Role of apoptosis in Duck Tembusu virus infection of duckling brains in vivo

Sheng Yang,* Jingxian Wu,* Yufei Huang,* Yafei Zhang,* Yonghong Shi ‡,,* Ping Yang,* and Qiusheng Chen* 1

†MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu Province, 210095, China; and 1Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, 200241, China

ABSTRACT The Duck Tembusu virus (DTMUV) is a novel flavivirus that occurs mainly in poultry. DTMUV infection can cause common neurological symptoms in ducklings, but the pathogenesis of DTMUV has not been elucidated yet. In this study, a DTMUV-infected duckling model was constructed to investigate the apoptosis in the duckling brains. After DTMUV infection, apoptotic cells were observed by transmission electron microscopy. It was found that the abundances of apoptosis-related genes and proteins were not obviously changed in the early stage of infection but significantly changed in the middle and late stages of the disease. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay staining results were also consistent with the above phenomena. Interestingly, although apoptosis occurred in the duckling brains infected by DTMUV, some antiapoptotic genes in the brain increased in varying degrees. In conclusion, DTMUV infection could induce apoptosis in ducklings’ brains, and the occurrence of apoptosis was accompanied by the virus infection process with certain regularity. This study provides a scientific basis for elucidating the apoptotic mechanism of brain lesions induced by DTMUV infection.

Key words: Duck Tembusu virus, brain, apoptosis

INTRODUCTION

The Duck Tembusu Virus (DTMUV) is a single-stranded plus-stranded RNA virus belonging to the Ntaya group, Flavivirus genus, Flaviviridae family. The DTMUV infection can cause an acute infectious disease, which is characterized by feed intake and growth rate restrictions, decreased egg production, and paralysis in laying ducks. It was reported that the laying rate might decrease from 70 to 90% to about 10%. The main neurological symptoms of ducklings, such as unsteady on the feet, rather uncoordinated, and falling to the ground can be observed after DTMUV infection. The probability of serious illness of ducklings is up to 80%, of which the mortality rate is 10 to 30% (Su et al., 2011). Thus, DTMUV disease can cause serious losses to the duck industry. DTMUV disease is endemic mainly in Asia affecting ducks of all strains, and even has also been detected in chickens and geese in the endemic areas (Yun et al., 2012).

Apoptosis, also known as programmed cell death, is a process of cell-autonomous death initiated by biological, chemical, and physical factors, which can remove infected cells and fight virus infection. Apoptosis and necrosis induced by viruses is a complex process involving diverse components, multiple signaling pathways, and complex factors. On the one hand, virus-infected host cells can reduce virus particles through apoptosis and necrosis. On the other hand, viruses can facilitate their own proliferation by preventing host cells from undergoing apoptosis or spread through promoting cell apoptosis (Aleman et al., 2001; Deruelle et al., 2010).

Apoptosis is a basic physiological response of virus-induced cell death, which is a self-defense mechanism of host cells against virus invasion (Ghosh Roy et al., 2014). A variety of flaviviruses (e.g., dengue virus (Jan et al., 2000) and Japanese encephalitis virus [JEV] (Lee et al., 2005a)) have been found to induce apoptosis. West Nile virus (WNV) induces neuronal injury and death in several brain regions, including the hippocampus, brainstem, cerebellum, and anterior horn of the spinal cord (Guarner et al., 2004). JEV infection leads to neuronal apoptosis, which is an important process owing to the pathogenesis of JEV in the central nervous system (Kleinschmidt-DeMasters et al., 2004). As a member of the flavivirus genus, DTMUV could also induce...
apoptosis (Pan et al., 2020; Zhang et al., 2021). However, there are few reports concerning the apoptosis induced by DTMUV, especially in the brain. Being the main component of the brain, neurons are the target cells where DTMUV may persist for the longest time. As a result, DTMUV infection in the brain leads to the main cause of neurological symptoms, sequelae, or even death. Ducklings of different ages have different susceptibility to this virus, among which the younger ones are more susceptible to it. Except for the brain, spleen and pancreas may also serve as the target organs for DTMUV to proliferate and spread (Sun et al., 2014). Therefore, in this study, ducklings were selected to explore the occurrence and dynamic trend of apoptosis in the brain after DTMUV infection. At present, there are few studies on the interaction mechanism between DTMUV and its host, especially on the mechanism of the apoptosis induced by the DTMUV. Therefore, it is of great significance to understand the mechanism of brain apoptosis induced by the DTMUV for formulating strategies to control its pathogenicity.

MATERIALS AND METHODS

Virus and Animals

The purified TMUV strain XZ-2012 was supported by the College of Veterinary Medicine in Nanjing Agricultural University (The virus titer used in this experiment was $5 \times 10^5$ TCID$_{50}$/mL). Seven-day-old Shaoxing Muscovy ducklings were acquired from Nanjing Qizai Biological Co., Ltd (Nanjing, China) and were randomly assigned to 2 groups. The experiment group (EG) ducklings were injected with 100 mL of virus solution through the left jugular vein. The control group (CG) animals received 100 mL sterile phosphate-buffered saline (PBS) by the same route. Ducklings’ brain tissues were collected at 3, 5, 7, 9, 12, and 15 d postinfection. All experiments were performed under approved protocols from the guidelines of the regional Animal Ethics Committee and the rules for experimental animals of Nanjing Agricultural University.

Antibodies

Rabbit anti-Bcl-2 (ab196495, Abcam Inc., Cambridge, MA; 1:1,000), Rabbit anti-Cleaved caspase-3 (AB3623, Merck Millipore, Billerica, MA; 1:200), Rabbit-FasL (A0234, ABclonal Biotechnology, Wuhan, China; 1:500), Rabbit-Fas (A0233, ABclonal Biotechnology; 1:500), Rabbit-Caspase-7 (A1524, ABclonal Biotechnology; 1:500), and rabbit-β-actin (4970, Cell Signaling Technology, Beverly, MA; 1:1,000) were prepared for protein analysis.

RNA Sequencing and Annotation of Differentially Expressed Genes

The RNA sequencing (RNA-seq) and following data analysis were performed as previously described (Yang et al., 2021). Briefly, the total RNA was obtained from brains tissues of ducklings of 9 dpi and extracted using Trizol kits (Invitrogen, Carlsbad, CA). All 6 mRNA samples (control and experiment groups, n = 3) were determined for integrity and quantified, enriched, and fragmented, then reverse transcribed into cDNA. Subsequently, cDNA libraries were constructed and sequenced using an Illumina HiSeq TM 2500. Following sequence mapping, differentially expressed genes were analyzed using the FPKM method, and annotation of differentially expressed genes (DEGs) were analyzed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations. The RNA-seq data have been uploaded in the Sequence Read Archive (SRA).

Transmission Electron Microscopy

The duckling brain sample blocks were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate-buffered saline (PBS, 4°C) for 24 h. After washed with PBS, samples were fixed in 1% (w/v) osmium tetroxide for 1 h at 37°C, rinsed in PBS, dehydrated in ascending concentrations of alcohol (25, 50, 75, 85, 95, and 100%), penetrated with a propylene oxide-Araldite mixture (50% propylene oxide: Araldite) and embedded in araldite. The ultrathin sections (50 nm) were stained with uranyl acetate and lead citrate. The electron micrographs were obtained with a transmission electron microscope (Hitachi H-7650, Japan).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End Labeling Assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL) assay was carried out to measure the apoptotic cells in duckling brain tissues. The staining was processed following the instruction of a Situ TUNEL Apoptosis Detection Kit (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China). After deparaffinization and aquation, the brain sections were treated with 20 mg/mL proteinase K for 20 min at room temperature. A mix of 45 μL equilibration buffer, 4 μL terminal deoxynucleotidyl transferase (TdT) enzyme and 1 μL fluorescence labeling was prepared to cover the samples and incubated in a dark and humidified chamber for 1 h at 37°C. Then, the slices were added 100 μL of antifluorescein antibody working solution for 30 min at 37°C, following incubated with diaminobenzidine for another 30 min at 37°C in a dark and humidified chamber. Finally, the slices were stained with hematoxylin and observed under the microscope.

Quantitative Detection of mRNA

The total RNA from the brains was extracted using the TRizol reagent (Invitrogen) and reverse transcribed to cDNA using a commercial kit (TaKaRa, China). The
real-time fluorescence quantitative PCR (Q-PCR) assay was conducted by the Light Cycler 96 Sequence Detection System (Roche, Switzerland) using an SYBR Green PCR Master Mix (Vazyme Biotech Ltd., China) based on the instructions. A complete list of Q-PCR primers is shown in the table (Table 1). Fold changes in expression were calculated by $2^{-\Delta\Delta CT}$ method and normalized with the control group.

**Western Blotting**

Samples of the brains were homogenized using the ice-cold radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, China) and centrifuged at 12,000 × g for 10 min at 4°C. Then the total protein concentration was detected using a BCA protein assay kit (Abcam, UK). Samples (40 μg of protein per lane) were resolved on a 10% SDS-PAGE gel suffering electrophoresis and then transferred to PVDF membranes (Millipore). After non-specific blocking in 5% (w/v) nonfat milk, the membranes were incubated with respective antibodies at 4°C overnight. The next day, after rinsing with TBST, the membranes were incubated with the peroxidase-linked goat anti-rabbit IgG (BS13278, Bioworld Technology Inc., Nanjing, China, 1:5,000) for 2 h. Then, High-sig ECL Western Blotting Substrate (Tannon, China) was used to visualize the bound antibodies. Immunoreactive bands were quantified using Quantity One software (Bio-Rad, Hercules, CA).

**Statistical Analysis**

All data were presented as means with a standard error of the mean (SEM). The normality and the equality of variances of data were analyzed by the Shapiro-Wilk test and Levene’s test, respectively. The data analysis was performed by the unpaired t test (or Welch test) method using SPSS for windows version 22.0 statistical package (SPSS Inc., Chicago, IL). $P < 0.05$ was considered to indicate statistical significance.

## RESULTS

**Apoptosis Activated During the Middle Stage of DTMUV Infection**

In previous RNA-seq studies, it was found that during the middle stage (9 dpi) of DTMUV infection in duckling’s brains, apoptotic pathways were significantly enriched (Yang et al., 2021). The changes in apoptotic pathway-related genes expression levels in the EG compared with the CG at 9 dpi (during which the infected ducklings showed obvious neurological symptoms) were described and summarized in Figure 1. As shown in Figure 1, most proapoptotic genes including Fas, Fas ligand (FasL), Caspase-8, Caspase-9, Caspase-3, and so on, were upregulated to a certain extent in the heat map of pathway enrichment, but some antiapoptotic genes such as B-cell lymphoma 2-related protein A1 (BCL2A1), Cellular inhibitor of apoptosis (cIAP1) also significantly increased during this period. These results suggested that both proapoptosis and antiapoptosis existed in the brain of ducklings after DTMUV infection.

**Detection of Duckling Brain Apoptosis by Transmission Electron Microscopy and TUNEL**

To confirm the occurrence of apoptosis in the brain after the challenge, the ultrastructural changes in the
brain were observed using transmission electron microscopy (TEM). During the early stage (3−5 dpi) of DTMUV infection, it was difficult to observe the obvious apoptosis under TEM. In the middle (7−9 dpi) and late stages (12−15 dpi) of infection, nuclear chromatin concentration (Figure 2C, 7 dpi), edge aggregation (Figure 2D, 7 dpi), and nuclear chromatin concentration into crescent shape were clearly observed (Figures 2E and 2F, 9 dpi).

To further understand the dynamics of apoptosis during DTMUV infection, the TUNEL assay was performed on the brain tissues after DTMUV infection. Cell nuclear DNA is fragmented in the early stage of apoptosis, and the fragmented DNA can be labeled with dUTP. The results showed that the TUNEL-positive cell was sparse in the early stage of infection, and the positive rates gradually increased at 7 to 12 dpi during which the pathological symptoms of the ducklings were gradually evident, and the apoptosis was gradually reduced at 15 dpi (Figure 3).

Changes of mRNA and Protein Expressions of Apoptosis-Related Genes During DTMUV Infection

Based on the results of RNA-seq, we investigated the dynamic changes of apoptosis-related genes and proteins expressions during DTMUV infection. The overall trend of changes in mRNA and proteins of apoptosis-related genes during DTMUV infection indicated that the changes of apoptosis-related genes were not evident in the early stage of DTMUV infection, and the significant changes mainly occurred during the middle and late stages of infection. As shown in Figure 4, the antiapoptotic gene BCL2 was slightly upregulated in the early stage of infection (3 dpi) and then decreased compared with the CG, and gradually increased in the late stage of infection (12−15 dpi). Another antiapoptotic gene, Bcl-2-like protein 1 (BCL2L1), was downregulated at the beginning of infection but significantly upregulated in the middle of infection. Proapoptotic genes such as Fas, FasL, Caspase-8, BID, BCL2 homologous antagonist/killer (BAK1), and Caspase-9 were significantly upregulated in the middle stage of infection, while Caspase-7 and Caspase-3 were upregulated during the early and middle stages of infection, especially the latter, and then decreased. As shown in Figure 5, during infection the variation tendency of apoptosis-related proteins was in line with the corresponding genes.
Apoptosis is a type of programmed cell death that regulates cellular homeostasis by removing damaged or unnecessary cells. It has been shown that DTMUV could induce apoptosis in a variety of cells in vitro, such as primary duck neurons (DN) (Kulprasertsri et al., 2021), duck embryo fibroblasts (DEFs; Shaozhou et al., 2015), and baby hamster kidney-21 (BHK-21) (Zhao et al., 2019), while its role in DTMUV’s interaction with the body is rare. Therefore, this study explored the brain apoptosis after DTMUV infection in duckling in vivo.

Apoptosis is one of the ways in which flaviviruses manipulate cellular responses to optimize viral production (Uno and Ross, 2018; Vicenzi et al., 2018). In this study, the results show that apoptotic cells were few in the early stage of DTMUV infection but then increased in the middle and late stages of virus infection. It was confirmed that DTMUV had entered the brains in the early stage of infection (3 dpi) in our prior study (Yang et al., 2021), but no apoptosis was observed at this time. With the prolonging of infection time, the copy number of the virus in the brain increased and the apoptosis was gradually obvious, which indicated that the copy number of DTMUV in the brain was positively correlated with the apoptosis. Similarly, the ratio of apoptosis was gradually increased with the time of virus infection in DTMUV-infected DEFs in vitro (Pan et al., 2020). The replication of flavivirus in target cells reduces cell viability and induces apoptosis, but it generally

Figure 3. Duckling brain photographs with TUNEL staining after DTMUV infection. Apoptotic cells were stained yellow-brown, and the black arrow points to TUNEL-positive cells. (A, B, C, D, F, G, and H) Represents control, 3 dpi, 5 dpi, 7 dpi, 9 dpi, 12 dpi, and 15 dpi, respectively. (E and I) The enlarged pictures of the corresponding parts of D and F, respectively. Abbreviations: DTMUV, Duck Tembusu virus; TUNEL, transferase-mediated dUTP-biotin nick end labeling assay.

Figure 4. Changes in mRNA expression of apoptosis-related genes in the brains of DTMUV-infected duckling. EG and CG represent experimental group and control group, respectively. The mRNA relative expression was detected by Q-PCR and calculated using the 2−ΔΔCT method. β-actin was used as a reference. Data represent the means ± SEM of three ducklings conducted triplicate. The statistical significance was assessed using a two-tailed Student t test. Asterisks indicate statistical significance (*, P < 0.05; **, P < 0.01). Abbreviation: DTMUV, Duck Tembusu virus.
occurs in the middle and late stages of infection (Liao et al., 1997; Courageot et al., 2003). Inhibition of apoptosis can inhibit cell death induced by WNV (Kleinschmidt et al., 2007). The PI3K/AKT pathway can be activated in the early stage of flavivirus invasion to enhance the anti-apoptotic effect of the cells (Airo et al., 2018), providing a favorable environment for virus replication. In the middle and late stages of virus infection, a large number of viruses replicate in the target cells, and activate mitochondrial and endoplasmic reticulum stress and other apoptosis pathways, leading to cell apoptosis. The flaviviruses infection is capable of triggering multiple apoptotic pathways, such as mitochondria-dependence (Suzuki et al., 2018), death receptor (Liao et al., 2010), and endoplasmic reticulum (ER) stress (Huang et al., 2016; Turpin et al., 2021). JEV triggered apoptosis of N18 (a mouse neuroblastoma cell line) cells through the mitochondrial pathway (Tsao et al., 2008), possibly by inducing proteolysis of endogenous p21 BAX to produce p18 BAX in SH-SY5Y cells (human neuroblastoma) (Wongchitrat et al., 2019). ZIKV induced neuronal apoptosis by increasing mitochondrial fragments (Yang et al., 2020). The NS4B protein of ZIKV could recruit BAX into mitochondria and induce conformational changes of BAX, thus inducing apoptosis of SY5Y cells (Han et al., 2021). JEV-infected transfected human brain microvascular endothelial cells could obviously increase expressions of BCL2-associated X (BAX), BID, Fas, and FasL (Al-Obaidi et al., 2017). Yellow fever virus could regulate cytokines’ mRNA expression and induce caspase 3/7 activation in HepG2 cells (Human Hepatocarcinoma cell line) (Holanda et al., 2019). In DNs and duck fibroblasts (CCL–141), DTMUV could promote apoptosis in a caspase-3 – dependent pathway (Kulprasertsri et al., 2021). In addition, previous studies have shown that the nonstructural proteins (NS, NS3, and NS2B-NS3) protease and helicase domains induced apoptosis via the caspase-9/-3 cascade pathway (Yang et al., 2009; Yiang et al., 2013). Flaviviruses were also able to trigger apoptosis through the ER pathway. JEV plays a role in cell apoptosis through p38-dependent and transcription factor C/EBP homologous protein (CHOP) mediated pathways (Su et al., 2002). ZIKV impaired the expression of CHOP/DDIT3, which is the main factor of ER-stress-driven apoptosis (Turpin et al., 2021). JEV infection decreased Forkhead box O (FoxO) expression both in vitro and in vivo. FoxO knockdown facilitated apoptosis, while its overexpression reduced apoptosis in JEV-infected Neuro-2a cells (Guo et al., 2018). In the present study, apoptosis-inducing pathways were activated to varying degrees during the infection of DTMUV, in particular, the death receptor pathway (e.g., representative gene Fas) and mitochondrial pathway (e.g., representative gene Caspase-7 and BID), which were significantly activated in the middle and late stages of DTMUV infection.

Flaviviruses could also activate the survival signaling pathway involved in phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Lee et al., 2005), whose activation may establish an advantageous environment for viral replication and virion assembly (François and Klotman, 2003; Esfandiairei et al., 2004). The gene products of viruses linked to polyomavirus (Talmage et al., 1989), Epstein-Barr virus (Dawson et al., 2003; Fukuda and Longnecker, 2004), human papillomavirus (Nair et al., 2003), hepatitis B virus (Shih et al., 2000), and hepatitis C virus (He et al., 2002) have been verified to stimulate PI3K/Akt-mediated cell survival, thus preventing the apoptosis of infected cells and resulting in viral survival.
In line with the above results, in this study, the expression levels of PI3KR5, PI3KCD, and AKT1 were upregulated to varying degrees in the middle stage of infection. There is a dual regulatory role between flavivirus and apoptosis (Pan et al., 2021), in which flavivirus uses various strategies to activate or inhibit cell apoptosis (Liao et al., 1998; Lee et al., 2005; Okamoto et al., 2017). Replication of JEV appeared to trigger apoptosis in infected culture cells and the expression of BCL2 protein was slowly stimulated by JEV in the early stage of infection (Lee et al., 2005). Over-expression BCL2 could delay the JEV-induced apoptosis process in the early stage and promote its persistent infection (Liao et al., 1998; Lee et al., 2005), but it did not affect viral production in THBMECs, BHK-21 cells, and N18 cells (Liao et al., 1997; Al-Obaidi et al., 2017). High concentrations of the viruses can inactivate proapoptotic proteins to prolong the duration of viral infection in JEV-infected THBMECs (Al-Obaidi et al., 2017). Cells infected with flaviviruses required BCLXL for survival (Suzuki et al., 2018). In the present study, the apoptosis of DTMUV-infected brain tissues occurred in the middle and late stage of infection, and antiapoptotic BCL2 mRNA and protein expression levels were upregulated in the early stage, both BCL2A1 and BCL2L1 were significantly upregulated during the middle stage of infection, which suggesting that the survival signal of host cells may block apoptosis during the virus infection process that will be beneficial to replication of the virus.

In conclusion, the infection of DTMUV in brain tissues can trigger the occurrence of apoptosis, but the regulation of apoptosis by the viruses is very complex during the process of infection. Clarifying the relationship between DTMUV and host apoptosis and the potential mechanism will help to understand the mechanisms of the occurrence, development, and outcome of DTMUV infection, which will enable people to understand the relationship between DTMUV and host from a more comprehensive perspective.

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DISCLOSURES

The authors who have taken part in this study declared that they have no competing interests.

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