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Development of transgenic mouse model expressing porcine aminopeptidase N and its susceptibility to porcine epidemic diarrhea virus

Jung-Eun Park[a,1], Eui-Soon Park[c,1], Jung-Eun Yu[c], Jaerang Rho[c], Sarita Paudel[a], Bang-Hun Hyun[d], Dong-Kun Yang[d], Hyun-Jin Shin[a,b,*]

[a] Laboratory of Infectious Diseases, College of Veterinary Medicine, Chungnam National University, Daejeon, Republic of Korea
[b] Research Institute of Veterinary Medicine, Chungnam National University, Daejeon, Republic of Korea
[c] Department of Microbiology & Molecular Biology, College of Bioscience & Biotechnology, Chungnam National University, Daejeon, Republic of Korea
[d] Animal and Plant Quarantine Agency, 175, Anyang-ro, Manan-gu, Anyang, Gyeonggi-do, Republic of Korea

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ABSTRACT

Porcine coronavirus infections have known as they are specific to pigs with predominantly enteric or respiratory diseases. No laboratory animal model is yet been developed in porcine coronaviruses study. Here, we report that development of a transgenic mouse model expressing porcine APN which is susceptible to porcine coronavirus infection. The porcine APN transgene was constructed by fusing with mouse proximal APN promoter at 5′ terminus and bovine growth hormone polyadenylation site at its 3′ terminus. After screen on pubs from the microinjected mice, we confirmed two transgenic lines expressing porcine APN in various organs. We confirmed the susceptibility to porcine epidemic diarrhea virus, one of the porcine coronaviruses. These transgenic mice will be an important tool for research into the porcine coronaviruses.

1. Introduction

The coronaviruses belong to the family *Coronaviridae* within the order *Nidovirales.* They are classified into four genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*, based on genetic similarities (Adams et al., 2014). The primary replication of the coronaviruses is often confined to respiratory- or gastrointestinal-tract epithelial cells, so they usually induce respiratory or enteric diseases, but also hepatic, renal and neuronal infections (Lednicky et al., 2013; Masters, 2006; Weiss and Navas-Martin, 2005).

The pathogenesis of several porcine coronaviruses, including transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCoV), has been broadly studied (Enjuanes et al., 1995; Saif, 2004a,b; Saif, 2004a,b; Weiss and Navas-Martin, 2005). TGEV is a major cause of viral enteritis and fetal diarrhea in swine, most severely in neonates and with a high mortality rate, which causes significant economic losses worldwide (Enjuanes et al., 1995). PRCoV is reported to be an attenuated variant of TGEV. PRCoV infects lung epithelial cells, and PRCoV antigen has been found in type I and type II pneumocytes and alveolar macrophages, and infection is followed by interstitial pneumonia (Halbur et al., 1993; Saif, 2004a,b). Porcine epidemic diarrhea virus (PEDV) is the causative agent of porcine epidemic diarrhea, which is characterized by watery diarrhea, as is TGEV infection (Chasey and Cartwright, 1978; Pensaert and de Bouck, 1978; Turgeon et al., 1980). PEDV-infected piglets usually show typical enteric signs, including profuse watery diarrhea, weight loss, and loss of milk uptake, entailing high mortality. PEDV has been reported in Europe and Asia, and also recently in the United States (Hess et al., 1980; Huang et al., 2013; Pan et al., 2012; Park et al., 2013; Wang et al., 2014). All these porcine coronaviruses belong to the genus *Alphacoronavirus*. A hemagglutinating enteric coronavirus, a member of the genus *Betacoronavirus*, is antigenically unrelated to the other porcine coronaviruses and uses a 5-N-acetyl-9-O-acetylneuraminic-acid-containing moiety as its cellular receptor.

* Corresponding author at: #302 Animal Hospital Building, College of Veterinary Medicine, Chungnam National University, Gungdong, Yuseong, Daejeon 305-764, Republic of Korea. Tel.: +82 42 821 6760; fax: +82 42 825 2993.
E-mail address: shin0089@cnu.ac.kr (H.-J. Shin).
[1] Jung-Eun Park and Eui-Soon Park, contributed equally to this work.

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Coronavirus infections are mediated by the spike (S) glycoprotein, a large surface glycoprotein on the viral envelope (Delmas and Laude, 1990; Masters, 2006). Coronavirus S glycoproteins recognize cellular receptors and mediate virus-cell fusion (Masters, 2006; Peng et al., 2011). Most *Alphacoronavirus*, including TGEV, PEDV, and PRCoV, use aminopeptidase N (APN/CD13) as their cellular receptor (Delmas et al., 1992; Li et al., 2007; Ren et al., 2010; Tresnan and Holmes, 1998; Yeager et al., 1992). APN/CD13 is a 150-kDa, zinc-dependent metalloprotease consisting of 967 amino acids (Rawlings and Barrett, 1995). Mammalian APN is ubiquitously expressed as a glycosylated homodimer on the surfaces of epithelial cells in the liver, intestine, kidney, and respiratory tract, and in fibroblasts and leukocytes, and plays multiple roles in many physiological processes, including coronavirus entry (Barnes et al., 1994; Luan and Xu, 2007; Mina-Osorio et al., 2008; Miura et al., 1983).

The natural hosts of porcine coronaviruses are young piglets, and clinical illness has only been observed in sucking piglets. However, *in vivo* studies using suckling piglets have many disadvantages, including their high cost, difficulty in handling the piglets, limited reagents, *etc.* Because the use of laboratory animal models (*e.g.*, mouse models) can circumvent these limitations, several transgenic animal models have been generated to study porcine viruses (Benbacer et al., 1998; Ono et al., 2006). Here, we generated transgenic mice expressing porcine APN under the control of the mouse proximal APN promoter. We tested their susceptibility to PEDV with reverse transcription-polymerase chain reaction (RT-PCR) and immunochemical analyses, and found that the porcine APN transgenic mice were susceptible to PEDV infection.

2. Materials and methods

2.1. Cells and viruses

Vero monkey kidney cells were maintained in minimal essential medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 250 μg/ml amphotericin B in a 5% CO_2 atmosphere at 37 °C. 293T human embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 250 μg/ml amphotericin B. All tissue culture reagents were purchased from Gibco (Carlsbad, CA, USA). KPEDV-9, a cell-adapted vaccine strain of PEDV, was grown and titrated in the Vero cells, as described previously, and stored at −80 °C until use (Cruz and Shin, 2007; Hofmann and Wyler, 1988).

2.2. Reagents and antibodies

The polyclonal antibodies specific for PEDV and porcine APN were generated in BALB/c mice immunized with KPEDV-9 and purified porcine APN, respectively, as described previously (Cruz et al., 2008). The antibody specificities were confirmed with enzyme-linked immunosorbent assays. The anti-Flag M2 monoclonal antibody (anti-Flag) and Anti-Flag M2 Affinity Gels (gel beads) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Construction of porcine APN transgene

The mouse proximal APN promoter region (starting at nucleotide-1044) was amplified from C57BL/6J genomic DNA by PCR with primers 5′-CCCGCGGCCGCAAGATTTGAAACAGTGGA-3′ and 5′-CCCAAGCTTGATGCCGGTGGACAGGGA-3′, containing flanking *Not*I and *Hin*dIII restriction endonuclease sites, respectively. The PCR product was cloned into the pBluescript KS (+) vector and the pGL3-Basic vector (Promega, Madison, WI, USA), which contains a promoterless luciferase reporter gene. The porcine APN gene was amplified from the total RNA isolated from porcine enterocytes with RT-PCR using specific primers and cloned into the pBluescript KS (+) vector. A sequence encoding the Flag epitope (DYKDDDDK) was fused to the 3′ terminus of porcine APN with PCR with primers 5′-CCCAAGCTTACCATGGCCAAGGGATTCTAC-3′ and 5′-CCCCTCGAGTCACTTGTCGTCATCGTCTTTGTAGTCGCTGTGCTCTAT-GAACCA-3′, which contain flanking *Hin*dIII and *Xho*I restriction endonuclease sites, respectively. The BGH-polyA sequence was amplified from the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) with PCR using primers 5′-CCCCTCGAGCGACTGTGCCTTCTAGTT-3′ and 5′-CCCGGTACCCCATAGAGCCCACCGCAT-3′, which contain flanking *Xho*I and *Kpn*I restriction endonuclease sites, respectively. The PCR product was cloned into the pBluescript KS (+) vector. All PCR products were confirmed with automated sequencing. To generate the porcine APN transgene, porcine APN-Flag and the mouse proximal APN promoter were ligated into pBluescript KS-BGH-polyA after they were digested with *Hin*dIII/*Xho*I and *Not*I/*Hin*dIII, respectively.

2.4. Promoter luciferase assay

293T cells (2.5×10^5 cells/ml) were plated in 24-well plates. After 24 h, the cells were transfected with pGL3-Basic-mouse proximal APN promoter (mAPN-luc) (0.2 μg or 1 μg). The cells were transfected in triplicate using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 24 h, the cells were lysed for assay with the luciferase assay system (Promega), according to the manufacture's protocol. All luciferase activities were normalized to β-galactosidase activity.

2.5. Generation and detection of porcine APN transgenic mice

The porcine APN transgene was linearized by restriction with *Not*I and purified with gel extraction (Qiagen, Valencia, CA, USA). Gain-of-function gene transfer was performed by microinjecting the purified DNA into the pronuclei of ICR mouse zygotes, which were then transferred into the oviducts of female recipient mice. The transgenic mice were identified with PCR analysis of tail genomic DNA with primers: PCR1-F: 5′-CCCAAGCTTACCATGGCCAAGGGATTCTAC-3′ and PCR1-R: 5′-GAAGTTGGAGAGCATCCT-3′; and PCR2-F: 5′-GGCGTCCTACTTGCATGC-3′ and PCR2-R: 5′-CCCCTC-GAGTCACTTGTCGTCATCGTCTTTGTAGTCGCTGTGCTCTATGAACCA-3′. The founder mice were backcrossed to the C57BL/6J background for five generations. All the mice used in this study were maintained in a specific-pathogen-free facility at the Biomedical Research Center at the Korea Advanced Institute of Science and Technology, Daejeon, Korea.

2.6. Infection of porcine APN transgenic mice with PEDV

All animals were cared for and the experiments were performed at the animal facility at Chungnam National University (CNU), Korea, with the permission of and according to protocols approved by the Institutional Animal Care and Ethics Committee of CNU (permission number 20110825). The porcine APN transgenic and nontransgenic wild-type mice were orally inoculated with 5×10^6 $TCID_{50}$ of KPEDV-9 or phosphate-buffered saline (PBS, pH 7.2) as the negative control. Their clinical signs were monitored and their feces collected for 5 days. Two mice from each group were killed on the indicated days after viral infection. The tissues were aseptically collected and prepared for RT-PCR and immunohistopathological analysis.