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Short Communication

Structural alteration of tight and adherens junctions in villous and crypt epithelium of the small and large intestine of conventional nursing piglets infected with porcine epidemic diarrhea virus

Kwonil Jung *, Bryan Eyerly, Thavamathi Annamalai, Zhongyan Lu, Linda J. Saif **

Food Animal Health Research Program, Ohio Agricultural Research and Development Center, College of Food, Agricultural, and Environmental Sciences, Department of Veterinary Preventive Medicine, The Ohio State University, Wooster, OH, USA

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ABSTRACT

Integrity of the intestinal epithelium is critical for proper functioning of the barrier that regulates absorption of water and restricts uptake of luminal bacteria. It is maintained mainly by tight junctions (TJs) and adherens junctions (AJs). We conducted immunofluorescence (IF) staining for *in situ* identification of zonula occludin (ZO)-1 proteins for TJ and E-Cadherin proteins for AJ in the small and large intestinal villous and crypt epithelium of nursing pigs infected with porcine epidemic diarrhea virus (PEDV). Twenty 9-day-old piglets [PEDV-infected ($n = 9$) and Mock ($n = 11$)] from PEDV seronegative sows, were orally inoculated [8.9 $\log_{10}$ genomic equivalents/pig] with PEDV PC21A strain or mock. At post-inoculation days (PIDs) 1–5, infected pigs showed severe watery diarrhea and/or vomiting and severe atrophic enteritis. By immunohistochemistry, PEDV antigens were evident in enterocytes lining the villous epithelium. At PIDs 1–5, PEDV-infected pigs exhibited mildly to extensively disorganized, irregular distribution and reduced expression of ZO-1 or E-Cadherin in villous, but not crypt epithelial cells of the jejunum and ileum, but not in the large intestine, when compared to the negative controls. The structural destruction and disorganization of TJ and AJ were extensive in PEDV-infected pigs at PIDs 1–3, but then appeared to reversibly recover at PID 5, as evident by increased numbers of ZO-1-positive epithelial cells and markedly improved appearance of E-Cadherin-positive villous epithelium. Our results suggest a possible involvement of structurally impaired TJ and AJ in the pathogenesis of PEDV, potentially leading to secondary bacterial infections.

* Corresponding author at: Food Animal Health Research Program, Ohio Agricultural Research and Development Center, College of Food, Agricultural, and Environmental Sciences, Department of Veterinary Preventive Medicine, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, USA. Tel.: +1 330 202 3569; fax: +1 330 263 3677.
** Corresponding author at: Food Animal Health Research Program, Ohio Agricultural Research and Development Center, College of Food, Agricultural, and Environmental Sciences, Department of Veterinary Preventive Medicine, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, USA. Tel.: +1 330 263 3744; fax: +1 330 263 3677.
E-mail addresses: jung.221@osu.edu (K. Jung), saif.2@osu.edu (L.J. Saif).

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1. Introduction

The intestinal epithelium provides a robust barrier that regulates absorption of nutrients and water and also restricts uptake of luminal bacteria (Su et al., 2011). The integrity of the intestinal epithelium is maintained by tight junctions (TJs), adherens junctions (AJs), desmosomes, and gap junctions. The TJ is the major paracellular barrier and functions to separate apical and basolateral compartments or membranes. The AJ forms a continuous belt between cells and is crucial for the maintenance of intercellular adhesion (Hartsock and Nelson, 2008; Su et al., 2011). The TJ and AJ are regulated by transmembrane TJ (occludin and claudin) and AJ (E-Cadherin and catenins) proteins on the opposing cells' plasma membranes and interactions mediated by actin binding proteins such as the zonula occludin (ZO) family, which link the AJ complex to actin microfilaments (Hartsock and Nelson, 2008; Su et al., 2011).

Porcine epidemic diarrhea virus (PEDV), which belongs to the genus *Alphacoronavirus* in the family *Coronaviridae*, causes high mortality of suckling pigs and substantial economic losses (Saif et al., 2012). Epidemic PEDV strains are highly enteropathogenic and acutely infect villous epithelial cells of the entire small and large intestines, but the jejunum and ileum are the primary sites of infection (Jung and Saif, 2015; Jung et al., 2014). During the early stages of PEDV infection, necrosis and exfoliation of infected villous epithelial cells is pronounced, resulting in acute, severe villous atrophy. To what extent the integrity and function of the PEDV-infected villous epithelium is restored by intestinal stem cells located at crypt cell layers is currently unclear. The aim of the present study was to determine whether PEDV infection causes structurally altered TJ and AJ in the villous and crypt epithelium of the small and large intestine by immunofluorescence (IF) staining for *in situ* identification of the related proteins, ZO-1 and E-Cadherin for TJ and AJ, respectively.

2. Materials and methods

2.1. Virus

The wild-type US PEDV strain PC21A was obtained from the intestinal contents of a diarrheic 1-day-old piglet on an Ohio farm in June 2013 (Jung et al., 2014). The original sample was serially passaged 2 times in Gn pigs. The original sample and Gn pig-passaged PC21A strain were confirmed to contain only PEDV, as reported in our previous study (Jung et al., 2014). The titer of Gn pig second-passaged PC21A strain was 11.8 $\log_{10}$ GE/ml and was used as virus inoculum after dilution in minimal essential medium (MEM).

2.2. Conventional specific-pathogen-free pigs and experimental pig infection

Two seronegative, Large White × Duroc crossbred, pregnant sows to acquire 20 nursing piglets, were obtained from a PEDV-free SPF (confirmed by history and seronegative sows; lack of qRT-PCR positive-fecal samples) swine herd of The Ohio State University. The SPF herd was seronegative for antibodies to PRRSV, PRCV, TGEV and porcine circovirus type 2. Twenty 9-day-old nursing piglets were randomly assigned to one of two groups: PEDV inoculated ($n = 9$) and Mock ($n = 11$). Each experimental group of pigs was housed in a separate room in a high-security isolation facility (biosafety level 2). Nursing pigs were inoculated orally [8.9 $\log_{10}$ GE (≈2.9 $\log_{10}$ plaque forming units)/pig] (Jung et al., 2014) with 1 ml of PC21A or mock inoculated with MEM. Inoculated and negative control pigs ($n = 3$–4/group at each time-point) were euthanized for pathological examination at an acute-stage (PID 1), at a mid-stage (PID 3), and at a later-stage (PID 5) of infection. After PEDV inoculation, the pigs were monitored for clinical signs 2–3 times daily until necropsy. The Institutional Animal Care and Use Committee (IACUC) of the Ohio State University approved all protocols related to the animal experiments and care in this study.

2.3. Gross and histological analysis and immunohistochemistry for the detection of PEDV antigen

Small (duodenum, proximal, middle and distal jejunum, and ileum) and large (cecum/colon) intestinal tissues and other major organs (lung, liver, heart, kidney, spleen, and lymph node) were examined grossly and histologically at PIDs 1, 3, and 5. Tissues were placed in 10% phosphate buffered formaldehyde (pH 7.0), dehydrated in graded alcohol, embedded in paraffin, and cut in 3-μm sections onto microscope slides, fixed and stained with hematoxylin and eosin (H&E) then analyzed for histopathological changes. Villous height and crypt depth were estimated by measuring at least 10 villi and crypts throughout the section. Mean ratios of jejunal villous height to crypt depth (VH:CD) were calculated as previously described (Jung et al., 2006). The formalin-fixed, paraffin-embedded tissues were prepared and tested by immunohistochemistry (IHC) for the detection of PEDV antigens, using monoclonal antibody 6C8-1 against the spike protein of PEDV strain DR13 (provided by Dr. Daesub Song, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). The antibody was diluted 1:200 in PBST [phosphate-buffered saline (PBS) containing Tween 20, 0.1%]. IHC was conducted, as described previously (Jung et al., 2009).

2.4. Immunofluorescence (IF) staining for the detection of ZO-1 and E-Cadherin

The frozen jejunal/ileal and cecal/colonic tissues were prepared in Tissue-Tek OCT compound (Sakura, Torrance, CA, USA) and tested by IF staining for the detection of ZO-1 and E-Cadherin using monoclonal antibodies against human recombinant ZO-1 and human E-Cadherin (Invitrogen, CA, USA). The anti-ZO-1 antibody was diluted 1:100 in PBST and incubated on the tissues at 4 °C overnight. The anti-E-Cadherin antibody was diluted 1:100 in PBST and incubated on the tissues at 4 °C overnight, and an anti-mouse antibody conjugated with Alexa Fluor®488 (Invitrogen) was used as the detection antibody and incubated on the tissues at 37 °C for 1 h. The