Apoptosis in the Lungs of Pigs During an Infection with a European Strain of Porcine Reproductive and Respiratory Syndrome Virus

G.G. LABARQUE, H.J. NAUWYNCK, K. VAN REETH, AND M.B. PENSAERT

Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

1. INTRODUCTION

It has been shown, *in vitro*, that porcine reproductive and respiratory syndrome virus (PRRSV) induces apoptosis during its replication in both MA-104 cells and porcine alveolar macrophages (Suarez *et al.*, 1996). Since transfection of mammalian cells with ORF5 of PRRSV leads to apoptosis it was suggested that the 25-kDa glycosylated membrane protein GP5 of PRRSV is responsible for this phenomenon. Apoptosis has also been demonstrated *in vivo* with North-American strains of PRRSV (Sur *et al.*, 1997, 1998; Sirinarumitr *et al.*, 1998). Apoptotic cells were localized in germinal epithelial cells of testicles (Sur *et al.*, 1997), in lungs and in lymphoid tissues of pigs (Sirinarumitr *et al.*, 1998; Sur *et al.*, 1998). The apoptotic cells were morphologically recognized as alveolar and pulmonary intravascular macrophages and mononuclear cells in the alveolar septa in lungs and as macrophages and mononuclear cells in lymph nodes. Apoptotic cells were more abundant than PRRSV-infected cells in all tissues and double-labeling experiments indicated that the majority of apoptotic cells were uninfected bystander cells.

In this study, apoptosis in lungs and bronchoalveolar lavage (BAL) cells was investigated with a European strain of PRRSV (Lelystad virus) and it

The Nidoviruses (Coronaviruses and Arteriviruses).
Edited by Ehud Lavi *et al.*, Kluwer Academic/Plenum Publishers, 2001.

was questioned whether apoptotic cells are virus-infected or not. The apoptotic cells were further phenotypically characterized using the monoclonal antibody (MAb) 41D3 (Duan *et al.*, 1998). This MAb reacts specifically with the 210-kDa putative PRRSV receptor, which is present on well-differentiated lung macrophages but not on peripheral blood mononuclear cells. Also, an attempt was made to clarify a possible role of apoptosis in the reduction of the population of differentiated macrophages during the first two weeks of a PRRSV infection (Labarque *et al.*, 2000).

2. MATERIALS AND METHODS

2.1 Virus strain

PRRSV (Lelystad) (Wensvoort *et al.*, 1991) was used in the present study. Virus used for inoculation was at the fifth passage in pulmonary alveolar macrophages (PAMs) from four- to six-week-old gnotobiotic pigs.

2.2 Pigs and inoculation

A total of twenty-four caesarean-derived colostrum-deprived (CDCD) pigs were used. They were housed in isolation facilities. Twenty-two pigs were intranasally inoculated at the age of 4 to 5 weeks with $10^{6.0}$ $TCID_{50}$ Lelystad virus in 3ml phosphate buffered saline (PBS) (1.5 ml in each nostril). The remaining two pigs were left uninoculated and served as negative controls. One to three of the PRRSV-inoculated pigs were euthanatized at 1, 3, 5, 7, 9, 14, 20, 25, 30, 35, 40 and 52 days post inoculation (PI) by intraperitoneal injection with an overdose of barbiturates (Natriumpentobarbital® 20%, IC KELA).

The control pigs were euthanatized at 4 and 5 weeks of age. The right lung was used for bronchoalveolar lavage and samples from the left lung lobes were collected for virological examinations and detection of apoptosis.

2.3 Collection of samples

The right lung was lavaged with 60 to 120 ml of Dulbecco's PBS without Ca^{2+} and Mg^{2+} via an 18-gauge blunt needle inserted through the trachea. The left main bronchus was cross-clamped to prevent lung lavage fluid from entering the left lung. About 75 to 90% of the initial volume of the lavage fluid was recovered. The BAL fluid was centrifuged (400xg, 10 minutes, 4°C) to separate the cells and the cell-free lavage fluid. Cell pellets were

resuspended in PBS and the total number of BAL cells was determined. Cytocentrifuge preparations of BAL cells were made by centrifuging at 140xg for 5 minutes. One preparation was fixed in 4% paraformaldehyde for 10 minutes at room temperature for detection of apoptosis and another was fixed in acetone for 20 minutes at –20°C to determine the percentage of infected cells using a streptavidin-biotin immunofluorescence technique, as described by Labarque *et al.* (2000).
Tissue samples from the left lung lobes were embedded in methylcellulose medium and frozen at –70°C. Cryostat sections (5 to 8 μm) were made and fixed in 4% paraformaldehyde for 10 minutes at room temperature for detection of apoptosis and in acetone for 20 minutes at –20°C for quantitation of PRRSV-infected cells.

2.4 Detection of apoptosis

In order to detect apoptosis, cytocentrifuge preparations of BAL cells and cryostat sections of lung tissue were processed for enzyme terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) using an In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, cytocentrifuge preparations and cryostat sections were treated with Triton X-100 (0.1%) at 4°C for two minutes. Then, the cytocentrifuge preparations and cryostat sections were subjected to an enzymatic incorporation of digoxygenin-labeled nucleotide with TdT. Finally, the preparations were washed with PBS, mounted in a glycerin-PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazabicyclo(2.2.2) octane (DABCO) (Janssen Chimica) and TUNEL-positive cells were detected and counted by fluorescence microscopy (Leica DM RBE, Wild Leitz).

2.5 Double-labeling experiments

A first double-labeling experiment was conducted to determine whether the apoptotic cells were PRRSV-infected or not. Briefly, cytocentrifuge preparations and cryostat sections were treated with Triton X-100 (0.1%) at 4°C for two minutes. The cytocentrifuge preparations and cryostat sections were first incubated with a pool of MAbs against the PRRSV nucleocapsid protein (dilution 1/100 of WBE1 and WBE4-6) (Drew *et al.*, 1995). Subsequently, the preparations were subjected to an enzymatic incorporation of digoxygenin-labeled nucleotide with TdT and incubated with 1/100 goat anti-mouse TexasRed (Amersham). Finally, the preparations were washed with PBS, mounted in DABCO and TUNEL-positive and/or PRRSV-infected cells were detected and counted by fluorescence microscopy (Leica DM RBE, Wild Leitz).

A second double-labeling experiment was conducted to determine whether the apoptotic cells were 41D3-positive cells. Briefly, cytocentrifuge preparations and cryostat sections were treated with Triton X-100 (0.1%) at 4°C for two minutes. The cytocentrifuge preparations and cryostat sections were first incubated with MAb 41D3 (dilution 1/100) (Duan *et al.*, 1998). Subsequently, the preparations were subjected to an enzymatic incorporation of digoxygenin-labeled nucleotide with TdT and incubated with 1/100 goat anti-mouse TexasRed (Amersham). Finally, the preparations were washed with PBS, mounted in DABCO and TUNEL-positive and/or 41D3-positive cells were detected and counted by fluorescence microscopy (Leica DM RBE, Wild Leitz).

3. RESULTS

The evolutions of the number of viral antigen- and TUNEL-positive cells in lung tissue and BAL cells throughout a PRRSV infection are presented in Figures 1 and 2.

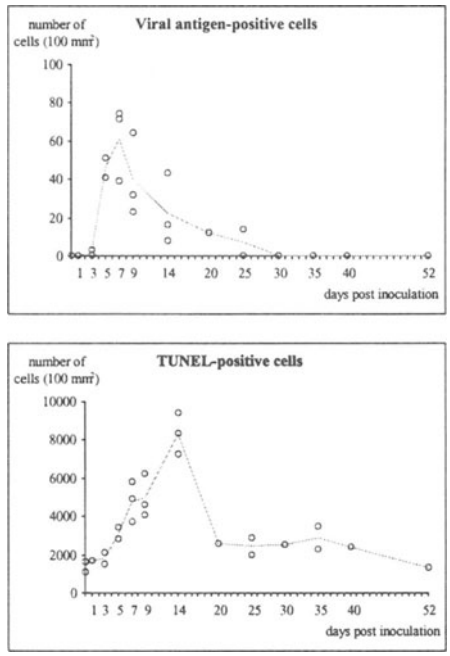

Figure 1. Evolution of the number of viral antigen-positive cells and the number of TUNEL-positive cells in lung tissue (100 mm^2) throughout a PRRSV infection.

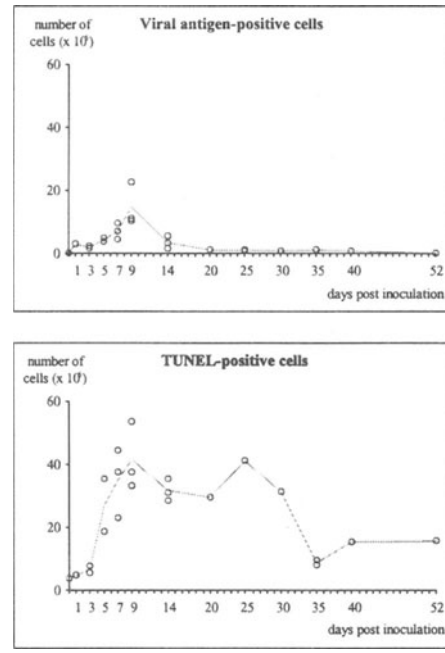

Figure 2. Evolution of the number of viral antigen-positive BAL cells and the number of TUNEL-positive BAL cells ($x10^6$) throughout a PRRSV infection.

Each point represents the individual value of one euthanized pig.
The continuous line represents the mean of the individual values at each time point.

Viral antigen-positive cells in lung tissue were observed from 3 until 25 days PI with a maximal number of 61 cells /100 mm^2 lung tissue at 7 days PI. PRRSV-infected BAL cells were first observed at 1 day PI (3 $x10^6$ cells), increased to a maximum of 15 $x10^6$ cells at 9 days PI, decreased to 3 $x10^6$ cells at 14 days PI and remained at levels of 0.5-1 $x10^6$ cells until 40 days PI. Viral antigen-positive cells were not observed in lung tissue and BAL cells of uninoculated control pigs.

TUNEL-positive cells were detected in lung tissue and BAL cells of both uninoculated and PRRSV-inoculated pigs. In uninoculated pigs, the mean number was 1350 cells /100 mm^2 lung tissue and 3.6 $x10^6$ in the BAL cell population. In PRRSV-infected pigs, the mean number in lung tissue was normal until 3 days PI, increased thereafter to a 5-fold at 14 days PI and sharply decreased to numbers similar to those of the uninoculated controls starting at 20 days PI. In BAL cells, the mean number was similar to that of the uninoculated controls during the first 3 days PI. Mean numbers increased from 6.6 $x10^6$ at 3 days PI to 41.4 $x10^6$ at 9 days PI and then remained at high levels until 30 days PI with numbers ranging between 29.5 and 41.2 $x10^6$. From 30 days PI, mean numbers dropped to numbers similar to those of the uninoculated control pigs. One pig, euthanatized at 25 days PI, had an extreme high number of TUNEL-positive BAL cells (85 $x10^6$). This value was not included in the calculation of the mean value.

Double-labeling experiments revealed that the majority of TUNEL-positive cells were uninfected cells. The mean percentages of PRRSV-infected cells, which were TUNEL-positive, ranged from 20 to 34% in lung tissue and from 21 to 25% in the BAL cell population between 5 and 14 days PI.

Double-labeling experiments with MAb 41D3 revealed that 74 to 85% of the apoptotic cells were expressing the putative PRRSV receptor on their cell membrane.

4. DISCUSSION

In the present study, it was shown that apoptosis occurs in lungs during a PRRSV infection in both infected and uninfected bystander cells.

A general feature of apoptotic cell death is that it does not induce severe inflammation and massive neutrophil infiltration into the lungs. This may explain why only a very mild lung inflammation is present during an infection with PRRSV (Lelystad virus) (Pol *et al.*, 1991) and why only low percentages of neutrophils are present in BAL fluids (Van Reeth *et al.,* 1999 ; Labarque *et al.*, 2000). Further, apoptosis of virus-infected cells may be an efficient mechanism by which the virus escapes from humoral immunity because progeny virus, which is present in membrane-bound apoptotic

bodies, can be taken up by neighbouring cells while protected from antibodies, favouring persistence of the virus in its host. This phenomenon has already been demonstrated for chicken anaemia virus (Jeurissen *et al.*, 1992). PRRSV may use this type of immune-evasion to persist in the respiratory tract until 40 (Labarque *et al.*, 2000) to 49 days after inoculation (Mengeling *et al.*, 1995) despite the presence of antibodies in sera and BAL fluids from 9 days PI (Labarque *et al.*, 2000).

PRRSV causes a reduction of the population of 41D3-positive well-differentiated lung macrophages during the first two weeks of infection (Labarque *et al.*, 2000). The reduction of this cell population can be largely attributed to cell lysis due to virus replication and apoptosis. The highest number of PRRSV-infected cells in lungs and BAL fluids was indeed detected between 3 and 14 days PI and a marked increase of apoptosis was demonstrated during the same time period in both lung tissue and BAL cells.

A relation was found between the peak of virus replication and the onset of apoptosis. The basis for the relation could be the production of a molecule that causes apoptosis. This can be a viral antigen, such as GP5 which has already been associated with the induction of apoptosis (Suarez *et al.*, 1996) or a product induced by the virus infection, such as Interleukine-1 (IL-1). IL-1 has already been demonstrated in BAL fluids of PRRSV-inoculated pigs starting from 3 days PI (Van Reeth *et al.*, 1999). Whether release of IL-1 is responsible for or coincides with the induction of apoptosis in the lungs of PRRSV-infected pigs needs further examination.

In lung tissue, the number of apoptotic cells dropped to a normal level starting from 14 days PI, while the number of apoptotic BAL cells remained at a rather high level until 30 days PI. The earlier time point at which the number of apoptotic cells dropped to normal values in lung tissue may be partly explained by the more rapid clearance of virus.

ACKNOWLEDGMENTS

The authors would like to thank Fernand De Backer, Tini De Lausnay, Chris Bracke and Lieve Sys for their excellent technical assistance. The authors would also like to thank Dr. T. Drew for his kind gift of the monoclonal antibodies WBE1 and WBE4-6 and Dr. G. Wensvoort for the supply of the Lelystad isolate of PRRSV.

REFERENCES

Drew, T.W., Meulenberg, J.J.M., Sands, J.J., and Paton, D.J., 1995, *J. Gen. Virol.*, **76**: 1361-1369.

Duan, X., Nauwynck, H.J., Favoreel, H.W., and Pensaert, M.B., 1998, *J. Virol.*, **72**: 4520-4523.

Jeurissen, S.H., Wagenaar, F., Pol, J.M., van der Eb, A.J., and Noteborn, M.H., 1992, *J. Virol.*, **66**: 7383-7388.

Labarque, G.G., Nauwynck, H.J., Van Reeth, K., and Pensaert, M.B., 2000, *J. Gen. Virol.*, **81**: 1327-1334.

Mengeling, W.L., Lager, K.M., and Vorwald, A.C., 1995, *J. Vet. Diagn. Invest.*, **7**: 3-16.

Pol, J.M.A., van Dijk, J.E., Wensvoort, G., and Terpstra, C., 1991, *Vet. Q.*, **13(3)**: 137-143.

Sirinarumitr, T., Zhang, Y., Kluge, J.P., Halbur, P.G., and Paul, P.S., 1998, *J. Gen. Virol.*, **79**: 2989-2995.

Suarez, P., Diaz-Guerra, M., Prieto, C., Esteban, M., Castro, J.M., Nieto, A., and Ortin, J., 1996, *J. Virol.*, **70(5)**: 2876-2882.

Sur, J.-H., Doster, A.R., Christian, J.S., Galeota, J.A., Wills, R.W., Zimmerman, J.J., and Osorio, F.A., 1997, *J. Virol.*, **71(12)**: 9170-9179.

Sur, J.-H., Doster, A.R., and Osorio, F.A., 1998, *Vet. Pathol.*, **35**: 506-514.

Van Reeth, K., Labarque, G., Nauwynck, H., and Pensaert, M., 1999, *Res. Vet. Sci.*, **67**: 47-52.

Wensvoort, G., Terpstra, C., Pol, J.M.A., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J.M., Moonen, P.L.J.M., Zetstra, T., de Boer, E.A., Tibben, H.J., de Jong, M.F., van 't Veld, P., Groenland, G.J.R., van Gennep, J.A., Voets, M.T., Verheijden, J.H.M., and Braamskamp, J., 1991, *Vet. Q.*, **13**: 121-130.

Participants of the VIIIth International Symposium of Nidoviruses.