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Angubindin-1, a novel paracellular absorption enhancer acting at the tricellular tight junction

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ABSTRACT

A limiting barrier for mucosal absorption of drugs is the tight junction (TJ). TJs exist between two adjacent cells (bicellular TJ, bTJ) and at the sites where three cells meet (tricellular TJ, tTJ). We present a novel approach which employs a physiologically regulated pathway for the passage of large molecules through the tTJ. Main barrier-relevant tTJ proteins are tricellulin and angulin-1 to -3. We developed an angulin binder from *Clostridium perfringens* iota-toxin (Ib) whose receptor is angulin-1. An Ib fragment corresponding to amino acids 421–664 (Ib421-664) of iota-toxin proved to bind in cells expressing angulin-1 and -3, but not angulin-2. This binding led to removal of angulin-1 and tricellulin from the tTJ which enhanced the permeation of macromolecular solutes. Ib421-664 enhanced intestinal absorption in rats and mice. Our findings indicate that Ib421-664, which we designate angubindin-1, is a modulator of the tTJ barrier and that modulation of that barrier qualifies for a new strategy of developing a mucosal absorption enhancer.

1. Introduction

Epithelia prevent the free movement of solutes between the body's inside and the outer environment. Thus, the most difficult step for drug absorption consists of passaging that barrier. Absorption across the epithelium can occur via transcellular or paracellular routes. For several substances transcellular transporters exist, but many compounds cannot permeate across cell membranes and are hindered to achieve desired therapeutic concentrations within the body. Therefore, methods to modulate the epithelial barrier have been developed to enhance the absorption of drugs long before the molecular composition of the tight junction was discovered [1]. As a first but finally too hazardous approach, a calcium chelator was identified as a mucosal absorption enhancer > 50 years ago [2].

The paracellular barrier of the epithelium comprises a multi-protein complex, the tight junction (TJ). In freeze-fracture electron microscopy, the TJ appears as a set of continuous, anastomosing, intramembranous particles forming a meshwork of strands [3]. Adjacent TJ strands associate with TJ strands of the opposing membranes of adjacent cells and more or less seal the intercellular space. There are two types of TJ arrangements, the bicellular tight junction (bTJ) and the tricellular tight junction (tTJ) [4,5]. Key components for the structural and functional properties of the bTJ of mammals are the family of 27 different claudins and occludin which both form tetraspan transmembrane proteins [6–8]. Expression of claudins varies among different tissues and the pattern of a given set of claudins determines the properties of the intercellular seal formed by TJ strands [9,10]. The concept of claudin-targeted drug absorption has been proven [11], and

Abbreviations: TJ, tight junction; bTJ, bicellular tight junction; tTJ, tricellular tight junction; Ib421-664, Ib fragment corresponding to amino acids 421–664 and designated angubindin-1; LSR, lipolysis-stimulated lipoprotein receptor; ILDR, immunoglobulin-like domain-containing receptor; pAb, polyclonal antibody; GST, glutathione S-transferase; mAb, monoclonal antibody; C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin; GFP, green fluorescent protein; FBS, fetal bovine serum; PBS, phosphate buffered saline; FACS, fluorescence-activated cell sorting; SDS-PAGE, sodium dodecyl sulfate gel-electrophoresis; CBB, Coomassie Brilliant Blue; BSA, bovine serum albumin; TER, transepithelial resistance; R^{epi}, epithelial resistance; R^{trans}, transcellular resistance; R^{para}, paracellular resistance; R^{sub}, subepithelial resistance; EGTA, ethylene glycol tetraacetic acid; FITC, fluorescein isothiocyanate; FD-4, fluorescein isothiocyanate-conjugated dextran with molecular mass of 4000 Da; AUC, area under the curve; TMR, tetramethylrhodamine

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absorption enhancers that target claudins have been developed recently [12–14].

The tTJ is a specialized structure at contacts of three cells [5,6,15]. Here, the elements of adjacent bTJ strands join and extend in basal direction forming a vertical central tube-like space at the tricellular contacts. These central tubes are critical points for the TJ barrier integrity and are sealed by at least two predominantly localized proteins, tricellulin and angulin. Tricellulin is a 65-kDa integral membrane protein with four transmembrane domains and structural homology to occludin [16]. It has been shown to tighten the tTJ barrier against the passage of macromolecules [17–19]. The family of angulins consists of angulin-1 (also known as lipolysis-stimulated lipoprotein receptor [LSR]), angulin-2, (also termed immunoglobulin-like domain-containing receptor 1 [ILDR1]) and angulin-3 (also known as ILDR2) [20–22]. Angulins recruit tricellulin to the tTJ, defining the tricellular contacts. Although tricellulin is ubiquitously expressed in the body, the expression profiles of the members of the angulin family differ among tissues [21]. Therefore, angulin proteins might be potent targets for drug absorption via the modulation of tTJ-seals because tissue specificity is associated with safety of absorption enhancers [23]. However, no angulin binder has yet been developed, and it remained open whether angulin proteins are appropriate targets for modulation of the tTJ.

Clostridium perfringens iota-toxin causes antibiotic-associated enterotoxemia [24] and is a binary toxin consisting of an enzymatic component (Ia) and a receptor-binding component (Ib). LSR, later named angulin-1, is a receptor for iota-toxin [22,25]. Thus, the receptor-binding domain of Ib may be suggested as an angulin-1 binder and could modulate the tTJ.

In this study, we investigated binding and tTJ barrier modulation abilities of an iota-toxin Ib fragment corresponding to amino acids 421–664, Ib421-664 (angubindin-1), and observe binding to angulin and subsequent removal of tricellulin which may lead to enhanced passage of mid-size and macromolecular passage.

2. Materials and methods

2.1. Reagents

Rabbit anti-angulin-1/LSR polyclonal antibody (pAb) was purchased from Atlas antibodies (Stockholm, Sweden). Rabbit anti-tricellulin (C-term) pAb and rabbit anti-FLAG pAb were purchased from Sigma-Aldrich (St Louis, MO). Mouse anti-occludin pAb, and mouse anti-glutathione S-transferase (GST) monoclonal antibody (mAb) were purchased from Invitrogen (Carlsbad, CA), and Wako Chemicals (Osaka, Japan), respectively. Anti-ZO-1 mAb was purchased from BD Transduction Laboratories (Franklin Lakes, NJ). Alexa Fluor 488-conjugated goat anti-rabbit pAb, Alexa Fluor 594-conjugated goat anti-mouse pAb, Alexa Fluor 647-conjugated goat anti-rabbit and R-phycoerythrin-conjugated goat anti-mouse pAb were purchased from Molecular Probes (Carlsbad, CA). All reagents were of research grade.

2.2. Cells

Mouse green fluorescent protein (GFP)-LSR-expressing L cells (GFP-LSR/L cells), mouse mammary epithelial cells (EpH4), LSR-knockdown EpH4 cells (LSR-kd cells), and ILDR1-FLAG- or ILDR2-FLAG-expressing LSR-kd cells (ILDR1-FLAG/LSR-kd cells or ILDR2-FLAG/LSR-kd cells, respectively) were kindly provided by Dr. M. Furuse (Kobe University, Japan) [21,22]. The human colon cell line HT-29/B6 was developed by Kreusel et al. [26] and Caco-2 cells were obtained from ATCC (Manassas, VA). GFP-LSR/L cells and Caco-2 cells, EpH4 cells, LSR-kd cells, ILDR1-FLAG/LSR-kd cells and ILDR2-FLAG/LSR-kd cells, and HT29/B6 cells were maintained in modified Eagle's medium, Dulbecco's modified Eagle's medium and RPMI1640, respectively, supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37 °C.

2.3. Green fluorescent protein-tagged tricellulin-expressing HT-29/B6 cells

HT-29/B6 cells were stably transfected by using the Lipofectamine Plus protocol provided by the manufacturer (Invitrogen) with either pCMV10 as a control or with pCMV10 containing the human tricellulin cDNA N-terminally tagged with eGFP and were grown in the presence of gentamicin G418. G418-resistant cell clones were screened for tricellulin overexpression by means of Western blotting and localization of GFP-tagged tricellulin was analyzed; mock-transfected cells served as controls.

2.4. Animals

Male Wistar rats (250–300 g) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). The rats were maintained in an environmentally controlled room with a 12 h light/12 h dark cycle and allowed access to standard rodent chow and water ad libitum. The rats were allowed a period of at least 1 week to adapt.

2.5. Preparation of the recombinant Ib fragment protein, Ib421-664 (angubindin-1)

The plasmid encoding the receptor-binding domain of Ib corresponding to amino acids 421 to 664 of iota-toxin (pGEX-Ib421-664) or the receptor binding domain of *Clostridium botulinum* C2 toxin corresponding to amino acids 592–721 of C2 toxin (pGEX-C2II592-721) was expressed as a fusion protein with GST in *Escherichia coli* strain BL21 (DE3) as described previously [27,28]. After growth at 25 °C and induction with isopropyl β-D-thiogalactopyranoside (Nacalai Tesque, Kyoto, Japan) of a large culture of 200 mL culture, the cells were harvested, resuspended in Buffer F (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$), and then lysed by sonication. The lysates were applied to a glutathione sepharose 4B column (GE Healthcare, Buckinghamshire, UK), and GST-tagged Ib421-664 (GST-Ib421-664) or GST-tagged C2II592-721 (GST-C2II592-721) was eluted with elution buffer (10 mM reduced glutathione, pH 8.0, 50 mM Tris-HCl). After the purified GST-Ib421-664 or GST-C2II592-721 was cleaved with thrombin, the solvent for Ib421-664 or C2II592-721 was changed to phosphate-buffered saline (PBS) by gel filtration by using a PD-10 column (GE Healthcare), and the purified proteins were stored at −80 °C until use. Concentration of the purified proteins was estimated by using a BCA protein assay kit (Pierce Chemical, Rockford, IL) with bovine serum albumin (BSA). as standard. Purification of the recombinant proteins was confirmed by use of sodium dodecyl sulfate gel-electrophoresis (SDS-PAGE), followed by staining with Coomassie Brilliant Blue (CBB).

2.6. Fluorescence-activated cell sorting (FACS) analysis

Mock/L cells or GFP-LSR/L cells, EpH4 cells, LSR-kd cells, ILDR1-FLAG/LSR-kd cells, or ILDR2-FLAG/LSR-kd cells were incubated with GST-Ib421-664 or GST-C2II592-721 for 1 h at 4 °C, and then incubated with an anti-GST-tag mAb. The cells were subsequently incubated with a fluorescein isothiocyanate (FITC)-labeled Ab, and the GST-Ib421-664- or GST-C2II592-721-bound cells were detected and analyzed with a flow cytometer (FACSCalibur, Becton Dickinson, NJ).

2.7. Immunofluorescence analysis

To investigate the interaction of angubindin-1 with angulin-expressing cells, purified GST-tag-free angubindin-1 or GST-tag-free C2II592-721 was coupled to DyLight 550 NHS ester (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Cells grown subconfluent on collagen-coated glass-bottom dishes (Matsunami, Osaka, Japan) were incubated with FITC-labeled angubindin-1 or C2II592-721 for 1 h at 37 °C. The cells were then washed