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Short communication

Dynamic changes of apoptosis in duck embryo fibroblasts induced by new type Gosling viral enteritis virus

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Abstract

The monolayer duck embryo fibroblast (DEF) cells were experimentally infected with new type Gosling viral enteritis virus (NGVEV) and the dynamic changes of apoptosis were detected at different time points after NGVEV infection by transmission electron microscopy (TEM), DNA agarose gel electrophoresis and Annexin V-FITC/PI stained fluorescence-activated cell sorter (FACS). The result shows that NGVEV can induce infected cells undergoing apoptosis and changing regularly. A series of characteristic apoptotic morphological changes including shrinkage of the cells, chromatin condensation and margination, as well as formation of apoptotic bodies, were observed by TEM. The typical ladder pattern of DNA fragmentation was demonstrated by agarose gel electrophoresis. And using flow cytometry analysis of Annexin V-FITC/PI staining, the dead, viable, apoptotic and necrotic cells could be analyzed quantitatively.

Keywords: New type Gosling viral enteritis virus (NGVEV); Duck embryo fibroblasts (DEF); Apoptosis

1. Introduction

Named as programmed cell death (PCD), apoptosis was first put forward by Kerr in 1972 [1]. In order to eliminate redundant, damaged, or infected cells, metazoan organisms have evolved the cell suicide mechanism in terms of apoptosis [1]. Apoptosis is an energy-dependent process of cell suicide in response to a variety of stimulators. It is characterized by a number of distinct morphological features and biochemical processes, including cell shrinkage and partial detachment from substratum, plasma membrane blebbing, chromatin condensation and intra-nucleosomal cleavage. And ultimately apoptotic bodies are formed without provoking an inflammatory response [2–8]. An increasing number of viruses or viral gene products have been reported to induce apoptosis both *in vitro* and *in vivo*, which contain adenovirus [9–11].

New type Gosling viral enteritis virus (NGVEV) is a new fowl adenovirus, which was first reported by Cheng et al. [12]. It can induce NGVE in Goslings less than 30 days of age with hemorrhagic, fibrinonecrotic, hyperaemic, necrotic and exudative enteritis in the small intestine [13,14]. NGVEV has been studied extensively [12–14], but little information is available on apoptosis. Although previous researches indicated that adenovirus can induce host cells undergoing apoptosis [15,16], few about NGVEV. In this study, to provide information on apoptosis evoked by this new fowl adenovirus, the dynamic changes of apoptosis induced by NGVEV in infected duck embryo fibroblast (DEF) cells were analyzed by TEM, DNA agarose gel electrophoresis and Annexin V-FITC/PI stained flow cytometry (FACS).

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2. Materials and methods

2.1. DEF and virus strain

Primary duck embryo fibroblast (DEF) cells were prepared from 11- to 13-day-old embryonated eggs (obtained from Yaan Duck Farm, Sichuan Province, China) and propagated in minimal essential medium (MEM, GIBCO) supplemented with 10% inactivated new born calf serum (NBCS, Hyclone), 0.22% $NaHCO_3$, 100 U/ml penicillin and streptomycin.

CN strain, a highly virulent field strain of NGVEV, was provided by the Avian Disease Research Center of Sichuan Agricultural University.

2.2. Infection of DEF with NGVEV

The monolayer DEF was washed with phosphate buffered saline solution (PBS, 0.15 M, pH 7.2) twice. Then the cells were exposed to stock NGVEV-CN on a rocker at 37.5 °C for 2 h. Stock virus was harvested from infected DEF when 75% cytopathic effect (CPE) was observed. After inoculation with NGVEV-CN, cells were maintained in MEM containing 2% NBCS at 37.5 °C. Mock-infected cells were treated following the identical method except for the omission of NGVEV.

2.3. Transmission electron microscopy

The NGVEV-infected and mock-infected cells were collected at 24, 48, 72, 96, 120 and 144 h post infection (p.i.), fixed in 2.5% glutaraldehyde at 4 °C for 2 h. The cells were scraped from the flasks and centrifuged at 3000 rpm/min for 5 min. Then the supernatant was discarded and the pellets were mixed with 2% low melting-temperature agarose at 37 °C, and centrifuged at 5000 rpm/min for 10 min. Samples were post-fixed in 1.0% aqueous OsO_4. After a stepwise dehydration in ethanol, samples were embedded in epoxy resin 618 and polymerized at 80 °C for 3 days. Then, 50 nm slices were stained with uranyl acetate and lead citrate for subsequent examination with the TEM (Hitachi H-600).

2.4. DNA ladder analysis

DNA was isolated from apoptotic and control cells, and the electrophoresis of the fragmented DNA used a slightly modified procedure as one previously described [17]. Briefly, NGVEV-infected and control cells that had grown in cell culture flask were washed twice with PBS (0.15 M, pH 7.2) and the cells scraped from the flask were centrifuged at 5000 rpm/min for 10 min. The pellets were resuspended in PBS, then treated with Proteinase K (10 mg/ml, Promega) and incubated for 1 h in a 50 °C water bath. Cellular DNA was extracted twice with phenol, followed by chloroform/isoamyl alcohol extraction for removal of protein and residual traces of phenol, then precipitated for 24 h in 2 volumes of ethanol at −20 °C. Precipitated DNA was resuspended in 50 μl double-distilled water and treated with RNase A (10 mg/ml, Sigma) for 30 min. DNA fragmentation was visualized under UV light after electrophoresis in 1.6% agarose gels and glodenview (SBS) staining in comparison of 100 bp DNA ladder (Tiangen).

2.5. Annexin V-FITC/PI stained FACS

At different infection time points (24, 48, 72, 96, 120 and 144 h) 3 NGVEV-infected and mock-infected cells were, respectively, collected by trypsin treatment, washed twice with cold PBS (0.15 M, pH 7.2) and resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. Then 100 μl of the sample solution was transferred to a 5 ml culture tube, incubated for 15 min with 5 μl of FITC conjugated annexin V (Annexin V-FITC, Pharmingen) at room temperature in the dark. And cells were additionally incubated for 15 min with 5 μl of propidium iodide (PI, Pharmingen). Then 400 μl of 1× binding buffer was added to each sample tube, the samples were analyzed by FACS (Becton Dickinson) and the Cell Quest Research Software.

3. Results

3.1. Morphological changes of apoptotic DEF cells induced by NGVEV

Infected cells were carefully examined under TEM, three distinct phases of morphological changes of apoptosis were observed. During 0–48 h p.i., the difference of apoptotic morphological change between NGVEV-infected and mock-infected cells was little. However in the later phase (72–120 h p.i.), the cells dehydrated and detached from their substratum and adjacent cells with a loss of desmosomes (Fig. 1), then nuclear chromatin condensed (Fig. 2) and the endoplasmic reticulum swelled (96–120 h p.i.). Meanwhile, the cells shrank and cytoplasmic contents increased in electron density. In the last phase (120–144 h p.i.), the condensation started at the nuclear periphery, which was accompanied by losing the nuclear envelope followed by nuclear fragmentation. Subsequently, nuclear fragments and the constituents of the cytoplasm were packaged into apoptotic bodies (Figs. 3 and 4). However, the apoptotic morphologic features were not observed in the mock-infected cells.

3.2. DNA fragmentation analysis

As shown in Fig. 5, electrophoretic separation of DNA from NGVEV-infected cells revealed that the ladder pattern of bands with multiple averaging was about 180 bp in length corresponding to oligonucleosomal fragments. The first sign of 180 bp could be detected at 48 h p.i. (lane 4), and the intensity of the ladder bands increased with infection time to reach a plateau at 96 h p.i. (lane 6). No

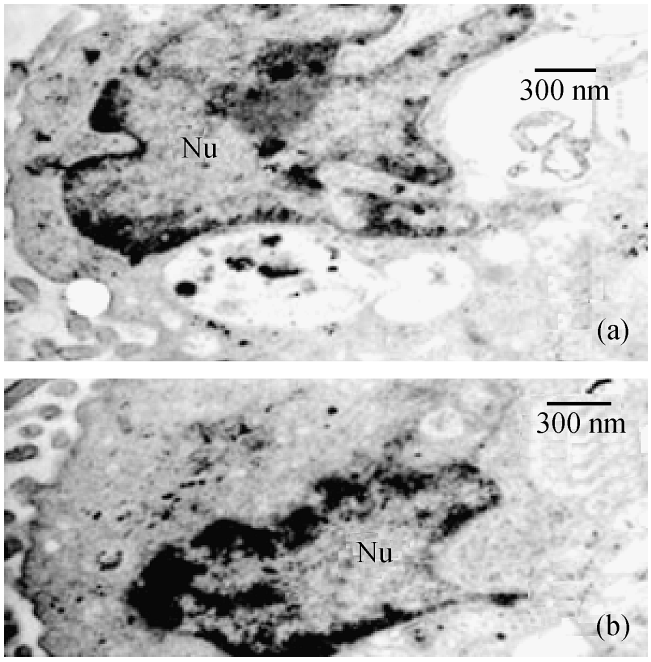

Fig. 1. Infected DEF cells at 72 h p.i.: shrinking of cells and nucleus, abnormal nuclear morphology, condensation of nuclear chromatin. Nu, nucleus.

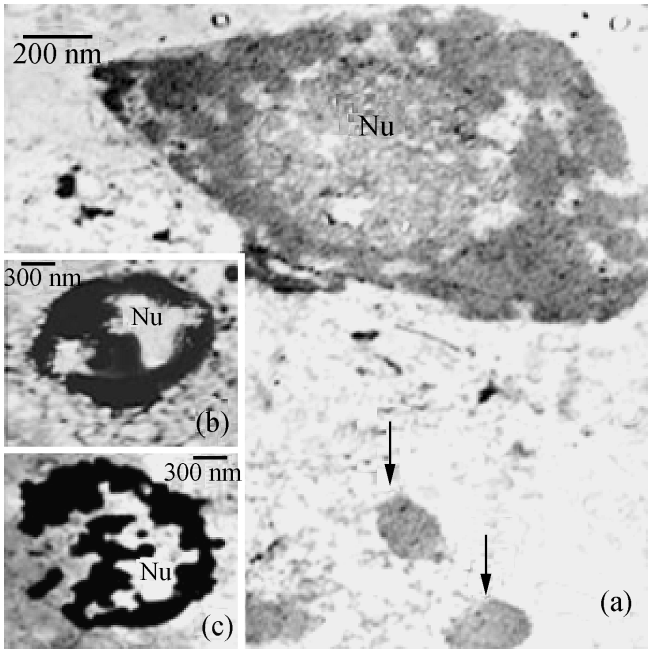

Fig. 2. Infected DEF cells at 96 h p.i. (a) and 120 h p.i. (b and c) Shrinking of nucleus and the condensation of nuclear chromatin. In (a) the arrowheads indicated typical apoptotic bodies.

obvious DNA ladder pattern was detected in mock-infected control (lane 2).

3.3. Annexin V-FITC/PI stained FACS

By staining cells with a combination of fluorescinated Annexin V-FITC and PI, FACS was used to quantitatively analyze dead, viable, apoptotic and necrotic cells after NGVEV infection (Fig. 6). As shown in Fig. 7, the percentage of apoptotic cells was found to increase with the incubation time until reaching a maximum at 120 h p.i. From Fig. 8, the proportion of secondary necrotic cells were increased greatly with infection time after 96 h p.i. The regular changes of the dead, necrotic and apoptotic cells with the infection time were shown in Fig. 9, and the basal level of apoptosis and necrosis was given in the mock-infected controls (Figs. 6–8).

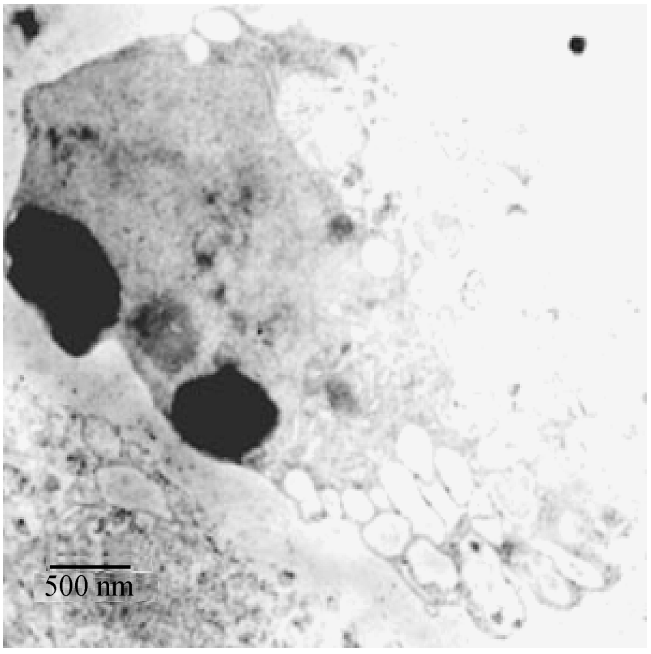

Fig. 3. Infected DEF cells at 120 h p.i.: the chromatin condensed, cytoplasmic shrank and plasma membrane blebbing.

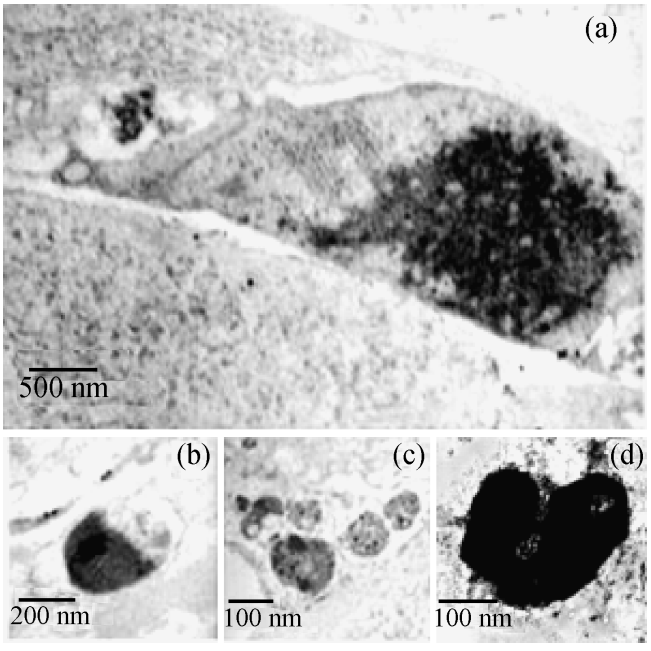

Fig. 4. Different shape of the membrane-bound apoptotic bodies.

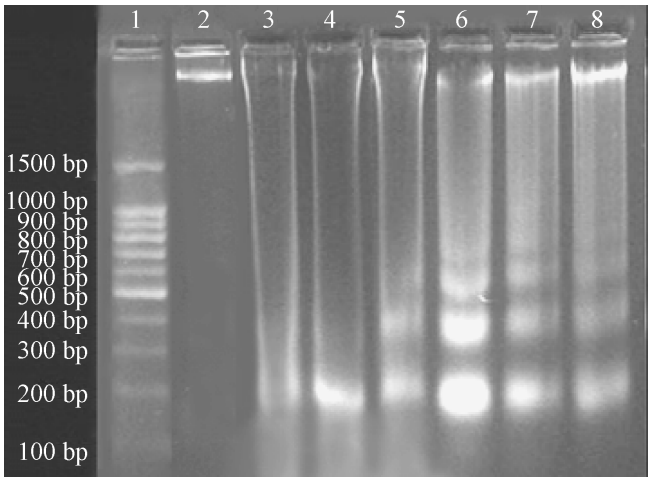

Fig. 5. DNA fragmentation analysis by agarose gel electrophoresis at different infection time points. Lane 1, a 100 bp DNA ladder marker; lane 2, DNA of mock-infected cells at 120 h incubation; lanes 3–8, the DNA of NGVEV infected cells at 24, 48, 72, 96, 120 and 144 h p.i., respectively.

4. Discussion

Virus infection and replication are usually associated with apoptosis, which is likely responsible for most of the pathology accompanied with infectious disease [18]. Early in 1968, Takemori [19] found that cyt mutants of human adenovirus could provoke more violent CPE. Ezoe [20] further proved that it could also induce the DNA degradation in the infected cells. Utenschlein [15] and Rautenschlein [16] also reported that HEV could induce B cells and spleen cells undergoing apoptosis [12–14], respectively. NGVEV is considered to be adenovirus, and whether NGVEV could induce host cells undergoing apoptosis has never been reported before.

In some situations, it is complicated in atypical apoptosis, which is characterized by the lack of internucleosomal DNA degradation [21,22]. The number of DNA strand breaking in such atypical apoptotic cells may be inadequate for their identification by agarose gel electrophoresis. On the other hand, false positive recognition of apoptosis may happen in a situation when internucleosomal DNA cleavage accompanies necrosis [23]. FACS can be applied for enumeration of apoptotic or necrotic cells [24,25]. However, regardless of the particular method that has been used to identify the mode of cell death, FACS analysis should always be confirmed by the inspection of cells under light or electron microscope. Morphological changes during apoptosis are unique and crucial factors when ambiguity arises regarding the mechanisms of cell death. Furthermore, apoptosis is originally defined based on the analysis of cell morphology [26,27]. Therefore, to get more evidence, DNA agarose gel electrophoresis and TEM were performed combined with FACS analysis to detect apoptosis induced by NGVEV in this research. The results demonstrated that the programmed cell death (apoptosis) of the DEF cells could be triggerred by NGVEV and also

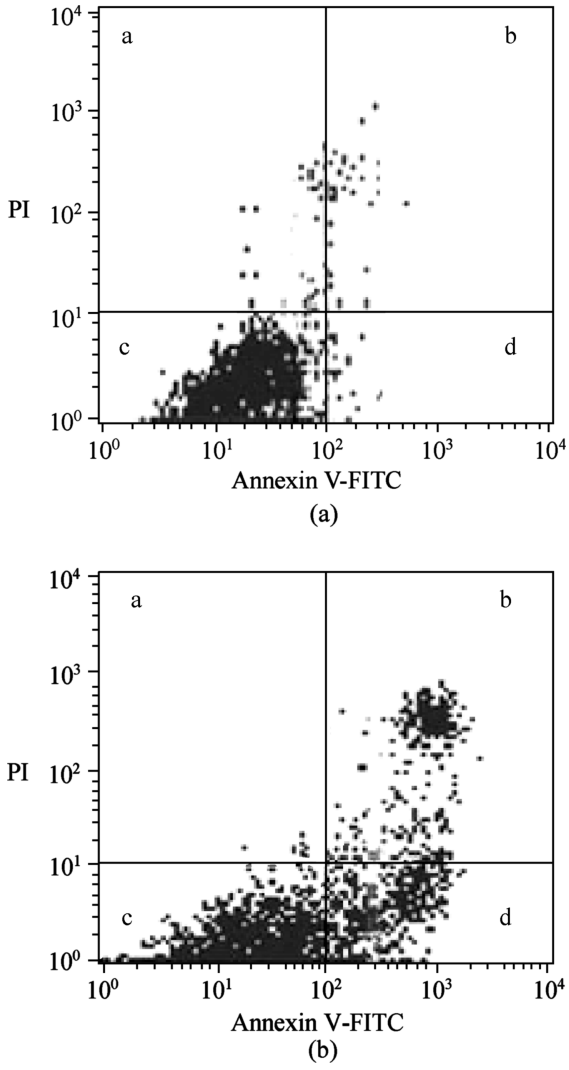

Fig. 6. Flow cytometry analysis of apoptosis in DEF cells detected by Annexin V-FITC/PI double staining. (a) Mock-infected DEF cells at 120 h incubation; (b) DEF cells infected with NGVEV at 120 h p.i. Quadrant a: dead cells (Annexin V^-/PI^+); Quadrant b: secondary necrosis (Annexin V^+/PI^+); Quadrant c: viable cells (Annexin V^-/PI^-); Quadrant d: early apoptotic cells (Annexin V^+/PI^-).

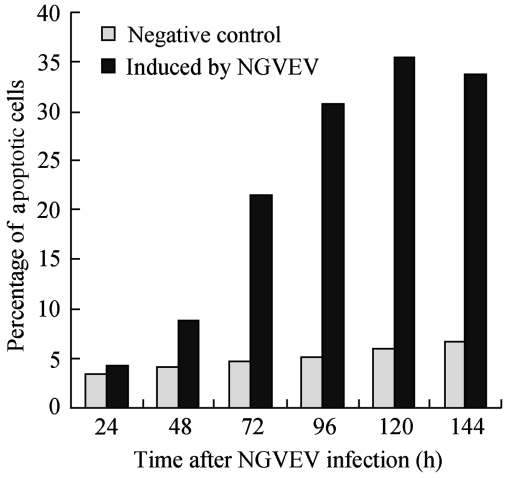

Fig. 7. The percentage of apoptotic DEF cells: negative control and induced by NGVEV.

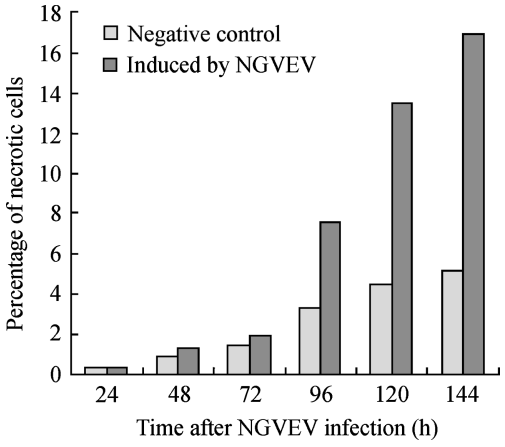

Fig. 8. The percentage of necrotic DEF cells: negative control and induced by NGVEV.

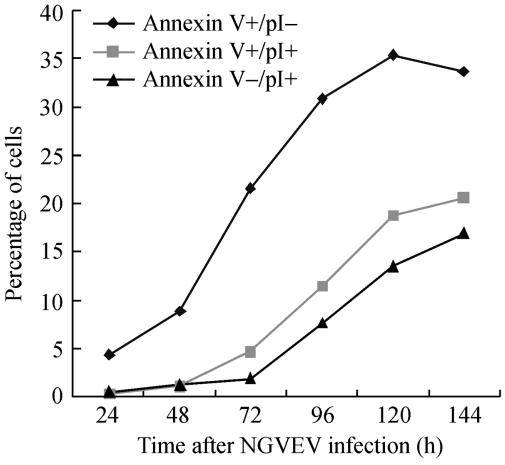

Fig. 9. Kinetics of induction of apoptosis in NGVEV infected DEF cells as analyzed by FACS.

displayed many characteristic apoptotic morphological and biochemical changes.

The results of our research indicate that the induction of apoptosis was barely detected in the early infection phase, the apoptotic changes (cell shrinkage and chromatin condensation, the first sign of DNA degradation, and Annexin V-FITC positive) could be observed from 72 h p.i., then the typical apoptotic features (apoptotic bodies and DNA ladders) were obvious in the later infection phase. Consequently, as presumed that the time from initial addition of an agent to the beginning of the first stages of apoptosis should be several days, the actual completion of apoptosis to the point of cell lysis required an additional day. By using cytochemical methods, we observed that the apoptosis was prior to necrosis post NGVEV infection. This may be due to the fact that apoptosis makes the cell remnants remain undisturbed *in vitro*, while they can be removed by phagocytes *in vivo*. The apoptotic cell debris interfers with the adjacent normal cells, leading to the necrosis. The present study demonstrates that the CPE caused by virus infection *in vitro* is mediated by apoptosis [28]. In our previous research, the typical CPE during NGVEV infection contains the detachment of infected cells from monolayer and the infected cell shrinkage [29], which were also observed as distinctive apoptotic morphological changes in this research. Therefore, it is hypothesized that apoptosis may be have some relationship with CPE during NGVEV infection.

For host, apoptosis plays a protective role in eliminating cells which might be harmful and also limits infection [30]. For virus, apoptosis facilitates persistent viral infection in host cells and is convenient for viral dissemination [9,31]. In view of this, virus may infect cell and remain undetected, preventing host cells from destruction [32,33]. The apoptotic bodies consumed by the phagocytic action of neighbouring cells provide a means for releasing the virus particles into the extracellular space without initiating a concomitant host response [34]. It is possible to presume that apoptosis induced by NGVEV may be an important mechanism for efficient dissemination of progeny, as well as the suicide of virally infected cells through apoptosis-limited infection, affording the host organism some degree of protection during infection.

In this research, NGVEV was demonstrated to cause apoptosis of DEF cells *in vitro*. And further investigation needs to be done on the intracellular events that trigger the apoptotic response.

Acknowledgements

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