Apoptosis in lymphoid organs of pigs naturally infected by porcine circovirus type 2

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The objective of the present study was to evaluate the involvement of apoptosis in the development of post-weaning multisystemic wasting syndrome (PMWS) lymphoid-depletion lesions. Twenty-one pigs that were categorized into three different lesional severity stages (S1, n = 5; S2, n = 7; S3, n = 9) and five healthy control pigs (stage S0) were used. From all pigs, samples of thymus, spleen, tonsil, ileum and superficial inguinal lymph node were processed for histological examination, in situ hybridization for porcine circovirus type 2 (PCV2) detection and cleaved caspase-3 (CCasp3) immunohistochemistry for detection of apoptotic cells.

PCV2 was quantified in serum samples by using TaqMan real-time PCR. CCasp3 labelling was measured in the different morphological compartments of all lymphoid tissues, using an automated system for quantification. Differences between each tissue compartment and lesional stage were assessed, as well as the correlation between apoptosis, lesional stage and viral load. Overall, the results indicated that the more intense the lymphoid depletion, the lower the rate of apoptosis. In the thymus, the cortex was the area where differences between PMWS-affected and control animals were more evident; it was found that all PMWS-affected pigs had significantly lower rates of apoptosis than the controls. In the secondary lymphoid organs, B-cell areas presented higher rates of apoptosis; similar apoptotic rates were found in this compartment in control and S1 pigs. In S2 and S3, B-cell areas were lost and the apoptotic pattern observed was a diffusely distributed low rate of positive cells. Significantly lower rates of apoptosis between PMWS-affected pigs and the control group were already evident in S1 for the thymus, spleen, superficial inguinal lymph node and Peyer’s patches, but not for the tonsils. Apoptotic rates in lymphoid tissues were correlated inversely with viral load in serum and with severity of lesions. In conclusion, the results indicate that apoptosis is not a remarkable feature in PMWS lymphoid lesion development.

INTRODUCTION

Post-weaning multisystemic wasting syndrome (PMWS) is a disease of nursery and fattening pigs that is associated with porcine circovirus type 2 (PCV2) infection (Allan et al., 1999; Kennedy et al., 2000; Bolin et al., 2001; Krakowka et al., 2001). Clinical signs of this syndrome are progressive weight loss, paleness, dyspnoea and, occasionally, diarrhoea and icterus. Histopathological findings include histiocytic infiltration and lymphocyte depletion of lymphoid tissues, interstitial pneumonia and, less frequently, hepatitis and nephritis (Rosell et al., 1999, 2000; Bolin et al., 2001; Segalés & Domingo, 2002; Darwich et al., 2003a, b; Kim & Chae, 2003). Experimental reproduction of PMWS has hardly been achieved by using PCV2 alone, infectious DNA clones or a pure form of PCV2 derived from infectious DNA clones (Allan et al., 1999; Albina et al., 2001; Bolin et al., 2001) and it is therefore assumed that PMWS is a multifactorial disease (Allan et al., 1999; Krakowka et al., 2001; Rovira et al., 2002).

Histological and haematological findings and cytokine profiles found in PMWS-affected pigs suggest that an immune system dysfunction, modulated by PCV2, underlies the pathogenesis of PMWS (Darwich et al., 2004). The main features of PMWS are T- and B-lymphocyte depletion in lymphoid tissues, peripheral blood lymphopenia and a specific downshift of B cells, CD4+CD8−, CD4+CD8−, CD4−CD8+, γδTCR+ T cells and natural killer cells (Darwich et al., 2002, 2003b; Chianini et al., 2003; Nielsen et al., 2003). Also, cytokine mRNA expression is altered in several lymphoid organs (Darwich et al., 2003a). It is speculated that viral load could be associated with the intensity of lymphocyte depletion and PMWS outcome (Liu et al., 2000; Rovira et al., 2002). Nevertheless, the mechanisms that lead to these alterations are not yet known.

Virus-induced apoptosis can contribute significantly to cytotoxicity, with the advantage of minimizing the host immune response (O’Brien, 1998); thus, this mechanism
can contribute to a successful viral host infection. Lymphocyte depletion can be induced in viral infections through apoptosis. For example, human immunodeficiency virus (HIV) uses apoptosis specifically for CD4+ T-cell depletion (O’Brien, 1998; Hay & Kannourakis, 2002). Apoptosis has been proposed as the mechanism that is responsible for B-cell depletion in naturally PMWS-affected pigs (Shibahara et al., 2000). However, this is not clear, as a recent report gave contradictory results (Mandrioli et al., 2004).

One of the best approaches to detecting apoptosis in paraffin-embedded material, particularly when tissues are of a variety of sources and have been subjected to different processing treatments, is an immunoperoxidase method for active detection of effector caspases (Huppertz et al., 1999; Dukers et al., 2002); this is also valid for porcine lymphoid tissue (Resendes et al., 2004). Caspase-3 is an effector caspase and its activation represents a 'point of no return' in the apoptotic pathway; therefore, its activation can be considered as a good marker for apoptosis (Hengartner, 2000).

The objective of this study was to determine rates of apoptosis in the thymus and several peripheral lymphoid organs of naturally PCV2-infected pigs with different lesional stages, and to correlate these rates with the level of viraemia in infected pigs.

**METHODS**

**Animals.** Study cases were 21 conventional pigs, 2–3-5 months old with a variable degree of growth retardation, that were selected from three high-health farms that were suffering from PMWS outbreaks. All pigs were positive serologically for PCV2 by immunoperoxidase monolayer assay (IPMA) (Rodríguez-Arrioja et al., 2000), with titres ranging from 1/320 to 1/20,480, and the PCV2 genome was detected in their serum by PCR (Quintana et al., 2002). The three farms were free of the most common pathogens that affect swine, including porcine reproductive and respiratory syndrome virus, Aujeszky disease virus, porcine parvovirus, swine influenza virus and Mycoplasma hyopneumoniae, as confirmed by serology. In addition, five healthy conventional pigs were selected from another farm that was free from PMWS and were seronegative for the above-mentioned pathogens. Selected pigs were negative for PCV2 by IPMA and PCR in serum.

**Categorization of animals in lesional stages.** Shortly after euthanasia by intravenous pentobarbital overdose, lymphoid tissue samples (spleen, thymus, tonsil, ileum and superficial inguinal lymph node) were collected from each pig and fixed in 10% neutral-buffered formalin for approximately 24 h. Afterwards, samples were dehydrated, paraffin-embedded, sectioned at 4 μm and stained with haematoxylin and eosin for histopathological examination. *In situ* hybridization to detect the PCV2 genome was performed in lymphoid tissues as described previously (Rosell et al., 1999; Kennedy et al., 2000). The positive control was a lymph node with PMWS histological lesions that was positive for PCV2 by PCR detection; the negative control was a histologically normal lymph node obtained from a pig that was negative for PCV2 by PCR. In lymphoid tissues from the study cases, the grade of lymphocyte depletion, histiocytic infiltration and amount of PCV2 DNA were scored according to Chianini et al. (2003). Pigs were classified into three lesional stages by considering the overall lymphoid depletion grade and the amount of PCV2 genome in lymphoid tissues. Lesional stage 1 (S1) included pigs with absent to mild PMWS lesions and low amounts of PCV2 in tissues (*n* = 5); S2 included pigs with moderate lymphoid lesions and moderate amounts of PCV2 in most tissues (*n* = 7); and S3 included pigs with severe lymphoid lesions and high amounts of PCV2 in most tissues (*n* = 9). Stage S0 comprised the pigs that were selected as controls, which had no lesions or PCV2 genome in lymphoid tissues.

**TaqMan real-time PCR.** PCV2 genomic load was quantified in serum samples by using the methodology described previously (Olvera et al., 2004).

**Cleaved caspase-3 (CCasp3) immunohistochemistry to detect apoptosis.** CCasp3 was investigated in paraffin wax-embedded tissues by the avidin–biotin peroxidase (ABC) method, as reported previously (Resendes et al., 2004). Briefly, 4 μm correlative tissue sections were placed on silane-coated slides [3-(triethoxysilyl)-propylamine]; then, they were dewaxed in xylene, rehydrated in graded alcohols and placed in dH2O. Afterwards, antigen retrieval was done by immersion of sections in 0.01 M citrate buffer (pH 6.0) in a steam bath at 98°C for 25 min, followed by rapid cooling over 20 min. After blocking endogenous peroxidase with 3% H2O2 in dH2O (30 min) and washing with 0.1 M Tris-buffered saline (TBS, pH 7.4), non-specific binding was blocked with 2% BSA (Sigma) for 30 min at room temperature. The anti-CCasp3 antibody used was an activation-specific polyclonal antibody (anti-Asp175; Cell Signalling), which does not recognize the caspase-3 precursor form (procaspase-3), but only the active form (CCasp3). The antibody was diluted 1/50 in 2% BSA and incubated overnight at 4°C. After washing in TBS, sections were incubated with a biotinylated goat anti-rabbit antibody (1/200 in TBS) (Dako) for 60 min at room temperature and subsequently treated with the ABC complex (1/100 in TBS) (Pierce) for 1 h at room temperature. Finally, sections were incubated in 0.05% diaminobenzidine plus 3% H2O2 in TBS for 10 min, rinsed in dH2O, counterstained with haematoxylin, dehydrated and mounted with DPX (Panreac). A porcine liver section with apoptotic hepatocytes was used as the positive control; as a negative control, the primary antibody was replaced by an irrelevant antibody (isotype-matched control antibody).

**Apoptotic cell quantification.** Morphometrical analysis was carried out by taking the histological compartments of each lymphoid organ into account, as described previously (Resendes et al., 2004). In tonsil and superficial inguinal lymph node compartments, follicular and interfollicular areas were considered; in the thymus, cortex and medulla; and in the spleen, white and red pulp were considered. In Peyer’s patches, only follicles were quantified, as dome areas were scant and interfollicular areas were small and difficult to outline. CCasp3 immunopositivity was quantified automatically by using a microscope equipped with an image analyser program (VIELOG 5.3; Noesys 2000). The software was programmed to quantify positivity with approximate lymphocyte size and to exclude labelled nuclear ‘dust’ (free apoptotic bodies). Ten random fields of 0.035 mm² (approximately one field of ×400 magnification) of each compartment were quantified per tissue and pig. When tissues lost morphological compartmentalization due to lymphocyte depletion, only 10 random fields were counted per tissue and pig. Morphometric results from each compartment were grouped into three categories, according to the mean number of apoptotic cells per field (0.035 mm²): low (< 5 cells); moderate (> 5 to ≤ 10 cells); and high (> 10 cells) (Resendes et al., 2004).

**Statistical analysis.** For each organ, mean apoptotic rates were compared between different compartments and lesional stages (S0–S3) by the Poisson regression model (Kleinbaum & Klein, 2002). Mean PCV2 loads were compared between the three PMWS stages.
lesional groups (S1–S3) by the Kruskal–Wallis test. In addition, overall numerical apoptotic rates were correlated with viral load by a simple linear regression model, and with lesional stages by the Tukey–Kramer test. For each organ, numerical apoptotic rates were also correlated with viral load by using Pearson’s correlation model. In addition, categorized apoptotic rates were correlated with lesional stages by using the χ² test and r x c tables, and lesional stages were correlated with viral load by the Tukey–Kramer test. Statistical analysis was performed with the Stats Direct and SAS programs. Differences were considered to be significant when $P<0.05$.

RESULTS

Apoptosis in control pigs

Positive labelling was detected mainly in the nuclei of lymphocyte-like cells that, in most cases, had evident apoptotic nuclear morphology. Diffuse cytoplasmic labelling was also detected in some large, ovoid follicular cells, mostly without apoptotic nuclear morphology. Faintly labelled apoptotic bodies were often found in follicular areas (this positivity was not quantified). Tonsil, superficial inguinal lymph node and Peyer’s patch follicles had moderate to high rates of positive cells. In the interfollicular areas, low rates of labelled cells were found to have a scattered distribution. Moreover, some positive cells were detected in the tonsillar crypt epithelium and high amounts were often found in the crypt lumen. The follicular areas had significantly higher amounts of apoptotic cells than did interfollicular areas (Fig. 1c). In the dome and lamina propria from Peyer’s patches, low numbers of positive cells were detected. In spleen, moderate to high rates were found in the white pulp; the higher amounts were found to be associated with follicular structures in these areas. Red pulp presented significantly lower rates of labelled cells than did white pulp. In thymus, significantly higher rates of positive cells were detected in the cortex than in the medulla, which had low to moderate rates (Fig. 1a). Overall, this stage, which was characterized by normal lymphoid tissues (S0), was mainly associated with high rates of apoptosis. CCasp3 quantification and statistical results between areas for each organ are displayed in Fig. 2.

Apoptosis in pigs in S1

The cell types that were labelled in this lesional stage were mainly the same as those found in control tissues. In addition, diffuse cytoplasmic labelling was detected occasionally in some spindle-shaped cells and in histiocytic/macrophage-like cells that had no apoptotic nuclear morphology. In tonsils and Peyer’s patches, the distribution of immunolabelling was similar in all animals and similar to that in the control pigs. Moderate to high rates of positive cells were found in follicles, whilst in the interfollicular areas, low rates were predominant and apoptosis was seen to have a scattered distribution. Superficial inguinal lymph nodes usually had low rates of apoptotic cells with a scattered distribution. Follicles could be seen only in the lymph node of one pig that had moderate rates of CCasp3 positivity;
in the other samples, follicles were lost. Tonsil follicular areas had significantly higher amounts of apoptotic cells than did interfollicular areas. In the spleen, low rates of scattered positive cells were found in both white and red pulp areas. In the thymus, significantly higher rates of positive cells had a scattered distribution in the cortex.

Fig. 2. Morphometric results. Mean and SD of positive CCasp3 cells for each area and pig in tonsil (a); superficial inguinal lymph node (b); Peyer’s patches (c); spleen (d); and thymus (e) for groups S0–S3. ●, Follicular/white pulp/cortex; ○, interfollicular/red pulp/medulla; △, no compartmentalized lymphoid tissue. Statistical results are indicated as: n.s., not significant; *, P < 0.05; **, P < 0.001.
Apoptosis in pigs in S2

In this lesional stage, the labelled cells were of the same type as that found in S1; however, the pattern of labelling distribution in tonsils, Peyer’s patches and spleen was different. In these tissues, low to moderate rates of CCasp3-positive cells had a scattered distribution in a tissue that had lost compartmentalization as a consequence of lymphocyte depletion. Occasionally, when follicular structures were present, moderate rates of apoptosis were found in follicles and low rates were found in interfollicular areas. Necrotizing lymphadenitis was found in the inguinal superficial lymph node, the tonsils, and Peyer’s patches from two pigs. In these tissues, focal areas with high rates of positivity were detected (>30 cells per field; data not shown), whereas areas that were adjacent to necrotic foci had low rates of labelled cells. In the spleen, low rates of scattered positive cells were detected in the red pulp; white pulp was not present. In the thymus, low to moderate rates of immunolabelled cells had a scattered distribution in the cortex, whilst low rates were found in the medulla. Overall, apoptotic rates for each organ were significantly lower than those in the control group. Moreover, apoptotic rates for tonsils and Peyer’s patches were significantly lower than those from S1 pigs. In general, S2 lymphoid lesions were associated mainly with low rates of apoptosis. CCasp3 quantification and statistical results are shown in Fig. 2.

Apoptosis in pigs in S3

The cell types labelled and the pattern of labelling distribution in this lesional stage were as noted for S2. In all pigs, tonsils, superficial inguinal lymph nodes (Fig. 1d) and Peyer’s patches had low rates of positive cells with a scattered distribution in tissue that had lost compartmentalization. In the spleen and thymus (Fig. 1b), CCasp3 labelling distribution and rates were similar to those found in S2. One pig presented with necrotizing lymphadenitis, where high amounts of apoptotic cells were observed focally, as described previously, and purulent splenitis with high rates of positive cells (>30 cells per field; data not included in statistical analysis) that were distributed diffusely. Apoptotic rates from all tissues were significantly lower than those from the control group. When compared with S1, significantly lower rates of apoptosis were found for tonsils and Peyer’s patches. When compared with S2, significantly lower rates were found only for Peyer’s patches. Overall, in most lymphoid tissues, S3 lesions were mainly associated with low rates of apoptosis. CCasp3 quantification and statistical results are displayed in Fig. 2.

Viral load from each animal is shown in Fig. 3. In pigs, the mean ± SD from S1 (n=5/5) were 1·6×10⁵ ± 1·2×10⁵ genomes ml⁻¹; from S2 pigs (n=6/7), 3·3×10⁷ ± 5·1×10⁷ genomes ml⁻¹; and from S3 pigs (n=7/9), 1·3×10⁸ ± 9·9×10⁷ genomes ml⁻¹. Significant differences among viral loads were found only between groups S3 and S1.

Statistical results

Overall, apoptotic rates or raw apoptotic numerical values were higher in S0 and S1 animals (P<0·05); the values for S2 and S3 lesional stages were not significantly different. In addition, the higher the viral load, the lower the apoptotic rates (r=-0·56; 95% confidence interval, −0·70 to −0·40). This correlation was also seen for each particular organ (P<0·05), except for thymus and inguinal superficial lymph node. Thus, the correlation coefficients for regression between apoptotic rates and serum viral load were r=-0·82 for tonsil, r=-0·72 for spleen and r=-0·92 for Peyer’s patches. Also, serum viral load was not significantly different (P>0·05) at low and moderate rates of apoptosis. The higher the blood viral load, the higher the lesional stage (P<0·001).

DISCUSSION

Lymphocyte depletion is an important feature of PMWS-affected pigs (Darwich et al., 2003a, b, 2004; Nielsen et al., 2003; Segalés et al., 2004a) and it is well-known that these lesions could be caused by apoptosis (Janeway, 2001), among other causes. However, the involvement of apoptosis in the development of PMWS lymphocyte-depletion lesions is not clear, as the two main published studies have conflicting results (Shibahara et al., 2000; Mandrioli et al.,...
Our study demonstrates that apoptotic rates are lower in the thymus and most peripheral lymphoid tissues in naturally affected PMWS pigs, compared with healthy pigs. We also found that the more severe the PMWS lesional stage and the higher the PCV2 load in serum, the lower the apoptotic rate. These results indicate that lymphocyte apoptosis is not a prevalent phenomenon in the development of PMWS lymphoid-depletion lesions.

Taking into account other viral infections (Summerfield et al., 1998; Sato et al., 2000; Sánchez-Cordón et al., 2002), it appears that when apoptosis is implicated in the pathogenesis of lymphocyte-depletion lesions, its incidence is usually increased and can be demonstrated by immunohistochemical methods in lymphoid tissues. Moreover, it appears that higher rates of apoptosis are found during the acute phase of infection, when active virus replication occurs. For example, this is the case in classical swine fever virus (Summerfield et al., 1998; Sato et al., 2000; Sánchez-Cordón et al., 2002), African swine fever virus (Oura et al., 1998; Salguero et al., 2004) and chicken anemia virus (Jeuriissen et al., 1992) infections. Nevertheless, in our study, no increased apoptotic rates were found in any lymphocyte-depletion lesional stage, and they were inversely proportional to the viral load.

The present study included pigs with the full spectrum of lymphoid-depletion lesions and a range of PCV2 load in serum, which is likely to be found during PMWS (Rosell et al., 1998; Shibahara et al., 1999; Olvera et al., 2004). Thus, we believe that the acute phase of PMWS infection was well-represented within the study cases. If apoptosis was massive or increased in PMWS, it should, at least, have been found in S1 and S2 pigs, which could be considered to be in the initial or early phases of disease. On the other hand, the control pigs had the expected apoptotic rates for healthy conventional pigs at this age (Resendes et al., 2004), which, in all cases, were higher than for diseased pigs. Our observations contrast with those of Shibahara et al. (2000), who found increased apoptotic rates in B-cell areas from PMWS-affected pigs with respect to control pigs. Compared with what are described as normal apoptotic rates for healthy conventional pigs (Resendes et al., 2004), the apoptotic rates observed in control pigs in the study of Shibahara et al. (2000) could be categorized as low. In view of these facts, the high apoptotic rates found in PMWS-affected pigs from this study should be revised, considering the full range of apoptotic rates that can be found in normal lymphoid tissues from healthy conventional pigs. In agreement with Mandrioli et al. (2004), who used the TUNEL assay for detection of apoptosis, we found similar results by using CCasp3 immunolabelling: an inverse correlation between apoptotic rates and PMWS lesional severity. Furthermore, the scarce cellularity we found in S2 and S3 cases must, in part, account for the low apoptotic rates observed. Nevertheless, interpreting the apoptotic rates from S1 thymic cortex, which was only mildly depleted, it seems feasible that in addition to cell reduction, inhibited apoptosis may also coexist, accounting for low apoptotic rates. It is important to understand that this study does not exclude partial involvement of apoptosis in the pathogenesis of lymphocyte depletion in PMWS, as this phenomenon could affect small regulator lymphocyte subpopulations or precursor cells, as can happen in HIV infection (O’Brien, 1998).

In normal lymphoid tissues, apoptosis and proliferation are known to be related phenomena through which the total lymphocyte population is kept at a fairly constant level (Janeway, 2001; Rathmell & Thompson, 2002). Thus, in an indirect way, apoptosis can be interpreted as an indicator of proliferative activity. Our results suggest that lymphocyte proliferation could be inhibited in several lymphoid tissues of PMWS-affected pigs. This hypothesis was also suggested by Mandrioli et al. (2004), who found low ratios of a proliferation marker in the lymph nodes of PMWS-affected pigs. In addition, we believe that thymocyte proliferation may also be decreased in early stages of the disease. Apoptotic rates in B-cell follicles apparently tend to decrease when compared with those from healthy pigs. It is possible that the loss of B-cell areas in S2 and S3 may also be associated with decreased proliferation of those cells. Inhibited lymphocyte proliferation in PMWS pathogenesis is supported by other studies. For example, PMWS-affected pigs show thymic interleukin 10 (IL10) mRNA overexpression (Darwich et al., 2003b), which could inhibit thymocyte proliferation (Rouleau et al., 1999). It is remarkable that, in vitro, PCV2 is able to induce IL10 overproduction in infected peripheral blood mononuclear cells (Darwich et al., 2003a); thus, it is not surprising that PCV2 may inhibit thymocyte proliferation in vivo. In addition, low IL4 mRNA expression is found in peripheral lymphoid tissues from PMWS-affected pigs, which may induce B cells to cease proliferation (Darwich et al., 2003b). The relationship between apoptosis and inhibition of lymphocyte proliferation should be investigated further, as it may be relevant to the development of PMWS.

The high variability in apoptotic rates found in some tissues from S2 pigs could be explained by considering the wide variability in lesion severity that can exist in this lesional category. This stage is also characterized by high variability in the loads of PCV2 in serum (Olvera et al., 2004). The areas with high apoptotic rates that are found in necrotizing lymphadenitis and splenitis, which are respectively associated with necrotic foci and diffuse neutrophilic infiltration, are probably a consequence of an undetermined bacterial infection. Most of the pigs used in this study suffered from secondary bacterial infections, which occur frequently in PMWS (Segalés et al., 2004b).

In conclusion, considering the relationship found between PCV2 serum load, apoptotic rates and severity of lymphocyte depletion, it appears that apoptosis is not a remarkable feature in PMWS lesion development. Further investigation is needed in order to evaluate the implication of apoptosis and defective production of lymphocyte subpopulations.
and precursor cells, and to exclude the potential of PCV2-infected antigen-presenting cells to induce defective stimulation of lymphocytes. The implications for defective lymphocyte homing should also be assessed.

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REFERENCES


Rodríguez-Arrioja, G. M., Segalés, J., Balasch, M., Rosell, C., Quintant, J., Folch, J. M., Plana-Durán, J., Mankertz, A. &


