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Review Article

The claudin family as a new approach for drug delivery

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CONTENTS

Abstract

Passing across epithelial and endothelial cell sheets is an inevitable step for the delivery of drugs to a target tissue. Tight junctions (TJs) exist between adjacent cells in these cell sheets, and TJs prevent the free movement of solutes through the cell sheets. Overcoming the barrier function of TJs is a pivotal issue in drug delivery, and modulation of TJs may be useful for drug delivery. However, the molecular mechanisms of TJ barriers were unclear and TJ-based drug delivery systems have never been fully developed. In 1998, claudin, an approximately 24-kDa tetratransmembrane protein, was identified as a structural and functional component of TJs. Interestingly, disruption of claudin causes dysfunction of the TJ barrier and influx of solutes into a particular tissue. A series of studies on claudin have provided us with new strategies for drug delivery systems. In this review, we discuss a novel drug delivery system based on claudin and the perspectives for claudin-based drug delivery.

Introduction

Epithelial cells surround mammalian organs and tissues, and epithelial cell sheets constitute the principal barrier to cellular uptake and transport. For absorption of a drug into systemic fluid, it must pass across epithelial cell sheets via epidermal and mucosal absorption. Likewise, for the transfer of a drug from the systemic fluid into a target tissue, it must pass through endothelial cell sheets. Thus, passage across epithelial and endothelial cells is a major determinant of the pharmacokinetic properties of a drug. Routes for passing through these cell sheets can be classified as transcellular or paracellular (Fig. 1) (1-4). Numerous efforts have focused on the development of drug delivery systems that would allow compounds to pass through the cell sheets via both the transcellular and paracellular routes.

In the transcellular route, a drug is taken up via a receptor and a transporter on the membrane, and the selection of a tissue-specific receptor and transporter enables drugs to be selectively delivered to a target tissue. For example, folate receptors are expressed to a greater extent in many human cancers as compared to normal cells, and targeting these receptors therefore represents a method for developing a tumor-targeting drug delivery system (5). Furthermore, asialoglycoprotein receptors are expressed on the membranes of mammalian hepatocytes and these receptors have therefore been used to target the liver (6).

Transporters are classified into influx and efflux transporters, and these transporters regulate the absorption, distribution and elimination of drugs (2). Many different drug transporters are also expressed in various tissues, including intestinal and hepatic epithelial cells and brain capillary endothelial cells (7-10). Some of these transporters determine the pharmacokinetic characteristics of a drug with regard to its intestinal absorption, tissue distribution and elimination. For example, efflux transporters, such as multidrug resistance-associated protein (MRP) or breast cancer resistance protein (BCRP), are expressed on the brush border membrane of enterocytes and excrete their substrates into the lumen, resulting in a potential limitation of net absorption (11-14).

Active secretion of the absorbed drug by efflux transporters is now recognized as a significant factor in oral drug bioavailability (15, 16). There is a relationship between the intestinal levels of MRP and the absorption of digoxin (17). Therefore, inhibition of intestinal efflux transporters is a useful way to improve the oral bioavailability of a co-administered drug (18). Much attention is being paid to drug discovery based on the transporter mechanisms and substrate specificities of drug transporters, together with *in silico* and high-throughput screening. The identification of compounds that are substrates for transporters is useful for the selection of new drug candidates.

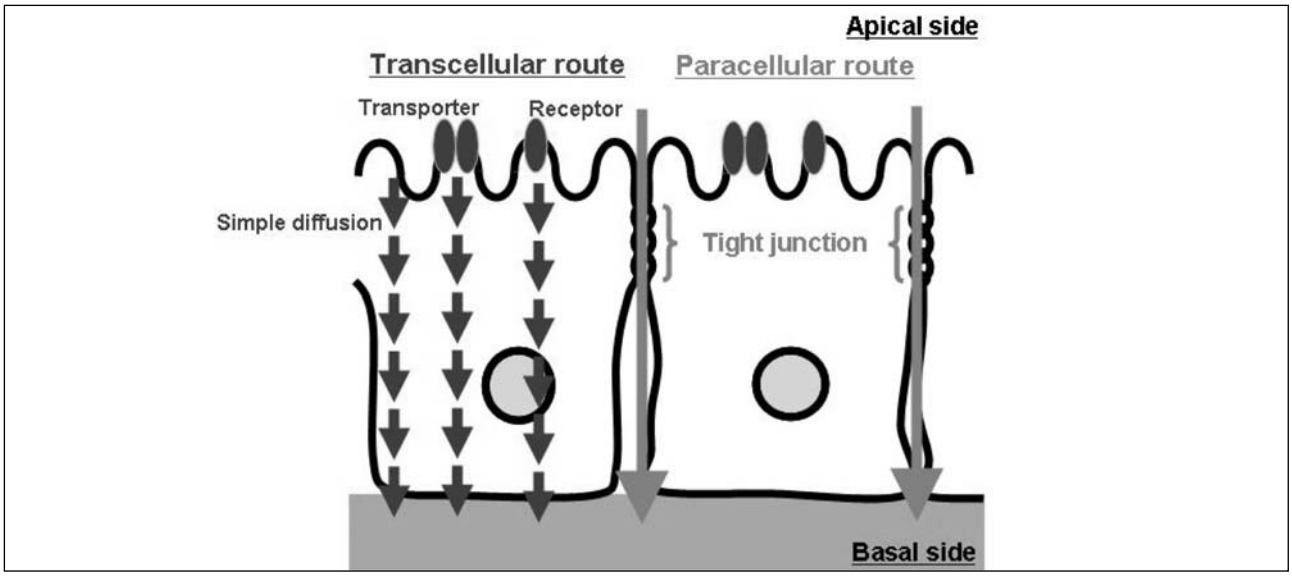

Fig. 1. The transport route within the epithelia.

Dramatic progress has been made in drug delivery via the transcellular route. In contrast, studies of drug delivery via the paracellular route have never been fully developed. Tight junctions (TJs) are found in the intercellular space between epithelial cells, and they act as a barrier that prevents substances from leaking across epithelial cells. In the paracellular route, alterations of the TJ barrier allow drugs to pass through the intercellular space. Indeed, since the 1960s, drug delivery systems via paracellular transport have been studied as absorption enhancers to loosen TJs. However, unacceptable side effects, such as low tissue specificity and high toxicity, were associated with most of the absorption enhancers. Absorption enhancers are classified as Ca^{2+} chelators or surfactants (19, 20). Ca^{2+} chelators disrupt adherent junctions, while surfactants cause exfoliation of the intestinal epithelium and irreversible compromise of the TJ barrier (19). Although surfactants, fatty acids, medium-chain glycerides and chitosan have been studied as absorption enhancers (21), sodium caprate is the only clinically used enhancer that works via the paracellular route. Some researchers have stated that it is extremely difficult to develop drug delivery systems via the paracellular route. However, until recently, the structure and function of TJs was unknown. Therefore, perhaps this delayed understanding of TJs rather than an inherent difficulty in the paracellular route may underlie the poor progress in the development of drug delivery systems through the paracellular route.

In this review, we introduce claudin, the key molecule in TJ barriers, and review recent progress in drug delivery through the paracellular route based on claudin.

Claudin family

Freeze-fracture replica electron microscopy analysis has revealed that TJs appear as a series of continuous, anastomotic and intramembranous particle strands or fibrils (Fig. 2), but the composition of TJs has been unclear. In 1993, Furuse *et al.* reported the first component of TJ strands, named occludin (22). Occludin is a four-transmembrane protein with a molecular mass of about 60 kDa. Overexpression of occludin increases the number of TJ strands and elevates the barrier function in epithelial cells, suggesting that occludin is responsible for TJ barrier function (23, 24). However, the structure and function of the TJ barrier were unaffected by disruption of the occludin gene, indicating that it is not a critical component of the TJ barrier (25).

In 1998, Furuse *et al.* isolated the key component of the TJ barrier, named claudin, in co-partitioned proteins with occludin (26). Claudin has a tetra-transmembrane domain and a molecular mass of about 23 kDa, with no sequence similarity to occludin. The claudins comprise a multigene family of 24 members. The expression profiles and barrier function of each member differ among tissues (27-29). For example, claudin-1 and claudin-5 are widely expressed in a variety of tissues, and the expression of claudin-6 is observed before birth (26, 30). Mice deficient in claudin-1 and claudin-5 exhibited dysfunction of the epidermal and blood-brain barriers, respectively (31, 32), and the barrier function boundary between blood and testis was eliminated in claudin-11 knockout mice (33, 34). Most cell types express more than two members of the claudin family, and each TJ strand contains several types of claudin.

Each claudin strand between adjacent cell membranes pairs in a heterotypic and homotypic manner, and it has therefore been proposed that TJ permeability is controlled by the combination and ratio of the 24 members of the claudin family (35-37). Indeed, the exogenous expression of claudin-11 or claudin-15 resulted in an elevated barrier function of TJs in MDCK II cells, but not in

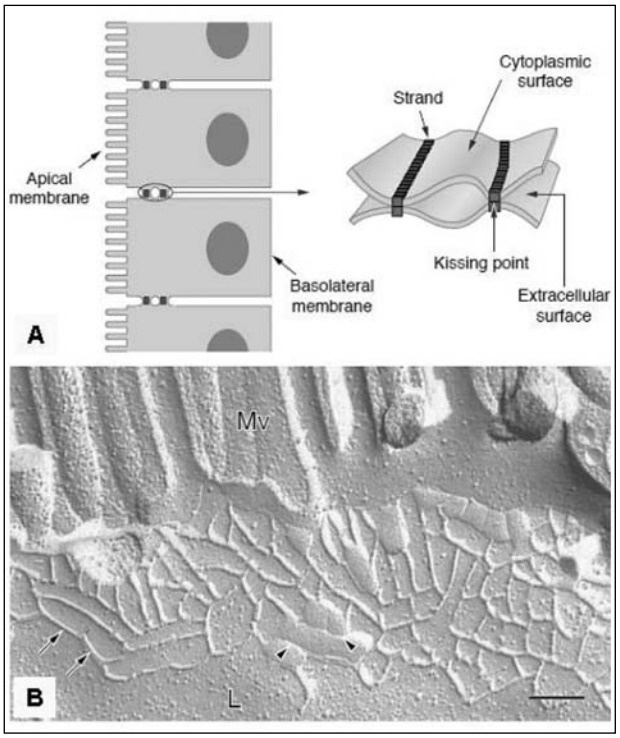

Fig. 2. Structure of tight junctions. **A**: Tight junctions (TJs; circled) are located at the apical part of lateral membranes. TJs comprise an elaborate network of paired strands, which form the so-called 'kissing points' that eliminate the extracellular space. TJ strands also function as a fence between apical and basolateral membrane domains. **B**: Freeze-fracture replica image of glutaraldehyde-fixed mouse intestinal epithelial cells. Between apical microvilli (Mv) and the lateral membrane (L), TJs appear as a set of continuous, anastomosing strands in the P-face (arrows), with complementary grooves in the E-face (arrowheads). Bar, 0.2 μm. Reproduced with permission from Ref. 30.

$LLC\text{-}PK_1$ cells (38, 39). Claudin-3 formed paired strands with claudin-1 and claudin-2, whereas claudin-1 did not form paired strands with claudin-2 (36). Epithelial cells surround tissues and organs, and they are responsible for the partition of every tissue and organ. The physiological requirements for paracellular permeability vary among the different types of tissues. Considering the complexity of intratissue circumstances in an organism, it is reasonable to presume that the properties of the TJ barrier in the tissue-surrounding cell sheets would be determined by a combination of all members of the claudin family.

The tissue specificity in the barrier function of the claudin family is an attractive characteristic for the development of drug delivery systems, since the selection of particular claudin members would allow drug delivery to a target tissue via a paracellular route.

Direct modulation of claudin

The claudin family has ideal characteristics to play a key role in TJ barriers; therefore, by modulating the claudin barrier, we would have a powerful strategy for drug delivery via the paracellular route. The claudin barrier can be modulated by both direct and indirect methods. In this section, we review the direct method of modulating the claudin barrier.

Claudin-4 modulator

In 1999, Morita *et al.* found that claudin-4 was identical to the receptor for *Clostridium perfringens* enterotoxin (CPE) (40). CPE is a 35-kDa single polypeptide that causes food poisoning in humans (41). The functional domains of CPE are divided into a receptor-binding region (*C*-terminal C-CPE) and a cytotoxic region (*N*-terminal C-CPE). Interestingly, treatment of cells with C-CPE decreased claudin-4 protein levels and disrupted TJ barriers, indicating that C-CPE is a modulator of claudin-4 (42). Based on these findings, we investigated whether claudin-4 could be a target for a drug delivery system using C-CPE. C-CPE concentration-dependently enhanced the absorption of fluorescein isothiocyanate-dextran, which has a molecular mass of 4000 Da (FD-4). This enhancement of activity by C-CPE was 400-fold greater than that observed for sodium caprate (C10), which is routinely used clinically as a drug absorption enhancer (Fig. 3) (42). There was no mucosal toxicity (*i.e.,* leakage of lactose dehydrogenase from intestinal mucosa and histological evaluation) observed with the C-CPE (0.1 mg/ml) treatments. The receptor-binding region of CPE is in its *C*-terminal 30-amino-acid region (43). Deletion of the binding region in C-CPE attenuated the ability to modulate the TJ barrier and bind to claudin-4, indicating that claudin-4 modulation is a novel strategy for drug delivery (42, 44, 45).

Claudin modulators

Because there are 24 members of the claudin family and each homozygous and heterozygous combination of claudin strands in the membrane affects the properties of TJ barriers, a huge number of strand-mediated effects are possible (36, 37). Although these findings suggest that claudin modulators may be promising for drug delivery, C-CPE is the only known modulator of claudin-4. Here, we discuss the possibility of claudin modulators for drug delivery.

The combination and ratio of the 24 claudin members in TJ strands are responsible for the properties of TJ barriers, which vary among tissues and cell types. For example, the expression of claudin-11 increases the integrity of the TJ barrier in MDCK II cells but decreases it in $LLC\text{-}PK_1$ cells (38). In contrast, knockdown of claudin-7 increases the TJ barrier in $LLC\text{-}PK_1$ cells but decreases the TJ barrier in MDCK II cells (46). Deletion of claudin-1 resulted in dysfunction of the epidermal barrier, followed by movement of an ~600-Da tracer molecule from the epidermis to the skin surface (31). Claudin-5-deficient mice showed size-selective loosening (~800 Da) of the blood-brain barrier (32). Overexpression of claudin-2 reduced TJ integrity in MDCK I cells (37), and overexpression of claudin-2,

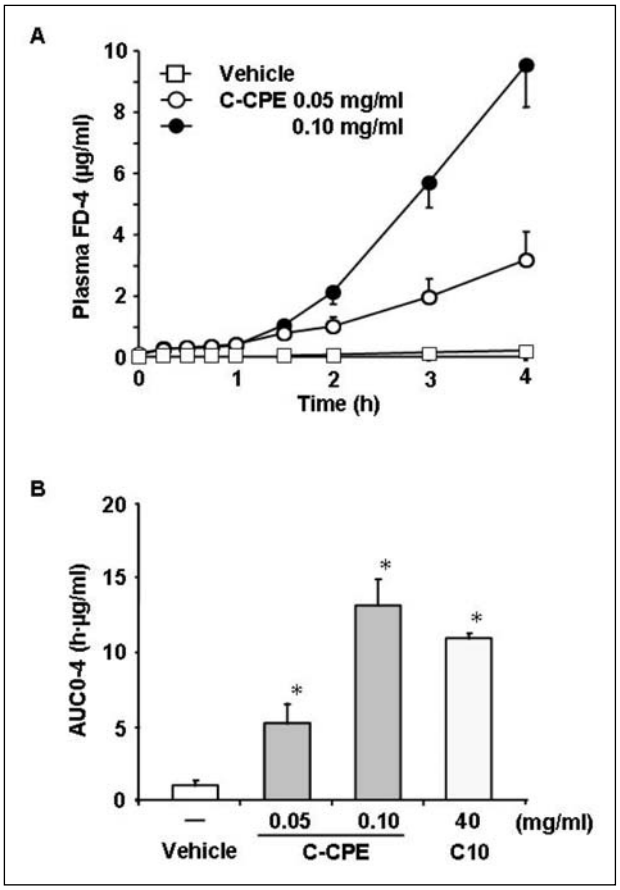

Fig. 3. C-CPE is a novel absorption enhancer. Rat jejunum was treated with FD-4 (10 mg/ml) in the presence of vehicle, C-CPE or C10. The FD-4 levels in plasma collected from the jugular vein were determined (**A**), and the $AUC_{0\text{-}4\ h}$ was calculated (**B**). Data are means ± SE (n=4). *Significant difference from the vehicle-treated group ($p < 0.05$). Reproduced with permission from Ref. 42.

-11 or -15 formed leaky TJ barriers in LLC-PK_1 cells (38). These *in vitro* and *in vivo* experiments strongly suggest that the modulation of claudin in a member-specific manner is a promising method for drug delivery through the paracellular route. To realize the development of a claudin-based drug delivery system, the preparation of a claudin modulator library that covers a spectrum of inhibitors would be useful.

RNA interference (RNAi) is a candidate technique for the modulation of claudin. Indeed, the knockdown of claudin by RNAi caused dysfunction of claudin in cells (46). Modulation of TJs by C-CPE is believed to be due to its binding to the extracellular loop region of claudin-4 (47), so a claudin binder could also modulate the TJ barrier. However, attempts to develop an antibody against the extracellular loop domain of claudin have not been successful. C-CPE is predicted to bind claudin-3, -4, -6, -7, -8 and -14 (44, 47). We are attempting to change and improve the binding properties of C-CPE in order to modulate TJs. The three-dimensional structure of claudin is unknown, but future research into the structural biology of claudin will provide information essential for the discovery of claudin modulators such as C-CPE derivatives, antibodies and chemical compounds.

Indirect modulation of claudin

As mentioned above, C-CPE is the only molecule known to directly modulate the claudin barrier (only the claudin-4 barrier). Another strategy for modulating the TJ barrier is indirect regulation of the claudin barrier function. Although there are no reports of drug delivery by the indirect regulation of the claudin barrier, we have identified possibilities for an indirect strategy based on research into signal transduction involved in the barrier function of claudin. Indeed, several reports have described the mechanism by which claudins regulate barrier function. Ikenouchi *et al.* found that Snail, a zinc finger transcription factor that regulates the epithelium-mesenchyma transition, directly suppresses expression of claudin-3, -4 and -7 (48, 49). Therefore, a chemical compound that activates Snail could be used to modulate the TJ barrier. MDCK I cells and the mouse cortical collecting duct cell line 94D show high integrity of TJs (50, 51). Treatment of MDCK I and 94D cells with an inhibitor of extracellular signal-regulated kinase (ERK) 1/2 induced expression of claudin-2, followed by dysregulation of TJ integrity (52). Claudin-3 and claudin-7 levels, but not claudin-1 and claudin-4 levels, were also decreased by the ERK1/2 inhibitor in MDCK I cells, suggesting that inhibition of the intracellular signaling pathway involved in controlling claudin levels may be useful for regulating claudin-mediated TJ barriers. Agents that elevate intracellular cAMP levels activate the TJ barrier function by inducing claudin-5 in brain endothelial cells (53, 54). An inhibitor of mitogen-activated protein kinase (MAPK) suppressed the barrier function of claudin-1 (55). Thus, chemical compounds that regulate intracellular signaling involved in the barrier function of claudins may be promising candidates for drug delivery systems.

Future directions for claudin-based drug delivery systems

TJs have two important roles: a barrier function and a fence function. In previous sections, we have described the barrier function of claudin and drug delivery. In this last section, we discuss critical problems in claudin-based drug delivery by modulation of the TJ barrier, and we provide some ideas on the fence function and drug delivery.

Drug delivery to open or modulate TJs is accompanied by the influx of substances other than the drug into the target tissue. This unregulated influx is the primary reason why the development of absorption enhancers was terminated. Some researchers believe that drug delivery through the paracellular route is unlikely to be practical for pharmaceutical therapy, whereas others believe that the transcellular pathway is a viable approach for the development of a drug delivery system. These opinions were especially true prior to 1998, when

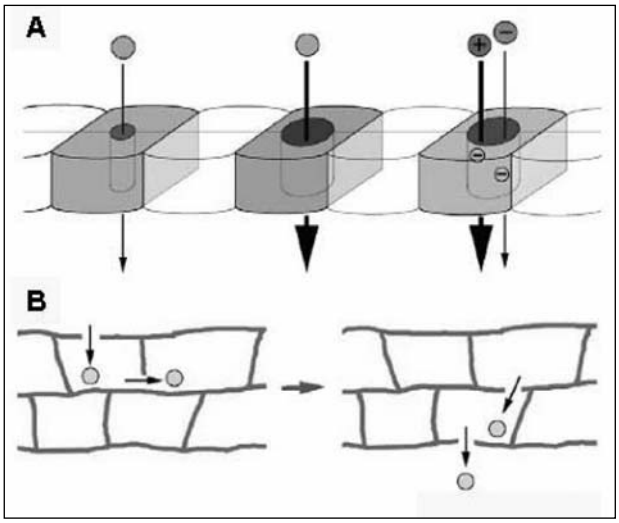

Fig. 4. Proposed mechanisms for paracellular transport of solutes across TJs. **A:** Aqueous pores for small molecules such as inorganic ions within claudin-based TJ strands. Depending on the diameters of their aqueous pores, TJ strands can exhibit size selectivity (left and center). When crucial sites within the pores of TJs are, for example, negatively charged owing to acidic amino acid residues in the extracellular loops of the claudins, cations can pass through the pores more easily than can anions, thereby generating charge selectivity (right). **B:** Dynamic reorganization of TJ strands. Local breaking and annealing of paired TJ strands (lines) enables solutes (circles) to pass across TJs, while maintaining the structural integrity of the TJs. Reproduced with permission from Ref. 27.

a key component of the TJ barrier, claudin, was identified. However, the transcellular route has great promise in light of more recent progress in the biology of TJs that has led to the identification of claudins and their interaction with the functional and structural components of TJ barriers (26, 27). For example, we now know that claudins in TJs are involved in the movement of ions; claudin-16 is involved in paracellular Mg^{2+} resorption in the thick ascending limb of Henle (56), and TJ strands are likely to act as charge-selective channels and sieve pores (Fig. 4) (27). TJ strands are occasionally broken and annealed, and they associate with each other in an end-to-side and side-to-side manner in fluorescein-labeled claudin-expressing cells (57). The dynamic characteristics of TJ strands are considered to contribute to the passage of substances across TJs while maintaining the structural integrity of TJs (27). Thus, tissue-specific TJ pores and dynamic reorganization of TJ strands could be promising for the development of tissue-specific delivery of drugs through TJs. These findings indicate that a claudin-based delivery system can overcome the long-pending problem of the influx of substances other than the drug during the opening of TJs.

TJs produce cellular polarity by division of the apical and basal domain faces to keep proteins in one domain from diffusing into the other (58). Polarized cells have an asymmetrical distribution of cell-surface proteins, such as ion channels, transporters and receptors (59, 60), indicating that TJ components may be indirectly involved in the transcellular delivery of drugs. How can we benefit from the fence function of TJs? A particular case may be tumor targeting. Perturbation of junctions and polarity can cause epithelial cells to proliferate wildly, leading to a multilayered architecture and the potential development of cancer. Notably, 90% of fatal malignancies in adult humans arise from epithelia. Indeed, downregulation of claudin was observed in some tumors (61-64). Thus, we could deliver antitumor agents to tumors by targeting a molecule that is specifically localized to the tumor.

In summary, recent progress in the understanding of TJ components revolving around claudin family members is providing breakthroughs in drug delivery. We are developing a novel strategy for drug delivery by using claudin modulators, and we will develop a novel tumor-targeting strategy based on the claudin fence function. The regulation and structure of claudin, as well as how claudin controls the TJ barrier, have not been fully elucidated. Future progress in the biology of TJs will provide new insight into drug delivery by using the claudin barrier and claudin fence functions of TJs.

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