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A toxicological evaluation of a claudin modulator, the C-terminal fragment of *Clostridium perfringens* enterotoxin, in mice

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Received December 2, 2010, accepted December 29, 2010

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Pharmazie 66: 543–546 (2011) *doi: 10.1691/ph.2011.0365*

Tight junctions (TJs) maintain cellular polarity between the apical and basolateral region of epithelial cells. Claudin, a tetra-transmembrane protein, plays a pivotal role in the barrier function of TJs. We previously found that a claudin modulator, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), may be a promising candidate for improving the mucosal absorption of drugs. C-CPE is a fragment of enterotoxin, and putative CPE claudin receptors are highly expressed in liver and kidney. The safety and antigenicity of C-CPE must be evaluated for future clinical application. Therefore, we evaluated whether C-CPE administration in mice leads to tissue injury or production of antibodies. Intravenous administration of C-CPE at 5 mg/kg, which is a more than 25-fold higher dose than that used in a murine mucosal absorption model, did not increase biochemical markers of liver and kidney injury even after 11 injections once a week. Nasal C-CPE administration (2 mg/kg) once a week for 11 administrations also did not increase these biochemical markers, but 6 administrations of C-CPE resulted in elevation of C-CPE-specific serum IgG. These results indicate that development of a less antigenic claudin modulator will be essential for future clinical application of a C-CPE-based mucosal absorption enhancer.

1. Introduction

The use of biologics, such as antibodies, peptides, and nucleic acids, in new drugs is becoming increasingly prominent. Biologics are biodegradable and poorly absorbed in the mucosa, and therefore they are often employed as injectable drugs. The development of a non-invasive system for delivery of drugs across the mucosal epithelium would improve quality of life and patient compliance. Since orally administered drugs can be degraded by digestive enzymes and first pass effects in the liver, developing ways to administer drugs through nasal and pulmonary transmucosal absorption has a high priority. However, passing biologics across the mucosal epithelium is extremely difficult because the mucosa's primary function is as a physical and biological barrier preventing the entry of pathogens and toxic substances into the body.

Epithelial cell sheets develop intercellular junctions to prevent the free movement of solutes between sheets. Adjacent epithelial cells adhere to one another via tight junctions (TJ), adherent junctions, and gap junctions. Among these, the TJ plays a key role in sealing the intercellular space and preventing leakage of solutes. Modulation of the TJ barrier has proven to be a promising strategy for enhancement of mucosal drug absorption. Tight junction modulators, such as surfactants, chelators, and nitric oxide donors, have been investigated as potential absorption enhancers since the 1960 s (Aungst 2000; Citi 1992; Engel and Riggi 1969; Tomita et al. 1996).

The detection and development of absorption-enhancers focuses on modulating activity of the TJ barrier. Such enhancers are called "the first generation TJ modulators" (Kondoh et al. 2008). The identification of claudin, a structural and functional TJ component, provided new insight into absorption-enhancers, and led to a TJ-components-based strategy for enhancer development, the second generation TJ modulators. Claudins are ∼23 kDa proteins bearing tetra-transmembrane domains and comprise a family of more than 20 members (Furuse and Tsukita 2006). The expression profiles and barrier function of the various claudin family members differ among tissues. For instance, claudins-1 and -5 are critical for epidermal barrier and blood-brain-barrier functions, respectively (Furuse et al. 2002; Nitta et al. 2003). Modulation of the claudin barrier has been proposed as a novel strategy for absorption enhancement (Furuse et al. 1998; Tsukita and Furuse 1998).

Clostridium perfringens enterotoxin (CPE) is a cause of food poisoning in humans (McClane and Chakrabarti 2004). A receptor of CPE is identical to claudin-3/4, and the C-terminal fragment of CPE (corresponding to amino acids 184–319) modulates the TJ barrier by its interaction with claudin-3/4 (Sonoda et al. 1999). We found that the claudin modulator the C-terminal fragment of CPE was 400-fold more potent at enhancing intestinal absorption than a clinically used absorption-enhancer, sodium caprate (Kondoh et al. 2005). However, the C-terminal fragment of CPE did not enhance intestinal absorption of a peptide drug when co-administered (Uchida et al. 2010). The N-terminal truncated fragment (C-CPE), comprising amino acids 194–319, did enhance intestinal, nasal, and pulmonary absorption of a biologically active peptide (Uchida et al. 2010). Thus, C-CPE may be a promising enhancer of mucosal drug absorp-

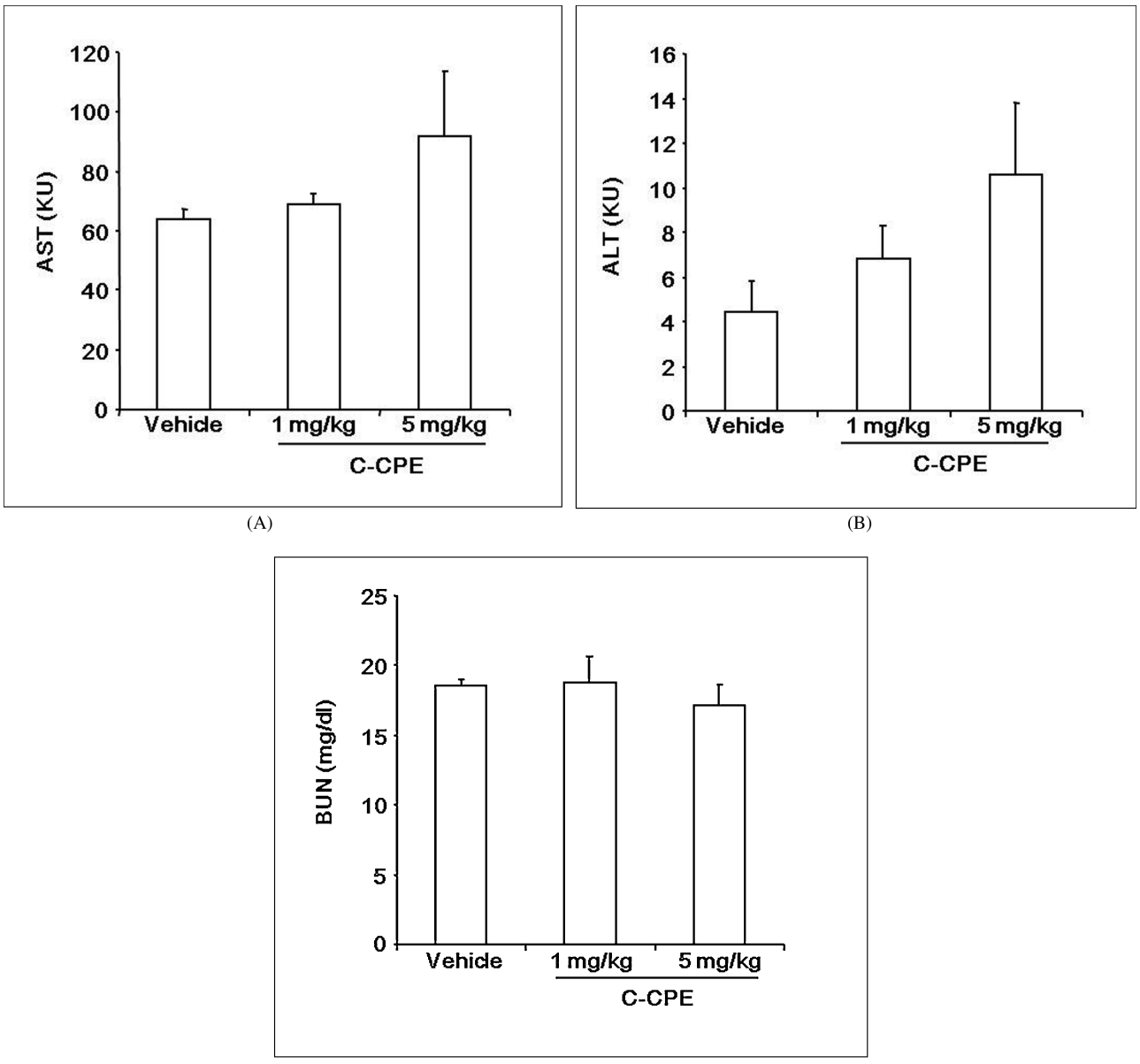


Fig. 1: Effect of systemic injection of C-CPE on biochemical markers for liver and kidney injury. Mice received intravenous injections of C-CPE at 0, 1, or 5 mg/kg once a week for 11 weeks. Blood was recovered 24 h after the last administration of C-CPE. Serum AST (A), ALT (B) and BUN (C) were measured using a commercially available kit as described in the Expreimental section. Data are presented as mean ± SEM (n = 3 or 5)

tion. Although data on safety and antigenicity is critical for any future clinical application of C-CPE, its potential side effects have not been investigated.

In the present study, we investigated the effect of C-CPE administration on liver and kidney tissues in which claudin-3/4 is expressed, as well as studied induction of anti-C-CPE antibodies.

2. Investigations, results and discussion

Administration of C-CPE enhanced the mucosal absorption of dextran with a molecular weight of 20 kDa, indicating that C-CPE (13 kDa) might enter into the systemic flow from the mucosal membrane with drugs (Kondoh et al. 2005). C-CPE constitutes the receptor-binding domain of CPE, and binds to claudin-3 and claudin-4 (Fujita et al. 2000; Katahira et al. 1997). Since claudins-3 and -4 are highly expressed in the liver and kidney (Morita et al. 1999), we evaluated the effect of C-CPE on these tissues. To investigate the potential effects of C-CPE on liver and kidney, we systemically injected C-CPE into mice once a week for 11 weeks and measured biochemical markers of liver (AST and ALT) and kidney (BUN) injury 24 h after the last injection. As shown in Figs. 1A, 1B and 1C, intravenous administration of C-CPE did not affect serum AST, ALT and BUN levels, even at a dose as high as 5 mg/kg. C-CPE was mucosally administered at 0.02–0.4 mg/kg (Uchida et al. 2010). Therefore, even if all C-CPE was absorbed, no side effects in liver or kidney are likely to occur.

Since C-CPE is a polypeptide, its antigenicity could interfere with its clinical use. We therefore investigated whether repeated mucosal administration of C-CPE activates serum C-CPE-specific IgG responses. Mice were surgically operated upon in jejunal and pulmonary absorption studies, and consequently could not be repeatedly treated with C-CPE. Therefore, to investigate the antigenicity of C-CPE following mucosal administration, C-CPE was intranasally administered to mice once a week for 10 weeks. Serum IgG production was monitored every week. C-CPE treatment did not increase C-CPE-specific serum IgG after 4 administrations of C-CPE at 2 mg/kg. However, 6 administrations of C-CPE did cause production of C-CPE-

specific serum IgG (Fig. 2). A dose of 1 mg/kg is equal to that used in a previously published study on mucosal absorption (Uchida et al. 2010). Repeated mucosal administration of C-CPE in our study at twice this dose (2.0 mg/kg) did not increase serum AST, ALT and BUN levels (Figs. 3A 3B and 3C). These findings indicate that while C-CPE does not cause tissue damage at clinically relevant doses, it may be limited in its clinical applications as a mucosal absorption enhancer only by its antigenicity.

There are two potential directions for clinical applications of claudin modulators. The first is preparation of a claudin modulator based on C-CPE. An antigenic determinant assay of CPE revealed that the C-terminal fragment corresponding to amino acids 286–305 was immunogenic (Sugii 1994). Mutating the antigenic domain while maintaining its claudin-binding activity would contribute to development of a low antigenic claudin modulator. In general, smaller peptides are less antigenic. The C-terminal fragment corresponding to amino acids 290–319 constituted the receptor-binding domain of CPE (Hanna et al. 1991). Preparation of a claudin-modulating peptide with low antigenicity and high claudin-modulating activity using this 30 amino acid fragment may lead to a claudin modulator useful as an enhancer of drug absorption.

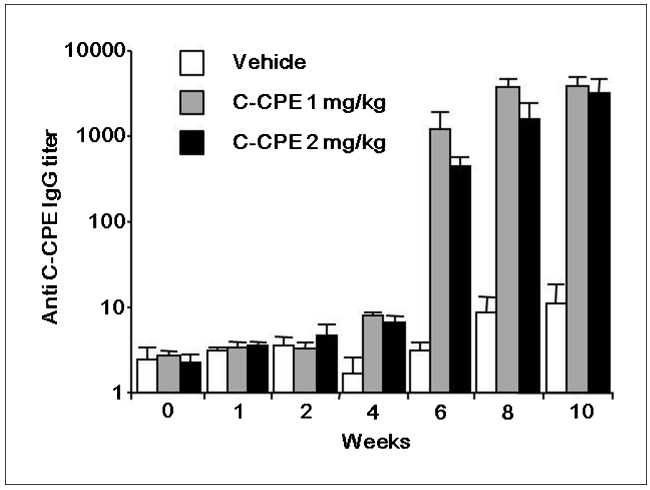
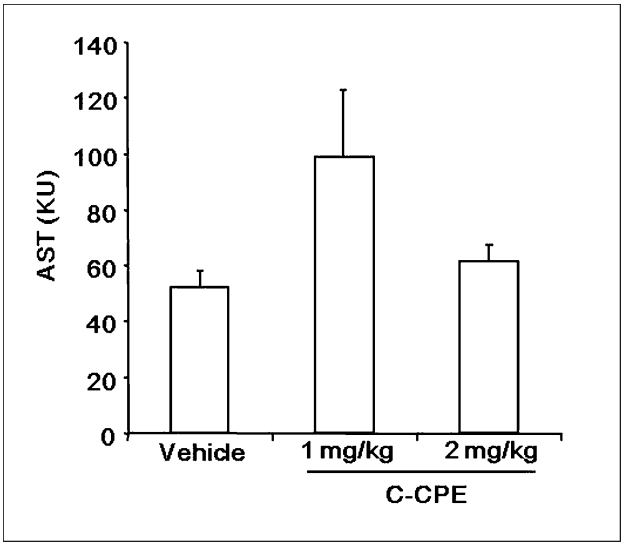
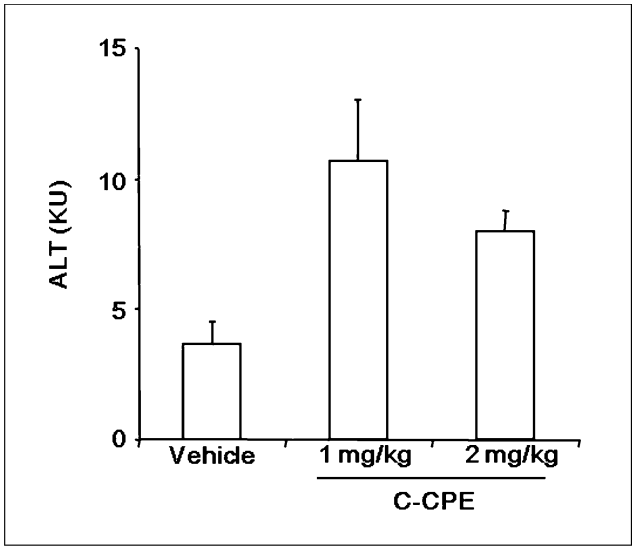


Fig. 2: Effect of mucosal administration of C-CPE on production of anti-C-CPE IgG. Mice received nasal injections of C-CPE at 0, 1, or 2 mg/kg once a week for 10 weeks. Blood was collected each week and serum C-CPE specific IgG levels were measured as described in Materials and methods. Data are presented as means ± SEM (n = 4 or 5)



(A)



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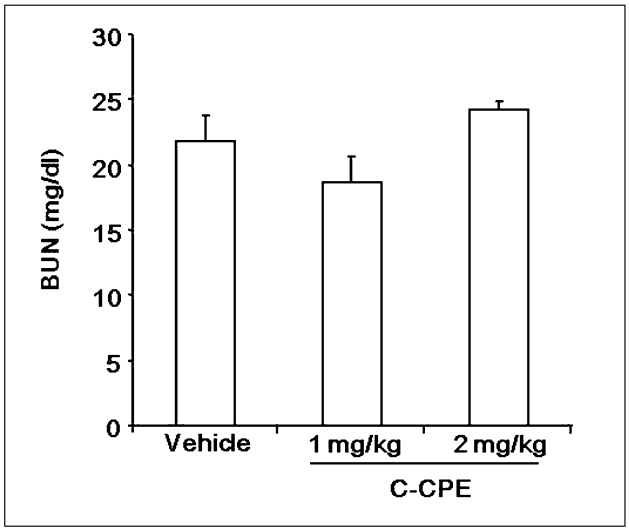

(C)

Fig. 3: Effect of repeated mucosal administration of C-CPE on biochemical markers of liver and kidney injury. Mice were nasally administered with C-CPE at 0, 1, or 2 mg/kg once a week for 11 weeks. Seven days after the last administration, blood was recovered, and serum AST (A), ALT (B) and BUN (C) were measured using a commercially available kit. Data are presented as mean ± SEM (n = 4 or 5)

The second potential direction for clinical applications of claudin modulators lies in the preparation of a human antibody to modulate the claudin-barrier. Claudin is characterized by low antigenicity, and it is therefore difficult to prepare an antibody to bind its extracellular domain (Evans et al. 2007). Recently, Romani et al. successfully prepared a human single-chain antibody to claudin-3, and Suzuki et al. also prepared a monoclonal antibody against claudin-4 (Romani et al. 2009; Suzuki et al. 2009). Development of a humanized antibody against claudin will contribute greatly to the clinical applications of claudin modulators.

In summary, we found that C-CPE administration does not result in significant tissue injury in mice. However, our findings also suggest that discovering a means of reducing C-CPE's antigenicity is critical for continued development of a C-CPE-based claudin modulator useful as an enhancer of drug absorption.

3. Experimentals

3.1. Animals

BALB/c female mice (6 wk) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), and were housed in an environmentally controlled room at $23 \pm 1.5\,^{\circ}C$ with a 12-h light/12-h dark cycle. The mice had free access to water and commercial chow (Type MF, Oriental Yeast, Tokyo, Japan). Experimental protocols involving mice were performed according to the ethics guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

3.2. Preparation of C-CPE

C-CPE was prepared as described previously (Uchida et al. 2010). Briefly, pET16b vector plasmids coding the C-terminal fragment of CPE (amino acids 194–319) were transduced into *E. coli* BL21 (DE3), and protein expression was stimulated by addition of isopropyl-1-thio-β-D-galactoside. Cell lysates were applied to HisTrap™ Chelating HP columns (GE Healthcare, Buckinghamshire, UK), and C-CPE was eluted with imidazole. The solvent was exchanged with phosphate-buffered saline by gel-filtration, and the purified proteins were stored at $-80\,^{\circ}C$ until use. Purification of the proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue. C-CPE was quantified by using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL) using bovine serum albumin as a standard.

3.3. Biochemical assay

C-CPE was administered to mice intravenously or nasally once a week for 11 weeks, and blood was collected from the mice by cardiac puncture one day after the last administration. Serum aspartate aminotransferase (AST), alanin aminotransferase (ALT) levels and blood urea nitrogen (BUN) were measured using commercially available Transaminase-CII and Blood Urea Nitrogen-B Test (WAKO Pure Chemical, Osaka, Japan) kits, respectively.

3.4. C-CPE-specific antibody production

C-CPE was administered to mice intravenously or nasally once a week. Serum was collected 7 days after each administration of C-CPE. The titers of C-CPE-specific antibody in serum were determined using an enzyme-linked immunosorbent assay. Briefly, an immunoplate was coated with C-CPE (1 μg/well in a 96-well plate). Ten-fold serial dilutions of samples were added to the wells, followed by reaction with horseradish peroxidase-conjugated anti-mouse IgG. The presence of C-CPE-specific antibodies was determined using TMB peroxide substrate. End point titers were expressed as the dilution ratio, which gave 0.1 above control values obtained for serum of naïve mice at an absorbance of 450 nm.

Acknowledgements: We thank the members of our laboratory for their useful comments and discussion. We would like to thank Dr. Y. Horiguchi (Osaka University, Osaka, Japan) for providing us C-CPE cDNA. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (21689006), by a Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan, by Takeda Science Foundation, by a grant from Kansai Biomedical Cluster project in Saito, which is promoted by the Knowledge Cluster Initiative of the Ministry of Education, Culture, Sports, Science and Technology, Japan. A.T. is supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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