NOTE *Pathology*

Infectivity of Porcine Circovirus 1 and Circovirus 2 in Primary Porcine Hepatocyte and Kidney Cell Cultures

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ABSTRACT. Infectivity of porcine circovirus (PCV) 1 and PCV2 was examined in primary porcine hepatocyte culture by comparing that of PCV in primary kidney cell culture. The virus titer of PCV2-infected hepatocyte cultures was higher than that of the PCV1-infected hepatocyte cultures and the PCV-infected kidney cell cultures. The number of virus-positive cells was most abundant in PCV2-infected hepatocyte cultures as determined by immunohistochemistry and/or *in situ* hybridization. The results of our data suggest that PCV2 preferably infects cultured hepatocytes as observed in the liver of pigs with postweaning multisystemic wasting syndrome.
KEY WORDS: infectivity, porcine circovirus, primary porcine hepatocyte cultures.

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Porcine circovirus (PCV) was first discovered in 1974 as a contaminant of the continuous porcine kidney cell line, PK-15 [18]. The PCV is now referred to as PCV type 1 (PCV1) and is known to be non- pathogenic in pigs [2, 17]. In contrast, PCV type 2 (PCV2) was isolated from pigs with naturally occurring and experimentally induced postweaning multisystemic wasting syndrome (PMWS) [1]. In most cases of PMWS, clinical signs appear at 5 to 12 weeks, and are characterized by progressive weight loss, dyspnea, palpable lymphadenopathy, and less frequently, pallor, diarrhea, and jaundice [1, 11]. Lesions associated with PCV2 infection include lymphohistiocytic to granulomatous lymphadenitis, interstitial pneumonia, hepatitis, nephritis, myocarditis, enteritis, and pancreatitis [1, 11, 14]. In many cases of PMWS, lymphadenitis and hepatitis are the commonly observed lesions. Since hepatic necrosis and/or apoptosis have been detected in the hepatic lesions of PMWS [4, 5, 7, 9, 10], hepatocytes also seem to be a target cell for PCV2 infection [5, 10]. During fetal life, cardiomyocytes, hepatocytes and macrophages were main target cells of PCV2 [12]. In our previous study, we reproduced lymphadenitis and hepatitis in a piglet experimentally inoculated with tissue homogenates from pigs with PMWS [5]. The aim of this study was to confirm if PCV2 can infect primary porcine hepatocyte cultures as observed in the liver of PMWS-affected pigs.

Male specific-pathogen-free (SPF) NIBS miniature pigs, one to 45-day-old, were obtained from the Laboratory Animal Research Station, Nippon Institute for Biological Science (NIBS) (Yamanashi, Japan). SPF NIBS miniature pigs were negative for PCV1 and PCV2 antibodies. All animal procedures in this study complied with the NIBS's Guidelines for Care and Use of Laboratory Animals (1999). Primary porcine hepatocytes were isolated by a modified method of the standard two-step collagenase-dispase perfusion and subsequent differential centrifugation [15]. Briefly, the liver was perfused with collagenase-dispase medium via the portal vein and hepatocytes were dispersed and sedimented in phosphate-buffered saline (PBS; pH 7.2) three times at 50 × g and seeded at a density of 5×10^5 cells per m*l* of Williams' medium E containing 10% fetal bovine serum (FBS) in 60-mm-diameter collagen-coated dishes and maintained at 37°C (5% CO_2). The cell suspension usually contains about 90–95% intact hepatocytes and only 1–2% mesenchymal cells [15]. Ninety-five percent of the cells were morphologically identified as hepatocytes. Four hours after plating, the medium was replaced and maintained with fresh Williams' medium E (10^{-7} M insulin, 10^{-9} M dexamethasone, 20 *n*g/m*l* epidermal growth factor, 10% FBS) and the cultures were incubated until the cells became semi-confluent before inoculation. Additionally, the infectivity of viruses in hepatocytes was compared to that in kidney cells. Tissue blocks of the kidney were minced, dissociated by Dispase and centrifuged. Pelletized cells were resuspended in Eagle's MEM containing 10 % FBS, seeded at a concentration of 5×10^5 cells/m*l*, and incubated at 37°C (5% CO_2) until the cells became semi-confluent before inoculation.

The PCV2 inoculum used in this study was the same as reported previously [5]. Briefly, the inoculum ($10^{3.0}$ $TCID_{50}$/m*l*) was derived from lymphoid tissue homogenates of pigs diagnosed as PMWS. The PCV2 inoculum was shown to be free from PCV1, porcine reproductive and respiratory syndrome virus (PRRSV) as determined by polymerase chain reaction (PCR). The inoculum was also free from porcine parvovirus (PPV) as determined by hemagglutination test. The PCV1 inoculum was prepared from the cultured medium of PK-15 cells persistently infected with PCV1 and adjusted to a titer of $10^{3.0}$ $TCID_{50}$/m*l*. The culture medium (Eagle's MEM) was used for mock infection.

Primary culture cells were incubated with viruses or with the same volume of culture media. After 1 hr of incubation at 37°C, the medium was removed and the cultures were washed once before the addition of fresh medium. The cells

were fixed with 4% paraformaldehyde (PFA) in PBS (pH 7.2) at 2, 3 and 4 days post-infection (dpi). For immunohistochemistry (IHC), hyperimmune swine sera raised against PCV1 or PCV2 was used. The procedures of IHC and *in situ* hybridization (ISH) were similar to those previously described [5, 6]. PCV-positive cells were counted in the dishes of hepatocyte and kidney cell cultures at the indicated time points after infection. The values were plotted as the percentage of positive cells among the total number of cells present in five fields under 40 × magnification. The titer of PCV was determined by inoculation of serial 10-fold dilution of the culture medium into PK-15 cells [6]. At 3 dpi, the cells were immunohistochemically stained by the hyperimmune swine sera raised against PCV1 or PCV2.

In order to examine cytopathic effect (CPE) in primary hepatocyte cultures by PCV infection, long-term cultivation of hepatocytes was performed. For this experiment, a 1-day-old, male SPF NIBS miniature pig was used. Hepatocytes were collected, as described above, and seeded in 60-mm-diameter collagen-coated dishes at 37°C (5% CO_2). At 4 hr after plating, hepatocytes were infected with either PCV1 or PCV2 inoculum. The same volume of Eagle's MEM media was used as a control. After 1 hr of incubation at 37°C, the cultures were washed once with medium before the addition of fresh medium. The cultures were maintained for up to 10 days with a medium change at every 2 days. At 0, 2, 4, 6, 8, 10 dpi, the cells were fixed with 4% PFA in PBS. By IHC, the percentage of PCV-positive cells among the total number of hepatocytes was calculated. γ-Glutamyltransferase (γ-GT) activity in the medium was determined by using an automated analyzer, a method recommended by the Japan Society of Clinical Chemistry.

Cell-free viruses were detected in media of both primary hepatocyte and kidney cell cultures. The titers of PCV in primary kidney cell cultures remained at low levels throughout the observation period (Fig. 1). In contrast, PCV titers in primary hepatocyte cultures began to increase by 2 dpi, and reached peaks on 3 dpi, decreasing gradually thereafter (Fig. 1). The virus titer of PCV2-infected hepatocyte cultures was higher than that of PCV1 throughout the experimental period. Consistent experimental results were obtained in cultures derived from different piglets.

PCV antigens were detected in both primary-cultured hepatocytes and kidney cells (Fig. 2). The number of positive cells and the amount of antigens were much higher in hepatocytes than in kidney cells (Fig. 2). The number of PCV2-infected hepatocytes was significantly higher than that of PCV1 (Fig. 3). The proportion of PCV2-positive hepatocytes was ranged from 13% to 17% (Fig. 2). In contrast, PCV1-labeled hepatocytes made up about 2% of the total cells. In cultured primary kidney cells, less than 2% of the cell population was labeled for PCV1 or PCV2. In PCV-positive hepatocytes and kidney cells, viral antigens were present in both the nucleus and cytoplasm. Uninfected cells were negative for PCV1 and PCV2 antigens. Cells containing PCV nucleic acids were observed in primary cultured hepatocytes in ISH studies (Fig. 3). Localization of positive

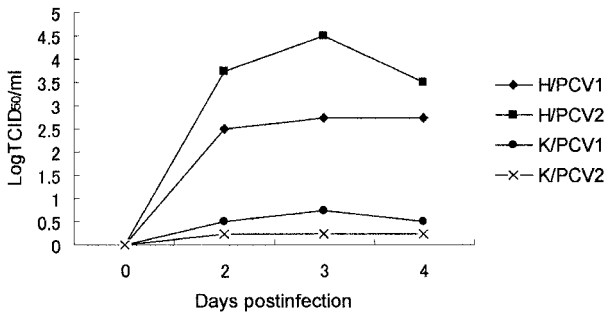

Fig. 1. Virus titers ($logTCID_{50}/ml$) in porcine primary cell cultures. PCV were infected in primary hepatocytes (H/PCV1 or H/PCV2) and kidney cells (K/PCV1 or K/PCV2).

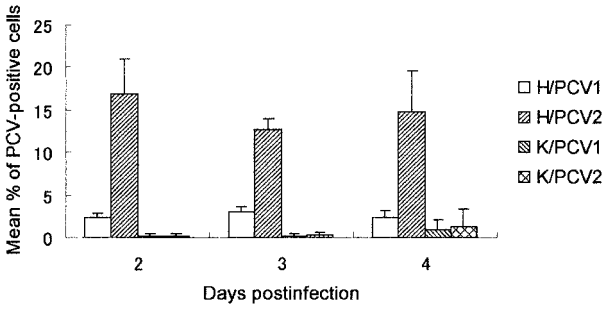

Fig. 2. PCV antigens in porcine primary cell cultures. Data are plotted as percentage of PCV-positive cells in cultured cell population identified in 5 randomly selected high-power fields.

hybridization signals was corresponded to those of the virus antigens. CPE was not detected in hepatocytes or kidney cells after inoculation with either PCV.

Although PCV1 or PCV2 antigens were detected in hepatocytes in long-term cultivation, the number of PCV2-positive cells was higher than that of PCV1-positive cells. The population of PCV2-positive cells gradually increased and reached approximately 20% of the total cells at 10 dpi (data not shown). In contrast, PCV1-positive cells remained less than 2% during the experimental period. γ-GT activity in PCV-infected hepatocyte cultures was similar to mock-infected control (data not shown). There were no apparent CPE in hepatocytes with or without PCV2-infection during cultivation of ten days.

The kidneys, liver and lungs have been described as major target organs of PCV2 in PMWS [1, 11]. *In vitro*, replication of PCV was best achieved by inoculation of PCV-free PK-15 cells [1, 3, 16]. When infectivity of PCV was examined in porcine primary hepatocytes and kidney cell cultures, hepatocytes were much more susceptible to PCV than kidney cells.

PCV1 is generally accepted to be a nonpathogenic virus [1, 2, 16]. Previous reports revealed that no gross or microscopic lesions were seen in pigs after experimental infection of PCV1 [1, 2]. However, PCV1 antigen was detected predominately in the spleen, thymus, lung and rarely in the liver

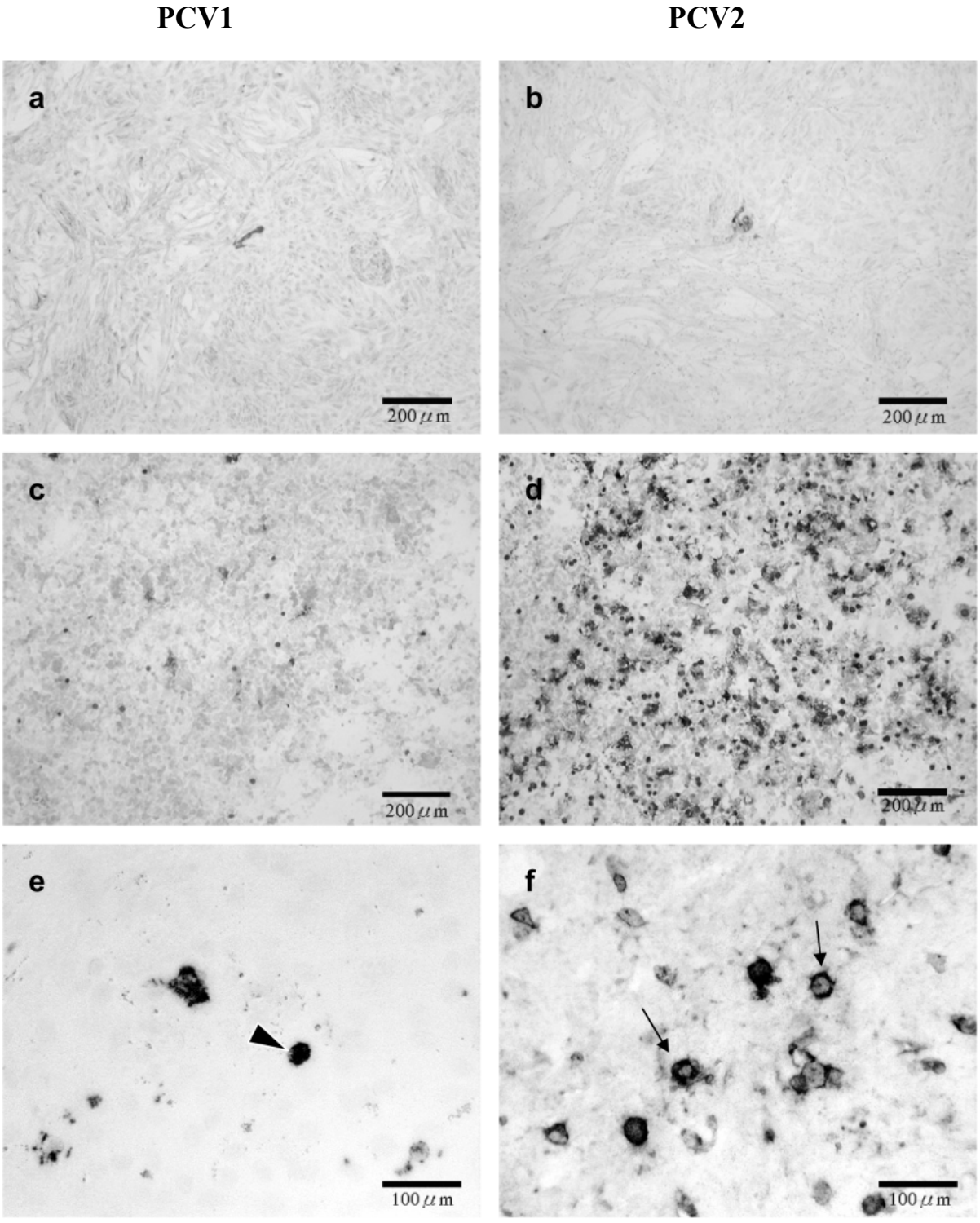

Fig. 3. Presence of PCV antigens and nuclei acid in cultured kidney cells (a, b) and hepatocytes (c-f) at 3 days post-infection. PCV-infected cells were subjected to immunohistochemical staining (a-d) and *in situ* hybridization assays (e, f). Positive hybridization signals are detected in both cytoplasm (arrows) and nucleus (arrowheads) of infected cells.

[2]. In contrast, 88% of pigs in natural cases of PMWS had a variable degree of hepatitis and 70% of the liver contained PCV2 nucleic acid [10]. These results revealed that the liver is one of the predominant target organs for PCV2 infection. The resulting liver damage may be a major cause of PMWS [10]. Cells of PCV2 infection included Kupffer cells, hepatocytes, and inflammatory infiltrates [5, 10]. The data obtained in the present study reflected those dynamics of PCV in pigs, namely a preferential infection of PCV2 in the liver cells and a higher magnitude of infection in PCV2 than PCV1.

PCV2 lacks its own DNA polymerase and is thought to replicate in cells with active cell polymerases, such as dividing cells, for optimal viral replication [16]. DNA synthesis in hepatocytes is active during fetal life [12]. Postnatally, hepatocytes still divide as part of the physiological turnover of these cells and at an increased rate in response to hepatic tissue loss [12]. Cultured hepatocytes that are undergoing division are most likely to favour viral replication. Susceptibility of hepatocytes to PCV2 may be related to the mitotic activity of these cells. In addition, hepatocytes may have other cellular factors such as receptors, which are essential for infection with PCV2.

Hepatic necrosis and apoptotic bodies (Councilman bodies) have been observed in the hepatic lesions of natural and experimental cases of PMWS [4, 5, 7, 9, 10]. In this experiment, there was no CPE and no abnormal increase of γ-GT activity after PCV2 infection. Therefore, it is speculated that PCV2 induces minimal or no hepatocellular damage *in vitro*. The reason why hepatocellular damage was not induced is unknown. One conceivable explanation is that ten days was an inadequate duration to induce CPE in PCV2-infected hepatocytes. Cells of monocyte-macrophage lineage including Kupffer cells are also susceptible to PCV2 infection [10]. Interaction between hepatocytes and Kupffer cells may play a crucial role in the pathogenesis of the hepatic disease of PMWS. Further studies are required to clarify the mechanisms of hepatocellular death by PCV2 infection in pigs.

PCV2 has been associated with a number of pathological conditions in pigs, including PMWS, reproductive failure, porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex, proliferative and necrotizing pneumoniae and congenital tremor type AII [13]. Although the genetic similarity between PCV2 strains isolated from cases of PMWS and abortion is high (>95%), differences in biological properties between strains have been considered [8]. In one study, a few abortion-associated strains showed different replication kinetics in PK-15 cells in comparison with PMWS or PDNS-associated strains [8]. Although the PMWS-associated strain used in this study showed high infectivity to primary hepatocytes, it is uncertain whether all strains from field cases have a similar infectivity to cultured hepatocytes. Our results suggest that PCV2 preferably infects cultured hepatocytes as observed in the liver of PMWS-affected pigs.

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