Endothelial tight junctions: permeable barriers of the vessel wall

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Summary
The endothelial lining of the vessel wall is a permeable barrier, which is located at the interface between the vascular and the perivascular compartments. Although the endothelium acts as an efficient barrier that strictly separates the two compartments, it may also act as a permeable filter which allows selective exchange of solutes and water between the luminal and abluminal sides of the barrier. Similarly to epithelia, also in the endothelium permeability follows two distinct routes, which have been termed transcellular pathway (across the apical and basolateral membranes of individual cells) and paracellular pathway (through the intercellular junctions and the lateral spaces between contacting cells). After an initial description of the two pathways, the review focuses on the cellular and molecular basis of the paracellular pathway, with emphasis on the role of intercellular tight junctions and tight junction-associated claudins. Finally, the signaling events that regulate paracellular permeability are discussed.

Keywords
Endothelial cells, permeability, tight junctions, ion channels

Permeability: transcellular and paracellular routes

The endothelium extends over a wide surface area which is located at the interface between vascular and perivascular compartments. In this location, endothelial cells serve two distinct functions. On one side, they separate strictly the two compartments, thus maintaining tissue homeostasis and preventing thrombosis. On the other side, they establish communications between circulating blood and underlying tissues, thus contributing to essential functions of the vessel wall. Hence, like the epithelial lining of several organs, the vascular endothelium acts as a barrier but, at the same time, behaves as a permeable filter. Permeability, which consists in the transport of solutes and water across endothelial and epithelial layers, follows two distinct pathways (Fig. 1). The transcellular route crosses apical and basal cell membranes and is mostly mediated by channels, carriers, pumps and vesicles. In contrast, the paracellular route passes through intercellular and lateral spaces between contacting cells and is mediated by the tight junctions (TJ). This review focuses on the molecular basis of paracellular permeability. Transcellular permeability will be discussed only with respect to its interactions with the paracellular system.

In addition to the cellular and molecular components, the two pathways also differ with respect to the physical properties (1, 2). First, transport across the transcellular pathway can be either passive or active, while passage through the paracellular pathway is exclusively passive, as it is driven by electrochemical and osmotic gradients (which are often established by the transcellular pathway). Second, compared with the transcellular route, the paracellular pathway is characterized by higher conductance and lower selectivity. Third, paracellular transport is not rectified, with similar conductance and selectivity in either apical-to-basal or basal-to-apical directions. Nonetheless, far from being a non-discriminating breach in the endothelium, also the paracellular pathway displays well-defined values of electrical conductance, as well as charge and size selectivity (3).

The transcellular route: carriers, channels and vesicles

Most of the communication that takes place between vascular and perivascular compartments relies on the transcellular pathway. For a detailed analysis of transcellular systems in the endothelium, the reader is referred to excellent reviews (4–6). Here, we will just highlight some important features of these systems. First, both apical (or luminal) and basolateral (or abluminal...
nal) membranes of the endothelial cells express several carriers for amino acids and glucose. The diverse systems for the transport of nutrients differ with respect to physical properties (e.g. selectivity and kinetics), location within the vascular tree (e.g. brain, retinal and peripheral vessels), and responsiveness to stimuli (e.g. vasoactive agonists, substrate deprivation and hyperglycemia). Interestingly, the effect of nutrients on paracellular conductance represents an important example (albeit derived from epithelial biology) of functional interactions between the two permeability pathways. Specifically, addition of glucose to the apical surface of a segment of the small intestine results in enhanced paracellular permeability and reduced electrical resistance (7). Although the complete pathway has not been entirely elucidated, it is likely that the effect of glucose involves signals originated by the apical Na\textsuperscript{+}-glucose co-transporter SGLT-1, which in turn induce Myosin Light Chain (MLC) kinase-dependent phosphorylation of MLC, contraction of the perijunctional actomyosin ring, partial opening of the TJ, and ultimately increased paracellular permeability (8, 9).

Second, endothelial cells (in spite of being non-excitatory cells) express several ion channels, which control important functions of the endothelial cell itself and of the vessel wall. For instance, ion channels actively regulate the production and release of vasoactive factors (e.g. nitric oxide and prostacyclin), the trafficking and secretion of haemostatic factors (e.g. von Willebrand factor and tissue-type Plasminogen Activator), as well as cell proliferation and angiogenesis. In addition, ion channels sense changes in hemodynamic forces, thus inducing changes in the shape of endothelial cells and in the contraction of vascular smooth muscle cells. At a mechanistic level, the major function of the endothelial ion channels consists in enhancing Ca\textsuperscript{2+} influx either directly (via Ca\textsuperscript{2+} channels, such as agonist-activated non-selective cation channels and store-operated Ca\textsuperscript{2+} channels) or indirectly (by stabilizing the driving forces for the Ca\textsuperscript{2+} influx, via Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels, the volume-regulated anion channels VRAC, and the inwardly rectifying K\textsuperscript{+} channel Kir2.1). Finally, ion channels provide another example of interactions between the two permeability pathways, as agents that increase endothelial [Ca\textsuperscript{2+}], by acting on the transcellular pathway (e.g. thrombin, histamine, cytokines and growth factors) also increase permeability along the paracellular pathway. Again, the response to these agonists involves a calcium- and calmodulin-dependent MLC kinase, which in turn induces phosphorylation of MLC and junction opening.

Third, the transcellular pathway is also mediated by vesicles. One of the most extensively studied examples is the transport of albumin (10). Vesicles form on the luminal surface following either binding of albumin to a specific gp60 receptor (11) or non-specific absorption to the membrane. Then, vesicles detach, cross the cytoplasm, fuse with the abluminal membrane, and finally expel their content in the subendothelial space.

**The paracellular route: TJ and TJ strands**

Together with adherens junctions, desmosomes and gap junctions, TJ form the junctional complex, which mediates adhesion and communication between adjacent and contacting cells. TJ were extensively analyzed by electron microscopy more than forty years ago. In thin sections (and at low magnification), TJ appear like fusions between the outer leaflets of the plasma membrane at points of cell-cell contact. At higher magnification, however, it becomes clear that the membrane leaflets are not fused, but merely in tight contact to each other. In freeze-fracture preparations, TJ look like anastomosis of fibrillar strands within the plasma membrane. In turn, these strands are composed of particles which are preferentially localized to the internal leaflet (P face). In correspondence to the strands, mostly on the external leaflet (E face), there are complementary grooves (12).

It is commonly accepted that cohesive interactions between TJ molecules localized on apposing strands occlude the lateral intercellular space, thus restricting the paracellular diffusion of solutes (“barrier” function). In addition, albeit not related to cell permeability, it should be mentioned that TJ strands also restrict the movement of membrane molecules between the apical and basolateral domains of the plasma membrane (“fence” function). Thus, the strands are the morphological and functional unit of the TJ. The junctional strands are primarily composed of proteins, and research in the past twenty years has identified a number of building blocks of the strands, thus setting the stage for the mechanistic explanation (at a molecular level) of the TJ barrier and fence functions (13).

**Molecular basis of paracellular permeability**

**Molecular architecture of the TJ**

The molecular architecture of the TJ has been recently reviewed in detail (14–18). TJ are composed of integral membrane proteins (e.g. occludin, claudins and Junctional Adhesion Molecule-A; JAM-A) and intracellular proteins (e.g. ZO-1 and cingulin, to name but a few). Here, we will briefly describe the transmembrane proteins and just mention that both transmembrane and intracellular proteins (and the complex network of molecular interactions among them) contribute not only to the establishment and maintenance of TJ structure, but also to the control of paracellular permeability, cell polarity, signal transduction and gene expression. So far, most research on the role of TJ in paracellular permeability has focused on the transmem-
brane proteins (and, in particular, on claudins), as it is conceivable that their extracellular domains (which lie alongside the intercellular space) potentially interact with the solutes which are transported along the paracellular route.

Occludin contains two extracellular loops, four transmembrane domains and two cytoplasmic termini (19). The first extracellular loop (which is highly conserved among species and enriched in glycine and tyrosine residues) contributes to intercellular adhesion, as demonstrated by the use of peptides corresponding to the loop (20). In contrast, a peptide encompassing the second loop affects transepithelial electrical resistance (21). Similarly, deletion of the carboxy-terminal cytoplasmic terminus enhances fluxes of non-electrolyte solutes (22), thus establishing a specific role for the second loop and the cytoplasmic tail of occludin in the regulation of paracellular permeability. To further support the role of occludin in permeability, it is noteworthy that its expression levels in the endothelial cells correlate with the permeability of different segments in the vascular tree, with higher expression (and a more continuous staining pattern) in the brain than in non-neural tissues (23).

Claudins belong to a family of more than twenty proteins. Like occludin, claudins have four membrane-spanning regions, two extracellular loops and two cytoplasmic termini. Compared to occludin (which has a molecular mass of 65 kDa), however, claudins are smaller (with a molecular mass of 20 to 25 kDa). Also, claudins display no sequence similarity with occludin. One of the first indications that claudins are the building blocks of the TJ strands came from the observation that, upon expression in L fibroblasts (which do not form TJ), claudin-1 and claudin-2 localize to sites of cell-cell contact and reconstitute structures which resemble the TJ strands described in epithelial and endothelial cells (24).

JAM-A (which was previously called JAM, JAM-1 and F11R) is a 32 kDa glycoprotein which is composed of an extracellular region, a transmembrane segment and a short cytoplasmic tail. Predictably, the extracellular region comprises two Ig-like domains, an amino-terminal (V1-type) and a carboxy-terminal (C2-type), respectively. The crystal structure of the extracellular domain of murine (25) and human (26) JAM-A has confirmed the prediction that the domain is composed of two Ig-like folds, which are connected by a short linker (with a bent conformation of 125°). These structural features, together with the observations that the extracellular domain forms dimers in solution and that the dimers bind homophilically (27), have led to the suggestion that, on the cell surface, parallel dimers of JAM-A expose an adhesive surface for homophilic interactions with other JAM-A dimers which are expressed on the surface of opposing cells. It remains to be determined whether these presumptive interactions contribute to the JAM-A-dependent regulation of paracellular permeability, which has been observed using JAM-A transfected cells (28) and blocking reagents (29, 30).

It should be mentioned that JAM-A belongs to an emerging family of Ig-like cell adhesion molecules, which comprises JAM-B, JAM-C, JAM-4 and JAM-L [reviewed in (17, 31)]. To a different extent, these molecules localize to the intercellular junctions of endothelial and epithelial cells (and also on the surface of circulating leukocytes and platelets) and display different patterns of homophilic and heterophilic adhesion. While convincing evidence supports a role for the members of the JAM family in the migration of leukocytes across endothelial and epithelial junctions, little evidence indicates at this time that these molecules also control paracellular permeability. Interestingly, in spite of localizing closely to the TJ of epithelial cells, exogenously expressed JAM-C increases permeability, which is probably related to JAM-C inability to bind homophilically (32).

Claudins as “walls” and “poles in the wall” As increasing evidence indicates that claudins are the building blocks of the TJ strands, one would expect that claudin expression would invariably reduce paracellular permeability. However, transfection experiments not always result in the expected decrease in paracellular conductance. For instance, expression of claudin-2 in the high-resistance kidney epithelial cells MDCK-I and MDCK-C7 does not reduce, but rather enhances paracellular permeability (33, 34). These observations led to the “poles in the wall” hypothesis. According to this hypothesis, although TJ strands represent an impermeable barrier (the “wall”), a series of permeable and aqueous “poles” perforate these walls (35). As discussed below, the differential ability of diverse claudins to primarily act as either “walls” or “pores” accounts for the different outcomes of transfecting different claudins.

Claudin expression regulates ion conductance and selectivity In contrast to the enhanced conductance that follows claudin-2 expression, transfection of other claudins (in the low-resistance MDCK-II cells) reduces conductance and consequently enhances electrical resistance. The expected reduction in conductance is obtained upon expression of claudin-1 (36), claudin-4 (37), claudin-8 (38), and claudin-15 (39). A major limitation of these transfection studies is the background of endogenous claudins in the recipient cell line. Thus, these studies do not define the absolute conductance of the exogenously transfected claudin, but rather the relative change in conductance with respect to the background.

In addition, the presence of endogenous claudins necessitates the development of theoretical models to explain the observed changes in conductance. As described by Yu (1), there are at least two possible ways, whereby exogenous claudins in theory can induce changes in conductance. First, in the series model, novel junctional strands (which are formed by exogenous claudins) behave as additional barriers in series with the preexisting strands (which are formed by endogenous claudins). According to this model, it can be predicted that claudin transfection invariably increases the number of junctional strands and decreases paracellular conductance, which is in keeping with the observation that electrical resistance (at least in epithelia) increases (in a logarithmic manner) with the number of strands (40). Actually, claudin-1 transfection increases both strand number and electrical resistance (36).

Second, in the parallel replacement model, exogenous claudins do not form novel junctional strands, but rather replace endogenous claudins in the preexisting strands. According to this model, it can be predicted that claudin transfection leaves the number of strands unchanged, as it has been reported in most
studies. However, the net effect on paracellular conductance is not predictable, as it depends upon the balance between the intrinsic conductance of endogenous and exogenous claudins (which are displaced from and inserted in the preexisting junctional strands, respectively). The reduced levels of endogenous claudin-2 (together with the increased number of junctional strands) which follow transfection of the exogenous claudin-8 provide a likely example of parallel replacement (38). In this framework, the ability of transfected claudin-8 to enhance electrical resistance and to reduce sodium conductance (in the low resistance MDCK-II cells) is likely due to the ability of exogenous claudin-8 to replace the endogenous and “leaky” claudin-2 from the existing strands, and not to the formation of novel strands made up of the “tight” claudin-8 (as it would be predicted by the series model).

A slightly different model predicts that the “pores” form, when apposing claudins (expressed on two contacting cells) introduce minor discontinuities in the context of two contacting strands. Actually, claudins may bind to each other not only within individual strands on one cell (side-by-side or in cis binding) but also within contacting strands on two adjoining cells (head-to-head, or in trans binding). However, although some claudins (e.g. claudin-1 and –2) can be assembled in cis into the same strand, the same claudins on two apposing strands cannot bind to each other in trans (41). Thus, such points of defective contact might correspond to the putative discontinuities between contacting strands, where leakage of solutes occurs (35).

Besides regulating conductance, some claudins can also regulate charge selectivity, by acting as an electrostatic barrier to either cations or anions. For instance, a mutation which replaces a positive to a negative charge in the first extracellular loop of claudin-4 increases the permeability to sodium. In contrast, mutations which replace three negative to positive charges in the first extracellular loop of claudin-15 increase the permeability to chlorine ions (39). Thus, claudin-4 and claudin-15 likely act as selective barriers for cations and anions, respectively. Finally, claudins can also regulate size selectivity, as best exemplified by in vivo studies of claudin-5, which is an important adhesive component of vascular TJ and is particularly well organized in brain vessels. Surprisingly, deletion of the claudin-5 gene in mice is not accompanied by altered morphology of brain vessels during development. Interestingly, however, there is selective impairment of the blood brain barrier function against molecules smaller than 800 Da (42).

Regulation of paracellular permeability

The regulation of paracellular permeability involves complex interaction between several agonist-activated signaling pathways and key structural components of the cell, which include (but are not limited to) the TJ. Schematically, the structural components mediate either adhesive forces or centripetal tension (which favor and hinder the endothelial barrier, respectively) or both. In general, adhesive forces are mostly exerted by the cell-cell junctions. In contrast, centripetal tension is mostly exerted by the peri-junctional actomyosin ring. Additionally, both adhesive and tensile forces are exerted by the cell-matrix (and integrin-based) focal adhesions. Specifically, integrin blockade increases basal permeability but attenuates agonist-induced permeability, thus implying that focal adhesions contribute to both maintenance of the basal barrier (likely via adhesive forces) and stimulated induction of barrier loosening (likely via tensile forces). Most of the permeability-related signaling pathways identified to date impinge on important effectors of each of the aforementioned structural components. For instance, phosphorylation of β-catenin, MLC and focal adhesion kinase are final events in the regulation of adherens junctions, contractile cytoskeleton and focal adhesions, respectively (43).

Similar to these structures, it has long been known that diverse agonists modulate TJ and the TJ-dependent control of paracellular permeability. However, a complete picture of the pathways which are involved in the process has not emerged yet. Nonetheless, several studies have demonstrated that TJ are involved in both outside-in signaling (i.e. from the TJ to the cell interior) and inside-out signaling (i.e. from the cell interior to the TJ). While the former signaling is related to the control of cell proliferation and differentiation, the latter is key to the regulation of junction formation and paracellular permeability. For a detailed description of the signaling pathways centered on the TJ, the reader is referred to other reviews (15, 44). Here, we will provide a brief overview of the most important pathways that regulate TJ components, TJ assembly and the establishment of paracellular permeability (which is coincident with the assembly of the TJ). As a caveat, one should take into account that several studies of TJ regulation refer to epithelial cells and to a specific subset of endothelial cells (i.e. the cerebral microvessels, in which TJ contribute to the formation and maintenance of the blood-brain barrier). The effects of the most important TJ regulators are schematically represented in Figure 2.

One of the most extensively studied regulators of TJ and TJ-mediated permeability is protein kinase C (PKC). As a general rule, PKC activation favors assembly of growing TJ, but impairs the barrier function of established TJ. For instance, during junction assembly in MDCK cells, phorbol esters (which stimulate PKC) induce recruitment of ZO-1 (45) and occludin (46) to nascent TJ. Accordingly, the PKC inhibitors calphostin C (47) and GF109203X (46) inhibit TJ formation. In contrast, in the presence of established junctions, addition of phorbol esters to brain endothelial cells (48) and expression of PKC-delta in the...
epithelial cell line LLC-PK1 (49) disrupt TJ and enhance paracellular permeability. Accordingly, the PKC inhibitor H-7 prevents the disassembly of established TJ which occurs in response to the depletion of extracellular calcium (50). Nonetheless, there are exceptions to the rule. For instance, in confluent monolayers of the epithelial T84 cells, PKC down-regulation (upon prolonged treatment with phorbol esters) enhances permeability (51). Also, in confluent Caco-2 cells, PKC activation reduces permeability by inhibiting MLC Kinase and consequently by decreasing the amount of phosphorylated MLC, which in turn might result in acto-myosin relaxation (52).

In addition to the conventional PKC, TJ assembly also involves an atypical PKC in complex with other intracellular proteins, such as the PDZ proteins Par-3 and Par-6, as well as the Rho-family GTPase Cdc-42. The molecular complex is conserved throughout evolution and is essential for establishing cell polarity. Expression of a dominant-negative atypical PKC in MDCK cells affects the junctional localization of Par-3 and enhances paracellular permeability (53). The well-known role of TJ in establishing apical-basal polarity (54) is in keeping with the involvement of a polarity-related complex in TJ assembly.

Similarly to PKC, also tyrosine kinases exert different effects on TJ assembly and TJ maintenance. In calcium-depleted MDCK cells, the tyrosine kinase inhibitors genistein and PP-2 prevent the reassembly of TJ which follows calcium repletion (55). In addition, CGP77675 (an inhibitor of the tyrosine kinase c-Yes) prevents TJ reassembly after removal of an oxidative stress (56). In contrast, in the presence of established junctions, the tyrosine phosphatase inhibitors pervanadate and phenylarsine oxide decrease the barrier function of the TJ (57). Finally, it is worth mentioning that another class of kinases, the MAPK (e.g. ERK1/2 and p38), is likely involved in disrupting the TJ-based barrier. For instance, the ERK1 inhibitor PD98059 prevents the increase in permeability and the TJ disassembly that occurs following exposure of endothelial cells to an oxidative stress (58).

As the membrane receptors for many permeability-inducing agents are coupled to heterotrimeric G-proteins and signal through cyclic nucleotides, several studies have addressed the role of these mediators in TJ function. Studies in MDCK cells have highlighted that the G protein subunits Goα (59), Go12 (60) and Goq (61) colocalize with ZO-1. In addition, expression of constitutively active mutants invariably accelerates establishment of transepithelial electrical resistance upon repletion of extracellular calcium. As to the cyclic nucleotides, evidence from either brain endothelial cells or in vitro models of the blood-brain barrier demonstrates that cAMP elevation enhances the barrier function (62, 63) and that the effect is associated with increased organization and complexity of the TJ strands (48), thus establishing a positive role for cAMP (and possibly for cAMP-dependent protein kinase A). In contrast to cAMP, elevation of cGMP in brain endothelial cells exerts opposite effects, resulting in increased paracellular permeability. Actually, the nitric oxide donor sodium nitroprusside enhances permeability (64), while the nitric oxide inhibitor L-NMMA prevents the permeability-inducing action of vascular endothelial cell growth factor (65). The production of cGMP is likely attributable to nitric oxide and is mediated by protein kinase G, as suggested by the observation that nitric oxide synthases, soluble guanylate kinase and cGMP-dependent protein kinase G type IB are all expressed in brain endothelial cells (66).

In addition to the heterotrimeric G proteins, there is ample evidence that GTPases of the Rho family are major regulators of TJ formation, TJ maintenance and paracellular permeability. However, as results have been obtained mostly using exogenous inhibitors and overexpression systems, it remains unclear whether the actual effect is either positive or negative. On one side, some data support a positive role, as Rho inhibition (for instance, upon C3 transferase microinjection) affects TJ assembly (67). On the other side, both constitutively active and dominant negative forms of RhoA alike affect TJ morphology and paracellular permeability (68). One possible explanation for these discrepancies is that the GTPase activity of these mediators has to be finely balanced and that TJ and paracellular permeability are sensitive to either excessive or insufficient levels of GTPase activity. To ensure this delicate balance, a series of regulators and effectors complement the GTPase activity of the small G proteins. As to the upstream regulators, it has been shown that overexpression of GEF-H1, a guanine nucleotide exchange factor for Rho, which normally localizes to the TJ, enhances paracellular permeability (69). In addition, as to the downstream effectors, the Rho-associated kinase ROCK is likely one of the major mediators of RhoA-dependent changes in paracellular permeability via effects on myosin-mediated contraction (70, 71). As already mentioned, modulation of myosin activity (at the level of the peri-junctional acto-myosin ring) is a common mechanism for controlling paracellular permeability (8, 9).

It is commonly accepted that the centripetal contraction of the actomyosin ring may exert pulling forces on the transmembrane proteins of the TJ. If this is indeed the case, it will be important to determine which intracellular molecules actually transmit these forces from the contractile cytoskeleton to the adhesive proteins at the cell surface. In this respect, it is noteworthy that several (if not all) transmembrane proteins of the TJ bind actin-associated proteins. For instance, occludin (72), claudins (73) and JAM-A (74, 75) bind ZO-1. In turn, ZO-1 associates with F-actin (76), the actin-associated proteins alpha-catenin (77) and corticalin (78) and the myosin-associated protein cingulin (79). In addition, JAM-A binds AF-6/Afadin (75) and CASK/Lin2 (80), which in turn associate with actin filaments in a direct and indirect manner, respectively. Interestingly, inflammatory cytokines that enhance endothelial and epithelial permeability also reduce the solubility of JAM-A and other TJ molecules in non-ionic detergents (81, 82), which likely reflects reduced association with actin filaments. Finally, JAM-A binds Par-3 (83, 84), thus possibly recruiting the aforementioned polarity complex to the cell membrane.

Concluding remarks

Although several studies have unveiled key issues concerning functional properties, molecular basis and regulatory mechanisms of endothelial permeability, several important questions remain unanswered. In particular, the real nature of the aqueous pores within the junctional strands has so far remained elusive, given the intrinsic difficulty of purifying transmembrane molecules (not to mention molecular assemblies) for structural
studies at high resolution. In addition, it is foreseeable that other molecules will join the (already long) list of the molecular components of the TJ. In this respect, interesting contributions are expected from studies in invertebrate animal models, such as Caenorhabditis elegans (85) and Drosophila melanogaster (86). Finally, one of the major post-genomic challenges consists in understanding how the complex networks of molecular interactions among the numerous TJ components bring about the finely tuned regulation of endothelial permeability.

**Abbreviations**

JAM-A: Junctional Adhesion Molecule-A; ML-C: Myosin Light Chain; PKC: Protein Kinase C; TJ: tight junctions

References

Vascular Cell Signalling

Bazzoni: Regulation of vascular permeability


