A pneumo-virulent United States isolate of porcine reproductive and respiratory syndrome virus induces apoptosis in bystander cells both in vitro and in vivo

Theerapol Sirinarumitr,† Yanjin Zhang,‡ John P. Kluge,¹ Patrick G. Halbur³ and Prem S. Paul², ⁴

Department of Veterinary Pathology¹, Veterinary Medical Research Institute², Department of Veterinary Diagnostic and Production Animal Medicine³, and Department of Veterinary Microbiology and Preventive Medicine⁴, College of Veterinary Medicine, Iowa State University, 1802 Elwood Drive, Ames, IA 50011, USA

Evidence of apoptosis was detected for the United States porcine reproductive and respiratory syndrome virus (PRRSV) in ATCC CRL11171 cells inoculated with strain ATCC VR2385 and in the tissues of pigs infected with the same strain. Apoptosis was detected by agarose gel electrophoresis, transmission electron microscopy and terminal deoxytransferase dUTP nick end labelling (TUNEL) techniques. By electron microscopy and double-labelling techniques, apoptosis was detected primarily in uninfected bystander cells in the continuous cell line rather than the PRRSV-infected cells. In the lungs, the apoptotic cells were predominantly alveolar and pulmonary intravascular macrophages, and mononuclear cells in the alveolar septa. In the lymph nodes, the apoptotic cells were predominantly tingible body macrophages and mononuclear cells. The induction of apoptosis in a large number of mononuclear cells in the lungs and lymph nodes appears to be a mechanism of PRRSV pathogenesis and might be an explanation for a dramatic reduction in the number of alveolar macrophages and circulating lymphocytes and monocytes in PRRSV-infected pigs.

Porcine reproductive and respiratory syndrome (PRRS), which has been recognized in the United States (US) since 1987 (Hill, 1990), is characterized by reproductive failure (stillborn, mummified and weak-born piglets) and respiratory disease in young and growing pigs (Christianson et al., 1992a; Halbur et al., 1995; Terpstra et al., 1991). The aetiological agent of PRRS was first isolated in 1991 in the Netherlands (Wensvoort et al., 1991), and then in 1992 in the US (Collins et al., 1992). The prototype European isolate is known as Lelystad virus (LV). PRRSV is a member of the family Arteriviridae in the order Nidovirales (Cavanagh et al., 1997). This classification is based on the similarity in morphology, genomic organization and the strategy of gene expression compared to lactic dehydrogenase elevating virus, equine arteritis virus and simian haemorrhagic fever virus (Cavanagh et al., 1997).

LV has been shown to induce apoptosis in both MA-104 cells and primary cultures of porcine alveolar macrophages. This has been attributed to the ORF 5 gene product, as the product of the ORF 5 of LV induced apoptosis in monkey BSC40 cells (Suarez et al., 1996). Recently, a US PRRSV isolate has been shown to induce apoptosis in germ cells in testes and the investigators have hypothesized that it may contribute to the pathogenesis of PRRSV in infected boars (Sur et al., 1997). However, several questions remain unanswered. It is not known whether US PRRSV induces apoptosis in virus-infected cells or uninfected bystander cells, or whether PRRSV isolates induce apoptosis in vivo in alveolar macrophages in the lungs, and macrophages and lymphocytes in lymph nodes, the main sites for virus replication. This information is needed to enable us to understand the mechanism of pathogenesis of PRRSV in infected growing pigs. In this paper, a highly pneumo-virulent US PRRSV isolate (VR2385) is shown to induce apoptosis both in vitro in a continuous cell line and in vivo in alveolar macrophages of the lungs and mononuclear cells of the lymph nodes of PRRSV-inoculated pigs. We also show that the primary cells that undergo apoptosis are the uninfected bystander cells and not the infected cells.

ATCC CRL11171 cells (Meng et al., 1996) were inoculated with ATCC VR2385 strain of PRRSV (10⁶.⁶ TCID₅₀/ml) at an m.o.i. of 0.0001 TCID₅₀ virus per cell for all experiments. PRRSV-infected and mock-infected cells, grown in 25 cm² flasks, were observed for cytopathic effect (CPE) and were collected with a cell scraper (Fishet) at 1, 2, 3, 4, 5 and 6 days post-inoculation (p.i.). Total cell DNA was isolated using a...
Qiagen tissue kit. Briefly, the cells were washed, resuspended and treated with 25 µl proteinase K (18 mg/ml) and 200 µl lysis buffer at 70 °C for 10 min. Cell lysates were treated with 210 µl absolute ethanol and the mixture was applied to a spin column. The columns were centrifuged and washed, and the DNA was eluted using preheated (70 °C) distilled water. Finally, 10 µl cell DNA was electrophoresed on a 1-2% agarose gel (Amresco). This assay was repeated three times using three different sets of cells. CPE, characterized by degeneration, cell rounding, clumping of cells and cell detachment, was observed at 2 days p.i. in PRRSV-infected cell cultures. About 30%, 80%, and 98% of the PRRSV-infected cells detached at 4, 5 and 6 days p.i., respectively. Multiple nucleosomal-sized DNA fragments increasing by 180 bases in size were observed at 2, 3 and 4 days p.i. (Fig. 1).

The PRRSV-infected and mock-infected cells were fixed at 1, 2, 3, 4, 5 and 6 days p.i. with 3% glutaraldehyde, and were used for electron microscopy. Cells were collected, and pelleted at 6000 g for 2 min in 1-5 ml microfuge tubes at 40 °C. The pellets were washed three times with 0-1 M sodium cacodylate buffer and post-fixed with 1% osmium tetraoxide in 0-1 M sodium cacodylate buffer for 2 h at room temperature, dehydrated in acetone, and embedded in plastic for electron microscopy according to Hayat (1989). Ultrastructural changes characteristic of both cell lysis and apoptosis were detected in PRRSV-infected cells. Apoptotic cells were characterized by condensation and sharp margination of nuclear chromatin, intact cell organelles and intact nuclear and cell membranes (Fig. 2c). Lytic cells were characterized by disrupted nuclear and cell membranes, degenerate cell organelles and the lack of sharp margination of nuclear chromatin (Fig. 2b). Most of the
lytic cells contained virus particles (Fig. 2b), whereas virus particles were not detected in apoptotic cells (Fig. 2c).

In order to analyse apoptosis in situ, cells on 8-well chamber slides were processed for enzyme terminal deoxynucleotidyl transferase (TdT)-mediated X-DUTP nick end labelling (TUNEL) using an ApoTag kit (Oncor) according to the manufacturer's instructions. Briefly, cell monolayers in 8-well chamber slides were treated with proteinase K at room temperature for 12 min and then treated with hydrogen peroxide to get rid of endogenous peroxidase. Cells on 8-well chamber slides and tissue sections were subjected to an enzymatic incorporation of digoxigenin-labelled nucleotide with TdT, washed and incubated with anti-digoxigenin peroxidase. After washing, slides were incubated with diamino-benzidine (DAB, Boehringer Mannheim) and counterstained with nuclear fast red. The TUNEL-positive cells were found in both mock-infected and PRRSV-infected cell monolayers; however, the number of TUNEL-positive cells in infected wells was higher than that in the mock-infected monolayers. The TUNEL-positive cells were detected as early as 1 day p.i., and their number increased until 4 days p.i. (data not shown). No data were collected on days 5 and 6 p.i. because most of the cells had detached from the wells.

A double-labelling experiment, using TUNEL and in situ hybridization (ISH), was conducted to determine whether the apoptotic cells were primarily virus-infected or were uninfected bystander cells. A specific fluorescein-labelled RNA probe for the US PRRSV isolate was prepared from plasmid pPSP 2385 containing the entire sequence from 50 nucleotides upstream of the ORF 5 stop codon to the end of ORF 7 (Haynes et al., 1997). Briefly, cells on 8-well chamber slides and tissue sections were used to perform ISH as described earlier (Haynes et al., 1997). The hybridization signal was detected using (1:400) anti-fluorescein alkaline phosphatase antibody (Boehringer Mannheim) and incubated with 45 µl (100 mg/ml) NBT (4-nitro blue tetrazolium, Boehringer Mannheim) and 35 µl (50 mg/ml) BCIP (5-bromo-4-chloro-3-indolyl phosphate, Boehringer Mannheim). Controls included mock-infected ATCC CRL11171 cells and RNase-treated ATCC CRL11171 cells infected with PRRSV. These slides were subjected to TUNEL as described above. PRRSV-infected cells in the virus-inoculated monolayers of CRL11171 cells had purple cytoplasmic staining, whereas apoptotic cells had brown-staining nuclei (Fig. 3a). There were several apoptotic cells and apoptotic bodies scattered throughout the slides (Fig. 3a). In contrast, cells in the mock-infected monolayer had no nuclear or cytoplasmic staining (Fig. 3b). The number of both PRRSV-positive and apoptotic cells increased with time. Most of the labelled cells were either positive for PRRSV nucleic acid or apoptosis, but not both. Only a few cells were positive for both PRRSV nucleic acid and TUNEL. However, the TUNEL-positive particles in the cytoplasm were apoptotic bodies from the neighbouring cells rather than the nucleus of the cells (Fig. 3a).

To determine whether PRRSV-induced apoptosis occurred in vivo, 24 5-week-old, caesarean-derived, colostrum-deprived pigs were randomly divided into PRRSV-infected and mock-infected groups of 12 pigs each. Pigs in the principle group were inoculated intranasally with 10^{8.5} TCID_{50} ATCC VR-2385. The negative control pigs were mock-infected with normal cell culture fluids. Two pigs from each group were necropsied at 1, 3 and 5 days p.i., and six pigs from each group were necropsied at 10 days p.i. Tissues were taken from all lung lobes, and mediastinal and tracheobronchial lymph nodes. All tissues were fixed in 10% buffered formalin for at least 24 h and processed and embedded in paraffin.

Lung sections from the PRRSV-inoculated pigs showed moderate multifocal interstitial pneumonia at 3 and 5 days p.i., which progressed to severe diffuse interstitial pneumonia at 10 days p.i. The interstitial pneumonia was characterized by septal infiltration with mixed mononuclear cells, hypertrophy and hyperplasia of type II pneumocytes, and accumulation of normal and necrotic macrophages in alveolar spaces. Lung sections from the mock-infected pigs were normal. Lymph node sections from PRRSV-inoculated pigs were hyperplastic compared to those from mock-infected control pigs. By days 3 and 5 p.i., PRRSV-induced lymphadenopathy was characterized by lymphoblastic follicular hyperplasia, swollen and vacuolated follicular macrophages and dendritic cells, and mononuclear cell infiltration in the subcapsular region. At 10 days p.i., lymphadenopathy was characterized by follicular necrosis and an increase in number of tingible body macrophages.

A double-labelling experiment was performed as described above using lung and lymph node tissues. The cells positive for viral nucleic acid had purple staining localized in the cytoplasm (Fig. 3c). The viral nucleic acid-positive cells were primarily alveolar macrophages (Fig. 3c) with fewer numbers of mononuclear cells in the alveolar septa. The viral nucleic acid-positive cells were detected as early as 1 day p.i. and the number of positive cells increased with time until 10 days p.i. The TUNEL-positive cells had a brown-staining nucleus (Fig. 3c, d). The TUNEL-positive cells were alveolar macrophages (Fig. 3d), and mononuclear cells which were morphologically consistent with pulmonary intravascular macrophages (Fig. 3c). The other TUNEL-positive cells were small mononuclear cells consistent with lymphocytes in the alveolar septa (data not shown). Lung sections from mock-infected pigs were negative for viral antigen and nucleic acids (Fig. 3e). The TUNEL-positive cells were also detected as early as 1 day p.i. and peaked at 10 days p.i. In lung sections, cells were either positive for PRRSV or apoptosis (Fig. 3c). There were a few areas in which the virus-positive alveolar macrophages were seen next to the apoptotic alveolar macrophages.

In lymph nodes, PRRSV nucleic acid-positive cells in the germinal centres resembled tingible body macrophages (Fig. 3f). Positive cells in the paracortical areas were either macrophages or interdigitating dendritic cells (data not shown).
The PRRSV nucleic acid-positive cells in the lymph nodes were detected as early as 1 day p.i. and peaked at 10 days p.i. The apoptotic cells in the lymph nodes were detected as early as 1 day p.i. and peaked at 10 days p.i. The TUNEL-positive cells were mostly tingible body macrophages and small mononuclear cells (Fig. 3g). Lymph node sections from mock-inoculated pigs were negative for viral nucleic acids (Fig. 3h). The viral nucleic acid-positive cells in both lungs and lymph nodes were multifocal in distribution. In contrast to viral nucleic acid-positive cells, the TUNEL-positive cells were scattered throughout the sections. In the lymph nodes, we also found that labelled cells were positive for either PRRSV nucleic
acid or apoptosis (Fig. 3f). There were a few cells in lymph nodes, especially tingible body macrophages, which were positive for both PRRSV nucleic acid and apoptosis (Fig. 3f). Evidence of apoptosis by TUNEL was present in tingible bodies in the cytoplasm rather than the nucleus of the double-positive tingible body macrophages. The double-labelling experiment using TUNEL and immunohistochemistry for PRRSV nucleocapsid protein was performed and showed the same results as the double-labelling experiment using TUNEL and ISH (data not shown).

Several viruses have been shown to induce apoptosis either directly (Lu et al., 1996; Suarez et al., 1996; Yamada et al., 1994; Zhuang et al., 1995) or indirectly (Finkel et al., 1995; Godfraind et al., 1995; Momoi et al., 1996; Ramiro-Ibáñez et al., 1996). This study demonstrates that the virulent US PRRSV strain ATCC VR2385 induces apoptosis both in vitro and in vivo in the lungs and lymph nodes of PRRSV-infected pigs. Moreover, the US PRRSV strain ATCC VR2385 induced apoptosis in the continuous cell line much earlier than that reported for the European PRRSV isolate. The nucleosomal-sized DNA fragments were detected 2 days p.i. with PRRSV strain VR2385 compared to 5 days p.i. with LV (Suarez et al., 1996). Whether this difference is related to the higher virulence of VR2385 is not known at this time. The VR2385 strain has been shown to produce more severe pneumonia and replicate to a higher titre in pigs than LV (Halbur et al., 1996). Another major finding was that most of the apoptotic cells in both in vitro and in vivo studies were uninfected bystander cells. PRRSV appeared to kill infected cells by cell lysis in vitro and induced apoptosis in uninfected bystander cells. The only double-positive cells in vivo were a few tingible body macrophages in the lymph nodes. However, the tingible bodies in the cytoplasm but not the nuclei of these macrophages were positive by TUNEL. Tingible bodies are believed to be the nuclear fragments of dying lymphocytes or plasma cells that are phagocytized by resident macrophages in the follicles (Hamilton, 1956; Swartzendruber & Congdon, 1963).

A large number of alveolar macrophages in the lungs and mononuclear cells in the lungs and lymph nodes were destroyed by apoptosis during the course of infection. The ability of PRRSV to induce apoptosis in macrophages and mononuclear cells might be the reason that PRRSV-infected pigs have a dramatic reduction in the numbers of the alveolar macrophages (Molitor et al., 1992) and circulating lymphocytes and monocytes (Christianson et al., 1992b; Zhou et al., 1992). This may in part explain why PRRSV-infected pigs appear to be prone to secondary infections with both bacteria (Galina et al., 1994; Kawashima et al., 1996; Done et al., 1995) and viruses (Groschup et al., 1993; Van Reeth et al., 1996).

At this time, the mechanism of PRRSV-induced apoptosis
in bystander cells is not known either in vitro or in vivo. Macrophages are capable of producing several mediators of apoptosis such as tumour necrosis factor α (Kizaki et al., 1993; Kolesnick et al., 1994), nitric oxide (Cui et al., 1994; Messmer et al., 1994), reactive oxygen species (Martin et al., 1993; Hansson et al., 1996) and interleukin (IL)-1 β (Kolesnick et al., 1994; Onozaki et al., 1985). Moreover, activated macrophages and lymphocytes are capable of expressing Fas and Fas ligand and become sensitive to Fas-mediated apoptosis (Eischen & Leibson, 1997). However, the alveolar macrophages from pigs infected with a US isolate of PRRSV have also been shown to express high levels of IL-1 β (Zhou et al., 1992), which may play a role in apoptosis induction in vivo. Interestingly, the pathological changes in the lungs and lymph nodes are often diffuse and a large number of apoptotic cells are observed diffusely in both lungs and lymph nodes. This is in contrast to the multifocal distribution of PRRSV antigen or nucleic acids in infected cells in these tissues. This suggests that cytokines, especially IL-1 β, reactive oxygen species or nitric oxide might have a role in PRRSV pathogenesis. Therefore, PRRSV-induced apoptosis in bystander cells might be the mechanism for amplifying the CPE of PRRSV. Differences in virulence of PRRSV isolates have also been demonstrated (Halbur et al., 1996). Since the virulence of PRRSV varies considerably among isolates, it is possible that the ability of PRRSV to induce apoptosis in bystander cells may be related to virus virulence. Whether release of IL-1 β or other cytokines is responsible for inducing apoptosis in PRRSV-infected pigs, and whether there is any correlation between virus virulence and the ability of PRRSV isolates to induce apoptosis need further study.

We would like to thank Drs Harley W. Moon and Lyle D. Miller for reviewing this article. This study was supported with a grant from the Iowa Livestock Health Advisory Council. The research described herein was performed at the College of Veterinary Medicine, Iowa State University, USA. and with a King’s scholarship from the Anandhamahidol Foundation, Thailand.

References


Received 23 January 1998; Accepted 11 August 1998