Apoptotic events induced by human rhinovirus infection

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HeLa and 16HBE14o bronchial epithelium cells infected with human rhinovirus serotype 14 (HRV14) were found to exhibit typical apoptotic morphological alterations, such as cell contraction and nuclear condensation. These events coincided with high-molecular-weight DNA fragmentation, activation of caspase-9 and caspase-3 and poly(ADP–ribose) polymerase cleavage. Caspase activation was preceded by cytochrome c translocation from the mitochondria to the cytoplasm, indicating that apoptosis caused by HRV14 infection was triggered predominantly via the mitochondrial pathway. Apoptosis did not affect HRV14 replication per se, but it facilitated the release of newly formed virus from cells. As apoptosis was fully induced at the time of maximal accumulation of progeny HRV14, it is postulated that apoptosis contributed to the destabilization of the cell and facilitated viral progeny release.

INTRODUCTION

Apoptosis, or programmed cell death, is a naturally occurring process that plays an essential role in development and the maintenance of homeostasis in multicellular organisms. It is fundamental to normal tissue physiology and the inability to undergo apoptosis is responsible for various disorders, such as genetic and autoimmune diseases, neurodegenerative disorders, immunodeficiencies and cancer. Apoptosis also plays a major role in the pathophysiology of viral infections (Rudin & Thompson, 1997). Cells undergoing apoptosis show highly characteristic morphological changes, including shrinkage, blebbing of the plasma membrane, chromatin condensation and DNA fragmentation (Kerr et al., 1972).

According to the classical scheme, apoptosis is induced by two main pathways. The extrinsic pathway is initiated by binding of death ligands, such as Fas or tumour necrosis factor, to their corresponding receptors. Upon complex formation between ligand and receptor, the signal for the induction of apoptosis is transmitted to the cell interior. In the intrinsic pathway, apoptosis is triggered by internal signals leading to permeabilization of the outer mitochondrial membrane, which causes the release of cytochrome c into the cytoplasm. However, there is crosstalk between the mitochondrial and the death receptor-mediated pathways; for example, death receptor-mediated production of a truncated form of Bid (tBid) leads to mitochondrial damage (Li et al., 1998; Luo et al., 1998).

The hallmark of apoptosis is the activation of caspases, a family of cysteine proteases that are produced by proteolytic cleavage of their inactive proforms. Caspases are the primary effectors of apoptosis and are responsible for the biochemical changes brought about by limited proteolysis of a number of cellular proteins (Alnemri et al., 1996; Kumar, 1999; Martin & Green, 1995). Signalling via the extrinsic pathway leads to the conversion of procaspase-8 to caspase-8. In contrast, the intrinsic pathway is characterized by the proteolytic cleavage of procaspase-9. In the next step of the activation cascade, the two pathways converge. Both caspase-8 and -9 activate caspase-3. Caspase-3 is then responsible for the cleavage of a large number of cellular proteins, including poly(ADP–ribose) polymerase (PARP), procaspase-6 and DNA fragmentation factor. Caspases-3 and -6 are also termed executioner caspases, as they constitute the main proteases in the activation cascade and are finally responsible for the establishment of the apoptotic state.

Many cells undergo apoptosis in response to viral infection. Some viruses induce apoptosis and use the disrupted cell remnants as vehicles for viral transmission and avoidance of the immune system (Thomson, 2001). Other viruses produce inhibitors of apoptosis in order to prevent premature cell death and to increase the amount of progeny virus. Mechanisms of induction and/or suppression of apoptosis during virus infection have been discussed in several review articles (Hay & Kannourakis, 2002; Teodoro & Branton, 1997; Tschopp et al., 1998). Infection by most picornaviruses leads to a cytopathic effect (CPE) in the host cell. Under certain conditions, some of these viruses have been shown to induce apoptosis, at least partially (Belov et al., 2003; Carthy et al., 2003;
Girard et al., 1999; Jelachich & Lipton, 1996; Saraste et al., 2003). Recently, apoptosis has also been demonstrated in HeLa cells infected with human rhinovirus (HRV) serotypes 1B and 9 (Taimen et al., 2004). However, no discrimination has been made between the different pathways of induction of apoptosis. In this study, we examined the morphological and biochemical changes occurring in HeLa cells and in 16HBE14o− cells following infection with HRV serotype 14 (HRV14). 16HBE14o− is an immortalized cell line derived from human bronchial epithelium and forms tight junctions in cell culture (Winton et al., 1998). It serves as a paradigm of bronchial epithelium and constitutes an excellent model to study viral infection of the human airway epithelium.

Here, we showed that, following HRV14 infection, apoptotic events observed in both cell lines, such as high-molecular-weight (HMW) DNA fragmentation, caspase activation and cleavage of cellular caspase substrates, were predominantly triggered via the mitochondrial pathway. The apoptotic process following HRV14 infection was accompanied by translocation of cytochrome c from mitochondria to the cytoplasm. Caspases-9 and -3, but not caspase-8, were activated and known caspase substrates, such as PARP, were cleaved. The general caspase inhibitor benzylxoycarbonyl-Val-Ala-Asp-(OMe) fluoromethylketone (zVAD.fmk) reduced the amount of released virus. At the same time, virus accumulated inside cells, suggesting that apoptosis may play a role by facilitating viral progeny release.

METHODS

Media, reagents and chemicals. All reagents and antibodies were purchased from Sigma unless otherwise indicated. Antibodies against cytochrome c and the caspase inhibitor zVAD.fmk were from BD Pharmingen, and anti-caspase-3, anti-caspase-8 and anti-caspase-9 antibodies were from Alexis. The CellTiter 96 AQueous non-radioactive cell-proliferation assay was purchased from Promega. Anti-α-COX IV (Molecular Probes), anti-PARP (Santa Cruz Biotechnology), MitoTracker (Molecular Probes) and protease-inhibitor cocktail (Boehringer Mannheim) were kind gifts from Dr Manuela Baccarini (Institute of Microbiology and Genetics, University of Vienna, Austria). Enhanced chemiluminescence Western blot detection reagents were from Pierce.

Tissue culture. Human cervix carcinoma HeLa cells (strain Ohio; European Collection of Cell Cultures) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 2 mM l-glutamine, 100 U penicillin ml−1 and 100 μg streptomycin ml−1 at 37 °C in a humidified atmosphere with 5% CO₂. Human bronchial epithelial 16HBE14o− cells (obtained from Dr Dieter C. Gruenert, San Francisco, CA, USA) were grown in minimal essential medium (MEM; Gibco) supplemented with 10% FCS, 2 mM l-glutamine, 100 U penicillin ml−1 and 100 μg streptomycin ml−1. Dishes were coated with 10 μg BSA (Sigma) ml−1, 30 μg bovine collagen type I ml−1 (Promocell) and 10 μg human fibronectin ml−1 (BD Pharmingen) in Ham’s F12 medium (HyClone).

Virus preparation and infection of cells with HRV14. HRV14 (ATCC) was grown in suspension cultures of HeLa cells and purified as described previously (Skern et al., 1984). HeLa and 16HBE14o− cells were infected at 80–90% confluency in MEM containing 2% FCS, 2 mM l-glutamine, 30 mM MgCl₂, 100 U penicillin ml−1 and 100 μg streptomycin ml−1 with purified HRV14 at 100 TCID₅₀ per cell. At 30 min post-infection (p.i.), unincorporated virus was removed by washing three times with MEM. At the indicated times, virus titres were determined as TCID₅₀ ml−1 on HeLa cells (Reed & Muench, 1938).

Cytosol and mitochondrial fractions. Cytosolic and mitochondrial fractions were prepared essentially as described by Jesenberger et al. (2000). Briefly, 1×10⁶ HeLa cells were infected with HRV14 or exposed to 50 μM puromycin. After 2, 4, 6, 8, 10 or 12 h, the attached cells were scraped off with a rubber policeman and collected, together with the floating cells. Cells were washed twice with ice-cold PBS, pelleted in a pre-cooled tube at 200 g for 10 min at 4 °C and resuspended in 250 μl of an ice-cold extraction buffer containing 200 mM mannitol, 20 mM HEPES (pH 7.4), 10 mM KCl, 1-5 mM MgCl₂, 1 mM EDTA and 1 mM EGTA. Immediately before use, the extraction buffer was supplemented with 1× protease-inhibitor cocktail, 10 μM PMSF and 1 mM dithiothreitol. After swelling for 30 min on ice, cells were forced through a 27-G hypodermic needle, avoiding the formation of air bubbles. Homogenates were centrifuged at 14 000 g for 20 min at 4 °C. The pellets containing mitochondria were resuspended directly in sample buffer. The supernatants containing the cytosolic fractions were dialysed overnight against water and concentrated for 2 h under vacuum. The quality of the cytosolic fractions was assessed by monitoring mitochondrial contamination by detection of cytochrome c oxidase subunit IV (COX IV) by Western blot analysis. Similarly, the mitochondrial fractions were checked for cytosolic contamination by testing for β-actin. Only preparations that showed no cross-contamination were used for further analysis. Fractions were stored at −70 °C. The amounts of cytochrome c retained in the mitochondrial and cytosolic fractions were determined by Western blot analysis.

Protein determination. Protein concentrations were determined by the method of Bradford (1976), using a protein-assay reagent (Bio-Rad).

Detection of caspase activation. To test for caspase activation, 5–20 μg total cell extract was separated by SDS-PAGE (15, 12.5 or 7.5% polyacrylamide) and transferred onto a nitrocellulose membrane (Protran; Schleicher & Schuell). Membranes were incubated overnight in PBS containing 1% milk powder, 1% polyvinylpyrrolidone and 0.1% Tween 20 at 4 °C with rabbit anti-PARP, or mouse anti-caspase-3, anti-caspase-9, anti-caspase-8 (all diluted 1:1000) or anti-β-actin (diluted 1:5000) antibodies. Membranes were stripped by incubation for 20 min at 56 °C in 30 mM Tris/HCl buffer (pH 6.7) containing 2% SDS and 0.7% β-mercaptoethanol. Immunoreactive proteins were visualized by using horseradish peroxidase (HRP)-coupled goat anti-mouse IgG (Bio-Rad) or HRP-conjugated goat anti-rabbit IgG (Bio-Rad) enhanced by the chemiluminescence detection system (Pierce).
Treatment of cells with caspase inhibitors. Stock solutions of zVAD.fmk (100 mM in DMSO) diluted in MEM to final concentrations of 200 μM were applied 1 h before infection. The final content of DMSO never exceeded 0.2%. For TCID_{50} measurements, cells were infected with HRV14 in MEM. Unincorporated virus was removed at 30 min p.i. by washing three times with MEM. Subsequently, complete Dulbecco’s medium containing 200 μM zVAD.fmk was added.

HMW DNA-fragmentation analysis. DNA fragmentation was analysed following the method of Gasiorowski et al. (2001). To prevent breakage of large DNA molecules, intact cells were embedded in low-melting-point (LMP) agarose (AppliChem), lysed and deproteinized in situ. Briefly, 1·3 × 10⁶ HeLa cells or 1·7 × 10⁶ 16HBE14o⁻ cells were harvested by trypsinization, centrifuged at 500 g for 3 min, resuspended in 60 μl PBS and mixed with equal volumes of molten 2% LMP agarose at 37°C. The liquid mixtures were poured into a multwell block former (Bio-Rad) and left at 4°C for 30 min to solidify. Agarose blocks were transferred into Eppendorf tubes and incubated overnight at 56°C in 1 ml buffer containing 10 mM NaCl, 10 mM Tris/HCl (pH 8.0), 50 mM EDTA and 1% N-lauroyl sarcosine, supplemented with 50 μg proteinase K ml⁻¹. The plugs were washed three times for 30 min with 50 mM Tris/HCl (pH 8.0) containing 50 mM EDTA and polymerized into a 1:2% agarose gel. HMW DNA molecules from HeLa and 16HBE14o⁻ cells were separated by PFGE in 0·5 × TBE on a Bio-Rad DR III apparatus for 27 h at 6 V cm⁻¹, with a 90 s switch time at an included angle of 120° at 10°C. Molecular-mass calibration (size range, 50–1000 kbp) was performed by using a Lambda Ladder PFG marker (New England Biolabs).

Proliferation assay. To determine cell viability, the CellTiter 96 AQdspec non-radioactive cell-proliferation assay (Promega) was performed as instructed by the manufacturer. Briefly, 2 × 10⁶ HeLa cells were seeded into a 96-well plate 1 day before performing the experiment. Where indicated, cells were pre-incubated for 1 h with 200 μM zVAD.fmk and subsequently infected with HRV14 at 100 TCID_{50} per cell. At 24 h p.i., cell viability was determined by the addition of tetrazolium compound to each well, followed by incubation for 2 h at 37°C and subsequent measurement of A_{492} in a Labsystem Multiscan RC plate reader. Each experiment was repeated three times.

Statistical analysis. Results were expressed as means ± SD.

RESULTS

Effect of HRV14 infection and puromycin treatment on cell viability and morphology

In order to evaluate the apoptotic changes brought about by HRV14 infection, the events were compared with those of an established inducer of apoptosis. From the various drugs known to induce apoptosis, treatment with puromycin was chosen because it inhibits translation and thus reflects the events taking place during rhinovirus infection. Although the mechanism of action of puromycin is quite different, there is a formal analogy, as HRV14 shuts off cellular protein synthesis during infection (Haghighat et al., 1996; Prévôt et al., 2003). In order to be able to compare the effects of HRV14 infection with those of puromycin, the viability of the cells was first determined by using a commercial non-radioactive cell-proliferation assay. In this assay, the activity of mitochondrial dehydrogenases is measured by using a colorimetric test. By following the A_{492}, the effect of virus infection and/or drug treatment on cell viability was determined in a CPE-reduction assay (Smee et al., 2002). As seen in Fig. 1(a), puromycin, at a concentration normally used for induction of apoptosis, had reduced viability to 60% by 4 h after drug treatment. Following HRV14 infection, cell viability dropped more slowly to about 60% at 8 h in HeLa cells and at 12 h in 16HBE14o⁻ cells. Corresponding morphological changes in the cells are shown in Fig. 1(b). In puromycin-treated cells, clear morphological alterations were visible after 4 h, whereas in HRV14-infected HeLa cells, typical CPE was visible at 10–12 h. In 16HBE14o⁻ cells, the CPE appeared later at 16 h. These differences had to be taken into account in the following experiments when comparing the kinetics of cell death after infection with HRV14 and after treatment with puromycin.

HRV14 infection induces nuclear fragmentation and HMW DNA fragmentation

Cells undergoing apoptosis exhibit distinct changes in nuclear morphology combined with a loss of genomic DNA integrity (Robertson et al., 2000; Willingham, 1999). These alterations are brought about by activation of cellular factors due to proteolytic cleavage by the executioner caspases (Enari et al., 1998). DNA degradation starts with the excision of large 300–500 kbp fragments and subsequent formation of characteristic 30–50 kbp fragments (Lagarkova et al., 1995; Oberhammer et al., 1993; Solovyan et al., 2002). These fragments are generated by the progressive cleavage of DNA in the chromatin loops associated with the nuclear scaffold (Filipski et al., 1990). Depending on the particular system, apoptosis-associated DNA degradation may stop after generating HMW fragments (Fournel et al., 1995) or may proceed further to low-molecular-weight DNA fragments, corresponding to oligo- and mononucleosomes (180 bp), that are detected as a ‘DNA ladder’ (Brown et al., 1993; Oberhammer et al., 1993). However, whereas degradation into HMW DNA fragments is considered to be indicative of apoptosis, internucleosomal fragmentation is a very late event and is even absent in some apoptotic cell systems (Collins et al., 1997; Lecoeur, 2002; Willingham, 1999).

Infection with HRV14 caused distinct changes in the nuclear morphology, which resembled that of typical apoptotic cells produced by puromycin treatment. These included nuclear shrinkage, membrane blebbing, chromatin condensation and finally nuclear segmentation. At about 10 h p.i., HRV14-infected cells showed the formation of dense particles, which are indicative of nuclear fragmentation (Fig. 2a). A similar process occurred at 4 h after puromycin treatment. The difference in the kinetics of appearance of nuclear fragmentation was not unexpected, as the viability of cells treated with puromycin dropped about twice as fast as after HRV infection (Fig. 1a). DNA degradation to large (≈50 kbp) DNA fragments was visible at about 8 h after HRV14 infection, compared with
6 h after puromycin treatment (Fig. 2b). HMW DNA fragmentation in HRV14-infected 16HBE14o− cells was initiated at 12 h p.i. This was in line with the slower rate of replication of HRV14 in 16HBE14o− cells compared with HeLa cells. We also looked for low-molecular-mass DNA fragmentation. However, there was no evidence of chromatin degradation to 180 bp DNA fragments up to 48 h after HRV14 infection or after exposure to puromycin.

Fig. 1. CPE and morphology in HRV14-infected or puromycin-treated HeLa and 16HBE14o− cells. (a) Viability of HeLa cells exposed to 50 μM puromycin, or HeLa and 16HBE14o− cells infected with HRV14, determined according to the proliferation rate measured by using the CellTiter 96 AQ aeous non-radioactive cell-proliferation assay as described in Methods. Mock control, uninfected/untreated cells. A$_{492}$ is shown, corresponding to relative mitochondrial activities. Values represent means ± SD of five samples. (b) Phase-contrast images of HeLa cells treated with 50 μM puromycin (upper panel) or infected with HRV14 at 100 TCID$_{50}$ per cell (middle panel, HeLa cells; lower panel, 16HBE14o− cells) at the indicated time points.
ladder-like DNA pattern of multiples of 180 bp was only obtained when DNA was isolated at times later than 48 h, i.e. after cell death had occurred (data not shown). This is in agreement with observations by other authors, who demonstrated that oligonucleosomal fragmentation of DNA is a very late event that is not necessarily related to apoptosis (Collins et al., 1997; Huang et al., 1995). However, the observed appearance of typical morphological changes and HMW DNA fragmentation to ~50 kbp fragments provided clear signs of an apoptotic process triggered by HRV14.

**HRV14 infection causes efflux of cytochrome c from mitochondria to the cytosol**

To investigate whether the mitochondrial pathway or the receptor-mediated apoptotic pathway becomes activated, the release of cytochrome c from mitochondria was investigated at the cellular level by immunofluorescence and by immunoblotting following subcellular fractionation. As seen from the diffuse staining pattern in cells marked with arrows in the immunofluorescence images in Fig. 3(a), cytochrome c was released from mitochondria of HeLa cells by 8 h after infection with HRV14 and by 4 h after treatment with puromycin, whereas in mock-infected cells, cytochrome c was associated exclusively with mitochondria. The same phenomenon was observed in HRV14-infected 16HBE14o− cells. To determine the kinetics of cytochrome c efflux from the mitochondria more precisely, we performed Western blot analysis following fractionation of cell homogenates. Translocation of cytochrome c from mitochondria to the cytoplasm in HRV14-infected HeLa cells started at about 6 h p.i. (Fig. 3b) and was complete by 12 h. In the puromycin-treated sample, a substantial release could already be seen by 2 h (Fig. 3c). By 4 h, most of the cytochrome c was in the cytoplasmic fraction. A loss of signal intensity of cytoplasmic cytochrome c was observed, particularly following puromycin treatment of unfractionated cells (total extract) (Fig. 3c, lower panel) and in HRV-infected cells by 12 h p.i. This could be due to efflux into the medium or degradation.

**Cytochrome c release leads to caspase activation and cleavage of PARP**

Although apoptosis and necrosis are very different events in terms of cell physiology and pathology, they share certain characteristics that were originally considered to be
exclusive markers of apoptosis. Recent studies have shown that cytochrome c release from mitochondria (Samali et al., 1999) and DNA degradation (Lecoeur, 2002; Saraste & Pulkki, 2000) can also occur under certain conditions in necrotic cells and thus are not sufficient as criteria for apoptosis. In contrast, caspases are activated exclusively in the execution of the apoptotic programme (Cryns & Yuan, 1998; Nicholson & Thornberry, 1997; Zhivotovsky et al., 1997).

In the apoptotic-activation cascade, caspase-9 is classified as the initiator caspase of the intrinsic pathway (Thornberry

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**Fig. 3.** Subcellular translocation of cytochrome c following HRV14 infection or puromycin treatment in HeLa and 16HBE14o- cells. (a) Cytochrome c release from mitochondria of HeLa and 16HBE14o- cells visualized by confocal immunofluorescence microscopy. HeLa and 16HBE14o- cells were labelled with MitoTracker probe (100 nM) and infected with HRV14 for 8 h or treated with 50 μM puromycin for 4 h, as described in Methods. Arrows indicate cells undergoing apoptosis in which cytochrome c has been released. (b, c) Western blot analysis of cytochrome c efflux from mitochondria to the cytoplasm in HeLa cells following HRV14 infection (b) or 50 μM puromycin treatment (c). At the times indicated, mitochondrial and cytoplasmic fractions were isolated as described in Methods and used for immunoblotting analysis. COX IV and β-actin antibodies were used for checking the purity of obtained subcellular fractions and as the loading controls. Cytochrome c (total extract) denotes unfraccionated, puromycin-treated samples to show the degradation of cytochrome c.
& Lazebnik, 1998). Cytochrome c released from mitochondria acts as the pro-apoptotic signal, causing autocatalytic activation of procaspase-9 (Li et al., 1997). In HeLa cells, after puromycin treatment, procaspase-9 processing takes place at 2–4 h (Fig. 4a), around the time of cytochrome c translocation to the cytoplasm. In HRV14-infected HeLa cells, it was observed that procaspase-9 cleavage to caspase-9 closely followed cytochrome c release at about 6–8 h p.i. (Fig. 4b). In 16HBE14o− cells, caspase-9 activation began at 8 h p.i. (Fig. 4c).

However, there was no evidence for specific cleavage of procaspase-8, indicating that the extrinsic pathway was not activated by HRV14 or puromycin. There was nonspecific degradation of caspase-8 at late time points, but no specific cleavage fragments of activated caspase-8 (p41/43) could be detected. Caspase-3 constitutes the central executioner in the apoptosis programme (Nicholson et al., 1995). It is obtained by cleavage of procaspase-3, which is the common target of caspase-8 and caspase-9. Thus, caspase-3 is positioned in the activation cascade at the site where the extrinsic and intrinsic pathways converge. As seen in Fig. 4(a), proteolytic cleavage of procaspase-3 to active caspase-3 had already started at about 2 h with puromycin treatment. In virus-infected HeLa cells, caspase-3 activation was initiated at about 6–8 h p.i. In HeLa cells (Fig. 4b) and less efficiently in 16HBE14o− cells. Additional proof for the presence of active forms of caspases has been provided by the demonstration that PARP, one of the characteristic targets of caspase-3, is also cleaved during HRV14 infection (Lazebnik et al., 1994). As shown in Fig. 4(a–c), the kinetics of PARP cleavage followed the pattern of caspase activation both for cells infected with HRV14 and for cells treated with puromycin. The corresponding virus titre of a single-cycle infection reached a peak in HeLa cells at 10–12 h, whereas in human bronchial epithelial cells, maximal virus production was obtained over a prolonged period from 16 to 20 h (data not shown).

Caspase inhibitor prevents cell death and affects virus release

In order to demonstrate that caspases were responsible for the cell death resulting from HRV14 infection, infected cells were treated with zVAD.fmk, an irreversible caspase inhibitor that prevents apoptosis in a variety of systems (Dolle et al., 1994; See et al., 1996; Thornberry et al., 1992). Cells were pre-incubated with zVAD.fmk to prevent side effects on virus replication due to inhibition of HRV14 2A protease by the methylated form of zVAD.fmk (Deszcz et al., 2004). As seen in Fig. 5(a), zVAD.fmk reduced the

![Fig. 4. Western blot analysis of caspase processing in HRV14-infected or puromycin-treated HeLa and 16HBE14o− cells.](http://vir.sgmjournals.org)
severity of CPE in HRV14-infected cells at 12 h p.i. and cell viability was retained (Fig. 5b). The addition of puromycin caused cell death by 8 h (Fig. 1a); however, cells pre-incubated with zVAD.fmk before antibiotic treatment remained fully viable for at least 12 h after exposure to puromycin (Fig. 5a).

To study the effect of zVAD.fmk on HRV14 replication and release, the proportion of virus inside the host cells and virus released into the medium was determined at 12 h p.i. At this time point, the maximal amount of progeny virus was reached in our system (data not shown). As seen in Fig. 5(c), about half of the HRV14 was released from cells under these conditions. This ratio changed dramatically when the titres of cells infected with HRV14 in the presence of zVAD.fmk were determined. Under these conditions, about 100-fold more virus was found inside the cells than in the supernatant. It is important to note that overall virus replication per se was not inhibited by zVAD.fmk. Thus, apoptosis did not have any dramatic effect on virus growth, but seemed to speed up the efficiency of viral progeny release from cells considerably.

**DISCUSSION**

In this study, we examined the apoptotic changes occurring in HeLa and 16HBE14o− bronchial epithelial cells following infection with HRV14 by biochemical and morphological methods. We showed that, following HRV14 infection, several signs of apoptosis could be detected. Apart from the appearance of apoptotic bodies, apoptosis could be detected clearly by HMW DNA fragmentation. Cytochrome c was translocated from mitochondria to the cytoplasm, which was followed by caspase-9 and caspase-3 activation, as well as by cleavage of the known caspase substrate PARP.

Previous studies on virus–cell interactions have focused mainly on the early stages of the infection cycle. It is only recently that the importance of viral enzymes from the late stages of the infection cycle has been recognized. Apoptosis is an essential process for the destruction of potentially harmful cells, including virus-infected cells. However, some viruses have apparently learned either to interfere with apoptosis in order to promote their own replication or to utilize apoptosis to their own advantage. In the case of
entero- and rhinoviruses, multiplication is apparently not affected directly by apoptosis, as caspase inhibitors neither stimulate nor reduce the total yield of viral progeny significantly. On the other hand, polioviral 2A and 3C proteinases have been shown to induce apoptosis when expressed as single proteins in transfected cells, suggesting that apoptosis is an active process induced by viral enzymes (Calandria et al., 2004; Goldstaub et al., 2000; Kuo et al., 2002). These proteinases are multifunctional enzymes. They are required for the multiple cleavages during processing of the viral polyprotein, as well as for the shutdown of cellular RNA and protein synthesis at the time of onset of virus replication (Haghighat et al., 1996; Prévôt et al., 2003).

In our assays, the translation inhibitor puromycin was used as a positive control for the induction of apoptosis. The apoptotic events following puromycin treatment were identical to those seen in HRV infection apart from differences in the kinetics, suggesting that the shut-off of protein synthesis might initially be responsible for the observed apoptotic events in HRV-infected cells.

In our study, we employed HeLa strain Ohio cells, a standard cervical carcinoma cell line used for HRV growth, and, in parallel, 16HBE14o− cells. This cell line is derived from human bronchial epithelium and should therefore reflect a natural HRV infection more closely. Indeed, following HRV14 infection, apoptosis was found to occur in both cell lines, based on the observation of the appearance of nuclear fragmentation, HMW DNA fragmentation, cytochrome c release and caspase activation. This confirms and extends previous findings by Carthy et al. (2003) on the role of apoptosis during picornavirus infection.

Apoptosis during infection with HRV14 is induced predominantly by the intrinsic pathway, as demonstrated by the extensive release of cytochrome c from mitochondria, the activation of caspase-9 and the absence of caspase-8 activation. This is in contrast to the findings of Taimen et al. (2004), who observed moderate cleavage of procaspase-8 in cells infected with HRV1B and HRV9, which would favour activation of the extrinsic pathway. In our experiments with HRV14, we were not able to detect any significant cleavage of procaspase-8. Although this discrepancy might be explained by the difference in HRV serotypes, it seems more likely that it reflects different experimental conditions. In our experiments with HRV14, a high m.o.i. (100 TCID50 per cell) was used to ensure that the maximum number of cells became infected initially. The time frame was such as would be used to study a one-step growth curve, in order to avoid potential side effects from factors released from lysed cells. In the experiments of Taimen et al. (2004), the m.o.i. used was only 10. Furthermore, the time points taken were between 10 and 24 h. As one replication cycle of HRV in HeLa cells takes about 10–12 h, most of the observed effects would actually have taken place in a second round of infection by progeny virus. Thus, it cannot be excluded that soluble factors released from lysed cells in the first round may actually cause activation of the extrinsic pathway of apoptosis.

The question arises of whether apoptosis induced by entero- and rhinoviruses is just a fortuitous event with no significance for virus production or whether the viruses actually benefit in some way from apoptosis. In the case of poliovirus, apoptosis was observed only under conditions of restricted replication and/or with certain HeLa cell lines (Agol et al., 1998; Graham et al., 2004; Tolskaya et al., 1995, 1996). The observed suppression of the apoptotic programme in poliovirus-infected cells might be explained, at least partially, by aberrant processing of procaspase-9 (Belov et al., 2003). Furthermore, anti-apoptotic functions have been assigned to the 3A protein and to poliovirus 3C protease [Blondel et al. (2004) and references therein].

In this paper, we demonstrated that apoptosis facilitates the release of virus from cells at the end of the infection cycle. The caspase inhibitor zVAD.fmk reduced the amount of released virus. At the same time, virus accumulated inside the cells, suggesting that apoptosis may play a role by facilitating viral progeny release and thus increasing pathogenicity by promoting virus spread.

Despite all the evidence demonstrating a role for apoptosis in HRV- and enterovirus-infected cells, cell death and lysis are definitely not caused by apoptosis alone. Both 2A and 3C proteinases also act on the cytoskeleton at the time of the CPE. The 2A proteinases of some rhino- and entero-viruses cleave within the head domain of cytokeratin 8 in epithelial cells and cleave dystrophin in cardial myocytes (Badorff et al., 1999; Seipelt et al., 2000). Similarly, 3C proteinase cleaves the microtubule-associated protein Map4 late in poliovirus- and in HRV14-infected cells (Joachims et al., 1995). At the same time, activated caspases cleave other important structural elements of the cytoskeleton, such as cytokeratin 18. Thus, lysis and release of virus are probably caused by at least two independent events that lead to destabilization of the cellular membrane and destruction of the cytoskeleton. It remains a challenge for the future to define more precisely the individual roles of apoptosis and cytoskeletal breakdown during cell death and release of virus, and to identify further targets of viral proteinases at the late stage of the infection cycle.

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