Characterizing the thymic lesions in piglets infected with attenuated strains of highly pathogenic porcine reproductive and respiratory syndrome virus

Gang Wang^{1†}, Ying Yu^{1†}, Yabin Tu¹, Yuming Li¹, Jie Tong¹, Chong Zhang^{1, 2},
Yonggang Liu¹, Aidong Li¹, Chenggang Jiang¹, Shujie Wang¹, Ye Tao¹, Xuehui Cai^{1#}

¹ *State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, 150001, PR China*

² *Jilin Agricultural University, Changchun 130118, PR China*

† Contribute equally

#Corresponding author: State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of Chinese Academy of Agriculture Science, Harbin, 150001, China; Tel: +86-18946066077; Fax: +86-451-82733132; E-mail: cai139@hvri.ac.cn.

Abstract

Piglets infected with the highly pathogenic PRRSV (HP-PRRSV) HuN4 strain develop severe thymus atrophy. However, the attenuated strain HuN4-F112 does not lead to lesions in organs. Here, we have characterized the thymic lesions in piglets infected with attenuated strains of HP-PRRSV HuN4 isolated at different passages in the attenuation process to produce HuN4-F112 from the parent HuN4 strain (HuN4-F5, HuN4-F15, HuN4-F23, HuN4-F30, and HuN4-F112). The thymic effects of infection were evaluated in terms of the thymus/body weight ratio, pathological changes, and thymocytes apoptosis. The ability of HP-PRRSV to induce thymus atrophy was reduced following attenuation after 23 passages; the HuN4-F23, but not HuN4-F30, caused thymus atrophy. The ability of the virus to induce thymocyte apoptosis decreased as it became attenuated. In addition, the viral load in the thymus was reduced as the virus was attenuated. The HuN4-F23 and HuN4-F30 strains might provide insight into the molecular mechanisms of HP-PRRSV pathogenesis. Taken together, our results indicate that the ability of HP-PRRSV to induce thymic atrophy is related to its pathogenicity.

Keywords: HP- PRRSV; HP-PRRSV attenuated strains; thymus atrophy

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first identified in the 1990s in the United States and Europe (Collins et al., 1992; Rossow, 1998; Terpstra et al., 1991; Wensvoort et al., 1991). It is widely recognized as an important viral pathogen in the swine industry worldwide. PRRS is characterized by reproductive failure in gilts and sows and severe respiratory dysfunction in young pigs. PRRS virus (PRRSV), the causative agent of PRRS, is an enveloped positive single-stranded RNA virus that belongs to the *Arteriviridae* family. In 2006, an outbreak of "high fever" disease caused by highly pathogenic PRRSV (HP-PRRSV) led to substantial economic losses in China (Tian et al., 2007; Tong et al., 2007).

We have previously shown that piglets infected with HP-PRRSV (HuN4) developed significant thymic atrophy (He et al., 2012; Wang et al., 2011). The atrophy observed during HuN4 infection was attributed to the large number of apoptotic thymocytes (He et al., 2012; Li et al., 2014a). Previous study has also shown that piglets immunized with an attenuated strain of HP-PRRSV (HuN4-F112) did not develop lesions in organs and developed a protective immune response against HP-PRRSV infection (Tian et al., 2009). Here, we have characterized the thymic lesions caused by HP-PRRSV and several HP-PRRSV strains isolated at different passages during the attenuation process to modify HuN4 to HuN4-F112. The thymic effects of infection with HP-PRRSV were evaluated in terms of the thymus/body weight ratio, pathological changes in the thymus, thymic viral load, and apoptotic thymocytes.

2. Materials and Methods

2.1. Virus Strains

The HP-PRRSV HuN4 strain (Genbank no. EF635006) used in this study has been previously described (Tong et al., 2007). The attenuated strains HuN4-F5, HuN4-F15, HuN4-F23, HuN4-F30, and HuN4-F112 are derivatives of HuN4 isolated after 5, 15, 23, 30, or 112 passages through Marc-145 cells, respectively (Tian et al., 2009). The tissue culture infective dose 50 (TCID₅₀) for each strain was determined using a microtiter infection assay and was calculated using the Reed-Muench method.

2.2 HP-PRRSV infection in piglets

Animal experiments were reviewed and approved by the Animal Ethics Committee of the Harbin Veterinary Research Institute at the Chinese Academy of Agricultural Sciences (CAAS) and conformed to animal ethics guidelines.

Twenty-four 28-day-old PRRSV negative piglets were randomly divided into six groups that were housed separately. The animals were given commercial feed and water at libitum throughout the experiment. The animals were acclimated to their surroundings for 1-week prior to infection. Five of the groups were infected with HuN4-F5, HuN4-F15, HuN4-F23, HuN4-F30, or HuN4-F112 intranasally at a dose of 3×10^5 TCID₅₀/piglet (3 mL volume). The negative control group received an equivalent volume of PBS (3 mL). Piglets were euthanized at 10 days post-infection (DPI).

2.3. Clinical signs and pathological observations

The piglets were evaluated daily for the onset of clinical symptoms including

anorexia, lethargy, fever, and emaciation prior to feeding. At necropsy, sections of the thymi were fixed in 10% neutrally-buffered formalin for histological examination using hematoxylin and eosin (H&E) staining.

2.4 Real-time RT-PCR quantification of viral loads in the thymus

The thymic viral load was quantified by TaqMan fluorescent quantitative RT-PCR (q-RT-PCR) using a previously described method (Liu et al., 2010).

2.5 Evaluation of apoptosis in thymocytes

The thymocyte isolation was performed as described previously (He et al., 2012). Briefly, the thymi were placed in ice-cold PBS supplemented with 2% fetal bovine serum (FBS). Thymocyte suspensions were prepared by pressing the tissue through a sieve in the ice-cold PBS supplemented with 2% FCS. The collected thymocytes were used for evaluation of apoptosis. The percentages of thymocytes undergoing apoptosis or necrosis were determined by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences; Franklin Lakes, NJ, USA) according to the manufacturer's instructions as we have previously described (Wang et al., 2015).

2.6 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 5.02 for Windows; GraphPad Software Inc.). Comparisons between groups were performed using the analysis of variance (ANOVA) test. A p -value less than 0.05 was considered statistically significant.

3. Results and Discussion

Piglets infected with the HuN4-F5 and HuN4-F15 strains displayed symptoms

typically associated with HP-PRRS such as a high fever (41–42°C), skin hemorrhage, and severe dyspnea. However, the piglets in the HuN4-F23, HuN4-F30, HuN4-F112, and negative control groups did not develop fever or other clinical signs of HP-PRRS. All of the piglets were euthanized at 10 DPI to evaluate the macroscopic thymic lesions. The piglets infected with the HuN4-F5 and HuN4-F15 strains showed severe thymic atrophy (Fig. 1A and 1B). Mild thymic atrophy was observed in piglets infected with HuN4-F23 (Fig. 1C). No apparent thymic atrophy was observed in piglets infected with HuN4-F30 or HuN4-F112 (Fig. 1D and 1E). As expected, the size of the thymus in the negative control piglets was normal (Fig. 1F). To quantify the degree of thymic atrophy, the average ratio of thymus/body weight (g/kg) was determined for each group. Consistent with the macroscopic evaluation, the lowest thymus/body weight ratio was observed in the HuN4-F5 and HuN4-F15 groups; and the highest thymus/body weight ratios were observed in the negative control, HuN4-F112, and HuN4-F30 groups. The thymus/body weight ratio of the HuN4-F23 group was significantly different from all of the other groups (Fig. 2A).

The thymi from piglets infected with the HuN4 strains (10 DPI) were then examined histologically following H&E staining to assess morphological changes. Severe atrophy was observed at 10 DPI in the thymic lobules of the HuN4-F5 strain infected piglets accompanied by blurred boundaries between the cortex and medulla (Fig. 1G). Similarly, loss of cortical thymocytes was observed in the thymi from the HuN4-F15 strain infected piglets (Fig. 1H). There were no apparent microscopic lesions in other groups (Fig. 1I-L).

To understand whether the thymic atrophy in the HuN4-F5 and HuN4-F15 groups was the result of apoptosis induction, the percentage of thymocytes undergoing apoptosis and necrosis was quantified by flow cytometry. At 10 DPI, more than 45% of the thymocytes harvested from piglets in the HuN4-F5 infected group were apoptotic. This was significantly higher than both the HuN4-F15 (29%) and HuN4-F23 (28%) groups. The lowest percentages of apoptotic thymocytes following infection were observed in the HuN4-F30 and HuN4-F112 groups, approximately 15% and 12%, respectively. All of the HP-PRRSV strains induced apoptosis above the level observed in the negative control group piglets (less than 10%; Fig. 2B).

The mean thymic viral load was assessed at 10 DPI. The highest mean viral loads were observed in the HuN4-F5 ($10^{8.12}$ copies/gram) and the HuN4-F15 ($10^{7.12}$ copies/gram) groups. Intermediate viral loads were observed in the HuN4-23 ($10^{5.86}$ copies/gram) and HuN4-F30 ($10^{5.63}$ copies/gram). The lowest mean viral load was observed in the HuN4-F112 infected piglets ($10^{3.18}$ copies/gram).

Taken together, our results suggest that as the parent HuN4 virus became attenuated through passaging, its ability to induce thymic atrophy was reduced. A recent study has reported that the nsp9 and nsp10 proteins contribute to the virulence of the HP-PRRSV strains emerging in China (Li et al., 2014b). We hypothesize that nsp9 and nsp10 may also be involved in the molecular mechanism leading to thymic lesions. Future studies will explore this hypothesis in detail. Two of the strains used here, HuN4-F23 and HuN4-F30, showed promise for use in studying the molecular mechanism(s) leading to thymic lesions induced by HP-PRRSV HuN4 infection. In

conclusion, the ability of HP-PRRSV to induce thymic atrophy is related to the pathogenicity of the HuN4 strain.

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Figure legends

Figure 1. Representative images showing the pathological changes in the thymus

of piglets infected with attenuated strains of HP-PRRSV HuN4. Piglets were infected with attenuated HuN4 strains and necropsied 10 days post-infection. The thymus was evaluated macroscopically (A-F) and sections were H&E stained for histological examination (G-L). The panels showing thymic changes for each attenuated HuN4 strain are: HuN4-F5- A and G; HuN4-F15- B and H; HuN4-F23- C and I; HuN4-F30- D and J; and HuN4-F112- E and K. The thymus of an age matched negative control piglet is shown in panels F and L.

Figure 2. The mean ($\pm$ SD) of the thymus/body weight ratio and levels of thymocyte apoptosis at 10 DPI. The thymus was collected from piglets infected with the indicated attenuated HuN4 strains 10 DPI. (A) Thymic atrophy was quantified as the ratio of the thymus to total body weight (g/kg). (B) The percentage of thymocytes undergoing apoptosis or necrosis was assessed by flow cytometry. Statistical analyses were performed using the GraphPad Prism software. The analysis of variance (ANOVA) test was used to compare between groups. A p -value less than 0.05 was considered statistically significant ($= p < 0.05$).

Fig. 1

Fig. 2