INTRODUCTION

Duck hepatitis A virus (DHAV) causes acute hepatitis with high morbidity and mortality in young ducklings. This disease was first reported in Long Island in 1949, but it had a worldwide outbreak later (Liu et al., 2011). Duck viral hepatitis was first described in China in 1963, and the pathogen was formally identified in 1984. Although 3 different serotypes of duck hepatitis virus (DHV; DHV-1, DHV-2, and DHV-3) have been described, there are no antigenic cross reactions between them (Haider and Calnek, 1979; Gough et al., 1985). The DHV-1 is the most important serotype because it could cause mortality higher than 80% in ducklings less than 3 wk old (Jin et al., 2008). Originally classified as an enterovirus, DHV-1 is reclassified as a member of a novel genus Avihepatovirus in the family Picornaviridae and renamed as DHAV by the International Committee on Taxonomy of Viruses. Based on phylogenetic analyses and neutralization tests, 3 variants of DHAV have been found in south Asia: DHAV-1 (the classical serotype 1), DHAV-2 (isolated in Taiwan recently), and DHAV-3 (isolated in South Korea and China recently; Gao et al., 2012).

ABSTRACT To investigate the role of apoptosis in duck viral hepatitis pathogenesis, 4- and 21-d-old ducks were inoculated with duck hepatitis A virus serotype 1 and killed at 2, 6, 12, 24, and 48 h postinfection. TdT-mediated dUTP nick-end labeling was used to detect apoptosis cells. Expression profiles of apoptosis-related genes including caspase-3, -8, -9, and Bcl-2 in spleen, bursa of Fabricius, liver, and the quantity of virus in blood were examined using real-time PCR. The TdT-mediated dUTP nick-end labeling analysis indicated there was a significant difference of apoptotic cells between treatments and controls. The same difference also appeared in virus amount variation in blood during infection. Gene expression analysis revealed that the apoptosis-related gene expression profile was different in the 2 groups, and also different between various organs. This study suggested that apoptosis may play an important role in duck hepatitis A virus serotype 1 infection, and apoptosis suppression might facilitate virus multiplication, resulting in the highest virus concentration in the host.

Key words: duck, apoptosis, gene expression, duck hepatitis A virus type 1

2014 Poultry Science 93:527–534
http://dx.doi.org/10.3382/ps.2013-03510

IMMUNOLOGY, HEALTH, AND DISEASE

Apopotosis induction in duck tissues during duck hepatitis A virus type 1 infection

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Many picornaviruses were associated with apoptosis, including foot and mouth disease virus, enterovirus, rhinovirus, poliovirus, and so on (Chen et al., 2007; Buenz et al., 2009; Son et al., 2009). In our research, cell apoptosis was also found in DHAV-1-infected ducklings, along with necrosis. Previous investigations indicated that several picornavirus proteins may act as inducers in apoptotic response, including viral proteins VP1, VP2, VP3 and 2A, 3C, 2C, or in antiapoptotic response, including the nonstructural proteins 2B and 3A (Henke et al., 2000; Neznanov et al., 2001; Campanella et al., 2004; Liu et al., 2004; Chau et al., 2007; Gullberg et al., 2010). Leader protein of Theiler’s murine encephalomyelitis virus could also induce apoptosis when transfected into mammalian cells (Fan et al., 2009). However, there were no data on DHAV-1-induced apoptosis.

Apoptosis was often associated with virus-induced human and animal diseases, and caused substantial morbidity and mortality. Apoptosis may also have a pathogenic role by contributing to cell death and tissue injury. Although it was well characterized in vitro, virus-induced apoptosis in vivo and its role in virus-induced disease were not well documented. Many molecules or genes involved in the regulation or induction of apoptosis have been identified, including the caspase family, Bcl-2 family, p53 tumor suppressor gene, TNF/NGF receptor superfamily, and so on (Thornberry and Lazebnik, 1998). In addition, the activation of caspas-
Histopathological Examination

The livers, spleens, and BF were fixed in neutral, buffered 10% formaldehyde solution, routinely processed, and embedded in paraffin. Four-μm sections were cut with a Leica SM 2000 R rotational microscope (Leica, Nussloch, Germany) and stained with the standard hematoxylin and eosin method. Stained tissue sections were examined under a microscope (Nikon, Tokyo, Japan).

TdT-Mediated dUTP Nick-End Labeling Assays

The apoptotic cells were stained with In Situ Cell Apoptosis Detection Kit I, POD (Wuhan Boster Biological Technology Ltd., Wuhan, China), according to manufacturer’s instructions with minor modification. Briefly, 4-μm sections were washed twice with PBS, then incubated in 3% H2O2 solution for 10 min and proteinase K solution for 7 min at room temperature. Sections were then washed twice with PBS and incubated with TdT-mediated dUTP nick-end labeling (TUNEL) reaction mixture (labeling buffer 18 μL, TdT 1 μL, and DIG-d-UTP 1 μL) at 37°C for 2 h in a humid box. After that, the sections were washed twice with PBS, and then incubated with blocking buffer, antibody solution, and streptavidin biotin complex solution, in order. Finally, the sections were stained with diaminobenzidine and hematoxylin and eosin, and then analyzed under a microscope. The number of TUNEL-positive cells relative to the total number of cells was determined from 7 randomly selected visual fields of the sections from 3 independent experiments.

Virus Quantitative Detection

The PCR primers of DHAV-1 were designed according to VP1 gene sequences issued on GenBank. Blood samples were collected from ducks at 2, 6, 12, 24, 48, and 72 h after inoculation, respectively. The amount of virus in blood was measured by quantitative PCR at each time point. Virus RNA was isolated from 50-μL samples of duck blood using Trizol reagent (Invitrogen, Carlsbad, CA). The RNA was dissolved in 50 μL of RNase-free water, and cDNA were synthesized by using M-MLV reverse transcriptase kit (Invitrogen) with random primer. Reverse-transcribed viral cDNA was quantified through real-time PCR by using Platinum SYBR Green qPCR Super MIX-UDG kit (Invitrogen). According to the manufacturer’s instructions, the final PCR mixture was 25 μL, containing 100 nM forward and reverse primers (listed in Table 1), 12.5 μL of Platinum SYBR Green qPCR Super MIX-UDG, and 1 μL of cDNA. Samples were analyzed 3 times. The reaction was performed in an IQTm5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA), and the reaction conditions were 95°C for 30 s; and then 45 cycles of 95°C for 5 s, 56°C for 30 s, and 72°C for 30 s.
Nuclease-free water was used as a negative control. The viral genome equivalents variation curve during infection was drawn based on the results of virus quantitative detection.

**Gene Differential Expression Analysis by Real-Time PCR**

To understand the mRNA expression profile in the liver and immune organs, 5 µg of RNA extracted from spleen, BF, liver tissues were reverse-transcribed into cDNA using a M-MLV reverse transcriptase kit (Invitrogen) primed with oligo-dT per the manufacturer’s protocol. The amount of cDNA corresponding to 20 ng of reverse-transcribed RNA was amplified by real-time PCR, using specific primers sets designed for caspase-3, caspase-8, caspase-9, Bcl-2, and β-actin, respectively. Platinum SYBR Green qPCR SuperMIX-UDG was used as the fluorescent dye for real-time PCR. Primers used in the PCR were listed in Table 1. Real-time PCR was conducted using an IQTm5 Multicolor Real-Time PCR Detection System. The thermal cycling program was 30 s denaturation at 95°C C, then 40 cycles of 5 s at 95°C, 30 s at 60°C, 30 s at 72°C, and 30 s at 79°C (plate reading). Each reaction was performed in duplicate in a 25-µL reaction volume. The expression of specific genes was normalized to the expression level of the β-actin gene. By using β-actin as housekeeping gene, the relative expression analysis of caspase-3, caspase-8, caspase-9, Bcl-2, and β-actin, respectively.

**Results**

**Clinical Symptoms and Histopathological Change**

After 22 h of infection with DHAV-1 in D4 ducklings, there were obvious typical clinical signs of duck virus hepatitis, including depression, wing drooping, and anorexia. All dead ducklings showed opisthotonus. Gross lesions tend to be restricted to the liver, which was swollen, fragile, and presented multiple punctate hemorrhages. Spleen and kidney also presented various levels of swelling. The surface of the spleen showed multiple punctate hemorrhages. There were no obvious clinical signs in the D21 ducklings. The mortality was 20% in D4 ducklings, and no dead ducks were found in the D21 group.

In the D4 group, liver cells appeared necrotic 24 h postinfection. Microscopically, the hepatic cells were small and shrunken with nuclear pyknosis and karyorrhexis. Some cells formed cytoplasmic blebs and broke down into small cellular fragments. After 48 h postinfection, the surviving ducks appeared with obvious epithelial hyperplasia surrounded by apoptotic cells (Figure 1). Histopathological changes in the spleen mainly referred to structural disorder, lymphoid depletion, and an increase of heterophilic granulocytes (Figure 2). However, in the D21 group, there were no significant histopathological changes and the number of apoptotic cells was low (Figure 3).

**Change of Virus in Blood During Infection**

Based on the results of quantitative PCR, in blood samples of the D4 ducklings group, virus copies increased after 2 h postinjection (p.i.), and reached the peak at 24 h p.i., and then decreased. The maximum of the virus in blood was $10^{6.3}$ viral genome equivalents at 24 h p.i. In the D21 group, the virus amount in blood samples increased from 2 to 48 h p.i., still lower compared with that in the D4 group (Figure 4).

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequence of primer</th>
<th>Length (bp)</th>
</tr>
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<tbody>
<tr>
<td>DHAV-F</td>
<td>5′-GAAAAGGTGAGGAAGCAAT-3′</td>
<td>244</td>
</tr>
<tr>
<td>DHAV-R</td>
<td>5′-AGCGTAGGCAGGCATAATAA-3′</td>
<td>200</td>
</tr>
<tr>
<td>Caspase-3F</td>
<td>5′-CCGGCTGTCATCTCGTTCAGGCAC-3′</td>
<td>178</td>
</tr>
<tr>
<td>Caspase-9F</td>
<td>5′-CCCCTTCATCCTGCTTCCATC-3′</td>
<td>219</td>
</tr>
<tr>
<td>Caspase-9R</td>
<td>5′-TTGCCGCTGAACCCTGTTGTC-3′</td>
<td>217</td>
</tr>
<tr>
<td>Bcl-2F</td>
<td>5′-GGAGGGCTCTGAAAGAAAAAG-3′</td>
<td>282</td>
</tr>
<tr>
<td>Bcl-2R</td>
<td>5′-TATGATGGGATGGGAGCTG-3′</td>
<td></td>
</tr>
<tr>
<td>β-actinF</td>
<td>5′-CCACTGATGGGAGCTGAG-3′</td>
<td></td>
</tr>
<tr>
<td>β-actinR</td>
<td>5′-GGCCGAGGCTTCTGATG-3′</td>
<td></td>
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Apoptosis Rate in Liver and Spleen Determined by TUNEL

Based on the TUNEL analysis, the apoptotic cells existed mainly in hepatocytes, macrophages, and lymphocytes (Figure 2D). To determine the relative levels of cell death in infected and noninfected ducklings, the numbers of TUNEL-positive cells were counted in liver and spleen at different times after infection. In the D4 group, the apoptosis rate in the liver increased significantly compared with controls from 6 h p.i., and then reached the peak at 24 h p.i. (Figure 5A). In spleen, apoptosis rate of infected group was higher compared with noninfected group from 6 h p.i., and significant at 6 and 12 h p.i. (Figure 5C). In the D21 group, apoptosis increased slightly in the liver from 6 h p.i., and no significant difference was observed between the infected and noninfected group (Figure 5B). In spleen, the percentage of apoptotic cells increased from 6 to 24 h p.i., and there was a significant difference between treatments and controls at 24 h p.i. At other time points, there were no obvious differences between treatments and controls (Figure 5D).
Apoptosis-Related Gene Expression in Immune Organs

In the spleen of the D4 group, the transcription of the Bcl-2 isoform was increased significantly in the first 2 h p.i., and then decreased from 6 h p.i., whereas the transcription of both caspase-9 and caspase-3 was induced from 6 to 24 h p.i. and kept a relatively moderate level (about 1- to 2.5-fold compared with control). The expression of caspase-8 was almost unchanged from 2 to 48 h, except for a slight induction at 2 and 12 h p.i. (Figure 6A, D, G, J). In the spleen of the D21 group, the expression of Bcl-2 was induced significantly from 6 h and reached a peak at 12 h p.i., and then decreased to the normal value at 48 h. Unlike Bcl-2, the transcription of caspase-8 was increased first and then decreased. Caspase-9 was decreased at all the time points tested. The transcription of caspase-3 was significantly increased from 2 to 24 h p.i. (Figure 6A, D, G, J).

In BF of the D4 group, the transcription of Bcl-2 was significantly upregulated at 24 h p.i. The transcription of caspase-8 and -9 was almost unchanged at all the time points. As for the expression of caspase-3, there was slight increase from 2 to 12 h p.i., and then a decrease. However, in the D21 group, the expression of Bcl-2 was induced from 2 h and reached a peak value at 6 h, with about 10-fold increase compared with the control, and then decreased to 48 h p.i. The transcription of caspase-3 kept a significantly higher level from 2 to 24 h p.i. compared with the controls. The expression of caspase-9 was almost at the level of the controls after the infection of the virus. As for the expression of caspase-8, it was significantly upregulated at 6 h p.i. (about 10-fold of the controls), and then decreased to the level of the controls at 48 h p.i. (Figure 6B, E, H, K).

Apoptosis-Related Gene Expression in Liver

In the liver of D4 ducks, the transcription of Bcl-2 was significantly upregulated at 2 h, and reached a peak value of about 6-fold of the control level, and then decreased to the normal level at 24 to 48 h p.i., whereas the expression of caspase-8 showed no obvious changes except for a significant increase until 48 h p.i. As for the transcription of caspase-3 and-9, there were almost no significant increases after the infection of DHAV-1, compared with the controls. On the contrary, their expression was both significantly inhibited at 24 h p.i. (Figure 6C, F, I, L).

In D21 ducks, the transcription of Bcl-2 was significantly induced at 6 h p.i., and then decreased. The transcription of caspase-8 was suppressed at almost all the time points in the treatment group compared with the controls. The transcription of caspase-3 kept the level of the controls except for a slight increase at 6 h p.i. in liver of D21 ducks. The transcription of caspase-9 isoform in liver was significantly upregulated from 6 h p.i., whereas at other time points, there were no obvious changes between treatment and controls (Figure 6C, F, I, L).
DISCUSSION

For most viral infections, the host immune system can induce apoptosis to promote virus clearance (Mori et al., 2006). On the other hand, to avoid host immunity, many viruses have developed strategies to prevent the apoptosis of infected cells and thereby increase viral proliferation at the first hours with infection (Clarke and Tyler, 2009). Cellular Bcl-2 family proteins regulate critical steps in programmed cell death pathway by modulating mitochondrial permeability and function. The anti-apoptotic members Bcl-2 can prevent cell death and pro-apoptotic elements such as Bax and Bak that promote apoptosis. Host cells exploit this apoptosis process as a primitive defense mechanism against viral infections. On the other hand, several vi-
ruses have evolved mechanisms to prevent or at least delay apoptosis of host cells for successful replication and to increase the production of viral progeny. Large DNA viruses, such as iridoviruses and adenoviruses, contain sequence homologs of Bcl-2 that have been shown to prevent apoptosis in the early stage of viral infection (Lin et al., 2008). The main mechanism of hepatocyte loss in liver disease was thought to be necrosis. In present study, histopathological examination revealed the occurrence of hepatocyte necrosis and inflammatory infiltrates, and apoptosis was also taking place in the necrotic area. Gene expression analysis indicated that the apoptosis suppressor genes Bcl-2 in the liver were upregulated during virus infection in the D4 group, although it was not a significant change. In the D21 group, proapoptotic genes and apoptosis suppressor genes were all upregulated, but the peak value time point was different. The result indicated that in D4 group, apoptosis suppression was a primary event, whereas the D21 group was apoptosis promoting. The possible mechanism of DHAV-1 infection is that apoptosis suppression facilitated virus multiplication, resulting in the highest concentration in the host. This hypothesis was confirmed by the virus amount of variation in blood during infection. The exact biological mechanisms underlying of DHAV-1 infection need to be explored in future research. Some evidence suggested that peak virus copy was associated with the appearance of activated caspase-3 during infection (Samuel et al., 2007).

Picornaviruses caused a wide range of animal and human diseases, based on their distinct tissue and cell type tropisms. The host response to picornavirus is complex, and the damage to tissues occurs not only from direct viral replication within infected cells. Picornavirus exhibited an exceptional ability to evade the early innate immune response, resulting in chronic infection and autoimmunity (Buskiewicz et al., 2012). Caspases play important roles in the initiation and execution of apoptosis; in addition, they also have nonapoptotic functions, including cytokine maturation (Martinon et al., 2002), T-cell activation (Chun et al., 2002), and monocyte differentiation (Sordet et al., 2002; Kang et al., 2004). Apoptosis is controlled by caspase-8 during picornavirus infection. Caspase-8 is one of cysteinyl aspartate specific proteinases that initiate apoptotic cell death in response to cell-surface death receptor activation such as Fas. Caspase-8 has been also implicated in the regulation of most pattern recognition receptor signaling pathways. Among the nonapoptotic functions of caspase-8 was the regulation of innate and adaptive immunity signaling pathways and the inhibition of an inflammatory form of cell death termed necroptosis. In this study, the activation of caspase-8 did not induce caspase-9 and -3, which could be attributed to the nonapoptotic functions of caspase-8. In D4 ducks infected with DHAV-1, apoptosis was taking place based on the TUNEL analyses, which might be the consequence of the expressions caspase-8, or the downregulation of Bcl-2 expression. Previous study showed that the expression of Bcl-2 could prevent the redistribution of cytochrome C in response to multiple death-inducing stimuli (Ott et al., 2007). Our result indicated that the activation of caspase-8 and Bcl-2 might correlate with the induction of apoptosis in the liver. In D4 ducks, the number of TUNEL-positive cells was increased and apoptosis gradually dominated from 24 h p.i. In addition, the trend of apoptosis in D21 ducks was similar to that in D4 ducks, and caspase-9 was the main apoptotic initiator.

The results of this study indicated that the activation of caspase-8 and -9 induced apoptosis in immune cells. However, there was a little difference in the 2 ages of ducks when apoptosis appeared. In spleen and BF, caspase-8, which could be initiated by the extrinsic apoptotic pathway following activation of cell-surface death receptors, was upregulated in D21 ducks. Bcl-2 was induced just at first phase of viral infection and inhibited the activation of caspase-9. In D4 ducks, intrinsic apoptosis was activated by increased expression of caspase-9 from the first 6 h p.i. At the same time, obvious necrosis was observed from 24 h. Taken together, these results suggested that there was a different initiator of apoptosis in the 2 ages and induction of immune cells’ apoptosis occurred before the visible immune injury. However, there was a little difference in the 2 ages of ducks when apoptosis appeared. The possible explanation might be that certain apoptotic genes were induced by other stimulating factors, such as ISG12b2 (Lu and Liao, 2011).

In conclusion, the results of this study indicated that the age-related apoptosis played an important role in DHAV-1 infection. Further studies in relational genes involved in the signal transduction of cell cycle and apoptosis pathway would be useful to better elucidate the complex molecular mechanism of apoptosis in DHAV-1 infection, and a detailed understanding of the apoptosis pathway may help to investigate the pathogenic mechanism.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grants 30972161), and the Fundamental Research Funds for the Central Universities (Program No. 2011QC045 and 2013QC006). We thank X. Liu (Tulane University) for revising our manuscript.

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