The 3C Protease Activity of Enterovirus 71 Induces Human Neural Cell Apoptosis

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The human glioblastoma SF268 cell line was used to investigate the induction of apoptosis by the 3C protease of enterovirus 71 (EV71). Transient expression in these cells of the wild-type 3C protein encoded by EV71 induced morphological alterations typical of apoptosis, including generation of apoptotic bodies. Degradation of cellular DNA in nucleosomes was also observed. When two of the amino acids in the catalytic motif of 3C were changed by mutagenesis, the 3C protein not only lost its proteolytic activity, but also its ability to induce apoptosis in the SF268 cells. Twenty-four hours after 3C transfection, poly(ADP-ribose) polymerase, a DNA repair enzyme, was cleaved, indicating that caspases were activated by the expression of EV71 3C. The 3C-induced apoptosis was blocked by the caspase inhibitors DEVD-fmk and VAD-fmk. Our findings suggest that the proteolytic activity of 3C triggers apoptosis in the SF268 cells through a mechanism involving caspase activation and that this apoptotic pathway may play an important role in the pathogenesis of EV71 infection.

INTRODUCTION

Enterovirus 71 (EV71) is a positive-stranded RNA virus in the family Picornaviridae. EV71 infection can cause hand, foot, and mouth disease (HFMD) or herpangina, common diseases in children. However, EV71 has also been associated with severe neurological complications, including encephalitis, meningitis, and a poliomyelitis-like syndrome as well as fatal pulmonary edema (Gibert et al., 1988; Alexander et al., 1994; Lum et al., 1998; Chang et al., 1999; Ho et al., 1999; Chen et al., 2001).

In 1998 there was a large HFMD outbreak caused by EV71 in Taiwan. It infected more than 120,000 children and left in its wake 78 dead (Ho, 2000). Postmortem studies using immunofluorescence and molecular assays during this outbreak clearly showed that EV71 infected the central nervous system (CNS) (Hseuh et al., 2000). In addition, EV71 was isolated from the medulla oblongata and spinal cord (Chang et al., 1998; Shih et al., 2000). Because of the severe consequences of this and other outbreaks in Bulgaria, New York, Australia, Europe, and Asia (Chumakov et al., 1979; Blomberg et al., 1974; Kennett et al., 1974; Chonmaitree et al., 1981; Nagy et al., 1982; Gilbert et al., 1988; Samuda et al., 1987) caused by EV71, further studies on the pathogenesis of disease associated with EV71 infection are needed, most notably on how it causes disease in the CNS.

The phenomenon of apoptosis is characterized morphologically by plasma membrane blebbing, cell shrinkage, and formation of membrane-bound apoptotic bodies (Teodoro and Branton, 1997; Roulston et al., 1999). Apoptotic cells are usually rapidly phagocytosed without the release of proinflammatory cytokines (Schwartzman and Cidlowski, 1993). Apoptosis may be an important host defense mechanism whereby virus-infected cells are eliminated, thus preventing the generation and spread of viral progeny during viral infection. On the other hand, viruses have evolved diverse strategies to evade or delay the early onset of apoptosis to ensure their continued propagation (O'Brien, 1998; Roulston et al., 1999; Tortorella et al., 2000; Everett and McFadden, 2001). However, an increasing number of viruses is known to induce apoptosis at the late stages of infection. This process may be important for the spread of progeny virus to neighboring cells and to protect the progeny virus from host enzymes and antibodies (Teodoro and Branton, 1997). In the family Picornaviridae, several viruses have been demonstrated to induce cell death by apoptosis. For example, coxsackievirus B3, like alphaviruses, another group of positive-strand RNA viruses, induces apoptosis through caspase activation (Carthy et al., 1998; Griffin and Hardwick, 1997; Nava et al., 1998); in the cases of Thelser’s murine encephalomyelitis virus (Jelachich and Lipton, 1996) and hepatitis A virus (Brack et al., 1998), the ability to induce apoptosis correlates...
with virulence and thus may determine whether the infection is persistent or acute; poliovirus can induce or inhibit apoptosis according to the conditions of viral infection (Tolskaya et al., 1995). Moreover, the CNS injury during paralytic poliomyelitis is associated with apoptosis induced by the virus infection (Girard et al., 1999). Previous studies have shown that cell damage in the CNS following virus infection could involve apoptosis (Shen and Shenk, 1995). This has been demonstrated, for example, with human immunodeficiency virus (HIV) (Petito and Robeerts, 1995), mouse hepatitis virus (Lin et al., 1997), dengue virus (Despres et al., 1998), and Sindbis virus (Levine et al., 1999). Therefore, it would be interesting to investigate whether the death of neural cells following infection with EV71 involves an apoptotic process.

Less is known concerning the specific viral proteins that are directly responsible for inducing apoptosis than of viral proteins that inhibit cell death. However, HIV transcription regulators Tat and Tax induce apoptosis via upregulation of the FasL promoter (Westendorp et al., 1995), HIV-1 Tat protein has also been shown to induce apoptosis in lymphocytes (Li et al., 1995), and human adenovirus-encoded E1A, E3, and E4 gene products have all been shown to induce the apoptotic pathway (Teodoro and Branton, 1997; Roulston et al., 1999; Rao et al., 1992). Among the Picornaviridae, the poliovirus 3C protease kills cells by apoptosis (Barco et al., 2000), although other viral proteins may also contribute to programmed cell death (Feduchi et al., 1995; Goldstaub et al., 2000).

The molecular mechanisms operative in virus-induced apoptosis involve the expression of a large number of genes. Among them, the ICE (interleukin-1-β-converting enzymes) family of cysteine proteases, caspases, are the central executioners in the apoptotic pathway (Shen and Shenk, 1995; Hengartner, 2000). All caspases contain the highly conserved QACRG sequence, which includes the active-site cysteine (Kuida et al., 1996). Caspases selectively cleave their target proteins, either activating or inactivating them, but in either case the result is apoptosis. Several caspase substrates have been identified recently. For example, the nuclease responsible for the generation of the DNA ladder in the apoptotic cells is activated by caspase cleavage (Enari et al., 1998).

Here we report that transient expression of the 3C protein encoded by EV71 can induce apoptosis in the human glioblastoma cell line, SF268. Our results also indicate that the induction of apoptosis by the 3C protein involves caspase activation, especially caspase-3.

RESULTS

The expression of EV71 3C protein in SF268 glioblastoma cells

Plasmids encoding wild-type EV71 3C (p3C/cDNA3.1) or mutant 3C (p3C-mut/cDNA3.1) were transfected into human glioblastoma SF268 cells. Figure 1 shows a Western blot in which lysates of SF268 cells expressing EV71 3C or 3C-mut were reacted with anti-3C monoclonal antibody. In both cases a 27k MW protein, which was not present in the control cells, was seen (lane 2).

The catalytic motif of the picornavirus 3C protease includes His39, Glu70, and Cys146. In order to evaluate the possible role of the 3C protease in apoptosis induced by EV71, we next wished to express a 3C protein lacking protease activity. We therefore expressed in vitro two fusion proteins, Δ3A-3B-3C-Δ3D and Δ3A-3B-3Cmut-Δ3D, with the latter containing two changes in 3C: His39Gly and Cys146Gly (Fig. 2A). The fusion proteins contain the autocleavage sites indicated by the arrows (Palmenberg, 1990; Dasmahapatra et al., 1992). In Fig. 2B, there is a distinct band whose size (∼52 kDa) corresponds to the full-length Δ3A-3B-3C-Δ3D (lane 1) and Δ3A-3B-3Cmut-Δ3D (lane 2). The larger bands are nonspecific. Major cleavage products were observed in the Δ3A-3B-3C-Δ3D sample but not in the Δ3A-3B-3Cmut-Δ3D sample. These products likely represent Δ3A-3B-3C (40.2 kDa), 3C-Δ3D (40.3kDa), and 3B-3C-Δ3D (43.6 kDa). Smaller cleavage products, which would be expected, such as 3ΔA (9.6 kDa), 3B (3.3 kDa), Δ3D (13 kDa), and Δ3A-3B (12.9 kDa), are probably not seen because they contain few or no [35S]methionine/cysteine residues. A low cleavage efficiency at the 3C-3D junction in this in vitro transcription and translation system (TNT) may account for the absence of prominent 3C (27.3 kDa) and 3B-3C (30.6 kDa) bands (Kean et al., 1990). The result indicates that changing two of the three amino acids of the protease motif of EV71 3C protein resulted in the loss of its proteolytic activity.

Morphological alterations induced by 3C of EV71

A number of alterations in cellular morphology were observed upon the transient expression of EV71 3C pro-
tease. As shown in Fig. 3A, 2 days after transfection, most cells became nonadherent and were floating in the culture medium. Cells in which the mutant 3C protease was expressed looked like the mock-transfected cells.

We then analyzed the ultrastructural modifications in 3C-transfected SF268 cells by transmission electron microscopy. EV71 3C, but not the 3C-mut protein, induced changes in both the cytoplasm and the nucleus typical of those seen in apoptotic cells (Earnshaw, 1995; Barco et al., 2000). These changes included extensive condensation of chromatin, nuclear fragmentation, and formation of membrane-enclosed apoptotic bodies (see arrow in Fig. 3B).

Genome fragmentation in 3C-transfected SF268 cells

To further examine apoptosis in 3C-transfected SF268 cells, we tested for internucleosomal DNA fragmentation.

DNA fragmentation was also examined using the TUNEL assay. SF268 cells were grown on Lab Tek chamber slides, fixed with formaldehyde, and assayed by the terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) method and observed under a fluorescence microscope (Fig. 4B). Both the 3C-transfected SF268 cells and the actinomycin D-treated cultures showed many fluorescent cells (Fig. 4B, b and c). In contrast no fluorescent staining was found in either the mock-transfected SF268 cells or the 3C-mut-transfected cells (Fig. 4B, a and d).

Cells labeled by the TUNEL method were also analyzed by flow cytometry. When EV71 3C was expressed, the SF268 cells showed a prominent new peak representing a 100-fold increase in fluorescence, consistent with 3C protease-induced apoptosis (Fig. 4C). When the proteolytic motif was inactivated by mutation, no such peak was observed. Taken together the morphological alterations and genomic DNA fragmentation indicate that EV71 3C-expressing SF268 cells undergo apoptosis.

Caspase activation and caspase inhibitors in 3C-transfected SF268 cells

Caspases, a family of cysteine proteases, play an essential role in the process of apoptosis (Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Green and Kroemer, 1998; Hengartner, 2000). They cleave several key structural proteins and result in the systematic and orderly disassembly of the cells. One of these caspase substrates is poly(ADP-ribose) polymerase (PARP), a zinc-dependent, eukaryotic, DNA-binding protein that specifically recognizes DNA strand breaks (Wang et al., 1997). The 113-kDa PARP is cleaved during apoptosis into 89- and 24-kDa fragments, a process which represents a specific marker for caspase activation. As shown in Fig. 5, PARP cleavage was seen 24 h after transfection in 3C-expressing, but not in 3C-mut-expressing SF268 cells (Fig. 5, lanes 3 and 4), indicating that caspase was activated by 3C expression.

Recently Barco et al. (2000) have shown that cellular caspases mediate poliovirus protease 3C-induced HeLa cell apoptosis. To determine whether caspases are crucial for EV71 3C-induced apoptosis in SF268 cells, we examined the effects of several different caspase inhibitors: DEVD-fmk, which blocks the activity of caspase-3 and caspase-8; VAD-fmk, which interferes with cas-
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Caspase-3 as well as other proteases in the ICE family; and YVAD-cmk, which blocks caspase-1. As shown in Fig. 6, both DEVD-fmk and VAD-fmk, but not YVAD-cmk, inhibited 3C-induced apoptosis in SF268 cells. The results indicate that caspase-3 is involved in 3C-induced SF268 cell apoptosis.
Although the picornavirus 3C protein functions as a cysteine protease, it has also been shown, in the case of poliovirus and rhinovirus, to interact specifically with the 5′-UTR of the viral RNA (Andino et al., 1990a,b, 1993; Walker et al., 1995). Gel shift assay was performed to examine whether the EV71 3C protein binds to the 5′-UTR of the viral RNA.
UTR of the viral RNA. As shown in Fig. 7, 3C protein of EV71 also bound specifically to the 5'-UTR of the viral RNA. Thus the EV71 3C protein likely possesses multiple activities.

**DISCUSSION**

Apoptosis is one of the common cellular responses to many viral infections. Such self-destruction of the host cells is considered a defense reaction to prevent generation and spread of the viral progeny. Although some viruses have evolved one or more ways to suppress this defense mechanism, other viruses trigger apoptosis in host cells at the late stages of infection to spread viral progeny (Teodoro and Branton, 1997; Roulston et al., 1999; Everett and McFadden, 2001). The overall cost to the viability of an organism of losing infected cells by apoptosis may be small if the dying cells can be replaced quickly. However, virus-induced apoptosis in nonrenewable cells, for example in the CNS, where most neurons are postmitotic and therefore cannot be replaced, may result in an irreversible pathology. Thus EV71-induced apoptosis may play an important role in the pathogenesis of disease in CNS.

Viral gene products that induce or suppress apoptosis have been identified for many DNA viruses as well as for retroviruses (Teodoro and Branton, 1997; Ikeda et al., 1998; Sundararajan and White, 2001). An increasing number of RNA viruses other than retroviruses have now been shown to induce apoptosis (Jan et al., 2000; Everett and McFadden, 2001). However, with these viruses, relatively little is known concerning which viral gene products cause apoptosis or about the mechanism involved. The viral proteases 2A and 3C have been demonstrated to contribute to poliovirus-induced apoptosis (Feduchi et al., 1995; Tolskaya et al., 1995; Barco et al., 2000). Poliovirus 2A induces apoptosis through the cleavage of translation initiation factor eIF4G (Feduchi et al., 1995), whereas poliovirus 3C kills cells by apoptosis through the activation of caspase (Barco et al., 2000). It has been shown that the death of poliovirus-infected cells may occur in two ways: canonical cytopathic effect in the productive infection and apoptosis when virus replication is restricted, in which case the caspase is activated (Agol et al., 1998). Coxsackievirus B3 induces cell death by caspase activation, but the specific gene product involved in this activation remains uncertain (Carthy et
In this study, we have shown that the EV71 3C protease induces apoptosis in the SF268 cells via the activation of caspase. In addition to proteolytic activity, the picornavirus 3C protein has been shown to function in the case of poliovirus and rhinovirus by interacting specifically with the 5′-UTR of the viral RNA (Andino et al., 1990a,b, 1993; Walker et al., 1995). We have also found that 3C of EV71 has RNA binding activity. Thus the viral 3C protein may also possess multiple activities. Which activity of 3C results in the induction of apoptosis? In this study we clearly demonstrated that the proteolytic activity of 3C is involved in the caspase-dependent apoptosis in SF268 cells. By changing two of the amino acids in the 3C protease catalytic motif, not only was the protease activity abolished, but the apoptosis-inducing effect was also lost. As the 3C protease cleaves a variety of host proteins, including various transcription factors (Clark et al., 1991; Yalamanchili et al., 1996), cleavage of one or more of these proteins or of other unidentified host factors could possibly activate caspase and consequently trigger apoptosis in the SF268 cells.

Caspases are a family of cysteine proteases involved in regulating cytokine maturation and apoptosis (Cryns and Yuan, 1998). Apoptosis is mediated by ‘effector’ caspases such as caspase-3 and caspase-7, which cleave crucial cellular polypeptides to trigger the apoptosis. These ‘effector’ caspases are activated through proteolytic cleavage by upstream ‘initiator’ caspases such as caspase-8 and caspase-9, which are launched to respond to pro-apoptotic stimuli (Nicholson, 2000). At least 14 caspases have been identified so far, of which 12 human homologues are known (Nicholson, 2000). Little is known about which specific caspase(s) is involved in virus-induced apoptosis; however it has been shown that caspase-3 is activated in poliovirus, HIV, adenovirus, and hepatitis C virus-infected cells (Lopez-Guerrero et al., 2000; Banki et al., 1998; Chiu and White, 1998; Ruggieri et al., 1997). In the present study, we investigated the molecular mechanism of EV71 3C-induced apoptosis. The demonstration that the caspase inhibitors DEVD-fmk and VAD-fmk prevent the apoptosis induced by the 3C of EV71, indicates that caspase-3 is involved in the 3C-induced cell death. Our finding is
consistent with a previous study showing that two major
caspases, caspase-3 and caspase-9, are involved in
neuronal cell death (Kuida et al., 1996, 1998; Yuan and
Yankner, 2000). Caspase-3 is thought to be a critical
executioner caspase that acts downstream in the apop-
totic pathway and is involved in cleaving important
substrates such as PARP (Cohen, 1997; Nicholson and
Thornberry, 1997) and ICAD (inhibitor of caspase-acti-
vated DNase), which activates the apoptotic DNA ladder-
forming activity of DNase (Enari et al., 1998; Sakahira et
al., 1998).

To understand the role that apoptosis plays in virus
infection and how it is induced, it is important to identify
the virus-triggered step in the signal transduction cas-
cade. Furthermore, understanding the mechanism in-
volved would potentially lead to rational approaches for
the development of methods to prevent cell death, espe-
cially of nonrenewable cells. Since recent observations
show that caspase-11 is responsible for the activation of
caspase-1 and caspase-3 only under pathological con-
ditions (Kang et al., 2000), it would be interesting to
investigate whether caspase-11 is activated by EV71 3C
expression; this would provide the prospect of being
able to inhibit pathological cell death therapeutically
without disturbing developmental and homeostatic apo-
tosis. We are currently using gene array analysis to
investigate the role of EV71 3C in this apoptotic pathway
as 3C-induced apoptosis might represent an efficient
mechanism which is able to spread EV71 into the CNS
and to evade host immune responses, including host
antibodies. In addition, it will be important to identify
3C-interacting cellular proteins which may be related to
the apoptotic pathway. What we learn will be helpful in
understanding in more detail the specific mechanisms
involved in EV71-induced apoptosis.

MATERIALS AND METHODS

Cell culture

SF268 (human glioblastoma) cells were grown in RPMI
1640 medium supplemented with 10% fetal calf serum
(BRL, Grand Island, NY).

Plasmid construction

All DNA manipulations were done by standard meth-
ods. To construct p3C/cDNA3.1, the EcoRI/XhoI fragment
from p3C/ET (Novagen, Madison, WI) was cloned into the
multiple cloning site of a pcDNA3.1/Zeo(+) vector down-
stream of the CMV promoter (Invitrogen, Groningen, The
Netherlands). This fragment contains the enterovirus 71
(TW/2231/98) 3C sequence and has been previously se-
quenced. For construction of p3C-mut/cDNA3.1 in which
two amino acids of the protease motif were changed
(His39Gly; Cys146Gly), an infectious clone of EV71 (per-
sonal communication with Dr. Mei-Shan Ho at Academia
Sinica, Taiwan) with the two required point mutations
was used as a template to amplify the sequence encoding
3C-mut. Pfu Turbo DNA polymerase (Stratagene, La
Jolla, CA) was used for the PCR; EcoRI and XhoI restric-
tion sites were added upstream and downstream, re-
spectively. The fragment was then digested with EcoRI and
XhoI and cloned into pcDNA3.1/Zeo(+) .

Transfection of SF268 cells

SF268 cells were grown in 35-mm plates to 70% con-
fluence and then transfected using the liposomal trans-
fection reagent, DOTAP (Roche, Basel, Switzerland) with
5 µg of p3C/cDNA3.1 or p3C-mut/cDNA3.1 according to
the DOTAP protocol. Cells were harvested 2 days after
transfection. Expression of 3C was detected by Western
blot.

Cleavage assay for 3C protease activity of
enterovirus 71

To demonstrate that alteration of two of the catalytic
residues (His39Gly; Cys146Gly) of the 3C protease led to
the loss of the proteolytic activity, we constructed two
plasmids, pΔ3A-3B-3C-Δ3D and pΔ3A-3B-3Cmut-Δ3D. PCR
fragments were generated so as to contain the EV71
genome covering parts of 3A and 3D and all of 3B and 3C
under the control of the T7 promoter. The expressed
protein contains 3C protease that is expected to have
autocatalytic activity. The proteins were produced and
labeled with [35S]methionine/[35S]cysteine in a rabbit re-
ticulocyte lysate in vitro transcription and translation sys-
tem (TNT) for 3 h. The labeled protein products were
separated on a 4–20% gradient SDS–PAGE gel. The gels
were dried and analyzed by autoradiography.

Cell death assays and microscopic techniques

Phase-contrast and electron microscopy were carried out
as described previously (Li et al., 1999). For analysis of
dNA fragmentation, DNA was isolated using the Blood
and Cell Culture DNA minikit (Qiagen, Hilden, Germany)
and analyzed by gel electrophoresis; the ApoAlert DNA
Fragmentation Assay kit (Clontech, Palo Alto, CA) was
used to perform the TUNEL assay according to the di-
rections of the manufacturer. Caspase activation and
inhibition were analyzed using the ApoAlert Caspase
Colorimetric Assay Kit (Clontech). The protease inhibi-
tors DEVD-fmk, YVAD-cmk, and VAD-fmk were pur-
chased from Clontech.

Poly(ADP-ribose) polymerase cleavage

SF268 cells were transfected with either p3C/cDNA3.1
or p3C-mut/cDNA3.1 as described previously. Cells were
harvested 24 h after transfection. Total protein extracts
were prepared according to the protocol provided by the
manufacturer. Full-length PARP as well as the large PARP
fragment generated by caspase cleavage were detected by Western blot using a polyclonal antibody against PARP purchased from Roche (Basel, Switzerland).

Preparation of labeled RNA and RNA binding assay

The 5’-terminal 126 nt of EV71 viral RNA was prepared and labeled with [α-32P]GTP using in vitro transcription. To assay for the binding of the viral RNA to the 3C protein, different amounts of 3C protein were incubated with 32P-labeled 126-nt viral RNA sequence (1 × 10⁴ cpm, 5 pmol) at 25°C for 30 min. The protein–RNA complexes were then separated from free RNA by electrophoresis on 4% nondenaturing polyacrylamide gels.

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