The Effect of Cytochalasin D on Outflow Facility and the Trabecular Meshwork of the Human Eye in Perfusion Organ Culture

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Purpose. To determine the effect of cytochalasin D on outflow facility in the human anterior segment, and the histologic changes that accompany the effect.

Methods. Human anterior segments were studied in perfusion organ culture. The anterior segment from one eye received cytochalasin D, and that from the fellow control eye received vehicle; doses ranged from 0.06 mg/ml to 27.7 mg/ml. The duration of action and the effect of repeated doses were studied, and the accompanying histologic changes were assessed in 12 pairs of anterior segments.

Results. Cytochalasin D in concentrations of 0.6 mg/ml and 1.1 mg/ml caused increases in outflow facility of 42% and 37%, respectively (P < 0.05), with a peak effect 2 to 6 hours after infusion and a duration of action of approximately 14 hours. Anterior segments were not responsive to repeated doses (24 hours apart). Compared with the effect of vehicle in control anterior segments, cytochalasin D caused scattered breaks in the inner wall endothelial lining of Schlemm's canal (4.6 ± 2.5% versus 0.7 ± 0.6%; P = 0.02; anterior segments fixed during maximum drug effect). No increase in the amount of optically empty space within the juxtacanalicular tissue was seen. Inner wall breaks persisted, even in eyes in which the outflow facility had returned to baseline; the basement membrane and subendothelial matrix of the inner wall remained intact. Final intraocular pressure was inversely correlated with the length of optically empty space immediately adjacent to the inner wall.

Conclusions. Cytochalasin D can increase outflow facility in the anterior segment of the human eye and causes ruptures of the inner wall of Schlemm's canal. These breaks persist, even when interocular pressure returns to baseline; the basement membrane and subendothelial matrix of the inner wall appear to remain intact. The final intraocular pressure was inversely correlated with the length of optically empty space immediately adjacent to the inner wall.


Cytochalasins have been studied as one of a number of classes of compounds that may produce a "pharmacologic trabeculocanalotomy." Pharmacologic agents that could potentially disrupt the inner wall of Schlemm's canal and the juxtacanalicular tissue (JCT) may allow washout of the extracellular matrix and its accumulated debris and may reduce the outflow resistance in glaucomatous eyes. Several classes of agents have been investigated, including enzymes that degrade the extracellular matrix, cytoskeleton agents that disrupt the internal microskeleton and cause changes in cell shape, and chelating agents that may loosen intercellular junctions and allow aqueous egress through alternate pathways.2–12 Cytochalasins are fungal metabolites that cause a depolymerization of cytoplasmic microfilaments by binding to the growing end of the microfilament and preventing further polymerization.1,13 These actin filaments are in an equilibrium of polymerization and depolymerization, and because cytochalasin interferes only with polymerization, the equilibrium is shifted toward eventual depolymerization of the microfilaments. This loss of the internal structural units of the cell causes "rounding up" of cells in monolayer cul-
TABLE 1. Effect of Single-Dose Cytochalasin D

<table>
<thead>
<tr>
<th>Dose (mg/ml)</th>
<th>n</th>
<th>Experimental (Cd/C0)</th>
<th>Control (Cd/C0)</th>
<th>Experimental (Cd/C0)/Control (Cd/C0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.06</td>
<td>4</td>
<td>1.14 ± 0.18</td>
<td>1.04 ± 0.13</td>
<td>1.13 ± 0.27</td>
</tr>
<tr>
<td>0.1</td>
<td>6</td>
<td>1.08 ± 0.16</td>
<td>0.92 ± 0.13</td>
<td>1.21 ± 0.59</td>
</tr>
<tr>
<td>0.6</td>
<td>5</td>
<td>1.48 ± 0.30</td>
<td>1.04 ± 0.10</td>
<td>1.42 ± 0.30</td>
</tr>
<tr>
<td>1.1</td>
<td>8</td>
<td>1.33 ± 0.40</td>
<td>0.97 ± 0.09</td>
<td>1.38 ± 0.43</td>
</tr>
<tr>
<td>11.0</td>
<td>6</td>
<td>1.27 ± 1.07</td>
<td>1.01 ± 0.10</td>
<td>1.27 ± 1.08</td>
</tr>
</tbody>
</table>

Values are mean ± SD.
Cd = facility of outflow after drug infusion; C0 = baseline facility of outflow.
*Statistical probability that ratio = 1.0 (i.e., no effect).

Cytochalasin and Trabecular Meshwork

TABLE 2. Effect of Repeated Doses of Cytochalasin D

<table>
<thead>
<tr>
<th>n</th>
<th>First Dose</th>
<th>Second Dose</th>
<th>Third Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.06 mg/ml</td>
<td>0.1 mg/ml</td>
<td>0.6 mg/ml</td>
</tr>
<tr>
<td>4</td>
<td>1.12 ± 0.24</td>
<td>1.06 ± 0.18</td>
<td>1.01 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>0.1 mg/ml</td>
<td>0.6 mg/ml</td>
<td>0.6 mg/ml</td>
</tr>
<tr>
<td>4</td>
<td>1.33 ± 0.44</td>
<td>0.91 ± 0.02</td>
<td>0.89 ± 0.16</td>
</tr>
<tr>
<td>8</td>
<td>1.1 mg/ml</td>
<td>11.0 mg/ml</td>
<td>27.7 mg/ml</td>
</tr>
<tr>
<td>8</td>
<td>1.33 ± 0.40</td>
<td>1.09 ± 0.31</td>
<td>1.00 ± 0.12</td>
</tr>
</tbody>
</table>

Values are mean ± SD of experimental (Cd/C0)/control (Cd/C0).
Cd = facility of outflow after drug infusion; C0 = baseline facility of outflow.

Drugs and presumably would cause similar changes in vivo.14,15 Kaufman et al studied cytochalasins B and D in living monkeys and found a rapid increase in outflow facility lasting several hours in many of the animals.16-17 Histologic examination revealed ruptures of the inner wall endothelium of Schlemm's canal, washout of extracellular materials, and distension of the JCT.7 Johnstone et al also found disruption of the inner wall and washout of extracellular materials in a series of 10 monkeys.8 Although cytochalasin has been proved effective in animals, its influence on human eyes has not been reported.

In the current experiment, cytochalasin D was studied in the anterior segments of human eyes, using the perfusion organ culture model. This allowed not only the study of effect on outflow facility, but also the determination of duration of action and the effect of repeated doses. Histologic examination was then performed to determine the mechanism of action.

MATERIALS AND METHODS

Culture Technique

Twenty-nine pairs of fresh, normal human eyes were studied (obtained within 20 hours after death). The average age of the donor eyes was 77 ± 8 years (range, 64 to 90 years). No eyes had glaucoma or uveitis, and none had received topical medications. Five eyes from five donors had undergone cataract surgery with posterior chamber intraocular lenses; no anterior chamber lenses had been used. The tenets of the Declaration of Helsinki were followed; the Mayo Institutional Review Board approved the project. The culture technique was similar to that described previously.16,17 Eyes were bisected at the equator, and the iris, lens, and vitreous were removed. The anterior segment was clamped in a modified Petri dish and the eye perfused with Dulbecco's modified Eagle's medium with added antibiotics (10,000 U penicillin, 10 mg streptomycin, 25 mg amphotericin B, and 17 mg gentamicin, in 100 ml medium; Sigma Chemical Co., St Louis, MO) at the normal human flow rate (2.5 μl/min). The anterior segments were cultured at 37°C in a 5% CO2 atmosphere. Intraocular pressures were continuously monitored, with a pressure transducer connected to the second access cannula built into the dish, and were recorded with an automated computerized system.

Drug Infusion

After an initial adaptation period in culture, usually lasting 2 to 4 days, the anterior segment of one eye was given cytochalasin D by an anterior chamber exchange, and that of the fellow control eye was given the dimethyl sulfoxide (DMSO) vehicle in a similar volume of fluid, also by anterior chamber exchange. Cytochalasin D (Sigma) was dissolved in 1% DMSO (medicinal grade; Sigma) in the culture medium. The anterior chamber exchanges were performed in a 10-minute period, using a gravity-driven, constant-pressure method. Pressure data from either eye were not used during the first hour after the anterior chamber exchange.

Cytochalasin D was given in concentrations of 0.06 mg/ml to 27.7 mg/ml in either single or sequential doses (Tables 1 and 2). In those anterior segments receiving sequential doses, the lowest concentrations of cytochalasin D were given first, followed in 24 hours by the next higher dose, and 24 hours later by the third dose. Data from these initial doses were included in the single-dose values in Table 1. Several of the
doses of cytochalasin D were chosen to be similar to those used by Kaufman and Erickson, allowing for differences in technique and dilutional effects.\(^5\) The osmolality of the cytochalasin D–DMSO mixture was 485 mmol/kg H\(_2\)O, pH 7.7. The osmolality of the 1% DMSO control solution was 478, pH 7.7. Outflow facility (\(C = F/P\)) was calculated every hour, beginning 3 hours before drug infusion and continuing for the duration of the culture. Results from each pair of eyes were combined into a group mean for each concentration of drug.

In two pairs of anterior segments, the tracer cationic ferritin (suspended in 5.2 mg/ml Dulbecco’s phosphate-buffered saline, pH 6.2; Sigma) was added by anterior chamber exchange 24 hours after the cytochalasin D had been given, but before fixation. Perfusion with culture medium was continued for 50 minutes, after which the infusion was switched to fixative.\(^18\)\(^19\)

All anterior segments were subsequently fixed by anterior chamber perfusion at a pressure level equal to that within the eye when the experiment was completed. Twelve pairs were fixed within 7 hours of receiving cytochalasin D, at a time when the intraocular pressure was under the effect of the cytochalasin D. The remaining anterior segments were fixed from 2 days to 2 weeks after receiving the final dose of cytochalasin D. Fixative was 4% paraformaldehyde in 0.1 M phosphate buffer.

Histologic Examination

All cultures were evaluated with light microscope to assess the appearance of the trabecular cells and to look for evidence of toxicity. In addition, the meshworks from 12 pairs of eyes were examined, using morphometric methods described later. Examination was performed in a masked fashion, using predetermined criteria including cell shape, nuclear shape, preservation of cellular and nuclear membranes, cell–cell attachments, cytoplasmic covering of trabecular lamellae, preservation of endothelial lining of Schlemm’s canal, and integrity of trabecular lamellae.\(^17\)\(^20\) Meshworks were considered normal in appearance if trabecular cells remained in their usual position on the lamellae, normal numbers of cells were seen (subjective assessment), and little disruption of the JCT and trabecular lamellae was seen.

The meshworks from 12 pairs of anterior segments were selected for morphometric examination with transmission and scanning electron microscopes. These were chosen to represent all stages of effect of the drug: fixation at the time of maximum lowering of IOP after a single dose (\(n = 6\) pairs), anterior segments with an initial lowering of IOP which then returned to baseline and did not change after two subsequent doses (\(n = 2\) pairs), anterior segments with no change in IOP after receiving drug (\(n = 2\) pair), and anterior segments with a paradoxical increase in IOP after drug infusion (\(n = 2\) pair). Wedges of tissue from the limbus were dissected from each quadrant. Two quadrants were prepared for transmission electron microscopic examination by dehydration in ascending concentrations of alcohol and embedding in epoxy resin. Two quadrants were prepared for the scanning electron microscope by dissection of wedges of tissue 3 mm wide and unroofing the inner wall of Schlemm’s canal.\(^21\) This entailed removal of the sclera overlying the canal and allowed a view of the inner wall cells from the lumen of the canal. Specimens then underwent critical-point drying and sputter coating with a gold–palladium mixture.

Quantitation

Transmission Electron Microscopic Examination. The amount of optically empty space within the JCT was measured by densitometry using the Zeiss IBAS 2000 system (Carl Zeiss, Thornwood, NY), as previously reported\(^22\)\(^23\) Initial examination involved low-magnification (\(\times600\)), overview photographs on the electron microscope for orientation and aid in determination of the boundaries of the JCT. Four micrographs of the JCT were then made at \(\times2500\), using a sampling protocol. These areas included the region underlying the anterior end of the canal, two areas from midcanal, and the region underlying the posterior end of the canal and covered \(\approx 154 \mu m\) of the length of the canal and JCT (roughly one half). In prior work, these samples provided a reasonable representation of the JCT.\(^22\)\(^23\) Measurements of optically empty space were expressed relative to the total area of the JCT examined, as measured in the four micrographs from each quadrant. At least two quadrants per eye were measured.

Other measurements included the length of the optically empty space adjacent to the inner wall of the canal, the length of breaks in the inner wall endothelium lining, and the length of “ballooning” (disinsertion of the inner wall cells from the underlying extracellular material of the inner wall).\(^24\) These were measured directly from the micrographs.

Scanning Electron Microscopic Examination. The inner wall was examined at \(\times1800\) magnification.\(^21\) The area of breaks or defects in the inner wall endothelium larger than 4 \(\mu m\) in diameter, which is the upper size limit of the physiologic pores,\(^21\)\(^25\) was measured from the entire region of inner wall present in that tissue wedge. This total area of inner wall was measured by counting the number of microscopic fields examined. Higher magnification was used to confirm the nature of the inner wall defects.
Statistical Analysis

Drug effects were expressed as the outflow facility after drug infusion ($C_d$) divided by the baseline facility ($C_o$) for each anterior segment. The percent change in outflow facility was expressed as the ratio of the facility in the experimental anterior segments to that in the control anterior segments of each pair. Results from each pair were combined into a group mean for each concentration of drug. Values are expressed as mean ± standard deviation. Statistical analysis was performed by a paired, two-tailed Student’s $t$-test.

Microscopic measurements from each of the four transmission electron micrographs per tissue section were combined to determine the value for each microscopic section. Data from the different quadrants were combined to give a mean value per anterior segment. Similarly, data from each wedge of tissue examined by scanning electron microscope were combined to give a mean value per anterior segment. Values of all data are mean ± standard deviation. Two-tailed, paired $t$-tests were used for comparisons between fellow anterior segments. Pearson’s correlation coefficient was used to test for correlations among measured facility and histologic measurements; nonparametric data were analyzed with Spearman’s correlation coefficient.

RESULTS

Facility of Outflow

Single Dose. A 42% increase in outflow facility was noted at a concentration of 0.6 mg/ml 2 hours after drug infusion ($P = 0.03$; Table 1). The onset of effect appeared within 20 minutes of drug infusion, peaked at 2 hours, and lasted at least 6 hours, at which time several of the anterior segments were fixed. Four of five anterior segments had increases in facility, whereas one had no change. At 1.1 mg/ml, a 37% increase in facility was found at 6 hours after drug infusion ($P = 0.04$; Table 1). This increase peaked at 6 hours after drug infusion, after which facility gradually declined during the next 8 hours, although it remained above the baseline even after 24 hours ($P = 0.02$). Five of eight anterior segments responded with an increase in facility, whereas three had no change. At lower doses (0.06 µg/ml and 0.1 µg/ml), no significant effect on outflow facility was apparent. Although two anterior segments in each of these groups of lower concentrations had facility increases of ~30%, the remainder had no change in facility. At the highest single dose used, 11 µg/ml, three of the six anterior segments tested had a decrease in outflow facility of ~27%, whereas one anterior segment had a large increase; no significant overall effect on outflow facility was observed in the group as a whole (Table 1).

Not all anterior segments responded to cytochalasin D with a change in facility, as discussed above. No differences could be found between responding and nonresponding specimens when considering age, sex, time from death to culture, days in culture before receiving cytochalasin D, or facility at the start of the experiment. The facility of outflow in the control anterior segments remained stable, in that no significant change in facility was noted after the initial anterior chamber exchange with 1% DMSO. Two additional pairs of anterior segments had been studied but were not included in the above 29 pairs, because the control anterior segments had large changes in facility (one decreased and one increased more than 100%).

Sequential Doses. No consistent effect on facility was observed with repeated doses, regardless of whether the eye had responded to the initial dose. This lack of effect was seen with low (0.6 µg/ml) and high (up to 27.7 µg/ml) sequential doses (Table 2). Control anterior segments maintained stable facilities of outflow.

Histologic Examination

Cytochalasin-D–treated meshworks had scattered areas of breaks or disruptions in the endothelial cells lining the inner wall of Schlemm’s canal, observed with transmission and scanning electron microscopes. These breaks and disruptions were larger and more numerous than those in the fellow control anterior segments in the group fixed during time of maximum drug effect (Table 3; 4.6 ± 2.5% of the inner wall versus 0.7 ± 0.6% of the inner wall; $P = 0.02$; $n = 6$) and also in the entire group of anterior segments analyzed, fixed at various time points after drug infusion (16.1 ± 21% versus 5.2 ± 6.6%; $P = 0.03$; $n = 12$). Study by transmission electron microscope revealed that these breaks were caused by the disruption of portions of cells or loss of cells, not merely by separation of cells (Fig. 1). In areas of loss of inner wall endothelial cells, the underlying basement membrane and subendothelial matrix material would usually remain, often maintaining a sharp linear border adjacent to the canal (Fig. 1). Ballooning of the inner wall tended to occur more often in the experimental than in the control meshworks, although the difference was not statistically significant (Table 3). In only a few areas did washout of extracellular material underlying the basement membrane seem to occur. No obvious differences were noted in the appearance of the basement membrane and underlying subendothelial matrix in meshworks from anterior segments with increased outflow facility when compared with those from fellow control eyes; however, immunohistologic labeling and other special techniques were not performed. In the two pairs of anterior segments receiving cationic ferritin, tracer was bound to the remnants of the cells and also to the bare basement membrane.
### TABLE 3. Morphometric Measurements

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Anterior Segment</th>
<th>Dose</th>
<th>C/P</th>
<th>Final IOP (%)</th>
<th>JCT: OES (%)</th>
<th>IW Breaks (%)</th>
<th>ES-SC (%)</th>
<th>Balloon SEM IW Breaks (%)</th>
<th>SEM IW Breaks (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fix at maximal effect</td>
<td>6</td>
<td>Experimental</td>
<td>0.6</td>
<td>1.00 ± 0.85</td>
<td>10.2 ± 2.8</td>
<td>33.8 ± 6.6</td>
<td>4.6 ± 2.5</td>
<td>22.9 ± 8.5</td>
<td>4.5 ± 5.0</td>
<td>2.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1.1</td>
<td>1.04 ± 0.09</td>
<td>11.7 ± 3.5</td>
<td>35.7 ± 6.2</td>
<td>6.7 ± 2.6</td>
<td>25.6 ± 5.9</td>
<td>5.0 ± 4.1</td>
<td>2.1 ± 2.3</td>
</tr>
<tr>
<td>Returned to baseline</td>
<td>2</td>
<td>Experimental</td>
<td>1.1,11</td>
<td>1.05 ± 0.04</td>
<td>17.5 ± 2.1</td>
<td>35.5 ± 4.7</td>
<td>6.5 ± 12.9</td>
<td>12.5 ± 5.5</td>
<td>5.0 ± 4.1</td>
<td>11.9 ± 15.9</td>
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<tr>
<td>No effect on C</td>
<td>2</td>
<td>Experimental</td>
<td>0.6</td>
<td>1.00 ± 0.00</td>
<td>16.0 ± 12.7</td>
<td>42.0 ± 2.4</td>
<td>14.0 ± 1.4</td>
<td>23.5 ± 5.5</td>
<td>0</td>
<td>2.0 ± 0.1</td>
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<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1.1,11</td>
<td>0.97 ± 0.04</td>
<td>17.5 ± 5.0</td>
<td>40.5 ± 3.5</td>
<td>1.5 ± 0.7</td>
<td>23.5 ± 6.0</td>
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<td>ND</td>
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<tr>
<td>Decrease in C</td>
<td>2</td>
<td>Experimental</td>
<td>11.0</td>
<td>0.94 ± 0.08</td>
<td>23.5 ± 16.9</td>
<td>48.0 ± 19.8</td>
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<td>15.0 ± 2.8</td>
<td>0</td>
<td>ND</td>
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<tr>
<td></td>
<td></td>
<td>Control</td>
<td>0.75 ± 0.10</td>
<td>24.5 ± 6.4</td>
<td>29.0 ± 4.2</td>
<td>35.0 ± 29.7</td>
<td>16.5 ± 15.4</td>
<td>0</td>
<td>5.5 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>Mean: all eyes</td>
<td>12</td>
<td>Experimental</td>
<td>1.00 ± 0.00</td>
<td>14.5 ± 5.0</td>
<td>29.0 ± 2.8</td>
<td>13.0 ± 7.1</td>
<td>20.0 ± 4.2</td>
<td>1.5 ± 2.1</td>
<td>10.5 ± 15.6</td>
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<tr>
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<td>Control</td>
<td>3.4 ± 5.9</td>
<td>16.1 ± 21.0</td>
<td>4.7 ± 6.9</td>
<td>16.1 ± 21.0</td>
<td>4.7 ± 6.9</td>
<td>16.1 ± 21.0</td>
<td>4.7 ± 6.9</td>
<td>16.1 ± 21.0</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

OES = optically empty space; ES-SC = optically empty space adjacent to Schlemm’s canal; IOP = intraocular pressure; IW = inner wall; JCT = juxtacanalicular tissue; SC = Schlemm’s canal; SEM = scanning electron microscopy; ND = not determined.

Examination by scanning electron microscope confirmed the loss of inner wall cells, and also revealed regions of bare basement membrane (Fig. 3). In one pair of anterior segments, platelets were present and appeared to bind to the bare basement membrane and underlying extracellular matrix (Fig. 4). In five experimental and two control meshworks, giant vacuoles appeared to have ruptured, allowing a view of the inside of the vacuoles and the underlying large circular pore in the endothelial cell ("meshwork pore" of Grierson) through which the vacuole presumably fills (Fig. 5). A limitation of scanning microscopic examination of the inner wall ap-

![FIGURE 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933421/ on 04/29/2017)

![FIGURE 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933421/ on 04/29/2017)
Cytochalasin and Trabecular Meshwork

FIGURE 3. (A) Scanning electron micrograph of inner wall. Loss of inner wall cell reveals underlying basement membrane with round openings ("meshwork pores") to underlying juxtacanicular tissue. Disrupted cell boundaries are indicated by arrows. Meshwork pores are indicated by arrowheads. (Magnification, ×4300; case 41-93L: single 0.6-μg dose of cytochalasin D; fixed 6 hours later at time of maximum effect). (B) Loss of inner wall cell reveals that the underlying basement membrane remains intact. Adjacent cell boundaries are shown with arrows. "Meshworks pore" are indicated with arrowhead. (Magnification, ×6000; case 120-92R; sequential doses of 1.1 μg, 11 μg, and 27.7 μg cytochalasin D; fixed 6 hours after final dose).

Examined to be the loss of the anterior and posterior portions of the canal during the dissection process, as was also noted by others.20,25 In addition, the inner wall endothelial cells were artifically disrupted near septa that had become torn during the dissection process.21,25

Examination of the groups of meshworks fixed at various times after receiving cytochalasin D revealed the smallest amount of inner wall disruption in anterior segments fixed soon after infusion of the drug, during the time of maximum effect (Table 3). Meshworks observed for longer periods after infusion of the drug tended to have larger areas of disruption of the inner wall cells. The inner wall breaks appeared

FIGURE 4. Scanning electron micrograph of inner wall reveals platelets (A, arrows) binding to bare basement membrane and extracellular matrix of juxtacanicular tissue in area of disrupted inner wall cell. (Magnification, ×11,000) Platelets binding to inside of disrupted giant vacuole (B; magnification, ×15,000; case 667-92R; single 11-μg dose cytochalasin D; fixed 2 hours later at time of maximum drug effect).

FIGURE 5. Disrupted giant vacuole reveals inner "meshwork pore" that connects with underlying juxtacanicular tissue (magnification, ×10,000; case 253-94R; control eye fixed 6 hours after receiving dimethyl sulfoxide vehicle).
FIGURE 6. Length of optically empty space adjacent to the inner wall and final intraocular pressure. Experimental and control eyes are combined.

Correlations

A significant inverse correlation was present between the final IOP and the length of optically empty space immediately adjacent to the inner wall endothelium for the combined group of cytochalasin-D–treated and control anterior segments. As the length of the optically empty space increased, the IOP decreased (Fig. 6; \( r = -0.38; P = 0.05 \)). No significant correlation was found between the length or area of disruptions of the inner wall endothelial cells and the final IOP or with the peak change in facility of outflow \((C_d)\).

DISCUSSION

Cytochalasin D increased outflow facility in the human eye by \( \sim 40\% \) at concentrations of 0.6 and 1.1 mg/ml. The onset of action appeared within the first 40 minutes, with maximum effect between 2 and 6 hours, and a duration of action of approximately 14 hours. Disruption of the inner wall endothelium was the major histologic change seen with cytochalasin D treatment and was present in larger amounts than in the fellow control meshworks in anterior segments fixed during time of maximum drug effect (4.6 ± 2.5% versus 0.7 ± 0.6%; \( n = 6 \)), and also for the entire group of eyes analyzed, fixed at various times after drug infusion (16.1 ± 21% versus 5.2 ± 6.6%; \( n = 12 \)). These findings are similar to those in primates, in which cytochalasin B was originally found to cause breaks in the inner wall of Schlemm’s canal,7,8 ranging from 6% to 83% of the inner wall.8 Cells in the uveal and corneoscleral meshwork remained intact, unlike findings in a study of monkeys in which separation, degeneration, and disappearance of meshwork cells in the uveal and corneoscleral regions was observed.7

The inner wall disruption appeared to persist in these cultured meshworks, even in eyes in which the facility had returned to baseline level. This differed from the reports of findings in primate eyes that de-
scribed platelets adhering to the regions of inner wall disruption within hours after drug infusion and repair of the disruptions by 6 days. Because there is no blood supply to the cultured human anterior segments, platelets were not expected to plug the inner wall disruptions. Instead, we expected that the inner wall disruptions would become repaired by migration and spreading of adjacent inner wall cells, especially in anterior segments in which outflow facility had returned to baseline level. This expectation was not seen. Of interest, platelets were observed in the canal of two cultured anterior segments, bound to the exposed basement membrane and underlying extracellular matrix in the regions of endothelial disruption, as described in the primate eyes. These platelets could have been trapped in Schlemm's canal or in the aqueous veins after death and were thus present despite the lack of a blood supply. In these two anterior segments, platelets were present in only small amounts and did not cover all of the inner wall disruptions. In the other 22 anterior segments, platelets were not observed.

The nature of the inner wall disruptions in the cultured anterior segments was investigated in three ways to discern whether the breaks occurred during culture or developed as artifacts during fixation and processing. First, cationic ferritin was added to two pairs of anterior segments and adhered to the broken inner wall cells and underlying basement membrane. Cationic ferritin is an electron-dense tracer that binds to negatively charged surfaces such as cell membranes, and has been used to study outflow pathways within the trabecular meshwork. Its binding to the cellular disruptions indicates that the disruptions occurred during the culture process, in that this was when the tracer was added, and not during histologic processing. Second, the finding of platelets bound to the exposed basement membrane and underlying extracellular matrix in two anterior segments also indicates that the disruptions occurred before fixation. Fixative would be expected to prevent binding. This binding of platelets is similar to reports of observations in living primates after treatment with cytochalasin B and also after disruption of the inner wall caused by other agents. Third, the remnants of the inner wall cells were rounded and usually encircled by cytoplasmic membrane in the regions of disruptions. Artifactual breaks would be linear and jagged, rather than smooth and rounded, as seen in these meshworks.

The inner wall of Schlemm's canal is usually thought to account for less than 10% of outflow resistance, based on Bill and Svedbergh's calculations. They found that the inner wall endothelial cells had too many pores opening to the canal to provide significant outflow resistance. Similarly, results in a recent study by Sit et al showed that the number of inner wall pores did not correlate with the facility of outflow. In results of the current study and in those of the studies in living monkeys, disruption of the inner wall endothelium was associated with the increase in outflow facility, yet this seems incompatible with the findings of Bill and Svedbergh and also with those of Sit et al. How could disruption of the inner wall cause a change in outflow facility if the inner wall has only a minor role in outflow resistance?

One speculation is that the inner wall and underlying basement membrane and subendothelial matrix function as a unit, and are not independent. In this scenario, proteins, proteoglycans, and other molecules bind to the basement membrane and also to the inner wall cells; specific cytoplasmic membrane receptors (integrins) are known to bind components of extracellular matrix, including laminin, collagen IV, entactin, and vitronectin. Because of this binding, loss of inner wall cells could cause loss of some of these components, and hence affect outflow facility. Although specific evidence for this was not found, because the basement membrane and subendothelial matrix did not appear different when examined with electron microscope, special fixative techniques were not used. Marshall et al noted that LR White embedding appeared to retain more extracellular matrix than conventional Araldite embedding methods. They found that the subendothelial matrix labeled with antibodies to collagen IV and laminin, materials common to basement membranes. This subendothelial matrix is composed of fine, fibrillar material and fibrogranular material, termed "type I plaques" by Rohen, and may be up to 4 μm thick. It is the third layer, or lamina reticularis, of the inner wall basement membrane and is thicker than the other two layers of the basement membrane, the lamina rara and the lamina densa. These layers are thin (~40 nm each), regular structures that parallel the endothelial cell contours.

A second explanation for the effect of cytochalasin D in affecting outflow resistance could be weakened cell–cell and cell–extracellular matrix junctions, which would create a separation of cells, or a disinsertion of inner wall cells from the underlying basement membrane (ballooning). Cell separation, rounding, or retraction were not observed, although they may have been early events, in that these anterior segments were fixed at the time of maximum drug effect. Ballooning of the inner wall was seen more often in the experimental meshworks, although the difference was not statistically significant (4.3 ± 5% of inner wall versus 1.8 ± 2.9%; P = 0.37).

The return of outflow facility to baseline levels would require replacement of the inner wall endothelial cells, plugging of the breaks with platelets, or synthesis of lost extracellular matrix components. Cells in the JCT and inner wall are known to synthesize
proteoglycans and other extracellular matrix materials.\(^{35}\) Proteoglycans undergo a continual turnover in the trabecular meshwork, with a half-life of 36 hours.\(^{35}\) The return of outflow facility, which took approximately 14 hours, is compatible with the synthesis of new components. The failure of a second dose of cytochalasin D to affect outflow facility may be related to the continued absence of the inner wall cells in specific regions. With no cell to disrupt, cytochalasin D would not be effective. This failure of the second dose would require that most culture medium would continue to flow through these regions, presumably because of preexisting preferential flow pathways within the meshwork.\(^{19,36-58}\) Such preferential flow pathways have been proposed from studies of the distribution of cationic ferritin within the meshwork\(^{19}\) and also surmised from the patchy distribution of giant vacuoles around the circumference of the eye.\(^{38}\)

A correlation was found between the final IOP of the anterior segments in this study and the length of the optically empty spaces, or gaps, in the basement membrane and subendothelial matrix of the inner wall. The basement membrane and subendothelial matrix are known to be discontinuous,\(^{38}\) with the optically empty spaces occupying ~25% of the length of the inner wall, as was measured in the control anterior segments. The relatively small correlation coefficient between the final IOP and the length of these optically empty spaces, however, \(r = -0.38,\) indicates that other factors are also involved in the creation of the outflow resistance; aqueous humor apparently does not flow freely through these gaps. These other factors are unknown but may involve proteoglycans or other substances within the gaps that are not visualized with conventional fixation and processing,\(^{39}\) the flow of aqueous through nongap regions of the basement membrane, resistance offered by the inner wall endothelial cells, or proteoglycans within the optically empty spaces of the entire JCT.\(^{39}\) It is interesting to speculate that if significant resistance to aqueous outflow were offered by the basement membrane and subendothelial matrix of the inner wall, which remained intact despite the persistent loss of the inner wall cells, these gaps would make the basement membrane of the inner wall leakier than basement membranes in other tissues. A comparison of the hydraulic conductivity of basement membranes in other tissues with that of the aqueous outflow system of the eye is in keeping with this: Bruch’s membrane\(^{40}\), \(0.6 \times 10^{-12}\) cm\(^4\) sec\(^{-1}\) dyn\(^{-1}\), renal glomerulus capillary wall\(^{41}\), \(0.47 \times 10^{-12}\) cm\(^4\) sec\(^{-1}\) dyn\(^{-1}\), an aqueous outflow resistance of 3 mm Hg/\(\mu\)l per minute converts to a hydraulic conductivity of \(11 \times 10^{-12}\) cm\(^4\) sec\(^{-1}\) dyn\(^{-1}\) (original data of Bruch’s membrane and renal glomerulus also converted to units of hydraulic conductivity).

In results of two other studies a correlation has been seen between ultrastructural findings in the JCT and outflow. Findings in a study in monkey eyes showed that the area of optically empty space immediately adjacent to the inner wall correlated with the outflow facility.\(^{56}\) In a study of human eyes, there was a significant inverse correlation between the area of amorphous basement membrane under the inner wall and the outflow facility in a series of 11 eyes with primary open-angle glaucoma.\(^{52}\) No correlation has been noted between the total amount of optically empty space within the entire juxtacanalicular region and the aqueous outflow facility in normal or glaucomatous human eyes.\(^{39,43-46}\) Similarly, no correlation was seen between the total amount of optically empty space and final IOP in the current study.

**Key Words**

aqueous humor outflow, cytochalasin, morphometry, organ culture, trabecular meshwork

**References**

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