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Progress in Natural Science 19 (2009) 47–53
Progress in Natural Science
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Host cell apoptosis induced by infection with duck swollen head hemorrhagic disease virus

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Received 5 March 2008; received in revised form 9 April 2008; accepted 24 April 2008

Abstract

This study aimed to examine the host cell apoptosis in the tissues of Peking ducks infected with duck swollen head hemorrhagic disease virus (DSHDV). The dynamic changes associated with apoptosis occurring in the internal tissues were evaluated at different time points postinoculation (PI) by performing hematoxylin and eosin (HE) staining, followed by light microscopy, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, and transmission electron microscopy (TEM). The results showed that DSHDV infection could induce apoptosis in host cells, including those of the bursa of Fabricius (BF), thymus, spleen, liver, intestinal tract, kidney, and esophagus. The apoptotic index (AI) values increased with time from 2 to 72 h PI, and the highest values were recorded at 72 h PI. Further, cell death due to classic necrosis was observed in the dying or deceased ducks after 72 h PI. In conclusion, host cell apoptosis can be induced by DSHDV and may play an important role in the pathogenesis of duck viral swollen head hemorrhagic disease (DVSHD).

Keywords: Duck swollen head hemorrhagic disease virus (DSHDV); Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL); Host cell; Apoptosis

1. Introduction

Duck swollen head hemorrhagic disease virus (DSHDV), a new type of reovirus that infects fowl, was first reported by Cheng et al. [1]. The virions are nonenveloped spherical or elliptical particles with a diameter of 80 nm, and their genomes are composed of RNA. DSHDV does not hemagglutinate the erythrocytes of chicken, duck, goose, pigeon, cattle, buffalo, or pig, and its titer stabilizes in the pH range of 4.0–8.0. Virus neutralization and cross-protection studies have not demonstrated the existence of any antigenic relationship between DSHDV and duck plague virus (DPV) or duck hepatitis virus (DHV). And, agar gel precipitin tests have not revealed any antigenic relationship between DSHDV and gosling plague virus (GPV), muscovy duck parvovirus (MDPV), avian influenza virus (AIV), infectious bursal disease virus (IBDV), avian viral arthritis virus (AVAV), or goose paramyxovirus. DSHDV is the causative agent of an acute contagious viral disease in ducks that is characterized by swelling of the head, diarrhea with green dejecta, 50% –100% morbidity, and 40%–80% mortality, which may occasionally increase to 100% [1]. Duck viral swollen head hemorrhagic disease (DVSHD), which is named after the causative virus DSHDV, has been observed in many regions including Sichuan Province, Chongqing Municipality, Guizhou Province, and Yunnan Province in China

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doi:10.1016/j.pnsc.2008.04.016

since 1998 and has resulted in tremendous economic loss due to the high mortality in domestic ducks [1]. Acute viral infection usually results in the death of the infected cells. Cell death occurs via two major mechanisms, namely, necrosis and apoptosis, which differ in both morphological and biochemical features [2–5]. Viral infection and replication are usually associated with apoptosis, and this may be responsible for most of the pathological findings associated with infectious diseases [6].

Currently, the induction of apoptosis is recognized as a type of general response in virus-infected cells. The number of animal viruses known to induce apoptosis in infected cells is increasing [7,8]. Previous research has indicated that reoviruses can induce apoptosis in host cells [9–13]. However, it should be noted that little information is available regarding the relationship between apoptosis and DSHDV infection. In this study, hematoxylin and eosin (HE) staining, transmission electron microscopy (TEM), and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay were used to evaluate host cell apoptosis induced by the new reovirus DSHDV and to gain novel insights into the pathogenesis of DVSHD.

2. Materials and methods

2.1. Experimental animals and virus

The present study was conducted using 72 Peking ducks at age of 28 days. They were procured from a DSHDV-free farm and tested negative for the DSHDV antibody. DSHDV strain HY-99, a highly virulent field isolate, was obtained from the Avian Disease Research Center, Sichuan Agricultural University, China. The 50% embryo lethal dose (ELD_{50}) for the allantoic fluid procured from a 10-day-old virus-infected duck embryo was determined to be $10^{-7.24}$/0.2 ml. An inoculum containing 100 ELD_{50}/0.2 ml was prepared.

2.2. Infection of the ducks

The study population of 72 Peking ducks was randomly divided into two groups. The ducks in the first group were inoculated intramuscularly with DSHDV at a dose of 100 ELD_{50}/0.2 ml (challenge group), while those in the second group were inoculated with phosphate-buffered saline (PBS) (control group). At each of the following time points, 10 min, 30 min, 60 min, 90 min, 2 h, 4 h, 12 h, 24 h, and 72 h postinoculation (PI) and every 3 days thereafter until the ducks began to die, two ducks in the challenge group were euthanatized and their tissue samples were collected. The dying or deceased ducks in the challenge group were necropsied. The number of ducks necropsied in the control group was identical to that in the challenge group. Tissue samples were collected from the heart, liver, spleen, lung, kidney, pancreas, brain, gland of Harder, thymus, bursa of Fabricius (BF), trachea, esophagus, glandular stomach, duodenum, jejunum, ileum, cecum, and rectum.

2.3. Histological observation

The tissue samples were fixed in 10% neutral phosphate-buffered formalin and embedded in paraffin. The tissue sections (thickness of 4 μm) were made, then stained with HE and observed under a light microscope (Olympus, Tokyo, Japan).

For ultra structural observation, the tissue samples were fixed in 3% glutaraldehyde, postfixed in 1.0% aqueous OsO_4, and stained with uranyl acetate. After stepwise dehydration in ethanol, the tissues were cleared in propylene oxide, embedded in Epon 816, and polymerized at 80 °C for 3 days. The tissue samples were then cut into 50-nm slices and stained with lead citrate and uranyl acetate for subsequent examination under a TEM (Hitachi H-600, Japan).

2.4. TUNEL assay and statistical analysis

Apoptosis was detected by performing the TUNEL assay [14–17]. The tissue samples were fixed in 4% paraformaldehyde (PFA) for 24 h, dehydrated, and embedded in paraffin in the conventional manner. The paraffin-embedded tissues were cut into 4-μm-thick sections. After deparaffinization in a graded alcohol series, the sections were processed using a TUNEL kit (*In Situ* Cell Death Detection Kit; Nanjing KeyGen Biotech Co., Ltd. China) according to the manufacturer's instructions. The positive control sections were pretreated with DNase I (Sigma, USA), while the negative control sections were subjected to the staining procedure without the terminal deoxytransferase (TdT) enzyme. The uninfected ducks were used as normal controls.

In each tissue specimen, five high-power fields (×400) were randomly selected; the apoptotic index (AI) was calculated in these fields as the percentage of positive cells, given by the following equation: AI = (number of positive cells/total number of cells) × 100% [18].

3. Results

3.1. Histological observation

Following inoculation, apoptosis occurred in different degrees in all types of tissues of the DSHDV-infected ducks. No apoptotic cells were detected during the time range of 10 min–4 h PI, while only a few apoptotic lymphocytes were observed in the immune organs from 4 to 24 h PI. After 24 h PI, apoptosis was induced in all types of lymphocytes, macrophages, and monocytes and in a few types of epithelial cells. In all the DSHDV-infected tissues, especially the BF, spleen, and thymus, significant apoptosis was observed at 72 h PI. However, it was noted that after 72 h PI, necrotic cells gradually replaced the apoptotic cells in

some tissues. Simultaneously, the DSHDV-infected ducks displayed typical symptoms of DVSHD. However, they began to die at 120 h PI. The morphological changes in the apoptotic cells, including cell shrinkage, crimson coloration in the cytoplasm, and isolation from the neighboring cells, were observed by light microscopy. In addition, nuclear chromatin margination, pyknosis, or karyorrhexis occurred, characterized by the formation of a crescent-, toroid-, or petaloid-shaped structure along the inner membrane of the nuclear envelope. With regard to the most crucial immune organs, apoptotic lymphocytes were mainly scattered in the medulla of the lymphatic nodule in the thymus and BF (Fig. 1a) and in the white pulp of the spleen (Fig. 1b).

3.2. Ultrastructural observation

The morphologies of all types of the DSHDV-infected tissues were examined by electron microscopy. No apoptotic cells were observed in the time range of 10 min–12 h PI, while only a few lymphocytes in the immune organs underwent apoptosis at 12 h PI. These apoptotic lymphocytes were characterized by progressive cytoplasmic shrinkage, isolation from the neighboring cells or surrounding matrix, and gradual disappearance of the nucleolus (Fig. 2a). Subsequently, at 24 h PI, chromatin condensation and margination formed a crescent-shaped structure along the inner membrane of the nuclear envelope (Fig. 2b). The number of apoptotic host cells increased from 24 to 72 h PI; these cells were characterized by lysis and disappearance of the mitochondria, excessive swelling and rupture of the plasma membrane, and fragmentation of the nucleus (Fig. 2c). The apoptotic host cells mainly included all types of lymphocytes and liver cells. In particular, apoptosis was more clearly visible in the lymphocytes in the immune organs than in the other host cells. During a later stage of apoptosis (after 72 h PI), the chromatin condensed or lysed and formed apoptotic bodies. Finally, the apoptotic bodies were phagocytized and digested by the macrophages present in the apoptotic lymphoid organs (Fig. 2d). Further, we found that cell death in the DSHDV-infected ducks predominantly occurred due to necrosis rather than apoptosis.

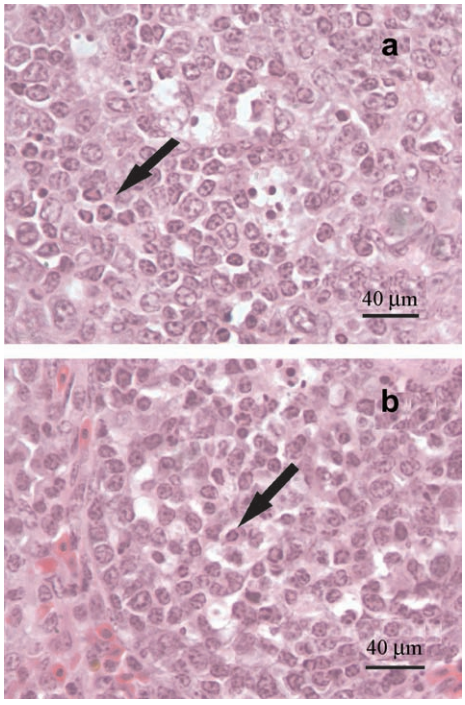

Fig. 1. The histological changes of apoptotic cells induced by DSHDV *in vivo* at the 72 h PI (HE staining, black arrows represent apoptotic cell). (a) Apoptotic lymphocytes of BF; (b) apoptotic lymphocytes of spleen.

3.3. Detection of apoptosis by TUNEL assay

3.3.1. Detection of apoptosis in the control group samples

In the positive control sample, the 3′-end of the DNA was exposed via fragmentation with DNase I and the DNA fragment obtained was ligated to biotin-labeled dUTPs by using the TdT enzyme. Diaminobenzidine (DAB) was used to trigger a specific binding reaction between streptavidin-conjugated horseradish peroxidase (HRP) and biotin-labeled dUTPs that produced intense nigger brown staining in the host cell nuclei (Fig. 3a). In the case of the negative control sample, the host cell nuclei were always stained amethyst due to the absence of the TdT enzyme (Fig. 3b). Normal physiological apoptosis was noted to occur in the normal tissues (Fig. 3c).

3.3.2. Detection of apoptotic cells

Apoptotic host cells were detected in a variety of tissues by performing the TUNEL assay at different time points. Although most but not all of the apoptotic or TUNEL-positive cells were characterized by visible morphological changes, all the apoptotic cells stained nigger brown during detection by the TUNEL assay. TUNEL-positive cells were detected in the BF, thymus, spleen, kidney, liver, heart, esophagus, glandular stomach, and intestinal tract (duodenum, jejunum, ileum, cecum, and rectum) (Fig.4a–f). The apoptotic host cells mainly included all types of lymphocytes, macrophages, monocytes, endothelial cells, and a few types of epithelial cells. Apoptosis was chiefly initiated in the endothelial cells lining the blood sinus, Kupffer cells and other hepatocytes, epithelial cells of the renal tubule, all types of lymphocytes, macrophages, and monocytes, and epithelial cells in the immune organs and digestive tract. In the spleen, the white pulp area – especially the acinus lienalis – exhibited more apoptotic cells than the red pulp area. Similarly, in the thymus, more apoptotic cells were present in the medulla than in the cortex. With regard to the BF, a larger number of host cells underwent apoptosis in the cortex and medulla than in the mucous membrane epithelium. Based on all the abovementioned results, we deduced that TUNEL assay could detect more species of apoptotic cells and tissues than HE staining and TEM.

Following viral inoculation, dynamic changes were noted in the AI values of the host cells *in vivo*. The

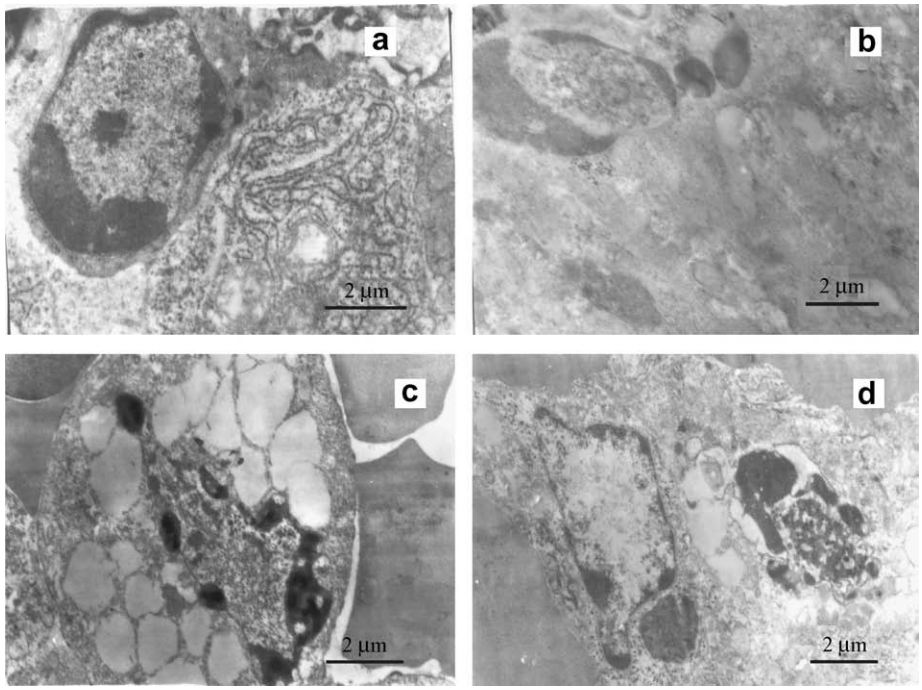

Fig. 2. TEM images showing the ultrastructural changes of apoptotic cells induced by DSHDV *in vivo*. (a) Apoptotic lymphocytes in spleen lost contact with neighboring cells or surrounding matrix and detached from the neighboring cells, cytoplasm condensed and nucleolus began to disappear at 12 h PI. (b) Chromatin condensation and margination characterized with crescent-shaped were located to the inner membrane of nuclear envelope in liver at 24 h PI. (c) Mitochondria were characterized by balloon-like swelling and nucleus of apoptotic lymphocytes in thymus broke into pieces at 72 h PI. (d) The apoptotic bodies were phagocytized and digested by macrophages in apoptotic lymphocytes in BF at 120 h PI.

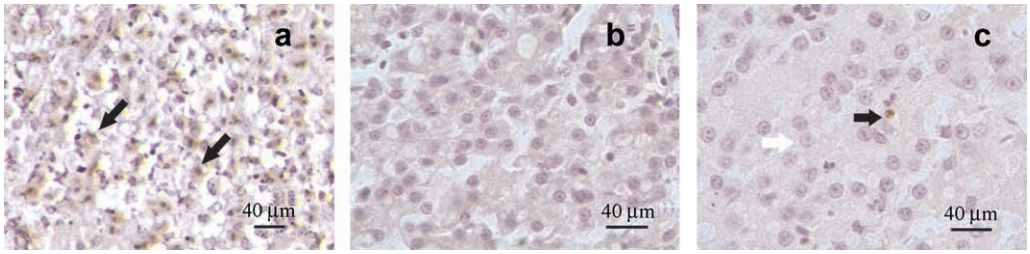

Fig. 3. The detection of control tissues by TUNEL assay. The nucleus in TUNEL-positive cells is nigger-brown; the nucleus in normal cell is amethyst (black arrows represent positive cells). (a) Positive samples dealt with DNaseI (liver); (b) negative samples without TdT enzyme treatment (liver); (c) liver from uninfected duck.

challenge group first exhibited higher AI values than the control group at 2 h PI; this was followed by a gradual increase in the AI values with time up to 72 h PI. In contrast, apoptotic host cells were first detected between 4 and 24 h PI by HE staining and at 12 h PI by TEM. The highest AI values were recorded at 72 h PI, and the BF exhibited a higher AI value than the other tissues. After 72 h PI, there was a gradual decrease in the number of apoptotic host cells. DSHDV infection induced cell death via apoptosis and necrosis, but necrosis played a crucial role in the depletion of host cells. This regulation of apoptosis detected by TUNEL was similar to that detected by the other two methods (HE staining and TEM) used in this study. The dynamic changes in the AI values of some tissues at different time points are shown in Fig. 5.

4. Discussion

4.1. Detection methods for apoptosis

The phenomenon of apoptosis or programmed cell death (PCD) was first described by Kerr in 1972 [19]. Apoptosis is a highly regulated process involved in cell depletion, and it plays a fundamental role in the maintenance of tissue homeostasis in multicellular organisms [20,21]. Currently, many effective methods are available to detect apoptosis; these include morphological studies, the TUNEL assay, electrophoresis using a DNA ladder, the caspase activity assay, enzyme-linked immunosorbent assay (ELISA), and flow cytometry. However, all these methods pose certain limitations. HE staining is a simple and rapid method for the detection of apoptosis; however,

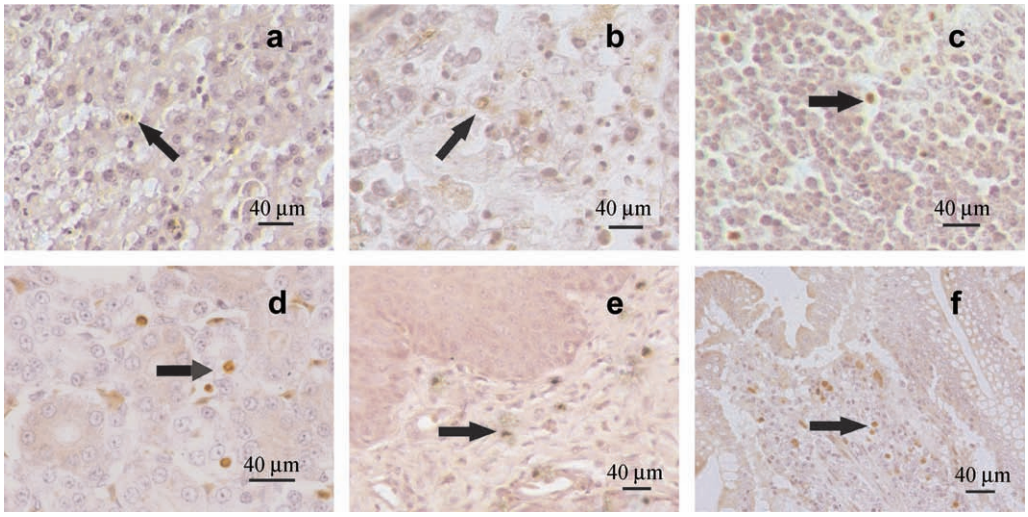

Fig. 4. TUNEL detection of partial organs at 72 h after *in vivo* infection with DSHDV. The nucleus in apoptotic cells is nigger-brown; the nucleus in normal cells is amethyst (black arrows represent positive cells). (a) Liver; (b) bursa of Fabricius (BF); (c) spleen; (d) kidney; (e) esophagus; (f) rectum.

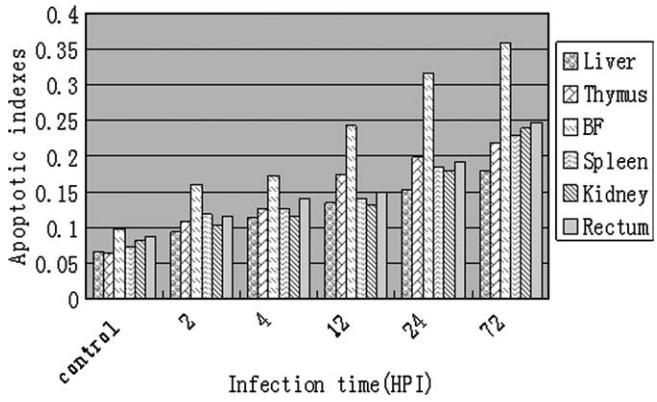

Fig. 5. The dynamic changes of the apoptotic indexes (AI) of some tissues at different time points.

lack of objectivity is considered a flaw or deficiency. Electron microscopic examination is a gold standard for identifying the characteristics of apoptosis. This method can be effectively used not only to examine the ultrastructural changes in apoptotic cells but also to distinguish between apoptotic and necrotic cells. However, the limitations of this approach are that it requires the use of a transmission electron microscope, is time-consuming, and does not permit quantitative evaluation. Further, although the two methods described above are easy to perform, they frequently underestimate the number of apoptotic cells; this is because DNA fragmentation occurs early during apoptosis, while typical morphological changes occur at later. A widely used quantitative method for detecting apoptosis is the TUNEL assay, wherein the degree of DNA fragmentation is measured. Therefore, in this study, we selected the TUNEL assay and not HE staining or TEM to observe the early apoptotic host cells. However, the TUNEL assay is also a slightly unsatisfactory method because of its limited sensitivity and specificity [22,23]. Thus, for reliable detection of apoptosis, it is generally recommended that at least two methods should be used, together with microscopic verification of the distinctive morphological alterations [24,25]. In the present study, the occurrence of host cell apoptosis in DSHDV-infected tissues was corroborated by three detection methods.

4.2. DSHDV infection and host cell apoptosis

It is well known that viruses belonging to different families can either induce or inhibit apoptosis [9,26–30]. However, it is also possible that the same virus can induce as well as inhibit cell apoptosis. For example, one type of viral protein may inhibit apoptosis, while another protein of the same virus may induce apoptosis. The inhibition or induction of apoptosis depends on the type of protein secreted during the period of viral infection [26]. RNA viruses are not considered to exhibit antiapoptotic activities since their dissemination appears to benefit from the rapid induction of cell death [31]. Cheng et al. have reported that DSHDV belongs to the family *Reoviridae* (RNA viruses) [1]. Consistent with this, in the present study, we demonstrated that DSHDV infection *in vivo* could significantly induce apoptosis, targeting the immune organs as well as the liver and digestive tract.

There are two types of views with regard to the significance of apoptosis in viral infection. On the one hand, apoptosis may contribute to the elimination of virus-infected host cells and may prevent viral propagation. Thus, apoptosis is likely to be one of the host defense mechanisms against viral infection [7]. It may be mediated by virus-specific cytotoxic T lymphocytes (CTLs) [32,33] or may occur spontaneously without any external signal or stimulus [34]. On the other hand, apoptosis may be beneficial for viral survival; and host cell apoptosis may be inhibited or delayed due to the expression of certain viral genes and the synthesis of the corresponding proteins [35]. In line with this view, fewer apoptotic cells were observed at an

early stage of DSHDV infection (before 24 h). This might be because DSHDV stimulated an antiapoptotic mechanism to circumvent or delay host cell apoptosis for its own survival. Thus, the virus primarily played an antiapoptotic role during the early stage of infection. However, from 24 to 72 h PI, apoptosis appeared to function as an important defense mechanism against viral infection because virus-induced apoptosis contributes to the elimination of pathogenic microorganisms [36]. At a later stage during infection (after 72 h PI), apoptosis might prove beneficial for viral replication by facilitating the dissemination of progeny viral particles toward the neighboring cells without being intercepted by the host immune cells.

4.3. Regulation of DSHDV-induced host cell apoptosis

Our results revealed that the AI values of the organs of the DSHDV-infected ducks changed dynamically with time (Fig. 5). The AI values of the partial organs among the challenge group ducks, which were recorded at as early as 2 h PI, were higher than those among the control group ducks. Moreover, Labrada clarified that apoptosis could be induced during viral uncoating following infection with an avian reovirus isolated from chickens [11]. Thus, the early apoptosis induced by DSHDV infection in our study might have occurred during viral uncoating. However, a recent report revealed that the avian reovirus σC protein could induce apoptosis in cultured cells [37]. This suggested that both the protein and gene expressions of avian reovirus also induce apoptosis. In our study, the number of TUNEL-positive signals gradually increased with time, and the highest values were recorded at 72 h PI. During this process, viral uncoating and viral gene expression may have initiated apoptosis in the host cells via different mechanisms.

4.4. Pathogenic and apoptotic mechanisms and DSHDV infection

Previous studies have indicated that apoptosis may be an important mechanism involved in the pathogenesis of viral infections if it is induced in the critical organs *in vivo*, such as immune organs and digestive organs [27–29]. For example, Ito et al. conducted a study on the influenza virus and found that the pathogenicity of this virus is closely related to virus-induced apoptosis [27]. Chicken infectious anemia is directly related to apoptosis of the thymus in chicken anemia virus (CAV)-infected chicken [28]. Previous research data have shown that reovirus-induced apoptosis is a critical mechanism underlying the development of lesions in the definite tissues of animals [9,30]. Consistent with this, the results of our study showed that apoptosis occurred in some critical organs, including the immune organs and digestive organs. This apoptosis in the immune organs as well as in other organs contributed to the development of a series of functional disorders in the immune system, digestive system, urinary system, etc. Thus, host cell apoptosis induced by DSHDV infection *in vivo* hampered cell function and resulted in a series of secondary pathological changes, leading to the emergence of clinical symptoms.

Apoptosis-induced depletion of lymphocytes in the immune organs was the most critical event involved in the pathogenesis of DVSHD. There are two critical views regarding the increase in the number of apoptotic lymphocytes. On the one hand, this increase is considered to be associated with lymphocyte proliferation and differentiation. The malfunction of these processes results in an increase in the number of premature cells or cells that do not enter into a proliferative phase. The cell death mechanisms associated with apoptosis are initiated when the ratio of the abovementioned cells reaches a suitable value. On the other hand, previous studies have indicated that changes occurring in the mitochondria play an important role in lymphocyte apoptosis [38]. The results of our study revealed that mitochondrial abnormalities played a crucial role in ultrastructural apoptotic changes. Free radicals, which are among the major inducers of apoptosis, are mostly released by the mitochondria [39]. Accordingly, an increase in the number of mitochondria during an early stage of infection may be associated with augmented energy metabolism or may have a direct effect on the process of apoptosis. The ultrastructural changes observed in the mitochondria included swelling, lysis of the cristae, and vacuolization (Fig. 2c). These changes may have been induced by impairment of the mitochondrial membranes during apoptosis. The resulting augmented permeability of the mitochondrial membranes permits the entry of moisture into the mitochondria during apoptosis [40]. In our study, DNA fragmentation and nuclear condensation were significantly detected by the TUNEL assay. Bcl-2 has been reported to inhibit nuclear condensation and DNA fragmentation, which depend on the presence of mitochondria [41]. Thus, Bcl-2 plays a critical role in mitochondria-induced apoptosis. The Bcl-2 family comprises both proapoptotic (e.g., Bax) and antiapoptotic (e.g., Bcl-2) proteins. Bcl-2 and Bax exhibit reciprocal expression patterns in the cytoplasm and form heterodimers to maintain homeostasis. The occurrence of apoptosis is determined by the balance between Bcl-2 and Bax. In our study, mitochondria were acutely destroyed; this resulted in the inhibition of Bcl-2 expression. Thus, the balance between Bcl-2 and Bax was disturbed, finally resulting in host cell apoptosis.

5. Conclusions

The results demonstrated that host cell apoptosis can be significantly induced by DSHDV infection. The AI values of the host cells *in vivo* dynamically changed at different time points PI. Apoptosis of the DSHDV-infected host cells is closely related to the pathogenesis of DVSHD. However, the exact pathogenesis of DVSHD remains unclear. Thus, further studies in this regard will provide insights into the mechanisms underlying the occurrence, development, and prognosis of DVSHD.

Acknowledgements

Program for Changjiang Scholars and Innovative Research Team in University, No. 0853. This work was supported by National Natural Science Foundation of China (Grant No. 30471297, 30771598), the New Century Excellent Scientist Research Program of China Education Department (No. NCET-04-0906/NCET-06-0818), the National Science and Technology Support Programs (No. 2007Z06-017), the Sichuan Excellent Scientist Research Funds (No. 03ZQ026-028, 05ZQ026-038, 07ZQ026-132), the China University Science and Technology Innovation Key Project Funds of Education Department (No. 706050), and the Sichuan Key Courses Construction Project (No. SZD0418).

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