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Vero cells infected with the Lederle strain of canine distemper virus have increased Fas receptor signaling expression at 15 h post-infection

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ABSTRACT. We evaluated the expression of the Fas receptor gene in Vero cells infected with the Lederle vaccine strain of canine distemper virus using RT-PCR. Vero cells were plated, and after being grown for 24 h in MEM with 5% FBS, 80-90% confluent monolayer cultures were infected with the virus. The cells were harvested at 3, 6, 9, and 15 h post-infection. Uninfected Vero cells were used as a control. Total RNA was isolated from Vero cells using 1 mL Trizol® LS, and RT was performed using 2 µg total RNA. Primer pairs for RT-PCR amplification for the canine distemper virus nucleocapsid gene, the S26 reference gene, and the Vero rFas gene were used to analyze expression in Vero cells. RT-PCR results revealed virus activity at 3, 6, 9, and 15 h in the virus-infected Vero cells. The S26 housekeeping gene was

amplified in virus infected and control samples. However, expression of the cell death receptor Fas was detected in Vero cells only at 15 h post-infection. We suggest that the Lederle vaccine induces apoptosis by Fas receptor signaling, possibly through caspase-8 signaling rather than through mitochondrial signaling in the infected cells.

Key words: Apoptosis; CDV; Fas receptor; RT-PCR; Vero cells

INTRODUCTION

Apoptosis is a regulated physiological process that plays a critical role in adult tissue homeostasis, and it is involved in embryonic development (Young et al., 1997). The process of apoptosis has also been implicated in the pathogenesis of many infectious diseases including those caused by viruses (Roulston et al., 1999). Apoptotic cells exhibit distinct biochemical and morphological changes such as nuclear shrinkage, chromatin condensation, plasma membrane blebbing, intra-nucleosomal cleavage and, ultimately, the formation of apoptotic bodies due to the fragmentation of DNA strands by activated endogenous endonucleases (Cohen, 1999).

Apoptosis can be triggered through two pathways: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway involves activation of caspase-8 and is initiated by ligand interaction with Fas or death receptors, while the intrinsic pathway is activated by an imbalance between proapoptotic and antiapoptotic proteins in mitochondria (Merry and Korsmeyer, 1997), resulting in the release of cytochrome *c* from mitochondria, which in turn activates caspase-9. Both caspase-8 and caspase-9 activate caspase-3, which along with other effector caspases cleave critical cellular proteins, resulting in apoptosis (Adams, 2003).

Canine distemper virus (CDV), a morbillivirus in the family Paramyxoviridae, causes canine distemper, a severe systemic disease in dogs characterized by a variety of symptoms, including fever, respiratory and enteric signs, and neurologic disorders. It has been reported that CDV causes apoptosis in lymphoid tissue and the cerebellum of infected dogs (Moro et al., 2003a,b; Kumagai et al., 2004), and also in monkey kidney (Vero) cells (Guo and Lu, 2000; Kajita et al., 2006).

It has been demonstrated that CDV infection activates the extrinsic pathway in CDV-infected Vero cells at 24 h post-infection (p.i.) with activation of caspase-8 and caspase-3 and gene expression of Fas death receptor (Kajita et al., 2006). The same mechanism was observed in cerebellum and lymph nodes of naturally infected dogs (Del Puerto et al., 2010). However, no study was performed before 24 h p.i. to clarify which initial mechanisms are triggered in apoptosis induced by CDV infection.

Thus, the main objectives of this study were to determine the initial mechanisms of apoptosis in cultured Vero cells, a monkey kidney cell line, infected by CDV Lederle strain (CDV-Lederle) and to identify the specific pathways.

MATERIAL AND METHODS

Cell and virus strains

Vero cells were plated on 6-well dishes (5 x 10^4 cells/cm^2), and after being grown for

24 h in modified Eagle's medium with 5% fetal bovine serum, 80-90% confluent monolayer cultures were infected with CDV-Lederle at a multiplicity of infection of 0.1. The cells were harvested for experiments at 3, 6, 9, and 15 h p.i. Non-infected Vero cells at 3, 6, 9, and 15 h were used as controls.

RNA isolation

Total RNA was isolated from CDV-infected Vero cells, and non-infected Vero cells using TRIZOL® reagent and protocol (Invitrogen Life Technologies, Carlsbad, CA, USA). Samples were treated using the TURBO DNA-free kit (AM1907 - Applied Biosystems), and RNA concentration was measured on Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA).

Reverse transcription (RT)

The first-strand complementary DNA (cDNA) was synthesized from 400 ng total RNA in a final reaction volume of 20 µL, using the Superscript first-strand synthesis system (Invitrogen Life Technologies). After denaturing the template RNA and primers (40 pmol Fas receptor, S26 and CDV reverse primers; Table 1) at 70°C for 10 min, 40 U reverse transcriptase was added in the presence of RT buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 4 µL dNTP mix (250 µM each), 40 U RNase inhibitor and RNase-free water to complete the final volume. The reaction mixture (20 µL) was incubated at 43°C for 1 h and then stopped at 4°C and used immediately for polymerase chain reaction (PCR).

Table 1. PCR primers selected.

	Primers	Sequence of the nucleotides (nt)	Nucleotide size	Fragment length
A	VCC01-F1	5'-CAG CAC CGT ACA TGG TTA TC-3'	20 nt	319 bp
	VVC02-R2	5'-TAG CAT AAC TCC AGA GCA ATG-3'	20 nt	
B	S26CF-F1	5'-CGT GCT TCC CAA GCT GTA CGT GA-3'	24 nt	75 bp
	S26CF-R2	5'-CGA TTC CGG ACT ACC TTG CTG TG-3'	23 nt	
C	FasVEROFor	5'-AAT AAA CTG CAC CCG GAC CCA GAA-3'	24 nt	93 bp
	FasVERORev	5'-GTG CAA GGG TCA CAG TGT TCA CAT-3'	24 nt	

Primers used for RT-PCR for canine distemper virus (CDV) diagnosis (A), reference gene S26 (B), and Fas death receptor (C).

Polymerase chain reaction

PCR amplification for Fas receptor, S26 and the CDV nucleocapsid gene was performed using 40 ng cDNA (2 µL RT reaction mix), using GoTaq® DNA Polymerase (Promega, WI, USA). PCR was performed in a final volume of 25 µL, for each target, as follows: 1.25 µL (12.5 pmol) of each primer (reverse and forward) (Table 1), 2 µL dNTP mix (0.2 mM each dNTP), 5 µL 5X GoTaq® DNA Polymerase buffer (final concentration of 1X (1.5 mM $MgCl_2$) 0.25 µL (2.5 U) GoTaq® DNA Polymerase and PCR grade water to complete the final volume.

Crystal violet staining

Vero cell monolayers were washed twice in 1X PBS, and 400 µL crystal violet solution

was added to each well and allowed to stand for 5 min. After staining, cells were gently washed three times with distilled water, preserving the monolayer. Vero cells were observed using an inverted light microscope, and apoptotic cells were counted using characteristics such as: nuclear shrinkage, chromatin condensation, plasma membrane blebbing, and apoptotic bodies.

RESULTS

RT-PCR

RT-PCR results revealed the presence of CDV at 3, 6, 9, and 15 h in CDV-Lederle-infected Vero cells, and there was no detection in Vero cell control groups. The S26 reference gene was amplified in all samples (Figure 1). However, the gene expression of the death receptor Fas was just detected in Vero cells at 15 h post-infection (Figure 2). There was no Fas receptor expression in Vero cells at 3, 6 and 9 h p.i.; also, no expression of Fas receptor was observed in Vero cell control groups at 3, 6 and 9 h (Figure 2).

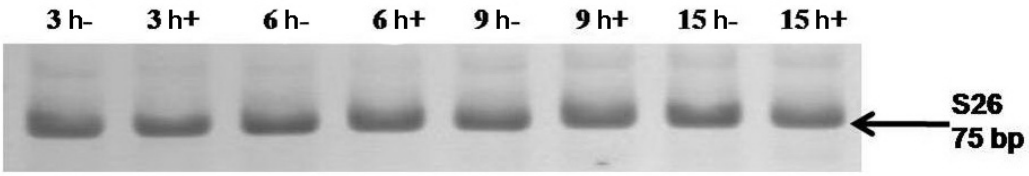

Figure 1. Silver-stained 8% polyacrylamide gel, after electrophoresis at 100 V for 50 min. S26 specific amplicon of 75 bp is indicated.

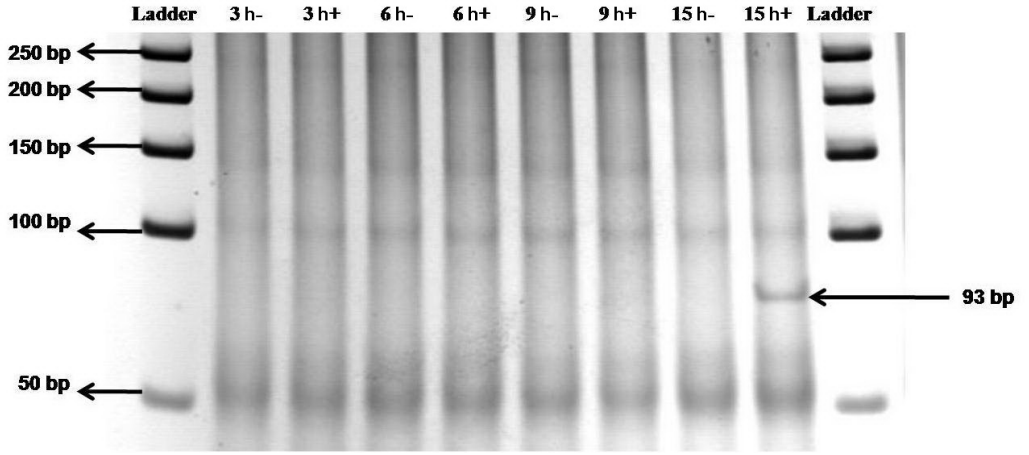

Figure 2. Silver-stained 8% polyacrylamide gel, after electrophoresis at 100 V for 50 min. Fas receptor specific amplicon of 93 bp is indicated, and it is shown at 15 h after CDV infection sample (15 h+). Ladder = 50-bp DNA (0.3 µg).

Crystal violet staining

Crystal violet staining revealed typical apoptosis morphology in Vero cells at 3, 6, 9, and 15 h p.i. (Figure 3). Upon quantification of approximately 100 cells, the apoptotic rate was found to be 11% at 3 h p.i., 16% at 6 h p.i., 23% at 9 h p.i., and 34% at 15 h p.i. Vero cell control groups did not show apoptotic cells. This indicated that CDV induces apoptosis of Vero cells in a time-dependent way.

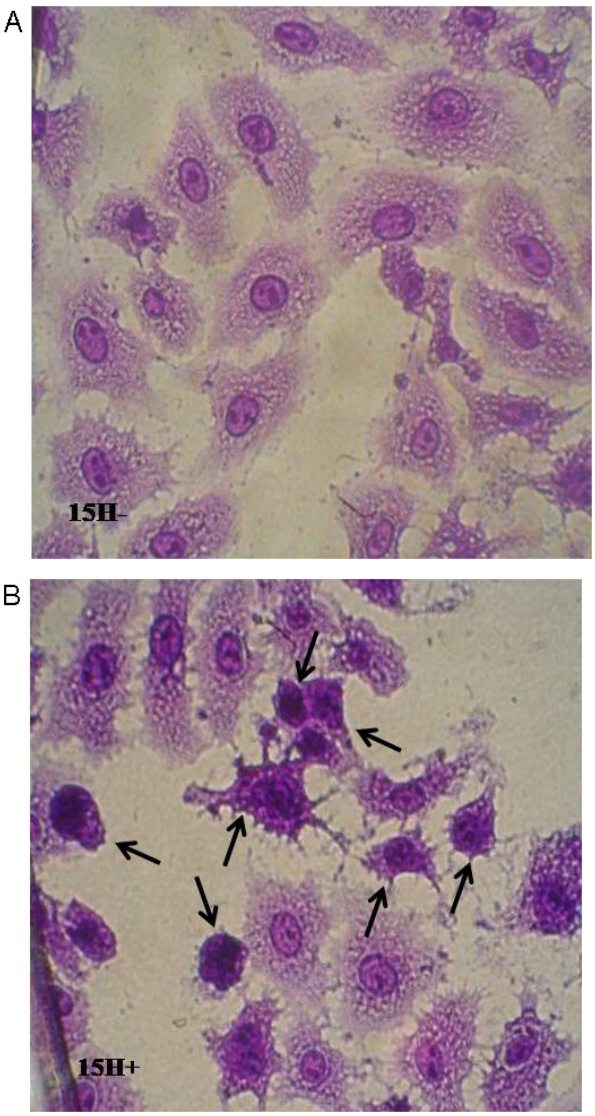

Figure 3. A. Control Vero cells (CDV non-infected) at 15 h. **B.** CDV-infected Vero cells at 15 h post-infection. Arrows indicate apoptotic cells.

DISCUSSION

In the present study, Vero cells infected by CDV-Lederle showed typical apoptosis morphology at different p.i. times, indicating that CDV-Lederle induced apoptosis in Vero cells, while no apoptotic cells were identified in control Vero cell groups.

In the present study, it was also found that CDV-Lederle cells at 15 h p.i. expressed Fas death receptor mRNA (Figure 2). Taken together, these findings indicate the possibility that CDV-Lederle infection may activate Fas-mediated caspase-8 signaling to induce apoptosis in Vero cells. Interestingly, there has been no report that Vero cells express FasL, so it seems unlikely that apoptosis in CDV-infected Vero cells was triggered by the FasL-bound Fas receptor. In the case of measles virus (MV), which belongs to the genus Morbillivirus, MV-infected peripheral blood mononuclear cells (Vuorinen et al., 2003) and dendritic cells (Servet-Delprat et al., 2000a) have been shown to induce apoptosis in uninfected T cells via the Fas/FasL pathway (Servet-Delprat et al., 2000b). With respect to the Sendai virus (Sv), which, like CDV, belongs to the subfamily Paramyxovirinae, it has been reported that induction of host cell apoptosis by Sv *in vitro* requires the activation of caspase-8 but does not require FasL (Bitzer et al., 1999). Our results revealed Fas receptor mRNA expression only at 15 h p.i. However, apoptotic cells were observed at 3, 6 and 9 h p.i., while no Fas receptor mRNA expression was demonstrated during these times. Therefore, it can be considered that during virus replication and infection, the cells' injury induced mRNA expression of the Fas receptor at 15 h p.i., and as reported by Kajita et al. (2006), this can persist up to 48 h p.i. Further studies will be needed to clarify the mechanisms of CDV-induced host cell apoptosis at initial times of infection and to determine if the initial apoptosis mechanisms induced by CDV trigger the intrinsic pathway, and later the extrinsic pathway.

In conclusion, CDV-Lederle was found to induce apoptosis in Vero cells by activating the extrinsic pathway at 15 h p.i., and the principal cascade for this induction occurred along the caspase-8 activation pathways. Based on the present findings, the mitochondrial pathway may not participate in the caspase-8/caspase-3-mediated induction of apoptosis after 15 h p.i., but it is not clear if the mitochondrial pathway participates at the initial times of CDV infection. Further studies will be necessary to clarify the detailed mechanisms by which apoptosis is induced in CDV-infected cells.

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