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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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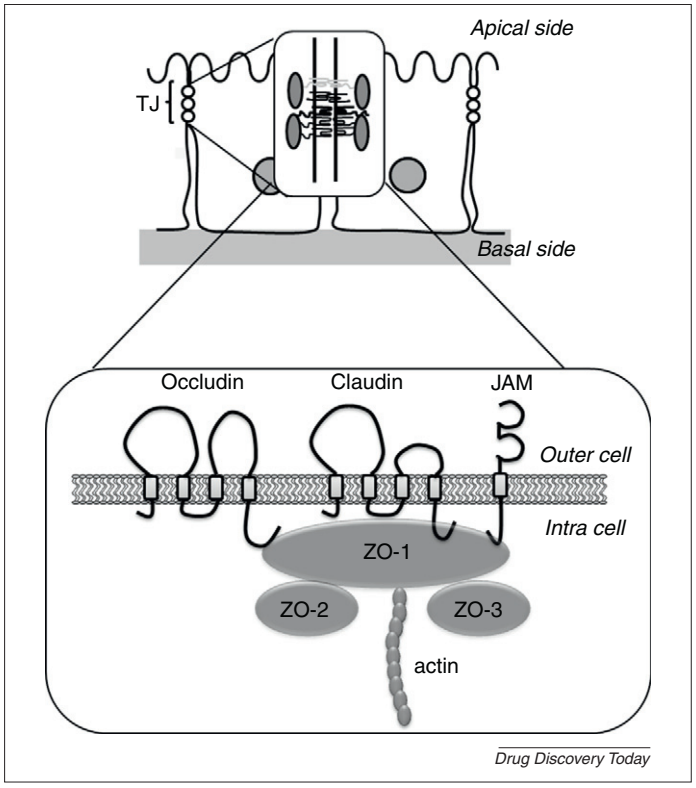

FIGURE 1

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 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.

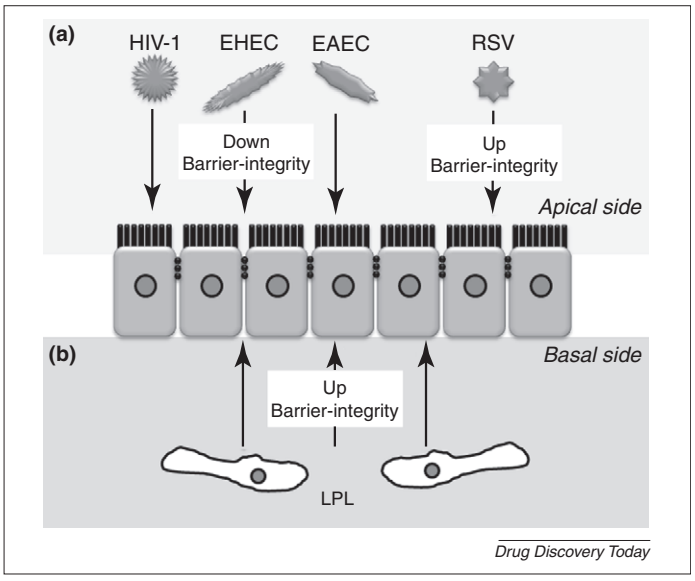

FIGURE 2

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.

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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