Elevated plasma-soluble CD16 levels in porcine reproductive and respiratory syndrome virus-infected pigs: correlation with ADAM17-mediated shedding

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Soluble CD16 (sCD16) is closely correlated with chronic diseases in humans. Here, plasma sCD16 levels in pigs were increased by infection with porcine reproductive and respiratory syndrome virus (PRRSV) but not with porcine epidemic diarrhea virus, porcine circovirus type 2 and pseudorabies virus. Of interest, PRRSV attached to blood neutrophils and reduced surface CD16 expression on neutrophils. In vitro data confirmed that PRRSV caused CD16 shedding in neutrophils. Further analyses revealed that ADAM17 was involved in porcine CD16 shedding. Thus, our findings suggest that increase in sCD16 levels may be an indicator of PRRSV infection.

Porcine reproductive and respiratory syndrome virus (PRRSV), a positive-stranded RNA virus in the family Arteriviridae, is the aetiological agent of porcine reproductive and respiratory syndrome (PRRS). Although first reported over 25 years ago, PRRS remains a severe disease and causes huge economic losses in pig production worldwide (Meulenberg et al., 1993; Nathues et al., 2014; Tian et al., 2007; Tornimbene et al., 2015; Zhou & Yang, 2010). The most important pathological characteristics of PRRS are lung inflammation, loss of epithelial barrier function, immune cell migration, and increased vascular permeability (Han et al., 2014; Weesendorp et al., 2014). PRRSV mainly replicates in lungs, tonsils and lymphoid organs, and induces a persistent viral infection. Although an important aspect of PRRSV infection is the persistence of viraemia (Allende et al., 2000; Wills et al., 2003, 1997), the link between viraemia and PRRS is poorly understood.

CD16, the type III receptor for IgG, is highly expressed on neutrophils and primarily recognizes IgG-containing immune complexes, providing an important link between innate and adaptive immunity. Our recent data suggested an important role of cellular CD16 in the antibody-dependent enhancement of PRRSV infection (Gu et al., 2015). The membrane-bound CD16 can be released in a soluble form (sCD16) in vivo and in vitro through proteolytic cleavage (shedding) (Galon et al., 1998; Huizinga et al., 1990; Wang et al., 2013). Several studies have shown that plasma sCD16 is involved in various inflammatory conditions, such as multiple myeloma (Mathiot et al., 1993), human immunodeficiency virus infection (Bouhlal et al., 2001) and coronary heart disease (Huang et al., 2012). Whether CD16 is involved in the pathogenesis of PRRSV, however, has not been reported.

In the current study, we evaluated the plasma levels of CD16 upon PRRSV infection. The animal experiment was approved by the Harbin Veterinary Research Institute and was performed in accordance with animal ethical guidelines and approved protocols. The animal ethics committee approval number is Heilongjiang-SYXK-2006-032.

Twelve healthy piglets obtained from a farrow free of PRRSV were randomly assigned to a mock control group (six piglets) or to a highly pathogenic PRRSV HuN4 (GenBank accession no. EF635006) infection group (six piglets) and were inoculated as previously described (Yang et al., 2012). Plasma samples were collected from all of the animals at 0, 1, 3 and 7 days post-inoculation (p.i.) and were assayed for sCD16 levels by ELISA. A well-established, commercially available porcine ELISA is not currently available. We developed a quantitative ELISA kit using a home-made rabbit polyclonal antibody against porcine CD16 and mouse anti-porcine CD16 mAb G7 (BioLegend) as previously described (Romee et al., 2013). At day 0 (before inoculation), the plasma levels of sCD16 in all piglets were below 1.2 μg ml⁻¹ (Fig. 1a). Throughout the study, plasma sCD16 levels in the six mock control piglets remained stable and did not significantly differ from 1.2 μg ml⁻¹. In contrast, plasma sCD16 levels significantly increased in virus-inoculated piglets at 3 and 7 days p.i.: two out of the six piglets in the virus-inoculated group...
PRRSV affects CD16 release

(a) Time (days p.i.)

Soluble CD16 (µg ml⁻¹)

Mock
PRRSV

0 1 3 7

(b) 0 day p.i. 7 days p.i.

Soluble CD16 (µg ml⁻¹)

PEDV PCV2 PRV

(c) CD16

Cell number

1 day p.i. 3 days p.i. 7 days p.i.

Mock control PRRSV HuN4

(d) CD16 staining (MF1)

Mock PRRSV

1 day p.i. 3 days p.i. 7 days p.i.

(e) Cell number

Mock control PRRSV HuN4

(f) PRRSV staining (MF1)

Mock PRRSV
began to show high levels of sCD16 at 1 day p.i.; and all six infected piglets showed much higher levels of sCD16 in comparison with the control group at 7 days p.i. Similar to our previous data (Yang et al., 2012), virus could be detected in the blood of PRRSV-infected piglets at 1, 3 and 7 days p.i. (data not shown), indicating that viraemia may initiate sCD16 production. To determine whether this increase in plasma sCD16 levels was specific to PRRSV, we measured the effects of other porcine viruses on plasma sCD16 levels. Plasma samples were collected at 0 and 7 days p.i. Neutrophils were stained for surface expression of CD16 with APC-conjugated anti-porcine CD16 mAb G7 (BioLegend) and were determined by flow cytometry. x-axis, log10 fluorescence. (d) Mean fluorescence intensity (MFI) for CD16 expression on neutrophils after PRRSV inoculation. The fluorescence density of CD16 on neutrophils was expressed as MFI. The line in each box represents the median MFI. (e) Levels of PRRSV on neutrophils after PRRSV inoculation. Neutrophils were stained for PRRSV with FITC-conjugated anti-PRRSV nucleocapsid protein mAb SDOW17 (Rural Technologies) and were examined by flow cytometry. x-axis, log10 fluorescence. (f) MFI for PRRSV density on neutrophils after PRRSV inoculation. The line in each box represents the median MFI. Flow cytometry data were analysed by FlowJo 8.7. The significance of the differences among groups was determined with Student’s t-test; *, P<0.05.

Huizinga et al. (1990) pointed out that the plasma sCD16 mainly originates from activation-induced release by neutrophils, suggesting that the increased sCD16 in the plasma of PRRSV-infected pigs might be produced by neutrophils. Therefore, we monitored the levels of surface CD16 on circulating neutrophils by flow cytometry. We found that PRRSV-inoculated animals had significantly lower levels of CD16 expression on their neutrophils than the mock controls at 3 and 7 days p.i. but not at 1 day p.i. (Fig. 1c). Fig. 1(d) summarizes the data for CD16 fluorescence intensity on porcine neutrophils. These results may be explained by the fact that the PRRSV viral load is higher at 3 and 7 days p.i. than at 1 day p.i. (Yang et al., 2012). Similarly, Gimeno et al. (2011) observed CD16 downregulation on bone marrow-derived dendritic cells following PRRSV treatment. Our data indicate that the levels of CD16 on the cell surface are inversely correlated with the levels of sCD16 in plasma, indicating a correlation between the amplitude of neutrophil activation and PRRSV infection. However, previous reports showed that PRRSV has a tropism for cells of the monocytic lineage, and there is no evidence that porcine neutrophils are susceptible to PRRSV (Duan et al., 1997; Han et al., 2014; Molitor et al., 1997; Shibata et al., 1997). Thus, we stained circulating neutrophils with anti-PRRSV nucleocapsid protein mAb and examined them by flow cytometry. The levels of PRRSV fluorescence intensity were greater on neutrophils from virus-inoculated pigs than from mock controls at 3 and 7 days p.i. (Fig. 1e, f). These data suggest that viraemia or PRRSV may directly activate porcine neutrophils and induce CD16 shedding.

To further evaluate the correlation between the CD16 levels and PRRSV, we examined porcine neutrophils following exposure to PRRSV in vitro. Consistent with the in vivo data, the in vitro findings showed that exposure of neutrophils to PRRSV induced the downregulation of CD16 expression and the release of sCD16 (Fig. 2a). To determine whether CD16 shedding by PRRSV depends on virus infectivity or simply on virus–cell surface interaction, neutrophils were treated with UV-inactivated virus. CD16 shedding occurred in cells treated with viral preparations, regardless of their infectivity (Fig. 2a). Thus, viable PRRSV is not required for CD16 shedding from porcine neutrophils. Additional experiments were performed to determine whether neutrophils are activated by PRRSV; these experiments measured cellular surface levels of CD11b, which is a marker of neutrophil activation (De Clerck et al., 1995). As shown in Fig. 2(b), CD11b was upregulated by both replicative PRRSV and UV-inactivated PRRSV, indicative of activation of neutrophils upon exposure to virus particles. Likewise, human neutrophils are not susceptible to influenza A virus, but the interaction between neutrophils and the virus can cause the cells to release inflammatory cytokines (Daigneault et al., 1992; Tate et al., 2011; Wang et al., 2008). Our results indicate that PRRSV interacts with porcine neutrophils, which causes the neutrophils to release sCD16.

Having found that sCD16 is produced by PRRSV-treated neutrophils, we were interested in determining the underlying production mechanism. Certain members of the A Disintegrin and Metalloprotease (ADAM) family are...
known to regulate the density of various surface molecules on different types of cells by ectodomain shedding (Reiss & Saftig, 2009). ADAM17 is a well-characterized ADAM sheddase and plays a broad role in cleaving various cell-surface proteins (Arribas & Esselens, 2009). We previously reported that human CD16a and CD16b are cleaved from activated natural killer cells or neutrophils by ADAM17 (Romee et al., 2013; Wang et al., 2013). Unlike in humans, only one gene in porcine immune cells encodes CD16, which is expressed as a transmembrane protein similar to human CD16a (Sweeney et al., 1996). Therefore, porcine neutrophils were pretreated with the ADAM17 inhibitor batimastat (BB94) (Rio et al., 2000) and then incubated with PRRSV. As indicated by flow cytometry and ELISA, the downregulation of CD16 surface expression and the release of sCD16 induced by PRRSV were prevented by BB94 (Fig. 3a), suggesting that ADAM17 is involved in porcine CD16 shedding. Previously, we found that targeting ADAM17 in neutrophils is problematic because their manipulation by transfection induced some level of activation (Wang et al., 2010), which may confound our results. Because CD16 was successfully expressed in HEK293T cells by transfection with porcine CD16 and FcRγ-chain DNAs (Fig. 3b), which is consistent with our previous report (Gu et al., 2015), HEK293T/CD16 cells were used to obtain additional evidence concerning the involvement of ADAM17 in porcine CD16 shedding. As was found when porcine neutrophils were pretreated with BB94, pretreatment of HEK293T/CD16 cells with BB94 significantly reduced CD16 down-regulation and sCD16 production, indicating that ADAM17 is involved in CD16 shedding. Western blot assay confirmed a significant increase in cell-associated CD16 and a corresponding decrease in the levels of sCD16 in the cell supernatant in the presence of BB94 (Fig. 3c). To validate the results obtained with pharmacological inhibitor BB94, we reduced the endogenous expression of ADAM17 by using small interfering RNA (siRNA) duplexes that targeted the ADAM17 gene. CD16 shedding was significantly lower in cells treated with specific siRNA rather than with control siRNA (Fig. 3d). Western blot analysis verified a significant reduction in the level of endogenous ADAM17 protein in cells transfected with the specific siRNA (Fig. 3e). Moreover, we evaluated the effect of ADAM17 on CD16 shedding in an overexpression assay and found that CD16 shedding was increased when ADAM17 was overexpressed (Fig. 3f). The overexpression of ADAM17 was also confirmed in the cells by Western blot (Fig. 3g). Together, these findings indicate that ADAM17 is involved in the shedding of porcine CD16 during PRRSV treatment.

In summary, our data are the first, we believe, to demonstrate that PRRSV infection is associated with plasma levels of sCD16. When neutrophils interact with PRRSV they are activated, resulting in an increase in sCD16 production. CD16 levels on the surface of neutrophils then decrease and sCD16 levels increase as a consequence of ADAM17-mediated shedding. Therefore, the plasma level of sCD16 may serve as an important biomarker of PRRSV infection. Although we have not yet determined the role of CD16 shedding in PRRSV-infected animals, several possibilities should be explored. The reduced CD16 expression in neutrophils may be the consequence of the inefficient removal of immune complexes from the

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**Fig. 2.** Expression of CD16 and CD11b on the surface of porcine neutrophils following exposure to PRRSV in vitro. (a) Neutrophils were incubated with mock, PRRSV or UV-inactivated PRRSV for 12 h at 37 °C. Relative levels of CD16 surface expression were determined by flow cytometry and were analysed by FlowJo 8.7 (upper panel). Levels of sCD16 in cell supernatant were examined by ELISA (lower panel). Values are the means ± SD of three independent experiments. P-values were calculated using Student’s t-test; *, P<0.05. (b) Surface expression of CD11b on neutrophils following exposure to mock, PRRSV or UV-inactivated PRRSV for 12 h. Neutrophils were stained for surface expression of CD11b with mouse anti-porcine CD11b mAb 3E10 (Antigenix). Data are representative of at least three independent experiments.
Fig. 3. Effect of ADAM17 on porcine CD16 shedding. (a) Inhibition of ADAM17 prevents the reduction of CD16 levels on neutrophils by PRRSV and reduces CD16 shedding. Neutrophils were incubated with PRRSV in the presence or absence of the ADAM17 inhibitor BB94 for 12 h at 37°C. Relative levels of CD16 surface expression were determined by flow cytometry (left panel). Levels of sCD16 in cell supernatant were determined by ELISA (right panel). (b, c) The ADAM17 inhibitor BB94 impairs CD16 shedding. HEK293T cells were transfected with porcine CD16 and FcRɣ-chain DNAs to express CD16. The HEK293T/CD16 cells were then treated with BB94 or control for 24 h before the relative levels of CD16 surface expression and sCD16 were determined (b). HEK293T/CD16 cells were treated as in (a), and detergent lysates (left panel) as well as media supernatants (right panel) from equivalent cell numbers were subject to Western blot with antibodies to CD16 (mAb G7) or β-actin (loading control; Santa Cruz) (c). (d, e) Specific siRNA targeting ADAM17 inhibits CD16 shedding. HEK293T/CD16 cells were transfected with siRNA duplexes specific to endogenous ADAM17 or with control siRNA for 24 h. Relative levels of CD16 expression and sCD16 in cell supernatant were determined (d). Detergent lysates from the cells in (d) were subjected to Western blot with antibodies to ADAM17 (Abcam) or β-actin (e). (f, g) ADAM17 overexpression induces CD16 shedding. HEK293T/CD16 cells were transfected with Flag-tagged ADAM17 DNA or vector control. Relative levels of CD16 expression on cell surface and relative levels of sCD16 in cell supernatants are shown (f). Detergent lysates
from these cells were subjected to Western blot with antibodies to Flag (Sigma) or β-actin (g). At least three independent experiments were performed, and representative results are shown. ELISA values are the means ± sd of three independent experiments. P-values were calculated using Student’s t-test; * P<0.05.

circulatory system, which might increase the susceptibility of animals with PRRS to further infection. On the other hand, activated neutrophils may be further activated to run against pathogen infection because as Galon et al. (1996) found, sCD16 can stimulate neutrophils to produce IL-8 by binding to complement receptors. Determining the effects of CD16 shedding in PRRS-infected pigs will require additional research.

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References


