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Singular PCV2a or PCV2b infection results in apoptosis of hepatocytes in clinically affected gnotobiotic pigs

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ABSTRACT

Porcine circovirus type 2 (PCV2) is clinically associated with respiratory disease, failure-to-thrive, hepatitis, and diarrhea; however, the precise pathogenesis of PCV2-associated disease and in particular its involvement in apoptosis is still controversial. The objectives of this study were (1) to determine whether PCV2 is associated with apoptosis by examining and comparing hepatic tissues from clinically affected or unaffected gnotobiotic pigs that were experimentally infected with PCV2, (2) to determine if there are differences between PCV2a and PCV2b in inducing hepatocyte apoptosis, and (3) to determine if there are differences between apoptosis detection systems. Forty-eight gnotobiotic pigs were separated into five groups based on inoculation status and development of clinical disease: (1) sham-inoculated, clinically-unaffected ($n = 4$), (2) inoculated with PCV2a, clinically-unaffected ($n = 10$), (3) inoculated with PCV2a, clinically-affected ($n = 6$), (4) inoculated with PCV2b, clinically-unaffected, ($n = 13$) and (5) inoculated with PCV2b, clinically-affected ($n = 15$). Formalin-fixed, paraffin-embedded sections of liver from all pigs were analyzed for signs of apoptosis [presence of single strand DNA breaks in the nucleus by the terminal transferase dUTP nick end labeling (TUNEL) assay or presence of intra-nuclear cleaved caspase 3 (CCasp3) demonstrated by CCasp3 immunohistochemistry (IHC)]. In addition, the liver tissues were also tested for presence of cytoplasmic and intra-nuclear PCV2 antigen by an IHC assay. Specific CCasp3 and TUNEL labeling was detected in the nucleus of hepatocytes in PCV2a and PCV2b infected pigs with significantly ($P < 0.05$) higher levels of apoptotic cells in clinically-affected pigs. Regardless of PCV2 subtype (PCV2a; PCV2b), there were higher levels of PCV2 antigen in clinically-affected pigs compared to clinically-unaffected pigs. There was no significant difference in detection rate of apoptotic cells between the TUNEL assay and CCasp3 IHC. When high amounts of PCV2 antigen were present, the incidence of CCasp3 and TUNEL staining also increased regardless of the PCV2 genotype. This suggests that PCV2-induced apoptosis of hepatocytes is important in the pathogenesis of PCV2-associated lesions and disease.

1. Introduction

Porcine circovirus (PCV) is a small, non-enveloped, circular, single stranded DNA virus. There are two main types of PCV defined as PCV1 and PCV2 (Meehan et al., 1998). Phylogenetic analysis of PCV2 isolates has shown that PCV2 can be further divided into two main genotypes, PCV2a and PCV2b, which are both distributed worldwide (Gagnon et al., 2007; Olvera et al., 2007).

Initially, PCV2 was identified in cases of post-weaning multisystemic wasting syndrome (PMWS) in Canada in 1991 (Ellis et al., 1998; Harding and Clark, 1997). PCV2 is associated with several disease manifestations in pigs commonly summarized as porcine circovirus associated disease (PCVAD) (Opriessnig et al., 2007). PCVAD includes systemic infection in nursery age pigs commonly characterized by wasting and general failure to thrive (Chae, 2004), hepatic disease (Rosell et al., 2000a), respiratory disease (Harms et al., 2002), enteric disease (Kim et al., 2004), reproductive failure (Ladekjær-Mikkelsen et al., 2001; O'Connor et al., 2001) and porcine dermatitis and nephropathy syndrome (PDNS) (Rosell et al., 2000b). PCVAD has since emerged globally (Segalés et al., 2005) and now has become one of the most economically important diseases throughout pig producing countries (Opriessnig et al., 2007).

Currently there are contradicting reports about whether or not PCV2 plays a vital role in inducing apoptosis in various pig tissues. Examples of viruses that induce apoptosis include: adenovirus, human immunodeficiency virus (HIV-1), porcine reproductive and respiratory syndrome virus (PRRSV), influenza virus A/B, reovirus,

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classical swine fever and many others (Roulston et al., 1999). Microscopically, apoptosis can be demonstrated by dUTP labeling of single strand DNA breaks in the nucleus (Krakowka et al., 2004) or by demonstrating the presence of cleaved caspase 3 (CCasp3) by immunohistochemistry (IHC) staining (Resendes et al., 2004a,b). A novel open reading frame (ORF3) was identified in PCV2 infected PK15 cells in 2005. It was discovered that the ORF3 protein is not essential for viral replication but is involved in activating caspase-8 and caspase-3 pathways which in turn induce apoptosis (Liu et al., 2005).

A study using naturally infected conventional pigs found that within lymphoid tissues, apoptosis of lymphocytes had occurred, which was determined by the use of the terminal transferase dUTP nick end labeling (TUNEL) stain (Shibahara et al., 2000). Within the same study PCV2 was stained via IHC and it was found that PCV2 was present in the nuclei of macrophages and apoptotic lymphocytes, as well as apoptotic bodies phagocytosed by macrophages, providing evidence that PCV2 was the cause of B lymphocyte depletion (Shibahara et al., 2000). Similarly in BALB/c mice experimentally inoculated with PCV2, it was found that PCV2 replicated in the spleen, lymph nodes and Peyer's patches of infected mice and that the replication of PCV2 within these tissues was associated with apoptosis which was confirmed by the TUNEL assay (Kiupel et al., 2005). It was also noted that upregulation of caspase 8 and 3 in the spleens of infected mice might be the mechanism through which PCV2 induces apoptosis in mice (Kiupel et al., 2005).

In contrast, a recent study using pigs naturally affected by PCVAD noted that there was an inverse relationship between amounts of PCV2 antigen and apoptosis in the thymus and most peripheral lymphoid tissues (Resendes et al., 2004b). It was also found that pigs with severe lymphoid depletion had less apoptosis (Resendes et al., 2004b). In Italy, pigs with varying clinical presentations of PMWS were classified into three classes based on the severity of the disease presentation (Mandrioli et al., 2004). Apoptosis in lymphoid tissues was analyzed and there was a significant decrease in apoptosis in lymphoid tissues when control pigs where compared to pigs at the initial stage of infection; however, no significant differences were found in pigs in the intermediate and final stages of the disease (Mandrioli et al., 2004).

PCV2 has been found to replicate in a number of different tissues and the induction of severe immunosuppression using cyclosporine in PCV2 infected gnotobiotic piglets increased the replication of PCV2 within hepatocytes (Krakowka et al., 2002). Livers from 100 pigs with naturally occurring PMWS were examined for lymphohistiocytic hepatitis, and apoptotic bodies; of these 100 livers 88% were found to have apoptosis and 12% had no microscopic liver lesions. It was also determined that hepatocytes were a target for PCV2 infection and replication (Rosell et al., 2000a). Similarly another study using 15 pigs from five different farms with previous confirmation of PMWS found PCV2 antigen and DNA in hepatocytes of the livers from all pigs that were PCV2 positive (Rosell et al., 1999).

The objectives of this study were to determine (1) whether PCV2 is associated with apoptosis of hepatocytes by comparing clinically affected or unaffected gnotobiotic pigs that were experimentally infected with PCV2, (2) if there are differences between PCV2a and PCV2b in inducing hepatocyte apoptosis, and (3) if there are differences between apoptosis detection systems.

2. Materials and methods

2.1. Pig source and housing

Forty-eight gnotobiotic piglets acquired surgically from cross-bred sows free of PCV2 were used. Directly after surgical removal pigs were placed into sterile stainless steel isolators which were mounted with polyvinyl canopies and maintained under positive pressure. The isolator units were cleaned with sodium hypochlorite and water before sterilization with ethylene oxide prior to the start of the experimental study. Isolators were sustained at a temperature of 32 °C and the temperature was progressively decreased to a subsistence temperature of 27 °C with HEPA filtration of the intake and exhaust air. The pigs were initially fed a pasteurized milk diet from a commercially available source (Esbilac® Kansas City, Kansas, USA) three times per day, with increasing quantities and a reduction to feeding twice a day to a maximum of 370 ml per feeding. All animal manipulations were approved by the Institutional Animal Care and Use Committee (IACUC) according to the guidelines set by the National Animal Disease Center. The pigs were tested on a weekly basis and found to be negative for aerobic and anaerobic bacteria from rectal swabs by routine culture methods. In addition, all pigs tested negative for porcine parvovirus (PPV), PRRSV, swine hepatitis E virus, and bovine viral diarrhea virus (BVDV) as determined by routine PCRs on serum collected on the day of necropsy.

2.2. Inoculation

The pigs were inoculated intranasally between seven and 10 days of age. Control pigs ($n = 4$) were inoculated with 1 ml PCV2-free cell culture medium. The remaining pigs were either inoculated with 1 ml of PCV2a strain DQ629114 (Cheung et al., 2007) at a dose of 10^{-2}–10^{-4} $TCID_{50}$/ml (PCV2a, $n = 16$) or with 1 ml of PCV2b strain DQ629115 (Cheung et al., 2007) at a dose of 10^{-2}–10^{-4} $TCID_{50}$/ml (PCV2b, $n = 28$). The infectious dose of the inocula was determined by IHC as previously described (Cheung, 2003).

2.3. Study design

For the purpose of this study, the pigs were separated based on inoculation status and development of clinical disease resulting in five groups: (1) Sham-inoculated, clinically-unaffected ($n = 4$), (2) PCV2a, clinically-unaffected ($n = 10$), (3) PCV2a, clinically-affected ($n = 6$), (4) PCV2b, clinically-unaffected, ($n = 13$) and (5) PCV2b, clinically-affected ($n = 15$). Liver tissue from all 48 pigs was collected at the time of necropsy between 23 and 41 days post inoculation, evaluated for microscopic lesions, and scored for presence of apoptotic cells as determined by the TUNEL assay or CCasp3 IHC stain and for presence of PCV2 antigen by IHC.

2.4. Tissue preparation

At necropsy several sections of different liver lobes were collected and immediately immersed in 10% buffered formalin. After the tissues were fixed for 24 h, the tissues were dehydrated, paraffin-embedded, sectioned at 4 μm and mounted on a glass slide followed by routine hematoxylin/eosin staining as described (Krakowka et al., 2004).

2.5. Histopathology

Microscopic lesions were evaluated by a veterinary pathologist (TO) blinded to treatment groups. The sections of liver were evaluated for the presence of lymphohistiocytic inflammation (0 = none; 3 = severe), suppurative hepatitis (0 = none; 3 = severe) and presence of hepatocyte degeneration (0 = none, 3 = diffuse, severe) and areas of necrosis (0 = none, 3 = diffuse, severe). The individual lesion scores were summed and the lesion sum scores were compared between groups.

2.6. Detection of PCV2 antigen

IHC for detection of PCV2-specific antigen was performed using a rabbit polyclonal antiserum as described previously (Sorden et al., 1999). PCV2-antigen scoring was done by a veterinary pathologist (TO) blinded to treatment groups. The following scoring protocol was used: 0 = no signal or no visible stain, 1 = staining was observed in individual inflammatory cells, 2 = staining was observed in moderate numbers of inflammatory cells and hepatocytes, 3 = abundant staining in inflammatory cells and hepatocytes. Although this system is subjective, the same veterinary pathologist scored treatment groups in an earlier study in which a high correlation was found with digital computer image analysis (Opriessnig et al., 2004).

2.7. TUNEL assay

In brief, liver tissue sections were dewaxed, rehydrated in graded alcohol, incubated in 3% hydrogen peroxide for 10 min at 22 °C and rinsed twice with phosphate buffered saline (PBS). This was followed by treatment with Proteinase K (Ambion, Inc., Foster City, USA) for 20 min at 30 °C. After two washing steps with PBS, the tissues were incubated in 10% sheep serum (Sigma, St. Louis, Missouri, USA) for 20 min at 22 °C and rinsed twice with the PBS. TUNEL staining was performed using a commercially available kit (*in situ* cell death detection kit, POD; Roche Diagnostics, Indianapolis, Indiana, USA). The manufacturers' instructions were followed and DAB chromogen (Dako North America Inc., Carpentaria, California, USA) was applied for 5 min and then counterstained with hematoxylin.

2.8. Cleaved Caspase-3 (CCasp3) immunohistochemistry (IHC)

The tissue sections were dewaxed, rehydrated in graded alcohol and incubated in 3% hydrogen peroxide for 10 min at 22 °C, rinsed three times in Tris buffered saline (TBS) treated with 0.05% protease (Sigma Aldrich) for 2 min and washed three times with TBS buffer. A CCasp3 IHC (Cell Signaling Technology®, Danvers, Massachusetts, USA) was applied at a concentration of 1:100 for 1 h at 22 °C. Further processing was done by using a streptavidin–biotin detection kit (Dako North America Inc.).

2.9. Apoptosis controls

Known negative and positive controls were included in each TUNEL and CCasp3 run. As a positive control, sections of murine intestines from a mouse experimentally inoculated with live mycobacterium paratuberculosis followed by dextran sulfate sodium treatment were used (Courtesy of Dr. C. Johnson). This procedure produced colitis in the mouse which resulted in epithelial cell apoptosis as described previously (Vetuschi et al., 2002).

2.10. Apoptotic cell scoring

Apoptotic cell counts were performed by scoring 10 random hepatic areas using a 25× lens and a 10× eyepiece, yielding a final magnification of 250× with an Olympus® microscope (Leeds Precision Instrument, Inc; North Minneapolis, Minnesota, USA). The apoptotic cell score ranged from 0 to 3 (0 = average of 0.0–0.9 apoptotic cells/10 fields, 1 = average of 1–4.9 apoptotic cells/10 fields; 2 = average of 5–14.9 apoptotic cells/10 fields; 3 = average of 15 or more apoptotic cells/10 fields).

2.11. Statistical analysis

The statistical analysis on the data was performed using the SAS software version 9.2, SAS system for windows (SAS Institute Inc., Cary, North Carolina, USA). A Kruskal–Wallis non-parametric analysis of variance (ANOVA) was used to determine differences in median scores between groups. A P value of 0.05 or less was considered statistically significant. If significant, pairwise Wilcoxon tests were conducted to determine which groups were different. An association between categorical variables in paired situations was evaluated by using Fisher's exact test.

3. Results

3.1. Microscopic lesions

Microscopic lesions in liver sections were observed in 88.6% (39/44) of the experimentally inoculated pigs, no microscopic lesions were observed in sham-inoculated pigs (Fig. 1). Hepatic lesions were characterized by infiltration with low-to-high numbers

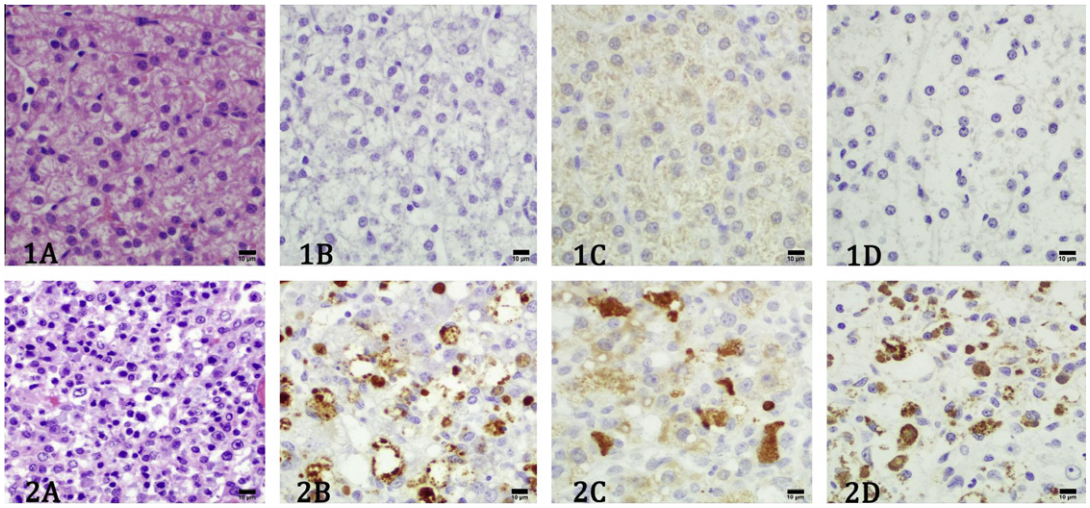

Fig. 1. Histological comparison of liver sections obtained from a negative control pig (1A–D) and a pig infected with PCV2b and severely affected by porcine circovirus associated disease (PCVAD) (2A–D) (Bars 10 μm). (1A) Hematoxylin and eosin (HE) stain: Normal hepatic architecture; (1B) PCV2 immunohistochemistry (IHC): No visible staining; (1C) Cleaved caspase-3 (CCasp-3) IHC: No visible staining; (1D) TUNEL staining: No visible staining. (2A) HE stain: Diffuse moderate multifocal degeneration of hepatocytes; (2B) PCV2 IHC: Diffuse moderate staining in nuclei and cytoplasm of hepatocyte-like cells; (2C) CCasp-3 IHC: Diffuse moderate to abundant staining of hepatocyte nuclei; (2D) TUNEL staining: Diffuse moderate to abundant staining of hepatocyte nuclei.

Table 1
Median group scores (minimum, maximum) for amount of PCV2 antigen as determined by PCV2 IHC stains and numbers of apoptotic cells as determined by cleaved caspase-3 (CCasp3) IHC and the TUNEL assay on liver tissues obtained from sham-inoculated control pigs and unaffected and clinically-affected pigs inoculated with PCV2a or PCV2b.

Clinical Status	Inoculation	PCV2 IHC*	CCasp3**	TUNEL**	Microscopic lesions***
Non-Affected	Sham (n = 4)	0 (0, 0)^{A}***	0 (0, 0)^{A}	0 (0, 0)^{A}	0 (0, 0)^{A}
	PCV2a (n = 10)	0 (0, 2)^{A}	1 (0, 2)^{B}	0.5 (0, 2)^{A}	3 (0, 4)^{B}
	PCV2b (n = 13)	0 (0, 1)^{A}	0 (0, 1)^{A}	0 (0, 1)^{A}	1 (0, 4)^{B}
Affected	PCV2a (n = 6)	3 (0, 3)^{B}	2.5 (1, 3)^{C}	2 (0, 3)^{B}	4 (3, 8)^{C}
	PCV2b (n = 15)	2 (0, 3)^{B}	2 (1, 3)^{C}	2 (0, 3)^{B}	3 (1, 9)^{C}

^{A,B,C}Different superscripts indicate significant ($P < 0.05$) differences between group medians within the same column.
* 0 = negative, 1 = low number, 2 = moderate numbers and 3 = large numbers.
** 0 = average of 0.0–0.9 apoptotic cells/10 fields, 1 = average of 1–4.9 apoptotic cells/10 fields; 2 = average of 5–14.9 apoptotic cells/10 fields; 3 = average of 15 or more apoptotic cells/10 fields.
*** The lesions sum score for microscopic lesions on liver tissues ranged from 0 = no lesions to 12 = severe lesions.

Table 2
Prevalence of scores ranging from 0 to 3 for PCV2 antigen as determined by PCV2 IHC and apoptosis as determined by cleaved caspase-3 (CCasp3) IHC and the TUNEL assay on liver tissues obtained from sham-inoculated controls or pigs inoculated with PCV2a or PCV2b.

Score range	PCV2 IHC*			CCasp3**			TUNEL**		
	Controls	PCV2a	PCV2b	Controls	PCV2a	PCV2b	Controls	PCV2a	PCV2b
0	4/4	9/16	10/28	4/4	1/16	7/28	4/4	6/16	12/28
1	0/4	1/16	5/28	0/4	9/16	10/28	0/4	5/16	6/28
2	0/4	2/16	6/28	0/4	3/16	6/28	0/4	3/16	7/28
3	0/4	4/16	7/28	0/4	3/16	5/28	0/4	2/16	3/28

* 0 = negative, 1 = low number, 2 = moderate numbers, 3 = large numbers.
** 0 = average of 0.0–0.9 apoptotic cells/10 fields, 1 = average of 1–4.9 apoptotic cells/10 fields; 2 = average of 5–14.9 apoptotic cells/10 fields; 3 = average of 15 or more apoptotic cells/10 fields.

of mixed inflammatory cells (mainly lymphocytes, macrophages, and a few neutrophils). In addition, there was mild-to-severe vacuolar degeneration and loss of hepatocytes in 58.3% (28/48) of the pigs (Fig. 1). Affected pigs also had severe lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues (tonsil, spleen, inguinal lymph node, tracheobronchiolar lymph node (data not shown). Regardless of clinical disease status and genotype, PCV2 inoculated pigs had significantly ($P = 0.003$) more severe lesions compared to sham-inoculated pigs (Table 1). The association between disease status (clinically-affected, unaffected) and lesion sum score was significant ($P = 0.003$) and clinically-affected pigs had significantly ($P = 0.001$) more severe lesions compared to clinically-unaffected pigs. No significant ($P = 0.228$) difference of lesion score was observed between pigs inoculated with PCV2a or PCV2b (Table 1).

3.2. PCV2 antigen in liver

Low to high amounts of PCV2 antigen was detected in 24/48 pigs (Table 1; Fig. 1). There were significantly ($P < 0.001$) higher amounts of PCV2 antigen in clinically-affected pigs when compared to clinically-unaffected pigs; however, there was no significant ($P = 0.402$) difference between pigs inoculated with PCV2a and PCV2b (Table 1). PCV2 antigen was detected in the cytoplasm of macrophage-like cells in areas of inflammation but also in the cytoplasm and nuclei of hepatocytes.

3.3. Detection of apoptotic cells by the TUNEL assay

The distribution of the final TUNEL scores in the different treatment groups (sham-inoculated, PCV2a, PCV2b) is summarized in Table 2 and representative images are shown in Fig. 1. Twenty-two of 48 (45.8%) liver tissues had a score of 0, 22.9% (11/48) had a score of 1, 20.8% (10/48) had a score of 2 and 10.4 % (5/48) had a score of 3. There was a significant ($P < 0.001$) association between disease status (clinically-affected, unaffected) and TUNEL scores and a significant ($P < 0.001$) association between lesion sum scores and TUNEL scores. Overall, TUNEL scores were significantly ($P < 0.001$) higher in clinically-affected pigs (Table 1). There was also a significant ($P < 0.001$) association of TUNEL scores and PCV2 IHC scores with an increase in TUNEL score associated with a higher PCV2 IHC score (Table 3). There was no significant ($P = 0.886$) association between TUNEL scores and PCV2 genotype (PCV2a, PCV2b) (Table 1). Comparison of apoptotic rates as determined by TUNEL assay at different DPIs demonstrated that there was no age dependent difference in the severity of apoptosis (data not shown).

3.4. Detection of apoptotic cells by CCasp3 immunohistochemistry

The distribution of the final scores in the different treatment groups is summarized in Table 2 and representative images are shown in Fig. 1. Twelve of 48 (25.0%) pigs had no detectable CCasp3 staining in sections of liver (score 0), 39.6% (19/48) of the pigs were found to have low amounts of CCasp3 staining (score 1), 18.8% (9/48) had moderate amounts of CCasp3 staining (score 2), and 16.7% (8/48) of the pigs had large amounts of CCasp3 staining in sections of liver (score 3). Clinically-affected pigs had significantly ($P < 0.001$) higher CCasp3 IHC scores compared to

Table 3
Mean CCasp-3, TUNEL and microscopic lesion scores (±SE) on liver tissues at different levels of PCV2 antigen as determined by PCV2 IHC.

PCV2 IHC scores*	CCasp3**	TUNEL**	Microscopic lesions***
0 (n = 23)	0.6 ± 0.1	0.3 ± 0.1	1.7 ± 0.4
1 (n = 6)	1.0 ± 0.3	0.5 ± 0.3	1.8 ± 0.5
2 (n = 8)	1.6 ± 0.2	1.4 ± 0.4	2.4 ± 0.4
3 (n = 11)	2.6 ± 0.2	2.3 ± 0.2	5.3 ± 0.7

* 0 = negative, 1 = low number, 2 = moderate numbers, 3 = large numbers.
** 0 = average of 0.0–0.9 apoptotic cells/10 fields, 1 = average of 1–4.9 apoptotic cells/10 fields; 2 = average of 5–14.9 apoptotic cells/10 fields; 3 = average of 15 or more apoptotic cells/10 fields.
*** The lesions sum score for microscopic lesions ranged from 0 = no lesions to 12 = severe lesions.

clinically-unaffected pigs. There was also a significant ($P = 0.03$) association between lesion sum scores and CCasp3 IHC scores. Although CCasp3 staining was significantly ($P = 0.023$) higher in clinically-unaffected pigs inoculated with PCV2a compared to clinically unaffected pigs inoculated with PCV2b and sham-inoculated pigs, a difference in CCasp3 staining was not observed between clinically-affected pigs inoculated with PCV2a or PCV2b (Table 1). However, there was a significant ($P < 0.001$) association of CCasp3 IHC scores and PCV2 IHC scores (Table 3). Comparison of apoptotic rates as determined by CCasp3 IHC at different DPIs demonstrated that there was no age dependent difference in the severity of apoptosis (data not shown).

4. Discussion

PCV2 infection in gnotobiotic pigs has been found to result in liver failure associated with icterus which becomes the imminent cause of death in pigs with this manifestation of PCVAD (Krakowka et al., 2004). The current study used experimentally PCV2-inoculated gnotobiotic pigs with and without signs of clinical disease and hepatic failure. The severity of microscopic lesions in hepatic tissue was significantly ($P < 0.05$) higher in the clinically-affected pigs when compared to the clinically-unaffected and sham-inoculated control pigs (Fig. 1) which is in agreement with previous studies in conventional pigs (Rosell et al., 2000a), further emphasizing the association of PCV2 with hepatic lesions and clinical disease.

The main goal of this study was to determine if there is an association between PCV2 and apoptosis of hepatocytes. We found that the numbers of apoptotic cells increased with increased lesion severity and increased amount of PCV2 antigen. The pigs used were essentially germ-free gnotobiotic pigs and the presence of other pathogens or factors can be ruled out. This is in contrast to field studies, where association of specific microscopic changes such as apoptosis with a specific pathogen is difficult as pigs in the field are commonly infected with more than one pathogen. Therefore, results obtained on field cases need to be viewed with caution. Studies conducted by several research groups have identified apoptosis by TUNEL assay (Shibahara et al., 2000; Krakowka et al., 2004; Mandrioli et al., 2004; Resendes et al., 2004a; Seeliger et al., 2007) and CCasp3 IHC (Resendes et al., 2004a,b; Seeliger et al., 2007) in conventional, clinically-affected pigs (Rosell et al., 1999, 2000b). A recent investigation on conventional pigs using the TUNEL assay, CCasp3 IHC and PCV2 *in situ* hybridization (ISH) provided evidence of virus-induced apoptosis in endothelial cells in pigs diagnosed with PCV2-associated cerebellar vasculitis (Seeliger et al., 2007). Similarly, a study using conventional clinically-affected pigs, the TUNEL assay and PCV2 ISH found that diminished lymphoid cell proliferation was the actual cause of cell depletion (Mandrioli et al., 2004). Another group investigating conventional PMWS-affected pigs using CCasp3 IHC determined that apoptosis was lower in clinically-affected pigs when compared to clinically non-affected pigs (Resendes et al., 2004b). More recently, a study looking at apoptosis in pigs with hepatitis caused by naturally-induced PCV2-associated PMWS concluded that there were higher levels of apoptosis in hepatocytes in pigs with higher PCV2 viral loads (Resendes et al., 2010).

While no information is currently available on PCV2-associated apoptosis in conventional specific pathogen free (SPF) pigs, colostrum-deprived (CD) pigs, or caesarian-derived colostrum-deprived (CDCD), investigations to associate PCV2 with apoptosis have been conducted in gnotobiotic pigs. Little to no apoptosis was found in archived paraffin-embedded blocks of formalin-fixed and cold ethanol-fixed liver, lymph nodes, spleen and thymus obtained from clinically-affected and unaffected gnotobiotic pigs (Krakowka et al., 2004). This is in contrast to the outcomes obtained in the current study and may be due to the fact that lower numbers of pigs were used in the previous study (Krakowka et al., 2004) as well as different challenge virus and different tissues were evaluated. In addition, virus dependent factors such as PCV2 isolates can be closely related and yet be exclusive, differences in detection systems for apoptosis and PCV2 may influence results, and host-dependent factors such as host susceptibility and immune response have been shown to be important (Opriessnig et al., 2006, 2007, 2009).

Differences in pathogenicity between PCV2 genotypes have been suggested; however, definitive experimental proof is lacking to date. While differences in virulence between PCV2 isolates exist (Opriessnig et al., 2006), this appears not to be confined to genotype (Opriessnig et al., 2008). To our knowledge, this is the first study comparing apoptosis in pigs infected with PCV2a or PCV2b. It has been shown through our study that PCV2 is involved in apoptosis; however, there was no significant difference found between the numbers of apoptotic cells within PCV2a or PCV2b inoculated pigs. PCV2a and PCV2b differ in a small region in ORF2 (Cheung et al., 2007) encoding the capsid protein (Nawagitgul et al., 2000), whereas ORF3 which has been demonstrated to be associated with apoptosis (Liu et al., 2005, 2006, 2007), is located within ORF1 and is more conserved among PCV2 isolates, which may explain the results of this study.

A recent study compared the differences between the TUNEL and the CCasp3 IHC assays on lymphoid tissues of healthy conventional pigs of different ages (Resendes et al., 2004a); however, to the authors' knowledge no experimental studies have been performed to compare these assays in hepatic tissues. Our study concluded that both methods detected significantly ($P < 0.05$) higher numbers of apoptotic cells in clinically-affected pigs compared to clinically-unaffected pigs and sham-inoculated control pigs. Nevertheless, when comparing the TUNEL assay directly with the CCasp3 assay we found no differences in detection rates of apoptotic cells (Fig 1) indicating that the same type of cell was labeled by both techniques as described by others using a double staining technique (Mirkes et al., 2001). However, in order to further confirm this observation, staining of serial sections would be necessary.

5. Conclusion

In summary, higher amounts of PCV2 antigen were demonstrated to be associated with more severe microscopic lesions and increased apoptosis in the hepatic tissues from PCV2a or PCV2b singularly-infected, clinically-affected gnotobiotic pigs. No differences were observed between PCV2a- or PCV2b-infected pigs. The TUNEL assay and CCasp-3 IHC appear to be equivalent in their ability to detect apoptotic cells in liver tissues from PCV2-infected gnotobiotic pigs.

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References

Chae, C., 2004. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. Veterinary Journal 168, 41–49.

Cheung, A., 2003. The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. Virology 313, 452–459.

Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Archives of Virology 152, 1035–1044.

Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin, K., McNeilly, F., Meehan, B., Todd, D., Haines, D., 1998. Isolation of circovirus from

lesions of pigs with postweaning multisystemic wasting syndrome. Canadian Veterinary Journal 39, 44–51.
Gagnon, C.A., Tremblay, D., Tijssen, P., Venne, M.H., Houde, A., Elahi, S.M., 2007. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. Canadian Veterinary Journal 48, 811–819.
Harding, J., Clark, E., 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health and Production 5, 201–203.
Harms, P.A., Halbur, P.G., Sorden, S.D., 2002. Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infections. Journal of Swine Health and Production 10, 27–30.
Kim, J., Ha, Y., Jung, K., Choi, C., Chae, C., 2004. Enteritis associated with porcine circovirus 2 in pigs. Canadian Journal of Veterinary Research 68, 218–221.
Kiupel, M., Stevenson, G.W., Galbreath, E.J., North, A., HogenEsch, H., Mittal, S.K., 2005. Porcine circovirus type 2 (PCV2) causes apoptosis in experimentally inoculated BALB/c mice. BMC Veterinary Research 1, 7.
Krakowka, S., Ellis, J.A., McNeilly, F., Gilpin, D., Meehan, B., McCullough, K., Allan, G., 2002. Immunologic features of porcine circovirus type 2 infection. Viral Immunology 15, 567–582.
Krakowka, S., Ellis, J., McNeilly, F., Meehan, B., Oglesbee, M., Alldinger, S., Allan, G., 2004. Features of cell degeneration and death in hepatic failure and systemic lymphoid depletion characteristic of porcine circovirus-2-associated postweaning multisystemic wasting disease. Veterinary Pathology 41, 471–481.
Ladekjær-Mikkelsen, A.S., Nielsen, J., Storgaard, T., Bøtner, A., Allan, G., McNeilly, F., 2001. Transplacental infection with PCV-2 associated with reproductive failure in gilt. Veterinary Record 148, 759–760.
Liu, J., Chen, I., Kwang, J., 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. Journal of Virology 79, 8262–8274.
Liu, J., Chen, I., Du, Q., Chua, H., Kwang, J., 2006. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. Journal of Virology 80, 5065–5073.
Liu, J., Zhu, Y., Chen, I., Lau, J., He, F., Lau, A., Wang, Z., Karuppannan, A.K., Kwang, J., 2007. The ORF3 protein of porcine circovirus type 2 interacts with porcine ubiquitin E3 ligase Pirh2 and facilitates p53 expression in viral infection. Journal of Virology 81, 9560–9567.
Mandrioli, L., Sarli, G., Panarese, S., Baldoni, S., Marcato, P.S., 2004. Apoptosis and proliferative activity in lymph node reaction in postweaning multisystemic wasting syndrome (PMWS). Veterinary Immunology Immunopathology 97, 25–37.
Meehan, B.M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V.A., Ellis, J.A., Hassard, L.E., Clark, E.G., Haines, D.M., Allan, G.M., 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. Journal of General Virology 79, 2171–2179.
Mirkes, P.E., Little, S.A., Umpierre, C.C., 2001. Co-localization of active caspase-3 and DNA fragmentation (TUNEL) in normal and hyperthermia-induced abnormal mouse development. Teratology 63, 134–143.
Nawagitgul, P., Morozov, I., Bolin, S.R., Harms, P.A., Sorden, S.D., Paul, P.S., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. Journal of General Virology 81, 2281–2287.
O'Connor, B., Gauvreau, H., West, K., Bogdan, J., Ayroud, M., Clark, E.G., Konoby, C., Allan, G., Ellis, J.A., 2001. Multiple porcine circovirus 2-associated abortions and reproductive failure in a multisite swine production unit. Canadian Veterinary Journal 42, 551–553.
Olvera, A., Cortey, M., Segalés, J., 2007. Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. Virology 357, 175–185.
Opriessnig, T., Thacker, E.L., Yu, S., Fenaux, M., Meng, X.-J., Halbur, P.G., 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with Mycoplasma hyopneumoniae and porcine circovirus type 2. Journal of Veterinary Pathology 41, 624–640.
Opriessnig, T., McKeown, N.E., Zhou, E.M., Meng, X.J., Halbur, P.G., 2006. Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. Journal of General Virology 87, 2923–2932.
Opriessnig, T., Meng, X.J., Halbur, P.G., 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. Journal of Veterinary Diagnostic Investigation 19, 591–615.
Opriessnig, T., Ramamoorthy, S., Madson, D.M., Patterson, A.R., Pal, N., Carman, S., Meng, X.J., Halbur, P.G., 2008. Differences in virulence among porcine circovirus type 2 isolates are unrelated to cluster type 2a or 2b and prior infection provides heterologous protection. Journal of General Virology 89, 2482–2491.
Opriessnig, T., Patterson, A.R., Madson, D.M., Pal, N., Rothschild, M., Kuhar, D., Lunney, J.K., Juhan, N.M., Meng, X.J., Halbur, P.G., 2009. Difference in severity of porcine circovirus type 2 (PCV2)-induced pathological lesions between Landrace and Pietrain pigs. Journal of Animal Science 87, 1582–1590.
Resendes, A.R., Majó, N., Segalés, J., Espadamala, J., Mateu, E., Chianini, F., Nofrarias, M., Domingo, M., 2004a. Apoptosis in normal lymphoid organs from healthy normal, conventional pigs at different ages detected by TUNEL and cleaved caspase-3 immunohistochemistry in paraffin-embedded tissues. Veterinary Immunology and Immunopathology 99, 203–213.
Resendes, A.R., Majó, N., Segalés, J., Mateu, E., Calsamiglia, M., Domingo, M., 2004b. Apoptosis in lymphoid organs of pigs naturally infected by porcine circovirus type 2. Journal of General Virology 85, 2837–2844.
Resendes, A.R., Majó, N., Van den Ingh, T., Mateu, E., Domingo, M., Calsamiglia, M., Segalés, J., 2010. Apoptosis in postweaning multisystemic wasting syndrome (PMWS) hepatitis in pigs naturally infected with porcine circovirus type 2 (PCV2). The Veterinary Journal. doi:10.1016/j.tvjl.2010.06.018.
Rosell, C., Segalés, J., Plana-Durán, J., Balasch, M., Rodriguez-Arrioja, G.M., Kennedy, S., Allan, G.M., McNeilly, F., Latimer, K.S., Domingo, M., 1999. Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. Journal of Comparative Pathology 120, 59–78.
Rosell, C., Segalés, J., Domingo, M., 2000a. Hepatitis and staging of hepatic damage in pigs naturally infected with porcine circovirus type 2. Veterinary Pathology 37, 687–692.
Rosell, C., Segalés, J., Ramos-Vara, J.A., Folch, J.M., Rodriguez-Arrioja, G.M., Duran, C.O., Balasch, M., Plana-Duran, J., Domingo, M., 2000b. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Veterinary Record 146, 40–43.
Roulston, A., Marcellus, R.C., Branton, P.E., 1999. Viruses and apoptosis. Annual Review of Microbiology 53, 577–628.
Seeliger, F.A., Brügmann, M.L., Krüger, L., Greiser-Wilke, I., Verspohl, J., Segalés, J., Baumgärtner, W., 2007. Porcine circovirus type 2-associated cerebellar vasculitis in postweaning multisystemic wasting syndrome (PMWS)-affected pigs. Veterinary Pathology 44, 621–634.
Segalés, J., Allan, G.M., Domingo, M., 2005. Porcine circovirus diseases. Animal Health Research Review 6, 119–142.
Shibahara, T., Sato, K., Ishikawa, Y., Kadota, K., 2000. Porcine circovirus induces B lymphocyte depletion in pigs with wasting disease syndrome. Journal of Veterinary Medical Science 62, 1125–1131.
Sorden, S.D., Harms, P.A., Nawagitgul, P., Cavanaugh, D., Paul, P.S., 1999. Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. Journal of Veterinary Diagnostic Investigation 11, 528–530.
Vetuschi, A., Latella, G., Sferra, R., Caprilli, R., Gaudio, E., 2002. Increased proliferation and apoptosis of colonic epithelial cells in dextran sulfate sodium-induced colitis in rats. Digestive Disease and Sciences 47, 1447–1457.