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Short Communication

Apoptosis in Vero cells infected with Akabane, Aino and Chuzan virus

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Akabane, Aino and Chuzan virus are arthropod-borne (arbo) viruses mainly associated with reproductive failures in cattle. We investigated apoptosis in Vero cells (C-1586) infected with Akabane, Aino and Chuzan virus. The fragmentation of chromosomal DNA was simultaneously detected with the progress of cytopathic effect from 48 hr to 72 hr post infection, depending on viruses. Although the treatment of cycloheximide blocked apoptosis in Vero cells infected with three viruses, actinomycin D did not prevent DNA oligomerization, thus indicating that *de novo* viral protein synthesis is critical for viral apoptosis. In addition, the activation of caspase-3 was also detected in Vero cells by indirect fluorescent assay. From the present results, it is of future interest whether apoptotic characteristics of these viruses are related to pathogenecity *in vivo*.

Key words: Aino virus, Akabane virus, Chuzan virus, apoptosis, Vero cells

Akabane, Aino and Chuzan virus are arthropod-borne (arbo) viruses transmitted by blood-sucking insects like mosquitoes, midges and ticks. Arbo viruses are a diverse group of RNA viruses that replicate in hematophagous arthropods (vectors) prior to transmission to human or animal host. Medically important arboviruses are classified within the families of *Togaviridae, Flaviviridae* and *Bunyaviridae* [19].

Akabane and Aino virus are *Bunyaviruses* in the family of *Bunyaviridae*, and Chuzan virus is *Orbivirus* in the family of *Reoviridae*. Three arbovirus infections are mainly associated with abortions, stillbirths and congenital defects in pregnant cattle, sheep and goat. The incidence of these viruses is widely distributed in Southest Asia and Australia [1,15,17]. Although Akabane, Aino and Chuzan virus cause clinically similar reproductive failures in cattle, the exact mechanisms on pathogenicity are not still clear.

Viruses from several genera and families are known to induce apoptosis in infected cells. Apoptosis is an energy-dependent process by which individual cells undergo [10, 24]. The process of apoptosis can be divided into three stages. The first stage is the initial signal for apoptosis by variety of stimuli. The second stage involves the classic morphological changes including condensation of chromatin and vacculization of cytoplasma, and biochemical changes including cellular proteases and endonucleases. The final stage involves the formation of membrane-bound apoptotic bodies [7,12,23].

In this study, we investigated apoptosis in Vero cells infected with Akabane, Aino and Chuzan virus. Vero cells (ATCC, C-1586) were regularly maintained in alpha-MEM, supplemented with 5% heat-inactivated fetal bovine serum, penicillin (100 unit/ml), streptomycin (100 unit/ml) and amphotericin (0.25 g/ml). A strain of Korean isolate of Akabane, Aino and Chuzan virus were plaque-purified once at the passage level of 6 in Vero cells. The viruses were inoculated at 1-10 multiplicity of infection (MOI) and maintained in alpha-MEM with 2.5% FBS in 5% CO_2 incubator at 37°C. Since DNA fragmentation is a hallmark of apoptotic cell death, the virus infected cells were scraped at regular time interval and harvested after centrifugation at 3,000 rpm for 10 min [5,10]. The DNA was extracted by the phenol-chroloform method [11]. The extracted DNA was suspended at 1/20 of original volume with TE buffer (0.01 M Tris, 0.001 M EDTA, 0.1 M NaCl, pH 8.0). The electrophoresis of DNA were carried out in 1.8% agarose gel. While DNA fragmentations were detected from 48 hr after the inoculation of Akabane and Aino virus, Chuzan virus induced DNA oligomerization was observed from 72 hr post inoculation as shown in Fig. 1. The fragmentation of chromosomal DNA was simultaneously detected with progress of cytopathic effect (CPE) from 48 hr to 72 hr post infection, depending on viruses. However, the CPE was completed in 96 hr in Vero cells infected Akabane, Aino and Chuzan virus.

In order to investigate whether *de novo* protein synthesis and RNA synthesis are required to induce apoptosis, we infected Vero cells with Akabane, Aino and Chuzan virus in the presence of cycloheximide (CHX, Sigma, USA) at the concentration of 0.5 μg/ml or 1 μg/ml. In addition, actinomycin

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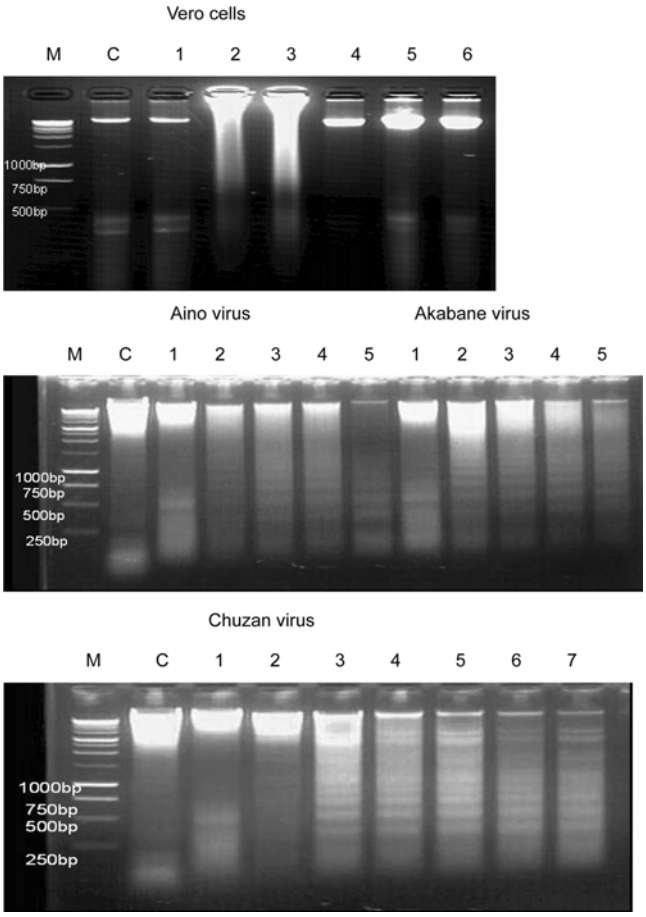

Fig. 1. Time course of DNA fragmentation in uninfected Vero cells (A) and Vero cells infected with Aino, Akabane (B) and Chuzan virus (C). The infected cells analyzed on 1.5% agarose gel electrophoresis. DNA fragmentations were detected from 48 hr (lane 2) after the inoculation of Akabane and Aino virus, Chuzan virus induced DNA fragmentations was observed from 72 hr (lane 3) post inoculation. Lanes M; 1 kb DNA marker, lane C; normal Vero cells, lanes 1~7; 24, 48, 72, 96, 120, 144, 168 hr post infection.

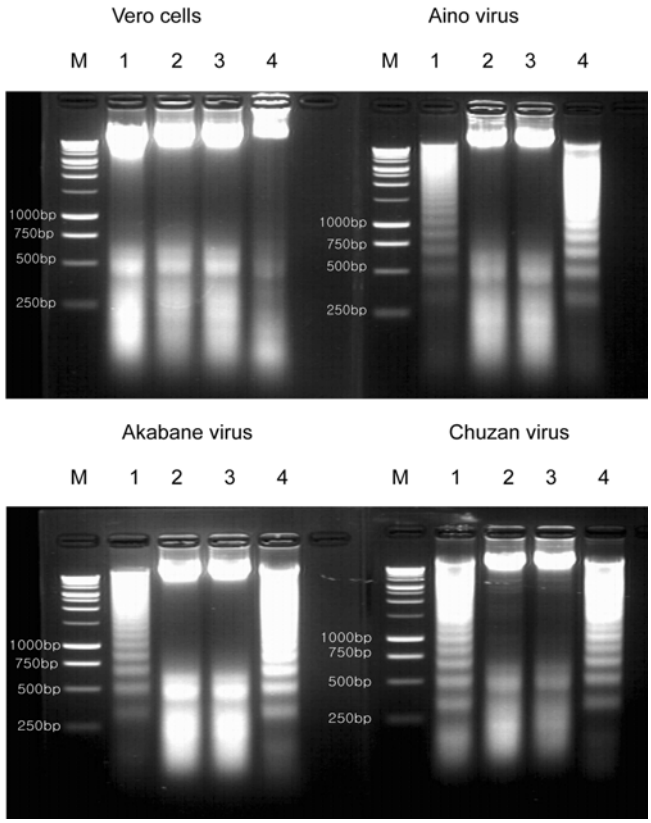

Fig. 2. Chromosomal DNA fragmentation of infected cells and treated with 0.5, 1 μg/ml cycloheximide, with 25 μg/ml actinomycin D. Lane M; 1 kb DNA marker, lane 1; a-MEM + 2.5% FBS (normal vero cell), lane 2; a-MEM + 2.5% FBS + cycloheximide (0.5 μg/ml), lane 3; a-MEM + 2.5% FBS + cycloheximide (1 μg/ml), lane 4; a-MEM + 2.5% FBS + actinomycin D (25 μg/ml).

D (Sigma, USA) was also examinded at the concentration of 25 μg/ml [21,22].

In this experiment, while DNA extracted from two different concentrations (0.5 and 1 μg/ml) of CHX-treated cells did not show any ladderring pattern, DNA extracted from actinomycin D-treated cells showed the characteristic apoptotic DNA fragmentation in Vero cells after 72 hr incubation (Fig. 2). Although viral titers were significantly decreased with the treatment of actinomycin D, apoptosis became clear in the presence of actinomycin D (Table 1). On the other hand, three viruses-induced apoptosis was blocked by the presence of CHX, indicating that apoptosis is required *de novo* viral protein synthesis in Vero cells infected with Akabane, Aino and Chuzan virus. At present, vesicular stomatitis virus, avian leucosis, Sindbis virus reovirus and vaccinia virus induce apoptosis in infected cells at an early stage of infection when virus particles interact with receptors on the cell surface or at the time of fusion with cell membrane and disassembly [3,4,6,10,14,18,22]. In addition, these viruses do not require protein translation or genome replication for induction of apoptosis. In order to investigate whether viral uncoating of three viruses are possible to induce apoptosis, we treated 10^7 $TCID_{50}$/ml of each virus with BEI as described previously [2]. The each inactivated viruses was inoculated at MOI of 10 per Vero cells for a week. However, any sign of apoptosis was not detected with three viruses (data not presented here).

The terminal events of apoptosis involve the activation of a specific series of cytoplasmic protease, caspases. The activation of these self-catalytic caspases in the cytoplasmic has been identified [21]. One pathway involves death receptors at the cell surface, and directly activates upstream caspase in the cytoplasm. Another pathway involves the participation of mitochondria through the induction of leakiness of the external mitochondrial membrane, leading the release of cytochrome c into the cytosol. Downstream from these initiator mechanisms are terminal caspases that

Table 1. Characteristics of apoptosis in Vero cells infected with Akabane, Aino and Chuzan virus

	Akabane				Aino				Chuzan			
	NT*	CHX† (μg/ml)		AD‡	NT	CHX (μg/ml)		AD	NT	CHX (μg/ml)		AD
		0.5	1			0.5	1			0.5	1	
L§	+	-	-	+	+	-	-	+	+	-	-	+
T¶	2.5	-	-	1.5	2.75	-	-	2	3.0	-	-	2.5

*; not treated, †; cycloheximide, ‡; actinomycin D, §; DNA laddering (+; positive, -; negative), ¶; virus titration in Vero cells, $Log_{10}TCID_{50}$/0.1ml.

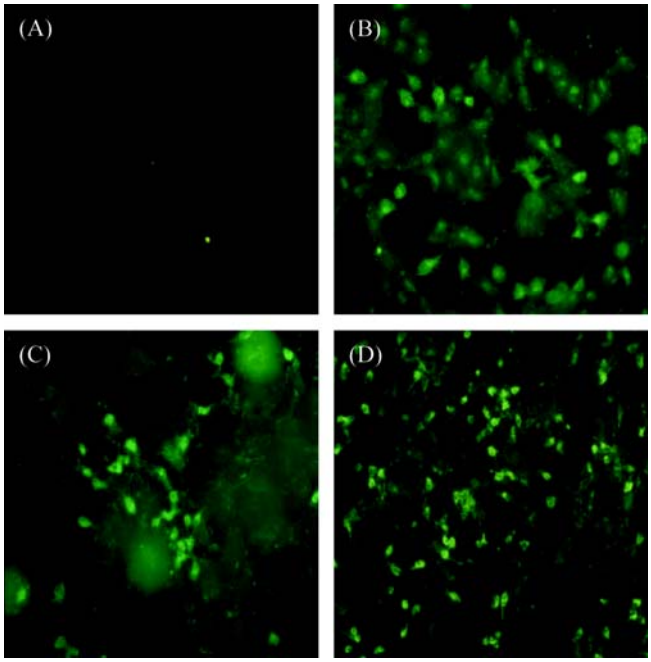

Fig. 3. Detection of activated caspase-3 by indirect immunofluorescence assay. Normal Vero cells (A) and Vero cells infected with Akabane (B), Aino (C) and Chuzan virus (D). ×100.

lead to the morphological and biochemical consequences of apoptosis [16,20].

Activation of caspase-3 was also analyzed to indentify a specific induction pathway of apoptosis in Vero cells infected with three viruses. Each virus was inoculated at 1 MOI in monolayer of Vero cells on cover slip and incubated in a 5% CO_2 incubator at 37°C for 3~4 days. The cells were fixed with 80% cold acetone and reacted with Rabbit anti-active caspase-3 polyclonal antibodies (Bioscience Phamingen, USA) by indirect immunofluorescence assay. In these experiment, all positive reactions of caspase-3 activities were detected in Vero cells-infected viruses at 72 hr postinoculation (Fig. 3). These results revealed that the activation of caspase-3 is induced for apoptosis by Akabane, Aino and Chuzan viruses. Caspase-3 is known to activate at early stage of apoptosis and being considered to be excellent marker of cells in the course of apoptosis [9,12,13,16].

At present, a number of DNA viruses, mainly undergoing latency in host cells, encode proteins inhibiting apoptosis. In contrast, various RNA viruses induce both apoptosis and lysis of cells for efficient dissemination of progency [8,21]. In present study, we demonstrated that apoptosis in Vero cells induced by Akabane, Aino and Chuzan virus with the development of CPE, suggesting for efficient dissemination of viral progeny. In addition, it was also demonstrated that the synthesis of viral proteins in infected cells is critical for the induction of apoptosis. From the present results, it is of future interest whether these apoptotic characteristics of these viruses are related to pathogenecity *in vivo*.

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