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Susceptibility of primary human endothelial cells to *C. perfringens* beta-toxin suggesting similar pathogenesis in human and porcine necrotizing enteritis

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ABSTRACT

Clostridium perfringens type C causes fatal necrotizing enteritis in different mammalian hosts, most commonly in newborn piglets. Human cases are rare, but the disease, also called pigbel, was endemic in the Highlands of Papua New Guinea. Lesions in piglets and humans are very similar and characterized by segmental necro-hemorrhagic enteritis in acute cases and fibrino-necrotizing enteritis in subacute cases. Histologically, deep mucosal necrosis accompanied by vascular thrombosis and necrosis was consistently reported in naturally affected pigs and humans. This suggests common pathogenetic mechanisms. Previous *in vitro* studies using primary porcine aortic endothelial cells suggested that beta-toxin (CPB) induced endothelial damage contributes to the pathogenesis of *C. perfringens* type C enteritis in pigs. In the present study we investigated toxic effects of CPB on cultured primary human macro- and microvascular endothelial cells. *In vitro*, these cells were highly sensitive to CPB and reacted with similar cytopathic and cytotoxic effects as porcine endothelial cells. Our results indicate that porcine and human cell culture based *in vitro* models represent valuable tools to investigate the pathogenesis of this bacterial disease in animals and humans.

1. Introduction

Clostridium perfringens type C strains cause fatal, segmental necrotizing enteritis in humans and animals (Songer, 2010). The disease occurs worldwide in various animal species including pigs, horses, goats, and sheep (Songer, 1996). It can lead to high morbidity and mortality in affected herds and therefore high economic losses (Songer, 1996). In humans the disease is named enteritis necroticans or pigbel, the latter name originating from Papua New Guinea (PNG) and relates to the association of the disease with feasts that include the consumption of pork. It is very rare in developed countries (Songer, 2010) but is more frequently reported in low income countries (Johnson and Gerding, 1997), where occasional outbreaks still occur (Mandrella, 2007; Poka and Duke, 2003). Pigbel was an important cause of child mortality in the Highlands of PNG (Lawrence, 1979) until a vaccination program in the early 1980s dramatically reduced its incidence (Lawrence et al., 1990). However, vaccination ceased in the mid 1990s when the vaccine was no longer commercially available and currently no pigbel vaccination is performed in PNG. The last documented survey on the occurrence of pigbel in PNG dates back to 2002 (Poka and Duke, 2003). Although the incidence of the disease was still low, regional clustering of cases was observed, raising

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the concern that pigbel could re-emerge in certain areas of the Highlands.

Pathological lesions of *C. perfringens* type C enteritis are similar among affected species and they are characterized by segmental hemorrhagic necrosis of the jejunum which can extend to the entire distal small intestine and sometimes to the colon in acute cases (Cooke, 1979; Songer, 1996). Subacute cases typically exhibit multifocal to diffuse fibrino-necrotizing enteritis with significant inflammatory response (Jäggi et al., 2009). Vascular thrombosis and necrosis of small mucosal and submucosal vessels in affected intestinal segments is consistently described in animals and humans (Cooke, 1979; Jäggi et al., 2009; Lawrence, 1979). Beta-toxin (CPB), the essential virulence factor of type C strains (Sayeed et al., 2008; Vidal et al., 2008), has been detected on the lining of mucosal and submucosal vessels in lesions of diseased piglets and a human patient from the Netherlands (Miclard et al., 2009a,b). Moreover, cultured primary porcine aortic endothelial cells were highly susceptible to CPB, suggesting that vascular endothelial cells in the pig intestine could be targeted by CPB (Gurtner et al., 2010). CPB was also shown to form multimeric complexes at the plasma membrane and release of arachidonic acid in primary human umbilical cord endothelial cells (HUVEC) (Steinthorsdottir et al., 2000). However, cytopathic effects were not further evaluated. Additionally, macrovascular endothelial cells, for example originating from aorta or umbilical cord veins, can differ from microvascular endothelial cells in their *in vitro* response to toxins. Therefore, cytopathic effects observed in culture should be confirmed in primary microvascular endothelial cells.

Due to the similarities between the natural diseases in different host species, porcine and human cell culture based studies and experimental infections of pigs could represent valuable models to study the pathogenesis of *C. perfringens* type C enteritis. The objectives of this study were to evaluate whether cultured primary human macro- and microvascular endothelial cells are similarly affected by CPB as primary aortic endothelial cells of porcine origin; thus enabling the porcine cells to be used for *in vitro* studies on the pathogenesis of this fatal clostridial disease.

2. Materials and methods

2.1. Cell cultures and media

Primary human umbilical cord endothelial cells were kindly provided by Prof. Haeberli (Faculty of Medicine, University of Bern). Primary human neonatal dermal microvascular endothelial (HMVEC-d) and primary human adult uterine microvascular endothelial cells (HMVEC-ut) were purchased from Lonza (Belgium). Cells were grown in EGM-2 plus supplements and growth factors (Lonza) at 37 °C with 5% CO_2. Cells in passage 3 were frozen as stock cultures in liquid nitrogen. Primary human foreskin fibroblasts were purchased from CellnTec (Bern, Switzerland) and grown in DMEM with glutamax, 10% FCS, 20 mM L-glutamine, 1 ng/ml aFGF, 1 ng/ml bFGF (GIBCO) 1% antibiotics/antimycotics (GIBCO). For all experiments, HUVEC, HMVEC-d, UtMVEC and fibroblasts were seeded at a density of $1.33 \times 10^4/cm^2$, and grown to confluency for 5 days. Cells from passage 4–6 exclusively were used for experiments.

2.2. Toxin preparation and antibody neutralization

Expression and purification of recombinant CPB (rCPB) was performed as previously described (Gurtner et al., 2010). In brief, rCPB was expressed as inactive Nus-Tag™ (Novagen) fusion protein (rCPB-Nus-Tag) and full length active rCPB was harvested after proteolytic cleavage. As controls rCPB-Nus-Tag and Nus-Tag protein expressed by *E. coli* transfected with the empty control vector were used (Gurtner et al., 2010). Neutralization of rCPB was performed using mAb-CPB. As a control monoclonal anti- alpha-toxin antibodies (mAb-CPA) were used (Gurtner et al., 2010).

2.3. Live cell imaging

Confluent HUVEC cultures in collagen coated glass bottom 6-well plates (MatTek) and confluent fibroblasts were incubated with medium containing 5 μg/ml propidium iodide and 30 ng/ml rCPB. Live cell imaging using a TE2000E-PFS microscope (Nikon) was carried out as described by Gurtner et al. (2010).

2.4. Immunofluorescence

Confluent cell cultures in labteks (Nunc) were incubated with 200 μl medium containing 30 ng/ml rCPB or rCPB-Nus-Tag. After 1 h or 3 h the cells were washed with PBS, fixed (1% paraformaldehyde, 20 min, RT), and made permeable (0.1% triton X-100, 5 min). F- and G-actin were stained and visualized as previously described by Gurtner et al. (2010).

2.5. Cell viability assays

Cells grown in 96-well-plates were incubated for 12 h with 150 μl of cell culture medium containing indicated amounts (Fig. 2) of rCPB, rCPB-Nus-Tag, or Nus-Tag. MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell viability tests were performed as described (Mosmann, 1983). Medium without toxin was used to determine reference values. MTT values for each individual concentration were determined in triplicate and each experiment was repeated independently three times. All measurements were determined as percentage of the baseline (from incubation with toxin free medium). To test the effect of group and dilution we used simple linear model one-way and two-way ANOVAs and, in addition, for group differences in each dilution, a post hoc Bonferonni Tukey Kramer Multiple Comparison test. Significant ANOVA values were set for $P < 0.05$. All statistical analyses were carried out with the statistical package NCSS 2007 (NCSS, Kaysville, UT, wwww.nccc.com).

3. Results

3.1. Rapid cytopathic effects of rCPB on HUVEC

Confluent HUVEC showed cell border retraction after 40 min of exposure to 30 ng/ml rCPB (Supplementary