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Metalloprotease ADAM17 regulates porcine epidemic diarrhea virus infection by modifying aminopeptidase N

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) is a causative agent of porcine epidemic diarrhea (PED). PED, characterized by acute diarrhea, vomiting, dehydration, has caused serious economic losses in pig industry worldwide. Here, we demonstrate that activation of a disintergrin and metalloprotease 17 (ADAM17) induced the decrease of PEDV infection in HEK293 and IPEC-J2 cells and the downregulation of cell surface aminopeptidase N (APN) expression, an important entry factor for PEDV infection. Furthermore, overexpression of ADAM17 suppressed PEDV infection in HEK293 and IPEC-J2 cells, whereas ablation of ADAM17 expression using ADAM17 specific siRNA resulted in a corresponding increase of PEDV infection and an upregulation of cell surface APN expression. Taken together, these data demonstrate that modulation of APN expression by metalloprotease ADAM17 regulates PEDV infection. Hence, the reduction in APN expression represents another component of the anti-PEDV infection response initiated by ADAM17.

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is a positive single-strand RNA virus in the family *Coronaviridae* and order *Nidovirales*. PEDV is the causative agent of porcine epidemic diarrhea (PED), which is characterized by acute watery diarrhea, vomiting, dehydration and high mortality in pigs of all ages, especially in neonatal piglets (Li et al., 2012). PED was first reported in England in 1971 (Wood, 1977), and subsequently spread to other swine production countries. Since 2010, PED outbreaks have swept throughout East Asia and North America and dramatically damaged pig industry (Ding et al., 2014; Kim et al., 2015; Li et al., 2012; Liu et al., 2015; Mole, 2013).

Porcine aminopeptidase N (APN), an important entry factor on cell surface, plays a crucial role in PEDV infection (Li et al., 2007; Oh et al., 2003). It is well known that Vero E6 cell is widely used for the isolation and passage of PEDV as well as the model for studies on virus pathogenesis *in vitro*. In addition, PEDV has also been confirmed to infect other kinds of cell lines, including PK15 cell (pig kidney), huh-7 cell (human liver), Tb1-Lu cell (bat lung) and so on (Liu et al., 2015). However, Vero cell line is recognized to be unable to secrete type I interferon when infected with viruses, due to loss of the type I interferon gene cluster (Ding et al., 2014; Le Bon and Tough, 2002). Therefore, Vero cells are not recommended as the ideal model to investigate viral infection-driven innate immune responses. Although porcine intestine epithelial cells are the target cells for PEDV infection *in vivo*, the immortalized porcine intestinal epithelial cell clone J2 (IPEC-J2) has a low susceptibility to PEDV infection *in vitro* (Guo et al., 2016; Luo et al., 2017). Our previous research has demonstrated that HEK293 cells can be efficiently infected by PEDV and APN is involved in the virus infection, which might provide a useful tool for understanding the fundamental mechanisms involved in PEDV infection *in vitro* (Zhang et al., 2017). Moreover, HEK293 cells have been widely used in cell biology research for many years because of their reliable growth and propensity for transfection. Thus, the HEK293 cell as a new cell model is essential to gain a better understanding of the mechanisms of PEDV infection *in vitro*.

Cell surface metalloproteases coordinate signaling during development, tissue homeostasis, and disease. A disintegrin and metalloprotease 17 (ADAM17) is a well-characterized member among ADAM family, which mediates the cleavage of various cell surface proteins (Black, 2002; Black et al., 1997; Dello Sbarba and Rovida, 2002; Wang et al., 2011). We have reported previously that ADAM17 can regulate pig CD163, TNFα and CD16 on the cell surface, playing an important role in modulating inflammation and infection of PRRSV (Gu et al., 2016; Gu et al., 2015; Guo et al., 2014; Li et al., 2016). However, whether and how this metalloprotease ADAM17 is involved in PEDV

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infection remains unknown. In this study, the role of ADAM17 on PEDV infection was examined in HEK293 and IPEC-J2 cells, and we found that modulation of APN expression as a consequence of ADAM17 sheddase activity regulates PEDV infection.

2. Materials and Methods

2.1. Cells and virus

HEK293 (Human embryonic kidney 293 cell), IPEC-J2 (porcine intestine epithelial cell clone J2) and Vero E6 (African green monkey kidney epithelial cell) cell lines were grown and maintained in Dulbecco minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher). Cells were cultured in a humidified atmosphere at 37 °C in 5% CO_2. PEDV strain CV777 (GenBank accession number: KT323979) was grown and titrated in Vero E6 cells.

2.2. Metalloprotease ADAM17 inhibition/activation assay

In order to regulate the activity of metalloprotease ADAM17, batimastat (BB94; Sigma-Aldrich, St. Louis, MO) and phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO) were selected as the inhibitor or activator, respectively. 10^6 cells were treated with BB94 or PMA prior to PEDV infection at MOI of 0.1 (HEK293) or 1.0 (IPEC-J2). At 24 h post infection, cells were subjected to western blot analysis for detection of PEDV nucleocapsid (N) protein or collected to determine the progenies titers by the method of 50% tissue culture infective dose ($TCID_{50}$).

2.3. Overexpression of ADAM17 in HEK293 and IPEC-J2 cells

The gene of ADAM17 has successfully cloned into the mammalian expression vector pCAGGS (pCAGGS/ADAM17) and kept in our laboratory (Guo et al., 2014). When cells reached 80% confluence, cell monolayers were transfected with recombinant pCAGGS/ADAM17 or empty pCAGGS vector as mock control for 24 h prior to PEDV infection. At 24 h post infection, cells were subjected to western blot analysis and progenies titration as indicated above.

2.4. RNA interference

Small interfering RNA (siRNA) was introduced to knock down the endogenous expression of ADAM17. Cells were transfected with ADAM17-specific (siADAM17) or negative control siRNA (siNC) duplexes at concentration of 100 nM using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. The sense strand sequence of siRNA against ADAM17 used in this study is 5'-GAGAGUACAACUACAAAUU-3'. The sequence of negative control siRNA is 5'-UUCUCCGAACGUGUCACGUTT-3'. At 24 h post transfection, PEDV infection was performed at MOI of 0.1 (HEK293) or 1.0 (IPEC-J2), respectively.

2.5. Western blot

Western blot analysis was performed as previously described with a slight modification (Gu et al., 2015). Typically, samples were separated by SDS-PAGE under reducing conditions and transferred onto a PVDF membrane. After blocking, the membranes were incubated with a primary antibody and then incubated with an appropriate IRDye-conjugated secondary antibody (Li-Cor Biosciences, Lincoln, NE). The membranes were scanned using an Odyssey instrument (Li-Cor Biosciences) according to the manufacturer's instructions. The anti-ADAM17 polyclonal antibody was purchased from Abcam (Abcam, Cambridge, MA). Mouse anti-Flag antibody was purchased from Sigma (Sigma-Aldrich, USA) and used to detect overexpression of ADAM17 fused with a Flag tag in the carboxyl terminal. Mouse mAb 2G3 against PEDV N protein was stocked in our laboratory. Anti-β-actin mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.6. $TCID_{50}$ assay

Collected virus samples were clarified by centrifugation prior to titration by the method of $TCID_{50}$. $TCID_{50}$ assays were performed on Vero E6 cells following the method of Reed & Muench as previously described (Chua et al., 2008). Briefly, cell monolayers in 96-well tissue-culture plates (Corning, USA) were inoculated with 100 μl 10-fold serial dilutions of each virus stock and incubated for 4 days prior to observation of the presence of cytopathic effect.

2.7. Flow cytometry

Flow cytometric analyses were performed on a FACSAria instrument (BD Biosciences) as described previously (Wang et al., 2009). Cells were fixed with a kit following the manufacturer's protocol (eBioscience). Isotype-matched negative control mAbs were used to evaluate levels of nonspecific staining. Typically, 10,000 labeled cells were analyzed. All samples were analyzed using FlowJo 8.7 (Tree Star) and FACS Diva (BD Biosciences).

2.8. Statistical analysis

Values are expressed as mean ± SD. Data were analyzed using Student's t test and analyzed using Prism 5. A p value of < 0.05 was considered significant.

3. Results and Discussion

3.1. Effects of metalloprotease inhibitor/activator on PEDV infection

Previous research has demonstrated that metalloprotease ADAM17 plays an important role in regulating cell surface proteins, such as CD163, CD16b, L-selectin, *etc.* (Droste et al., 1999; Wang et al., 2013; Wang et al., 2010). In order to evaluate the involvement of ADAM17 on PEDV infection, HEK293 and IPEC-J2 cell monolayers were treated with a metalloprotease inhibitor BB94 or carrier control DMSO as previously described (Guo et al., 2014). As shown in western blot, higher levels of PEDV N protein were detected in HEK293 cells with BB94 treatment than that with DMSO treatment (Fig. 1A). Moreover, BB94 treatment significantly increased the virus titer compared to that in DMSO mock treatment ($p < 0.05$) (Fig. 1A), suggesting that inhibition of ADAM17 activity facilitates PEDV infection. Meanwhile, a metalloprotease activator PMA was also introduced to examine the effect of ADAM17 on PEDV infection. As shown in Fig. 1B, PMA treatment downregulated the PEDV N protein expression in HEK293 cells and led to a lower progeny virus titer than control treatment ($p < 0.05$), indicating that the metalloprotease ADAM17 activation can inhibit PEDV infection. As was the case with HEK293 cells, PEDV infection of IPEC-J2 cells was upregulated by treatment with BB94 and was downregulated with activator PMA treatment as determined by $TCID_{50}$ ($p < 0.05$) (Fig. 1C). To rule out the side effect of inhibitor or activator, the levels of endogenous ADAM17 expression were analyzed by western bot. The results showed that neither BB94 nor PMA had an effect on ADAM17 expression (Fig. 1D), suggesting that the differences in PEDV infectivity are associated to ADAM17 activity. The inhibitory effect of ADAM17 on PEDV infection is similar to that on porcine reproductive and respiratory syndrome virus (PRRSV) infection (Guo et al., 2014; Li et al., 2016).

3.2. ADAM17 suppresses PEDV infection

Preliminary results have been conveyed that the metalloprotease

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