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Porcine epidemic diarrhea virus infects and replicates in porcine alveolar macrophages

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) is a causative agent of porcine epidemic diarrhea; consequently, the small intestine was believed to be its only target organ. In this study, we found that PEDV infected not only the small intestines, but also the respiratory tract. Infection and replication of PEDV in the respiratory tract from naturally PEDV-infected piglets were examined by reverse transcription polymerase chain reaction, immunohistochemistry, and virus re-isolation. Our observations were confirmed by experimental inoculation, and we found that PEDV infection in the respiratory tract was specifically associated with alveolar macrophages in the lung. Vero cell-adapted PEDV was able to replicate in both primary alveolar macrophages and continuous porcine alveolar macrophage cells. Sequencing analysis of the spike (S) glycoprotein revealed that mutations in S might be a potential determinant of auxiliary targets for PEDV. The discovery that PEDV infects and replicates in alveolar macrophages provides new insights into its pathogenesis.

1. Introduction

Coronaviruses infect many animals, including humans, in a species-specific manner. Most coronaviruses in farm animals cause respiratory or gastrointestinal tract infections in young animals, resulting in huge economic losses (Saif, 2004a,b; Stoddart et al., 1988; Weiss and Navas-Martin, 2005). Several viruses in the family *coronaviridae* infect pigs, including transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCoV), porcine hemagglutinating encephalitis virus, and porcine epidemic diarrhea virus (PEDV) (Cartwright et al., 1969; Greig et al., 1962). TGEV mainly infects epithelial cells in the small intestine and causes fatal enteritis in piglets (Enjuanes et al., 1995). Additionally, TGEV replicates in porcine respiratory tract tissues but does not induce primary respiratory disease (Kemeny et al., 1975; Saif, 2004a,b). PRCoV, a spontaneously occurring variant of TGEV, replicates more extensively in the respiratory tract without causing clinical signs (Wesley et al., 1991). It has been reported that PRCoV replicates in different types of lung cells, including alveolar, bronchiolar and bronchial epithelial cells and in bronchoalveolar macrophages, but not in enterocytes (Cox et al., 1990).

PEDV is one of the major etiological agents of seasonal acute diarrhea in piglets (Debouck and Pensaert, 1980). The pathology following PEDV infection resembles that of TGEV, which is mainly characterized by acute watery diarrhea and dehydration in suckling piglets (Hess et al., 1980; Pensaert et al., 1981; Takahashi et al., 1983). Based on these symptoms, the enterocytes have largely been thought of as the target cells of PEDV infection. Indeed, experimental infection with the prototype strain CV777 showed that replication in epithelial cells covering small and large intestinal villi resulted in villous atrophy, malabsorption and diarrhea (Debouck and Pensaert, 1980). Similarly, Korean strains of PEDV caused severe villous atrophy in the duodenum, jejunum and ileum of experimentally infected pigs (Kim and Chae, 2000, 2003). Replication was mainly localized in the cytoplasm of villous epithelial cells of the small intestine and colon (Sueyoshi et al., 1995). Microscopically, marked cytoplasmic vacuolation and exfoliation of enterocytes with subsequent considerable shortening and fusion of villi were observed (Coussement et al., 1982; Pospischil et al., 1981). However, according to immunohistochemistry (IHC) results reported by Lee et al., PEDV antigens were detected not only in the cytoplasm of the villous enterocytes but also in macrophages that had infiltrated the lamina propria (Lee et al., 2000). PEDV infection in macrophages may reflect the possibility of widespread pathology or the presence of extra-gastrointestinal infection and replication,

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as observed in other species infected with coronaviruses (Gu et al., 2005). Most recent studies on the pathogenesis of PEDV have been limited to partial autopsies of the gastrointestinal tract. Therefore, a comprehensive approach to identify pathogenesis in different organs or cell types was needed.

In this study, we used histological and molecular approaches to acquire a better understanding of PEDV pathogenesis. The presence of PEDV genome and antigens in both the small intestines and respiratory tracts of PEDV-infected piglets suggests that, in addition to the small intestine, the porcine respiratory tract could also be a replication site for PEDV.

2. Materials and methods

2.1. Cells and viruses

African green monkey kidney cells (Vero, CCL-81) were prepared in minimum essential medium (MEM, Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL). 3D4 cells are a porcine monomyeloid cell line, which was established following transfection of primary porcine alveolar macrophage cultures with plasmid pSV3neo carrying genes for neomycin resistance and SV40 large T antigen (Weingartl et al., 2002). 3D4 cells were maintained in RPMI 1640 (Gibco-BRL) supplemented with 10% FBS, 10 mM HEPES (Gibco-BRL), 1.0 mM sodium pyruvate (Gibco-BRL) and 0.1 mM nonessential amino acids (Gibco-BRL) (Weingartl et al., 2002). PK-15 and MARC-145 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% FBS. All cells were incubated in a humidified incubator at 37 °C with 5% CO_2.

The Korean Vero cell-adapted PEDV isolates, KPEDV-9 and SM98LVec, were propagated as described previously (Hofmann and Wyler, 1988). Briefly, Vero cells were inoculated with virus at a multiplicity of infection (m.o.i.) of 1 and cultured in serum-free MEM for 48 h at 37 °C with 5% CO_2. The progeny virions were titrated using the $TCID_{50}$ method. CSFV (LOM strain) and PRRSV (VR-2332 strain) were propagated in PK-15 cells and MARC-145 cells, respectively.

2.2. RT-PCR and sequence comparison

Clinical samples were obtained from piglets in the Chungman province that were exhibiting watery diarrhea and were screened by RT-PCR. Viral RNA was extracted from tissue homogenates using the Viral gene-spin viral DNA/RNA extraction kit (iNtRON Biotechnology, Sungnam, Korea) following the manufacturer's instructions. For first-strand cDNA synthesis, M-MLV reverse transcriptase (iNtRON Biotechnology, Korea) was used along with 10 μl of extracted viral RNA in a 50 μl randomly primed reaction. To exclude possible infection by other swine enteric viruses, specific primer sets were used for the detection of PEDV, TGEV and rotavirus (Song et al., 2006).

The specific primers used for the sequencing analysis of spike (S) protein were PEDV-S forward (GTGATGTTGTGTTAGGCTTGTTGAAG, 20541–20566) and PEDV-S reverse (CATCACTGCACGTGGACCTTTTC, 24769–24791). RT-PCR was performed at 94 °C for 2 min, followed by 40 cycles of 94 °C for 20 s, 54 °C for 10 s, 72 °C for 4 min, and a final extension was performed at 72 °C for 10 min. Amplified products were purified using the PCR clean-up kit following the manufacturer's instructions (GeneAll, Seoul, Korea) and sequenced. The nucleotide and deduced amino acid sequences of S protein were compared with those from published reference strains; CV777 (NC003436), DR13 (DQ462404), JS-2004-2 (AY654204), Chinju99 (AY167585), and KNU-0901 (GU180144). The multiple-sequencing alignments were generated using the MultAlin (http://multalin.toulouse.inra.fr) program, and similarities between nucleotide sequences were further assessed using the same software.

2.3. Experimental inoculation

With permission (permission number 20110345) and following the protocols approved by the Institutional Animal Care and Ethnic in Chungnam National University (CNU), Korea, all animals were cared for and experiments were performed in the animal facility at CNU. Healthy 3-day old colostrum-free piglets were inoculated orally with 2 ml of tissue homogenates from PVED infected animals or KPEDV-9. Controls were inoculated with phosphate buffered saline (PBS, pH 7.2). All piglets were examined daily for clinical signs such as diarrhea, anorexia, weight loss, vomiting, and dehydration. At 72 hpi, all piglets were euthanized and tissue samples processed for histological and molecular analysis.

2.4. Tissue sampling and histopathology

After the piglets were euthanized, necropsies were performed. Small intestines, lungs, livers, kidneys, and spleens were collected and each divided into two parts. For histological studies, tissue specimens were fixed in 10% (w/v) buffered formaldehyde for 48 h, and embedded in paraffin according to standard laboratory procedures. For virus re-isolation, tissue specimens were homogenized in PBS containing protease inhibitors (Sigma) using Tissue Lyser II homogenizer (Qiagen).

For histopathologic studies, sections were prepared from each of the formalin-fixed, paraffin-embedded tissues. These sections were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed in retrieval buffer (pH 6) using a pressure boiler as a heat source. The endogenous peroxidase was quenched by treating sections with 3% H_2O_2 in methanol for 30 min. All slides were incubated with 2% skim milk for 30 min at room temperature (RT) to saturate nonspecific protein-binding sites and then incubated with primary antibodies overnight at 4 °C. Mouse polyclonal anti-PEDV, which was obtained by immunizing mice with KPEDV-9, was used as the primary antibody. After three washes with PBS, sections were incubated for 30 min with biotinylated horse anti-mouse IgG diluted 1:200 in PBS. These were washed 4 times with PBS and incubated for 30 min with alkaline phosphatase-conjugated streptavidin. The slides were then washed 4 times with PBS and processed by immersing sections in 3,3′-diaminobenzidine substrate solution (Vector Laboratories, Burlingame, CA) at RT. Sections from all tissues were also stained with hematoxylin and eosin for histopathological examination.

2.5. Virus re-isolation

Tissue homogenates from PEDV-infected piglets were centrifuged and the resulting supernatant was used for virus isolation. Each sample resulting from these tissue homogenates was diluted (1:100 for small intestinal samples, 1:5 for other organ samples) in MEM containing 10 μg/ml of trypsin and added to a Vero cell monolayer and incubated for 1 h. After adsorption, cells were washed twice with PBS and then incubated in MEM containing 10 μg/ml of trypsin at 37 °C for 48 h. PEDV-infected cells were confirmed by an indirect immunofluorescence assay using mouse polyclonal anti-PEDV antibody. Cells were stained with Texas red-conjugated goat anti-mouse IgG and observed under a fluorescence microscope.

2.6. Susceptibility of porcine alveolar macrophages to PEDV

3D4 cells were seeded in 96-well Plates 24 h prior to infection with PEDV (KPEDV-9 strain), CSFV (LOM strain) or PRRSV (VR-2332 strain) at a MOI of 1. After virus adsorption, the inoculums were