Implication of caspases during maedi–visna virus-induced apoptosis

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Maedi–visna virus (MVV) causes encephalitis, pneumonia and arthritis in sheep. In vitro, MVV infection and replication lead to strong cytopathic effects characterized by syncytia formation and subsequent cellular lysis. It was demonstrated previously that MVV infection in vitro induces cell death of sheep choroid plexus cells (SCPC) by a mechanism that can be associated with apoptotic cell death. Here, the relative implication of several caspases during acute infection with MVV is investigated by employing diverse in vitro and in situ strategies. It was demonstrated using specific pairs of caspase substrates and inhibitors that, during in vitro infection of SCPC by MVV, the two major pathways of caspase activation (i.e. intrinsic and extrinsic pathways) were stimulated: significant caspase-9 and -8 activities, as well as caspase-3 activity, were detected. To study the role of caspases during MVV infection in vitro, specific, cell-permeable, caspase inhibitors were used. First, these results showed that both z-DEVD-FMK (a potent inhibitor of caspase-3-like activities) and z-VAD-FMK (a broad spectrum caspase inhibitor) inhibit caspase-9, -8 and -3 activities. Second, both irreversible caspase inhibitors, z-DEVD-FMK and z-VAD-FMK, delayed MVV-induced cellular lysis as well as virus growth. Third, during SCPC in vitro infection by MVV, cells were positively stained with FITC-VAD-FMK, a probe that specifically stains cells containing active caspases. In conclusion, these data suggest that MVV infection in vitro induces SCPC cell death by a mechanism that is strongly dependent on active caspases.

Introduction

Lentiviruses cause persistent infections and chronic disease after a prolonged incubation period in their hosts. Virus infection culminates in multiorgan pathology with an invariably fatal outcome (Clements & Zink, 1996; Pépin et al., 1998). Maedi–visna virus (MVV) was first identified by Sigurdsson (1954) in sheep affected by degenerative progressive encephalitis (‘visna’) and interstitial pneumonitis (‘maedi’) characterized by a slowly progressive disease. MVV is related to human immunodeficiency virus type-1 (HIV-1) (Sonigo et al., 1985) and causes a systemic infection in sheep (Petursson et al., 1976). Host–virus interactions in visna disease show several features similar to those observed in the early stages of HIV-1 infection (Georgsson, 1994; Petursson et al., 1989). The main target cells in infection with MVV are, as in HIV-1 infection, monocytes/macrophages (Narayan et al., 1982) but lymphocytes are apparently not permissive for MVV, which is in contrast to infection with HIV-1, and infection does not cause an overt immunodeficiency. Initial pathological alterations in the central nervous system in MVV are meningitis, periventricular inflammation and infiltration of the choroid plexus. Occasionally, at late stages of infection, plaques of primary demyelination are observed (Georgsson et al., 1982). In vitro, MVV replicates efficiently to a high titre in monolayer cultures of sheep choroid plexus cells (SCPC). Replication of MVV is associated on SCPC with a specific cytopathogenic effect (CPE), characterized firstly by syncytia formation generated through fusion of infected cells with surrounding susceptible cells and secondly by the appearance of cell death characterized by cellular lysis. We demonstrated previously that the major mechanism of cell death occurring during MVV infection in vitro is associated with the induction of apoptosis (Duval et al., 2002).

Apoptosis is defined as an active physiological process of cellular self-destruction, with specific morphological and biochemical changes (Steller, 1995). Apoptotic death is known to involve a cascade of proteolytic events accomplished mainly by a family of cysteine proteases called caspases (Alnemri et al., 1996; Salvesen & Dixit, 1997). These proteases are synthesized as inactive proenzymes that are activated after cleavage at
specific aspartate residues (Nicholson & Thornberry, 1997). A crucial role for caspases in the execution phase of programmed cell death is supported by genetic (Metcstein et al., 1998), biochemical (Hengartner, 2000) and physiological (Meier et al., 2000) evidence. A current model proposes a cascade of events by which caspases proteolytically activate other caspases (Kidd, 1998). More recent evidence suggests that different death stimuli trigger the activation of a subset of upstream caspases that possess long prodomains at their N termini (Earnshaw et al., 1999). These prodomains serve to target proteases to specific protein complexes, where the prodomains are removed by proteolysis to produce active proteases. These caspases proteolytically activate other downstream caspases (with shorter prodomains) that cleave key substrates to ultimately produce the characteristic apoptotic phenotype of cell shrinkage, membrane blebbing, chromatin condensation and fragmentation and cell death (Kerr et al., 1972). A growing list of proteolytic substrates of the caspases has been identified (Earnshaw et al., 1999) and these substrates are all cleaved after an aspartate residue (P1 position). The precise role of these cleavage events is not known but they may either inactivate key cellular functions or produce cleavage products with prodeath activity. These proteolytic events serve as biochemical markers of apoptosis. Furthermore, synthetic, cell-permeable, irreversible peptide inhibitors of different caspases, like z-VAD-FMK and others, prevent the development of apoptosis in many systems (Nicholson, 2000).

Mechanisms controlling and executing virus-induced apoptosis are, however, not completely understood because of the complexity of underlying biochemical cascades (Budihardjo et al., 1999) and because not all of the participating host factors have been identified (Teodor & Branton, 1997). It has been demonstrated for a growing number of RNA and DNA viruses in cell culture and in vivo that apoptosis is a common consequence of virus replication. It has also been suggested that virus-induced apoptosis contributes to the CPE of these viruses (O’Brien, 1998; Roulston et al., 1999). A role for caspases in virus infections is suggested by the finding that baculovirus infection activates an apoptotic cysteine protease in insect cells (Bertin et al., 1996). Similar work has suggested that adenovirus protein E1A activates caspase-3 (Boulakia et al., 1996). In addition, HIV-1 is actually the most studied model of retrovirus-induced apoptosis and inappropriate apoptosis of CD4+ T lymphocytes in HIV-1 disease is well established (McCune, 2001).

In this work, we show that caspases play a key role in the effector phase of MVV-induced cell death, since we demonstrate the activation of caspase-3, -8 and -9. Furthermore, we found that inhibition of caspases by z-VAD-FMK (a broad spectrum caspase inhibitor) or z-DEVD-FMK (an inhibitor of caspase-3-like activities) (i) maintained plasma membrane integrity during the time-course of infection and (ii) diminished virus particle production. Thus, our results suggest that MVV-induced CPE involves activation of several apoptotic caspases, mediated either by the death-receptor pathway or by the mitochondrial pathway, as in the HIV-1 model (Gougeon et al., 2000; Gougeon & Montagnier, 1999).

Methods

- **Reagents.** Eagle’s minimum essential medium (MEM) and MEM non-essential amino acid solution were purchased from Sigma. Foetal bovine serum (FBS) was obtained from Gibco. DAPI was obtained from Sigma. The Cytotoxicity Detection kit for lactate dehydrogenase (LDH) detection was from Roche. The CaspACE Assay (Fluorometric) (for caspase-3 activity) and the CaspACE FITC-VAD-FMK in situ Marker Assay systems were purchased from Promega. z-DEVD-FMK and z-VAD-FMK were from Calbiochem. Ac-LEHD-AMC/Ac-LEHD-CHO (caspase-9 substrate/inhibitor) and Ac-IETD-AMC/Ac-IETD-CHO (caspase-8 substrate/inhibitor) were obtained from BACHEM Biochimie.

- **Cell culture and virus.** Primary cell cultures were established from normal sheep brains by explants of choroid plexus cultured on clots of chicken plasma and were grown in MEM supplemented with 20% FBS. Monolayer cultures of SCPC were maintained in MEM supplemented with 10% FBS (2.5% FBS per virus infection) at 37°C in a humidified 5% CO₂ atmosphere. MVV strain K1514, kindly provided by Institut Pourquier (Montpellier, France), was used in all experiments. Viruses were passaged on SCPC and the titre of the virus stocks was quantified according to the method of Reed & Muench (1938).

- **Virus infection.** SCPC were cultured at a density of 2.5 to 3 x 10⁶ cells/cm² in (i) 75 cm² culture flasks (Corning) for the in vitro assessment of caspase activities, (ii) Nunclon Δ Surface microwell plates (Nunc, Life Technologies) to assess the in situ effects of caspase peptide inhibitors (e.g. z-VAD-FMK and z-DEVD-FMK) and (iii) in Nunclon SonicSeal slide wells (Nalge Nunc) for FITC-VAD-FMK in situ labelling. SCPC were infected with MVV at an m.o.i. of 0.1 in an appropriate volume of culture medium with 2.5% FBS at 37°C.

- **Detection of caspase catalytic activity.** The activity of various caspases was studied using the CaspACE Assay (Fluorometric) system. Assays are based on fluorometric measurement of fluorescent 7-amino-4-methylcoumarin (AMC) after cleavage from the AMC-label peptide substrates, Ac-DEVD-AMC, Ac-IETD-AMC and Ac-LEHD-AMC, for caspase-3, -8 and -9 activities, respectively. At different times after MVV infection, cells were collected and homogenized in lysis buffer according to the manufacturer’s protocol. Lysates were stored at 4°C until experimentation. Fluorometric assays were conducted in white, opaque, tissue culture plates (Falcon, Becton Dickinson) and all measurements were carried out in duplicate. First, 100 µl of assay buffer (10 mM DTT, 2% DMSO and caspase buffer) were added to each well. Peptide substrates for caspase-3, -8 or -9 were added to each well to a final concentration of 5 x 10⁻⁵ M. Caspase inhibitors Ac-DEV-D-CHO, Ac-IETD-CHO, Ac-LEHD-CHO, z-DEVD-FMK and z-VAD-FMK were used at a concentration of 5 x 10⁻⁵ M immediately before the addition of the substrate. Cell lysates were added to the reaction mixture to start the reaction. Background fluorescence was determined in wells containing assay buffer and substrate without cell lysate. Assay plates were incubated at 37°C for 1 h for the measurement of caspase activities. Fluorescence was measured with a microplate reader (Fluorolite 1000, Dynatech Laboratories) using 360 nm excitation and 460 nm emission filters. Raw data (relative unit of fluorescence (RUF)) correspond to the concentrations of AMC released.

- **Effects of caspase peptide inhibitors on infected SCPC monolayers.** Cells were cultured in Nunclon Δ Surface microwell plates and either infected or not with MVV (m.o.i. of 0.1) and simultaneously exposed, or not, to peptide inhibitors (e.g. z-DEVD-FMK and z-VAD-
FMK) at a concentration of $5 \times 10^{-5}$ M. Microtitreplates (MTPs) were placed at 37°C in a humid chamber. At various times of treatment, supernatants were collected, fractionated into two samples and then placed at 4°C or -80°C for subsequent experiments. Fresh medium supplemented with 2.5% FBS was added to all wells and MTPs were replaced at 37°C in a humid chamber until the next time of sample. Samples at 4°C were used for the assessment of LDH release (to examine cell lysis) and samples at -80°C were used to titre the production of new virions during the time-course of experiments.

- **LDH release.** Detection procedures used for LDH release were in accordance with the manufacturer's instructions. Briefly, supernatants stocked at 4°C were warmed slowly, centrifuged at 3000 g for 10 min and removed from the tubes to generate cell-free samples. Then, 100 µl of each sample were transferred to a MTP. Freshly prepared reaction mixture was added to each well and incubated for up to 30 min in the dark at room temperature. Absorbance values were measured at a wavelength of 490 nm using an ELISA plate reader (MRX, Dynatech Laboratories) with a reference wavelength of 690 nm. Cytotoxicity was calculated using the following formula: cytotoxicity (%) = [(experimental $A_{490}$ - control $A_{490}$/positive control $A_{490}$ - control $A_{490}$)] × 100.

- **Virion production.** Detection procedures for virus release were carried out in accordance with the method of Reed & Muench (1938). The resulting values were substituted in the following equation:

inhibition (%) = $[1 - (\text{experimental value}/\text{positive control value})$.

- **In situ FITC-VAD-FMK labelling.** The FITC-VAD-FMK In situ Marker Assay system was used to detect active caspases in situ, in accordance with the manufacturer's instructions. Briefly, at different times after virus infection, medium from samples was removed and replaced with fresh medium supplemented with 2.5% FBS and FITC-VAD-FMK peptide at a final concentration of $1 \times 10^{-5}$ M. Then, samples were returned to the humidified chamber at 37°C until the appearance of fluorescence. Cells were fixed with 4% paraformaldehyde in PBS, pH 7-4, for 30 min at room temperature. Coverslips were rinsed and then mounted onto glass slides using gelatin/glycerol (Sigma). Slides were examined by fluorescence microscopy (Leica), set and photographed at ×400 magnification.

Double staining experiments were done as follows: after FITC labelling, cells were washed in PBS and stained with 0.5 ng/µl of fluorescent DNA-binding dye (DAPI) for 1 min. Cells were simultaneously examined by fluorescence microscopy with appropriate filters.

- **Statistical analyses.** Data are expressed as the mean of three or more separate experiments ± SD. Statistical analysis of the data included an overall analysis of variance (ANOVA) followed by Post-Hoc PLSD Fisher's test using the statistical functions in the SAVT program (SAS Institute). A probability of $P < 0.05$ was considered significant.

### Results

**Detection of the activity of individual caspases**

To investigate the involvement of caspase-3 during MVV-induced apoptosis, cell lysates from infected and mock-infected cells were subjected to a fluorescence assay with the specific substrate relevant for caspase-3 activity, Ac-DEVD-AMC. Caspase-3 activity was significant from 54 h post-infection, reached a peak at 68 h and then decreased (Fig. 1A).

To determine which apoptotic pathway was involved during SCPC infection with MVV, we examined the involvement of caspase-8 and -9 during MVV infection. In the same fluorometric protease assay, we replaced the caspase-3 substrate with the specific substrates for measuring caspase-8 and -9 activities (Ac-IETD-AMC and Ac-LEHD-AMC, respectively). In both cases, and under the same conditions, we detected significant caspase-8 (Fig. 1B) and caspase-9 (Fig. 1C) activities. The activities of caspase-8 and -9, like those of caspase-3, increased to reach a peak at 68 h post-infection. However, caspase-8 activity, as well as caspase-9 activity, was always weaker than caspase-3 activity.

**z-DEVD-FMK and z-VAD-FMK inhibit caspase activities**

We investigated further the spectrum of inhibition of z-DEVD-FMK and z-VAD-FMK on caspase-3, -8 and -9 activities. As described previously, cell lysates from infected and mock-infected cells were, once again, subjected to the fluorometric protease assay with the specific substrates for caspase-3, -8 and -9 (Ac-DEVD-AMC, Ac-IETD-AMC and
Ac-LEHD-AMC, respectively). However, in this latter case, the reversible peptide inhibitors, Ac-DEVD-CHO, Ac-IETD-CHO and Ac-LEHD-CHO, were replaced by two irreversible inhibitors, z-DEVD-FMK and z-VAD-FMK. As reported previously, we detected significant caspase-3, -8 and -9 activities, which reached a peak at 68 h post-infection (Fig. 2). More interestingly, we demonstrated that the two cell-permeable, irreversible caspase inhibitor peptides, z-DEVD-FMK and z-VAD-FMK (5 × 10\(^{-5}\) M), showed the same spectrum of inhibition: they both inhibit caspase-3 (Fig. 2A) as well as caspase-8 (Fig. 2B) and caspase-9 (Fig. 2C) activities in the same manner.

Inhibition of caspases delays MVV-induced CPE

To determine if caspase activation plays a role in cell killing (e.g. cell lysis) induced during MVV infection, mock-infected or MVV-infected SCPC were treated with either z-DEVD-FMK or z-VAD-FMK.

First, our results showed that there was no significant leakage of LDH in supernatants from mock-infected cells during the time-course of experiments (Fig. 3, ◇): levels of cytotoxicity never exceeded 2%. Second, in both cases, i.e. following treatment with z-DEVD-FMK (Fig. 3A) or z-VAD-FMK (Fig. 3B), there was no difference between the levels of LDH detected in supernatants from mock-infected cells (Fig. 3, ◇) and cells treated with the inhibitors at 5 × 10\(^{-5}\) M (Fig. 3, ○). Third, LDH release in supernatants from MVV-infected SCPC (Fig. 3, ◇) reached maximum levels at approximately 120 h post-infection with, respectively, 98% cytotoxicity for cells treated with 5 × 10\(^{-5}\) M z-DEVD-FMK (Fig. 3A) and 76% cytotoxicity for cells treated with 5 × 10\(^{-5}\) M z-VAD-FMK (Fig. 3B). Fourth, treatment of MVV-infected SCPC with either z-DEVD-FMK or z-VAD-FMK at 5 × 10\(^{-5}\) M (Fig. 3, ■) delayed the time of maximum release of LDH in both cases (approximately 150 h) compared to MVV-infected SCPC controls (Fig. 3, ◇), with notably decreased cytotoxicity at 120 h: 98–65% for infected cells treated with 5 × 10\(^{-5}\) M z-DEVD-FMK (Fig. 3A) and 76–47% for infected cells treated with 5 × 10\(^{-5}\) M z-VAD-FMK (Fig. 3B).
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Fig. 4. Effect of caspase inhibitors on virus production. TCID50 was determined from supernatants collected at different times (see Methods). Cells were infected with MVV (m.o.i. of 0–1) or infected with MVV and simultaneously treated with $5 \times 10^{-5}$ M of caspase inhibitor (●). (A) z-DEVD-FMK; (B) z-VAD-FMK.

z-DEVD-FMK and z-VAD-FMK affected the titre of virus particles released during MVV infection

To test whether inhibition of caspases by z-VAD-FMK or z-DEVD-FMK had an effect on virus release, the titre of virus particles released from infected cells was measured in cultures treated with z-VAD-FMK or z-DEVD-FMK and compared to that for untreated cultures.

During MVV infection, we determined virus progeny release by a TCID50 assay of culture supernatants. These studies revealed that the TCID50 from supernatants of MVV-infected SCPC increased during the time-course of infection to reach maximum levels at approximately 120 h (Fig. 4A) or 144 h (Fig. 4B) post-infection, then it declined. However, it is noteworthy that, in the case of MVV-infected SCPC treated with either z-DEVD-FMK or z-VAD-FMK (at $5 \times 10^{-5}$ M), even if we had the same time-profile (with a maximum TCID50 between 120 and 144 h post-infection), the level of released infective progeny virus was always less (Fig. 4), with TCID50 values decreased by 70% for infected cells treated with $5 \times 10^{-5}$ M z-DEVD-FMK (Fig. 4A) and 80% for infected cells treated with $5 \times 10^{-5}$ M z-VAD-FMK (Fig. 4B). These results suggested that z-VAD-FMK or z-DEVD-FMK treatment reduced virus release.

In situ labelling of caspase

To specifically follow in situ active caspases in SCPC infected with MVV, we performed FITC-VAD-FMK assays on cultured cells. Mock-infected cells showed no FITC-positive cells (Fig. 5A). Numerous FITC-stained cells, showing intense green staining, were detected in SCPC (Fig. 5B) at 82 h post-infection. The FITC-positive apoptotic cells detected during MVV infection showed a more intense nuclear fluorescence (Fig. 5B, arrows).

Figs. 5(C, D) shows parallel DAPI and FITC staining on the same field. There was a correlation between FITC-positive and apoptotic nuclei harbouring intense blue fluorescence (DAPI-positive cells), as each FITC-positive cell exhibited chromatin morphology characteristic of apoptotic cells (chromatin condensation), while cells showing normal nuclear staining had no FITC staining.

Discussion

Mechanisms of virus-induced cell injury play an important role in our understanding of the pathogenesis of virus infection. In this study we show that MVV infection leads to apoptotic cell death in SCPC, in vitro. The strong CPE that can be observed after MVV infection can thus be attributed to the induction of a MVV-triggered apoptotic cell death programme.

Apoptosis is a natural, genetically controlled process of self-destruction that is vital to the development of an organism.
and for its life-long maintenance free from aberrant cells that can be generated by various factors such as radiation, oncogenesis and virus infection (Kroemer et al., 1997). The importance of caspase activation during apoptosis is well established (Hengartner, 2000). Caspases play a central role in the effector phase during apoptotic cell death. To date, 14 mammalian caspases have been described (Earnshaw et al., 1999). Still, it is not clear how many different caspases have to be activated for the successful execution of an apoptosis programme (MacFarlane et al., 1997; Takahashi & Earnshaw, 1996).

Over the past few years, a growing number of RNA and DNA viruses have been found to induce apoptosis in host cells and the involvement of caspase-3 activity in virus-infected cells has been shown recently for HIV (Banki et al., 1998), adenovirus (Chiou & White, 1998) and hepatitis C virus (Ruggieri et al., 1997).

The replication of many viruses has been shown to lead to apoptosis and some viruses are believed to actively induce apoptosis. Blebbing of apoptotic cells is speculated to facilitate the dissemination of non-enveloped, non-lytic viruses. Other viruses have evolved mechanisms to suppress apoptosis via interference with the apoptotic cascade by certain virus-specific proteins in order to prolong the life of the host cell (Shen & Shenk, 1995; Teodoro & Branton, 1997; O'Brien, 1998; Roulston et al., 1999). It has been shown that prevention of apoptosis enhances production of HIV (Chinnaiyan et al., 1997), simian immunodeficiency virus (Chuang et al., 1993) and adenoviruses (Chiou & White, 1998). Induction of apoptosis by specific viral genes near the end of infection may promote cell-to-cell spread and virus release and interfere with inflammatory responses. The adenovirus E3 gene promotes virus release and enhances virus growth in cultured cells by promoting cell death that is distinct from apoptosis (Tollefson et al., 1996). However, little is known about which individual caspases are activated during virus infections leading to apoptosis in host cells. It has been shown that the final execution process (e.g. the ‘caspase cascade’) of apoptosis can be inhibited by pseudosubstrates of these proteases. A chemically modified oligopeptide called z-VAD-FMK has been shown to efficiently and specifically inhibit apoptosis, including Sindbis virus-induced apoptosis (Nava et al., 1998; Zhivotovsky et al., 1995), rubella virus-induced apoptosis (Pugachev & Frey, 1998), Sendai virus-induced apoptosis (Bitzer et al., 1999) and poliovirus-induced apoptosis (Agol et al., 1998).

In our previous assays we detected only the terminal stages of apoptosis. In the present study, we investigated the molecular mechanism as well as the consequences of MVV-induced apoptosis for virus propagation. As a step to obtain insight into these complex virus–cell relationships, we attempted to define the role of caspases in these processes. To define the relevant steps employed in the MVV-triggered apoptotic signal transduction pathway, we investigated the activation of individual caspases during MVV infection. In order to improve emission fluorescence collected from the AMC released upon cleavage, the experiments presented here were performed in white, opaque, tissue culture plates, thus preventing leakage of fluorescence. As demonstrated by the fluorescence assay, caspase-3 was found to be activated upon MVV infection (Fig. 1A). The cellular protease caspase-3 is expressed as a 32 kDa precursor protein, which, upon activation, is processed into p17 and p12 subunits (Nicholson et al., 1995). Our caspase activity assay employed the substrate -DEVD-, which is a fairly selective substrate for caspase-3 (versus other caspases). Among the different members of the caspase family, caspase-3 appears to play a particularly prominent role in apoptosis. Earlier work implicated caspase-3 as a central executioner protease because it is activated by a multitude of apoptotic stimuli and is able to cleave various cellular substrates (Cohen, 1997; Nicholson & Thornberry, 1997). Recently, novel members of the caspase family, designated caspase-8 and -9, have been shown to directly cleave caspase-3 (Roy & Nicholson, 2000). In fact, caspase-8 and -9 activation is thought to represent the most upstream event in the CD95-mediated apoptotic cascade (extrinsic pathway) and mitochondria-mediated apoptotic cascade (intrinsic pathway), respectively. To study caspase-8 and -9 activities during MVV infection, we applied the same protocol as that for caspase-3 but with Ac-DEVD-AMC and Ac-DEVD-CHO (caspase-3 substrate and inhibitor) replaced by specific substrate and inhibitor pairs for caspase-8 (Ac-IETD-AMC and Ac-IETD-CHO) and caspase-9 (Ac-LEHD-AMC and Ac-LEHD-CHO). Thus, we demonstrated that caspase-8 and -9 are activated during MVV infection (Fig. 1B, C); we could therefore hypothesize either that caspase-8 and -9 were activated independently of each other or that caspase-9 activation was a consequence of caspase-8 activation (Hengartner, 2000; Roy & Nicholson, 2000).

These findings prompted us to determine if caspases have an active role in facilitating cell death during in vitro infection of SCPC. Therefore, we tested z-DEVD-FMK and z-VAD-FMK for their effects on the outcome of MVV infection. z-DEVD-FMK is described as a potent inhibitor of caspase-3-like activities (Nicholson et al., 1995) and z-VAD-FMK is a broad spectrum caspase inhibitor that prevents apoptosis mediated by a variety of stimuli (Cain et al., 1996; Slae et al., 1996). Both are cell-permeable and irreversible caspase inhibitors. We used them as inhibitors in the fluorometric protease assay and tested their inhibition capacities on caspase-3, -8 and -9. Thus, we demonstrated that z-VAD-FMK, as well as z-DEVD-FMK, present the same inhibitor competency for caspase-3, -8 and -9 activities (Fig. 2). We concluded that these two oligopeptides are potent caspase inhibitors in our cell–virus system.

To further delineate the contribution of caspases to MVV-induced apoptosis, we decided to infect SCPC with MVV and to simultaneously treat the cells with z-DEVD-FMK or z-VAD-FMK. At different times post-infection and treatment,
supernatants were collected and replaced by fresh culture medium without z-VAD-FMK or z-DEVD-FMK. We first investigated the influence of apoptosis and especially caspase activation on CPE observed during MVV infection. Since the release of LDH from infected cells in culture supernatants is a reliable marker for monitoring CPE development of MVV-infected SCPC, by treating infected SCPC with the two inhibitors we could estimate what role, if any, caspases played during the development of CPE. When infected SCPC were incubated with either z-VAD-FMK or z-DEVD-FMK, maximum LDH release was delayed compared to infected SCPC without inhibitor treatment (Fig. 3). Moreover, LDH release was notably reduced between 72 and 120 h post-infection. Thus, we hypothesized that caspase activation led to MVV-induced CPE. Secondly, we investigated the influence of apoptosis on the release of MVV progeny. We determined the infectivity of progeny viruses in relation to the total number of virion particles (TCID50) from infected and/or treated (or not) SCPC. Progeny release was reduced when cells were treated with z-VAD-FMK or z-DEVD-FMK (Fig. 4); thus we could assume that caspase activation or other events led to virus assembly or release. Therefore, apoptosis inhibition by caspase inhibitors did not lead to dramatically enhanced virus replication. Our results obtained with MVV were in accordance with those described for HIV-1 (Taddeo et al., 2000). But, in the case of HIV studies, conflicting data were published, as other authors showed that apoptosis inhibition lead to dramatically enhanced virus replication (Antoni et al., 1995; Chinnaiyan et al., 1997; Sandstrom et al., 1996); these discrepancies are probably due to different protocols as well as the systems used to prevent apoptosis.

Finally, we used an in situ marker of active caspases, FITC-VAD-FMK. In effect, this peptide, which has the same properties as z-VAD-FMK, was coupled to the fluorescent dye FITC. We showed that FITC-VAD-FMK specifically labelled infected SCPC harbouring active caspases (Fig. 5B), while mock-infected SCPC were not labelled (Fig. 5A). Moreover, we demonstrated that DAPI-positive, condensed nuclei were also FITC-positive for active caspases (compare Fig. 5C, D). Thus, we demonstrated that SCPC infected with MVV have active caspases, with a more intense fluorescence at the nuclear level (Fig. 5B, arrow).

Collectively, the results reported here demonstrate (i) that apoptosis developing in a MVV infection depended on the activities of caspase-3, -8 and -9, (ii) that apoptosis induced during in vitro infection of SCPC could occur both from the extrinsic pathway and the intrinsic pathway, and (iii) that CPE and cell death in SCPC, which are triggered by MVV, are due to the induction of caspase activation, given the ability of z-VAD-FMK and z-DEVD-FMK to prevent or delay the appearance of characteristic CPE without affecting virus replication but diminishing virus release. Our findings were not surprising and instead serve as evidence to support our conclusion that MVV can cause apoptosis, because, to our knowledge, no caspase-independent apoptotic pathway has been described in vitro.

Taken together, these results show that there is no common role for the apoptotic process during different virus infections; rather, it has to be determined individually for each different virus species.

To understand further the role that apoptosis plays in MVV infection, it is crucial to define the virus-triggered steps of the apoptosis signal transduction cascade.

We thank Dr Jeanne Cook-Moreau for her critical reading of the manuscript.

References


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Received 23 April 2002; Accepted 18 July 2002