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Dynamic Regulation of Barrier Integrity of the Corneal Endothelium

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Abstract

The corneal endothelium maintains stromal deturgescence, which is a prerequisite for corneal transparency. The principal challenge to stromal deturgescence is the swelling pressure associated with the hydrophilic glycosaminoglycans in the stroma. This negative pressure induces fluid leak into the stroma from the anterior chamber, but the rate of leak is restrained by the tight junctions (TJs) of the endothelium. This role of the endothelium represents its barrier function. In healthy cornea, the fluid leak is counterbalanced by an active fluid pump mechanism associated with the endothelium itself. Although this Pump-Leak hypothesis was postulated several decades ago, the mechanisms underlying regulation of the balance between the pump and leak functions remain largely unknown. In the last couple of decades, the ion transport systems that support the fluid pump activity have been discovered. In contrast, despite significant evidence for corneal edema secondary to endothelial barrier dysfunction, the molecular aspects underlying its regulation are relatively unknown. Recent findings in our laboratory, however, indicate that barrier integrity (i.e., structural and functional integrity of the TJs) of the endothelium is sensitive to remodeling of its peri-junctional actomyosin ring (PAMR), which is located at the apical junctional complex. This review provides a focused perspective on dynamic regulation of the barrier integrity of endothelium vis-à-vis plasticity of the PAMR and its association with cell signaling downstream of small GTPases of the Rho family. Based on findings to date, it appears that development of specific pharmacological strategies to treat corneal edema in response to inflammatory stress would be possible in the near future.

Keywords

cornea; endothelium; pump-leak hypothesis; tight junctions; actomyosin contraction

As a monolayer of cells forming a regular hexagonal mosaic at the posterior surface of the cornea, the corneal endothelium forms a critical epithelial interface between the anterior chamber and the corneal stroma. From a cell physiologic perspective, despite its name, the corneal endothelium is quintessentially a leaky epithelium. It is considered to be of neural crest origin, ~ 5 μm thick, and typically exhibits a cell density of 2500 cells/ mm^2 in adult human eyes.¹ In the early 1970s, the physiological activity of the corneal endothelium became well known from a series of experiments by Maurice et al.,^{2, 3} using rabbit cornea *in vitro*. Even to this day, their experimental paradigm is routinely employed in studies on the endothelial physiology. Their experiments demonstrated, without a doubt, that the corneal endothelium

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acts as a metabolic fluid pump. Specifically, it was shown that the endothelium drives fluid from stroma into the anterior chamber and thereby prevents corneal edema, which would have otherwise ensued in response to the inherent fluid leak into the stroma (Fig. 1). This mechanism, referred to as the Pump-Leak hypothesis,^{1, 4–6} is undisputed to date and remains an accepted physiological basis for stromal hydration control. In the absence of the latter, a strict organization of the collagen fibrils in the stroma, critical for transparency of the cornea, would not be possible. Given this vital role for the endothelium, there has been a sustained interest in the molecular basis of endothelial fluid transport.^{6–14} In parallel, details about the molecular and pathophysiological aspects of the barrier function have also been accrued, but to a limited extent.^{1, 15–23} The main purpose of this review is to provide a fresh perspective on the regulation of barrier function of the corneal endothelium, and thereby provide impetus for further progress. In the interest of brevity, however, we highlight sensitivity of the barrier integrity to plasticity of a pool of actin cytoskeleton at the apical junctional complex (AJC) after a brief consideration into salient aspects of endothelial fluid transport.

Fluid Transport by the Corneal Endothelium

The fluid pump activity, akin to that in other fluid transporting epithelia, is associated with a host of ion channels/exchangers/pumps positioned strategically at the apical and basolateral membranes. These mechanisms are driven by ionic gradients set up by the sodium pump (i.e., Na^+/K^+ -ATPase), which is in the basolateral membrane of the endothelium.^{6, 11} The role of Na^+/K^+ -ATPase in the endothelium and the importance of the fluid pump function toward stromal hydration control becomes readily apparent by prompt stromal swelling when the monolayer is challenged with the cardiac glycoside ouabain.^{1, 11, 21} Similar pharmacological maneuvers seem to suggest that the corneal endothelium brings about a net $\text{Na}^+/\text{HCO}_3^-$ movement across its apical membrane.⁶ This elicits fluid secretion into the anterior chamber, presumably through resultant local osmotic gradients. Accordingly, several ion transport mechanisms that are likely to contribute to the vectorial ionic movement have been examined extensively, and such studies have been reviewed.⁶

Although the molecular identity of the transport mechanisms and their putative role toward net ionic secretion are being established with relative flair,⁶ the exact nature of the solute-solvent coupling leading to fluid secretion remains uncertain.^{5, 23, 24} With the discovery of aquaporins and the specific involvement of AQP1 in endothelial osmotic water permeability,^{25, 26} there is impetus to accept the classical standing gradient model²⁷ as a plausible mechanism underlying the solute-solvent coupling (Fig. 2). In contrast, however, Fischbarg et al., have recently invoked the theory of electro-osmotic coupling to explain fluid transport by the endothelium.²³ Interestingly, in the realm of electro-osmotic coupling, water secretion can also assume the paracellular pathway.^{5, 28–31} As we strive to further delineate the nature of the solute-solvent coupling, it appears that mechanisms of cell volume regulation in response to acute osmotic shocks are invaluable.³² This is because cell volume changes, which can be measured in realtime,^{7–10, 33} are also brought about by ion transport mechanisms that are implicated in fluid transport (Fig. 3). Hence, measurement of cell volume regulation provides a novel window to observe the regulation of ion transport mechanisms by extracellular stresses and bioactive factors found in the aqueous humor.

Barrier Integrity: Leakiness and Relationship to Stromal Deturgescence

As suggested earlier, the barrier function represents resistance to facile permeability to solutes, and most importantly, to fluid leak through the paracellular route. This resistance is conferred by the tight junctions (TJs) of the endothelium. In general, the TJs are supramolecular assemblies forming the intercellular junctions and are found close to the apical domains of the epithelial/endothelial cells in a monolayer (Fig. 4A). The trans-membrane molecules of the

TJs (namely, occludins, claudins, and the junctional adhesion molecule, or JAM) interact with their homophilic counterparts in the neighboring cells.³⁴ Such interactions, facilitated by the intercellular tethering forces evoked by the cadherin-dependent adherens junctions (AJs), occlude the paracellular space. Accordingly, AJs are proximal to TJs, and together, for the most part, form the apical junctional complex (AJC). In the cytoplasm, each of the components of the AJC forms a microdomain comprising a large number of linker proteins.³⁴ The components of such microdomains possess significant ties to several major cell signaling molecules as well as a proximal pool of actin cytoskeleton (Fig. 4A).

In the corneal endothelium, the TJs offer only a very weak resistance to paracellular passage of solutes and water, presumably due to fewer TJ strands. The leakiness of the endothelium is reflected in the very small trans-endothelial resistance (TER) of $15\text{--}25\ \Omega\cdot\text{cm}^2$,^{1, 16, 21} and also by a significant permeability to macromolecules of MW > 150 kDa.³⁵ Nonetheless, a breakdown of the barrier integrity by pharmacological or pathological insults is known to readily cause stromal swelling.¹ The biophysical basis for the stromal edema lies in the swelling pressure put forth by the hydrophilic glycosaminoglycans of the stroma (50 mm Hg). The corneal epithelium poses a significant barrier for potential water influx from tears, so the main challenge for stromal deturgescence is at the posterior surface of the cornea in the form of the fluid entry across the endothelium from the anterior chamber. The fluid pump activity in the opposite direction, as discussed earlier, balances this leak (Fig. 1). In summary, the barrier function of the endothelium restrains facile leak into the stroma so that a state of deturgescence of the tissue is maintained.

In addition to a direct block of the paracellular route for water influx from the anterior chamber (i.e., the “paracellular gate” function of TJs), the TJs in the endothelium could be envisaged to influence the fluid pump activity by at least two other mechanisms (Fig. 4B). First, stable TJs are essential for preventing dissipation of electrolyte gradients set up by the ion transport mechanisms; they restrain potential futile solute back-flux through the paracellular space. In other words, if the TJs break down and consequently occlusion of the paracellular space is lost, it is unlikely that sustained local osmotic gradients would be developed to promote trans-endothelial fluid movement. The second mode of coupling to fluid transport lies in the so-called “molecular fence” function of TJs (Fig. 4B). Specifically, it is known that intact TJs are indispensable for the maintenance of apical-basal polarity of the integral membrane proteins (including those of the ion transport mechanisms) by limiting their lateral diffusion.^{36–38} Therefore, a breakdown of the TJs would adversely impact the polarity of the membrane proteins and thereby possibly reduce the rate of fluid pump in the face of increase in paracellular fluid leak. In summary, the barrier integrity of the corneal endothelium not only restrains the rate of fluid leak into stroma secondary to the swelling pressure of the glycosaminoglycans, but may also modulate the molecular determinants of the endothelial ion transport and the solute-solvent coupling. Therefore, barrier integrity of the endothelium is a prerequisite for the maintenance of corneal transparency (Fig. 5).

Pathophysiology of the Corneal Endothelium

Despite a continuous age-related loss of endothelial cells (~0.6% per year), corneal transparency is maintained as long as the cell density is > 700 cells/mm², a minimum density needed to counteract the basal fluid leakage.¹ Thus, a cell density of 2500 cells/mm², which is found in adult human corneas, suggests that a “functional reserve” is associated with the endothelium. Nevertheless, when the corneal endothelium is subjected to inflammation (e.g., uveitis allograft rejection), disease (e.g., Fuchs’ dystrophy), or surgical trauma, there is a loss of stromal hydration control (Fig. 6). Among the pathological insults that result in corneal edema, the potential for barrier dysfunction in response to inflammation poses a clinically significant threat in the context of corneal transplantation and anterior uveitis. Given a lack of

pharmacological approaches to a variety of corneal disorders including decompensated endothelium (e.g., Fuch's dystrophy and bullous keratopathy), corneal transplantation is being carried out in large numbers (~ 40,000 annually in the US), and this may increase further given new approaches such as DSEK.^{39–42} Even after transplantation, survival of the endothelium is a major concern. The central endothelial cell loss of the donor cornea over the first post-operative year exceeds 30%.^{43–47} At the present time, there are no pharmacological strategies to prevent the progressive loss of endothelial function in response to acute or chronic allograft rejection. Therefore, understanding the basic mechanisms of endothelial barrier in healthy conditions and in the presence of pro-inflammatory factors, which are released secondary to immune response during allograft rejection and uveitis, is of prime importance.

Plasticity of the Actin Cytoskeleton and its Influence on the Barrier Integrity

A plethora of mechanisms are implicated in the dynamic regulation of the barrier integrity in response to extracellular stresses, neurohormonal factors, growth factors, bacterial/viral products, cytokines, and nutrients in a variety of epithelial/endothelial monolayers.^{34, 48–55} For example, as a direct effect, altered expression and/or changes in the tyrosine phosphorylation of the components of TJs and AJs are known to break down the barrier integrity.³⁴ On the other hand, an indirect mechanism frequently noted in response to inflammatory stress involves plasticity of the actin cytoskeleton.^{34, 52, 56–59} In this mechanism, agents that induce actomyosin contraction and/or depolymerization of the cortical actin cytoskeleton at the AJC bring about disruption of the barrier integrity.^{34, 57, 58, 60–62} The pool of actin cytoskeleton at the AJC, which forms a dense band around the cells, has been referred to as the peri-junctional actomyosin ring (PAMR).^{56, 58, 62} The PAMR is structurally and functionally linked to the trans-membrane components of AJs and TJs through a variety of linker proteins such as ZO-1 (zonula occludens-1).^{56, 58, 62} Accordingly, many molecules involved in inflammatory stress (e.g., thrombin, histamine, TNF- α) break down the barrier integrity through mechanisms that increase contractility of the PAMR. The breakdown occurs with concomitant disruption of the PAMR, redistribution of the components of the AJs and TJs, and formation of intercellular gaps. In agreement with these findings, agents that promote relaxation of the PAMR (e.g., agents known to elevate cAMP, such as adenosine and adrenomedullin) are known to oppose the loss of barrier integrity by agents such as thrombin and histamine, and also oppose structural rearrangements alluded to above. These observations have led to the notion that a significant increase in the actomyosin contraction of the PAMR is detrimental to the integrity of AJs and TJs owing to onset of a centripetal force that would oppose the intercellular tethering forces at AJC. In the absence of cell-cell tethering, trans-membrane proteins of the AJs and TJs cannot maintain a stable interaction, and consequently results in the breakdown of the barrier integrity. Interestingly, although direct actomyosin contraction of the PAMR is not implicated, cytokines (e.g., TNF- α) and oxidative stress are known to induce profound redistribution of the components of the TJs and AJs, along with loss of barrier integrity and significant disruption of actin cytoskeleton. In essence, PAMR, which has structural interactions with components of AJs and TJs on one hand and coupling to cell signaling on the other, is able to focus diverse extracellular signals into regulation of the barrier integrity.

In light of the above observations, we began characterizing the actomyosin contraction in corneal endothelium in the context of model agents and drugs to establish potential involvement of actin cytoskeleton (specifically, PAMR) in the regulation of the barrier integrity. We are now investigating how the cytokines and proteinases found in the aqueous humor and cornea during uveitis and allograft rejection disrupt the barrier integrity (Fig. 5). In the following, after a general introduction to cell signaling that couples to actomyosin contraction, we highlight our recent experimental findings on the regulation of the barrier integrity of corneal endothelium.

PAMR and the Actomyosin Contraction in the Corneal Endothelium

As in smooth muscle cells, a variety of endothelial and epithelial cells show increased contractility of their actin cytoskeleton in response to the phosphorylation of myosin light chain (MLC), a regulatory protein associated with the motor protein Myosin II (Fig. 6). MLC phosphorylation is determined by two opposing pathways: MLCK (myosin light chain kinase)-driven phosphorylation and MLCP (myosin light chain phosphatase)-driven dephosphorylation.^{63–65} MLCK, activated upon binding to the Ca^{2+} -calmodulin complex, is dedicated to MLC phosphorylation (Fig. 6A). MLCP is a trimeric complex consisting of PP1C δ (catalytic subunit), MYPT1/2 (myosin-binding subunit), and M20 (unknown function) (Fig. 6C). Phosphorylation of MYPT1 by Rho kinase, a downstream effector of RhoA, inhibits the phosphatase activity of PP1C δ . Elevated cAMP inhibits the activation of RhoA by phosphorylation of the small GTPase itself by cAMP-dependent protein kinase (PKA).⁶⁶ This is thought to increase affinity of the RhoA-GDP to RhoA-GDI (Fig. 6D). In contrast with Rho kinase, PKC inhibits the activity of MLCP by phosphorylation of CPI-17 (PKC-activated 17 kDa inhibitor protein of type 1 phosphatase) and consequent inactivation of PP1C δ . Thus, activation of Rho kinase and/or PKC results in contraction of the actin cytoskeleton while elevated cAMP promotes relaxation.⁶⁶ These mechanisms surrounding MLC phosphorylation, which is well characterized in vascular endothelial cells, are found in corneal endothelial cells, as shown in our studies, 15, 16, 18, 19, 22, 66–76 described below.

Effect of Actomyosin Contraction on the Barrier Integrity of the Endothelium

Based on pharmacological agents to modulate MLC phosphorylation, we have examined the influence of actomyosin contraction on the barrier integrity of corneal endothelium. In our first study,¹⁸ (Fig. 7) we demonstrated that the thrombin activates RhoA–Rho kinase axis through $\text{G}\alpha_{12/13}$ -coupled PAR-1 receptors in bovine corneal endothelial cells. The resulting increase in MLC phosphorylation led to a disruption of the PAMR with a concomitant breakdown of the barrier integrity. In two subsequent studies,^{16, 19} (Fig. 8) we showed that thrombin-induced MLC phosphorylation and the breakdown in the barrier integrity could be suppressed through cAMP-mediated inhibition of RhoA signaling. We have also investigated the Ca^{2+} - and PKC-dependent mechanisms involved in MLC phosphorylation upon activation of H1 receptors,¹⁵ which are not known to activate RhoA significantly. Our data showed that histamine-induced MLC phosphorylation and the resulting loss of endothelial and epithelial barrier integrity are suppressed by ML-7 and a PKC inhibitor, indicating a role of MLCK and PKC as well as actin cytoskeleton.^{15, 70} Taken together, our published findings to date emphasize a strong role for actomyosin contraction along the locus of the AJC in the regulation of the endothelial barrier integrity. These data have thus provided insights into how many bioactive factors found in the aqueous humor may influence the barrier function of the corneal endothelium and thereby alter the stromal hydration control.

A High Throughput Assay for Barrier Integrity

In our early studies, we assessed the barrier integrity in terms of flux of FITC Dextran (10 kDa) and/or horse radish peroxidase (40 kDa), applied at the apical side of the corneal endothelial cells (Fig. 9A). In order to increase the throughput of our experiments and enable rapid testing of other bioactive factors in the aqueous humor, we began to examine trans-endothelial electrical resistance (TER) by measurement of electrical impedance (Fig. 9B) as a measure of barrier integrity of confluent monolayers. We used electrical cell-substrate impedance sensing (ECIS, Applied Biophysics, NY), a commercial device designed to assess TER in up to 16-independent monolayers at a time. For such measurements, cells are grown to confluence on small, gelatin-coated gold electrodes. A small AC current is applied across the cells. The impedance for the current flow is measured by a lock-in amplifier at ~ 0.1 Hz. The resistive

component of the impedance for current flow is taken as TER since the plasma membrane resistance is relatively large.⁷⁷ Fig. 9C shows evolution of the resistive component of the measured impedance with cell spreading on the gold electrodes. The steady state resistance is indicative of the barrier integrity. Fig. 9D shows the dynamics of TER measurements in response to cytochalasin D, which is known to break down barrier integrity in epithelia.⁶⁰ As an inhibitor of actin polymerization, cytochalasin D breaks apart the PAMR, and consequently the barrier integrity is lost. These results clearly show that TER, as measured using the ECIS system, reflects the status of the barrier integrity. Similar findings have been reported contemporaneously by Yin and Watsky.⁷⁸ Moreover, the ECIS protocol offers a sensitive and high-throughput approach to assess the barrier integrity of the leaky corneal endothelium.

Reformation of Adherens and Tight Junctions in the Endothelium

As opposed to the indirect influence of enhanced actomyosin contraction of the PAMR, cell loss during Fuch's dystrophy, iatrogenic injury (e.g., phacoemulsification), and keratoplasty present a direct threat to the barrier property of the corneal endothelium. Given that the corneal endothelium is non-mitotic in human eyes, reassembly of cell-cell junctions following cell spreading becomes crucial for preserving the barrier integrity. In order to investigate the influence of actin cytoskeleton on the dynamics of disassembly and reassembly of AJs and TJs, we employed the so called Ca^{2+} switch maneuver. In this protocol, Ca^{2+} depletion breaks down the AJs and subsequent Ca^{2+} add-back allows them to re-assemble. Disassembly of the AJs, and as a consequence TJs, was induced by replacing Ringers (2 mM Ca^{2+}) with Ca^{2+} -free Ringers (with 2 mM EGTA). On the same cells, reassembly of the AJs, and thus resealing of TJs, was induced by exposing to Ca^{2+} -rich Ringers (2 mM). As shown in Fig. 10A, Ca^{2+} depletion leads to precipitous loss in TER. Repletion of Ca^{2+} led to a recovery of TER (Fig. 10B).

In order to probe the role of actomyosin contraction, we employed Y-27632 and blebbistatin, the selective inhibitors of Rho kinase and Myosin II ATPase, respectively. Pre-exposure to Y-27632 and blebbistatin led to significant inhibition in the rate of decline in TER (Figs. 10C–D). In addition, the same agents also reduced the rate of recovery in TER in response to Ca^{2+} add-back (Figs. 10E–F). Since cadherin-dependent adherens junctions require external Ca^{2+} , the reduction in the rate of decline in TER upon Ca^{2+} removal in the presence of blebbistatin and Y-27632 indicates that disassembly of AJs is dependent on actomyosin contraction. Significant inhibition in the rate of increase in TER following Ca^{2+} add-back by blebbistatin and Y-27632 suggests that a contractile tone is necessary for assembly of tight junctions. These findings, summarized in Fig. 11, indicate the need to determine whether AJ assembly, which precedes TJ assembly, is also influenced by actomyosin contractility and inflammatory stress.

Effect of TNF- α on Barrier Integrity of the Corneal Endothelium

As noted above, after corneal transplantation, survival of the endothelium is a major concern because of cytokines produced secondary to allograft rejection. Among the cytokines found at significant levels in the cornea and aqueous humor, TNF- α is thought to play a major role in corneal allograft rejection.^{39, 44, 45, 79, 80} TNF- α is known to increase permeability of epithelial and endothelial monolayers by mechanisms that are independent of apoptosis. Its influence on the corneal endothelium has also been demonstrated in an *in vitro* model of rabbit eyes.⁸¹ This study by Watsky et al., showed that exposure of the corneal endothelium to the cytokine led to increase in the permeability to hydrophilic dye carboxyfluorescein with a concomitant disruption of the actin cytoskeleton. A recent studies on (TNF- α)-induced loss of barrier integrity in vascular endothelium have implicated microtubule disassembly.^{82, 83} One mechanism shown in vascular endothelium involves activation of RhoA through a release of

certain RhoA-specific GEFs (guanine nucleotide exchange factors), which are anchored to the microtubules and released in response to its disassembly.⁸³ In a recent study from our laboratory,²² we showed that microtubule disassembly by exposure to nocodazole results in disruption of the actin cytoskeleton, leading to a loss of barrier integrity in corneal endothelium (Fig. 12). Based on nocodazole findings and given the importance of TNF- α in allograft rejection, we have begun to investigate the effects of the cytokine on barrier integrity.^{17, 74} Exposure to TNF- α led to disruption of microtubules and dispersion of ZO-1 (Figs. 13A–B). These events occurred in parallel with a sustained reduction in TER, indicating a loss of barrier integrity. All effects of TNF- α were opposed by pretreatment with paclitaxel (microtubule stabilizing agent) (Fig. 13)¹⁷ and SB-203580, a p38 MAPK (mitogen-activated protein kinase) inhibitor (Fig. 14).⁷⁴ The latter inhibited the effect of TNF- α completely.⁷⁴ Based on these findings, we have suggested that the loss of barrier integrity in response to TNF- α involves activation of p38 MAP kinase and cytoskeletal reorganization, and that these can be inhibited to rescue barrier dysfunction in corneal endothelium.

Summary and Future Perspective

The corneal endothelium is a leaky epithelium, but its barrier integrity is critical to corneal transparency. As in many other epithelia, corneal endothelial cells possess a dense band of actin cytoskeleton at the AJC. This peri-junctional actomyosin ring (PAMR) is structurally and functionally associated with AJs, TJs, and gap junctions. These intercellular junctions are strongly influenced by the status of MLC phosphorylation. Increased MLC phosphorylation, which induces increased contractility of the PAMR, breaks down the barrier integrity, presumably by opposing cell-cell adhesion at the AJs. Elevated cAMP, which opposes MLC phosphorylation by inhibiting activation of RhoA, opposes loss of barrier integrity induced by enhanced actomyosin contraction. Measurement of TER by ECIS is a sensitive approach for high throughput assessment of the barrier integrity of the corneal endothelial monolayers. Similar to the data in this review, there are reports to suggest that connexin-dependent gap junctions and connexin hemichannels^{84–86} are significantly impacted by actomyosin contraction in corneal endothelium.^{67–69, 72} Given this understanding on the plasticity of the actin cytoskeleton and its kaleidoscope of its effects on intercellular junctions including TJs, AJs, and connexin-dependent gap junctions/hemichannels, the challenge ahead of us is to sort out the bioactive factors whose influence are likely to converge on the variety of signaling pathways that impact the PAMR and consequently modulate the barrier integrity (Fig. 15). We have already embarked on such an investigation of the impact of TNF- α on the corneal endothelium, and it appears that tweaking the tone of the PAMR to modulate the leak could be one approach to overcome corneal edema in response to inflammation.

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Figure 1.

The Pump-Leak hypothesis in the regulation of stromal deturgescence. Fluid leak into the stroma through the paracellular pathway is driven by a hydraulic gradient equivalent to swelling pressure (SP) $\sim 50 \text{ mm Hg}$ at normal stromal thickness. Despite the leaky nature of the endothelium ($\text{TER} < 30 \Omega \cdot \text{cm}^2$), stromal thickness is held constant by the fluid pump mechanism, which counterbalances the fluid leak.

Figure 2.

Model of fluid secretion by the corneal endothelium. Trans-endothelial ionic flux is osmotically coupled to trans-endothelial water flux. Swelling pressure of the glycosaminoglycans draws water into the stroma through the paracellular route. The trans-endothelial water flux is facilitated by water channels expressed in both basolateral and apical membranes. Notation: Jv – Fluid flux.

Figure 3.

Ion transport mechanisms during cell volume regulation. RVD (regulatory volume decrease) represents the dynamic of cell volume response following acute hypo-osmotic shock. On the other hand, RVI (regulatory volume increase) is the dynamic cell volume response following acute hyper-osmotic shock. Mechanisms underlying RVD include efflux of K^+ and Cl^- through their respective ion channels. Water loss during RVD can be envisaged to be analogous to fluid transport (i.e., trans-endothelial route) across the apical membrane involving osmotic coupling. Mechanisms underlying RVI include $Na^+-K^+-2Cl^-$ cotransport and coupled Na^+/H^+ and Cl^-/HCO_3^- exchangers. Water gain during RVI can be thought of as analogous to fluid transport across the basolateral membrane.

Figure 4.

Schematic of the apical junction complex. Tight junctions (TJs), which occlude the paracellular route, consist of several trans-membrane and cytoplasmic components. The trans-membrane components include occludin, claudin, and JAM. These maintain homophilic interactions with their counterparts in the neighboring cells and thereby occlude the paracellular space. They are also linked to the actin cytoskeleton via adapter proteins such as ZO-1. Adherens junctions (AJs) confer cell-cell adhesion through cadherins. These are coupled to cytoplasmic components such as catenins, which are indirectly linked to the cytoskeleton in a dynamic manner. The actin cytoskeleton at the AJs and TJs contribute to the formation of the PAMR.

Figure 5.

Pathophysiology of the barrier Integrity in the regulation of stromal deturgescence. Corneal edema could be induced in response to many factors including aging, iatrogenic injury, allograft rejection, and genetic disorders such as Fuch's dystrophy. When the barrier integrity breaks down, the pump mechanism cannot cope with the leak, and hence, stromal edema would be induced. Furthermore, when the tight junctions are abrogated, the fluid pump mechanism cannot continue since the local osmotic gradients generated by ion transport are dissipated by futile solute back-flux. Cytokines such as TNF- α that are implicated in allograft rejection may also directly reduce fluid transport activity by modulating the ion transport mechanisms.

Figure 6.

Overview of signaling pathways underlying actomyosin contraction. **(A)** MLC phosphorylation is catalyzed by Ca^{2+} -Calmodulin-dependent MLCK. The expression of both endothelial and smooth muscle isoform is known in corneal endothelium. **(B)** MLC phosphorylation promotes actomyosin interaction, leading to increased cellular contractility. **(C)** MLCK activity is opposed by MLCP, which catalyzes dephosphorylation of pMLC. MLCP is a heterotrimeric complex consisting of MYPT1 (a regulatory subunit), PP1C δ (the catalytic subunit), and M20 (function unknown). **(D)** Rho kinase, effector of RhoA, phosphorylates MYPT1. This inhibits PP1C δ . RhoA is phosphorylated by PKA at Ser-188. This prevents dissociation of RhoA-GDI from RhoA-GDP. Notes: [1]. GEFs promote the release of GDP and subsequent binding of RhoA to GTP; GAPs stimulate the GTPase activity of RhoA; GDIs stabilize the inactive state of RhoA. Several isoforms of PKC phosphorylate CPI-17 lead to inhibition of PP1C δ .

Figure 7.

Response to thrombin through PAR-1 receptors. **(A)** MLC phosphorylation in response to thrombin is inhibited by Y-27632 (Rho kinase inhibitor; Y) and Chelerythrin (PKC inhibitor; Ch). C: Control (Untreated cells). T: Thrombin; **(B)** Bar graph of data similar to experiments shown in Panel A. **(C)** Schematic showing PAR-1 mediated RhoA activation and MLC phosphorylation. Summarized from our previous publications.^{15, 16, 18, 19}

Figure 8.

Effect of elevated cAMP on MLC phosphorylation: (A) MLC phosphorylation in response to thrombin is inhibited by adenosine (A2B receptor agonists; Ado), NECA (A2B receptor agonists; NECA), Forskolin (activator of adenylate cyclase; Fsk), C: Control (Untreated cells). (B) Bar graph of data similar to experiments shown in Panel A. (C) PAR-1 mediated RhoA activation is inhibited by elevated cAMP. T: Thrombin; One possible mechanism is phosphorylation of Ser188 of RhoA by PKA. Summarized from our previous publications.^{15, 16, 18, 19, 66}

Figure 9.

Assessment of barrier integrity in corneal endothelial monolayers. **(A)** Loss of barrier integrity shown by increased flux of HRP (horseradish peroxidase) across endothelial monolayer grown on Anopore™ filters. OD on the Y-axis represents optical density proportional to permeability. T: thrombin; C: Control; Y: Y-27632 (Rho kinase inhibitor). **(B)** Measurement of TER by electrical impedance across corneal endothelial monolayers grown on Anopore™ filters. Ado: Adenosine. **(C)** TER measurements using ECIS-1600R (Applied Biophysics, Inc., Troy, NY), including attachment and spreading of cells on the gold electrodes. After inoculation of the electrodes, impedance was measured at 3 frequencies at > 1 Hz. The plot shows the increase in the resistive component of the impedance at 4 KHz. A steady state is reached at about 18 hrs after cells grow to confluence. The resistance thus measured is a measure of the TER, which is taken to specify the barrier integrity quantitatively. **(D)** Loss of barrier integrity in response to cytochalasin D. After the normalized resistance had reached a steady state, the serum-rich medium in the electrode wells was replaced with a serum-free medium. At up-arrow shown at about 1 hr, cells were exposed to different levels of cytochalasin D, which is well known to break down the barrier integrity by depolymerization of actin cytoskeleton. As expected, cytochalasin D led to a decrease in the normalized resistance in a dose dependent manner. Summarized from our previous publications.^{15–19, 22, 73–76}

Figure 10.

Breakdown and reassembly of apical junctions during Ca^{2+} Switch: **(A)** Disassembly of AJs by Ca^{2+} removal: Cells grown on gold electrodes were exposed to a Ca^{2+} -free Ringers (w/ 2 mM EGTA). This led to an immediate precipitous fall in TER consistent with the breakdown of AJs. **(B)** Neoformation of AJs and TJs: Ca^{2+} add-back led to a gradual recovery of TER. The rate and extent of recovery was dependent on the level of extracellular Ca^{2+} . **(C)** Disassembly of AJs is opposed by Y-27632 (Rho kinase inhibitor). Cells pretreated with Y-27632 were exposed Ca^{2+} -free Ringers. The rate of decrease in TER was smaller compared to the rate observed in the absence of Y-27632. **(D)** Disassembly of AJs is opposed by blebbistatin (Myosin II ATPase inhibitor): Cells pretreated w/ blebbistatin for 10 min were

exposed Ca^{2+} -free Ringers. The rate of decrease in TER was smaller compared to the rate observed in the absence of blebbistatin. Data in Panels B–D, taken together, suggest a role for actomyosin contraction in the disassembly of AJs. Summarized from our previous publications.

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Figure 11.

Simplified overview of disassembly and reassembly of the AJC in the corneal endothelium during Ca²⁺ switch maneuver. We have shown that Ca²⁺ depletion results in disengagement of cadherins (i.e., breakdown of AJs) as well as outside-in signaling, resulting in activation of RhoA. Our results based on Y-27632 and blebbistatin further show that the consequent increase in actomyosin contraction of the PAMR accelerates breakdown of the TJs, which is induced by the disassembly of the AJs. Our Ca²⁺ add-back data have highlighted that F-actin is essential for the formation of AJs and that actomyosin contraction is crucial for the reassembly of TJs. Taken from our previous publication.⁷³

Figure 12.

Effect of microtubule disassembly on MLC phosphorylation: (A) MLC phosphorylation in response to nocodazole (NDZ) is inhibited by Y-27632 (Rho kinase inhibitor; Y), C: Control (Untreated cells). (B) Bar graph of data similar to experiments shown in Panel A. (C) Nocodazole-mediated RhoA activation. One possible mechanism may involve release of GEF-H1 bound to microtubules. Taken from our previous publication.²²

Figure 13.

Effect of paclitaxel on the (TNF- α)-induced response in corneal endothelial monolayers. **(A)** Effect of microtubule stabilization on TNF- α -induced microtubule disassembly. Cells were pretreated with 10 μ M paclitaxel (PTX) for 1 hr with or without 20 ng/mL TNF- α for 6 hrs. Cells were then stained for α -tubulin (green) and counterstained for nucleus with DAPI (blue). In untreated cells, microtubules exist as fibrillary extensions from around the nucleus to the cell periphery (indicated by arrows). TNF- α induced microtubule disassembly is characterized by the loss as well as condensation of fibrillary extensions (indicated by arrows). Paclitaxel stabilize microtubule assembly and its pretreatment opposes microtubule disassembly by TNF- α . **(B)** Effect of microtubule stabilization on the (TNF- α)-induced dispersion of ZO-1. The influence of TNF- α on integrity of TJs was assessed by visualizing localization of ZO-1, a marker of TJ assembly, by immunostaining. Cells were pretreated with paclitaxel as in Panel A. ZO-1 at the plane of apical junctional complex is continuous throughout the cell periphery (shown by arrows) in untreated cells. TNF- α induces dispersion of ZO-1 from its normal locus. Upon treatment with paclitaxel, the pattern of ZO-1 localization is similar to those of untreated cells. **(C)** and **(D)**: Effect of paclitaxel on TNF- α -induced decline in TER. Cells were pretreated with 10 μ M paclitaxel for 1 hr with or without 20 ng/ml TNF- α . Paclitaxel attenuates the decrease in TER by TNF- α . Panel D shows bar graph of experiments similar to that shown in

Panel C. The % reduction in TER induced by TNF- α is significantly greater than control beyond 8 hrs. Note: TNF- α + PTX: TNF- α + Paclitaxel. Error bars represent the SEM (n = 6). *Indicates significant difference from the control. C vs. TNF- α : p<0.001. Paclitaxel significantly opposes TNF- α -induced reduction in TER beyond 12 hrs. **Indicates significant difference TNF- α vs. TNF- α + PTX: p<0.001. Summarized from our previous publication.⁷⁵

Figure 14.

p38 MAP kinase-dependent TNF- α response in corneal endothelial monolayers. **(A)** Effect of inhibition of p38 MAP kinase on TNF- α -induced microtubule disassembly. Cells were pretreated with 20 μ M SB-203580 (SB) for 1 hr with or without 20 ng/mL TNF- α for 6 hrs. TNF- α induced microtubule disassembly is opposed by SB-203580 pre-treatment. **(B)** Effect of inhibition of p38 MAP kinase on the (TNF- α)-induced dispersion of ZO-1. Cells were pretreated with SB-203580 as in Panel A. Upon treatment with SB-203580, the pattern of ZO-1 localization is similar to those of untreated cells. **(C)** and **(D)**: Effect of SB-203580 on TNF- α -induced decline in TER. Pretreatment with SB-203580 attenuates the decrease in TER by TNF- α . Panel D shows a bar graph of experiments similar to that shown in Panel C.

**Significantly different than TNF- α , $P < 0.001$. Summarized from our previous publication.

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Figure 15.

Plasticity of the actin cytoskeleton. Extracellular stresses involving cytokines and neurohormonal stimuli mobilize the activity of small GTPases of Rho family and/or stress kinases (e.g., p38 MAP kinase) and, in turn, produce actin remodeling, actomyosin contraction, and/or microtubule disassembly. These changes enable remodeling of the apical junctional complex leading to changes in barrier integrity, cell-cell adhesion, and intercellular communication.