Research in Veterinary Science 110 (2017) 12–15

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Goblet cell depletion in small intestinal villous and crypt epithelium of conventional nursing and weaned pigs infected with porcine epidemic diarrhea virus

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ARTICLE INFO

Article history:
Received 21 April 2016
Received in revised form 26 August 2016
Accepted 22 October 2016

Keywords:
PEDV
Pathogenesis
Goblet cell
Pig
Virus

ABSTRACT

Intestinal goblet cells secret mucins to form mucus layers critical for maintaining the integrity of the intestinal epithelium. Porcine epidemic diarrhea virus (PEDV) causes watery diarrhea and high mortality of suckling pigs. PEDV mainly infects villous epithelial cells of the small intestine, and infected cells undergo acute, massive necrosis, followed by severe villous atrophy. Conventional 9-day-old nursing pigs [PEDV-inoculated ($n = 9$); Mock ($n = 11$)] and 26-day-old weaned [PEDV-inoculated ($n = 11$); Mock ($n = 9$)] were inoculated orally [8.9 $\log_{10}$ genomic equivalents/pig] with PEDV strain PC21A or mock. We used alcian blue or Periodic-Acid-Schiff staining for the detection of acidic or neutral mucin-secreting goblet cells in the small intestine. We demonstrated that PEDV infection of the nursing pigs at post-inoculation days (PIDs) 1–5 and weaned pigs at PIDs 3–5 led to depletion or significant reduction in the number of goblet cells (and also the number of villous goblet cells normalized by jejunal villous crypt height to crypt depth ratios) in the villi or crypts. These findings coincided with the development of intestinal villous atrophy. By immunohistochemistry, a few PEDV antigen-positive goblet cells were identified in the jejunal or ileal villous epithelium of the infected nursing or weaned pigs. During the early stages of PEDV infection, goblet cell mucins in the small intestine may be decreased, possibly leading to an impaired mucus layer and increased susceptibility to secondary enteric bacterial infection.

Intestinal villous epithelium lining the gastrointestinal tract mainly consists of the absorptive enterocytes and specialized secretory cells such as goblet cells (McCauley and Guasch, 2015). For both lubrication and barrier function of the intestinal epithelium against enteric pathogens, the intestinal mucosal surface is covered by the mucus layer(s) that mainly consist of mucins secreted by goblet cells (McCauley and Guasch, 2015). Goblet cells are critical to maintain intestinal homeostasis and integrity of intestinal epithelium. Loss or dysfunction of intestinal goblet cells is implicated in enteric disease (McCauley and Guasch, 2015).

Porcine epidemic diarrhea virus (PEDV) (family *Coronaviridae,* genus *Alphacoronavirus*) causes acute watery diarrhea, dehydration and high mortality of suckling pigs and substantial economic losses (Saif et al., 2012; Jung and Saif, 2015). PEDV is highly enteropathogenic and acutely infects 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, villous epithelial cells are infected, followed by acute, massive necrosis and exfoliation of infected cells, resulting in severe villous atrophy (Madson et al., 2016). The severity of clinical disease in suckling pigs may be exacerbated by secondary co-infections of other enteropathogens, such as *Escherichia coli* or other viruses (Jung and Saif, 2015). Another enteropathogenic *Alphacoronavirus*, transmissible gastroenteritis virus (TGEV), binds to goblet cells via sialic acid receptors expressed on the surface (Schwegmann-Wessels et al., 2003; Schwegmann-Wessels et al., 2011). Whether or how, after attaching to the surface sialic acids, TGEV enters them via a cellular receptor to replicate in goblet cells is unclear. Further studies are also needed to investigate whether the sialic acid binding activity of TGEV contributes to stabilizing and anchoring the virus in the intestine or mainly to providing the virus access to the cellular receptor, porcine aminopeptidase N (APN), expressed on the surface of enterocytes (Schwegmann-Wessels et al., 2011). However, similar to TGEV, whether PEDV also binds to goblet cells is unknown. A previous study showed that the number of goblet cells per intestinal villi of gnotobiotic pigs infected with an original US PEDV strain PC21A was reduced at post-inoculation hours 30–72, compared to the corresponding negative controls (Jung and Saif, 2015), suggesting a potential effect of PEDV infection on intestinal goblet cells. Therefore, our aim was to determine whether PEDV infection causes a reduced number of goblet cells in the villous and crypt epithelium of the small intestine of infected conventional nursing and weaned pigs. We used alcian blue (pH 2.5) (AB) and Periodic-Acid-

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http://dx.doi.org/10.1016/j.rvsc.2016.10.009
0034-5288/

Schiff (PAS) staining for the detection of the acidic and neutral sialylated mucin-secreting goblet cells, respectively.

All tissue samples tested were archival formalin-fixed, paraffin-embedded tissues acquired from twenty 9-day-old [PEDV infected ($n = 9$) and Mock ($n = 11$)] and twenty 26-day-old [PEDV infected ($n = 11$) and Mock ($n = 9$)] conventional pigs, inoculated orally with 8.9 $\log_{10}$ genomic equivalents of PEDV strain PC21A, or mock (modified Eagle's medium) (Jung et al., 2015). The clinical disease, fecal virus shedding, and gross and histopathology, including measurement of mean jejunal villous height: crypt depth (VH:CD) ratios to evaluate the severity of intestinal villous atrophy, were reported in a previous paper (Jung et al., 2015). Pigs ($n = 3$–4/time-point) were euthanized for pathologic examination at post-inoculation days (PIDs) 1, 3, and 5. The mid-jejunal and ileal tissues were stained with AB or PAS. Only well-orientated, intestinal tissue sections were evaluated to count the number of goblet cells per intestinal villus or crypt. Mean numbers of AB or PAS-stained goblet cells per villus or crypt were estimated by measuring at least 10 villi and crypts for each PID and from infected or control pigs. All values were expressed as the means ± standard deviation of the means (SDM). Mean numbers of AB or PAS-stained goblet cells per villus or crypt between PEDV-infected and uninfected nursing or weaned pigs at the same time-points were analyzed and compared by a Student's *t*-test using GraphPad Prism software (GraphPad Prism Inc.). A value of $P < 0.05$ was considered statistically significant.

The intestinal tissues were also tested by immunohistochemistry (IHC), as described previously (Jung et al., 2007; Jung et al., 2014), for the detection of PEDV antigen, using monoclonal antibody 6C8-1 against the spike protein of PEDV strain DR13 that was shown previously to cross-react with the US PEDV strain PC21A (provided by Daesub Song, Korean Research Institute of Bioscience and Biotechnology, Daejeon, Korea) (Jung et al., 2014). Briefly, endogenous alkaline phosphatase in rehydrated tissues was quenched with 3% glacial acetic acid 20% for 20 min at room temperature. Antigen retrieval was performed using 100 μg/ml of proteinase K (Invitrogen, Carlsbad, CA). The tissue slides were then washed in PBTS [phosphate-buffered saline (PBS) containing Tween 20, 0.1%] three times and blocked with 1× buffered solution of casein (Universal Blocking Reagent; Biogenex, Fremont, CA) in distilled water for 30 min at room temperature. Sections were coated with monoclonal antibody 6C8-1 diluted 1 in 200 in PBTS and incubated overnight at 4 °C in a humid chamber. After three washes with PBTS, the sections were incubated for 1 h at 36 °C with goat anti-mouse IgG labeled with alkaline phosphatase (Dako, Glostrup, Denmark) diluted 1 in 200 in PBTS. After three washes with PBTS, the final reaction was generated by immersing the tissue sections in a staining solution [1 tablet of Fast Red in 2 ml of 0.1 M Tris-buffer (pH 8.2); Roche Applied Science, Mannheim, Germany] for 10 min at room temperature. Sections were lightly counterstained with Gill's hematoxylin.

In infected nursing pigs, mean numbers of AB (acidic mucin)-stained goblet cells per villus and crypt were significantly ($P < 0.05$) reduced in mid-jejunum and ileum at PIDs 1, 3, and 5 (except for crypt in ileum at PID 5), compared to the corresponding negative controls (Table 1A; Fig. 1A and B). Similarly, mean numbers of PAS (neutral mucin)-stained goblet cells per villus and crypt were also significantly ($P < 0.05$) reduced in the mid-jejunum and ileum of the infected nursing pigs at PIDs 1, 3, and 5, but not in crypt in ileum at PIDs 1, 3, and 5 and in jejunum at PID 5 (Table 1A; Fig. 1C and D). The reduction rate, ranging from 1.8 to 16.5%, in the number of AB or PAS-stained goblet cells per villus in the jejunum of infected nursing pigs at PIDs 1–5 was also less than that (15.2 to 48.0%) in the ileum during the same period. Relative to the jejunal crypts of infected nursing pigs, goblet cells in the ileal crypts were less depleted at PIDs 1–5 (Table 1A), possibly contributing to more efficient compensation for loss of villous goblet cells.

Additionally to investigate if the reduced numbers of goblet cells observed per villus could be an expected outcome of the shortened villus following PEDV infection, mean numbers of goblet cells per villus were normalized by dividing by the mean intestinal VH:CD ratios acquired from infection and non-infection groups at each PID. All infected nursing pigs at PIDs 1–5 exhibited severe watery diarrhea and jejunal villous atrophy (mean VH:CD ratios of 1.1–1.4), whereas none of the uninfected control nursing pigs showed clinical signs. Their mean jejunal VH:CD ratios were 7.1–8.7 during the same period, as reported previously (Jung et al., 2015). In the jejunal villous epithelium of uninfected nursing pigs, the numbers of goblet cells normalized by mean jejunal VH:CD ratios were 2.3 (AB) or 2.5 (PAS), 2.6 (AB) or 3.0 (PAS), and 3.1

Table 1
Mean numbers (±SDM) of alcian blue (acidic mucin) or Periodic Acid Schiff (neutral mucin)-stained goblet cells per villi or crypt in the mid-jejunum and ileum of conventional 9-day-old nursing (A) and 26-day-old weaned (B) pigs infected with the original US PEDV strain PC21A at post-inoculation days (PIDs) 1, 3, and 5.

(A) Conventional 9-day-old nursing pigs

	PID 1				PID 3				PID 5			
	Jejunum		Ileum		Jejunum		Ileum		Jejunum		Ileum	
	Villus	Crypt	Villus	Crypt	Villus	Crypt	Villus	Crypt	Villus	Crypt	Villus	Crypt
Alcian blue-stained goblet cells												
Uninfected[a]	19.9 (6.8)	3.4 (1.8)	30.5 (6.7)	8.9 (3.8)	19.9 (6.0)	4.7 (1.9)	33.6 (5.6)	10.0 (3.3)	21.8 (7.4)	4.7 (1.9)	31.9 (11.6)	12.3 (6.0)
Infected[a]	**0.5 (0.8)**[b]	**1.4 (1.3)**	**10.8 (8.8)**	**6.3 (3.4)**	**1.1 (1.3)**	**2.9 (1.4)**	**5.1 (4.3)**	**5.8 (1.7)**	**1.6 (1.1)**	**2.5 (1.9)**	**15.3 (5.0)**	9.5 (1.4)
Periodic Acid Schiff-stained goblet cells												
Uninfected[a]	21.7 (5.0)	4.9 (1.6)	34.1 (6.8)	8.7 (3.6)	23.0 (5.7)	4.6 (2.0)	34.3 (6.3)	10.8 (3.6)	25.5 (9.0)	4.6 (1.9)	35.6 (6.6)	12.4 (4.2)
Infected[a]	**0.4 (0.7)**[b]	**1.9 (1.8)**	**12.5 (14.1)**	6.7 (3.2)	**2.0 (1.5)**	**2.9 (1.9)**	**9.6 (2.6)**	11.4 (5.3)	**4.2 (3.8)**	6.2 (3.5)	**15.2 (4.3)**	13.8 (5.3)

(B) Conventional 26-day-old weaned pigs

	PID 1				PID 3				PID 5			
	Jejunum		Ileum		Jejunum		Ileum		Jejunum		Ileum	
	Villus	Crypt	Villus	Crypt	Villus	Crypt	Villus	Crypt	Villus	Crypt	Villus	Crypt
Alcian blue-stained goblet cells												
Uninfected[c]	10.2 (3.2)	5.6 (2.2)	18.9 (8.1)	10.3 (3.6)	7.2 (3.4)	9.6 (4.7)	13.9 (6.3)	9.7 (3.2)	11.4 (2.9)	7.6 (3.7)	23.1 (7.7)	10.1 (3.3)
Infected[c]	8.1 (3.4)	7.7 (4.3)	20.0 (5.8)	8.5 (2.8)	**4.0 (3.9)**[b]	**5.2 (3.5)**	21.9 (4.5)	10.7 (5.7)	**1.8 (1.6)**	7.1 (2.6)	**15.9 (6.1)**	11.8 (5.0)
Periodic Acid Schiff-stained goblet cells												
Uninfected[c]	11.9 (4.2)	7.5 (3.4)	24.6 (11.0)	10.2 (4.6)	12.4 (4.1)	6.9 (5.0)	24. 2 (9.2)	11.0 (5.0)	19.8 (5.8)	9.1 (2.7)	27.3 (4.8)	10.8 (3.1)
Infected[c]	11.7 (4.9)	9.4 (4.6)	18.7 (6.7)	12.5 (4.9)	**4.3 (4.1)**[b]	6.8 (2.5)	21.0 (9.5)	13.1 (3.5)	**3.2 (2.6)**	**4.5 (3.0)**	**13.6 (8.2)**	9.3 (5.0)

[a] Uninfected, $n = 3$ at PID 1 and $n = 4$ at each PID 3 and 5; PEDV infected, $n = 3$ at each PID.
[b] Bold numbers, $P < 0.05$ (statistically significant differences between the PEDV-infected and uninfected pigs by Student's *t*-test).
[c] Uninfected, $n = 3$ at each PID; PEDV infected, $n = 3$ at PID 1 and $n = 4$ at each PID 3 and 5.