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Microtubule Stabilization Opposes the (TNF- α)-Induced Loss in the Barrier Integrity of Corneal Endothelium

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Abstract

Microtubule disassembly breaks down the barrier integrity in a number of epithelial and endothelial monolayers. This study has investigated effects of TNF- α , which is implicated in corneal allograft rejection, on microtubules and barrier integrity in cultured bovine corneal endothelial cells. Exposure to TNF- α led to disassembly of the microtubules, and also caused disruption of the perijunctional actomyosin ring (PAMR). As a measure of barrier integrity, trans-endothelial electrical resistance (TER) was determined based on electrical cell-substrate impedance sensing in realtime. Exposure to TNF- α caused a slow decline in TER for > 20 h, and a similar exposure to cells grown on porous culture inserts led to a significant increase in permeability to FITC dextran. These changes, indicating a loss of barrier integrity, were also reflected by dislocation of ZO-1 at the cell border and disassembly of cadherins. These effects of TNF- α were inhibited upon stabilization of microtubules by pre-treatment with paclitaxel or epothilone B. Microtubule stabilization may be a useful strategy to overcome (TNF- α)-induced loss of the barrier integrity of corneal endothelium during inflammation associated with transplant rejection and uveitis.

Keywords

Corneal endothelium; TNF-alpha; Barrier Integrity; Microtubules; Paclitaxel

1. Introduction

Dysfunction of the corneal endothelium secondary to iatrogenic injury, inflammation, and genetic disorders (e.g., Fuchs' dystrophy) can lead to a loss of corneal transparency (Joyce 2003; Edelhauser 2006). Currently, there are no pharmacological strategies to treat endothelial dysfunction, and the alternatives include endothelial or full-thickness corneal transplantation. In USA alone, there are nearly 40,000 corneal transplants performed annually (George and Larkin 2004). Even after transplantation, survival of the endothelium is a major concern because of inflammatory response secondary to allograft rejection (Armitage et al. 2003; Edelhauser 2006; Niederkorn 2007). Among the cytokines found in significant levels in the cornea and aqueous humor, TNF- α is also assumed to play a major role in corneal allograft rejection (George and Larkin 2004; Niederkorn 2007). Thus, it is important to investigate the influence of the cytokine on the barrier integrity of corneal endothelium, and to assess strategies to oppose its adverse influence.

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TNF- α is known to increase permeability of epithelial and endothelial monolayers by mechanisms that are independent of apoptosis (Goldblum et al. 1993; Watsky et al. 1996; Bruewer et al. 2003; Ma et al. 2005; Ye et al. 2006; McKenzie and Ridley 2007; Kimura et al. 2008). Accordingly, the involvement of the cytokine in the breakdown of the barrier integrity is well known in many disorders including pulmonary edema (Petrache et al. 2003) and Crohn's disease (Ma et al. 2004). Its influence on corneal endothelium has also been demonstrated in an *in vitro* model of rabbit eyes (Watsky et al. 1996). This study by Watsky et al., showed that exposure of the endothelium to the cytokine results in an increase in permeability to the hydrophilic carboxyfluorescein. In parallel with the loss of the barrier integrity, the actin cytoskeleton was disrupted. Furthermore, the study showed that elevated cAMP opposed the influence of TNF- α . Despite these early observations, molecular mechanisms involved in the breakdown of the actin cytoskeleton as well as the barrier integrity remain unknown.

Several recent studies on the (TNF- α)-induced loss of barrier integrity in vascular endothelium have implicated microtubule disassembly (Petrache et al. 2003; Kakiashvili et al. 2009). The microtubules, as part of the cytoskeleton, are essential for structural integrity of cells. Although their involvement in trafficking of membrane proteins, migration, polarity, mitosis, and maintenance of cell shape (Musch 2004) are relatively well understood, the mechanism(s) by which microtubule disassembly induces actomyosin contraction is not well established. One mechanism that is shown in vascular endothelium involves activation of RhoA (Birukova et al. 2005) through a release of RhoA-specific GEFs (guanine nucleotide exchange factors), which are anchored to the microtubules and released in response to its disassembly (van Horck et al. 2001; Birukova et al. 2006). In a recent study from our laboratory, we have shown that microtubule disassembly by exposure to nocodazole results in disruption of the actin cytoskeleton and thereby induces a loss of barrier integrity in bovine corneal endothelial monolayers (Jalimarada et al. 2009). In current study, we have investigated the role of microtubules in the (TNF- α)-induced loss of barrier integrity in corneal endothelium.

In studies involving pulmonary artery endothelial cells, it has been shown that microtubule stabilization effectively opposes loss of barrier integrity induced by TNF- α (Petrache et al. 2003). Among the agents known to stabilize microtubules, the efficacy of paclitaxel is well established (Amos and Lowe 1999). Epothilones are a new class of antineoplastic agents known to induce tubulin polymerization, and enhance microtubule stability (Reichenbach and Hofle 2008). Here, we have examined the ability of both paclitaxel and epothilone B to oppose the loss of barrier integrity induced by TNF- α . Our results show that (TNF- α)-induced microtubule disassembly contributes to barrier dysfunction of corneal endothelium, and that the stabilization of microtubules effectively opposes the TNF- α response.

2. Materials and Methods

2.1. Materials

TNF- α (biological activity of 2×10^7 U/mg; free of endotoxin), paclitaxel, α -tubulin antibody (anti-mouse), monoclonal pan-cadherin antibody (anti-mouse) and FITC dextran (10 kDa) were purchased from Sigma Aldrich (St. Louis, MO). Epothilone B was obtained from EMD Biosciences (La Jolla, CA). Texas red conjugated phalloidin, goat anti-mouse Alexa-488 and anti-fade agent were purchased from Molecular Probes (Eugene, OR). ZO-1 antibody (anti-mouse) was from Zymed (Long Island, NY). Electrodes (8W10E+) for TER measurements were from Applied Biophysics, Inc. (Troy, NY).

2.2. Cell Culture and Treatments with Drugs

Primary cultures of BCEC from fresh eyes were established as previously described. (Satpathy et al. 2004; Srinivas et al. 2004) The growth medium contained Dulbecco's Modified Eagle's

Medium (DMEM), supplemented with 10% bovine calf serum and an antibiotic-antimycotic mixture (Penicillin 100 U/ml, Streptomycin 100 µg/ml and amphotericin-B 0.25 µg/ml). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. The medium was replaced every 2–3 days. Cells of the first and second passages were harvested and seeded onto glass coverslips and porous filters, then allowed to grow to confluence for 3–4 days before use. Cell culture supplies were from Invitrogen (Long Island, NY). For all the experiments, TNF-α (20 ng/ml), paclitaxel (10 µM), and epothilone B (0.1 µM) were used to treat the cells. Paclitaxel or epothilone B was pretreated for 1 h before the addition of TNF-α.

2.3. Immunocytochemistry

Cells grown on coverslips were washed with PBS after desired drug treatment, fixed with 3.7% paraformaldehyde and permeabilized using 0.2% Triton X-100 for 5 min. This was followed by staining for F-actin by phalloidin conjugated to Texas Red (1:1000) for 45–60 min at room temperature. Cells were stained for microtubule, ZO-1 and cadherins by fixing the cells followed by permeabilization with 0.01% saponin in PBS. The cells were exposed to blocking buffer for 45 min at RT, and then incubated with the antibody for α-tubulin (1:1000)/ZO-1 (1:25)/pan-cadherin (1:1000) in a mixture of 0.01% saponin in PBS-goat serum (1:1) overnight at 4°C. This was followed by washing with 0.01% saponin in PBS and incubation with secondary antibody (goat anti-mouse IgG Alexa Fluor 488 at 1:1000) for 1 h at RT. Stained cells were mounted using anti-fade medium with DAPI and visualized using an epifluorescence microscope equipped with a 60/100x oil immersion objective and 1.2 NA (Nikon, Tokyo, Japan).

2.4. Transendothelial Electrical Resistance

TER was measured by ECIS (electric cell substrate impedance sensing) device (ECIS 1600R, Applied Biophysics, Troy, NY). The electrodes (8W10E+, Applied Biophysics, Inc.) were stabilized with aqueous solution of L-cysteine (10 mM) for 15 min in the incubator at 37°C, followed by washing with regular media two times before seeding of cells. Cells were seeded at a density of 5×10^5 cells/ml on electrodes and grown to confluence. The approach to confluence was monitored through spreading data acquired overnight. After the monolayers reached steady resistance values, cells were washed twice with serum-free media and allowed to equilibrate for 1 h before drug treatment. The resistive portion of impedance (TER) normalized to its initial value at time zero was monitored continuously and taken as a measure of barrier integrity.

2.5. Measurement of Permeability to FITC Dextran

Cells were grown on 0.2 µm pore-size collagen IV (1 mg/ml)-coated tissue culture inserts (Nunc, Fisher Scientific, Pittsburgh, PA) to confluence. The monolayers were then serum-starved for 1 h and either left untreated or exposed in triplicate with desired agents. Cells were pretreated with paclitaxel or epothilone B for 1 h before exposure to TNF-α. Following treatment, FITC-dextran (10 kDa) dissolved in the Ringer solution was placed in the apical compartment at 0.4 µg/mL and the cells were placed back in the incubator for 2 h. Samples were then taken from the basolateral chamber for fluorescence measurements. Fluorescence was measured by excitation at 492 nm and the emission was collected at 520 nm.

2.6. Data Analysis

Statistical comparisons were made by one-way analysis of variance (ANOVA) with Bonferroni's post test analysis using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA; Version 5.0). A value of $p < 0.05$ was considered statistically significant. Data are expressed as mean ± SE. "n" represents number of independent experiments.

3. Results

3.1. Effect of TNF- α on Microtubules

Fig. 1 shows the influence of TNF- α on microtubules with and without paclitaxel or epothilone B pre-treatment. In untreated cells (Fig. 1A), microtubules extend from around the nucleus as fibrillary structures towards the cell periphery. Treatment with TNF- α for 6 h led to loss of the fibrillary extensions, and condensation of microtubules noticeable around the nucleus (Fig. 1B). Treatment with paclitaxel or epothilone B stabilized microtubule assembly (Figs. 1C and 1E, respectively). This loss is not apparent when cells were pre-treated with paclitaxel or epothilone B (Figs. 1D and 1F, respectively). These results show the ability of TNF- α to induce microtubule disassembly, and that the response is masked upon microtubule stabilization.

3.2. Influence of TNF- α on the Barrier Integrity: FITC Dextran Permeability

In order to establish the effect of (TNF- α)-induced microtubule disassembly on barrier integrity, we first examined its influence on permeability to FITC dextran (10 KDa) across cells grown to form a monolayer on porous culture inserts. After growing the cells to confluence, as established previously (Jalimarada et al. 2009), for 7 days, we challenged the apical chamber to TNF- α for 20 h with or without pre-treatment with paclitaxel or epothilone B. We then assessed flux of FITC dextran to the basolateral chamber. TNF- α significantly increased flux of FITC dextran when compared to untreated cells (Fig. 2). Furthermore, cells that were pre-treated with paclitaxel or epothilone B showed significantly attenuated flux in response to TNF- α compared to those treated with the cytokine alone. In addition, it may be noted that the opposition of epothilone B to the TNF- α response is significantly greater than that of paclitaxel.

3.3. Influence of TNF- α on the Barrier Integrity: Measurement of TER

In order to gain further insights into the loss of barrier integrity and its protection by microtubule stabilization, we examined trans-endothelial electrical resistance (TER). As shown by us (Jalimarada et al. 2009) and Watsky et al (Yin and Watsky 2005) for corneal endothelial cells, we employed electrical cell substrate impedance sensing (ECIS) approach, which is especially sensitive and therefore highly suitable for leaky epithelia. This method, which is widely employed with vascular endothelial cells, (Petrache et al. 2003; Birukova et al. 2004) is especially useful for the corneal endothelium. As shown in Fig. 3A, exposure to TNF- α led to a slow but a persistent decline in TER that continued beyond 20 h. Pre-treatment with paclitaxel attenuated the decline in TER induced by TNF- α . However, it did not alone influence TER significantly. Data from experiments similar to that shown in Fig. 3A are summarized in terms of % reduction in TER (Fig. 3B) assessed at 4 h intervals. It is clear that paclitaxel significantly opposes the TNF- α response beyond 12 h.

As an alternative microtubule stabilizing agent, we tested the efficacy of epothilone B. Similar to paclitaxel, pretreatment with epothilone B also opposed the TNF- α response (Fig. 4A). Furthermore, epothilone B alone led to a small increase in TER. Histogram analysis as before is shown in Fig. 4B. It is evident that epothilone B significantly opposes TNF- α response starting from 4 h itself. Thus, the effect of epothilone B on the (TNF- α)-induced decline in TER is pronounced compared to paclitaxel. But, however, it is evident that both microtubule agents elicit similar influence to the TNF- α response, which induces disassembly of microtubules. Therefore, (TNF- α)-induced microtubule disassembly underlies the loss in barrier integrity.

3.4. Influence of TNF- α on the Barrier Integrity: Reorganization of the Apical Junctions

In order to confirm that the disassembly of microtubules contributes to the observed loss in barrier integrity, we also investigated the apical junctional complex by immunofluorescence. Specifically, we examined disposition of peri-junctional actomyosin ring (PAMR), ZO-1, and cadherins in response to TNF- α with and without the microtubule stabilizing agents.

ZO-1, a cytoplasmic marker of tight junctions and localized contiguous with that of the neighboring cells, stains uniformly along the cell borders when the monolayer exhibits significant barrier integrity (Fig 5A). This disposition was disturbed upon treatment with TNF- α for 6 h (Fig. 5B). However, upon pre-treatment with paclitaxel or epothilone B, the distribution of ZO-1 was largely unaltered in response to TNF- α (Figs. 5D and 5F respectively). This finding is consistent with the efficacy of both agents in opposing TNF- α response in terms of reduced TER and increase in FITC dextran flux. In contrast with ZO-1, which is closely associated with the tight junctions, cadherins represent transmembrane domains of the adherens junctions. Hence, to examine the influence of TNF- α on the adherens junctions, we examined localization of cadherins by immunofluorescence using a pan-cadherin antibody. In untreated cells, similar to ZO-1, cadherins distribution is contiguous with that of the neighboring cells, and accordingly densely stained at the intercellular borders (Fig. 6A). Exposure to TNF- α for 6 h led to a considerable dispersion in the distribution of cadherins at the intercellular regions leading to a compromise in cell-cell contacts (Fig. 6B). On treatment with paclitaxel or epothilone B, the cadherins distribution was largely unaltered and similar to that of untreated cells (Fig. 6C and 6E, respectively). Pre-treatment with paclitaxel or epothilone B (Fig. 6D and 6F, respectively) opposed the TNF- α response. These results are also in concordance with the observation that the microtubule disassembly underlies the loss of barrier integrity in response to TNF- α .

A significant component of the apical junctions in the polarized epithelia, such as the corneal endothelium, is the presence of a dense band of actin cytoskeleton proximal to tight and adherens junctions, which has been referred to as the peri-junctional actomyosin ring (PAMR). When challenged by agents that enhance actomyosin contraction, the structural integrity of the PAMR is disturbed. The disruption of the PAMR, which is structurally linked to the proteins of the TJs and AJs, is known to reorganize the apical junction. This instability presumably is responsible for the breakdown of the barrier integrity in addition to loss of cell-cell tethering secondary to centripetal forces generated in the PAMR following actomyosin contraction. Thus, in order to determine if the (TNF- α)-induced redistribution of ZO-1 and cadherins could be attributed to the disruption of the PAMR, we characterized its reorganization following exposure to the cytokine. As shown in Fig. 7B, exposure to TNF- α resulted in significant disruption of the PAMR. Treatment with paclitaxel or epothilone B did not alter the organization of PAMR to a significant extent (Figs. 7C and 7E respectively). However, TNF- α failed to show a similar disruption of the PAMR upon pre-treatment with paclitaxel or epothilone B (Fig. 7D and 7F, respectively). Taken together, these findings suggest that TNF- α response includes significant alteration of the apical junctional complex concomitant with the disassembly of the microtubules.

4. Discussion

The corneal endothelium, as a monolayer at the posterior surface of the cornea, is responsible for keeping the cornea transparent. The maintenance of transparency requires that hydration of the corneal stroma to be held constant. The endothelium is able to actively pump fluid that leaks into the stroma from the aqueous humor which bathes its apical surface. The leakage of fluid into the stroma is dependent on the barrier function of the endothelium, which is the focus of this study. When the barrier integrity fails, cornea becomes edematous since the fluid pump, which drives fluid flow in the direction opposite to that of the leak, is overwhelmed. In several

recent studies, we have investigated the mechanisms by which inflammatory mediators elicit breakdown in barrier integrity and intercellular communication through altered cytoskeleton (Satpathy et al. 2004; Satpathy et al. 2005; Srinivas et al. 2006; D'Hondt et al. 2007; D'Hondt et al. 2007; Ponsaerts et al. 2008). In this study, our major goal was to investigate how TNF- α breaks down the barrier integrity in a commonly used cell culture model of the corneal endothelium. In this study, for the first time, we have demonstrated that (TNF- α)-induced loss of barrier integrity in the corneal endothelium occurs largely through disassembly of microtubules and accordingly, we have also shown that microtubule stabilization effectively opposes the response to the cytokine.

A number of studies have examined the influence of TNF- α on barrier integrity in a variety of epithelial and endothelial monolayers (Goldblum et al. 1993; Nwariaku et al. 2002; Petrache et al. 2003; Ma et al. 2004; McKenzie and Ridley 2007). As a pleiotropic cytokine, it is known to induce barrier dysfunction through a variety of mechanisms including disruption of actin cytoskeleton (Goldblum et al. 1993; Watsky et al. 1996), activation of ROS (reactive oxygen species) (Natarajan et al. 1998; Usatyuk et al. 2003), RhoA GTPase (McKenzie and Ridley 2007), MAPKs (Nwariaku et al. 2002; Petrache et al. 2003), transcriptional activation of MLCK (myosin light chain kinase) (Petrache et al. 2001; Ma et al. 2005; McKenzie and Ridley 2007) and/or Hsp27 (heat shock protein 27) (Kierner et al. 2002), and microtubule disassembly (Petrache et al. 2003). Although Watsky et al documented the disruption of the actin cytoskeleton following exposure to TNF- α in the rabbit corneal endothelium, their study did not consider a role for microtubules (Watsky et al. 1996). In this study, we focused on microtubule disassembly since it has been recently reported that cytokines can induce the disassembly leading to disruption of actin cytoskeleton (Petrache et al. 2003; Xu et al. 2008).

In consistence with our main goal, we began this study by examining whether TNF- α can induce microtubule disassembly. As expected, we consistently observed loss of microtubule staining in the cellular periphery, which indicates its depolymerization. The extent to which depolymerization occurs in response to TNF- α is not evident when cells are pretreated with paclitaxel and epothilone B (Fig. 1D and 1F, respectively). It is possible that excessive polymerization induced by these drugs may have masked the TNF- α response, but we note that the net level of microtubule polymerization is significantly greater in cells pretreated with paclitaxel and epothilone B. Thus, we conclude from Fig. 1 that TNF- α induces microtubule disassembly and that the response to the cytokine is not noticeable in cells pre-treated with microtubule-stabilizing agents. Similar observations have been noted in pulmonary artery endothelial cells (Petrache et al. 2003).

Having established the influence of TNF- α on microtubules, we next examined the barrier integrity by two distinct methods. We first confirmed the TNF- α response on barrier integrity by examining the flux of FITC dextran in cells treated with the cytokine and compared with those pre-treated with paclitaxel and epothilone B. While TNF- α treatment alone increased the permeability significantly, microtubule stabilization masked the response to the cytokine (Fig. 2). This is our first indication to suggest that microtubule disassembly at the cellular periphery is responsible for loss of the barrier integrity. Despite this clear indication, the temporal response to TNF- α with and without microtubule stabilization is not evident. Hence, we took recourse to the ECIS approach, which we had employed to investigate the loss of barrier function upon deliberate microtubule disassembly by nocodazole (Jalimarada et al. 2009). As demonstrated in Figs 3 and 4, it is clear that exposure to TNF- α results in a sustained decline in TER, which is attenuated by both paclitaxel and epothilone B (Figs. 3 and 4, respectively). It may be noted that the opposition to TNF- α response by epothilone B is significantly more profound than paclitaxel. Although we did not perform a dose response study, it is important to note that epothilone B is known to have higher potency in stabilizing the microtubules compared to paclitaxel (Kowalski et al. 1997; Altmann et al. 2000).

In order to gain further insights into the (TNF- α)-induced loss of barrier integrity, we investigated structural instability at the apical junction by immunofluorescence. In addition to ZO-1 and cadherins as markers of tight and adherence junctions, we examined PAMR, which lies proximal to the tight and adherens junctions. Furthermore, PAMR is structurally linked to both tight and adherens junctions and hence its disruption results in their remodeling which may underlie the loss in barrier integrity. In Figs. 5 and 6, we have demonstrated that TNF- α induces dislocation of ZO-1 and cadherin, respectively, in consistence with the loss of barrier integrity induced by the cytokine. In agreement with masking of the effect of TNF- α on barrier integrity by paclitaxel and epothilone B, we also show that the cytokine-induced dispersions of ZO-1 and cadherins are also attenuated by microtubule stabilization (Figs. 5D, 5F, 6D, and 6F, respectively). These results reaffirm that the cytokine-induced loss in barrier integrity is brought about by microtubule disassembly. Of special significance is the finding in Fig. 7 in which we demonstrate that microtubule disassembly by the cytokine also disrupts the PAMR. This is in consistence with the dispersion of ZO-1 and cadherins from their normal locus as shown in Fig. 5 and Fig. 6.

The disruption of the PAMR secondary to microtubule disassembly caused by the cytokine is reminiscent of our recent work in which we demonstrated a similar effect in response to nocodazole (Jalimarada et al. 2009). As reported with pulmonary artery endothelial cells, we had found increased MLC phosphorylation secondary to microtubule disassembly. This was attributed to activation of small GTPase RhoA. While it is tempting to speculate similar downstream mechanisms secondary to microtubule disassembly induced by TNF- α , the pleiotropic nature of the cytokine and lack of response to Rho kinase inhibitors (data not shown) to subdue its response prevented us from further emphasis on RhoA-Rho kinase pathway. Similar to uncertainty in the downstream targets of the (TNF- α)-induced microtubule disassembly, the upstream mechanism(s) are also not well established. It has been reported that increased oxidative stress can increase iNOS activity, leading to decreased tubulin polymerization and consequent disruption of the microtubules (Banan et al. 2003). A study also suggests that ROS (reactive oxygen species) activation is mediated by TNF- α signaling (Rajashekhar et al. 2007). Hence, the TNF- α -induced microtubule disassembly may also involve upregulation of ROS and iNOS. Recent studies suggest that p38 MAP kinase is involved in TNF- α -mediated increase in endothelial permeability (Petrache et al. 2003). It has also been shown in human and bovine pulmonary artery endothelial cells that pharmacological inhibition of p38 MAP kinase attenuates nocodazole-induced microtubule depolymerization, actin remodeling, and barrier dysfunction (Birukova et al. 2005). Studies also suggest that p38 MAP kinase activation can induce phosphorylation of microtubule associated proteins such as tau, thereby reducing its ability to promote microtubule assembly (Goedert et al. 1997). It remains to be seen whether activation of p38 MAP kinase by TNF- α is responsible for microtubule disassembly and thereby the downstream events associated with the loss of corneal endothelial barrier function.

In conclusion, for the first time, we have shown that microtubule disassembly by TNF- α contributes to the barrier dysfunction in corneal endothelium. Hence, inhibition of microtubule disassembly through its stabilization may be effective in reducing corneal edema occurring as a result of acute graft rejection or anterior uveitis. However, further studies are necessary to delineate the signaling pathways activated by TNF- α , leading to microtubule disassembly and investigate potential adverse effects of microtubule stabilizing agents.

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References

- Altmann KH, Wartmann M, et al. Epothilones and related structures--a new class of microtubule inhibitors with potent in vivo antitumor activity. *Biochim Biophys Acta* 2000;1470(3):M79–91. [PubMed: 10799747]
- Amos LA, Lowe J. How Taxol stabilises microtubule structure. *Chem Biol* 1999;6(3):R65–9. [PubMed: 10074470]
- Armitage WJ, Dick AD, et al. Predicting endothelial cell loss and long-term corneal graft survival. *Invest Ophthalmol Vis Sci* 2003;44(8):3326–31. [PubMed: 12882777]
- Banan A, Farhadi A, et al. The delta-isoform of protein kinase C causes inducible nitric-oxide synthase and nitric oxide up-regulation: key mechanism for oxidant-induced carbonylation, nitration, and disassembly of the microtubule cytoskeleton and hyperpermeability of barrier of intestinal epithelia. *J Pharmacol Exp Ther* 2003;305(2):482–94. [PubMed: 12606598]
- Birukova AA, Adyshev D, et al. GEF-H1 is involved in agonist-induced human pulmonary endothelial barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2006;290(3):L540–8. [PubMed: 16257999]
- Birukova AA, Birukov KG, et al. Involvement of microtubules and Rho pathway in TGF-beta1-induced lung vascular barrier dysfunction. *J Cell Physiol* 2005;204(3):934–47. [PubMed: 15828024]
- Birukova AA, Smurova K, et al. Microtubule disassembly induces cytoskeletal remodeling and lung vascular barrier dysfunction: role of Rho-dependent mechanisms. *J Cell Physiol* 2004;201(1):55–70. [PubMed: 15281089]
- Bruewer M, Luegering A, et al. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. *J Immunol* 2003;171(11):6164–72. [PubMed: 14634132]
- D'Hondt C, Ponsaerts R, et al. Thrombin inhibits intercellular calcium wave propagation in corneal endothelial cells by modulation of hemichannels and gap junctions. *Invest Ophthalmol Vis Sci* 2007;48(1):120–33. [PubMed: 17197525]
- D'Hondt C, Srinivas SP, et al. Adenosine opposes thrombin-induced inhibition of intercellular calcium wave in corneal endothelial cells. *Invest Ophthalmol Vis Sci* 2007;48(4):1518–27. [PubMed: 17389480]
- Edelhauser HF. The balance between corneal transparency and edema: the Proctor Lecture. *Invest Ophthalmol Vis Sci* 2006;47(5):1754–67. [PubMed: 16638979]
- George AJ, Larkin DF. Corneal transplantation: the forgotten graft. *Am J Transplant* 2004;4(5):678–85. [PubMed: 15084160]
- Goedert M, Hasegawa M, et al. Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases. *FEBS Lett* 1997;409(1):57–62. [PubMed: 9199504]
- Goldblum SE, Ding X, et al. TNF-alpha induces endothelial cell F-actin depolymerization, new actin synthesis, and barrier dysfunction. *Am J Physiol* 1993;264(4 Pt 1):C894–905. [PubMed: 8476021]
- Jalimarada SS, Shivanna M, et al. Microtubule disassembly breaks down the barrier integrity of corneal endothelium. *Exp Eye Res.* 2009
- Joyce NC. Proliferative capacity of the corneal endothelium. *Prog Retin Eye Res* 2003;22(3):359–89. [PubMed: 12852491]
- Kakiashvili E, Speight P, et al. GEF-H1 mediates tumor necrosis factor-alpha-induced Rho activation and myosin phosphorylation: role in the regulation of tubular paracellular permeability. *J Biol Chem* 2009;284(17):11454–66. [PubMed: 19261619]
- Kiemer AK, Weber NC, et al. Inhibition of p38 MAPK activation via induction of MKP-1: atrial natriuretic peptide reduces TNF-alpha-induced actin polymerization and endothelial permeability. *Circ Res* 2002;90(8):874–81. [PubMed: 11988488]
- Kimura K, Teranishi S, et al. Delayed disruption of barrier function in cultured human corneal epithelial cells induced by tumor necrosis factor-alpha in a manner dependent on NF-kappaB. *Invest Ophthalmol Vis Sci* 2008;49(2):565–71. [PubMed: 18235000]
- Kowalski RJ, Giannakakou P, et al. Activities of the microtubule-stabilizing agents epothilones A and B with purified tubulin and in cells resistant to paclitaxel (Taxol(R)). *J Biol Chem* 1997;272(4):2534–41. [PubMed: 8999970]

- Ma TY, Boivin MA, et al. Mechanism of TNF- α modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. *Am J Physiol Gastrointest Liver Physiol* 2005;288(3):G422–30. [PubMed: 15701621]
- Ma TY, Iwamoto GK, et al. TNF- α -induced increase in intestinal epithelial tight junction permeability requires NF- κ B activation. *Am J Physiol Gastrointest Liver Physiol* 2004;286(3):G367–76. [PubMed: 14766535]
- McKenzie JA, Ridley AJ. Roles of Rho/ROCK and MLCK in TNF- α -induced changes in endothelial morphology and permeability. *J Cell Physiol* 2007;213(1):221–8. [PubMed: 17476691]
- Musch A. Microtubule organization and function in epithelial cells. *Traffic* 2004;5(1):1–9. [PubMed: 14675420]
- Natarajan V, Scribner WM, et al. Reactive oxygen species signaling through regulation of protein tyrosine phosphorylation in endothelial cells. *Environ Health Perspect* 1998;106(Suppl 5):1205–12. [PubMed: 9788899]
- Nieder Korn JY. Immune mechanisms of corneal allograft rejection. *Curr Eye Res* 2007;32(12):1005–16. [PubMed: 18085464]
- Nwariaku FE, Chang J, et al. The role of p38 map kinase in tumor necrosis factor-induced redistribution of vascular endothelial cadherin and increased endothelial permeability. *Shock* 2002;18(1):82–5. [PubMed: 12095140]
- Petrache I, Birukova A, et al. The role of the microtubules in tumor necrosis factor- α -induced endothelial cell permeability. *Am J Respir Cell Mol Biol* 2003;28(5):574–81. [PubMed: 12707013]
- Petrache I, Verin AD, et al. Differential effect of MLC kinase in TNF- α -induced endothelial cell apoptosis and barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2001;280(6):L1168–78. [PubMed: 11350795]
- Ponsaerts R, D'Hondt C, et al. The myosin II ATPase inhibitor blebbistatin prevents thrombin-induced inhibition of intercellular calcium wave propagation in corneal endothelial cells. *Invest Ophthalmol Vis Sci* 2008;49(11):4816–27. [PubMed: 18614806]
- Rajashekhar G, Grow M, et al. Divergent and convergent effects on gene expression and function in acute versus chronic endothelial activation. *Physiol Genomics* 2007;31(1):104–13. [PubMed: 17566077]
- Reichenbach H, Hofle G. Discovery and development of the epothilones : a novel class of antineoplastic drugs. *Drugs R D* 2008;9(1):1–10. [PubMed: 18095749]
- Satpathy M, Gallagher P, et al. Extracellular ATP opposes thrombin-induced myosin light chain phosphorylation and loss of barrier integrity in corneal endothelial cells. *Exp Eye Res* 2005;81(2):183–92. [PubMed: 16080912]
- Satpathy M, Gallagher P, et al. Thrombin-induced phosphorylation of the regulatory light chain of myosin II in cultured bovine corneal endothelial cells. *Exp Eye Res* 2004;79(4):477–86. [PubMed: 15381032]
- Srinivas SP, Satpathy M, et al. Adenosine induces dephosphorylation of myosin II regulatory light chain in cultured bovine corneal endothelial cells. *Exp Eye Res* 2004;79(4):543–51. [PubMed: 15381038]
- Srinivas SP, Satpathy M, et al. Histamine-induced phosphorylation of the regulatory light chain of myosin II disrupts the barrier integrity of corneal endothelial cells. *Invest Ophthalmol Vis Sci* 2006;47(9):4011–8. [PubMed: 16936117]
- Usatyuk PV, Vepa S, et al. Redox regulation of reactive oxygen species-induced p38 MAP kinase activation and barrier dysfunction in lung microvascular endothelial cells. *Antioxid Redox Signal* 2003;5(6):723–30. [PubMed: 14588145]
- van Horck FP, Ahmadian MR, et al. Characterization of p190RhoGEF, a RhoA-specific guanine nucleotide exchange factor that interacts with microtubules. *J Biol Chem* 2001;276(7):4948–56. [PubMed: 11058585]
- Watsky MA, Guan Z, et al. Effect of tumor necrosis factor alpha on rabbit corneal endothelial permeability. *Invest Ophthalmol Vis Sci* 1996;37(9):1924–9. [PubMed: 8759364]
- Xu SQ, Mahadev K, et al. Adiponectin protects against angiotensin II or tumor necrosis factor alpha-induced endothelial cell monolayer hyperpermeability: role of cAMP/PKA signaling. *Arterioscler Thromb Vasc Biol* 2008;28(5):899–905. [PubMed: 18292388]

- Ye D, Ma I, et al. Molecular mechanism of tumor necrosis factor- α modulation of intestinal epithelial tight junction barrier. *Am J Physiol Gastrointest Liver Physiol* 2006;290(3):G496–504. [PubMed: 16474009]
- Yin F, Watsky MA. LPA and SIP increase corneal epithelial and endothelial cell transcellular resistance. *Invest Ophthalmol Vis Sci* 2005;46(6):1927–33. [PubMed: 15914605]

Fig. 1.

Effect of microtubule stabilization on the (TNF- α)-induced microtubule disassembly. Cells were pretreated with 10 μ M paclitaxel or 0.1 μ M epothilone B for 1 h with or without 20 ng/mL TNF- α for 6 h. Cells were then stained for α -tubulin (green). The nucleus (blue) is stained with DAPI. **A–F:** Representative images of three independent experiments showing organization of microtubules. **A:** In untreated cells, microtubules exist as fibrillary extensions from around the nucleus to the cell periphery (indicated by arrows). **B:** TNF- α induces microtubule disassembly, which is characterized by the loss of fibrillary extensions (indicated by arrows). **C and E:** Paclitaxel and epothilone B stabilize microtubule assembly, as evident

by the dense organization of microtubule network around the nucleus. **D and F:** Pretreatment with paclitaxel or epothilone B opposes microtubule disassembly by TNF- α .

Fig. 2.

Effect of microtubule stabilization on the (TNF- α)-induced increase in permeability. In vitro permeability in response to TNF- α with or without pretreatment with paclitaxel/epothilone B was ascertained by quantifying the flux of FITC dextran (10 KDa) across cells grown on porous culture inserts. Treatment with 20 ng/mL TNF- α for 20 h significantly increases permeability to FITC dextran in comparison to control. **Notes:** C: control; T: TNF- α ; T+PTX: TNF- α + Paclitaxel; T+Epo B: TNF- α + Epothilone B. Error bars represent the SEM (n = 6). *Indicates significant difference from the control. C vs T: p<0.001. Pretreatment with 10 μ M paclitaxel or 0.1 μ M epothilone B for 1 h significantly attenuates the (TNF- α)-induced increase in permeability. **Indicates significant difference. T vs T+PTX: p<0.001; # Indicates significant difference. T vs T+Epo B: p<0.001.

Fig. 3.

Effect of paclitaxel on the (TNF- α)-induced decline in TER. **A:** Cells were pretreated with 10 μ M paclitaxel for 1 h with or without 20 ng/ml TNF- α . Paclitaxel attenuates the decrease in TER by TNF- α . Treatment with paclitaxel alone does not affect TER and remains steady. **B:** Histogram analysis of experiments similar to that shown in **A**. The % reduction in TER induced by TNF- α is significantly greater than control beyond 8 h. 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 the (TNF- α)-induced reduction in TER beyond 12 h. **Indicates significant difference. TNF- α vs TNF- α + PTX: p<0.001.

Fig. 4.

Effect of epoithilone B on the (TNF- α)-induced decline in TER. **A:** Cells were pretreated with 0.1 μ M epoithilone B for 1 h with or without 20 ng/ml TNF- α . Epoithilone B attenuates the decrease in TER by TNF- α . Epoithilone B by itself increases TER when compared to control. **B:** Histogram analysis of experiments similar to that shown in **A**. The % reduction in TER induced by TNF- α is significantly greater than control beyond 8 h. Notes: Epo B: Epoithilone B; TNF- α +Epo B: TNF- α +Epoithilone B. Error bars represent the SEM (n = 6). *Indicates significant difference from the control. C vs TNF- α : p<0.001. Epoithilone B significantly opposes the (TNF- α)-induced reduction in TER beyond 4 h. **Indicates significant difference. TNF- α vs TNF- α + Epo B: p<0.001

Fig. 5.

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 10 μ M paclitaxel or 0.1 μ M epothilone B for 1 h with or without 20 ng/ml TNF- α for 6 h. **A–F:** Representative images showing localization of ZO-1 at the plane of apical junctional complex. **A:** In untreated cells, ZO-1 localization is continuous throughout the cell periphery (shown by arrows). **B:** TNF- α induces dispersion of ZO-1 from its normal locus, as evident by discontinuities in ZO-1 localization (shown by arrows). **C and E:** Upon treatment with paclitaxel or epothilone B, the pattern of ZO-1 localization is similar to those of untreated cells. **D and F:** Pretreatment with paclitaxel or epothilone B opposes the dispersion of ZO-1 by TNF- α .

Fig. 6.

Effect of microtubule stabilization on the (TNF- α)-induced redistribution of cadherins. The influence of TNF- α on the integrity of adherens junction with or without pretreatment with paclitaxel or epothilone B was ascertained by visualizing localization of cadherin, an AJ protein, by immunostaining. **A–F:** The representative images showing localization of cadherin. **A:** In untreated cells, increased localization of cadherins at the region of cell-cell contacts is evident (shown by arrows). **B:** Treatment with 20 ng/mL TNF- α for 6 h induces compromise in cell-cell contacts through reduced localization of cadherins at the intercellular regions (shown by arrows). **C and E:** Upon treatment with paclitaxel or epothilone B, the localization of cadherins is similar to untreated cells. **D and F:** Pretreatment with paclitaxel or epothilone B opposes the influence of TNF- α on localization of cadherins.

Fig. 7.

Effect of microtubule stabilization on the (TNF- α)-induced disruption of cortical actin. Cells were pretreated with 10 μ M paclitaxel or 0.1 μ M epothilone B for 1 h with or without 20 ng/ml TNF- α for 6 h. **A–F:** Representative images showing organization of cortical actin at the plane of apical junctional complex. **A:** In untreated cells, the characteristic organization of cortical actin referred to as the perijunctional actomyosin ring (PAMR) is evident (shown by arrows). **B:** TNF- α induces disruption of PAMR and formation of actin stress fibers (shown by arrows). **C and E:** Upon treatment with paclitaxel or epothilone alone, the organization of PAMR is similar to that of untreated cells. **D and F:** Pretreatment with paclitaxel or epothilone B opposes the influence of TNF- α on PAMR.