Microtubule Disruption Leads to Cellular Contraction in Human Trabecular Meshwork Cells

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PURPOSE. To determine whether microtubule- and actin-altering drugs, which have been shown to increase aqueous humor outflow, cause cellular contraction in human trabecular meshwork (HTM) cells.

METHODS. HTM cells were plated in culture dishes containing a polymerized deformable silicone substrate. After 48 hours, the dishes were placed on an inverted microscope and treated with ethacrynic acid, colchicine, vinblastine, cytochalasin B, or 1-(5-isouquinolinylsulfonyl)-2-methylpiperazine (H-7) and then recorded on videotape for 15 minutes. An increase in silicone substrate wrinkle size and/or number indicated a contraction. Sham controls were used.

RESULTS. Cellular contraction was observed with ethacrynic acid, colchicine, and vinblastine in the 10⁻⁵ to 10⁻⁴ M dosage range. Pretreatment with H-7 blocked these effects. Cytochalasin B did not produce cellular contraction.

CONCLUSIONS. Microtubule disruption causes cellular contraction in HTM cells, and this effect depends on an intact actin cytoskeleton network. Contraction of trabecular meshwork cells in response to various stimuli is an attractive hypothesis for possible homeostatic mechanisms in the outflow pathway, and this may serve as a focus for novel glaucoma drug development. (Invest Ophtalmol Vis Sci. 1998;39:653-658)

We have observed pharmaceutically produced, cytoskeleton-related changes in cell shape and cell-to-cell attachment in trabecular and other endothelial cells in culture, which we have related to the measured aqueous outflow effects observed in vitro and in vivo. These observed changes in cell shape and attachment in vitro could be produced simply by some form of cellular reorganization or, alternatively, by cellular contraction. Previous studies have implied that trabecular meshwork cells contract and respond in this contraction to pharmacologic or other signaling, but direct evidence for force generation in isolated cells has not yet been presented. In other systems, this question has been approached by growing cells on a silicone membrane and assessing the amount of induced wrinkling. In the present study, we developed this method to directly evaluate trabecular meshwork cell contraction. We also chose to study the potent outflow agent, ethacrynic acid (ECA), because of its potential relevance to the therapy of glaucoma and because its mechanism of action may be multifold and not yet fully clarified. One hypothesis for one of ECA's outflow actions involves induced depolymerization of microtubules, therefore, we also chose to investigate other microtubule-acting drugs in this system.

MATERIALS AND METHODS

Human donor eyes were obtained from the National Disease Research Interchange (Philadelphia, PA) within 48 hours of death. Human trabecular meshwork (HTM) cell culture and actin cytoskeletal analyses, cell shape staining, and viability studies were performed as previously described.

Contraction Assay

Cellular contraction was determined by visualizing wrinkles in a thin polymerized silicone substrate. Dimethylpolysiloxane (12,500 centistokes [cs]; Sigma Chemical, St. Louis, MO) was spread evenly over a glass coverslip with the aid of a bent Pasteur pipet. The silicone was polymerized by a brief exposure to flame, producing a polymerized deformable silicone substrate. HTM cells were trypsinized and plated onto the silicone substrate and cultured for 48 to 72 hours at 37°C in a 7% CO₂ atmosphere. One hour before use in an experiment,
the media on the cells was replaced with serum-free Dulbecco's modified Eagle's medium that was buffered with 10 mM Hepes (Sigma), and the cells were allowed to equilibrate for 1 hour in a 37°C room air incubator. After the equilibration period, the cells were placed on the warmed stage (37°C) of an inverted microscope (IM 35; Zeiss, Thornwood, NY) and observed at a magnification of 100×. Each experimental dish was examined completely, and an area was chosen for observation during the experiment.

We chose observation areas that contained cells that had already wrinkled the silicone substrate (Fig. 1A), indicating that the silicone substrate was polymerized correctly and that the cells were healthy and potentially contractile. Each dish was used for only one experiment. After a baseline period of 5 minutes, the experimental or control solution was added to the dish, and the cells were observed for an additional 15 minutes. This was recorded on videotape (Super VHS) using a video computer, Cupertino, CA) and the U82 VCR.

Results

Contraction

We observed that untreated HTM cells spontaneously produced new wrinkles in our assay system beginning at periods usually longer than 30 minutes. We also observed that pharmacologically produced new wrinkles were stable over short, defined time windows of less than 15 minutes. We therefore chose the 15-minute time period for this assay. With these parameters, we still observed positive wrinkling in 11% (n = 57) of vehicle control dishes (Table 1).

There was no difference in mean cell number between control dishes (178 ± 11 cells/mm²) and those treated with ECA (182 ± 31 cells/mm², P = 0.9), colchicine (183 ± 22 cells/mm², P = 0.8), vinblastine (138 ± 21 cells/mm², P = 0.1), and bradykinin (213 ± 42 cells/mm², P = 0.4). Additionally, controls with no spontaneous contraction (181 ± 11 cells/mm²) were not different from those with spontaneous contractions (193 ± 36 cells/mm², P = 0.7). Cytochalasin was not analyzed because it induced loss of cell adhesions and no contractions.

Bradykinin (1 μM) produced wrinkles in the silicone substrate in 83% of experimental dishes (Table 1). Typically, bradykinin produced wrinkles either immediately or within the first 3 minutes of exposure, and this was followed by a gradual decrease. No loss of cellular adhesion to the silicone substrate was observed.

Colchicine caused wrinkling of the silicone substrate (Table 1) that began at a later time—beginning at 3 to 7 minutes after treatment—than did wrinkling with bradykinin. No subsequent loss of wrinkles or loss of cellular adhesion to the silicone substrate was observed.

Vinblastine produced wrinkles (Table 1) that began 2 to 5 minutes after treatment. In contrast to colchicine, some vinblastine-induced wrinkles disappeared after 10 to 15 minutes. We also observed that vinblastine induced a loss of cellular adhesion to the silicone substrate at all concentrations tested. This increased with the concentration of vinblastine and was accompanied by a loss of observed wrinkles.

ECA displayed a somewhat biphasic dose-response effect (Table 1) that began 3 to 7 minutes after drug treatment. However, these differences were not statistically significant. In some of the positive dishes, a loss of silicone substrate wrinkles was also observed late in the time course. As the dosage of ECA
TABLE 1. Cellular Contraction Assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage Positive for Wrinkles</th>
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<tbody>
<tr>
<td><strong>ECA</strong></td>
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<tr>
<td>100 μM ECA</td>
<td>12</td>
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<tr>
<td>50 μM ECA</td>
<td>14</td>
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<tr>
<td>25 μM ECA</td>
<td>15</td>
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<tr>
<td>10 μM ECA</td>
<td>3</td>
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<tr>
<td>200 μM Col</td>
<td>4</td>
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<tr>
<td>100 μM Col</td>
<td>8</td>
</tr>
<tr>
<td>50 μM Col</td>
<td>8</td>
</tr>
<tr>
<td>5 μM Col</td>
<td>14</td>
</tr>
<tr>
<td>80 μM Vin</td>
<td>4</td>
</tr>
<tr>
<td>40 μM Vin</td>
<td>8</td>
</tr>
<tr>
<td>20 μM Vin</td>
<td>6</td>
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<tr>
<td>10 μM Cyto B</td>
<td>2</td>
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<tr>
<td>5 μM Cyto B</td>
<td>3</td>
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<tr>
<td>2 μM Cyto B</td>
<td>10</td>
</tr>
<tr>
<td>1 μM Cyto B</td>
<td>8</td>
</tr>
<tr>
<td>1 μM bradykinin</td>
<td>6</td>
</tr>
<tr>
<td>Control</td>
<td>57</td>
</tr>
</tbody>
</table>

**Sham control**

- → Sham control 7 14
- → 1 μM bradykinin 1 50
- → 50 μM Vin 4 100
- → 0.1 mM ECA 4 100
- → Sham control 4 0
- → 100 μM H-7 4 0
- → 300 μM H-7 4 0
- → 600 μM H-7 4 0
- → 300 μM H-7 4 0
- → 300 μM H-7 5 0
- → 300 μM H-7 4 0

ECA, ethacrynic acid; Col, colchicine; Vin, vinblastine; Cyto B, cytochalasin B; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine.

Increased, the incidence of late loss of silicone substrate wrinkles increased, the time of occurrence shortened, and a loss of cellular adhesion was observed, all similar to that seen with vinblastine.

Cytochalasin B produced no wrinkling of the silicone substrate at any dosage tested (5-100 μM) (Table 1) and, in fact, produced a rapid loss of the endogenous baseline wrinkles. Cytochalasin B also caused a loss of cellular adhesion to the silicone substrate and produced a change in trabecular meshwork cell shape similar to what we have observed in other cell culture model systems. Ethanol (1%) sham controls, run in parallel, demonstrated no loss of preexisting silicone substrate wrinkling or cellular adhesion.

**Contraction Inhibition**

In the controls for the contraction inhibition studies (sham treatment followed by another sham treatment), 14% of the experimental dishes showed spontaneous wrinkle formation in each observation period (Table 1). In the positive controls for these sequential studies (sham treatment then drug treatment) 1 μM bradykinin, 50 μM vinblastine, and 0.1 mM ECA induced wrinkle formation in 50%, 100%, and 100% of the experimental dishes, respectively (Table 1). We observed that H-7 caused a loss of endogenous wrinkles beginning at 8 to 10 minutes, and this progressed to total loss, similar to that seen with cytochalasin B. This H-7-induced loss of wrinkles was not accompanied by a loss of cellular attachment to the silicone substrate, unlike what was observed with cytochalasin B, vinblastine, and ECA.

When the dishes were first treated with 300 μM H-7 and then 1 μM bradykinin, 50 μM vinblastine, or 0.1 mM ECA during the second observation period, no wrinkle formation (or loss of cellular adhesion) was observed (Table 1).

**Cell Shape and Actin Staining**

In separate experiments, cells treated with 300 μM H-7 showed a rapid loss of filamentous actin staining beginning as early as 10 minutes after treatment and progressing to a nearly complete loss at 60 minutes. This is similar to that seen with cytochalasin B (data not shown); in contrast to cytochalasin B, there were no changes in cell shape (Fig. 2C, 3C).

HTM cells treated with 0.1 mM ECA for 2 hours showed a dramatic change in cell shape with narrowing of the cell profile and retraction of cells from neighbors, with a subsequent attenuation of cell-substratum attachments. Cells showed an almost complete loss of filamentous actin staining (Fig. 2B). In contrast to this, when the HTM cells were pretreated with 300 μM H-7 for 1 hour before treatment with 0.1 mM ECA for 2 hours (300 μM H-7 was maintained in the medium during the ECA treatment), the cells demonstrated a complete loss of filamentous actin staining. However, no loss of cell-cell or cell-substratum attachments was observed, and there was little change in cell shape (Fig. 2D).

HTM cells treated with 50 μM vinblastine demonstrated changes in cell shape that began between 30 minutes and 60 minutes and progressed with time to a maximum at 2 hours (Fig. 3B). This effect was smaller in magnitude than that seen with 0.1 mM ECA. The vinblastine-treated cells appeared to have pulled apart from their neighbor cells. Some cell-to-cell attachments were still maintained, and no loss of cell substrate attachment was observed (in contrast to what was observed with vinblastine on the silicone substrate in contraction experiments). The cells were retracted to approximately two thirds of their initial spread surface area. Vinblastine did not appear to change the filamentous actin staining pattern (Fig. 3B). In contrast, when HTM cells were pretreated with 300 μM H-7 for 1 hour and then treated with 50 μM vinblastine for 2 hours (H-7 was maintained in the medium during the vinblastine exposure), the cells demonstrated no cell shape or attachment changes. The cells did show the same loss of filamentous actin staining that was seen with H-7 alone (Fig. 3D).

All cytoskeletal and cell shape changes induced by exposure to ECA, vinblastine, and H-7 were completely reversible within 24 hours with reintroduction of the control medium. (This could not be assessed for cytochalasin B because of the loss of cell-substratum attachments.)

Sham-treated control cells and those treated with ECA, vinblastine, colchicine, and H-7 showed positive staining with fluorescein diacetate and were negative for staining when incubated with propidium iodide. As a positive control, cells treated with 0.8 mM ECA for 2 hours, a concentration known to be cytotoxic, showed no fluorescein diacetate staining and were positive for propidium iodide staining.
FIGURE 2. Filamentous actin stain of human trabecular meshwork cells. (A) Sham-treated control. (B) 0.1 mM ECA for 2 hours. (C) 300 μM 1-(5-isquinolinylsulfonyl)-2-methylpipera-zine (H-7) for 3 hours. (D) 300 μM H-7 for 1 hour and then 0.1 mM ECA for 2 hours (H-7 was maintained in the medium during the ECA incubation). Note the loss of filamentous actin staining in the H-7-treated cells (C) and the lack of an ECA-induced change in cell shape in the H-7-pretreated cells (D). All effects were fully reversible. (Original magnification = 1000X.)

DISCUSSION

In this study we observed that HTM cells exhibit a low level of spontaneous contractile activity. We observed that, as expected, bradykinin, which produces calcium release from internal stores and calcium influx from external spaces and cellular contraction, induced contraction of HTM cells in our assay system in 83% of the experimental dishes tested (Table 1). The contraction observed with bradykinin was typically early, peaking within the first 3 minutes, with a subsequent loss of induced wrinkling at 5–8 minutes. This time course of contraction and relaxation coincides with that of induced calcium fluxes in vascular endothelial cells.

ECA also induced contraction in HTM cells (Table 1), but the onset was delayed compared with bradykinin and there was no subsequent relaxation observed. In separate studies we have determined that ECA does not produce an intracellular calcium rise in trabecular meshwork cells (unpublished data), and this may therefore explain this different time profile. In previous experiments we had observed that ECA altered cell-substratum adhesion at concentrations and incubation times higher or greater than those required to produce changes in cell shape and cell-to-cell attachment. These latter submaximal effects were accompanied by alterations in cytoskeletal protein staining patterns, in particular, those for β-tubulin.

We were therefore interested in learning whether other microtubule-disrupting drugs would cause cellular contraction in our HTM system. We chose to evaluate colchicine and vinblastine, because these drugs have been observed to increase aqueous humor outflow in different model systems. We observed that colchicine and vinblastine produced contraction in our assay system that was more similar to ECA than bradykinin. Our data documents that three separate drugs with microtubule-depolymerizing actions, which can increase aqueous humor outflow facility, all produce HTM cellular contraction in vitro.

Microtubule-depolymerizing agents have been observed in other systems to produce cellular contraction. The mechanism for this has not been fully clarified but possibly involves some form of secondary signaling. Alternatively, it has been proposed in a tensegrity hypothesis that cell shape is determined, in general, by a balance between outwardly directed and inwardly directed cytoskeletal forces. Microtubules, which are constantly undergoing polymerization (lengthening) and depolymerization (shortening) reactions in a cell, a phenomenon that has been termed dynamic instability, are conceptualized to provide outward force and filamentous actin inward force. With the loss of microtubules resulting from drug action, the now unopposed inwardly directed actin forces may cause cellular contraction and, hence, cell-to-cell separation and changes in cell shape. In possible support of such a concept in outflow pathway cells, we have observed that the microtubule-stabilizing drug paclitaxel (Taxol; Bristol–Meyers Oncology, Inc.)
Figure 3. Filamentous actin stain of human trabecular meshwork cells. (A) Sham-treated control. (B) 50 μM vinblastine for 2 hours. (C) 300 μM 1-(5-isouquinolinylsulfonyl)-2-methylpiperazine (H-7) for 3 hours. (D) 300 μM H-7 for 1 hour and then 50 μM vinblastine for 2 hours (H-7 was maintained in the medium during the vinblastine incubation). Note the change in cell shape and the intact filamentous actin-staining pattern in the vinblastine-treated cells (B). Note the lack of vinblastine-induced change in cell shape in the H-7-pretreated cells (D). All effects were fully reversible. (Original magnification, 1000X.)

New York, NY) interferes with the ECA-induced increase in outflow and the alteration of cell shape (and microtubule disruption) observed in vitro. We have hypothesized that tensegrity-related microtubule actions could be involved in the normal regulation of outflow resistance. Contraction of trabecular meshwork cells in the juxtacanalicular tissue (JCT) in vivo, in response to pressure gradients or cellular signaling, could alter the dimensions of the flow pathway for aqueous humor in the JCT. Alternatively, contraction of JCT cells, which insert cellular processes onto the inner wall of Schlemm's Canal cells, could exert traction on inner wall cells and alter the paracellular and transcellular flow pathways. Contraction of trabecular meshwork cells in response to various stimuli is an attractive hypothesis for possible homeostatic mechanisms in the outflow pathway.

We observed that the actin-disrupting drug cytochalasin B did not produce cellular contraction. This may be expected from the above tensegrity considerations in which an intact actin organizational structure would be required to act as the inward force. Conversely, the effects of cytochalasin on actin are complex. Cytochalasin B does produce cell-to-cell separation and changes in cell shape in vitro, and we postulate that these result from other interactions with adhesive cellular junctions that are distinct from cell contraction. Nevertheless, the induced changes in cell attachment would explain the observed increase in outflow.

It is noteworthy that the actin-disrupting drug H-7 blocked the in vitro changes in cell shape induced by ECA (Fig. 2) and vinblastine (Fig. 3) and their induced HTM cellular contractions (Table I). This is consistent with the above tensegrity hypothesis, which requires an intact actin organizational structure for induced microtubule depolymerization to secondarily cause cell contraction. Unlike cytochalasin B, H-7 does not produce a change in cell shape by itself (Figs. 2, 3) in vitro. We postulate that H-7 disrupts actin without inducing other dynamic changes in the cell-to-cell junctions.

The fact that ECA and two other microtubule-depolymerizing drugs, colchicine and vinblastine, increase aqueous humor outflow and cause HTM cellular contraction is important in understanding ECA's mechanism of action and in investigating other potential trabecular meshwork-directed glaucoma therapy. Regardless of class, other drugs that cause HTM cell contraction may be worthwhile to investigate, and other microtubule-acting drugs also deserve study. This cell contraction assay may be an important new
method to screen for glaucoma therapy compounds that could be directed at the outflow pathway.

References


Mice Deficient in Tumor Necrosis Factor Receptors p55 and p75, Interleukin-4, or Inducible Nitric Oxide Synthase Are Susceptible to Endotoxin-Induced Uveitis

Justine R. Smith,1 Prue H. Hart,2 Douglas J. Coster,1 and Keryn A. Williams1

PURPOSE. To investigate the roles of tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and inducible nitric oxide synthase (iNOS) in endotoxin-induced uveitis (EIU) using gene knock-out mice.

METHODS. Mice (C57BL/6 x 129) either of normal phenotype or deficient in the genes encoding one or both tumor necrosis factor receptors (TNF p55 and TNF p75), IL-4, or iNOS were given footpad injections of 400 μg Escherichia coli lipopolysaccharide. Animals were killed 24 hours later, and infiltrating cells were counted on 5-μm ocular cross-sections through the optic nerve.

RESULTS. All abnormal mouse phenotypes were susceptible to EIU. Yet, TNF p55 and IL-4 gene knock-out mice experienced less ocular inflammation than control animals (P = 0.021 and 0.007, respectively), whereas disease was not reduced for iNOS-deficient mice. Mice deficient in TNFR p55 and TNF p75 experienced milder EIU than mice lacking TNF p75 alone (P = 0.046).

CONCLUSIONS. Mice deficient in TNF p55 and TNF p75, IL-4, or iNOS retain the susceptibility to EIU, but TNF-α and IL-4 influence the influx of inflammatory cells to the eye during this disease. (Invest Ophthalmol Vis Sci. 1998; 39:658-661)

Acutely anterior uveitis causes distressing ocular symptoms and, when recurrent, may lead to sight-threatening disease, including cataract, glaucoma, and cystoid macular edema. Current therapy involves relatively nonspecific suppression of inflammation using topical corticosteroids. Although frequently effective, these agents may induce cataract or raise intraocular pressure, and patients are prone to microbial infection and rebound inflammation after drug withdrawal. The basic mechanisms operating in acute anterior uveitis have not yet been clarified. Little human material is available for research, but animal models of the disease provide opportunities to study the pathogenic process and devise more specific treatment options. In one well-established model1 known as endotoxin-induced uveitis (EIU), rodents injected with bacterial lipopolysaccharide develop an evanescent form of acute anterior uveitis. Cytokines are presumed to drive uveal inflammation. Studies in the rat have implicated tumor necrosis factor-α (TNF-α) as a critical early mediator of uveitis. Reverse transcription-polymerase chain reaction (RT-PCR) detects an increase in TNF-α gene expression within 1 hour of systemic endotoxin injection, and the TNF-α mRNA has been localized to histiocyte-like cells by in situ hybridization.2 The expressed product has been detected in the anterior segment before and during EIU by bioassay.3 Macrophages and monocytes, the major producers of this cytokine, respond to stimuli such as lipopolysaccharide, interleukin-1 (IL-1), and TNF-α itself. The multiple inflammatory activities of TNF-α are signaled through two distinct cell surface tumor necrosis factor receptors, designated TNF p55 and TNF p75, respectively.