubated at 50 °C for 3 h. The cell extract was then centrifuged at equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol. DNA in the aqueous phase was precipitated with high salt and 100% ethanol at −80 °C overnight (Sambrook et al., 1989). For analysis, the DNA pellet was washed with 70% ethanol, vacuum-dried and resuspended in a solution of phenol/chloroform/isoamyl alcohol, vacuum-dried and resuspended in a solution of sterile/deionized water and 4 μl RNaseA, then loaded onto a 1·8% agarose gel. Electrophoresis was performed at 70 V for 3 h. Gels were stained with ethidium bromide and photographed under UV transillumination.

**Fragment end-labelling assay for apoptosis.** SHK-1 and TO cells were grown in slide flasks (Nunc). After 24 h, the cell monolayer was infected with ISAV strain NBISA01 at an m.o.i. of 10. Slides with TO cells and SHK-1 cells were processed for a fragment end-labelling assay for apoptosis at 24 and 48 h p.i., respectively. Cells were washed with PBS once before fixation with 4% paraformaldehyde for 10 min at room temperature. Fragment end-labelling of DNA and staining of apoptotic cells were carried out by using a TdT-FragEL DNA-fragmentation detection kit (Oncogene) according to the manufacturer’s instructions. Briefly, terminal deoxynucleotidyl transferase (TdT) enzyme was used to add biotin-labelled and unlabelled deoxynucleotides to the exposed 3’-OH ends of the DNA fragments that were generated in response to apoptotic signals. Biotinylated nucleotides were detected by using a streptavidin–horseradish peroxidase (HRP) conjugate. Diaminobenzidine was used to generate an insoluble, coloured substrate at the site of DNA fragmentation. Slides were mounted before viewing with a light microscope.

**Indirect fluorescent antibody test (IFAT) for necrosis.** SHK-1 and TO cells were grown in slide flasks (Nunc). After 24 h, the cell monolayer was infected with ISAV strain NBISA01 at an m.o.i. of 10. Slides with TO and SHK-1 cells were processed for IFAT for necrosis at 24 and 48 h p.i., respectively. Slides were fixed with fresh 4% paraformaldehyde for 10 min at room temperature and washed twice with PBS, then the cells were permeabilized by adding 0·2% Triton X-100 in PBS for 5 min at room temperature. Slides were washed three times with 0·2 % Triton X-100 in PBS, then incubated with a 1·50 dilution of anti-HMGBl polyclonal IgG antibody (Abcam) for 1 h at room temperature. Slides were again washed for 5 min with three changes of 0·2% Triton X-100 in PBS and then incubated with a 1·40 dilution of anti-rabbit IgG (whole molecule)–fluorescein isothiocyanate conjugate (Sigma) for 20 min at room temperature. The slides were washed as described above and examined under a fluorescent microscope. Intensity of fluorescent staining in the nuclei was assessed by spot densitometry using AlphaDigiDoc AD-1200 software in the AlphaEase FC imaging system (Alpha Innotech). On the digital images of cells, two-dimensional areas were created around the nuclei and fluorescence intensity was obtained through the corresponding pixel intensity values, which were expressed as integrated density value. Thus, intensity of fluorescent staining in the nuclei of 30 cells from three different fields (10 cells per field) was measured. The difference between the mean integrated density values from the ISAV-infected and uninfected control cells was assessed by using an unpaired, two-sample t-test.

**Assay for caspase-3 activity.** SHK-1 and TO cells were grown in growth medium at a concentration of 10⁶ cells per well in six-well tissue-culture plates (Nunc). After 24 h, the cell monolayer was infected with ISAV strain NBISA01 at an m.o.i. of 10. Infected cells and uninfected control cells were harvested at 6 and 12 h and 1, 2, 4 and 6 days p.i. For this, the cell monolayer was washed two times with PBS. The cells were trypsinized, then the trypsin was neutralized with 1 ml growth medium. Cells were harvested by centrifugation at 1000 g for 10 min, counted, and 1 × 10⁵ cells were lysed with 50 μl lysis buffer (Caspase-3 assay kit; Calbiochem). The cell lysate was centrifuged at 10 000 g for 10 min and the supernatant was collected and frozen immediately at −80 °C until used. ELISA for caspase-3 activity was performed according to the manufacturer’s instructions (Calbiochem).

**Treatment of ISAV-infected SHK-1 cells with caspase inhibitor.** SHK-1 cells were grown in 3 ml growth medium (1·5 × 10⁶ cells ml⁻¹) in six-well tissue-culture plates. After 24 h, the confluent cell monolayers were treated with a cell-permeable preparation of the pan-caspase inhibitor Z-VAD-fmk at a concentration of 20 μM for 4 h. Control cells were treated with DMSO (used as diluent of Z-VAD-fmk). After replacing with fresh medium containing caspase inhibitor, cells were infected with ISAV strain NBISA01 at an m.o.i. of 10. Cells were harvested after 4 days infection when there was >50% CPE. Cellular DNA was isolated from virus-infected cells in the presence and absence of caspase inhibitor, and from uninfected control cells treated with either caspase inhibitor or DMSO. Isolated DNA was analysed for apoptotic DNA fragmentation by agarose gel electrophoresis as described above.

**Interaction of ISAV proteins with cellular caspases.** Poly-styrene microtitre plates (Falcon Pro Bind Assay plates) were used to investigate the interaction of ISAV proteins with caspase-8, following the method described by Zhironov et al. (2002a) with minor modifications. For the production of viral proteins, ORFs of
10 putative proteins of ISAV were amplified from purified ISAV RNA by RT-PCR and PCR products were cloned into pCRII vector, which has dual promoters (T7 and Sp6) in opposite orientation (Invitrogen Life Technologies). Plasmids were sequenced to check for the correct orientation and to verify each viral cDNA sequence. ISAV proteins were then produced by an in vitro transcription—translation reaction using the TNT T7/SP6 coupled reticulocyte lysate system (TNT-RLS; Promega), following the manufacturer’s instructions and as described previously (Kibenge et al., 2004), except that the translation mix used cold methionine instead of [35S]methionine. Each well of the microtitre plate was coated overnight with 15 μl reaction product in 85 μl ELISA coating buffer (0.2 M bicarbonate buffer, pH 9.6) at 4 °C. Three replicates were set up for each protein. A non-related protein, luciferase, also produced by the TNT reaction, was used as a negative control. The well surface was then saturated with 3 % goat serum prepared in 1 × Dulbecco’s PBS with 0.05 % (v/v) Tween 20 for 1 h at room temperature. The caspase-8 (BioVision) solution at a concentration of 2 U (100 μl)−1 in 1 × T-PBS containing 1 % goat serum was then added to each well on the plate (100 μl per well) and incubated at 15 °C for 2 h. Rabbit polyclonal anti-caspase-8 antibody (BioVision) at a concentration of 1 μg ml−1 in 1 × T-PBS containing 1 % goat serum was then added (100 μl per well) and the microtitre plate was further incubated. The concentrations of caspase-8 and anti-caspase-8 antibody for use were determined in a preliminary experiment by checkerboard titrations of recombinant human caspase-8. The immune complex formed was detected by a reaction with secondary antibody conjugated to HRP (Bio-Rad) diluted 1:3000. Each step was separated by extensive washing using 1 × T-PBS. The substrate for HRP, ABTS [2,2′-azino-di(3-ethylbezthiazoline-6-sulfonic acid)] (Bio-Rad), was then added and the extent of the reaction was assessed by colour intensity, as registered with an automated microtitre ELISA reader (SpectraMax 340) at 415 nm.

RESULTS

Apoptosis in ISAV-infected cells is cell type-specific

To determine whether cellular DNA of ISAV-infected cells undergoes fragmentation that is characteristic of apoptosis, DNA was isolated from SHK-1, CHSE-214 and TO cells at different time points after infection with ISAV and analysed by gel electrophoresis. DNA fragmentation was observed in SHK-1 cells that were infected with ISAV strain NBISA01, beginning on day 3 p.i., and with ISAV strain U5575-1, beginning on day 6 p.i., when CPE was apparent. Intensity of the DNA fragmentation increased (Fig. 1a) as CPE increased. No DNA fragmentation was observed in uninfected SHK-1 cells (Fig. 1b). DNA fragmentation was also observed in CHSE-214 cells that were infected with NBISA01, beginning on day 9 p.i., which corresponded with the appearance of CPE. No DNA fragmentation was observed in uninfected CHSE-214 cells. CHSE-214 cells that were infected with U5575-1 did not show CPE or DNA fragmentation. No DNA fragmentation was observed in ISAV-infected and uninfected TO cells, although the infected cells showed CPE by day 2 with NBISA01 and by day 4 with U5575-1, which progressed to completion by 5–7 days p.i. Consistent with these results, the fragment end-labelling assay for apoptosis also revealed apoptotic cells in ISAV-infected SHK-1 cells (Fig. 2b1–b3). Extensive condensation of nuclei (Fig. 2b1–b3, arrows) was observed in the apoptotic cells when compared with intact, rounded nuclei of uninfected cells (Fig. 2a). By using this assay, apoptotic cells were detected in virus-infected SHK-1 cells after 48 h p.i. No apoptotic staining was detected in uninfected (Fig. 2c) or infected (Fig. 2d) TO cells.

TO cells undergo necrosis during ISAV infection

Previous studies have shown that the HMGB1 protein is a specific marker for necrotic cells, as this protein leaks out rapidly into the extracellular space when membrane integrity is lost during necrosis (Scaffidi et al., 2002). An indirect fluorescent antibody test using an anti-HMGB1 antibody showed bright fluorescence only in the nuclei of uninfected TO cells (Fig. 3a), whereas there was reduced fluorescence in the nuclei of ISAV-infected TO cells (Fig. 3b), which is indicative of HMGB1 protein leakage. Neither uninfected (Fig. 3c) nor ISAV-infected (Fig. 3d) SHK-1 cells showed leakage of HMGB1 protein. Spot densitometry analysis showed the reduction in fluorescence between ISAV-infected and uninfected TO cells to be statistically significant (P=0.018), whereas no significant difference was observed between ISAV-infected and uninfected SHK-1 cells (P=0.597) (Fig. 4). These observations suggest that necrosis was the predominant mechanism of cell death in ISAV-infected TO cells, in contrast to ISAV-infected SHK-1 cells, which died by apoptosis.

Fig. 1. Agarose gel electrophoresis showing apoptosis in ISAV-infected cells. (a) SHK-1 cells infected with ISAV strain NBISA01, showing apoptotic DNA fragmentation. Lane 1, 1 kb+ DNA ladder; lanes 2–4, DNA isolated from ISAV-infected SHK-1 cells at 3, 4 and 6 days p.i., respectively. (b) Uninfected SHK-1 cells. Lane 1, 1 kb+ DNA ladder; lanes 2–4, DNA isolated from uninfected control SHK-1 cells harvested at 3, 4 and 6 days, respectively.
Apoptosis in ISAV-infected cells is caspase-dependent

Caspase-3 is a central player in apoptosis regulation and its activity is often measured to determine the impact of a given apoptotic stimulus (Watanabe et al., 2002). We therefore performed a colorimetric assay using a commercial kit (Calbiochem) in order to determine caspase-3-like proteolytic activity in ISAV-infected SHK-1 and TO cells. No caspase-3-like activity was detected in either infected or control SHK-1 and TO cells. In order to establish whether other caspases are involved in ISAV-induced apoptosis, a cell-permeable preparation of a pan-caspase inhibitor, Z-VAD-fmk, was used to determine whether it could inhibit the induction of apoptosis in ISAV-infected SHK-1 cells. Use of the pan-caspase inhibitor Z-VAD-fmk at a concentration of 20 μM inhibited apoptosis, as well as CPE, in ISAV-infected SHK-1 cells, suggesting that ISAV-induced apoptosis in these cells is caspase-dependent.

Fig. 2. Fragment end-labelling of chromosomal DNA in cells undergoing apoptosis. (a) Uninfected SHK-1 cells (× 160) after TdT-FragEL staining. (b1–b3) ISAV strain NBISA01-infected SHK-1 cells (× 250) undergoing apoptosis at 48 h p.i., showing TdT-FragEL staining in the nucleus (arrows). (c) Uninfected TO cells (× 160) and (d) ISAV strain NBISA01-infected TO cells (× 160) (24 h p.i.) after TdT-FragEL staining.
apoptosis occurs through the caspase-activation pathway. In order to establish whether any of the ISAV proteins could interact with the caspase-activation pathway, the eight RNA genomic segments of ISAV were expressed in vitro with rabbit reticulocyte lysates and the products were tested for binding with caspase-8, an upstream initiator caspase. Only three of the 10 putative ISAV proteins that were tested bound caspase-8. A specific, strongly positive signal was observed with the RNA segment 7 ORF2 product, and a weaker positive signal was observed with both RNA segment 7 ORF1 product and ISAV putative PB2 protein (Table 1).

**DISCUSSION**

Viruses can kill cells by either necrosis or apoptosis (Hardwick & Griffin, 1997). The significance of virus-induced apoptosis has not yet been elucidated fully. Previous studies on apoptosis of virus-infected cells revealed that, in some cases, apoptotic cell death brings on concomitant abortion of progeny virus production, suggesting that virus-induced apoptosis can have some role in the host defence mechanism against virus infection (Clem & Miller, 1994). There are several potential mechanisms by which viruses activate the apoptotic pathway. Some viruses may do so through the direct action of specific viral proteins,
Mean apoptosis and uninfected SHK-1 cells.

such as adenovirus E1A protein (Roulston et al., 1999). Other viruses induce apoptosis indirectly through their effects on cellular functions, for example by shutting down protein synthesis or by activating pro-apoptotic proteins, such as upregulation of Fas in influenza virus infection (Hardwick & Griffin, 1997; Balachandran et al., 2000). In some disease states, apoptosis occurs only in those cells that are infected with the virus, as in Sindbis virus infection of neurons (Lewis et al., 1996). It is also conceivable that a virus-infected cell could cause apoptosis in an adjacent uninfected cell by a number of mechanisms, including secretion of factors that activate the death programme. A good example is the apoptosis of CD4$^+$ T cells in human immunodeficiency virus infection (Hardwick & Griffin, 1997). A recent study by Chu & Ng (2003) also documented one virus, West Nile virus, as killing Vero cells by either apoptosis or necrosis, depending on the initial infectious dose. Mechanisms of cell death that are caused by ISAV infection in cultured cells have not previously been studied.

To determine whether CPE caused by ISAV is associated with apoptosis, we used three different permissive fish cell lines, TO, SHK-1 and CHSE-214, and analysed them for the fragmentation of cellular DNA that results in a characteristic ‘DNA ladder’ when cells undergo apoptosis. During apoptosis, endonucleases are activated, which cleave the DNA into fragments at sites between the nucleosomes, resulting in regularly sized DNA fragments that separate as regularly spaced bands of multiples of approximately 200 bp on an agarose gel. In necrosis, the DNA fragments are cleaved randomly and dispersed, rather than concentrated in membrane-bound packages (Watanabe et al., 2002). The DNA-fragmentation assay showed that ISAV strain NBISA01 induced apoptosis in SHK-1 cells at 3 days p.i. (Fig. 1a), whereas a less virulent ISAV strain, isolate U5575-1, induced apoptosis by 6 days p.i. ISAV strain NBISA01, which replicates in CHSE-214 cells with production of CPE (Kibenge et al., 2000), also induced apoptosis in infected CHSE-214 cells, beginning at 9 days p.i. Apoptotic DNA fragmentation in SHK-1 cells was also confirmed by using a fragment end-labelling assay (Fig. 2). By using this assay, apoptotic cells could be detected in ISAV-infected SHK-1 cells as early as 48 h p.i. In both SHK-1 and CHSE-214 cell lines, the intensity of DNA fragmentation increased as CPE progressed (Fig. 1a). This indicated to us that the CPE in these cells was due to virus-induced apoptosis.

The absence of DNA fragmentation in ISAV-infected TO cells, which developed complete CPE, indicated to us that ISAV may kill TO cells by necrosis. In order to confirm this, we studied the release of HMGB1 protein, which is a chromatin-binding factor. Release of HMGB1 protein from the nucleus is used as a specific marker for necrosis, as cells undergoing apoptosis do not release HMGB1 (Scaffidi et al., 2002; Chu & Ng, 2003). Spot densitometry analyses showed a statistically significant reduction in fluorescent staining of HMGB1 protein in the nucleus of ISAV-infected TO cells (P=0·018; Fig. 4). This observation confirmed the release of this protein from the nucleus, due to the necrotic type of cell death that is induced by ISAV. Uninfected TO and SHK-1 cells, as well as SHK-1 cells undergoing apoptosis during ISAV infection, retained this protein in their nuclei (Fig. 3a, c, d). Brighter fluorescence, which is known to occur in the nucleus of cells undergoing apoptosis (Scaffidi et al., 2002), might have caused the slight, but not statistically significant, increase of fluorescence (P=0·597) in ISAV-infected SHK-1 cells (Fig. 4). This observation further confirmed that ISAV induces apoptosis in SHK-1 cells and necrosis in TO cells.

Further investigation of the ISAV-induced apoptosis

Table 1. Comparison of binding of caspase-8 by ISAV proteins

<table>
<thead>
<tr>
<th>Protein used to coat ELISA microtitre plate</th>
<th>OD$_{415}^*$</th>
<th>Interpretation</th>
</tr>
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<tr>
<td>Active recombinant human caspase-8 ISAV gene segment (ORF)</td>
<td>0·709</td>
<td>Positive control</td>
</tr>
<tr>
<td>1</td>
<td>0·0327 ± 0·05</td>
<td>Weak</td>
</tr>
<tr>
<td>7 (ORF 1)</td>
<td>0·0607 ± 0·05</td>
<td>Weak</td>
</tr>
<tr>
<td>7 (ORF 2)</td>
<td>0·1010 ± 0·09</td>
<td>Strong</td>
</tr>
</tbody>
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*Mean ± SEM OD readings after subtracting mean OD of negative control. Goat serum (3%) and luciferase were used as negative controls for caspase-8 and ISAV proteins, respectively.

Fig. 4. Quantification of nuclear fluorescence following immunofluorescent antibody staining for HMGB1 protein, using spot densitometry. Fluorescence intensity was obtained through the corresponding pixel intensity values and expressed as an integrated density value. Statistically significant reduction in fluorescence intensity was observed in the nuclei of ISAV-infected TO cells (P=0·018), compared with uninfected TO cells. No significant difference (P=0·597) was observed between the fluorescence intensity of ISAV-infected SHK-1 cells undergoing apoptosis and uninfected SHK-1 cells.

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revealed that it was not associated with caspase-3-like activity. Caspase-3 is a cysteine protease that is activated in the terminal apoptosis cascade. It is sequestered in the cytoplasm as a zymogen in its inactive form, and activated when cleaved by other caspases. Caspase-3-like activity is often used as a quantitative measure of apoptosis. Thus, its absence in ISAV-infected SHK-1 cells undergoing apoptosis was unexpected. As caspase-3 is part of the downstream effector caspases (Thornberry & Lazebnik, 1998), its absence in ISAV-infected SHK-1 cells undergoing apoptosis could suggest that the apoptosis occurs independently of the caspase-activation pathway. However, we were able to prevent apoptosis in ISAV-infected SHK-1 cells by using a broad-spectrum caspase inhibitor, Z-VD-fmk. The tripeptide Z-VD-fmk is an effective pan-caspase inhibitor (Leist & Jäättelä, 2001), which inhibits caspase activity optimally by fitting into the catalytic pocket of all caspases and cross-linking the fluoromethylketone (fmk) group to the cysteine in the active site (Nicholson, 1999). Alternatively, failure to detect caspase-3 activity could be because the caspase-3 assay reagents used were developed for use in mammalian cells, not fish cells. However, Guo et al. (2003) used a similar assay for caspase-3-like activity in sea bass (SB) cell lines and showed that activation of fish caspase-3 can be detected by using assays that were developed for mammalian cells. Z-VD-fmk has also been used successfully to inhibit virus-induced apoptosis in other fish cell lines, such as Grunt Fin (GF) cells (Imajoh et al., 2004). Therefore, these findings suggest that ISAV-induced apoptosis in SHK-1 cells occurs via the caspase-activation pathway, but may not involve activation of caspase-3. We further showed that ISAV proteins, particularly the protein encoded by segment 7 ORF2, have the potential to bind caspase-8 specifically, which might have implications in ISAV-induced apoptosis.

The absence of DNA fragmentation and the release of HMGB1 protein in ISAV-infected TO cells that developed complete CPE indicated that ISAV kills TO cells by necrosis, and that the apoptosis induced by ISAV is cell type-specific. The TO cell line consists of uniformly epithelial-like cells (Wergeland & Jakobsen, 2001), whereas the SHK-1 cell line contains at least two subpopulations of cells, one of which is fibroblast-like (Rolland et al., 2002) and the CHSE-214 cell line consists of fibroblast-like cells; only the ISAV-infected SHK-1 and CHSE-214 cells developed apoptosis. Although it is possible that the CPE observed in vitro may not be relevant to events in vivo, it is generally accepted that the execution of either apoptosis or necrosis in virus-infected cells reflects the pathogenicity of viruses (Hay & Kannourakis, 2002). We therefore speculate that ISAV infection leads to total destruction of highly susceptible cells such as TO cells, possibly by necrosis. Infection of such cell types in vivo (e.g. leukocytes) may lead to inflammatory reactions, due to the release of HMGB1 protein and subsequent immune response, and this may explain the clinical disease and pathology during a natural infection. At the same time, ISAV is capable of inducing apoptosis in cells such as SHK-1 and CHSE-214 cells. Infection of such cell types in vivo (e.g. in the heart and haematopoietic portion of the kidney) may cause no inflammatory reactions, and therefore subclinical disease and virus persistence, during a natural infection. Identification of the cellular factors that are involved in ISAV-induced apoptosis might lead to a better understanding of the mechanisms of persistence and pathogenesis of ISAV infection in vivo.

In conclusion, our study has demonstrated for the first time that ISAV is capable of inducing apoptosis and that this apoptosis is cell type-specific, as it occurred only in ISAV-infected SHK-1 and CHSE-214 cells. We further show that ISAV induced a necrotic type of cell death in TO cells. These findings suggest that the mechanism of cell death during ISAV infection is dependent on cell type, which may have implications for the pathogenesis and persistence of ISAV in Atlantic salmon.

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REFERENCES


ISAV causes cell type-specific cell death


