**Clostridium perfringens** beta-toxin targets endothelial cells in necrotizing enteritis in piglets

J. Miclard, M. Jäggi, E. Sutter, M. Wyder, B. Grabscheid, H. Posthaus*

Institute of Animal Pathology, Vetsuisse Faculty, University of Bern, PO-Box 8466, 3001 Bern, Switzerland

**1. Introduction**

*Clostridium perfringens*, a Gram-positive, anaerobic, spore-forming bacterium is an important cause of enteric disease in animals and humans (Johnson and Gerding, 1997; Songer, 1996). Based on the production of 4 major toxins, alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX), *C. perfringens* isolates are classified into 5 toxino-types (A–E) (Petit et al., 1999). *C. perfringens* type C strains are defined by the production of CPA and CPB and cause severe segmental, necro-hemorrhagic enteritis in pigs, sheep, goats, and calves (Songer, 1996). The most commonly affected species is the pig, in which necrotizing enteritis leads to high mortality rates amongst newborn piglets (Songer and Uzal, 2005). Experimental infections in pigs and the rabbit ileal loop model showed that *C. perfringens* type C initiates necrosis of enterocytes at the apices of jejunal villi which progresses to mucosal necrosis (Songer, 1996; Vidal et al., 2008). Damage to microvilli, mitochondria and terminal capillaries was however reported to occur prior to bacterial adhesion and the molecular basis for this remains unknown (Songer, 1996). CPB is both required and sufficient to induce typical necrotizing intestinal lesions in the rabbit ileal loop model (Sayeed et al., 2008; Vidal et al., 2008). Additionally, CPB increases capillary permeability upon intradermal injection in mice, indicating a direct vascular interaction (Nagahama et al., 2003b; Sakurai and Nagahama, 2006). CPB is secreted as a monomeric toxin, which subsequently forms multimeric, cation-selective, pores in susceptible membranes (Shatursky et al., 2000; Nagahama et al., 2003a). So far only human endothelial cells (Steinthorsdottir et al., 2000) and a human leukaemia cell line (HL60) (Nagahama et al., 2003a) demonstrated susceptibility to CPB, indicating cell specificity of the toxin. Detailed studies on the cell tropism of CPB in naturally occurring or experimentally induced lesions are however lacking. Thus, the natural target cells of CPB are unknown. The goal of our

---

* Corresponding author. Tel.: +41 316312399; fax: +41 316312635. E-mail address: horst.posthaus@itpa.unibe.ch (H. Posthaus).

0378-1135/$ – see front matter © 2009 Elsevier B.V. All rights reserved. doi:10.1016/j.vetmic.2009.01.025
study was to localize CPB in lesions of naturally acquired *C. perfringens* type C enteritis in piglets. To achieve this, we retrospectively evaluated tissues from 52 piglets with confirmed *C. perfringens* type C enteritis and 14 control animals by immunohistochemistry.

2. Materials and methods

2.1. Animals

Formalin fixed, paraffin embedded tissue sections of sixty-five 1 day to 3 weeks old piglets and one 2 months old fattening pig submitted for routine diagnostic necropsy were selected for the study. 52 of these piglets were selected because they showed classical morphological lesions of *C. perfringens* type C enteritis (Taylor, 1999) in addition to the identification of *C. perfringens* type C by 2 separate approaches. First, routine diagnostic bacteriological cultivation of intestinal samples for *C. perfringens* followed by 100% ethanol (twice for 15 min). Endogenous cytochemistry was confirmed *C. perfringens* type C enteritis in piglets. To achieve this, we retrospectively evaluated tissues from 52 piglets with confirmed *C. perfringens* type C enteritis and 14 control animals by immunohistochemistry.

Following the PCR based genotyping of 5 pooled colonies (Albini et al., 2008) identified *C. perfringens* type C in each case. Secondly, multiplex real-time PCR investigations (Albini et al., 2008) on DNA extracts of small intestinal case, confirming *C. perfringens* type C infection.

Fourteen animals (13 suckling piglets and 1 fattening pig), not affected by necrotizing enteritis, were chosen as controls. The cause of death of the suckling piglets was *Escherichia coli* diarrhea (K88) in 4, *Isospora suis* infection in 2, inanition in 2 and accidental crushing by the sow in 2 animals. Three piglets were littermates of animals affected by necrotizing enteritis and were euthanized for diagnostic purposes. The fattening pig died of small intestinal volvulus (hemorrhagic intestinal syndrome). The 3 control piglets which derived from herds with recent outbreaks of necrotizing enteritis were culture positive for *C. perfringens* type C. In the remaining 11 animals (10 suckling piglets and the fattening pig) *cpb2* positive *C. perfringens* type A but no *C. perfringens* type C were identified by culturing and direct multiplex real-time PCR on intestinal DNA extracts.

2.2. Histopathology

Representative samples of small and large intestine were fixed in 10% buffered formalin for 24 h. From 5 animals with necrotizing enteritis additional samples of lung, liver, spleen, kidney and brain were taken. All tissues were routinely processed for histology and stained with Haematoxylin & Eosin (H&E). Histologically, the extend of infiltration with neutrophilic granulocytes was graded as 0 (no infiltration), 1 (small amounts of perivascular neutrophilic granulocytes), 2 (moderate amounts of neutrophilic granulocytes underneath the necrotic mucosa), or 3 (large amounts of neutrophilic granulocytes demarcating the necrotic mucosa).

2.3. Immunohistochemistry

Histological sections were deparaffinized with xylene followed by 100% ethanol (twice for 15 min). Endogenous peroxidase activity was inhibited by H$_2$O$_2$ (0.1% in PBS, 15 min) and slides were incubated with 0.1% proteinase (Sigma-Aldrich, in TBS, pH 7.6, 15 min at 37 °C). Primary antibodies (Center for Veterinary Biologics, Ames, Iowa) were: mouse monoclonal anti-CPB (mAb-CPB, 10A2), mouse monoclonal anti-CPA (mAb-CPA, 6H7 1F3) and mouse monoclonal anti-Clostridium chauvoei flagella protein (7D11). Slides were incubated for 2 h at room temperature (RT) with primary antibodies (1:100 in PBS, pH 7.5). The LSAB and AEC Kit (DakoCytomation) were used for secondary antibody incubation and signal detection. Slides were counterstained with Haemalaun. The distribution and intensity of positive signals at the endothelial lining, within the necrotic debris and within inflammatory cells was assessed by light microscopy and graded as 0 (no staining), 1 (low), 2 (moderate), or 3 (strong signal intensity).

2.4. Immunofluorescence

Histological sections of the small intestine from 20 animals with moderate or strong mAb-CPB immunolabelling were processed as described for immunohistchemistry. Slides were incubated with mAb-CPB and rabbit anti-factor-8 antibody as a marker for endothelial cells (DakoCytomation, 1:100 in PBS, 0.1% bovine serum albumin, 2 h at RT). Secondary antibodies were: Alexa Fluor 488 labelled goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG (1:1000, Molecular Probes, 60 min at RT). Slides were viewed under a Nikon Eclipse 800 fluorescence microscope. Digital photographs were processed using the OpenLAB software package (Improvision).

3. Results

3.1. Histopathology

3.1.1. Necrotizing enteritis cases

Histologically, 18 out of the 52 animals with typical segmental necro-hemorrhagic enteritis showed no inflammatory reaction (grade 0), compatible with a peracute course of the disease. Lesions were characterized by deep coagulation necrosis of the jejunal mucosa associated with marked hemorrhages into the lamina propria, submucosa and muscular layers (Fig. 1A). Numerous small and medium sized vessels in the lamina propria and submucosa contained fibrin thrombi and there was multifocal acute vascular necrosis (Fig. 1B). Numerous, clostridial-like organisms lining the necrotic villi were present. 12 piglets showed in addition to the above described lesions a grade 1 inflammatory reaction.

In 22 animals, lesions compatible with a more protracted, acute to subacute course of the disease were present. 13 of these 22 animals showed grade 2 inflammatory reactions (Fig. 1E), 9 grade 3. Lesions in these animals were characterized by complete coagulation necrosis of the mucosa and demarcation of the necrotic zone by infiltration with moderate to large numbers of mainly degenerated neutrophilic granulocytes (Fig. 1E). Within the lamina propria and submucosa,
there was extensive vascular necrosis with widespread loss of vascular endothelium (Fig. 1F). Many of the remaining vessels contained fibrin thrombi. Segments of macroscopically non-affected duodenum, terminal ileum and colon of each animal with necrotizing enteritis did not show histopathological alterations. No additional lesions were found in the liver, spleen, lung, kidney or brain of 5 piglets.
3.1.2. Control cases

Three control C. perfringens type C carrier piglets (Supplementary Fig. A and B) as well as the piglets which died from starvation or crushing from the sow showed no intestinal lesions. E. coli diarrhea cases were characterized by attachment of cocccoid bacteria on the surface of enterocytes and I. suis infected animals depicted marked villous atrophy with intracytoplasmic protozoa in enterocytes of the ileum. Lesions of the small intestinal volvulus (hemorrhagic intestinal syndrome) of the 2 months old fattening pig were characterized by massive transmural hemorrhages and edema accompanied by widespread acute mucosal necrosis (Supplementary Fig. C–H).

3.2. Immunohistochemistry

3.2.1. Necrotizing enteritis cases

In small intestinal tissue sections with a grade 0 or 1 inflammatory reaction, the mAb-CPB consistently outlined the endothelial lining of vessels within the lamina propria and submucosa (Fig. 1C). The endothelial signal intensity was generally strong in animals with a grade 0 inflammatory reaction (Fig. 2). The mean intensity of the endothelial signal diminished with increasing infiltration by neutrophilic granulocytes and destruction of vessel walls (Fig. 2). In addition, CPB signals were detectable in some inflammatory cells around necrotic blood vessels of lesions with a grade 1 or 2 inflammatory reaction (Figs. 1G and 2). Few clostridial-like organisms and multiple poorly circumscribed spots within the necrotic debris stained positive for CPB. This staining pattern remained constant throughout all stages of the disease (Fig. 2). None of the non-affected small and large intestinal tissue sections nor lung, liver, kidney, spleen, or brain sections of animals with necrotizing enteritis showed positive staining for CPB at vascular endothelia. Control mAb-CPA (Fig. 1D and H) and the anti-C. chauvoei antibody (data not shown) did not label the endothelium in any investigated tissue section. Positive spotty signals within necrotic areas and positive clostridial-like organisms were also visible, albeit to a lesser extent as with mAb-CPB.

3.2.2. Control cases

None of the sections from control animals revealed any specific labelling using mAb-CPB or control antibodies (Supplementary Fig. C–H).

3.3. Immunofluorescence

Immunofluorescent double labelling on 20 cases confirmed endothelial localization of CPB (Fig. 3).

4. Discussion

C. perfringens type C causes fatal necrotizing enteritis in domestic animals, such as pigs, sheep, goats and calves as well as humans. Using the rabbit ileal loop model it was recently established that CPB is the major virulence factor in this disease (Sayeed et al., 2008; Vidal et al., 2008). The exact mode of action of CPB in the intestine is however still not fully understood. In particular, the cellular targets of CPB in naturally affected species have not been identified yet.

Our study revealed binding of CPB to vascular endothelial cells in lesions of spontaneously acquired necrotizing enteritis in piglets. Using a monoclonal anti-CPB antibody, strong immunohistochemical signals were consistently detected at endothelial cells in peracute and acute necrotizing enteritis lesions. Multiple positively stained vessels showed acute endothelial degeneration and vascular necrosis. The immunohistochemical signal intensity diminished in more protracted lesions which were characterized by widespread vascular necrosis and marked inflammatory reactions. This demonstrates that CPB binding to endothelial cells occurs during the early phase of the disease and precedes vascular necrosis. Our findings indicate that the vascular necrosis and hemorrhages result from a direct interaction of CPB with vascular endothelial cells. These results are supported by the finding that CPB forms multimeric complexes in plasma membranes of human endothelial cells (Steinthorsdottir et al., 2000). Piglets surviving the peracute stage of necrotizing enteritis develop a marked inflammatory reaction and the diminishing of the immunohistochemical signal is most likely due to widespread necrosis of the endothelial cells, phagocytosis of cellular debris by inflammatory cells and proteolytic degradation of CPB. A previous report of a human C. perfringens type C enteritis case demonstrated CPB localization at or around clostridial-like organisms (Matsuda et al., 2007) but binding of CPB to particular cells was not demonstrated. This finding is compatible with our results in subacute cases.

The mechanism by which CPB reaches the vascular endothelium remains to be investigated. In the rabbit ileal loop model, purified CPB induced destruction of jejunal and ileal villous tip epithelium after 15 min (Vidal et al., 2008). Damage increased to necrosis of the intestinal
epithelium and lamina propria, villous blunting, hemorrhage and neutrophilic infiltration in the mucosa and submucosa at 6 h. These results suggest a primary CPB induced destruction of the intestinal epithelium. However, the cellular localization of CPB was not investigated. Additionally, a morphological evaluation of endothelial or vascular damage was not reported. It is noteworthy that CPB was initially reported to be weakly toxic to a fetal human intestinal epithelial cell line I407 (Gibert et al., 1997). This could however not be reproduced by other groups (Shatursky et al., 2000; Nagahama et al., 2003a). Thus, a definite proof of a direct toxic effect of CPB on the intestinal epithelium is lacking.

In our study, we used tissue sections from naturally diseased piglets. The disease in these animals had already progressed to the fatal stage and they were thus comparable to those at the termination of the ileal loop assays performed by Sayeed and Vidal (Sayeed et al., 2008; Vidal et al., 2008). Due to already existing mucosal necrosis we were unable to thoroughly investigate initial binding of CPB to epithelial cells in situ. This would require standardized, experimental infectious trials.

CPB toxaemia was hypothesized to significantly contribute to the lethality of type C infections (Fisher et al., 2006). Due to the destruction of the intestinal barrier in necrotizing enteritis other toxins present in the intestine would however also readily be absorbed. Our evaluation of macroscopically unaffected intestine and internal organs neither revealed histopathological lesions nor immunohistochemical CPB signals. Thus, we were unable to detect morphological evidence of a systemic effect during the natural disease in piglets.

In summary, our results demonstrate that the pathogenesis of CPB induced necrotizing enteritis involves binding of CPB to vascular endothelial cells in the small intestine during the peracute phase of the disease which precedes widespread vascular necrosis. This indicates that CPB targeting of endothelial cells specifically induces vascular necrosis, hemorrhage and subsequent hypoxic tissue necrosis. With regard to similar necro-hemorrhagic lesions caused by other C. perfringens strains in different host species and tissues, exotoxin induced endothelial damage followed by hypoxic tissue necrosis might represent a common pathogenic mechanism in histotoxic clostridial diseases.

Conflict of interest statement

The authors confirm that the submitted manuscript does not provide any conflict of interest.

Acknowledgement

This project was supported by the grant BE/05 (M. Jäggi) from the Vetsuisse Faculty, University of Bern.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2009.01.025.

References


