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

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Abstract
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Keywords
Apoptosis, Cleaved caspase-3, Gnotobiotes, Porcine circovirus type 2, Terminal transferase dUTP nick end labeling assay

Disciplines
Large or Food Animal and Equine Medicine | Statistical Methodology | Veterinary Infectious Diseases

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

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ABSTRACT
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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 was 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,
2. Materials and methods

2.1. Pig source and housing

Forty-eight gnotobiotic piglets acquired surgically from crossbred 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 PCV2 strain DQ629114 (Cheung et al., 2007) at a dose of 10^{2–10} 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} 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
of mixed inflammatory cells (mainly lymphocytes, macrophages, and a few neutrophils). In addition, there was mild-to-severe vac-ular degeneration and loss of hepatocytes in 58.3% (28/48) of the pigs (Fig. 1). Affected pigs also had severe lymphoid depletion and histiocyctic 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 1. Two 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 clinically-unaffected pigs (Fig. 1). There was also a significant (P = 0.003) 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 clinically-unaffected pigs. No significant (P = 0.228) difference of lesion score was observed between pigs inoculated with PCV2a or PCV2b (Table 1).

<table>
<thead>
<tr>
<th>Clinical Status</th>
<th>Inoculation</th>
<th>PCV2 IHC</th>
<th>CCasp3</th>
<th>TUNEL</th>
<th>Microscopic lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Affected</td>
<td>Sham (n = 4)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td></td>
<td>PCV2a (n = 10)</td>
<td>0 (0, 2)</td>
<td>1 (0, 2)</td>
<td>0.5 (0, 2)</td>
<td>3 (0, 4)</td>
</tr>
<tr>
<td></td>
<td>PCV2b (n = 13)</td>
<td>0 (0, 1)</td>
<td>0 (0, 1)</td>
<td>1 (0, 4)</td>
<td>1 (0, 4)</td>
</tr>
<tr>
<td>Affected</td>
<td>PCV2a (n = 6)</td>
<td>3 (0, 3)</td>
<td>2.5 (1, 3)</td>
<td>2 (0, 3)</td>
<td>4 (3, 8)</td>
</tr>
<tr>
<td></td>
<td>PCV2b (n = 15)</td>
<td>2 (0, 3)</td>
<td>2 (0, 3)</td>
<td>3 (1, 5)</td>
<td>3 (1, 5)</td>
</tr>
</tbody>
</table>

** Different superscripts indicate significant (P < 0.05) differences between group medians within the same column.
* 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 on liver tissues 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, colostrodeprived (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 (Nawagtitul 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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