Virus Research 189 (2014) 29–33

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Identification of apoptotic cells in the thymus of piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus

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ARTICLE INFO

Article history:
Received 4 March 2014
Received in revised form 10 April 2014
Accepted 11 April 2014
Available online 29 April 2014

Keywords:
Highly pathogenic PRRSV
Thymus
Apoptotic cells

ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is an immunosuppressive disease that is characterized by respiratory distress and poor growth in piglets and by severe reproductive failure in sows. PRRS was first recognized in the 1990s in Europe and the United States. In 2006, highly pathogenic (HP)-PRRS caused enormous economic losses in China. Our previous studies demonstrated that the HP-PRRS virus (HP-PRRSV) induced the apoptosis of numerous thymocytes in infected piglets, leading to severe thymus atrophy. To further identify the subset of apoptotic cells in thymus of HP-PRRSV-infected piglets, different cell types, apoptotic cells, and HP-PRRSV were marked with the corresponding markers. Results of the colocalization demonstrated that the apoptotic cells were not infected by HP-PRRSV, and most of them were $CD3^+$ T cells. No apoptosis was observed in the epithelial cells, and only few $CD14^+$ cells were apoptotic. HP-PRRSV was only found in $CD14^+$ cells, and epithelial cells and $CD3^+$ cells were not infected by HP-PRRSV. This is the first study to report the apoptotic and infected cells in the thymuses of HP-PRRSV-infected piglets.

Porcine reproductive and respiratory syndrome (PRRS) is an important disease of pigs that is responsible for significant economic losses in the swine industry worldwide (Li et al., 2007; Polson et al., 1992). The PRRS virus (PRRSV), which is the causative agent of PRRS, is a small, enveloped, positive single-stranded RNA virus of the Arterivirus group of viruses (Cavanagh, 1997). PRRS emerged in China in 1996, and has spread across the global swine industry since then (Chen et al., 2006). A highly pathogenic PRRS (HP-PRRS) outbreak occurred in China in May 2006, resulting in the death of more than one million pigs (Tian et al., 2007; Tong et al., 2007).

Different PRRSV strains cause different thymic lesions: the SD 23983 strain induces severe thymus atrophy in newborn piglets, leading to immunosuppression within a month (Feng et al., 2002); HP-PRRSV (HuN4) has been reported to have much stronger tropism to the thymus than low-pathogenicity PRRSV (CH-1a) (He et al., 2012; Wang et al., 2014, 2011). These studies demonstrated that thymus atrophy may be related to the apoptosis of thymocytes. However, no studies have investigated the apoptotic cells and the type of infected cells in the thymus of PRRSV-infected piglets. In this study, we used double immunofluorescence labeling to investigate the types of thymic cells that were infected and underwent apoptosis in HP-PRRSV-infected piglets.

The thymus samples used in this study were obtained from a previous experiment (He et al., 2012). Here, we collected thymus samples from three HuN4-infected piglets (HuN4 group) and uninfected piglets (control group) after they were euthanized at 10 days post-inoculation (DPI). The thymus samples were collected during necropsy and cut into 8-μm sections on a cryostat. The cryostat sections were used for double immunofluorescence staining. Two sections were obtained from different parts of each thymus specimen for each detection.

Double immunofluorescence staining was used to examine whether PRRSV was colocalized with apoptotic cells. Briefly, thymus sections were incubated with SDOW-17 monoclonal

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http://dx.doi.org/10.1016/j.virusres.2014.04.011
0168-1702/

antibodies (mAbs; Rural Technologies, Inc., Brookings, SD) against the PRRSV N protein, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibodies (ZSGB-BIO, China). Apoptosis was detected using terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling (TUNEL) according to the instructions provided in the In Situ Cell Death Detection Kit (Roche, Germany), and the nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI; Sigma, USA). As shown in Fig. 1, PRRSV$^+$ or TUNEL$^+$ cells exhibited only single staining, indicating that HP-PRRSV-infected cells did not undergo apoptosis. Meanwhile, the apoptotic cells were not infected by HP-PRRSV (Fig. 1). These results demonstrated that HP-PRRSV induces apoptosis in bystander cells in the thymus of infected piglets.

To further investigate the type of cells undergoing apoptosis, CD14$^+$ cells were stained with FITC-conjugated mouse anti-pig CD14 antibodies (AbD Serotec, USA), CD3$^+$ cells were stained with mouse anti-human T cell (CD3) antibodies (DakoCytomation, Switzerland) and FITC-conjugated goat anti-mouse antibodies (ZSGB-BIO, China), and thymus epithelial cells were stained with rabbit anti-pan-cytokeratin (P-CK) antibodies (Bioss, China) and FITC-conjugated goat anti-rabbit antibodies (ZSGB-BIO, China). The apoptosis staining and the nuclear staining were carried out as described for last detection. As shown in Fig. 2, most apoptotic cells were colocalized with CD3$^+$ cells (Fig. 2A), only a few apoptotic cells were colocalized with CD14$^+$ cells (Fig. 2B), and none of the thymus epithelial cells were found to have undergone apoptosis (Fig. 2C).

To further investigate the type of cells that were infected with HP-PRRSV, PRRSV antigen was stained with SDOW-17 mAbs and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse antibodies (ZSGB-BIO, China) or FITC-conjugated goat anti-mouse antibodies (ZSGB-BIO, China). After incubation with 10% mouse serum, the corresponding sections were incubated with FITC-conjugated mouse anti-pig CD14 antibodies (AbD Serotec) or rabbit anti-P-CK antibodies (Bioss, China) and FITC-conjugated goat anti-rabbit antibodies (ZSGB-BIO, China) or spectral red (SPRD)-conjugated mouse anti-pig CD3 antibodies (Southern Biotech, USA). The nuclei were stained with DAPI. As shown in Fig. 3, only CD14$^+$ cells were found to be colocalized with PRRSV N protein (Fig. 3B), while CD3$^+$ cells and thymus epithelial cells were not infected by HP-PRRSV (Fig. 3A and C).

The thymus is the site of T lymphocyte development, and therefore contains many T cells at various stages of differentiation. Most of these T cells express CD3 (Pearse, 2006). During the development of thymocytes, thymus epithelial cells play an important role in positive and negative selection of thymocytes via the MHC antigens on the cell surface (Pearse, 2006). In addition, macrophages, which are targeted by the PRRSV, are also found in the thymus. Previous studies have reported that some viruses, including PRRSV, can induce apoptosis in epithelial cells (Lu et al., 1996; Sur et al., 1998), and PRRSV can induce apoptosis of pulmonary alveolar macrophages (Thanawongnuwech et al., 1997). Therefore, we investigated CD3$^+$ T cells, thymus epithelial cells, and CD14$^+$ cells in the thymuses of piglets. The results showed that most apoptotic cells were CD3$^+$ T cells (Fig. 2), and only CD14$^+$ cells were infected by HP-PRRSV in thymus (Fig. 3).

It has been shown that PRRSV induces apoptosis in bystander cells in the lungs of infected pigs (Choi and Chae, 2002). Other studies have reported that HP-PRRSV induced apoptosis of large numbers of thymocytes (He et al., 2012; Wang et al., 2011). In the current study, we carried out double immunofluorescence staining to detect HP-PRRSV antigen and apoptotic cells in order to determine the relationship between infected cells and apoptotic cells in the thymuses of infected piglets. Results of the colocalization revealed that a large number of the apoptotic cells were not infected by HP-PRRSV (Fig. 1), suggesting that the virus induces an indirect pathway of cell apoptosis. These results suggest that HP-PRRSV-induced apoptosis in bystander cells may be related to the release of cytokines; these findings are consistent with the results of previous studies (Nagata, 1997; Zheng et al., 1995). Further studies are required to investigate the mechanism of cell apoptosis in the thymus of PRRSV-infected piglets.

The thymus is the first lymphoid organ to develop, and it shows considerable growth immediately after birth in response to postnatal antigen stimulation, producing large numbers of mature T cells (Pearse, 2006). Furthermore, it may be the primary organ of PRRSV replication (Benson et al., 2002; Rowland, 2010). Previous studies have reported that PRRSV can injure the thymus; however, the subset of apoptotic and infected cells has not yet been identified. This study is the first to report that the majority of apoptotic cells in PRRSV-infected thymuses were CD3$^+$ T cells, and only the CD14$^+$ cells in the thymus of piglets were infected with HP-PRRSV.

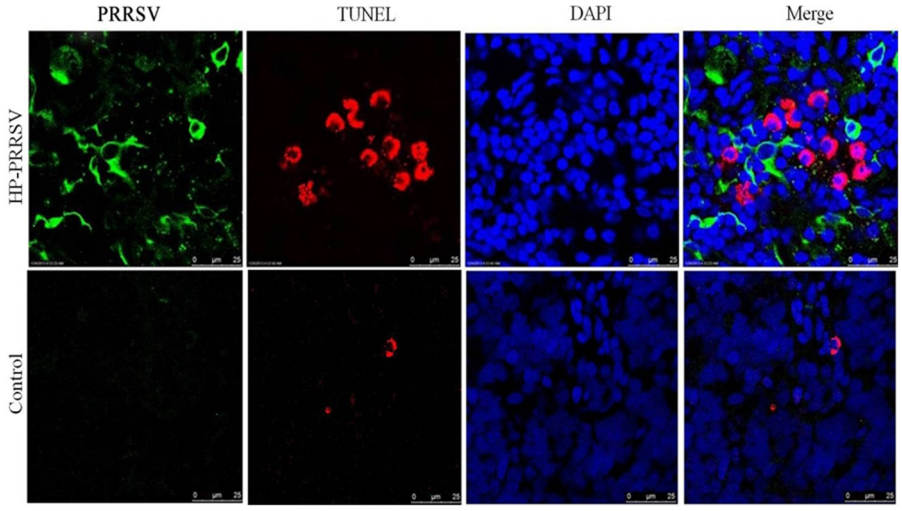

Fig. 1. Colocalization of porcine reproductive and respiratory syndrome virus (PRRSV)-infected cells, apoptotic cells, and nuclei. Thymus sections were incubated with SDOW-17 monoclonal antibodies (mAbs) against PRRSV N protein and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibodies. Apoptotic cells were detected according to the instructions provided with the In Situ Cell Death Detection Kit, and the nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI).