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

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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

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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

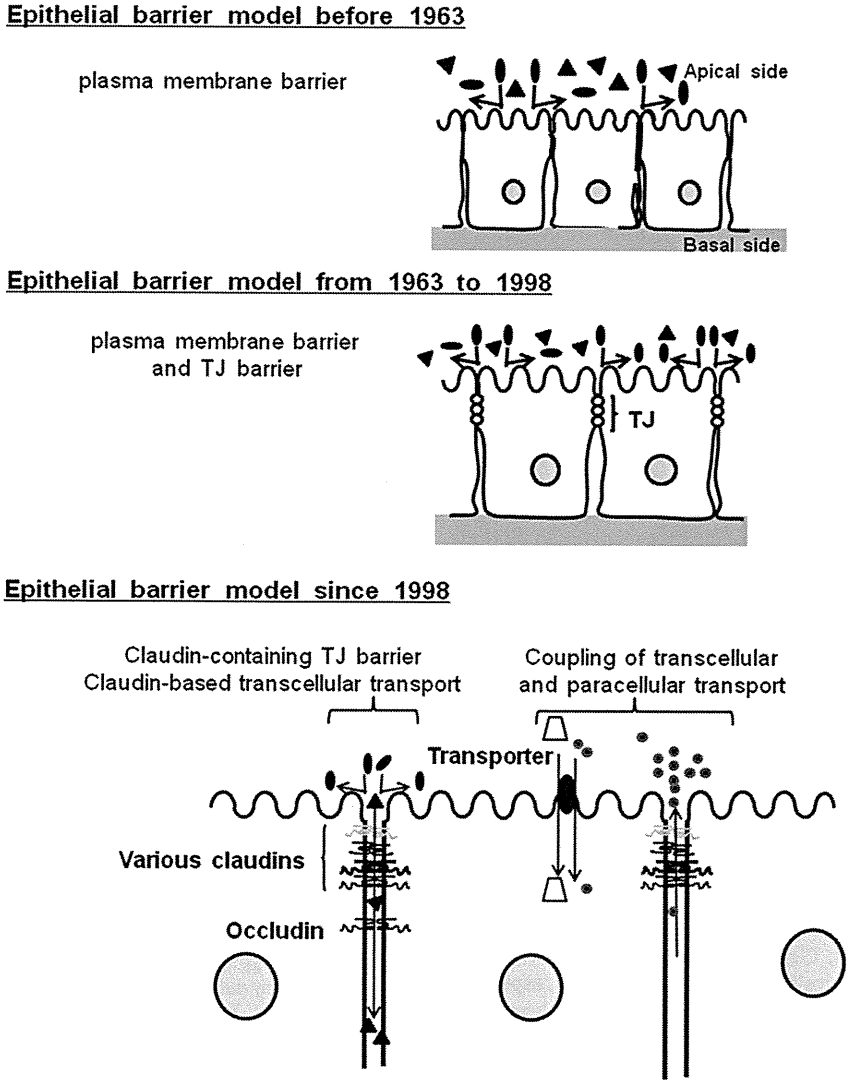

Fig. 1. Progression in paracellular transport research through understanding of the biology of the epithelial barrier. The plasma membrane was originally considered to be the primary barrier in epithelial cell sheets. TJ strands were subsequently identified as the intercellular sealing components [4,5]. 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].

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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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Effect of 70-nm silica particles on the toxicity of acetaminophen, tetracycline, trazodone, and 5-aminosalicylic acid in mice

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Exposure to nano-sized particles is increasing because they are used in a wide variety of industrial products, cosmetics, and pharmaceuticals. Some animal studies indicate that such nanomaterials may have some toxicity, but their synergistic actions on the adverse effects of drugs are not well understood. In this study, we investigated whether 70-nm silica particles (nSP70), which are widely used in cosmetics and drug delivery, affect the toxicity of a drug for inflammatory bowel disease (5-aminosalicylic acid), an antibiotic drug (tetracycline), an antidepressant drug (trazodone), and an antipyretic drug (acetaminophen) in mice. Co-administration of nSP70 with trazodone did not increase a biochemical marker of liver injury. In contrast, co-administration increased the hepatotoxicity of the other drugs. Co-administration of nSP70 and tetracycline was lethal. These findings indicate that evaluation of synergistic adverse effects is important for the application of nano-sized materials.

1. Introduction

Nano-sized particles, which have a diameter of less than 100 nm, are widely used in medicine, food, and machinery. With their smaller size, the physical and chemical properties of their constituents change, so that they may be toxic, for example to the lungs or liver, even though macro-particles of the same materials are not (Byrne and Baugh 2008; Nishimori et al. 2009b). Some nano-sized particles show long-term accumulation or a wide distribution in the body (Byrne and Baugh 2008; Nishimori et al. 2009b; Xie et al. 2009; Yang et al. 2008).

Recent reports indicate that some nano-sized particles can generate reactive oxygen species (ROS) on their surfaces, leading to cellular injury (Jin et al., 2008; Sharma et al. 2007; Ye et al. 2010). There are also many drugs that cause adverse effects through the generation of ROS (Ali et al. 2002; Kovacic 2005; Xu et al. 2008). Thus, nano-sized particles might enhance the side-effects of some pharmaceutical drugs. Indeed, we have shown that 70-nm silica particles (nSP70) cause liver injury but that macro-sized silica particles with a diameter of 300 and 1000 nm do not (Nishimori et al. 2009b). Also, when co-administered to mice, nSP70 but not the macro-sized silica particles enhance the toxicity of cisplatin and paraquat (Nishimori et al. 2009a). Surprisingly, co-administration of cisplatin and nSP70 was lethal, suggesting that each chemical may have different synergistic effects in the presence of nano-sized materials. In the current study, to clarify the influence of nano-sized materials on the adverse effects of chemicals, we assessed the toxicity in mice of 5-aminosalicylic acid (an agent for treating inflammatory bowel disease), tetracycline (a broad-spectrum antibiotic), trazodone (an antidepressant), and acetaminophen (a common antipyretic analogue) in the presence or absence of nSP70.

2. Investigations and results

Several reports indicate that 5-aminosalicylic acid, which is used to treat inflammatory bowel disease, causes liver injury and interstitial nephritis (Deltenre et al. 1999; Margetts et al. 2001). Administration of 5-aminosalicylic acid caused an increase in ALT, AST and BUN levels (Fig. 1). Also, nSP70 dose-dependently elevated ALT and AST levels. Co-treatment with 5-aminosalicylic acid and nSP70 resulted in higher levels of ALT and AST than nSP70 alone. In contrast, changes in BUN levels in response to 5-aminosalicylic acid were not affected by nSP70.

Next, we investigated effect of nSP70 on tetracycline, a broad-spectrum antibiotic. As shown in Fig. 2A and 2B, administration of tetracycline did not elevate biochemical markers for liver injury. In contrast, co-administration with nSP70 resulted in the synergistic induction of liver injury. However, nSP70 alone did not cause kidney injury. Importantly, co-administration of 30 and 50 mg/kg nSP70 with tetracycline resulted in the death of 1 of 4 and 2 of 4 mice, respectively.

Finally, we investigated effect of nSP70 on toxicity of the antidepressant trazodone and the antipyretic analgesic acetaminophen. We found that nSP70 did not have a synergistic effect on the toxicity of trazodone (Fig. 3). In contrast, co-administration of acetaminophen with nSP70 caused synergistic liver injury (Fig. 4).

3. Discussion

In this study, we showed that nSP70 synergistically enhance the toxicity of 5-aminosalicylic acid, tetracycline, and acetaminophen but not trazodone. To avoid direct interac-

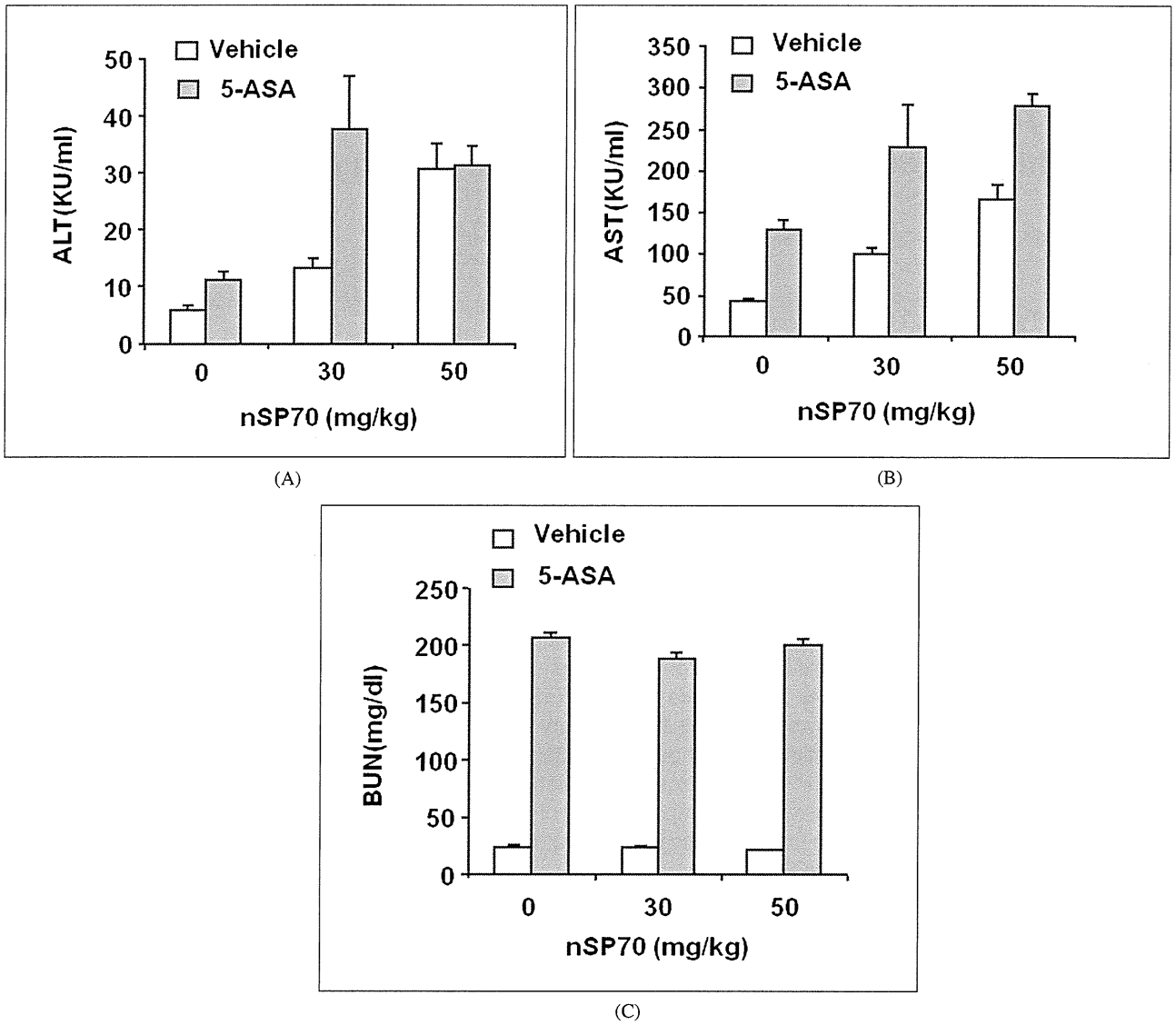
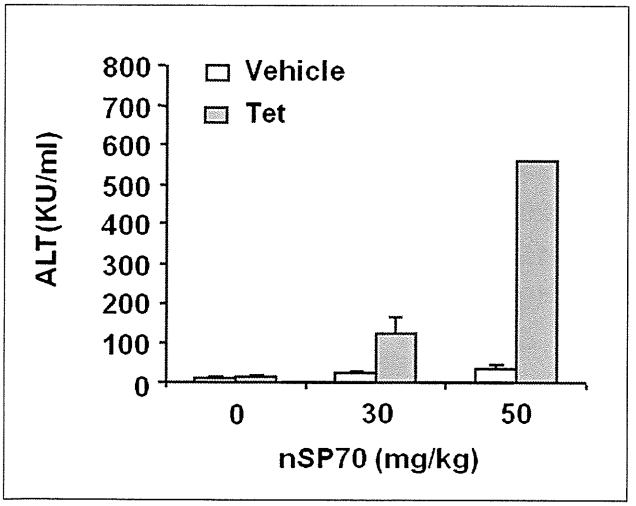


Fig. 1: Effect of nSP70 on 5-aminosalicylic acid (5-ASA)-induced toxicity Mice were injected intraperitoneally with 5-ASA at 0 (open column) or 500 mg/kg (gray column) and intravenously with nSP70 at the indicated doses. After 24 h, the serum was collected. Shown are the levels of ALT (A), AST (B), and BUN (C). Data are means ± SEM (n = 4)

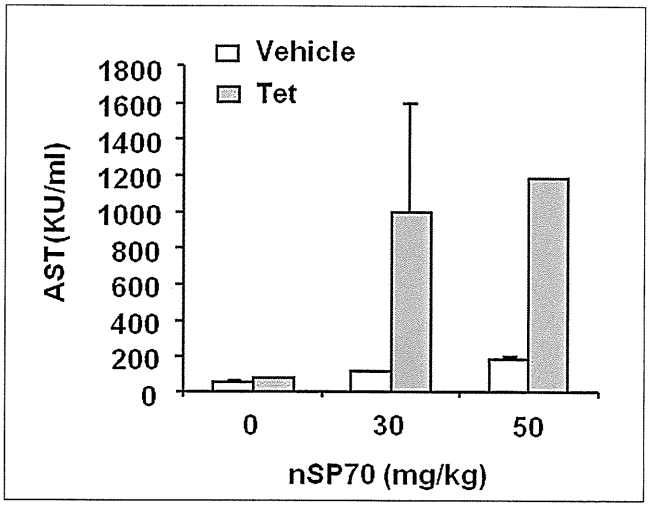
tions between nSP70 and chemicals in their administration and absorption, nSP70 and chemicals were administered intravenously and intraperitoneally, respectively. Administration of nSP70 alone has been shown to cause liver injury but not kidney injury (Nishimori et al. 2009b). Also, in this study, nSP70 did not enhance kidney injury induced by 5-aminosalicylic acid or tetracycline, two drugs known to be nephrotoxic (Grisham et al. 1992; Kunin 1971). The renal toxicity of cisplatin, another nephrotoxic chemical, was unaffected by nSP70 (Nishimori et al. 2009a). Like 5-aminosalicylic acid, tetracycline, and acetaminophen (Chun et al. 2009; Herzog and Leuschner 1995; Kunin 1971), nSP70 is hepatotoxic (Nishimori et al. 2009b), and we showed here that its co-administration synergistically enhanced liver injury. These findings indicate that nSP70 may enhance the toxicity of certain chemicals. Therefore, it will be important to assess the tissue-specific risk of nano-sized materials.

The nSP70 particles had a lethal effect when combined with tetracycline. The 50% lethal dose of tetracycline is 318 mg/kg by intraperitoneal injection in mice. A previous report showed that 100 mg/kg nSP70 is lethal in 100% of mice (Nishimori et al. 2009b). A single injection of tetracycline (100 mg/kg) or nSP70 (30 or 50 mg/kg) alone was not lethal in this study but a combination of the two was. Co-administration of cisplatin and nSP70 showed a similar synergistic lethal effect. This could be due to an interaction between nSP70 and serum albumin. Tetracycline in the bloodstream can bind to albumin (Popov et al. 1972; Powis 1974). Likewise, serum albumin adsorbs onto nano-sized silica particles (Dutta et al. 2007). When injected intravenously, 100-nm anionized albumin-modified liposomes are taken up by hepatic endothelial cells and Kupffer cells (Kamps et al. 1997), which normally clear chemically modified albumin (Jansen et al. 1991). Thus, tetracycline-bound serum albumin may adsorb onto nSP70, causing it to be taken up by the hepatic endothelial cells and Kupffer cells in the liver where it may accumulate and cause lethal liver damage.

Indirect interactions between chemicals and nano-sized particles mediated by serum albumin may be useful for estimating the toxicity of nano-sized materials. In this study, co-treatment of mice with nSP70 (50 mg/kg) and tetracycline decreased BUN levels compared to tetracycline alone or nSP70 (30 mg/kg) and tetracycline. A similar decrease in BUN levels



(A)



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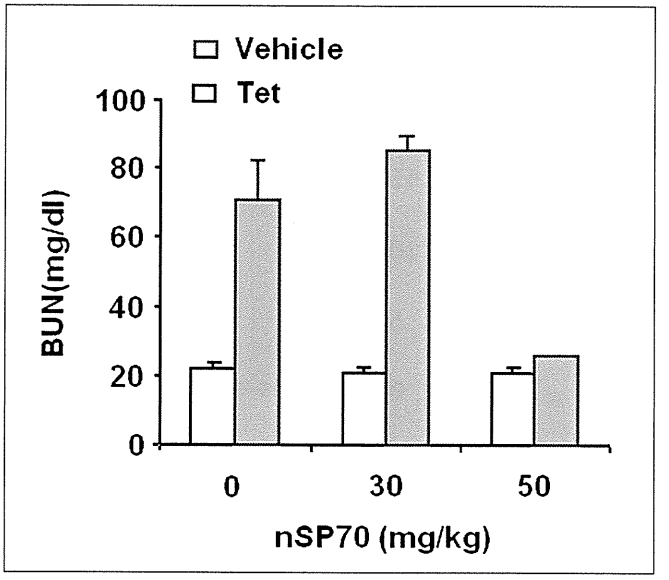

(C)

Fig. 2: Effect of nSP70 on tetracycline (Tet)-induced toxicity Mice were injected intraperitoneally with Tet at 0 (open column) or 100 mg/kg (gray column) and intravenously with nSP70 at the indicated doses. After 24 h, the serum was collected. Shown are the levels of ALT (A), AST (B), and BUN (C). One of 4 mice died when co-treated with nSP70 (30 mg/kg) and Tet (100 mg/kg), and 2 of 4 mice died when co-treated with nSP70 (50 mg/kg) and Tet (100 mg/kg). Data are means or means ± SEM (n = 2–4)

was also reported in mice co-treated with nSP70 and cisplatin (Nishimori et al. 2009a). However, the mechanism by which these decrease the BUN level remains to be determined.

In conclusion, we found that nSP70 cause synergistic toxicity when combined with some clinically used drugs, although the synergistic effects differ between chemicals. One combination was lethal, and the others resulted in tissue injury. These studies suggest that evaluation of possible synergistic adverse effects with pharmaceutical drugs may be important for assessing the safety of nano-sized particles.

4. Experimental

4.1. Materials

The nSP70 nanoparticles were obtained from Micromod Partikeltechnologie GmnH (Rostock, Germany). The mean diameter of the particles, as analyzed by a Zetasizer (Sysmex Co., Kobe, Japan), was 55.7 nm, and the particles were spherical and nonporous. The particles were stored at 25 mg/ml as an aqueous suspension. The suspensions were thoroughly dispersed by sonication before use and diluted in water. An equal volume of solution was injected for each treatment. Acetaminophen, tetracycline, and trazodone were dissolved in saline solution, and 5-aminosalicylic acid was suspended in 1% sodium salt of carboxy methyl cellulose. All reagents were of research grade.

4.2. Animals

Eight-week-old BALB/c male mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). Mice were maintained in controlled environment (23 ± 1.5 °C; 12-h light/12-h dark cycle) with free access to standard rodent chow and water. The mice were given 1 week to adapt before experiments. All of the experimental protocols complied with the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

4.3. Biochemical analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN) were measured using commercially available kits according to the manufacturer's protocols (WAKO Pure Chemical, Osaka, Japan).

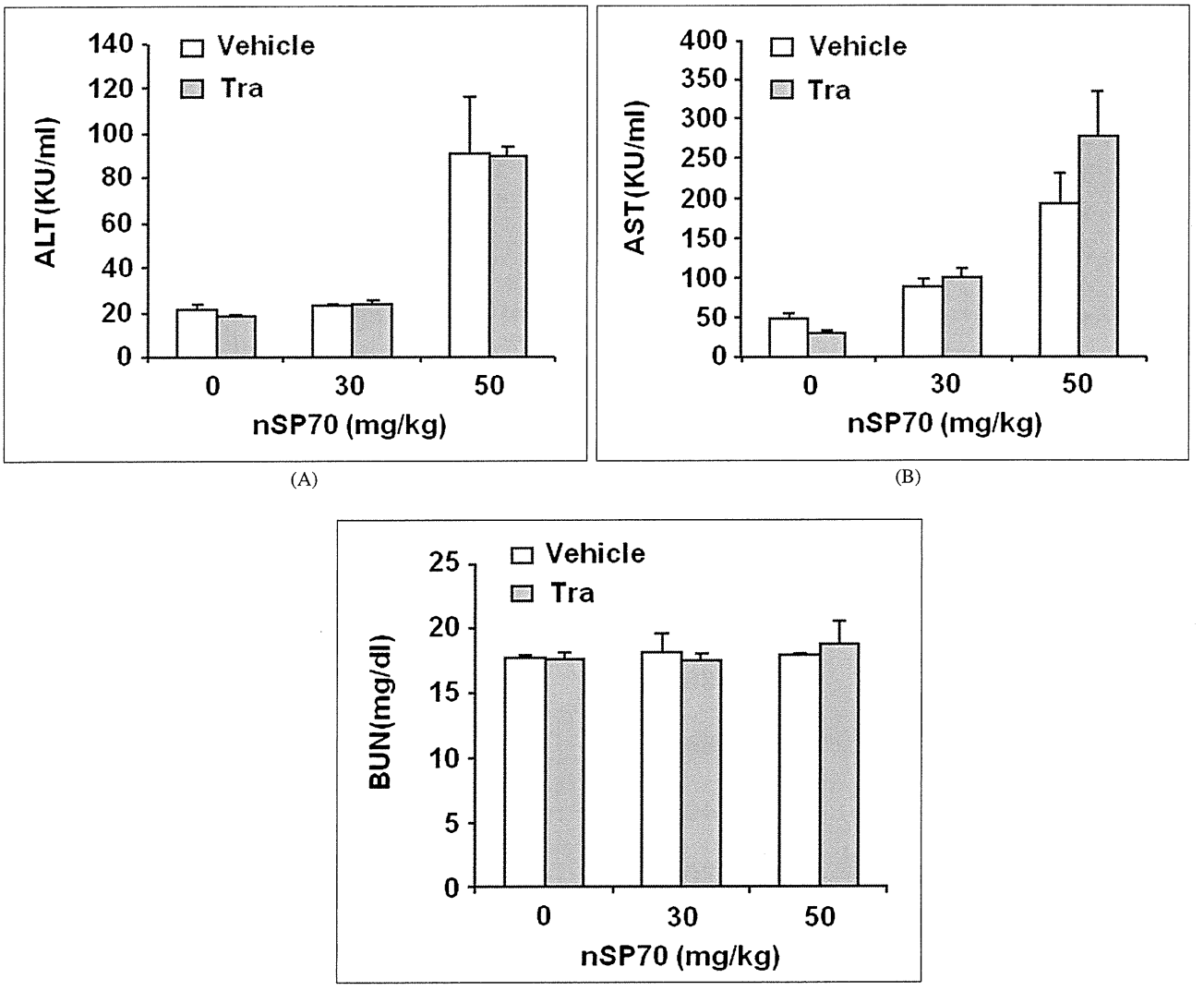

Fig. 3: Effect of nSP70 on trazodone (Tra)-induced toxicity Mice were injected intraperitoneally with Tra at 0 (open column) or 100 mg/kg (gray column) and intravenously with nSP70 at 30 or 50 mg/kg. After 24 h, the serum was collected. Shown are the levels of ALT (A), AST (B), and BUN (C). Data are means ± SEM (n = 4)

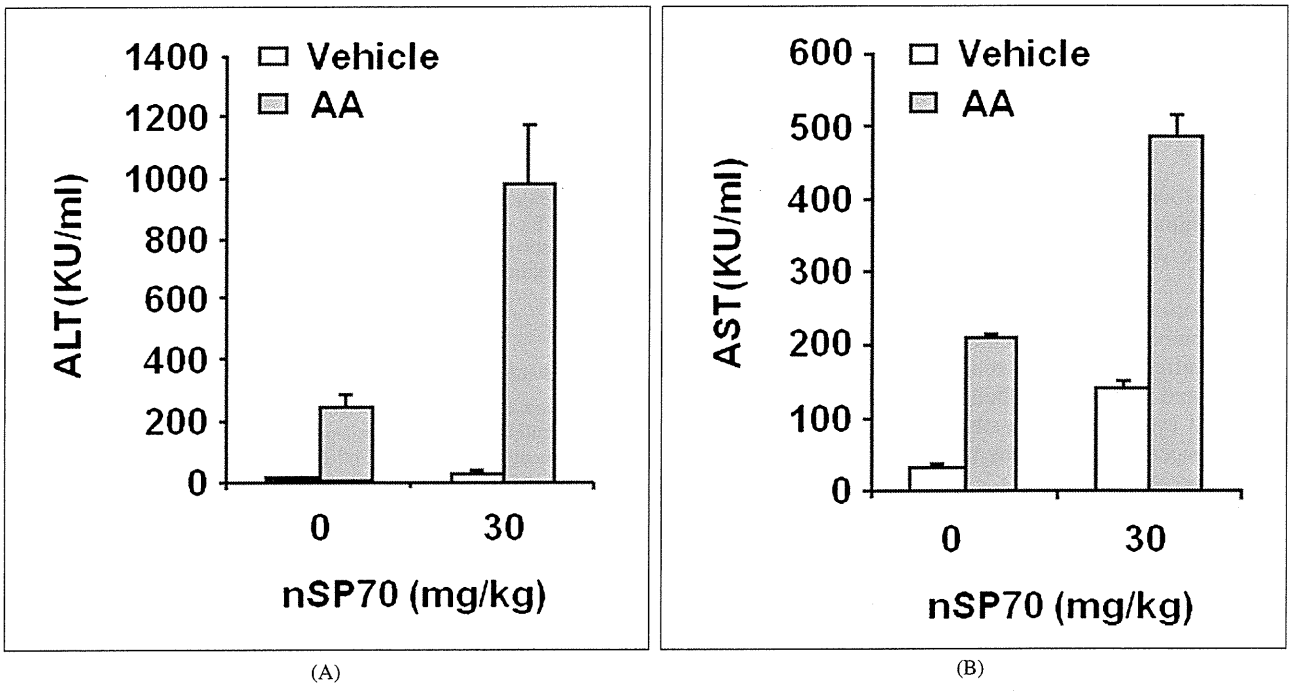

Fig. 4: Effect of nSP70 on acetaminophen (AA)-induced toxicity Mice were injected intraperitoneally with AA at 0 (open column) or 500 mg/kg (gray column) and intravenously with nSP70 (30 mg/kg). After 24 h, the serum was collected. Shown are the levels of ALT (A) and AST (B). Data are means ± SEM (n = 4)

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—Review—

Claudinを標的とした非侵襲性投与技術の開発

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A Non-invasive Drug Delivery System Using Claudin Binder

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The intercellular spaces between adjacent epithelial cells are sealed by tight junctions (TJs). Modulation of TJ-seal is a potent strategy for drug absorption. Claudin is a key structural and functional component of TJ-seal. Claudin comprises a tetra-transmembrane protein family consisting of more than 20 members, whose expression profiles and barrier-function differ among tissues. For instance, claudin-1 plays roles in the epidermal and mucosal barriers, and claudin-4 regulates the mucosal barrier. Claudin forms homo- and hetero-type TJ strands. Properties of TJ-seal are determined by combination of the claudin family members. Some claudin strands work as size-selective or charge-selective paracellular routes for solutes. Thus, claudin modulators will make it possible to deliver drugs in a solute- and tissue-specific manner. The C-terminal fragment of the *Clostridium perfringens* enterotoxin (C-CPE) is the most characterized claudin modulator. In this review, we describe potency of claudin-targeting mucosal absorption, and we mentioned development of a novel claudin modulator using C-CPE as a prototype.

Key words——tight junction; claudin; *Clostridium perfringens* enterotoxin; drug delivery

1. はじめに

昨今のゲノム・プロテオーム創薬の進展に伴い，ペプチド・タンパク質・核酸などのバイオ医薬が次世代医薬品として台頭しつつあり，2010年代末までに，世界の新薬の約30%がバイオ医薬品で占められると言われている．一般的に，バイオ医薬は消化酵素等によって分解を受け易いこと，生体膜透過性に乏しいことから，臨床応用に際しては多くのバイオ医薬が侵襲性の注射による投与を余儀なくされているのが現状である．投与の利便性及び患者の生活の質（QOL）を考慮すると，経口・経鼻・経皮投与などの非侵襲性投与が理想的な投与方法であるものの，元来粘膜や皮膚は生体内外を隔てるバリアとして機能しており，ここに非侵襲性投与法開発の難しさがある．粘膜を介した吸収促進法は30年余りに渡り吸収促進剤として研究開発されてきているが，吸収促進活性の特異性に乏しく粘膜傷害性も観察されるなどの理由から実用化されているのはカプリン酸ナトリウムなどに過ぎない．既存の吸収促進剤は1980年代に開発されたものが大半を占めており，当時，上皮細胞バリアの分子基盤は未解明であった．

皮膚及び粘膜面には上皮細胞層が存在し，隣接する細胞間隙に存在するtight junction（TJ）によって細胞間隙はシールされており物質透過が抑制されていることから，TJバリア制御が非侵襲性投与法開発の基本戦略の1つとなっている．TJの発見から約40年の歳月を経て，1998年に京都大学月田博士のグループによりTJシールの本体としてclaudinが同定された.[1] Claudinは分子量23 kDaの4回膜貫通タンパク質であり，現在までに27種類の分子が同定されている.[2] 興味深いことにclaudinの発現及びバリア機能には組織特異性が観察され，claudin-1は粘膜や皮膚バリア，claudin-4は粘膜バリアを担っていることが明らかになっている．さらにclaudinはTJにおいてホモ及びヘテロストランドを形成していること，27種類存在する分子の組

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本総説は，平成22年度日本薬学会近畿支部奨励賞（医療系薬学）の受賞を記念して記述したものである．

み合わせによって多種多様な生体バリアを形成していること，claudin が分子量依存的・荷電選択的な物質透過経路として機能していること，claudin ストランドのダイナミックな再構成により物質輸送がなされていることが明らかになっている.[3] このことは，claudin を自由自在に制御することができれば，従来の吸収促進剤にはない組織特異性及び透過物質特異性を兼ね備えた，細胞間隙経路を介した新たな薬物送達法の開発が可能になることを示唆している.[4]

以上の背景を踏まえ，筆者らは，claudin-4 binder であるポリペプチド（C-CPE）を用い，claudin を利用した非侵襲性投与の proof of concept（POC）を確立してきた．本総説では，claudin を標的とした粘膜吸収促進法の開発，及び C-CPE を prototype として用いた新規 claudin binder の創製について紹介させて頂く.

2. Claudin を利用した吸収促進法の開発

Claudin の発見から 10 年もの月日が経過したが，いまだ claudin binder の開発はほとんど進展していない．Claudin のバリア機能を阻害する分子としては，*Clostridium perfringens* enterotoxin の C 末断片（C-CPE）が claudin-4 のバリア機能を阻害する分子として唯一報告されていた.[1] そこで，筆者らは C-CPE を claudin modulator のモデル分子として用い，claudin を標的とした粘膜吸収促進法の可否について検証を試みた.

まず，モデル薬物として分子量 4000 のデキストラン（FD-4）を用い，*in situ* loop assay によりラット空腸における粘膜吸収促進効果を解析した．C-CPE 処理により血漿中 FD-4 濃度の上昇が観察され，臨床応用されている吸収促進剤であるカプリン酸ナトリウム（C10）40 mg/ml と同程度の吸収促進効果を C-CPE はわずか 0.1 mg/ml で示していた.[5] 過去の報告により，CPE は C 末 30 アミノ酸を介して受容体と相互作用することが知られていた.[6] そこで，C-CPE の C 末 30 アミノ酸，16 アミノ酸を欠損させた C-CPE289, C-CPE303 を作製したところ，いずれの欠損体も claudin-4 結合性を消失していた.[5] C-CPE289, C-CPE303 では吸収促進活性が観察されなかったことから，C-CPE は claudin-4 に作用することで吸収促進活性を発揮しているものと推察される（Fig. 1）.[5]

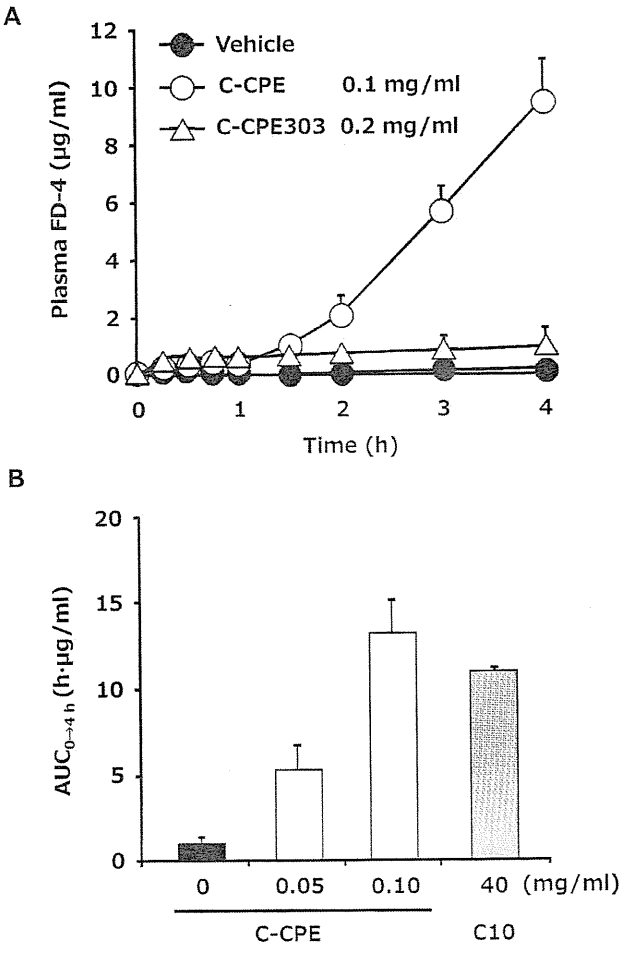

Fig. 1. Effect of C-CPE on Jejunal Absorption in Rats

Rat jejunum was treated with FD-4 (2 mg) in the presence of C-CPE, C-CPE303 or C10. The FD-4 levels in plasma collected from the jugular vein were determined (A), and the $AUC_{0\to4\,h}$ was calculated (B). Data are means ±S.E. (n=4).

次に，モデル薬物として現在骨粗鬆症治療薬として使用されているヒト副甲状腺ホルモン hPTH を用いて，腸管・経鼻・経肺吸収促進効果を解析した．C-CPE 処理により，鼻粘膜からの hPTH 吸収が観察されたが，腸管及び肺胞粘膜からの吸収は観察されなかった．C-CPE を前投与することで，hPTH の吸収が観察されていたことから，腸管及び肺胞粘膜では，C-CPE が claudin-4 バリアを阻害する前に hPTH の分解が生じている可能性が考えられた.

そこで次に，claudin を標的としたバイオ医薬の非侵襲性投与法を開発するために，物性に優れた新たな claudin-4 modulator の創製を試みた．2008 年，Anderson のグループは C-CPE の N 末 10 アミノ酸欠損体を作製し立体構造の解明に成功していた.[7] そこで，C-CPE の立体構造情報を基に N 末欠損体

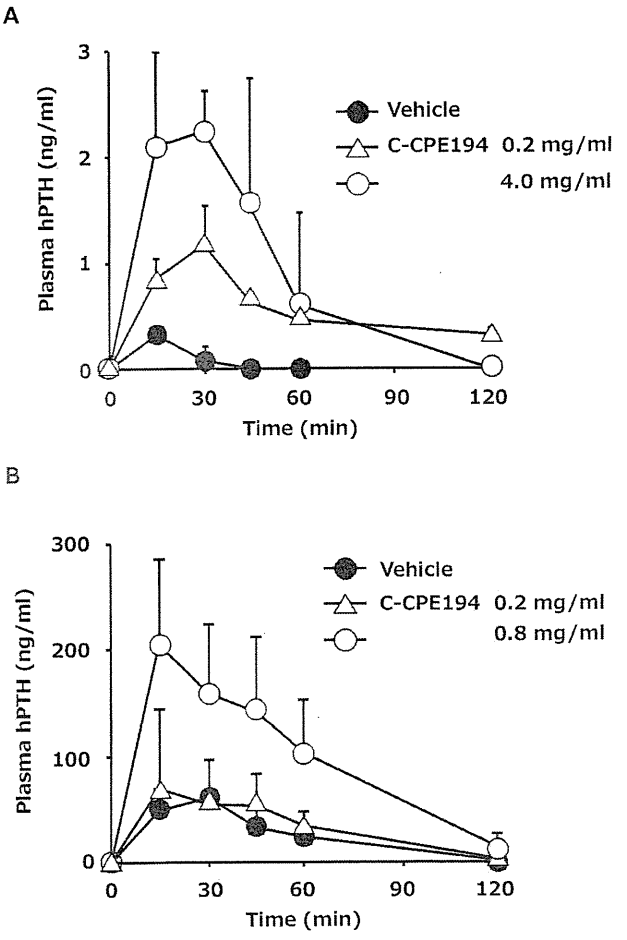

Fig. 2. Mucosal Absorption of hPTH by C-CPE194

(A) Jejunal absorption of hPTH. Rat jejunum was treated with hPTH (100 μg) in the presence of C-CPE194 at the indicated doses. Time course changes of plasma hPTH were assayed. (B) Pulmonary absorption of hPTH. hPTH (150 μg) and C-CPE194 at the indicated doses were pulmonary administered. Time course change of plasma hPTH were analyzed. Data are means±S.E. (n=4).

を作製し，claudin-4 結合性及び溶解性を解析したところ，C-CPE の N 末を 10 又は 21 アミノ酸欠損させることで，claudin-4 結合性を保持したまま溶解性が 10 倍以上向上することを見い出した.[8] N 末 10 アミノ酸欠損体（C-CPE194）の溶解性が最も高かったことから，C-CPE194 を用いて hPTH の吸収促進効果を解析したところ，高濃度処理することにより腸管及び肺胞粘膜において hPTH の顕著な吸収促進効果が観察され（Fig. 2），[8] claudin-4 modulator を利用したバイオ医薬の非侵襲性投与の POC を初めて確立した.

3. C-CPE 変異体ライブラリの作製

前述したように，粘膜及び皮膚バリア制御が非侵襲性投与法開発の基本戦略の 1 つとなっている．これまでの上皮細胞バリア研究の進展により，claudin-1 が粘膜や皮膚，claudin-4 が粘膜バリアを担っていることが明らかになっていることから，claudin-1 及び claudin-4 の制御が非侵襲性投与の鍵を握っていると考えられる．C-CPE を用いた検討から claudin binder がバリア制御能を有していることが明らかになっているものの，claudin は抗原性が低い上にタンパク質精製が困難であり，抗体を含め claudin binder の開発は立ち遅れているのが現状である．そこで筆者らは，claudin-4 binder である C-CPE を prototype として用い，claudin-1 及び claudin-4 に結合性を有する分子の創製を試みた.

まず，ファージ表面提示法を用いて C-CPE 変異体ライブラリを作製するために，C-CPE の機能ドメインの解析を試みた．C-CPE の C 末 16 アミノ酸を欠損させると claudin-4 結合性を消失していたことから，C 末 16 アミノ酸中に claudin-4 結合ドメインが存在していると考えられる．そこで，C 末 16 アミノ酸をアラニンスキャンにより解析したところ，Y306, Y310, Y312, L315 をアラニンに置換することで claudin-4 結合性が低下していた（Table 1）．一方，興味深いことに，S304, S305, S307, N309, S313, K318 をアラニンに置換した変異体では C-CPE に比して claudin-4 結合性の亢進が認められた（Table 1）.[9-11] そこで，アラニン置換により claudin-4 結合性が亢進していたアミノ酸をランダムなアミノ酸に置換した 1.38×10^7 種類の C-CPE 変異体提示ファージライブラリを作製した.

4. Claudin-1 and -4 binder の創製

さて，claudin binder のスクリーニングには claudin タンパク質が不可欠であるものの，claudin は膜貫通領域が多くタンパク質の精製に成功した例は依然として claudin-4 のみである．そこで，claudin の細胞外領域のペプチドや claudin 発現細胞を用いたスクリーニングを試みたものの，非特異的な結合が多く claudin binder の取得には成功しなかった．浜窪博士（東京大学先端研）は，発芽バキュロウイルス（BV）膜上に膜タンパク質が intact な状態で提示されることを見い出し，BV を利用した膜タンパク質発現系を開発している.[12] そこで，claudin 提示 BV を利用した claudin binder スクリーニング系の構築を試みた．Claudin-4 提示 BV は claudin-4 binder とのみ特異的に結合すること，ネガティブコントロールファージと C-CPE 提示ファージの混

Table 1. Competitive Inhibition of C-CPE-PSIF-induced Lactate Dehydrogenase Release by Mutant C-CPEs

C-CPE mutants	Inhibitory ratio (% of C-CPE)
C-CPE	100
S304A	125.6±0.7
S305A	126.8±0.2
Y306A	63.8±0.4
S307A	123.6±2.2
G308A	99.1±2.7
N309A	125.6±1.8
Y310A	72.2±2.3
P311A	114.9±0.5
Y312A	73.1±2.4
S313A	132.9±0.8
I314A	94.3±3.1
L315A	69.1±2.7
Q317A	96.8±1.6
K318A	126.2±2.1
F319A	111.5±3.9

After a 1 h treatment with C-CPE or mutant C-CPEs at 5 μg/ml, claudin-4-expressing L cells were treated with C-CPE-PSIF as a claudin-4-directed toxin (0.2 μg/ml) for 36 h, and the release of lactate dehydrogenase (LDH) was determined. The results are shown as the inhibitory ratio of C-CPE, and the values are the means±S.D. (n=4).

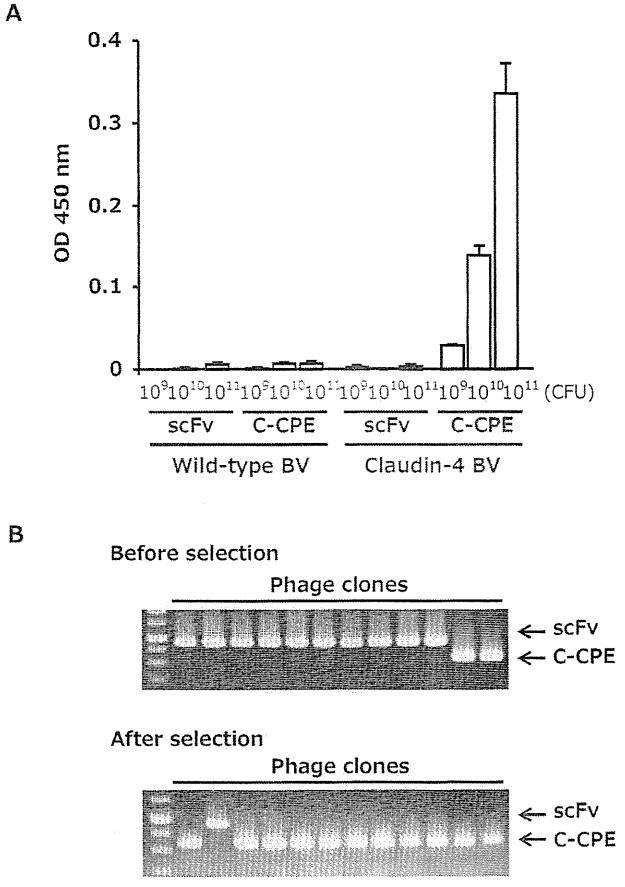

Fig. 3. Selection of C-CPE-displaying Phage by Using the Claudin-4-displaying Baculovirus System

(A) Interaction of C-CPE-displaying phage with claudin-4-displaying baculovirus (BV). Wild-type BV or claudin-4 BV was coated on an immunoplate, and then scFv-displaying phage or C-CPE-displaying phage was added to the BV-coated immunoplate at indicated concentrations. The BV-bound phages were detected by ELISA with anti-M13 antibody. Data are means±S.D. (n=3). (B) Enrichment of C-CPE-phage by the BV system. A mixture of scFv-phage and C-CPE-phage (mixing ratio of scFv-phage to C-CPE-phage=2 : 10) was incubated with a claudin-4 BV-coated immunotube, and the boud phages were recovered. Each phage clone was identified by PCR amplification, followed by agarose gel electrophoresis.

合液から効率的に C-CPE 提示ファージが濃縮されたことから，claudin 提示 BV を用いることで claudin binder のスクリーニングが可能になるものと推察された（Fig. 3）.[13]

そこで，claudin-1 提示 BV を用いて C-CPE 変異体ライブラリの中から claudin-1 結合性ファージのスクリーニングを試みたところ，claudin-1 に対して高い結合性を示すファージクローンが複数認められた．高い claudin-1 結合性を示したファージクローンのシークエンス情報を基にタンパク質を作製し，claudin 結合性を解析したところ，C-CPE 変異体（m19）は claudin-1 結合性を有していた（投稿準備中）．m19 は，claudin-4 結合性を保持していたことから，m19 は claudin-1/-4 binder であるものと推察された（投稿準備中）．Caco-2 細胞の単層膜培養系を用いて膜電気抵抗値 TEER を指標に，m19 の TJ バリア制御活性を解析し，m19 は優れた TJ バリア制御活性を有することを見い出した（投稿準備中）．さらに，モデル薬物として FD-4 を用いて吸収促進効果を解析したところ，m19 は C-CPE に比して優れた粘膜吸収促進活性を有していた．今後は本 binder を druggable claudin modulator の lead として用い，非侵襲性投与技術の顕在化を試みていきたい．

5. おわりに

以上，これまでに，TJ シールの分子基盤 claudin を標的としたバイオ医薬の粘膜吸収促進技術を世界に先駆けて開発し，優れた粘膜吸収促進活性を有する dual specific claudin binder の創製に成功した．Claudin modulator の実用化に際しては，claudin バリア制御活性を有する低分子化合物の開発，及び薬物吸収後に可及的速やかに claudin シールを回復させる方法論の確立も必要であり，今後は claudin バリアを自由自在に制御する技術を創出することで，患者に優しい非侵襲性投与の実現に貢献していきたい．

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タイトジャンクションを標的とした創薬研究のらせん的発展

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1 はじめに

約300年前，ドイツの観念哲学者ヘーゲルは，物事は直線的に発展するのではなく，あたかもらせん階段を上るように発展していくという「事物のらせん的発展の法則」を提唱した．らせん階段を上る人を横から見ると上って行くように見えるが，上から眺めると元の位置に戻ってくる．すなわち，事物の発展では，古く懐かしいものが新しい価値観を伴って再び現れてくると考えられている．

さて周知のように，薬物治療では上皮細胞における薬物透過が不可避であり，1950年代には粘膜上皮細胞を介した薬物吸収促進研究が開始されている．1960年代に入り，隣接する上皮細胞間にタイトジャンクション(tight junction；TJ)が存在しTJによって細胞間隙がシールされていることが示され，TJシール制御による薬物吸収促進法が開発され始めた．これが，TJを標的とした創薬研究の始まりである．

本拙稿では，1960年代に生まれたTJを標的とした創薬の萌芽が，上皮細胞生物学の進展に伴い辿ってきたらせん的発展について概説したい(図1)．

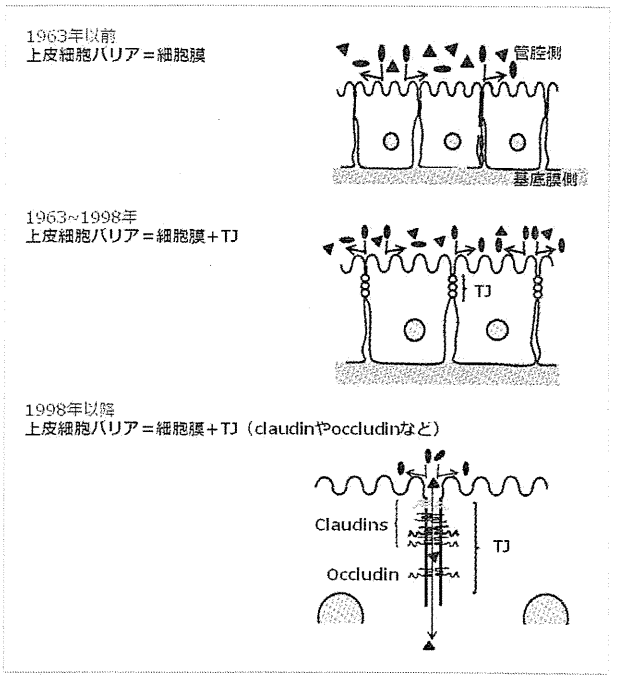

図1 上皮細胞バリアの分子基盤

1963年以前は細胞膜，1963~1998年は細胞膜およびTJが上皮細胞バリアの基盤であると考えられていた．1993年のoccludin，1998年のclaudinの発見により，claudinなどから構成されているTJが細胞間隙における物質透過を制御していることが明らかになった．

2 TJを標的とした創薬研究の始まり

進化の過程において，多細胞生物は生体内外・組織内外を隔てる生体バリアとして，上皮細胞を発達させてきた．薬物吸収では，この障壁が透過・吸収障壁として機能することから，50年以上前から上皮細胞における薬物透過促進法の開発が進められていた．1961年，*Nature*誌にEDTAが粘膜吸収促進作用を有しているという報告がなされた．[1] 振り返ると，この論文がTJを標的とした創薬の端緒となっていたものの，当時はTJの存在が知られておらず，その作用機構は不明なままであった．1963年にTJが同定，1967年にTJシール制御による薬物吸収促進のproof of concept(POC)が確立され，粘膜上皮における透過促進活性を指標にした吸収促進剤の開発が進められた．[2,3]

3 TJを標的とした吸収促進研究のらせん的発展

TJシール制御による薬物吸収のPOCが確立され，中鎖脂肪酸，ポリカチオンなどが吸収促進剤と

して研究開発されたものの，粘膜障害性を伴うこと，TJシール開口に伴う非特異的な物質流入が不可避であることから，臨床応用されているのはカプリン酸ナトリウムなどに限られている．TJシールの分子基盤解析遅延に伴い，1990年代以降，安全性・特異性を兼ね備えた経細胞内経路(トランスポーターなどの利用)が上皮細胞の薬物透過研究のメインストリームとなった．

TJの分子基盤については，1982年*Nature*誌に脂質ミセル説が提唱されたものの，10年余りにわたり構成成分すら未解明なままであった(表1)．[4] 1993年に京都大学月田グループにより，TJ構成タンパク質として4回膜貫通タンパク質オクルディン(occludin)が同定され，TJシールがタンパク質によって形成されていることが初めて実証された．[5] その後，オクルディンの細胞外領域ペプチドがTJのバリア機能を低下させること，マンニトールなどの透過性を亢進できることなどが見いだされ，TJシールの分子基盤を標的とした吸収促進法が提唱された．[6] しかしながら，オクルディンを欠損させてもTJシールに異常が観察されなかったことから，“真のTJシール分子基盤”の解析が進められた．そしてついに1998年，TJを標的とした創薬研究のらせん的発展の起爆剤となるクローディン(claudin)が，月田グループ古瀬らによって報告された．[7] クローディンは分子量約23 kDaの4回膜貫通タンパク質であり，現在までに27種類の分子が見いだされており，claudin-1は皮膚バリア，claudin-5は血液脳関門，claudin-11は血液精巣関門を担っていることが明らかにされている．[8] また，1999年にウエルシュ菌下痢毒素(CPE)の受容体がclaudin-3, -4と同一分子であることが見いだされた．[9] CPEは*N*末側の下痢活性ドメインと*C*末側の受容体結合ドメイン(C-CPE)を含んでおり，C-CPEを上皮細胞に作用させると細胞障害性を伴うことなくTJバリアを弱める活性を示していたことから，クローディン結合分子(claudin binder)を利用した粘膜吸収の可能性が示唆された．[10] 2005年に，C-CPEがカプリン酸ナトリウムの400倍余りの粘膜吸収促進活性を有すること，本吸収促進活性にはclaudin-4との相互作用が関与していることが示され，クローディン結合分子を利用した粘膜吸収促進のPOCが確立された．[11] さらに，クローディン結合分子は副甲状腺ホルモン(現在注射剤として使用)の経肺・経鼻吸収促進活性も有しており，クローディンを標的としたバイオ医薬の非侵襲性投与法も提唱されている．[12]

TJシール開口作用を介して薬物吸収を促進する場合，薬物以外の物質の非特異的な流入が副作用発現につながる可能性が指摘されており，このことが細胞間隙経路を介した薬物吸収促進の実用化に立ちはだかる大きな壁となっている．TJには複数のクローディンが含まれていること，構成するクローディンの組合わせによってTJシールとしての機能が異なること，クローディンが細胞間隙経路を介したイオン透過に関与していること，クローディン制御による物質透過に分子量依存性が観察されることから，クローディンバリアを自由自在に制御することができれば，組織特異性および透過物質特異性を兼ね備えた新たな概念の吸収促進剤の開発が可能になると期待されている．[8]

表1 上皮細胞バリアの生物学の進展

年代	上皮細胞バリアに関する発見
1963	TJの発見
1973	TJストランドの発見
1982	TJの脂質ミセル説が提唱
1993	オクルディンの発見
1998	クローディンの発見
1999～	クローディンのTJバリア機能が証明
2005	トリセルリン(tricellulin)の発見
2011	細胞間隙経路および細胞内経路の共役物質輸送にクローディンが関与

4 TJを標的とした創薬研究のらせん的発展

上述したように，吸収促進の観点から研究が進められてきたTJを標的とした創薬研究は，オクルディンやクローディンの発見によりらせん的発展を遂げつつある．

悪性腫瘍の90%は上皮由来であること，上皮細胞は多くの病原性微生物の侵入門戸となっていること，炎症性腸疾患では上皮細胞バリアの破綻が観察

されることから，上皮細胞は薬物吸収・薬物送達のみならず，がん・感染症・炎症性疾患に対する創薬ターゲットとして高い可能性を有している．しかしながらターゲット分子の解析遅延から，上皮細胞を標的とした創薬研究は進展していなかった．クローディンの発見に端を発した上皮細胞生物学の進展に伴い，ヒトでは膵臓がんや膀胱がんをはじめとした12種類余りのがんでクローディンの高発現が認められること，粘膜免疫組織を覆う上皮細胞層にclaudin-4が高発現していること，claudin-1がC型肝炎ウイルス(HCV)の感染受容体の1つになっていること，炎症性腸疾患患者においてクローディンの発現異常が観察されることなどが報告され，TJを標的とした創薬においてクローディンがターゲットになる可能性が示唆されている(図2)．[13~16]

2001年にclaudin-4指向性毒素であるCPEが膵臓がんに対して抗腫瘍活性を有することが見いだされ，クローディンを標的としたがん治療の可能性が初めて実証された．[17] クローディンを標的としたがん治療法の構築に際しては，クローディン結合分子の作製が不可欠であるものの，クローディンは抗原性が低い上にタンパク質精製が困難であり，抗体を含めクローディン結合分子の開発は立ち遅れていた．最近，我が国において自己免疫疾患マウスを用いたクローディン抗体作製法が確立され，クローディンを標的としたがん治療法開発の可能性がようやく見え始めている．[18] さらに，クローディン結合分子により腫瘍組織内の細胞間接着を弱めることで低分子抗がん剤の腫瘍内浸透性を改善できる可能性も指摘されており，クローディンはがんターゲティング療法のみならず，がん化学療法においても注目されている．[18,19]

さて，粘膜面における病原体侵入防御網構築能および生体内に侵入した病原体や感染細胞排除能を併せ持つ粘膜ワクチンは，感染症対策の切り札として期待されているものの，抗原を粘膜面に投与しただけでは免疫反応は惹起されず，粘膜面に存在している免疫組織に抗原を効率的に送達する技術の開発が粘膜ワクチン開発における最重要課題の1つとなっている．[20] 2003年に粘膜免疫組織を覆う上皮細胞層にclaudin-4が高発現していることが示され，既にCPEの受容体結合領域を用いてclaudin-4を標的とした粘膜ワクチンのPOCが確立されている．[16,21]

現在，我が国には約170万人のHCV感染者がおり，肝がんの8割がHCV感染に起因している．進行した肝がんでは肝移植が行われているが，HCVの再感染率は99%となっており，HCV感染阻害が肝がん患者における肝移植の成否を握っている．2007年にclaudin-1がHCVの感染受容体であること，2010年に抗claudin-1抗体がHCVの感染阻害活性を有することが報告され，claudin-1 binderを利用したHCV感染阻害のPOCが確立された．[14,22] インターフェロン療法では副作用に伴い治療の中断を余儀なくされることが多いことから，抗claudin-1

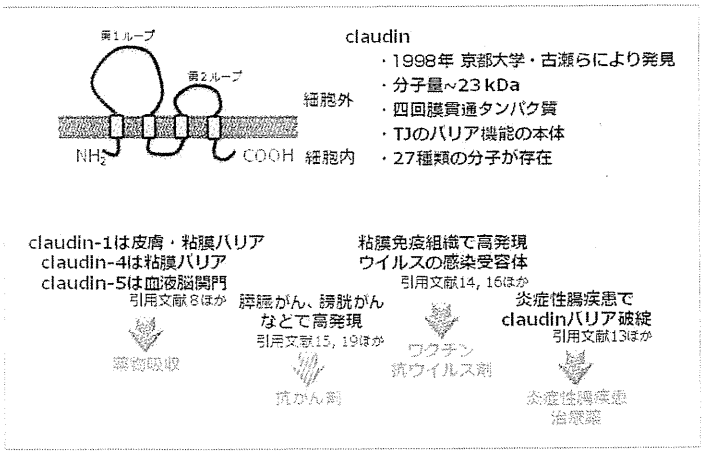

図2 Claudinを標的とした創薬の可能性