Detection of apoptosis induced by new type gosling viral enteritis virus in vitro through fluorescein annexin V-FITC/PI double labeling

Shun Chen, An-Chun Cheng, Ming-Shu Wang, Xi Peng

AIM: To achieve a better understanding of the pathogenesis of new type gosling viral enteritis virus (NGVEV) and the relationship between NGVEV and host cells.

METHODS: The apoptosis of duck embryo fibroblasts (DEF) induced by NGVEV was investigated by fluorescence-activated cell sorter (FACS) and fluorescence microscope after the cells were stained with Annexin V-FITC and propidium iodide (PI).

RESULTS: By staining cells with a combination of fluorescein annexin V-FITC and PI, it is possible to distinguish and quantitatively analyze non-apoptotic cells (Annexin V-FITC negative/PI negative), early apoptotic cells (Annexin V-FITC positive/PI negative), late apoptotic/necrotic cells (Annexin V-FITC positive/PI positive) and dead cells (Annexin V-FITC negative/PI positive) through flow cytometry and fluorescence microscope. The percentage of apoptotic cells increased with the incubation time and reached a maximum at 120 h after infection, while the percentage of non-apoptotic cells decreased.

CONCLUSION: NGVEV can induce the infected DEF cells to undergo apoptosis and the apoptosis occurs prior to necrosis.

Key words: Gosling viral enteritis; New type; Virus; Duck embryo fibroblasts; Apoptosis; Fluorescein annexin V-FITC/PI

INTRODUCTION

In order to eliminate the redundant, damaged, or infected cells, metazoan organisms evolve the cell suicide mechanism termed apoptosis[1]. Apoptosis is a physiological process defined by a number of distinct morphological features and biochemical processes[2,3], which distinguish from necrosis[4,5]. Apoptosis is recognized as an important process in different biological systems, including embryonic development, cell turnover, and immune response against tumorigenic or virus-infected cells[6]. An increasing number of viruses or viral gene products were reported to induce apoptosis both in vitro and in vivo[7,8].

The new type gosling viral enteritis (NGVE) is a new infectious disease and firstly recognized by Cheng et al, and it was observed in goslings less than 30 d of age in various areas of Sichuan Province[9,10]. The mortality from acute NGVE is high, and it is clinically characterized by respiratory, digestive, and neurological symptoms and...
sudden death[17–19]. Catarrhal hemorrhagic fibrinonecrotic enteritis of the small intestine and coagulative embolus in the lower middle part of the intestine are the typical pathological changes of the NGVE in infected goslings[18]. NGVE virus was recognized as an adenovirus, which was round or oval, and characteristic icosahedral in shape, containing double-stranded DNA genome and fifteen structural proteins[18–24]. There are many researches on the histopathology, epizootiology, clinical signs, diagnoses, and immunity of the NGVE[17–24]. Interestingly, the apoptosis induced by NGVE virus infection is poorly documented.

In the early stage of apoptosis, which occurs at the cell surface, one of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell[25–27]. Annexin V-FITC is a phospholipid-binding protein with a high affinity for PS, which can be used as a sensitive probe for PS exposure to the cell membrane[28–30]. However, it has also been reported that it binds to the inner face of the plasma membrane that has lost its integrity during the late stage of apoptosis, also known as secondary necrosis[31]. During the early apoptosis, the cells become reactive with annexin V-FITC after the onset of chromatin condensation, but prior to the loss of the plasma membrane ability to exclude PI[32]. Hence, necrotic cells are both stained by annexin V-FITC and PI, whereas early apoptotic cells are only stained by annexin V-FITC. Double staining of the infected DEF cells with annexin V-FITC and PI in this research could distinguish apoptotic cells from necrotic cells[33]. In this way, live, early apoptotic, late apoptotic/necrotic and dead cells can be discriminated on the basis of a double-labeling for annexin V-FITC and PI, and analyzed by either flow cytometry or fluorescence microscopy[34,35].

### MATERIALS AND METHODS

**Primary duck embryo fibroblast (DEF) and viral strain**

DEF cells were prepared using 11 to 13-d-old embryonated specific pathogen-free (SPF) eggs and propagated in minimal essential medium (MEM; Gibco) containing 100 mL/L new born calf serum (NBCS; Hyclone), 2.2 g/L NaHCO₃, 100 U/mL penicillin/streptomycin (Gibco).

The NGVEV-CN strain with a high virulence field was adopted as above except that 1.0 × 10⁴ cells/mL of the sample solution was transferred to a 5 mL culture tube, and incubated with 5 µL of FITC-conjugated annexin V (Pharmingen) and 5 µL of PI (Pharmingen) for 15 min at room temperature in the dark. Four hundred µL of 1 × binding buffer was added to each sample tube, and the samples were analyzed by FACS (Becton Dickinson) using Cell Quest Research Software (Becton Dickinson).

**Experimental NGVE virus infection of DEF**

The monolayer DEF cells were washed twice with phosphate buffered saline solution (PBS; 0.15 mol/L, pH 7.2) and subsequently exposed to stock NGVEV-CN on a shaker at 37.5°C for 2 h. Stock virus was harvested from infected DEF when 75% cytopathic effects (CPEs) were observed. After inoculation with NGVEV-CN, cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in MEM supplemented with penicillin/streptomycin and 20 mL/L NBCS. Mock-infected cells were processed in the same way except that the virus was excluded.

**Annexin V-FITC/PI staining and analyzed by FACS**

At 24, 48, 72, 96, 120 and 144 h after infection (p.i.), 3 infected and mock-infected cells were harvested through trypsinization, and washed twice with cold PBS (0.15 mol/L, pH 7.2). The cells were centrifuged at 3000 r/min for 5 min, then the supernatant was discarded and the pellet was resuspended in 1 × binding buffer at a density of 1.0 × 10⁴-1.0 × 10⁵ cells per mL. One hundred µL of the sample solution was transferred to a 5 mL culture tube, and incubated with 5 µL of FITC-conjugated annexin V (Pharmingen) and 5 µL of PI (Pharmingen) for 15 min at room temperature in the dark. Four hundred µL of 1 × binding buffer was added to each sample tube, and the samples were analyzed by FACS (Becton Dickinson) using Cell Quest Research Software (Becton Dickinson).

**Annexin V-FITC/PI stained fluorescence microscopy**

The annexin V-FITC/PI staining procedure of the sample was adopted as above except that 1.0 × 10⁶ cells/mL were centrifuged onto glass slides and studied under fluorescence microscope (Nikon 80i).

### RESULTS

**Annexin V-FITC/PI stained FACS**

By staining cells with annexin V-FITC and PI, FACS was used to distinguish and quantitatively determine the percentage of dead, viable, apoptotic and necrotic cells after NGVE virus infection (Figure 1 and Table 1). At 72 h p.i., the percentage of apoptotic cells increased from 4.6% in the mock-infected control culture to 21.5% (Figure 2). The percentage of early apoptotic cells increased with incubated time until 120 h p.i. reaching the maximum 35.3%, and the proportion of the late apoptotic/necrotic cells increased from 0.3% to 17.7% (Table 1). A high level of the early apoptosis was detected from 72 h p.i. and high level of the late apoptosis/necrosis was detected after 96 h p.i., while the basal level of apoptosis and necrosis was shown in the mock-infected controls (Table 1).

**Annexin V-FITC/PI stained fluorescence microscopy**

When examined under fluorescence microscopy, different
labeling patterns in this assay enabled us to identify different cell populations: live cells (Annexin V-FITC negative/PI negative), early apoptotic cells (the intactness of the cell membrane, affinity for annexin V-FITC and devoid of PI staining) (Figure 3A a, b, 3B), late apoptotic/necrotic cells (the cell membrane loses its integrity,
the cell becomes both annexin V-FITC and PI staining) (Figure 3A c-f, 3B) and dead cells (Annexin V-FITC negative/PI positive) (Figure 3A g, h, 3B).

DISCUSSION

Modulation of apoptosis is a common feature of infection by animal viruses and it also contributes to the pathogenesis process[16]. A variety of animal viruses have been identified to induce apoptosis in cultured cells[12,14-16], which contained adenovirus. Early in 1968, Takemori[17] found that cyt mutants of human adenovirus could provoke more violent CPEs. Ezoe[18] further proved that it could also induce the DNA degradation in infected cells. Rautenschlein[19,20] respectively reported that the hemorrhagic enteritis virus (HEV) (fowl adenovirus) could induce B cells and spleen cells undergoing apoptosis. This research indicated that NGVE virus recognized as an adenovirus[17,19,20] could induce DEF undergoing apoptosis, which has never been reported before.

FACS is frequently used to monitor early apoptosis[26-29], which should always be confirmed by the inspection of cells under electron or fluorescence microscope. Annexin V-FITC positive cells were first observed in NGVE-infected DEF cells at 72 h.p.i. under fluorescence microscopy, while it can be detected early from 24 h.p.i. through FACS. The small number of apoptotic cells presented in mock-infected controls, which may be attributed to physiological cell death in vitro. The cells stained by annexin V-FITC alone obviously increased from 72 h.p.i., indicating the induction of apoptosis rather than necrosis due to NGVE virus infection. The cells that stained positive for both annexin V-FITC and PI were increased from 96 h.p.i. indicated the end stage of apoptosis or necrosis, which also suggested that apoptosis occurs prior to necrosis. This may be due to the fact that apoptosis makes many cell remnants undisturbed in vitro, which can be removed by phagocytes in vivo. The apoptotic cell debris interfered with the adjacent normal cells, leading to the necrosis. Furthermore, the lysis that eventually occurred at the end of apoptosis, which had essentially the same membrane permeability that occurred in necrosis. Further experiments are needed for a definite the intracellular events that trigger the apoptotic response during NGVE virus infection.

Recent studies demonstrate that the CPEs caused by virus infection in vitro is mediated by apoptosis[41-43]. Our previous research had revealed that the CPEs became obvious after 72 h.p.i.[24] and TCID<sub>50</sub> reached a maximum at 120 h.p.i., which was consistent to the results of this research: apoptotic cells obviously increased from 72 h.p.i. and the apoptotic peak reached at 120 h.p.i.. Therefore, it seems likely that apoptosis is related to CPEs during NGVE virus infection.

Virus-induced apoptosis is a complex and important aspect of the pathogenesis of viral infection[44-46]. In fact, in the case of virus-infected cells, the induction of cell death can reduce viral spread in the host by early killing of infected cells. In the case of virus itself, apoptosis facilitates persistent viral infection in host cells and is convenient for viral dissemination[47-49]. Quantitative assay of the apoptosis in the present study indicated that the apoptosis was largely induced in the late phase of NGVE virus infection. During late NGVE virus infection, the virus almost completes its replication, therefore, the apoptosis provided a means for releasing the virus particles into the extracellular space without initiating a concomitant host response. It is assumed that NGVE virus induction of apoptosis may be an important mechanism of the efficient dissemination of progeny and the suicide of virally infected cells through apoptosis can limit infection, affording the host organism a certain degree of protection.

Many questions regarding NGVE virus-induced apoptosis remain unanswered, and future studies should be carried out.

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