Acute Hepatitis in a Piglet Experimentally Inoculated with Tissue Homogenates from Pigs with Postweaning Multisystemic Wasting Syndrome

Takuya HIRAI1), Tetsuo NUNOYA1), Takeshi IHARA1), Kouichi KUSANAGI1), Tetsuo KATO1) and Kazumoto SHIBUYA1)

1)Nippon Institute for Biological Science, 9–2221–1, Shinmachi, Ome, Tokyo 198–0024, Japan

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ABSTRACT. Five 2 day-old colostrum-deprived piglets were inoculated with tissue homogenates from pigs with postweaning multisystemic wasting syndrome. One of the five piglets developed icterus and died 23 days post-inoculation. Histologic examination revealed acute hepatitis. Porcine circovirus type 2 (PCV-2) antigen and nucleic acid were detected in hepatocytes and phagocytic cells. Ultrastructurally, hepatocytes and phagocytic cells had large numbers of cytoplasmic inclusions, which were composed of electron-dense paracrystalline arrays of small non-enveloped viral particles approximately 17 nm in diameter. Apoptotic hepatocytes were confirmed by the TUNEL method and electron microscopic examination. These findings may indicate that hepatocellular necrosis is associated with replication of PCV-2. Apoptosis of hepatocytes also contributes to the pathogenesis of hepatic lesions in this case.

KEY WORDS: hepatitis, PCV-2, PMWS.
transferase-mediated dUTP-nick end labeling (TUNEL) procedure (Apoptosis in situ Detection Kit, Wako Pure Chemical Industries, Ltd., Osaka, Japan) [3].

For in situ hybridization (ISH), deparaffinized sections were treated with 0.2 N HCl at room temperature for 20 min and digested with proteinase K (Sigma-Aldrich Japan Inc., Tokyo, Japan) 0.75 mg/ml in PBS at 37°C for 20 min. After postfixation with 4% PFA in PBS for 5 min, the sections were immersed in 2 mg/ml glycine in PBS for 30 min and kept in 40% deionized formamide in 4× standard saline citrate (SSC; pH 7.0) until use for hybridization. A digoxigenin (DIG)-labeled probe which was derived from ORF1 region of PCV-2 was PCR-amplified with primers 5′ GGG TGT TCA CGC TGA ATA ATC CTT CCG 3′ and 5′ TCC GAT AGA GAG CTT CTA CAG C 3′ using DIG labeling mix. The probe was dissolved in a hybridization buffer which consisted of 2× SSC containing 50% formamide, 5% dextran sulfate solution and 0.2% skim milk. Each probe/tissue preparation was covered with a clean cover slip, and placed in a 90°C oven for 10 min. Hybridization was carried out at 42°C for 12–16 hr. After repeated washes, sections were soaked once in 1% blocking reagent in PBS at 37°C for 1 hr and rehydrated with PBS and processed for immunohistochemistry.

Results

Hepatic lesions were observed only in one piglet (No. 6) inoculated with tissue homogenates from pigs with PMWS. Piglets are summarized in Table 1. PCV antigen and nucleic acid were demonstrated in lymph nodes and spleen of inoculated piglets by PCR.

Table 1. Summary of gross and microscopic lesions in piglets inoculated with tissue homogenates from pigs with PMWS

<table>
<thead>
<tr>
<th>Piglets No.</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Heart</th>
<th>Lung</th>
<th>Lymph nodes</th>
<th>Ileum</th>
<th>Colon</th>
</tr>
</thead>
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<tr>
<td>1* (23)</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
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<tr>
<td>2* (35)</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td>3* (49)</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td>4 (7)</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
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<td>-/-</td>
<td>1+/2+-</td>
</tr>
<tr>
<td>5 (7)</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td>6 (23)</td>
<td>3+/3+/3+</td>
<td>3+/3+</td>
<td>-/-</td>
<td>2+/3+</td>
<td>3+/3+/3+</td>
<td>1+/-/3+3</td>
<td>-/-</td>
<td>-/-</td>
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<td>7 (35)</td>
<td>3/-/-</td>
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<td>8 (49)</td>
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<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/3+</td>
</tr>
</tbody>
</table>

* Uninfected controls. Days post-inoculation stated in parenthesis.

Gross lesions (a-g): a) congested, flaccid small intestine, b) mesocolonic edema, c) icterus, d) hydropericardium, e) pulmonary edema, f) lymphadenopathy, g) pale heart.

Microscopic lesions (h-n): h) mesocolonic edema with catarhal colitis, i) acute hepatitis, j) granulomatous splenitis, k) edema of subpleural and interstitial tissue, l) granulomatous lymphadenitis, m) granulomatous nephritis, n) nonsuppurative myocarditis.

For electron microscopy, small pieces of the PFA-fixed liver were postfixed in 1% osmium tetroxide, and embedded in epoxy resin (Epok 812, Okenshoji Co., Ltd., Tokyo, Japan). Semithin sections were stained with 1% toluidine blue. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined with a Hitachi H-600L transmission electron microscope.

Two piglets (Nos. 6 & 7) had lesions in the lymph nodes, spleen, tonsil and Peyer’s patches. These lymphoid lesions were characterized by multifocal to diffuse infiltrates of epithelioid macrophages with basophilic cytoplasmic inclusion bodies, formation of syncytial cells, depletion of lymphocytes, and necrosis of follicular centers. Lesions in other piglets are summarized in Table 1. PCV antigen and nucleic acid were detected in the lesions in liver, spleen, kidney, heart, lungs and lymph nodes (Nos. 6–8). PCV-2 DNA was detected in the lymph nodes, tonsils, liver, spleen, heart, and lungs of three piglets (Nos. 6–8) by type specific PCR. PCV-1 was not demonstrated in any tissues of inoculated and control piglets by PCR.
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and were characterized by centrilobular necrosis of hepatocytes, dissociation of hepatic plates, Kupffer cell hyperplasia, and increased number of macrophages in hepatic sinusoids. Hepatocytes were often swollen and occasionally contained eosinophilic intranuclear inclusion bodies in their vesicular nuclei. In the cytoplasm of hepatocytes and Kupffer cells, there were often small, granular and basophilic to amphophilic inclusion bodies (Fig. 1). Scattered acidophilic bodies (Fig. 2), which sometimes contained several pyknotic fragments, were observed. It was sometimes difficult to distinguish apoptotic bodies from inclusions in anuclear hepatocytes by HE staining. A large amount of PCV antigen and nucleic acid were detected in the hepatocytes, Kupffer cells, and infiltrating macrophages (Fig. 3). PCV antigen and nucleic acid in hepatocytes were found frequently in the nucleus and sporadically in the perinuclear cytoplasm. In contrast, PCV antigen and nucleic acid in Kupffer cells and infiltrating macrophages were mainly detected in the cytoplasm and, to a lesser extent, in the nuclei. The distribution of PCV antigen and nucleic acid closely mimicked that of the inclusions in hepatocytes and Kupffer cells. The number of ISH positive cells was higher than IHC positive cells.

Many nuclei of hepatocytes and some apoptotic bodies were positive by the TUNEL method. TUNEL-positive hepatocytes were present in and around the necrotic areas (Fig. 4). Electron microscopic examination revealed that cytoplasmic inclusion bodies, which were 0.3–3 μm in diameter, were scattered in the degenerative or anuclear necrotic hepatocytes as well as in phagocytic cells (Fig. 5). Inclusion bodies were electron dense and heterogeneous and contained non-enveloped viral particles, approximately 17 nm in diameter, which usually formed paracrystalline arrays (Fig. 5). There were apoptotic hepatocytes characterized by condensed chromatin surrounded by a nuclear membrane. Pyknotic fragments sometimes observed in the acidophilic bodies were composed of homogeneously electron-dense matrix without viral particles and were considered to be apoptotic bodies (Fig. 6). No intranuclear viral particles were observed in any hepatocytes, Kupffer cells, or infiltrating macrophages.

Clinical disease, gross and histological lesions observed in No. 6 piglet were consistent with those in field and experimental cases of PMWS [1, 7, 12, 14]. PCV-2 infection was confirmed by immunohistochemistry, electron microscopy, ISH technique, and PCR test. These findings demonstrated that PMWS could be experimentally reproduced in a colostro-num-deprived piglet by inoculation of tissue homogenates from pigs with PMWS. In previous studies, coinfections with other porcine viruses or immunostimulation have been shown to be important in the development of clinical PMWS [1, 7, 8]. In contrast, a recent report demonstrated that PCV-2 could induce clinical PMWS in piglets in the absence of other swine pathogens [10]. In the case presented here, the inoculum of tissue homogenates was shown to be free from PCV-1, PPV and PRRSV. No evidence of either environmental or laboratory contamination was observed in the No. 6 piglet.

There were some difference between the hepatic lesions of natural cases and the present No. 6 piglet. Severe hepatic lesions observed in the natural cases of PMWS were characterized by extensive loss of hepatocytes, diffuse inflammatory infiltration, and perportal fibrosis [1, 12, 13]. These changes occurred in a later stage of the disease [13]. In the No. 6 piglet, lymphoplasmacytic infiltration was extremely mild and fibrosis was absent. These findings were probably related to a more acute disease accompanying the severe damage of lymphoid tissues in the present case. The severity and nature of the hepatic lesions may also be influenced by the age of piglet exposed to PCV-2 and the size of the infective dose.

A pathognomonic microscopic finding of PMWS is the formation of cytoplasmic inclusions, which were characterized by round, homogeneous, and magenta to basophilic, and botryoid clusters of variable sizes (5–25 μm in diameter) [1, 6, 12]. Cytoplasmic inclusions were formed in the cells of monocyte/macrophage lineage [1, 6, 12]. In the present study, cytoplasmic inclusions were detected in the liver as well as lymphoid tissues. Unlike lymphoid tissues, the inclusions in the hepatocytes were smaller in size (0.3–3 μm in diameter) of different stainability, and did not form botryoid clusters. To our knowledge, there are no reports on the formation of inclusion bodies in the hepatocytes. PCV antigen and nucleic acid were similarly detected in the hepatocytes with inclusion bodies. Intranuclear inclusions found in hepatocytes also contained PCV-2 antigen and nucleic acid but no distinct viral particles were detected in them by electron microscopy. It is not clear whether these inclusions are nuclear structures including immature viral components. Our findings may indicate that hepatocellular necrosis is due partly to replication of PCV-2. Furthermore, apoptotic hepatocytes were confirmed by TUNEL method and electron microscopy. Therefore, apoptosis may be also involved in hepatic lesions in this case. While the immune-mediated hepatocyte death was not excluded completely, lymphocytic infiltrates were rarely observed. This was reflected by a crippled immune status, evidenced by lymphocytic depletion in the lymphoid tissues in the piglet. The reason why only one of five piglets developed severe hepatitis remained unexplained. Conceivable explanations are differences in health status or immune response among piglets at the time of PCV-2 infection. Further studies are required to clarify the pathogenesis of hepatic damage in PMWS.

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REFERENCES