Case Report

Clostridium perfringens β -toxin binding to vascular endothelial cells in a human case of enteritis necroticans

Julien Miclard,¹ Joop van Baarlen,² Marianne Wyder,¹ Benno Grabscheid¹ and Horst Posthaus¹

Correspondence

Horst Posthaus

horst.posthaus@itpa.unibe.ch

¹Institute of Animal Pathology, Vetsuisse Faculty, University of Bern, Switzerland

²Laboratorium Pathologie Oost Nederland, Enschede, The Netherlands

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Clostridium perfringens type C-induced enteritis necroticans is a rare but often fatal disease in humans. A consistent histopathological finding is an acute, deep necrosis of the small intestinal mucosa associated with acute vascular necrosis and massive haemorrhage in the lamina propria and submucosa. Retrospective immunohistochemical investigations of tissues from a diabetic adult who died of enteritis necroticans revealed endothelial localization of *C. perfringens* β -toxin in small intestinal lesions. Our results indicate that vascular necrosis might be induced by a direct interaction between *C. perfringens* β -toxin and endothelial cells and that targeted disruption of endothelial cells plays a role in the pathogenesis of enteritis necroticans.

Introduction

Enteritis necroticans (pigbel) is an acute, life-threatening disease caused by *Clostridium perfringens* type C. It occurs sporadically in Western countries and is endemic in the highland regions of Papua New Guinea (Johnson & Gerding, 1997). The disease is characterized by segmental haemorrhagic necrosis of the jejunum, which rapidly progresses to full-thickness necrosis of small and occasionally large intestinal segments (Lawrence & Walker, 1976; Johnson & Gerding, 1997). Histopathological hallmarks are deep mucosal necrosis with vascular necrosis and extensive haemorrhage (Lawrence & Walker, 1976). Risk factors for developing this disease are diabetes mellitus, malnourishment, low protein diets, sudden intake of proteinaceous meals, ingestion of *C. perfringens* type C-contaminated pig meat, and high amounts of trypsin inhibitors in the nutrition (Lawrence & Walker, 1976; Johnson & Gerding, 1997; Matsuda *et al.*, 2007). *C. perfringens* type C strains are defined by the production of α - (CPA) and β -toxin (CPB). The pathogenesis of type C enteritis is mainly attributable to CPB, a highly trypsin-sensitive exotoxin, causing cellular damage through multimeric pore formation at plasma membranes of susceptible cells (Smedley *et al.*, 2004; Sayeed *et al.*, 2008). The exact role of CPB in the pathogenesis of enteritis necroticans, in particular its target cells, has not been resolved, however. So far, only human endothelial cells (Steinthorsdottir *et al.*, 2000) and a promyeloblastic cell line (HL-60) (Nagahama *et al.*, 2003) have been shown to be susceptible to CPB. Results from immunohistochemical evaluations of *C. perfringens* type C enteritis in pigs in our

laboratory (Miclard *et al.*, 2009) indicated that CPB targets endothelial cells and induces vascular necrosis. The aim of the present study was to evaluate CPB localization in lesions of an archived human case of *C. perfringens* type C enteritis by immunohistochemistry.

Retrospective case

Archived, paraffin-embedded tissue blocks of small intestinal lesions as well as non-affected colon and stomach were available from a diabetic adult who died from enteritis necroticans in 1983. The clinical presentation and macroscopical, histopathological and microbiological findings have been described previously (Severin *et al.*, 1984). Briefly, the patient was a 24-year-old diabetic male who died of a severe, acute, haemorrhagic enteritis 2 days after initial onset of bloody diarrhoea and 24 h after being admitted to the hospital.

Methods

Histopathology. Tissues were routinely processed for histology and stained with haematoxylin and eosin.

Real-time PCR. DNA from paraffin-embedded small intestine was extracted as described by Miclard *et al.* (2009). Simplex real-time PCR amplification of the genes of *C. perfringens* α - (cpa), β - (cpb), β -2- (cpb2), ϵ - (etx) and ι -toxin (itx) and enterotoxin (cpe) was performed as described by Albini *et al.* (2008). All reactions were repeated three times.

Immunohistochemistry. Histological sections were deparaffinized with xylene, endogenous peroxidase activity was inhibited by H₂O₂ (0.1% in PBS, 15 min), and slides were incubated with 0.1% proteinase (Sigma-Aldrich; in TBS, pH 7.6, 15 min, 37 °C). Primary antibodies (Center for Veterinary Biologics, Ames, Iowa; 1:100 for

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2 h at room temperature) were: mouse monoclonal anti-CPB (mAb-CPB; 10A2) and mouse monoclonal anti-CPA (mAb-CPA; 6H7 1F3). Slides were developed using the LSAB and AEC kit (Dako) and counterstained with haemalaun.

Results and Discussion

Histopathological findings are briefly summarized in the legend of Fig. 1. The absence of a substantial inflammatory reaction in combination with the reported rapid deterioration of the patient's condition (Severin *et al.*, 1984) corresponds to a peracute to acute course of the disease. Real-time PCR on DNA from tissue extracts detected low

levels of *cpb* (C_t value 36.4), *cpb2* (C_t value 39) and *cpe* (C_t value 36.5), but not *etx* or *itx*. Although primers and fluorescent probes were designed in highly conserved gene regions of both human and animal isolates (Albini *et al.*, 2008), *cpa* could not be amplified. Overall, the low levels of *cpb* and *cpb2* indicated low yields of clostridial DNA from the paraffin blocks. Because these genes are usually detectable in higher copy numbers than *cpa* in *C. perfringens* isolates (Albini *et al.*, 2008), it is conceivable that *cpa* was beyond the detection threshold in our samples. Despite this, amplification of *cpb* and two additional *C. perfringens* toxin genes reconfirms the initial diagnosis of *C. perfringens* type C infection achieved

Fig. 1. Histopathology and immunohistochemical localization of CPB. (a) Haematoxylin and eosin stained section of jejunum depicting deep coagulation necrosis of the small intestinal mucosa and haemorrhage in underlying layers. (c, e) Higher magnification of (a) showing remnants of necrotic vessels with fibrin thrombi in the necrotic upper zone (c) and haemorrhage but no substantial inflammatory reaction in the submucosa (e). (b) Immunohistochemical signals for CPB outlining vascular structures in areas affected by necrotic lesions. CPB-positive signals were present at vascular remnants in the necrotic zone (d) and at the endothelium of submucosal vessels (f).

through bacteriological culturing and biochemical and guinea pig skin neutralization tests (Severin *et al.*, 1984).

Immunohistochemically, CPB signals consistently and preferentially outlined the vascular endothelium in the lamina propria and submucosa and vascular remnants in necrotic small intestinal sections (Fig. 1b, d, f). Control mAb-CPA did not produce any specific staining (Supplementary Fig. S1 in JMM Online). Non-affected large intestinal segments of the same patient did not exhibit any CPB-specific staining at endothelial cells (Supplementary Fig. S2). Thus we were able to localize CPB at endothelial cells in the necrotizing lesions. Our findings suggest that vascular necrosis, a hallmark of enteritis necroticans (Lawrence & Walker, 1976), results from a direct interaction of CPB with endothelial cells. This conclusion is supported by a previous study (Steinthorsdottir *et al.*, 2000) which demonstrated that CPB forms multimers in plasma membranes of human endothelial cells and induces arachidonic acid and ion release from these cells *in vitro*. Thus we hypothesize that CPB targeting of endothelial cells could induce vascular necrosis, haemorrhage, and subsequent hypoxic tissue necrosis.

A previous report on immunohistochemical localization of CPB in lesions of *C. perfringens* type C in a human case of enteritis necroticans demonstrated CPB at or around clostridial-like organisms but did not demonstrate binding of CPB to particular cells (Matsuda *et al.*, 2007). Although we also multifocally detected such signals in our samples, it is not clear whether this represents active CPB, which is known to be rapidly inactivated by proteases in the gut content. As a pore-forming toxin, CPB is supposed to cause cellular damage by inserting into plasma membranes of target cells (Steinthorsdottir *et al.*, 2000), thus detection of extracellular CPB does not necessarily confirm a pathogenic role. The difference in results from previous studies and our study could be explained by the use of different antibodies and antigen retrieval methods. Additionally, widespread necrosis of the endothelial cells and proteolytic degradation of CPB could have hampered immunohistochemical detection of CPB at endothelial cells. The detected CPB signals most likely represent the multimeric, membrane-inserted form (Nagahama *et al.*, 2003; Steinthorsdottir *et al.*, 2000), which seems to be present in detectable quantities during this stage of the disease, and coincide with widespread vascular necrosis within the lesions. Our results cannot exclude initial effects of CPB at the intestinal epithelium, because of the limitation to one case which represents the fatal stage of the disease. At this stage, necrosis of enterocytes had already occurred and epithelial cells were largely destroyed and absent in our tissue sections.

Spontaneous *C. perfringens* type C enteritis rarely occurs in Western countries. Several reported cases were associated with either diabetes mellitus, as in this patient, or diet-induced low levels of proteolytic enzymes in intestinal contents leading to reduced proteolytic inactivation of CPB (Matsuda *et al.*, 2007). Despite different predisposing

factors, the recent evidence of CPB as the major virulence factor in inducing necrotizing lesions in an experimental model (Sayeed *et al.*, 2008; Vidal *et al.*, 2008) clearly demonstrates that elevated CPB levels in the intestine play a key role in the pathogenesis of enteritis necroticans. *C. perfringens* type C enteritis is frequently observed in veterinary medicine, especially in newborn piglets, in contrast to in human medicine, and the diseases share remarkable morphological similarities between human and veterinary cases (Songer, 1996). Our findings in this case in a human patient are consistent with results of our recent larger scale study in neonatal piglets, where we consistently demonstrated endothelial localization of CPB in peracute to acute lesions of *C. perfringens* type C enteritis (Miclard *et al.*, 2009). Taken together, our findings suggest that targeting and destruction of vascular endothelial cells through CPB might play an important role in the pathogenesis of *C. perfringens* type C-induced enteritis necroticans.

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