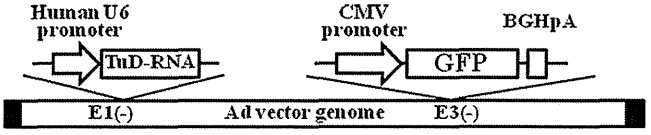

Fig. 1. Structure of Ad vectors used in this study. The human U6 promoter-driven TuD-RNA expression cassette was inserted into the E1-deleted region of the Ad vector genome. The CMV promoter-driven GFP expression cassette was inserted into the E3-deleted region of the Ad vector genome.

Systems which efficiently deliver or express anti-miR-122a drugs in the liver are necessary to efficiently treat HCV-related diseases.

Recently, tough decoy (TuD)-RNAs against miRNAs, which efficiently and specifically inhibit miRNAs, were developed by Haraguchi et al. (2009). TuD-RNAs are composed of two miRNA-binding sequence (MBS) regions and two stem structures with 3-nucleotide linkers. The MBS in the TuD-RNA is considered to tightly bind to miRNAs, leading to the inhibition of miRNAs. The inhibition activity of the TuD-RNA against miRNAs is higher than that of LNA oligonucleotides and miRNA sponges (Haraguchi et al., 2009). Another advantage of the TuD-RNA is that it can be expressed by viral and non-viral vectors. miRNAs can be persistently suppressed by lentivirus vector- and retrovirus vector-mediated expression of the TuD-RNA. Furthermore, liver-specific expression of the TuD-RNA is thought to be achievable by an adenovirus (Ad) vector and adeno-associated virus vector, because these vectors can express transgenes in a liver-specific manner after systemic administration. These properties of the TuD-RNA are highly promising for inhibition of miR-122a in the liver and suppression of HCV replication.

In the present study, we developed an Ad vector expressing the TuD-RNA against miR-122a (TuD-122a) to efficiently inhibit miR-122a and to suppress the HCV replication. Transduction with an Ad vector expressing TuD-122a efficiently inhibited miR-122a in vitro. In HCV replicon-expressing cells, HCV replicon levels were significantly reduced by Ad vector-mediated TuD-122a expression.

First, in order to examine the transduction efficiencies of the Ad vectors constructed in this study in the HCV replicon-expressing cells, Huh-7.5.1 1bFeo cells, which is a genotype 1b HCV replicon cell line (Yokota et al., 2003), were transduced with an Ad vector expressing TuD-122a (Ad-TuD-122a) or the control TuD-RNA (Ad-TuD-NC). Ad-TuD-122a and Ad-TuD-NC were prepared as described in Supplemental materials and methods. Structure of Ad vectors used in this study is shown in Fig. 1. The ratio of particles-to-biological titer was between 6 and 9 for each Ad vector used in this study. Both Ad-TuD-122a and Ad-TuD-NC carry the TuD-RNA expression cassette and the green fluorescence protein (GFP) expression cassette in the E1-deleted and E3-deleted region, respectively (Fig. 1). Both Ad-TuD-NC and Ad-TuD-122a efficiently transduced Huh-7.5.1 1bFeo cells (Fig. 2). More than 80% of the cells were found to be GFP-positive following transduction with Ad-TuD-122a and Ad-TuD-NC, respectively, at a multiplicity of infection (MOI) of 100. The averages of GFP-positive cells following transduction with Ad-TuD-NC were slightly higher than those with Ad-TuD-122a; however, statistically significant differences were not found for either group. Apparent cellular toxicity was not found following transduction with Ad-TuD-122a or Ad-TuD-NC (data not shown). These results indicate that Ad-TuD-122a and Ad-TuD-NC efficiently transduce Huh-7.5.1 1bFeo cells.

Next, in order to examine the inhibitory effects of TuD-122a expressed by the Ad vector on miR-122a, a reporter gene assay using the miR-122a complementary sequence-encoded plasmid, psiCheck-122aT, was performed in Huh-7 cells. Huh-7 cells endogenously express a high level of miR-122a (Suzuki et al., 2008). Huh-7 cells were transduced with the Ad vectors at MOIs of 25 and 100 for 1.5 h. After a 24-h incubation, the cells were transfected with

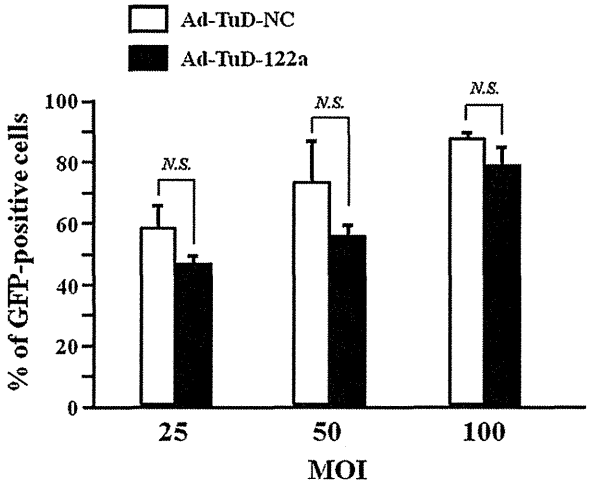

Fig. 2. Transduction efficiencies of Ad-TuD-122a and Ad-TuD-NC in Huh-7.5.1 1bFeo cells. The cells were transduced with Ad-TuD-122a or Ad-TuD-NC at multiplicities of infection (MOIs) of 25, 50, and 100 for 1.5 h. At 48 h after transduction, GFP expression was evaluated by flow cytometry. The data are expressed as the means ± S.D. ($n = 3$). The percentage of GFP-positive cells in the mock-transduced group was less than 0.2%. N.S.: not significant.

psiCheck-2 or psiCheck-122aT. The renilla and firefly luciferase expression was evaluated 48 h after transfection with the plasmid DNA. psiCheck-122aT, plasmid DNA containing the two copies of miR-122a complementary sequences in the 3′-UTR of the renilla luciferase gene, was constructed by ligation of *NotI*/*XhoI*-digested psiCheck-2 (Promega, Madison, WI) with the oligonucleotides 122aT-F and 122aT-R. The sequences of the oligonucleotides 122aT-F and 122aT-R are described in the Supplemental information. In mock-transduced cells, the relative renilla luciferase expression level by psiCheck-122aT was about 5-fold lower than that by the control plasmid psiCheck-2, which does not possess miR-122a target sequences, due to the endogenous expression of miR-122a in Huh-7 cells (Fig. 3). The renilla luciferase expression profiles following transfection with psiCheck-122aT were similar in the mock-transduced cells and Ad-TuD-NC-transduced cells, indicating that expression of the control TuD-RNA does not inhibit the miR-122a. Ad-TuD-122a did not alter the renilla luciferase expression level by psiCheck-2; on the other hand, psiCheck-122aT-mediated renilla luciferase expression was significantly restored by Ad-TuD-122a. The cells transduced with Ad-TuD-122a exhibited 2.8-fold and 3.5-fold higher renilla luciferase expression at MOIs of 25 and 100, respectively, than the mock-transduced cells following transfection with psiCheck-122aT. These results indicate that miR-122a is efficiently inhibited by Ad-TuD-122a. We also performed quantitative RT-PCR analysis for miR-122a following transduction with Ad-TuD-122a and Ad-TuD-NC in Huh-7 cells. No significant differences in the miR-122a expression levels were found in the cells transduced with Ad-TuD-122a and the cells transduced with Ad-TuD-NC (data not shown), probably because TuD-RNA does not induce degradation of miRNA, although TuD-RNA tightly binds to the target miRNA (Haraguchi et al., 2009).

Next, in order to examine whether TuD-122a-mediated inhibition of miR-122a suppresses the HCV replicon, Huh-7.5.1 1bFeo cells were transduced with Ad-TuD-122a and Ad-TuD-NC at the indicated MOIs. Huh-7.5.1 1bFeo cells express an mRNA consisting of the HCV 5′-UTR and the upstream part of the core region, connected in-frame with the firefly luciferase gene, which allows the simple evaluation of the HCV replicon levels by measuring the firefly luciferase activity in the cells (Yokota et al., 2003). Huh-7.5.1 1bFeo cells were transduced with the Ad vectors at MOIs of 25, 50, and 100 for 1.5 h. After a total 48-h incubation,

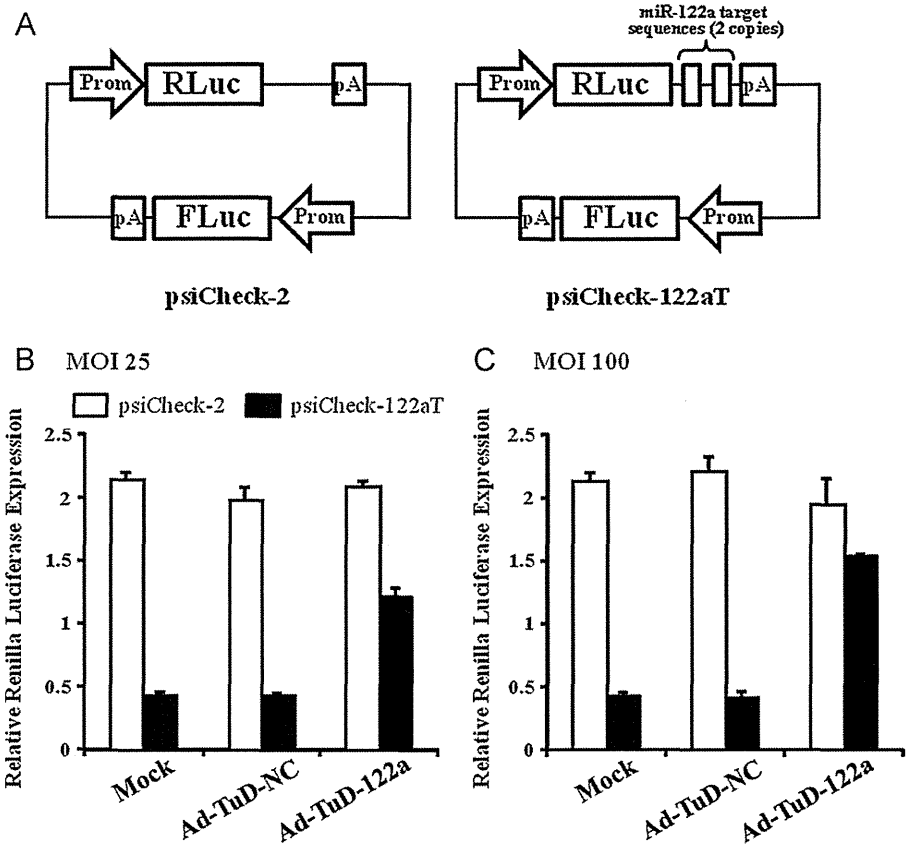

Fig. 3. Inhibition of miR-122a by Ad vector-meditated TuD-122a expression. (A) Structure of the reporter gene-expressing plasmids psiCheck-2 and psiCheck-122aT. (B and C) Relative renilla luciferase expression levels following transduction with Ad-TuD-NC or Ad-TuD-122a at MOIs of 25 (B) and 100 (C). The data are expressed as the means ± S.D. ($n = 4$)..

firefly luciferase expression levels were determined. Ad-TuD-122a significantly reduced the firefly luciferase expression levels in a dose-dependent manner (Fig. 4a). The firefly luciferase expression level was reduced to 29% of that in the cells transduced with Ad-TuD-NC at MOI of 100 by transduction with Ad-TuD-122a at MOI of 100. In contrast, no significant changes in the firefly luciferase expression were found by transduction with Ad-TuD-NC.

To examine whether inhibition of miR-122a by Ad vector-mediated TuD-122a expression leads to a reduction in HCV replicon RNA levels, strand-specific real-time RT-PCR analysis was performed to determine the HCV replicon RNA levels. Briefly, Huh-7.5.1 1bFeo cells were transduced with the Ad vectors as described above, and the total RNA was isolated 48 h after transduction. Real-time RT-PCR analysis for the HCV positive-strand RNA genome was performed as follows. Briefly, 2 μg of total RNA was reverse-transcribed to cDNA using the primer specific for the HCV positive-strand genome (RC21; 5′-ctc ccg ggg cac tcg caa gc-3′). Real-time RT-PCR was performed using the primers (RC21 and RC1; 5′-gtc tag cca tgg cgt tag ta-3′) and SYBR Premix Ex Taq II (Takara Bio Inc., Kyoto, Japan). Similarly to the results for the firefly luciferase expression in Fig. 4A, HCV replicon RNA levels were significantly reduced by Ad-TuD-122a (Fig. 4B). There was an approximately 2.2-fold decline in the HCV replicon RNA level in the cells transduced with Ad-TuD-122a at an MOI of 100, compared with the HCV replicon RNA level in the cells transduced with Ad-TuD-NC at an MOI of 100. Ad-TuD-NC did not apparently decrease the HCV replicon RNA levels. These results indicate that the inhibition of miR-122a by Ad vector-mediated TuD-122a expression efficiently suppresses the replication of the HCV replicon.

The present study demonstrates that Ad vector-mediated TuD-122a expression significantly inhibits the function of miR-122a and replication of the HCV replicon. Replication of the HCV genome is promoted by the direct interaction between miR-122a and the complementary sequences in the 5′-UTR of the HCV genome (Henke et al., 2008; Jangra et al., 2010), indicating that sequestration of miR-122a leads to suppression of the HCV replication. In order to suppress the HCV replicon by inhibiting miR-122a, TuD-RNA was selected as an inhibitor of miRNA in this study, because TuD-RNA potently inhibits miRNA by strongly binding to miRNA (Haraguchi et al., 2009). In addition, TuD-RNA can be expressed by conventional gene delivery vectors, including virus vectors. One drawback of TuD-RNA is that TuD-RNA does not discriminate miRNA members that belong to the same miRNA family (Haraguchi et al., 2009); however, miR-122a does not constitute a family of miRNA, suggesting that TuD-122a would not inhibit other miRNAs.

As described above, an Ad vector is suitable for liver-specific expression of TuD-RNA due to the strong hepatotropism. Previous studies demonstrated that Ad vectors expressing short-hairpin RNA (shRNA) or antisense RNA against the HCV genome successfully exhibited the suppressive effects on HCV infection in vivo (Gonzalez-Carmona et al., 2011; Sakamoto et al., 2008). Another advantage of using an Ad vector for treatment of HCV-related diseases is that in vivo administration of an Ad vector induces type I interferon (IFN) production via innate immune responses (Huarte et al., 2006; Zhu et al., 2007). Our group previously demonstrated that VA-RNA, which is a small non-coding RNA expressed from a replication-incompetent Ad vector as well as wild-type Ad, stimulates type I IFN production in an IFN-β promoter stimulator-1 (IPS-1)-dependent manner (Yamaguchi et al., 2010). Ad vector-induced type I IFN would contribute to suppression of HCV infection. The anti-HCV activity of Ad-TuD-122a can also be up-regulated by insertion of an expression cassette of an

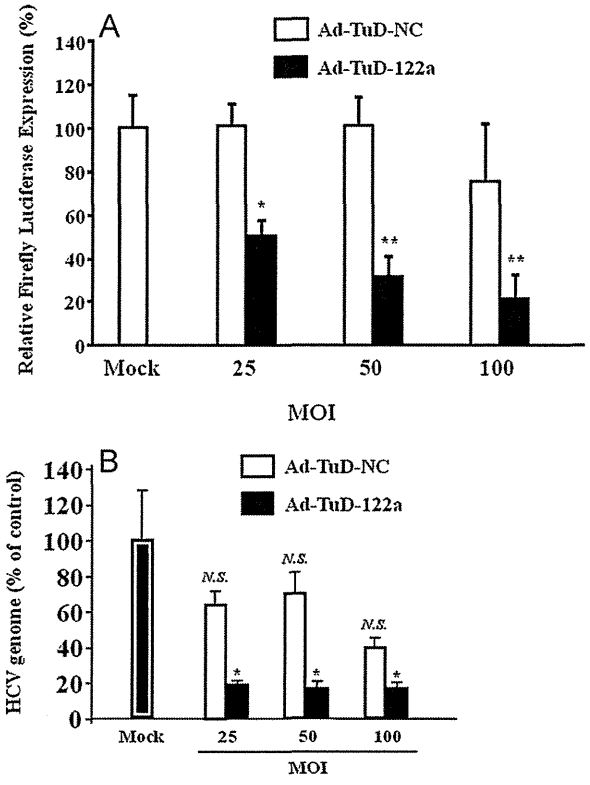

Fig. 4. Suppression of the HCV replicon by Ad vector-mediated TuD-122a expression. (A) Firefly luciferase expression levels and (B) HCV replicon RNA levels in Huh-7.5.1 1bFeo cells following transduction with the Ad vectors. All the data are shown as the means ± S.D. ($n = 3$). N.S.: not significant. $^{*}P < 0.05$, $^{**}P < 0.005$ between mock-transduced cells and cells transduced with Ad-TuD-122a.

anti-HCV gene, including type I IFN genes and short-hairpin RNA (shRNA) or antisense RNA against the HCV genome, into the Ad vector genome. Our group has developed various types of Ad vectors in which two or three transgene expression cassettes can be inserted into a single Ad vector genome (Mizuguchi et al., 2001, 2005, 2003).

Previous studies have demonstrated that lipid droplets, which are lipid-storage intracellular organelles, are crucial for the production of infectious HCV particles (Hinson and Cresswell, 2009; Miyanari et al., 2007). Miyanari et al. demonstrated that HCV capsid proteins recruit the non-structural proteins and the replication complex to the lipid droplet-associated membrane (Miyanari et al., 2007). miR-122a is an important factor that regulates cholesterol and fatty-acid metabolism in the hepatocytes (Esau et al., 2006; Iliopoulos et al., 2010). Intravenous administration of the antisense oligonucleotide against miR-122a resulted in a reduction in the plasma levels of cholesterol and triglycerides (Esau et al., 2006; Lanford et al., 2010). In addition to the enhancement of accumulation and translation of the HCV genome, miR-122a might up-regulate HCV infection by regulating lipid metabolism in the hepatocytes.

Almost similar levels of reduction in the HCV replicon RNA copy numbers were found for Ad-TuD-122a at MOIs of 25, 50, and 100, although there was dose-dependent reduction in the firefly luciferase expression following transduction with Ad-TuD-122a. It remains unclear why dose-dependent reduction in the HCV replicon RNA copy numbers was not found, however, miR-122a plays a crucial role in the enhancement of both translation and stability of HCV genome (Henke et al., 2008; Jopling et al., 2005; Randall et al., 2007; Shimakami et al., 2012). Stability of HCV genome might be more susceptible to inhibition of miR-122a than translation. The averages of HCV replicon RNA levels were also reduced following transduction with Ad-TuD-NC, although statistically significant differences were not found, compared with the mock-transduced cells. Replication-incompetent Ad vectors express non-coding small RNA (VA-RNA), which forms RNA-induced silencing complex (RISC) with argonaute 2 (Ago2) (Xu et al., 2007). Ago2 is an important factor for miRNA processing (Diederichs and Haber, 2007). Processing of miR-122a might be slightly disturbed by Ad vector-expressed VA-RNA, leading to the reduction in the HCV replicon RNA levels.

In summary, we efficiently suppressed the HCV replicon levels by Ad vector-mediated expression of TuD-122a, which blocks the function of miR-122a. This study indicates that Ad vector-mediated expression of TuD-122a in liver hepatocytes would offer an alternative approach for the treatment of HCV infection.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2012.02.003.

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Spiral progression in the development of absorption enhancers based on the biology of tight junctions ☆

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ABSTRACT

Epithelium covers the body and, therefore, separates the inner body from the outside environment. Passage across the epithelium is the first step in drug absorption. Tight junctions (TJs) seal the space between adjacent epithelial cells and prevent the free movement of solutes through the paracellular space. Modulation of the epithelial barrier is the most important strategy for enhancing drug absorption. Development of the strategy has accelerated with progress in understanding of the biology of the TJ seal. The first-generation absorption enhancers were screened on the basis of their absorption-enhancing activity in vivo. However, TJs were not well understood initially. The identification of TJ components, including those based on occludin and claudins, has led to the development of new strategies for drug absorption. Accumulation of knowledge of claudins has provided new insights into the paracellular transport of drugs. This review examines the relationship between advances in understanding of TJ biology and paracellular transport of drugs and discusses progress in the development of mucosal absorption enhancers.

Contents

1. Introduction

The philosopher Hegel proposed that "change moves in spirals, not circles." In other words, things of the past will reemerge with progress.

Drugs are administered by routes that include oral, nasal, pulmonary, and epidermal routes or injection. Noninvasive routes are better than invasive ones, but drug administration via the nasal, pulmonary, and epidermal routes requires specialized equipment.

Abbreviations: TJ, tight junction; EDTA, ethylenediaminetetraacetic acid; AJ, adherens junction; DS, desmosome; GP, gap junction; CPE, *Clostridium perfringens* enterotoxin.

☆ This review is part of the *Advanced Drug Delivery Reviews* theme issue on "Advances in Oral Drug Delivery: Improved Bioavailability of Poorly Absorbed Drugs by Tissue and Cellular Optimization".

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These noninvasively administered drugs are expensive. Drugs administered orally without using specific equipment are the least expensive. Patients are often compliant in taking oral medication because this method of drug delivery is easy and pain-free. Therefore, orally administered drugs are ideal for drug development. However, the intestinal mucosa functions as a biological barrier, separating the outside environment from the inner body and preventing the free movement of solutes. Modulation of the epithelial barrier must be considered in the development of orally administered drugs.

The first report of mucosal absorption of drugs was published approximately 50 years ago [1]. Ethylenediaminetetraacetic acid (EDTA) was shown to enhance intestinal absorption of heparin and synthetic heparinoids, but the mode of action was unclear. Chelation of magnesium and calcium by EDTA was later shown to increase intestinal permeability to solutes, but the mechanism was not clarified [2]. EDTA was found to widen the intercellular junctional seal, enhancing intestinal permeability to solutes [3]. This was the first evidence that modulation of the intercellular seal in the mucosal epithelium may be a potent strategy for mucosal absorption of drugs.

The first breakthrough in the biology of the epithelial barrier was the identification of tight junctions (TJs) between adjacent epithelial cells, in 1963 [4]. This finding promoted the development of novel strategies for mucosal absorption of drugs by modulating the TJ seal. Development has progressed further with our understanding of the biology of the epithelial barrier.

Strategies for mucosal absorption of drugs are developing, accompanied by advancements in understanding of the biology of epithelial cells. This review describes the development of strategies for mucosal absorption of drugs as a result of the accumulation of knowledge of the epithelial barrier. We also describe the future directions in research on mucosal absorption of drugs.

2. First-generation mucosal absorption enhancers

The first advancement in the biology of the epithelial barrier was the discovery of the TJ (Table 1) [4]. High-resolution replica analysis revealed that TJs form a continuous band-like meshwork. Globular components bridge the width of the adjoining membranes linked together in the plane of the intercellular space [5]. However, it was unclear whether the TJ components are proteins or lipids. Membrane lipids, rather than membrane proteins, were thought to be the structural elements of TJ seals [6,7]. Therefore, the initial stage of the development of novel strategies for mucosal absorption of drugs was based on the knowledge that the intestinal mucosa is a barrier for drug absorption and that TJ components are responsible for intercellular sealing, but no molecular level information about TJ components was available.

The first advancements evoked the idea that disruption of the mucosal epithelial barrier or TJ seal would lead to the development of novel strategies for mucosal absorption of drugs (Table 2). Nonionic, anionic, and cationic surfactants are also intestinal absorption enhancers [8,9]. These surfactants were believed to disturb the integrity of the plasma membrane [9]. Surfactant–surfactant interactions occur at high surfactant concentrations in the plasma membrane and can result in dissolution of the plasma membrane into surfactant–membrane mixed micelles. Surfactants can also extract proteins from the plasma membrane. Surfactant-enhanced membrane permeability is generally assumed to be nonspecific and cytotoxic [9]. Some surfactants, such as polyoxyethylene esters and dodecylmaltoside, exhibit absorption-enhancing effects and toxic effects in the intestine [10–13].

EDTA, a calcium chelator, enhances mucosal absorption of drugs [1]. EDTA modulates TJ barrier integrity by opening intracellular TJ seals [14]. Some surfactants also enhance intestinal absorption by sequestering calcium ions [10].

Various fatty acids, including caprate, caprylate, and laurate, enhance membrane permeability [15]. Mucosal absorption of insulin and cefmetazole is increased with 1% caprate treatment, but only absorption of insulin is enhanced by treatment with 0.25% caprate. One possible explanation for the differential effects on mucosal absorption of drugs was that the electrically repulsive effects of the paracellular route might affect paracellular absorption of a neutral molecule, insulin, and an acidic molecule, such as cefmetazole [15]. This finding indirectly suggested that modulation of the paracellular route can lead to mucosal absorption of solutes in a solute-specific manner. A series of analyses aimed at determining the mode of action of sodium caprate indicated that the compound activates phospholipase C, elevates intercellular calcium levels, and subsequently stimulates contraction of calmodulin-dependent actin–myosin filaments, thereby opening TJ seals [16,17].

Cationic chitosan increases epithelial paracellular permeability [18]. Chitosans bind to the epithelial cell membrane through a charge-dependent interaction, resulting in F-actin depolymerization and separation of TJ components. This event triggers enhanced epithelial permeability. Polylysine also enhances epithelial paracellular permeability by opening TJ seals [19].

There are two issues in the development of absorption enhancers: the toxicity of these substances and the risk of opening TJ seals. The first-generation absorption enhancers disrupted the cell membrane and modulated the TJ seal. Transient modulation of TJ seals by EDTA, fatty acids, and polycations would be less toxic than disruption of the cell membrane by surfactants. Opening the intercellular TJ seal might lead to the influx of solutes other than drugs, including undigested food, metabolites of intestinal microorganisms, and bile salts. Mucosal

Table 1
Progressive elucidation of TJ biology.

Year	Event
1963	Identification of TJ [4]
1973	Identification of TJ strands [5]
1982	Membrane lipid hypothesis [6,7]
1986	Identification of ZO-1 [96]
1993	Identification of occludin[24]
1998	Identification of claudin [33]
1999 onward	Clarification of TJ barrier function of claudins [47] Identification of paracellular ion transport via claudins [94]
2011	Identification of transcellular transport coupled to claudin-based TJ strands [95]

TJ: tight junction.

Table 2
Progress in the development of absorption enhancers.

Category	Enhancer	Possible mode of action
First-generation absorption enhancers	EDTA	Sequestration of Ca^{2+}[14][a]
	Surfactants	Perturbation of the plasma membrane [8,9]
	Sodium caprate	Phospholipase C [16,17][b]
	Chitosan	Depolymerization of actin [19]
Second-generation absorption enhancers	Occludin peptide	Perturbation of occludin [26–29][c]
	C-CPE	Binding to claudin-4 [47,50]
	FSH-fused occludin peptide	Perturbation of occludin in BTB [30]
	Claudin peptide	Binding to claudins and occludin [77]

EDTA: ethylenediaminetetraacetic acid; FSH: follicle-stimulating hormone; BTB: blood–testis barrier.

[a] Activation of protein kinase C is partly involved in modulation of the TJ barrier by chelation of Ca^{2+}[97,98].

[b] Activation of phospholipase C increases intracellular calcium levels, followed by contraction of calmodulin-dependent actin–myosin filaments and subsequent opening of the TJ-seal [16,17].

[c] Occludin peptides cause a decrease in the cellular content of occludin or perturbation of localization of occludin [26–28]. An occludin peptide interacts with occludin and claudin-1 [99].

absorption through the paracellular route requires opening of the intercellular seal, and most researchers believed (and may still think) that such a clinical application is impossible. Transcellular drug delivery via transporters has been widely investigated.

3. Second-generation mucosal absorption enhancers

3.1. Impact of occludin

3.1.1. Occludin

Mammalian cells have four types of intercellular junctions: adherens junctions (AJs), desmosomes (DSs), gap junctions (GPs), and TJs. Although specific types of integral membrane proteins – cadherins, desmogleins and desmocollins, and connexins – had been identified in AJs, DSs, and GPs, respectively [20–22], biochemical information about TJs remained sparse.

An electron microscopic analysis in 1973 revealed that TJs appear as a linear series of individual intramembranous particles [5]. Evidence later indicated that TJs might be pairs of inverted cylindrical lipid micelles [6,7]. Freeze fracture analysis revealed that TJ components are not exclusively lipids [23]. Progression in the understanding of TJ biology led to identification in 1993 of the integral membrane protein occludin located in TJs (Table 1) [24]. Furuse et al. isolated the AJ fraction from chicken livers and immunized rats with the fraction. They prepared monoclonal antibodies and found antibodies that recognized an approximately 65-kDa membrane protein enriched in the junctional region of endothelial and epithelial cells. The approximately 65-kDa protein containing four transmembrane domains was identified as occludin. Occludin homologs have been identified in humans, mice, dogs, and rat-kangaroos, and the development of drug delivery systems targeting occludin was proposed by Tsukita et al. in 1996 [25]. This was the first suggestion of a molecular TJ seal-based drug delivery system.

3.1.2. Occludin and drug delivery

The extracellular loop domains of occludin are rich in tyrosine and glycine residues [24]. The nonpolar nature of the extracellular domains and conservation of their sequences among humans, mice, dogs, chickens, and rat-kangaroos suggest that the extracellular domains have important functional roles in the formation of intercellular TJ seals. These findings suggest the possibility of modulating TJ seals by using a synthetic peptide corresponding to the extracellular domain of occludin (Table 2). The TJ seal is modulated by targeting occludin with a synthetic peptide corresponding to the second extracellular loop domain of occludin [24]. The TJ barrier in *Xenopus* kidney epithelial A6 cell lines is reduced by the occludin peptide but not by a peptide containing scrambled amino acid sequences [26]. The occludin peptide enhances the epithelial permeability of A6 monolayer cell sheets to mannitol, insulin, and 3- and 40-kDa dextran. Another synthetic peptide corresponding to the first loop domain of occludin decreases TJ barrier integrity and increases the permeability of human colon carcinoma Caco-2 cells to mannitol [27]. Treatment with a synthetic peptide corresponding to the first extracellular loop domain of occludin in human airway epithelial cells reversibly decreases the integrity of the TJ barrier. Permeability to 70-kDa dextran is transiently and reversibly increased in human airway epithelial cell sheets [28]. An occludin peptide also modulates TJ barrier integrity in rat Sertoli monolayer cell sheets [29]. Receptors for follicle-stimulating hormone (FSH) are expressed in Sertoli cells. An inactive FSH mutant was used as a ligand for the in vivo blood–testis barrier, and injection of the FSH mutant-fused occludin peptide led to reversible disruption of the blood–testis barrier [30]. Therefore, identification of occludin as a TJ component resulted in a shift in the mucosal absorption paradigm from a phenomenon- to a molecular-based approach. In addition, the discovery of occludin led to major progress in understanding of the biology of the epithelial barrier.

3.2. Impact of claudin

3.2.1. Claudin

Gene knockout analyses were used to clarify the roles of occludin in the TJ barrier. Occludin-deficient embryonic stem cells differentiate into polarized epithelial cells [31]. Paracellular influx of biotin is prevented in both wild-type and occludin-deficient epithelial cells. The morphology of the intestine, liver, and kidney in occludin-deficient mice is normal [32]. No dysfunction of the TJ barrier is observed in the intestinal epithelial cells of occludin-deficient mice. These findings indicate that occludin is not the only integral membrane protein component of TJs. Furuse et al. identified another integral membrane protein, claudin, by using occludin as a probe (Table 1) [33]. They isolated the junctional fraction containing occludin in chickens, removed the peripheral proteins, and obtained a fraction containing only integral membrane proteins by guanidine extraction. The isolated fraction was sonicated and fractionated by centrifugation in a stepwise discontinuous sucrose density gradient. The fraction containing occludin contained two novel integral membrane proteins, claudin-1 and -2, with no sequence homology with occludin. Claudin-1 and -2 are tetratransmembrane proteins with molecular masses of approximately 22-kDa. These proteins are directly incorporated into TJ strands [33]. Expression of claudins in L cells lacking TJs induced the formation of TJ networks and claudin polymerization in the plasma membrane [34]. These findings indicate that claudins are structural components of TJ seals. A genomic analysis revealed that claudins are a family containing at least 27 proteins [35–37].

3.2.2. Claudin and the TJ barrier

Interestingly, the expression profiles and barrier functions of claudins differ among tissues. Paracellulin-1 (identical to claudin-16), which is expressed in the thick ascending limb of the loop of Henle, controls paracellular magnesium ion transport [38]. Claudin-19 expression in Schwann cells may be involved in the electrophysiological sealing function of Schwann cells [39]. Claudin-1-deficient mice exhibit dysfunction of the epidermal barrier [40]. A solute with a molecular mass of approximately 600 Da appears to pass through TJs in the epidermis of these mice. These mice possess normal morphological and biochemical structures in the epidermis, and claudin-1 can be specifically removed from the TJs of stratified epithelial cells without affecting TJ morphology. These findings indicate that claudin-based TJ strands are crucial for the barrier function of mammalian skin but not for maintenance of epidermal morphology. The cornified cell envelope and lipid lamellae in the stratum corneum were assumed to be the only epidermal barriers before the identification of claudin-1. Identification of claudin-1 allowed researchers to confirm that both the stratum corneum and stratified epithelial cells in the skin are epidermal barriers [41]. Therefore, modulation of the claudin-1 barrier could act as a potent strategy for epidermal absorption of drugs. Claudin-5-deficient mice exhibit a dysfunctional blood–brain barrier [42]. Small molecules, with molecular masses of <800 Da, pass through the blood–brain barrier of these mice. The mice exhibit normal blood vessel morphology and no bleeding or edema. TJs are often composed of more than two distinct species of claudin; the removal of one claudin species markedly changes the TJ barrier without affecting its continuous structural integrity. This suggests the possibility of improving drug delivery to the central nervous system by modulating claudin-5 [42].

Clostridium perfringens enterotoxin (CPE) is a 35-kDa polypeptide that causes food poisoning in humans [43]. The C-terminus of CPE is involved in binding to target cells, whereas the N-terminus is responsible for the cytotoxicity [44]. A receptor for CPE (CPE-R) was cloned from an expression library of CPE-sensitive cells [45]. Rat

ventral prostate-1 (RVP-1) possesses a sequence and function similar to those of CPE-R [46]. Morita et al. revealed CPE-R and RVP-1 to be claudin-4 and claudin-3, respectively [37]. CPE is cytotoxic to claudin-3- and -4-positive cells, but this effect is lost if its N-terminus is removed. Treatment of cells with the C-terminal fragment of CPE, corresponding to amino acids 184–319 (C-CPE184) reduces transepithelial electrical resistance, which is a typical indicator of the barrier function of TJs. TJ barrier integrity recovers after the removal of C-CPE184. Treatment of cells with C-CPE184 decreases the level of claudin-4 protein [47]. Claudin-4 levels recover after the removal of C-CPE184. C-CPE184 treatment does not affect claudin-1 protein levels. Claudin contains the clathrin-sorting signal, and endocytosis of claudin occurs during the remodeling of TJ strands [48,49]. C-CPE184-bound claudin-3/-4 may be taken up by endocytosis; this is followed by degradation of claudins. Two possible mechanisms were proposed to explain C-CPE184-induced modulation of the TJ-barrier [47]. C-CPE184 may bind directly to claudin-3/-4 within TJ strands and then evoke depolymerization of TJ strands. Alternatively, C-CPE184 may bind to claudin-3/-4 in the non-junctional area, and this binding to claudin may suppress the polymerization of TJ strands. Furthermore, C-CPE184 increases the paracellular permeability of 4- and 10-kDa dextran in epithelial cell sheets. Inhibition of the claudin-based TJ-barrier by using claudin binders would provide a new means to modulate the TJ barrier and improve the bioavailability of drugs to target organs.

3.2.3. Claudin-targeted drug delivery

The efficacy of claudin binders as a novel strategy for mucosal absorption of drugs was demonstrated 6 years after the idea was first proposed (Table 2). C-CPE184 dose-dependently enhances intestinal absorption of 4-kDa dextran [50]. The absorption-enhancing activity is more than 400 times that of capric acid, an enhancer used clinically. A C-CPE184 mutant lacking the claudin-3/-4-binding domain does not exhibit absorption-enhancing activity. C-CPE184 enhances intestinal absorption of 4-, 10-, and 20-kDa dextran but not 40-kDa dextran. There is no mucosal injury in the intestines of C-CPE184-treated animals. Therefore, the use of a claudin-3/-4 binder is a novel method to enhance mucosal absorption. CPE also binds to claudin-6, -7, -8 and -14, and CPE interacts with the second extracellular loop of claudins [51]. Electrostatic interaction could be involved in the interaction between CPE and the second extracellular loop of claudins [52]. Claudin-7 and -8 are also expressed in the intestine, but any interaction between C-CPE184 and claudin-7 and/or -8 in the intestine remains unclear [53].

Recent progress in genomic and proteomic technologies has yielded some new biologics, including peptides, proteins, and nucleic acids, as pharmaceutical candidates. Most of these compounds are hydrophilic molecules that are poorly absorbed by the mucosa. Although injection is a compelling route for the administration of biologics, a transmucosal delivery system would be an ideal route because it is noninvasive and therefore would enable easy, pain-free administration by patients. C-CPE184 enhances nasal, but not jejunal and pulmonary, absorption of a peptide drug [54]. The solubility of C-CPE184 is <0.3 mg/ml. C-CPE194 is a derivative of C-CPE184 with a deletion of 10 N-terminal amino acids. C-CPE194 has greater solubility (10 mg/ml) than C-CPE184. C-CPE194 is also a claudin-3/-4 binder that enhances jejunal and pulmonary absorption of a peptide drug [54]. These findings support the idea, proposed by Sonoda et al. in 1999, that claudin binders may improve drug bioavailability. Modulation of the claudin barrier could be useful for development of non-invasive administration of biologics, most of which must be injected in patients.

3.2.4. Development of claudin binders

A series of studies using C-CPEs have revealed the possibility of developing claudin-targeted drug absorption [50,54]. The claudin family has at least 27 members. Several claudins are expressed in the duodenum, jejunum, ileum, and colon, and the expression profiles of claudins differ throughout the segments of the intestinal tract (see review in [53]). Development of claudin binders with broad and narrow specificity for claudins will be needed for the development of oral drug delivery systems based on modulation of the claudin barrier. However, claudins have four transmembrane domains, are hydrophobic, and have small extracellular loop domains. Therefore, preparation of recombinant proteins and antibodies to these extracellular domains is difficult. Only claudin-4 protein has been purified [55], and the development of specific claudin binders has been delayed. Claudin binders are classified into three categories: C-CPE derivatives, antibodies, and claudin peptides.

3.2.5. C-CPE derivatives

C-CPE184 is the first claudin binder and the only one discovered before 2005. C-CPE184 is a toxin fragment, and overcoming its antigenicity is critical for its clinical application [56]. Two approaches have been utilized to develop a claudin binder by using C-CPE184. One approach is the development of a binder by using C-CPE184 as a prototype. A deletion analysis revealed that the C-terminal segment, corresponding to 30 or 16 amino acids, is essential for the interaction of C-CPE184 with claudin-4 [57,58]. Synthetic peptides, corresponding to the 30 amino acids, bind to claudin-4 and modulate the TJ barrier in human intestinal cell lines [59]. Deletion of a portion of the N-terminal segment of C-CPE184 improves its solubility, and the improved C-CPE enhances mucosal absorption of a bioactive peptide drug [54]. Therefore, the deletion approach is one method of developing claudin binders, but there are no reports of a synthetic short peptide that can enhance mucosal absorption. Random mutagenesis of C-CPE184 is another approach. A site-directed mutagenesis analysis identified the functional amino acids of C-CPE184, and a C-CPE mutant library was formed by randomly mutating functional residues into other amino acid residues [60,61]. Recombinant claudin proteins are needed for the selection of claudin binders. It is generally difficult to obtain recombinant membrane proteins, including claudins, with an intact structure, and a protocol has been established only for recombinant claudin-4 [55]. Functional membrane proteins are heterogeneously expressed on the budded baculovirus, and interactions between membrane proteins can be detected by using receptor- and ligand-displaying budded baculoviruses [62–64]. Very recent studies suggest that claudins on budded baculovirus possess the native form and the claudin-displaying baculovirus functions as a screening system for claudin binders [60]. Indeed, claudin binders with broad specificity for claudins were isolated from the C-CPE mutant library by using the baculoviral display system, and the binders enhance mucosal absorption (our unpublished data). Chemical claudin binders are ideal for clinical application in terms of costs and antigenicity. Structural information about claudins and C-CPEs can provide the theoretical basis for claudin binders, but the three-dimensional structure of claudins has never been identified. An X-ray diffraction analysis revealed that C-CPE194 contains nine beta-sheets and one alpha helix and that the loop between beta-sheets 8 and 9 is a binding site of C-CPE194 to claudin-3 and -4 [65]. CPE interacts with the second loop of claudin-3 [51,66]. A series of analyses of the interaction between CPE and claudins indicated that the negatively charged cleft of C-CPE surrounded by Tyr306, Tyr310, Tyr312, and Leu315 might interact with the positively charged CPE-sensitive region [52]. Future determination of complex interactions between claudins and C-CPEs will facilitate the development of chemical claudin binders.

3.2.6. Antibodies and claudin peptides

The first antibodies to the extracellular domains of claudins were prepared by immunizing chickens with synthetic peptides corresponding to the extracellular domains [67]. Some of the polyclonal antibodies bound to claudin-3 and -4. Single-chain Fv fragments that bound to claudin-3 were isolated through selection from a human recombinant

antibody library containing $>10^9$ possible antibody combinations by using synthetic peptides corresponding to the extracellular domain of claudin-3 [68]. Immunization of immune-deficient mice or mice with claudin-4-expressing cells results in the production of monoclonal antibodies that bind claudin-4 or both claudin-3 and -4 [69–71]. Genetic immunization of mice with claudin-1-expressing DNA vectors led to the successful production of anti-claudin-1 monoclonal antibodies [72]. These findings did not include any data on modulation of the TJ barrier by antibodies. Therefore, progress has been made in the development of anti-claudin antibodies that recognize the extracellular domain.

Claudins have two extracellular loop domains. The intercellular interaction of the extracellular domains is thought to regulate paracellular permeability [73,74]. There may be homophilic interaction between claudins. Claudins have a *cis* interaction within the plane of the membrane to form dimers, followed by *trans* interactions between claudins in adjacent cells and additional *cis* interactions to assemble claudin oligomers into intramembrane TJ strands [75,76]. These findings suggest that peptides mimicking the extracellular loop domains of claudins interfere with the interactions of claudins, leading to modulation of the TJ barrier. A specific claudin-1 extracellular loop peptide mimetic corresponding to a 53- to 80-amino acid domain reversibly decreases the TJ barrier integrity and increases paracellular permeability to solutes in epithelial cells through its interaction with claudin-1 and -3 and occludin [77]. The peptide mimetic also enhances mucosal absorption in vivo [77].

3.2.7. Progression in development of absorption enhancers mediated by claudins

Some claudin binders truly enhance mucosal absorption of solutes through the paracellular route in the epithelium. What is the difference between first- and second-generation absorption enhancers? Can second-generation absorption enhancers overcome the problems associated with first-generation absorption enhancers? What progress has there been in the development of absorption enhancers mediated by claudins?

One problem with first-generation absorption enhancers is the influx of solutes other than drugs across epithelial cell sheets after the TJ seals are opened. The enhancers widen the paracellular space by inhibiting TJ seals, leading to the movement of solutes. Solutes with molecular masses of 600–800 Da were found to cross the epidermal and blood–brain barriers in claudin-1- and -5-deficient mice, respectively [40,42]. C-CPE184 enhances the epithelial permeability and mucosal absorption of dextran in a molecular size-dependent manner [47,50]. Claudin-based TJ strands also function in paracellular

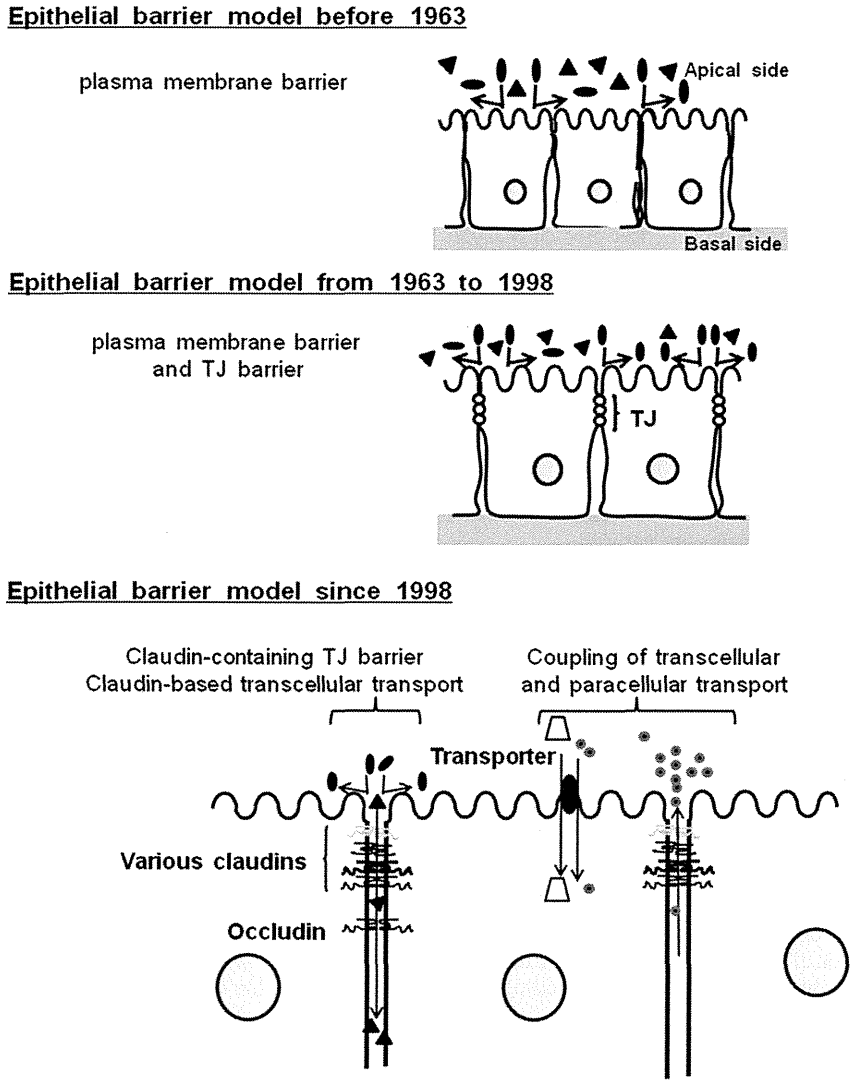

Fig. 1. Progression in paracellular transport research through understanding of the biology of the epithelial barrier. The plasma membrane was originally considered to be the primary barrier in epithelial cell sheets. TJ strands were subsequently identified as the intercellular sealing components [4,5]. Claudins were later found to be key structural and functional components of TJ seals. They function as intercellular seals and are also involved in the intercellular transport of ions. Claudins are coupled to both transcellular and paracellular transport. Claudin-based paracellular transport can be charge and size selective [35].

ion transport. Claudin-16 is required for paracellular magnesium resorption in the kidney [38]. Claudin-2 forms a leaky, sodium ion-selective paracellular channel in the kidney [78]. Paracellular influx of cations or anions is regulated by the expression of different claudins [79]. Claudins represent a family consisting at least 27 proteins, and TJ strands contain several types of claudins in a tissue-specific manner. The combination and mixing ratios of claudins are important for the tightness of TJ strands [80]. Incorporation of claudin-4 and -8 into TJ strands requires a paracellular anion channel in the kidney [81]. Interactions between claudin-16 and -19 are required for their assembly into TJ strands and renal reabsorption of magnesium [82]. Therefore, claudins function as a biological barrier that prevents the influx of solutes and pathogens, and they also function in the paracellular transport of solutes across TJ strands. Charge- and molecular size-dependent aqueous pores within claudin-based TJ strands for small molecules such as inorganic ions have been proposed [35]. Modulation of claudin-based aqueous pores suitable for a drug using claudin binders might allow the specified drug to pass through the mucosal epithelium only through paracellular routes. Future combinations of structural biological approaches involving claudin and the development of claudin binders will lead to a breakthrough in the development of a paracellular drug transport system that overcomes the drawbacks of first-generation absorption enhancers.

4. Future direction of research in transmucosal absorption

Hegel proposed the concept of "interpenetration of opposites" in his dialectic; this means that opposites will develop to interpenetrate each other. The final section will discuss the coupling of transcellular and paracellular drug delivery systems.

Routes for passing through the mucosal epithelium are classified as transcellular and paracellular [83–87]. Early research in this process focused on the paracellular routes, leading to the development of first-generation absorption enhancers. Transcellular uptake of drugs is mediated by simple diffusion and receptor- and transporter-dependent transcellular routes. Many different drug transporters are expressed in various tissues, including intestinal and hepatic epithelial cells and brain capillary endothelial cells [88–91]. Some of these transporters are involved in intestinal absorption and tissue distribution of drugs and can be used to determine the pharmacokinetic characteristics of various drugs. Although a number of receptors and transporters are available for drug delivery, each drug needs to be modified for recognition by these receptors and transporters. These findings indicate that the safety of transcellular transport may be superior to that of paracellular transport, because nonspecific influx of solutes other than drugs does not occur through carrier-recognized transport. Therefore, transcellular transport is thought to be the ideal intestinal absorption system. Paracellular and transcellular drug delivery systems will be coupled, on the basis of Hegel's "interpenetration of opposites," and the coupling will result in progression in the development of mucosal absorption enhancers.

Coupling of transcellular and paracellular transport can be categorized as production of the driving forces for solutes moving through either route and regulation of cellular signaling that controls permeability to solutes [92]. Intestinal absorption of nutrients, including sugars and amino acids, is coupled with Na^+ absorption [93]. Claudin-based TJ functions as a charge-selective channel in the paracellular route [74,79,94]. Claudin-15 is responsible for transepithelial permeability to extracellular monovalent cations, especially Na^+. Claudin-15-deficient mice exhibit low luminal Na^+ levels and low glucose absorption in the intestine, indicating that paracellular transport of Na^+ through claudin-15-based TJ strands may be coupled to the transcellular transport of glucose through a glucose transporter [95]. Modulation of the claudin barrier may be a novel mode of action of mucosal absorption enhancers that can modulate both the paracellular and transcellular transport of drugs.

In summary, strategies for intestinal absorption of drugs through the paracellular route have made dramatic progress because of progress in our understanding of the cell biology of the epithelial barrier. Two paradigm shifts have occurred in the consideration of paracellular route-mediated epithelial absorption of drugs (Fig. 1). The first paradigm shift was from modulation of the plasma membrane to the opening of TJ seals, leading to the development of absorption enhancers with lower cytotoxicity. The second paradigm shift was from the opening of TJ seals to the modulation of TJ components, leading to the development of molecular-based absorption enhancers. Moreover, the second shift revealed the possibility of enhancing intestinal absorption of drugs by regulating the nonspecific influx of substances other than drugs. A subsequent third paradigm shift may occur: coupling of paracellular and transcellular transport. The third shift suggests that future absorption enhancers will regulate both the transcellular and paracellular transport of drugs. Orally administered drugs are ideal pharmaceutical agents because they are less expensive and facilitate high patient compliance. The TJ-based strategy for mucosal absorption will lead to progress in the development of strategies for oral absorption of drugs.

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Pathological changes in tight junctions and potential applications into therapies

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Epithelial cells are pivotal in the separation of the body from the outside environment. Orally administered drugs must pass across epithelial cell sheets, and most pathological organisms invade the body through epithelial cells. Tight junctions (TJs) are sealing complexes between adjacent epithelial cells. Modulation of TJ components is a potent strategy for increasing absorption. Inflammation often causes disruption of the TJ barrier. Molecular imaging technology has enabled elucidation of the dynamics of TJs. Molecular pathological analysis has shown the relationship between TJ components and molecular pathological conditions. In this article, we discuss TJ-targeted drug development over the past 2 years.

During evolution from single-celled to multi-celled organisms, a compartment system developed to separate the inside of the body from the outside environment. This compartment system is made up of epithelial and endothelial cell sheets. Sealing of the intercellular space between individual epithelial or endothelial cells is crucial for compartmentalization.

Tight junctions (TJs) are the apical-most component of intercellular seals. TJs are directly involved both in the sealing of paracellular spaces and in two major functions of membranes: the barrier function and the fence function [1,2]. The barrier function is the first line of defense against pathogenic microorganisms and xenobiotics, and the fence function regulates cellular polarity. Deregulation of these functions is often observed in infectious diseases, inflammation and carcinogenesis.

Freeze-fracture electron microscopy analysis has shown that TJs are a set of continuous and anastomosing strands [3]. A series of analyses revealed that TJ-seals contain integral membrane proteins, such as occludin, claudins and junctional adhesion molecules (Fig. 1) [4–6]. The claudin protein family comprises 27 members and the junctional adhesion molecule (JAM) family comprises 3 members [4,7]. A tricellular junction-sealing component, tricellulin, has also been identified in epithelial cell sheets [8]. Occludin and tricellulin contain the tetra-spanning and other related proteins for vesicle trafficking and membrane line (MARVEL) domain. Occludin and tricellulin are members of the MARVEL protein family [9]. MarvelD3, another member of the MARVEL protein family, has been identified as a component of TJs [10]. The intracellular constituents of TJs, ZO-1 and ZO-2, determine where the claudin-based strands are formed [11]. Lipolysis-stimulated lipoprotein receptors define where tricellular junctions are formed [12]. These biochemical components of TJ-seals were all clarified within a single decade [5,6,13]. Our understanding of TJ-components has provided us with a new perspective on drug delivery and drug discovery for infectious diseases, inflammations and cancers [14–16].

There have been two main progressions in our understanding of the biology of TJs within the past 2 years: mucosal barrier homeostasis and TJ barrier homeostasis. Proof-of-concepts for TJ-targeted drug delivery have been demonstrated. In this article, we discuss recent topics in TJ biology and TJ-targeted therapy.

Biology of the epithelial barrier

Tight junctions

Epithelium is central to the construction of multicellular animals. More than 60% of the cell types in the vertebrate body are epithelial cells. Epithelia enclose and partition the animal body, line all of its surfaces and cavities, and create internal compartments. Epithelial cells are structurally polarized into a basal side that is anchored to other tissue, and an apical side that is

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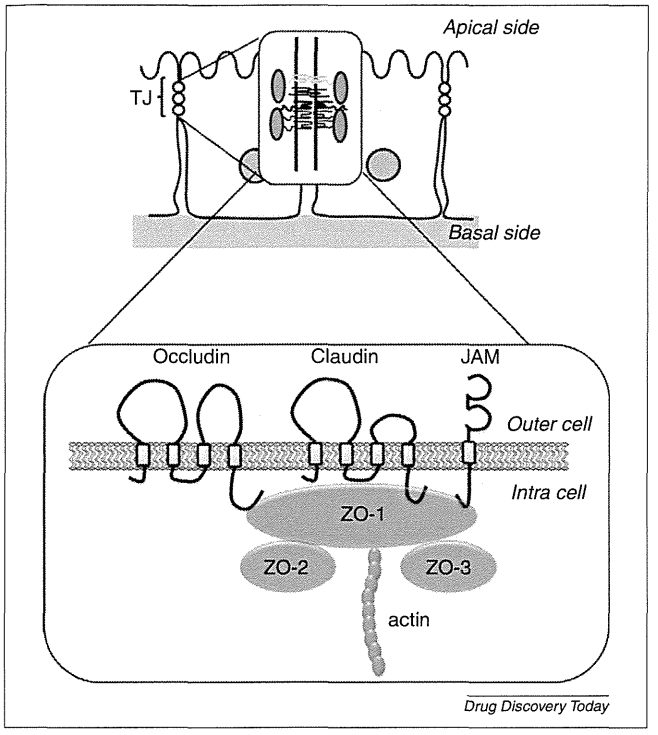

FIGURE 1

The epithelial barrier. Occludin, a tetra-transmembrane protein, was the first TJ-constituting protein identified [19]. Claudin was the second [21]. Claudins comprise a tetra-transmembrane protein family of 27 members. JAMs are glycosylated transmembrane proteins that belong to the immunoglobulin superfamily [4]. ZO-1, ZO-2 and ZO-3 are membrane-associated guanylate kinase proteins composed of a PSD95/Dlg/ZO-1 domain, an SH3 domain, a guanylate kinase domain, an acidic domain and an actin-binding region [68]. *Abbreviations*: JAMs: junctional adhesion molecules; TJ: tight junction;

unanchored. Adjacent epithelial cells are joined by occluding junctions called TJs. TJs have pivotal roles in separating the inside of the body from the outside environment, and in separating the inside and outside of tissues. TJs also function as a fence by preventing the free movement of apical membrane components and basal membrane components in epithelial cells.

TJs are intercellular sealing components located at the apical-most part of lateral membranes between adjacent epithelial cells and endothelial cells [17]. Adjacent TJ strands laterally associate with each other to form a paired strand thereby eliminating the intercellular space. Freeze fracture electron microscopy analysis revealed that TJs are continuous anastomosing intramembranous particle strands or fibrils with complementary grooves [3]. TJs are composed of transmembrane proteins, such as claudins, occludin and JAMs, in addition to cytoplasmic plaque proteins, including ZO-1, ZO-2, ZO-3 and cingulin [18].

Integral membrane proteins

Occludin was the first integral membrane protein identified in TJs [19]. Occludin has four transmembrane domains and has a molecular mass of approximately 65 kDa. Deletion of occludin does not affect the structure and function of TJs [20]. Claudins were the second integral membrane proteins identified in TJs [21]. Claudins comprise a multigene family with at least 27 members [7]. Claudins are 21–28-kDa proteins with tetratransmembrane domains. Claudins are key components in the structure and function of TJs [5,6]. A series of cellular analysis and knockout mouse analysis has clarified the roles of claudins in TJs [5,22].

Cytoplasmic proteins

ZO-1 was the first identified TJ-associated protein [23]. ZO-1, ZO-2 and ZO-3 contain PDZ-domains and the membrane-associated guanylate kinase domain. ZO-1, ZO-2 and ZO-3 are involved in formation of the TJ seal; they bind to the C-terminal cytoplasmic domain of occludin and claudins through the ZO PDZ domains [13]. ZO-1 and ZO-2 are crucial components for the definition of TJ formation [11].

Tricellular tight junctions

There are two types of TJs in epithelial cell sheets: bicellular and tricellular [2,24,25]. Occludin, claudins and JAMs are components of bicellular TJs. Tricellulin (approximately 65 kDa) is the only integral membrane component in tricellular TJs [8]. Tricellulin contains four transmembrane domains and shows structural similarity with occludin. Tricellulin is highly concentrated in tricellular TJs, but it is also localized in bicellular TJs [8,26]. Lipolysis-stimulated lipoprotein, a tricellular TJ-associated protein, defines tricellular contacts in epithelial cell sheets [12].

Mucosal barrier

The intestinal epithelium is where nutrients derived from food are absorbed, and it is also the first line of defense against microorganisms and xenobiotics. Regulation of the epithelial barrier is crucial for mucosal homeostasis. Recently, two intestinal epithelium proteins that regulate the intestinal barrier were identified.

The first protein is guanylyl cyclase C (GCC), which is a transmembrane receptor for the endogenous peptides guanylin and uroguanylin and for bacterial heat-stable enterotoxins [27]. GCC signaling has a pivotal role in the regulation of intestinal fluid and electrolyte homeostasis [28]. GCC-knockout mice show increased intestinal permeability, and GCC-knockdown in Caco-2 cells disrupts TJ integrity. This disruption of the TJ barrier is accompanied by phosphorylation of myosin II regulatory light chains, which induces TJ disassembly. GCC signaling is therefore involved in regulation of the TJ barrier [29].

The second intestinal membrane protein is matriptase. Matriptase is an integral membrane protein with trypsin-like serine protease activity and is a member of the type II transmembrane serine protease family [30]. It is widely expressed in all epithelia, and it is expressed in epithelial cells in the gastrointestinal tract [30]. Loss of matriptase reduces epithelial barrier integrity and enhances paracellular permeability. Matriptase facilitates claudin-2 loss from TJ complexes by indirect regulation of claudin-2 protein turnover by atypical protein kinase C zeta. Interestingly, matriptase does not affect some of the other TJ components, such as claudin-1, claudin-3, claudin-4, claudin-8, ZO-1, or E-cadherin [31].

These findings indicate that GCC signaling and matriptase might be potent targets for the treatment of intestinal disorders whose pathogenesis is disruption of the intestinal barrier function leading to mucosal inflammation and immune activation.

TJ dynamics

TJs are complexes of transmembrane and peripheral membrane proteins, including occludin, claudins, ZO-1 and ZO-2 [6]. The TJ structure is highly dynamic and undergoes continuous remodeling through unique kinetics [32]. The properties of TJs are determined by these dynamics [33].

Occludin S408 dephosphorylation reduces paracellular cation influx by stabilizing the occludin–ZO-1 interaction, leading to enhancement of claudin-1 and claudin-2 exchange and reduction of their pore formation at the TJ. By contrast, occludin S408 phosphorylation enhances homotypic occludin–occludin interactions, leading to the release of ZO-1 and formation of claudin-1- and claudin-2-based pores. Therefore, occludin S408 phosphorylation is a key factor in the remodeling of the claudin–occludin–ZO-1 interaction [34].

Claudin-1 is stably localized in TJs [35]. Most occludin is mobile and diffused within the junctional membrane. By contrast, most ZO-1 is continuously exchanged between the membrane and cytosol pools [34]. Fluorescence recovery after photo-bleaching (FRAP) analysis provided new insights into the dynamics of TJs. The perijunctional actomyosin ring contributes to myosin light chain kinase (MLCK)-dependent TJ regulation. FRAP analysis showed that TJ-associated ZO-1 exists in three pools: a fixed pool, a fast exchangeable pool associated with the cytosolic pool, and a slow exchangeable pool associated with the cytosolic pool. The exchange between the TJ pools and the cytosolic pool is regulated by MLCK [36]. Claudin dynamics differ depending on the particular claudin. Claudins forming TJ strands showed slower dynamics than those not forming TJ strands. Distinct claudin stabilities might affect how TJs regulate paracellular permeability by altering paracellular flux and paracellular ion permeability [37].

These insights into the dynamics of TJs address the molecular mechanism of paracellular homeostasis and will hopefully lead to the development of TJ-targeted tissue-specific and solute-specific drug delivery systems.

Epithelial barrier as the first line of defense against pathological microorganisms

The human mucosa has a surface area equivalent to 1.5 tennis courts. This large surface area means that there is significant risk of infection by pathological microorganisms; therefore, homeostasis of the epithelial barrier is important. Indeed, some pathogens modulate the epithelial barrier to facilitate easy and widespread infection (Fig. 2a).

Modulation of the epithelial barrier by pathogens

Human immunodeficiency virus-1 (HIV-1) infection is often associated with increased permeability of mucosal epithelial cells. Viral envelope glycoprotein (gp)120 is a crucial viral protein that increases the permeability of the epithelial barrier. When HIV-1 binds to cells it induces production of TNF-α, leading to a decrease in mucosal epithelial barrier integrity and spread of HIV-1 infection [38].

Atopic dermatitis (AD) is the most common inflammatory skin disease [39], and susceptibility to cutaneous infections is increased in AD patients. Widespread skin infection by the herpes simplex virus (HSV) causes severe viral complications, such as eczema herpeticum in AD patients. Defects in the epidermal TJ barrier

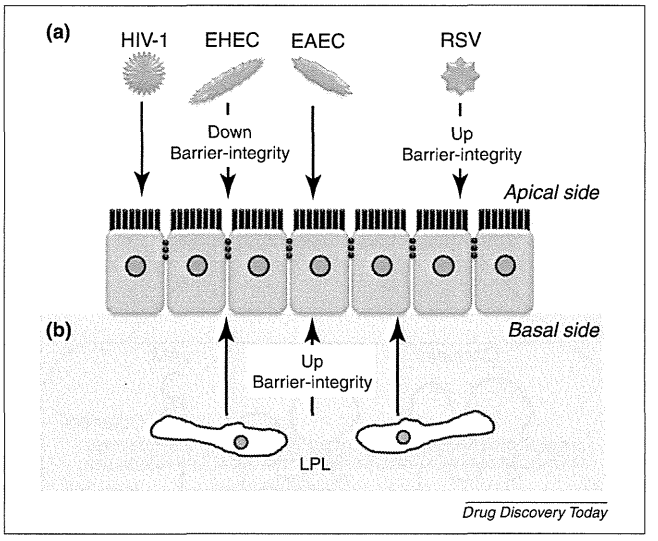

FIGURE 2

Regulation of the first line of defense, the epithelial barrier. **(a)** Pathological microorganism–epithelial barrier interaction. Infection of epithelial cells by HIV-1, EHEC, or EAEC decreased epithelial barrier integrity [38,41,42]. By contrast, RSV infection increased the barrier function [44]. **(b)** Lymphocyte–epithelial barrier interaction. LPLs regulate the integrity of the epithelial barrier via direct interaction with epithelial cells through notch signaling [49]. *Abbreviations*: EAEC: enteroaggregative *Escherichia coli*; EHEC: enterohemorrhagic *Escherichia coli*; HIV-1: human immunodeficiency virus-1; LPLs: lamina propria lymphocytes; RSV: respiratory syncytial virus.

increase the susceptibility of patients with AD to widespread subcutaneous infection with HSV or other viral pathogens [40]. In the early stage of infection with enterohemorrhagic *Escherichia coli* (EHEC), non-bloody diarrhea occurs in the absence of shiga toxin. EHEC infection increases expression of claudin-2 and redistribution of claudin-3 and occludin. These changes correlate with increased intestinal permeability [41]. Infection by enteroaggregative *Escherichia coli* (EAEC) causes dissociation of claudin-1 from the TJs between epithelial cells, leading to disruption of the TJ barrier [42]. By contrast, respiratory syncytial virus (RSV) increases TJ integrity. RSV is the major cause of bronchitis, asthma and severe lower respiratory tract diseases in infants and young children [43]. RSV infection induces expression of claudin-4 and occludin in human nasal epithelial cells. Induction of TJ components has a crucial role in epithelial cellular polarity, leading to budding of the virus from the epithelial apical surface [44]. Therefore, prevention of TJ barrier modulation by pathogens might be a viable therapeutic strategy.

Lymphoepithelial cross talk in the epithelial barrier

Mucosa-associated lymphoid tissues (MALTs) are lymphoid immune tissues that are located in the mucosal epithelium. By activating mucosal immune responses, they function as the first line of defense against pathogens invading the body through the epithelium [45]. MALTs comprise gut-associated lymphoid tissues, nasopharynx-associated lymphoid tissues and bronchus-associated lymphoid tissues. MALTs contain lymphocytes, M cells, T cells, B cells and antigen-presenting cells. Recently, lamina propria lymphocytes (LPLs) underlying the intestinal epithelium have

been shown to have a crucial role in the homeostasis of the epithelial barrier (Fig. 2b). Direct interaction of LPLs with intestinal epithelial cells is essential for the barrier function of the intestinal epithelium [46]. The notch signaling pathway regulates cell fate decisions through cell–cell interactions [47]. Notch signaling determines the differentiation of intestinal stem cells into secretory cells, absorptive cells, or enterocytes [47,48]. The absence of LPLs in mice causes increased intestinal permeability and a lack of activation of notch in colonocytes [49]. Transfer of LPLs to LPL-deficient mice decreased intestinal permeability and activated notch signaling in colonocytes. In Caco-2 cells, knockdown of notch mRNA reduced the epithelial barrier function, and was accompanied by upregulation of claudin-2 proteins, reduction of occludin and cytoplasmic localization of claudin-5 [49]. Therefore, lymphoepithelial cross talk might regulate epithelial differentiation and barrier integrity. Notch signaling is highly activated in the mucosa of patients with Crohn's disease, leading to dysregulation of the differentiation of epithelial cells [49]. Normalization of disruption of this cross talk might be a potent strategy for treating immune-mediated intestinal disorders.

Proof-of-concept for TJ-targeted drug development

As mentioned in the introduction, epithelial cells are a potent target for drug development. TJ-targeted drug development has been attempted [14,50], and proof-of-concepts for TJ-targeted drug absorption, cancer targeting and mucosal vaccination have been established. Recent findings indicate that TJ-targeted therapy for hepatitis C virus (HCV), diabetes and inflammatory diseases might be possible.

HCV infection

A total of 170 million people worldwide are infected with the HCV. Hepatitis C is the leading cause of chronic liver inflammation, cirrhosis and cancer. Claudin-1 and occludin are co-receptors for HCV infection, indicating that binders to claudin-1 or occludin might be potent inhibitors of HCV entry [16]. DNA immunization enabled successful preparation of monoclonal anti-claudin-1 antibodies against the extracellular loop of claudin-1, and these anti-claudin-1 antibodies prevented HCV infection. Antibodies effectively blocked cell entry of highly infectious escape variants of HCV that were resistant to neutralizing antibodies [51]. When hepatitis C patients reach end-stage liver failure, liver transplantation is the only choice for curative treatment; however, reinfection of the transplanted liver by HCV often occurs. There is a significant correlation between hepatic levels of claudin-1 and occludin and HCV reinfection after liver transplantation [52]. Inhibition of HCV reinfection of the transplanted liver by using anti-claudin-1 antibodies might be a potent treatment for patients with liver transplantation.

Diabetic retinopathy

Breakdown of the blood–retinal barrier (BRB) is a hallmark of diabetic retinopathy [53]. Alterations to the BRB occur early in the progression of diabetic retinopathy and eventually lead to macular edema, which is responsible for vision loss [54]. Diabetic patients show elevated levels of TNF-α in the vitreous humor. TNF-α increases the permeability of retinal endothelial cells. TNF-α decreases ZO-1 and claudin-5 expression and alters cellular localization of ZO-1 and claudin-5 [55]. Thus, regulation of BRB-integrity might be a potent strategy for treating vision loss owing to diabetes. Indeed, a chemical already in clinical use for the treatment of diabetic retinopathy, calcium dobesilate, attenuates the decrease in occludin and claudin-5 and prevents BRB breakdown [56]. Berberine, a plant alkaloid, has also been used for the treatment of diabetes. Berberine prevents barrier defects in retinal epithelial cells [57]. Inducers of occludin and claudin-5 or promoters of TJ integrity could be a potent treatment for diabetic retinopathy.

Inflammatory diseases

Berberine has been also used in the treatment of gastroenteritis and diarrhea. TNF-α disrupts TJ integrity in inflammatory bowel diseases (IBD). Regulation of the TNF-α-dependent signaling pathway is a potent strategy for the treatment of IBD. TNF-α removes claudin-1 from TJs and induces claudin-2 expression, leading to disruption of the TJ barrier. Attenuation of TNF-α signaling is a potent strategy for IBD therapy. Berberine also attenuates TNF-α-induced TJ barrier defects by removing claudin-1 and inducing claudin-2 expression [58]. Spontaneous colitis was observed in interleukin (IL)-10−/− mice in which paracellular permeability was increased in conjunction with decreased expression and redistribution of ZO-1, occludin and claudin-1. Treatment with a probiotic, *Lactobacillus plantarum*, restored expression of TJ components and TJ integrity, resulting in prevention of bacterial translocation and proinflammatory responses in IL-10−/− mice [59]. Recovery of TJ integrity might be a potent strategy for inflammatory intestinal diseases. Ouabain, which is an inhibitor of Na^+, K^+-ATPase, increased TJ integrity through signaling pathways involving c-Src and ERK1/2 and by modulating the expression of claudin-1, claudin-2 and claudin-4 [60,61]. Several natural products have been found to be therapeutically useful against epithelial barrier defects.

Paracellular drug transport

The claudin protein family comprises 27 members [7]. Claudins form homo- and hetero-type strands in the lateral membrane. Adjacent claudin-based TJ strands associate with each other, leading to sealing of the intercellular space. The combination of the claudin members is a determinant factor for the properties of the TJ barrier [5]. These findings suggest that optimization of claudin modulators with narrow-specificity in certain cases, or broad-specificity in other cases, might regulate solute- and tissue-specificity in paracellular transport. The most important issue in TJ-targeted drug absorption is the development of claudin modulators. Claudin is an integral membrane protein with a tetra-transmembrane domain. Claudin binders are the first choice for claudin modulators. The first extracellular loop contains approximately 50 amino acids and the second contains approximately ten amino acids. Claudins are hydrophobic proteins, and preparation of a recombinant protein is only currently possible for claudin-4 [62]. Therefore, the development of claudin binders, including antibodies, has been slow. Budded baculoviruses display functional forms of membrane proteins on their surface [63]. Claudin-displaying budded baculoviruses possess a native form of claudin and can be used as a screening system for claudin binders [64]. Functional membrane proteins are heterogeneously expressed on

budded baculoviruses [63]. Functional information using FRAP analysis will enable development of a screening system for claudin modulators with narrow- or broad-specificity using the heterogenous claudin-displaying baculoviral system. We predict that, in the near future, proof-of-concept for tissue- and solute-specific paracellular transport by modulating the claudin-barrier will be demonstrated.

Coupling of transcellular and paracellular transport systems controls permeability to solutes [65]. Claudin-based TJs function as charge-selective paracellular channels [6]. Claudin-15 is responsible for transepithelial permeability to extracellular monovalent cations, especially Na^+. Claudin-15-deficient mice exhibit low luminal Na^+ levels and low glucose absorption in the intestine, indicating that paracellular transport of Na^+ through claudin-15-based TJ strands might be coupled to transcellular transport of glucose through a glucose transporter [66]. These findings suggest that modulation of the claudin-mediated paracellular transport of solutes might regulate the transcellular transport of drugs through a transporter.

Concluding remarks

To our knowledge, the first report of TJ-targeted drug development was the discovery in 1961 of enhanced mucosal absorption of drugs by co-administration of ethylenediaminetetraacetic acid [67]. TJs were identified in 1963 [17]. Modulation of the TJ-barrier has been a major strategy for enhancing mucosal absorption; however, the biochemical structure of TJs was unclear until 1998. Until that year, absorption enhancers were screened mainly by modulating epithelial cell sheets. Recent imaging studies have begun to reveal the dynamics of TJs and also how these dynamics are regulated [36,37]. Future detailed analyses using FRAP will provide us with new insights into strategies for modulation of the TJ barrier. In addition to TJ-modulated drug absorption, TJ-targeted therapy for HCV infection and diabetic retinopathy has recently been proved effective [51,56]. The questions of how TJ dynamics are regulated, and how expression of TJ components is regulated are still to be answered. The molecular pathology of deregulation of the TJ barrier is not yet fully understood. TJ-targeted drug development has been spearheaded by rapid progress in our understanding of the biology of the TJ barrier.

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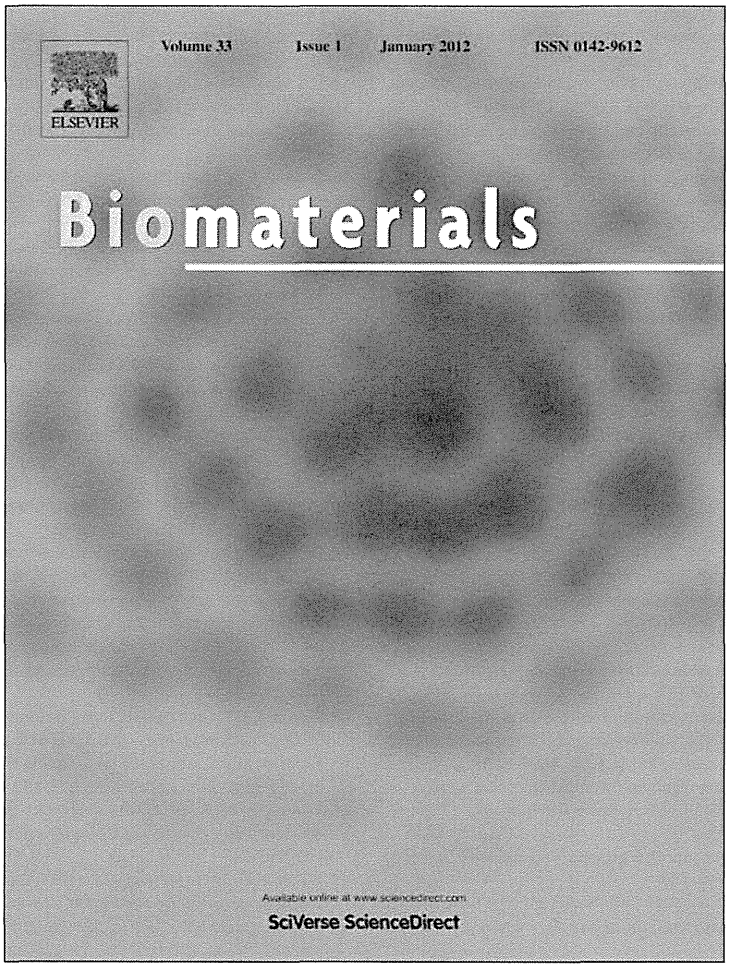

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The application of an alanine-substituted mutant of the C-terminal fragment of *Clostridium perfringens* enterotoxin as a mucosal vaccine in mice

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ABSTRACT

Efficient delivery of antigen to mucosal immune tissues is an essential part of mucosal vaccination. Claudin-4 is expressed on the epithelial cells that cover the mucosal immune tissues. We previously found that claudin-4-targeting is a promising strategy for mucosal vaccination by using a claudin-4 binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE). Substitution of Asn and Ser at positions 309 and 313, respectively, with alanine increased the affinity of C-CPE for claudin-4. However, application of the C-CPE mutant as a mucosal vaccine has never been tried. Here, we investigated whether the C-CPE mutant could serve as a mucosal vaccine. We used ovalbumin (OVA) as a model antigen and fused the C-CPE mutant to it. The resultant fusion protein was bound to claudin-4. When mice were immunized with the C-CPE mutant-fused OVA, OVA-specific serum IgG and nasal IgA increased relative to levels in mice immunized with a C-CPE-fused antigen. Immunization with the C-CPE mutant-fused OVA activated Th1- and Th2-type responses and led to increased anti-tumor activity against OVA-expressing thymoma cells relative to that of mice immunized with the C-CPE-fused antigen. These findings suggest that the alanine-substituted C-CPE mutant shows promise as a claudin-targeted mucosal vaccine.

1. Introduction

Vaccination is a potent therapeutic strategy for infectious diseases and cancers. Vaccines are classified as either parental or mucosal; parental vaccines are injected into patients, whereas mucosal vaccines are nasally, pulmonarily, or orally administered. Parental vaccines activate systemic immune responses but do not induce mucosal immunity. For parental vaccines, patient compliance is low and the preventive effects in response to invasion by pathological microorganisms into the body from the mucosal epithelium are poor. Mucosal vaccines, however, potentiate both systemic and mucosal immune responses. They eliminate invading pathogens and infected cells, and prevent the entry of pathogens from mucosal epithelium by producing antigen-specific IgA. Thus, mucosal vaccine is an ideal strategy for vaccination. However, mucosal administration of antigen alone does not activate immune responses; the delivery of antigens to the mucosal immune tissues is critical for the mucosal vaccine response.

Lymphoid immune tissues, called mucosa-associated lymphoid tissues (MALTs), reside in the mucosal epithelium, where they function as a first line of defense against pathogens invading the body via the epithelium by activating mucosal immune responses. MALTs comprise gut-associated lymphoid tissues (GALT), nasopharynx-associated lymphoid tissues (NALT), and bronchus-associated lymphoid tissues (BALT). MALTs contain lymphocytes, M cells, T cells, B cells, and antigen-presenting cells (APCs). Delivery of antigens to MALTs is essential for the activation of mucosal immune responses, and microparticles, liposomes, lectins, and chitosans have been used to deliver antigens to MALTs [1–5]. Follicle-associated epithelium

Abbreviations: C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin; OVA, ovalbumin; MALT, mucosa-associated lymphoid tissue; GALT, gut-associated lymphoid tissue; NALT, nasopharynx-associated lymphoid tissue; APC, antigen-presenting cell; FAE, follicle-associated epithelium; CPE, *Clostridium perfringens* enterotoxin; C-CPE184, C-terminal fragment of *Clostridium perfringens* enterotoxin from amino acids 184 to 319; C-CPE194, C-terminal fragment of *Clostridium perfringens* enterotoxin from amino acids 194 to 319; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BV, budded baculovirus; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; IFN, interferon; IL, interleukin.

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