The barrier function of endothelia lining Schlemm’s canal has received recent interest due to its role in the potential obstruction of aqueous humor flow (27, 29). Alterations in cell contraction, cell volume and shape, phagocytosis, and other activities that could potentially affect transendothelial fluid flow have been described (20, 22, 51, 56, 59, 69, 75). However, there are no reports in which direct effects of glucocorticoids on the barrier function of SCE and TM cells have been studied. This article seeks to measure the effect of glucocorticoids on the barrier function of SCE and TM cells and to investigate the underlying cellular mechanisms.

Fluid flow through the paracellular pathway of endothelial cells appears to take place through preferential flow channels within the intercellular space (ICS) known as interendothelial “gaps” (1, 13, 14, 25, 40, 53, 57, 60, 74, 76). Gaps have been shown to undergo changes that accurately mirror alterations in endothelial permeability after the application of vasoactive agents and the activation of cytoskeletal elements (11, 14, 46, 74, 77). Intercellular junctions, especially tight junctions (TJs), play an important role in the regulation of the permeability of the paracellular pathway (53–26, 33, 37, 47, 49, 61), at least in part by modulating gap formation (14, 53, 74). Therefore, our experiments measure the number and size of gaps and TJs present within the paracellular pathway of SCE and TM cells.

Recently, the protein composition of TJs has also been related to the resistance of some cellular barriers. TJs contain a complex array of proteins, including ZO-1,ZO-2, occludin, cingulin, rab 13, and 7H5 (8, 19, 25, 26, 35, 37, 41, 81). Increased expression of ZO-1 (68, 79) and occludin (10) has been correlated with increased paracellular resistance. In addition, in some cell types the presence of a greater proportion of one of two ZO-1 isoforms is associated with higher resistance (9). Of particular interest is the observation that, in mouse mammary epithelial cells, glucocorticoid treatment results in the increased expression of ZO-1 and a greater transepithelial electrical resistance (68, 79). To begin to assess the importance of ZO-1 in the dexamethasone (Dex)-mediated regulation of fluid flow, its expression was blocked using antisense oligonucleotides, and the effect on transendothelial fluid flow, TJs, and gaps was assessed.

Here we report that the glucocorticoid Dex acted directly on monolayers of cultured human SCE and TM cells to increase the resistance to transendothelial fluid flow. The increased resistance was associated with the following molecular and cellular findings: 1) increased synthesis of the ZO-1 protein in both cell types, with a different isoform expression in the more resistive SCE and TM cells; and 2) increased resistant associated with glucocorticoid exposure.
than in TM cells, 2) increased representation of TJ s, and 3) a narrowing of the paracellular route measured as a marked decrease in the number and size of gaps. Moreover, we report that an antisense oligonucleotide sequence that suppressed ZO-1 expression blocked the Dex-induced increase in transendothelial fluid flow resistance as well as accompanying changes in TJ s and gaps.

MATERIALS AND METHODS

Cell Culture

TM cell lines obtained from eyes of 14- and 30-yr-old nonglaucomatous donors and SCE cell lines obtained from eyes of 17- and 45-yr-old nonglaucomatous donors were used for these studies. The TM cells were dissected from the corneoscleral and uveoscleral meshworks according to methods previously described and stored as frozen stocks of fourth-passage cells (2, 4, 5, 57, 66, 78). The SCE explants were dissected from Schlemm's canal in the following manner. Several small blocks (e.g., 3 × 6 mm) of deep corneoscleral tissues were cut with a 45° "super" blade (I-Knife, Alcon Surgical, Fort Worth, TX). Schlemm's canal was identified by cannulating its lumen with a 9-0 nylon suture under microscopic observation. The corneoscleral and uveoscleral TM tissues were removed as completely as possible by dissection with sharpened jeweler's forceps. In each of the small blocks the nylon suture was then moved forward toward the anterior-most portion of the canal lumen (i.e., toward the deep corneolimbus) to tear the canal longitudinally (4). The 9-0 nylon suture was then pulled inwardly to open the canal along its anterior extension. A super blade was used to cut the inner from the outer wall.

The inner wall explants were placed on dishes coated with extracellular matrix secreted by cultured bovine corneal endothelial cells, as originally described by Gospodarowicz et al. (36). This matrix was used to promote selective growth of SCE cells and retard growth of TM and other cell types. The inner wall explants were grown in medium 199 supplemented with 15% FCS and fibroblast growth factor (250 ng/ml). Sheets of confluent monolayers of SCE cells were grown separately on 10-cm plastic dishes containing DMEM with 15% fetal bovine serum (Hyclone Laboratories, Bedford, MA) for hydraulic conductivity (HC) measurements, and at the fourth passage the cells were stored as frozen stocks.

The TM and SCE stocks were defrosted, and each cell type was grown separately on 10-cm plastic dishes containing DMEM with 15% fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 50 µg/ml gentamicin, and 2.5 µg/ml amphotericin B. Just before confluence, these fifth-passage cells were dissociated using 0.05% trypsin, 0.02% EDTA, 0.1% glucose, and 0.58 g/l NaHCO3, and 5.0 µg/ml amphotericin B. Just before confluence, these fifth-passage cells were dissociated using 0.05% trypsin, 0.02% EDTA, 0.1% glucose, and 0.58 g/l NaHCO3, and 5.0 × 104 cells/cm2 were seeded onto 1) permeable methylcellulose filters (0.45-mm pores; catalog no. PIHA01250, Millipore, Bedford, MA) for hydraulic conductivity (HC) measurements, 2) four-well plastic slides (Lab-Tek 177437, Nunc, Naperville, IL) for immunofluorescence, and 3) 6-cm dishes for Western blots. When cells formed confluent monolayers (~day 14), the fetal bovine serum concentration was decreased to 10% and the cells were fed every 2nd day with fresh media with or without Dex.

HC

The Millipore filters containing confluent monolayers of SCE or TM were perfused with media by use of the flowmeter instrument diagrammed in Fig. 1 to measure transendothelial flow. This flowmeter was modified from one previously developed in our laboratory (2, 4, 57). The perfusion device has three components arranged in series consisting of a fluid column, a flowmeter, and the cell monolayer to be tested, as shown in Fig. 1. The fluid column has a siphon to maintain its height at a constant hydrostatic level (usually 5.5 mmHg). The flowmeter consists of two pressure gauges, P1 and P2 (Honeywell, Minneapolis, MN), and a "resistor" made up of a section of microbore Teflon tubing (Cole-Parmer, Vernon Hills, IL) of a standard length (230 mm) and bore (0.008 in.). This resistor has a resistance of ~0.5 mmHg·µl−1·min, which optimizes the sensitivity of the meter when flow rates are in the microliter per minute range. P1 reads the pressure generated by the fluid column, and P2 reads the pressure at the surface of the cell monolayer. P2 reflects the rate of flow through the resistor: with low flow rates, P2 will approach P1; with high flow rates, P2 < P1, P2 also represents transendothelial pressure. Since atmospheric conditions exist on the basal side of the filter.

Flow (Q, µl/min) through the resistor was calculated using a derivation of Poiseuille's equation: Q = ΔP/R, in which ΔP is P1 − P2, and R is the known resistance of the Teflon resistor (~0.5 mmHg·µl−1·min). Because the entire system is arranged in series, flow through the resistor equals that through the cell monolayer. HC was determined as follows: HC = Q/(P2 − A), where A is the surface area of the cell monolayer (0.6 cm2) and HC is expressed as microliters per minute per mmHg per square centimeter, a unit that is equivalent to 1.25 × 10−5 cm·s−1·cmH2O−1 or 1.25 × 10−8 cm2·s−1·dyn−1. The contribution of the filter (0.04 mmHg·µl−1·min) was subtracted to yield the HC of the cell monolayer, as previously described (4, 57). Pressure gauge outputs were collected 10 times per second, digitized, and analyzed by a microcomputer, and flow measurements were averaged over 5-min intervals.

The flowmeters were calibrated before and after each experiment, and their function was monitored throughout each study. P1 and P2 were calibrated to hydrostatic pressure levels. The accuracy of the flowmeters was tested by compar-
ing flow rates for a given time and pressure obtained by weighing the water output with flow rates provided by the flowmeter instrument. In a typical comparison, in five Teflon resistors of standard dimensions, the average flow rate was 1.7 ± 0.09 (SD) µl/min measured by the flowmeter instrument and 1.71 ± 0.09 µl/min measured by weighing the water output. The two methods yielded measurements that were not significantly different (P = 0.8). During each experiment, one of the flowmeter stations was connected to a test resistor of known HC to ascertain the stability and accuracy of the flowmeter device. The other stations were monitored at the end of the experiment in a similar manner to confirm that the flowmeters had functioned properly.

When HC is determined in the cultured SCE and TM cells, the values obtained depend on a number of factors, including length of time in culture, direction of perfusion, and cell type. For instance, both cell types tend to reach their most resistive state ~2 wk after confluence. After a variable number of days, conductivity may increase as a function of time (3, 4, 55). At the most resistive state, when the cells were perfused from the apex toward the base, the HC was 0.5–1.5 and 1.0–3.0 µm²/mg · mm · h−1 · cm−2 for SCE and TM cells, respectively. In the studies reported here, cells were perfused 2 or 7 wk after seeding.

The perfusion direction was particularly important for SCE cells, because perfusion from the base toward the apex results in the formation of giant vacuoles and pores (54). These structures provide the SCE cells with a transcellular pathway in addition to a paracellular route and alter their HC. Because the present study focused on flow through the paracellular pathway, cells were perfused from the apex to the base, the same direction used to determine permeability in blood vessels.

**Morphometry**

The Millipore filters containing confluent monolayers of SCE and TM cells were fixed with 1% paraformaldehyde and 1% glutaraldehyde containing 1 mM MgCl₂ and 0.067 M sodium cacodylate buffer at pH 7.3 (4, 57). The filters were removed from their mountings with a super blade, postfixed in 1% OsO₄, stained en bloc with 0.5% uranyl acetate in 70% ethanol, dehydrated with ethanol of increasing concentrations, and embedded in LX112 (Ladd, Burlington, VT). The filters were cut in cross section, and the thin sections were mounted on one-slot grids, stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope (TEM; model 100C, J EOL, Tokyo, J Japan). The micrographs were printed at magnifications of ×12,500, ×25,000, ×35,000, and ×50,000.

**Intercellular junctions.** The intercellular junctions were studied and classified using TEM. In an initial evaluation, intercellular junctions of all types present within a given ICS were identified. The frequency of TJ's was assessed by determining the number of profiles that contained structures in which the outer leaflet of adjacent cell membranes were fused (33). The length of each TJ was determined by measuring the aggregate length of only those portions of a TJ where the ICS was occluded by fusion of the outer cell membrane leaflets. The number of macula adherens and gap junctions was also counted on each profile of the ICS. For morphometry, segments of the paracellular route in transmission electron micrographs (at >25,000) taken at random by an individual who was masked to treatment and cell type were evaluated in the following manner. In the initial set of experiments, ~40 transmission electron micrographs were taken of each filter for DEX-treated and untreated SCE and TM preparations. In a second set of experiments, in which the cells had been treated with antisense phosphorothioate oligonucleotides (PONs) to ZO-1, ~20 transmission electron micrographs were taken for each treatment condition.

The scanning and transmission electron micrographs were digitized using a Sigma Scan program (Jandel Scientific, Corte Madera, CA). For comparison of treated with untreated controls, ² and t-tests were performed (4).

**Intercellular gaps.** The ICS was evaluated using scanning electron microscopy (SEM). The filters, fixed according to the protocols used for TEM, were postfixed in 1% OsO₄, dehydrated in ethanol, critical point dried, sputter coated with Au/Pd, and photographed (model J SM-840A, J EOL (4). Survey scanning electron micrographs of the ICS in both cell types were taken at magnifications of ×1,000–3,000. Subsequently, 24 micrographs from randomly selected portions were taken at ×3,000 by an individual masked as to cell type and treatment from one filter prepared each for DEX- and antisense-treated and control SCE and TM samples.

The scanning electron micrographs for all treatment groups were mixed, and then an individual masked as to cell type and treatment traced the cell perimeters for a standard length of 2,800 µm (4). Then intercellular gaps or preferential flow channels (1, 57) consisting of rounded intercellular openings along this standard length were counted and measured to determine gap incidence (number/photograph), size (area/opening in µm²), and aggregate area (µm²/2,800 µm).

**ZO-1 Localization and Expression**

Immunofluorescence. Cells grown on four-well plasticslides were washed in PBS with calcium, fixed for 10 s in methanol chilled to −20°C, and washed twice in PBS. The slides were further washed in 0.1 M glycine for 1 min, ovalbumin (1 mg/ml in PBS; Sigma Chemical, St. Louis, MO) for 30 min, 3% Carnation nonfat dry milk in PBS for 30 min, and 5% sheep serum (Dako, Carpenteria, CA) for 30 min. Then the slides were incubated for 1 h with rabbit anti-human ZO-1 antibody (Zymed, South San Francisco, CA) diluted 1:200 in PBS. Cells were washed four times with PBS over a 30-min period, incubated for 60 min with a 1:12,000 dilution of biotinylated donkey anti-rabbit antibody IgG (Jackson Immuno Research Labs, Westgrove, PA) in PBS, and washed four times in a 30-min period in PBS. Samples were then incubated for 1 h in a 1:200 dilution of Cy3-conjugated streptavidin (J. Jackson Immunology, Westgrove, PA), washed several times in PBS, and kept in darkness until examined with a Zeiss photomicroscope equipped with epifluorescence, as described previously.

PCR and Western blots. RT-PCR assays were performed according to the following protocol. RNA was extracted from SCE and TM cells, and mRNA was reverse transcribed to generate cDNA (65). The following primers were used for PCR amplification of ZO-1 sequences: 5’-CATAGAATAGACTCCCCTGG-3’ and 5’-GCCCTGAAGCTGATCCTGCACT-3’ (31). These primers yielded two bands (234 and 474 bp), which represent the two ZO-1 isoforms, ZO-1α and ZO-1α* (10). PCR amplification for actin was done according to published methods (65). Compared samples were adjusted to contain equivalent amounts of total RNA and actin mRNA.

Cells grown in 6-cm dishes were rinsed with Tris-buffered saline (TBS; 10 mM Tris, pH 7.5, 150 mM NaCl) and 500 µl of TBS containing the following proteinase inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin, 2 µg/ml leupeptin, 4 µg/ml aprotinin, 10 µg/ml antipain, 50 µg/ml benzamidine, 10 µg/ml soybean trypsin inhibitor, 100 µg/ml iodoaceticamide, and 1 mM EDTA. The cells were scraped from the dishes with a rubber policeman and centrifuged at 2,000 rpm for 3 min. TBS (50–150 µl) with the above inhibitors was added to the pellets (72), which were then vortexed. The
protein concentration was determined using the DC Protein Assay (Bio-Rad, Hercules, CA), and then equivalent amounts of protein were added to each well. Sample buffer (50 mM Tris, pH 6.8, 10% SDS, 20% β-mercaptoethanol, 20% sucrose) was added to each treatment group (10 µl sample buffer/100 µl cell extract). The cell extract was boiled for 2 min, forced through a 27-gauge needle several times, applied to a 7.5% polyacrylamide gel, and transferred to nitrocellulose.

The nitrocellulose membrane was washed in PBS, incubated in 10% Carnation nonfat dry milk with 0.1% Tween 20 in PBS for 1 h, washed in PBS, and incubated overnight with a 1:2,000 dilution of rabbit anti-human ZO-1 antibody (Zymed) in PBS. The membrane was washed in PBS with 0.1% Tween 20 three times, each for 10 min, and incubated for 1 h with the secondary antibody goat anti-rabbit-peroxidase (Hyclone Laboratories). The membrane was rinsed again in PBS three times, each for 10 min. The ZO-1 bands were visualized on X-ray film with use of enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL). The same protocol was used on the same cell extracts but probed with mouse anti-β-actin antibody (catalog no. A5441, Sigma Chemical) diluted 1:5,000 in PBS. The nitrocellulose was incubated with the mouse anti-β-actin antibody, then with the peroxidase-conjugated anti-mouse antibody (Jackson Immuno Research Laboratories). The bands were visualized on X-ray film with use of enhanced chemiluminescence reagents (Amersham). Intensity of the bands was quantified by densitometry with use of a densitometer (LKB Bromma, Pharmacia).

**Dex Treatment**

Preparations of the SCE and TM cells grown over Millipore filters, plastic slides, and 6-cm dishes were treated with Dex (Sigma Chemical). Dex was dissolved in absolute ethanol to make a 0.01 M stock solution, which was further diluted in culture media to make the 500 nM Dex used to treat the cells every other day. This concentration of Dex has also been used by other investigators in experiments with cultured human TM cells (2, 58). In studies similar to ours, cultured mammalian epithelial cells were treated with 1,000 nM Dex (68, 79). Investigators studying cytoskeletal effects associated with Dex exposure have used as little as 100 nM (21, 22) or as much as 100 mM Dex (56).

**ZO-1 Antisense Treatment**

The cultured cell preparations were also treated with ZO-1 antisense PONs and sense and nonsense PONs. The sequence selected for synthesis of antisense PONs, 5′-CTCTTTCTGTTGAGGGTCT-3′, corresponds to the segment from base pair 3154–3169 in MUSZO1 accession number D14340I. The complementary sense PON, 5′-AGCTCTCAACAGAAATGCAG-3′, and a random-order nonsense PON, 5′-ATTGCTACGGTGCTCTTGGT-3′, were used as controls (PONs synthesized by Oligo, Portland, OR). In these experiments, treatment was begun 1 h after seeding before the establishment of cell-cell interactions and the assembly of TJ s.

Preliminary experiments were done to select the concentration of antisense PONs (8 µM) that would induce at least a twofold change in HC above untreated controls. Sense and nonsense PONs at this dose did not produce a change in HC.

**RESULTS**

**Dex Treatment**

Confluent monolayers of cells were treated with 500 nM Dex every other day for 2–7 wk. At the end of the treatment period, the HC, cell junctions, intercellular gaps, and expression of a junction-associated protein, ZO-1, were compared in treated and control cells.

HC. In multiple experiments in which 500 nM Dex was administered for ≥2 wk, the HC of SCE and TM cells was consistently reduced. The results from one experiment in which cells were treated for 7 wk are shown in Fig. 2 (n = 6–8 millicells in each group). HC (µl/min·mmHg·cm−2) was significantly reduced in both cell types (P < 0.001), with a three- to fivefold increase in resistance in the cultured cells. Similar results from other experiments in cells treated with Dex for 2 wk are described below.

**Intercellular junctions.** Three types of junctions, including TJs, macula adherens, and gap junctions, were present in the cultured SCE and TM cells (Fig. 3). The same three junction types have been described in Schlemm’s canal and the TM tissues in vivo (16, 63). In the cultured cells, TJs were the most commonly observed type of junction. TJs were located along the apicolateral region (Fig. 3A), where their presence markedly reduced the width of the ICS (25, 26) (Fig. 3B). The TJs did not form a continuous belt or zonula around the cell periphery; instead, they comprised short segments or, according to the nomenclature of Farquhar and Palade (33), fascia that were interspersed between areas devoid of junctions. Macula adherens and gap junctions (Fig. 3, C–E) were usually located toward the midpoint or basal side of the ICS.

The prevalence of TJs was assessed using morphometric approaches, as described in MATERIALS AND METHODS (Table 1). TJ s could be identified in 50% of ICS profiles in control SCE cells (19 of 38); this proportion increased to 82% (32 of 39) with Dex treatment. A similar relationship was observed in TM cells, where in controls TJs were present in 1% of ICS profiles (37 of 53) compared with 85% in Dex-treated cells (41 of 48). Thus nearly twice as many ICS profiles contained TJs in Dex-treated cells as in controls regardless of the cell type (χ², P < 0.005).

SCE cells had longer TJs than TM cells, and this difference was accentuated by Dex treatment, so that TJs were significantly longer in Dex-treated SCE than in TM cells (P < 0.001; Table 1). When the number and length of TJs s were considered together as the mean

![Fig. 2](http://ajpcell.physiology.org/)
aggregate length, there was a twofold increase in Dex-treated SCE and TM cells compared with controls (Table 1).

Adherens and gap junctions were observed less frequently than TJs, and Dex treatment also appeared to have an effect on their number (Table 1). The proportion of the ICS containing adherens junctions increased significantly in the SCE cells treated with Dex (10 vs. 44%), but this increase was not significant in TM cells (42 vs. 52%, P = 0.09). There were many more adherens junctions in untreated TM cells (22 of 53) than in untreated SCE cells (4 of 38). Regarding gap junctions, we noted very few such junctions in all treatment groups; however, significantly more gap junctions were observed in Dex-treated cells than in controls (P < 0.001).

Intercellular gaps. The ICS along the apical cell borders, examined by SEM, was quite narrow, except at sites of round openings, through which the underlying filter support was clearly visible (Fig. 4A).

Table 1. Frequency of each junction type assessed by determining number of ICS profiles containing that structure

<table>
<thead>
<tr>
<th></th>
<th>SCE Cells</th>
<th></th>
<th>TM Cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Dex</td>
<td>P</td>
<td>Control</td>
</tr>
<tr>
<td>TJs frequency</td>
<td>19/38 (50)</td>
<td>32/39 (82)</td>
<td>&lt;0.005*</td>
<td>27/53 (51)</td>
</tr>
<tr>
<td>TJs length, µm</td>
<td>0.541 ± 0.765</td>
<td>0.819 ± 0.582</td>
<td>&lt;0.15†</td>
<td>0.336 ± 0.363</td>
</tr>
<tr>
<td>TJs aggregate length, µm</td>
<td>10.3</td>
<td>26.0</td>
<td>&lt;0.001*</td>
<td>9.0</td>
</tr>
<tr>
<td>Macula adherens frequency</td>
<td>4/38 (10)</td>
<td>17/39 (44)</td>
<td>&lt;0.001*</td>
<td>22/53 (42)</td>
</tr>
<tr>
<td>Gap junction frequency</td>
<td>1/38 (3)</td>
<td>6/39 (15)</td>
<td>&lt;0.001*</td>
<td>1/53 (2)</td>
</tr>
</tbody>
</table>

Values for tight junction (TJ) length are means ± SD; values in parentheses are percentages. TJ length in endothelia lining Schlemm's canal (SCE) and trabecular meshwork (TM) cells treated with and without dexamethasone (Dex) was determined by measuring and adding together individual segments where intercellular space (ICS) was occluded by apposition of membrane outer leaflets. Only TJ's within first 3 µm of apical side of cells were counted. SCE cells were treated for 60 days; TM cells were treated for 20 days. TJ's were measured in transmission electron microscopes at ×25,000. P values are for χ² (*) or unpaired t-tests (†) between means of treated and untreated groups.
ings are referred to as gaps and closely resemble those of other endothelial cells, in that they were found between overlapping filipodia or filopodia (14, 53, 74). The filopodia extended from one cell border, across the ICS, and onto the surface of the neighboring cell (Fig. 4A). TEM showed that these filopodia were bound to the underlying apical surface of neighboring cells by numerous TJ s (Fig. 3A).

Simple observation of the gaps by SEM suggested more gaps in control cells than in Dex-treated cells of both types (Fig. 4). Morphometric analyses showed marked differences, inasmuch as Dex treatment was associated with an eightfold reduction in the number of gaps in SCE cells (76 vs. 9) and a fourfold reduction in TM cells (118 vs. 29, P < 0.005) compared with controls (Table 2). Gaps were also more frequent and larger in the more conductive TM cells than in SCE cells, and their size decreased three- to fourfold in both cell types with Dex treatment (P < 0.002). Given the reduction in gap number and size, the mean aggregate area of gaps was reduced 30-fold in SCE cells and 13-fold in TM cells with Dex treatment (Table 2).

ZO-1 expression. To investigate the role of the junction-associated protein ZO-1 in the Dex-induced increase in TJ s and in fluid flow resistance, the location and expression of this protein were assessed by immunofluorescence and Western blots.

ZO-1 was localized by immunofluorescence at the borders of TM and SCE cells (Fig. 5). Slight differences were observed in staining in Dex-treated (D) cells, where the staining appeared to be more intense than in controls (C). Nuclear staining also appeared to be more intense in Dex-treated cells.

RT-PCR demonstrated that one ZO-1 isoform was present in TM cells, whereas two isoforms were present in SCE cells, suggesting a splicing event in the mRNA of SCE cells (Fig. 6). The presence of different ZO-1 isoforms in SCE and TM cells was confirmed by Western blots (Figs. 6 and 7).

Dex treatment appeared to induce an increase in ZO-1 expression in SCE and TM cells (Fig. 7). Comparison of bands among lanes with equivalent amounts of actin in the cell extracts, as shown in Fig. 7, demonstrated more intense ZO-1 bands in Dex-treated cells (Fig. 7, lanes C and D). Band densitometry showed that the induction amounted to a three- to fivefold increase in ZO-1 expression with Dex treatment.

In summary, data from this set of experiments showed that the administration of Dex to SCE and TM cells resulted in the increased expression of ZO-1, the formation of a greater number of TJ s, and a decrease in the width of the paracellular pathway measured as an alteration in gaps as well as an increase in fluid flow resistance.

ZO-1 Antisense Treatment

To consider the involvement of ZO-1 apart from other steroid influences, its expression was blocked using antisense PONs. Treatment of SCE and TM cells was begun 1 h after the cells were seeded and before the formation of cell junctions (25). The cultured cells received antisense, sense, and nonsense PONs with and without Dex for 2 wk, then morphological, physiological, and biochemical analyses were performed.

ZO-1 expression. ZO-1 expression in cells treated with antisense and sense PONs was compared with cells that were not exposed to PONs in Fig. 5. Antisense treatment clearly reduced the intensity of ZO-1 staining around the periphery of the cells (CA and DA, arrow) compared with that of cells treated with sense PONs (CS and DS), as shown by immunofluorescence. There also appeared to be an increase in nuclear

| Table 2. Number of interendothelial gaps or rounded openings in paracellular route and their size in cultured SCE and TM cells treated with and without Dex |
|---------------------------------|--------|--------|--------|--------|
|                                | SCE Cells | TM Cells |
|                                | Control  | Dex    | P      | Control  | Dex    | P      |
| Gap number                      |          |        |        |          |        |        |
|                                  | 76       | 9      | <0.005*| 118      | 29      | <0.005*|
| Gap size, µm²                   | 1.25 ± 1.14 | 0.35 ± 0.22 | <0.002† | 4.68 ± 8.89 | 1.48 ± 2.80 | <0.002† |
| Gap aggregate area, µm²         | 95       | 3      |        | 551      | 43      |        |

Values for gap size are means ± SD. In 24 scanning electron micrographs per treatment group or 96 micrographs for each cell type, number of gaps was counted and normalized to a standard length (2,800 µm) of paracellular pathway. Area covered by each gap was measured, and mean gap area and aggregate area were calculated. P values are for χ² (*) and unpaired t-tests (†) between mean gap areas in treated and untreated groups. Both cell types were treated with Dex for 20 days.
staining with Dex exposure, which could mean that Dex also induces a change in ZO-1 localization.

There was a marked inhibition of ZO-1 expression after 2 wk of antisense treatment, as shown in Western blots (Fig. 7, A and B). In SCE cells, treatment with antisense PONs (Fig. 7A, CA) resulted in a three- to fivefold reduction in the intensity of the ZO-1 bands compared with untreated (C) or sense-treated controls (CS). The addition of Dex to antisense-treated cells (DA) yielded a band of greater intensity than its control (CA) but of lesser intensity than untreated controls (C). The intensity of actin bands in these cell extracts did not change significantly. These findings indicate that antisense PONs inhibit the induction of ZO-1 by Dex, whereas sense PONs do not.

In TM cells, treatment with antisense PONs also resulted in a three- to fivefold reduction in the intensity of the ZO-1 band compared with untreated (Fig. 7B, CA and C) or sense- and nonsense-treated controls (Fig. 7B, CS and CN). Again, there was a partial suppression of the glucocorticoid induction of ZO-1, inasmuch as Dex treatment yielded a signal of greater intensity than its control (Fig. 7B, CA). Dex treatment increased the expression of ZO-1 by as much as fivefold in the presence of sense and nonsense PONs (CS and DS, CN and DN). The intensity of actin bands did not signifi-
Fig. 6. RT-PCR for ZO-1 (left) yielded 2 ZO-1 bands in SCE cells, suggesting presence of a splicing event. Only 1 ZO-1 band was detected in TM cells. Total RNA extracted from treated and untreated SCE and TM cells was adjusted so that equal amounts of actin RNA were added to each PCR reaction. Western blots for ZO-1 (right) showed that SCE cells expressed 2 ZO-1 bands ([ZO-1α+ (~225 kDa) and ZO-1α- (~215 kDa)] and that TM cells expressed only 1 ([ZO-1α-]). Increased intensity of bands in Dex-treated cells suggested an increase in ZO-1 expression as equal amounts of protein (25 μg) were added to each well. CON, control.

Fig. 7. Western blots in SCE and TM cells suggested increased ZO-1 expression in response to Dex treatment and decreased ZO-1 expression in response to antisense PONs treatment. A standard amount of protein (25 μg) from each treatment group was analyzed by SDS-PAGE and visualized by immunochemical analysis. Immunoblots for protein (25 μg) from each treatment group was analyzed by SDS-PAGE in response to antisense PONs treatment. A standard amount of protein (25 μg) were added to each well. CON, control.

TJ formation. The impact of ZO-1 inhibition on TJs was assessed by morphometric methods (Table 3). Antisense PONs treatment of SCE cells for 2 wk resulted in a reduction in the percentage of ICS profiles with TJs from ~52% in untreated and sense-treated controls (C and CS) to ~28% in antisense-treated cells (CA and DA; Table 3). When alterations in the number and size of TJs were considered together, the mean aggregate TJ length decreased 6- to 10-fold with antisense treatment compared with controls. These data suggest that the antisense PONs had a pronounced effect on the formation and development of TJs.

Antisense treatment also blocked the induction of TJs by Dex (Table 3). When Dex was used together with antisense PONs, there was no significant difference in the percentage of ICS profiles with TJs or in their aggregate length (CA and DA). These findings support an important role for ZO-1 in the glucocorticoid-induced increase in TJ formation.

In cells treated with Dex alone, the percentage of ICS profiles with TJs was increased (Table 3) in a manner similar to that described in the previous experiment (Table 1). The percentage of ICS profiles with TJs increased twofold from ~50% in controls (C and CS) to ~90% in Dex-treated cells (D and DS; χ², P < 0.01), whereas the mean aggregate TJ length increased three- to fivefold.

The TJs in the present experiment with SCE cells differed from those in the previous experiment in several respects. Treatment with Dex for 2 wk (Table 3) resulted in TJs that were shorter than those treated for 60 days (Table 1). Dex treatment resulted in a doubling of the TJ length compared with untreated controls (0.186 vs. 0.34) or sense-treated preparations (0.147 vs. 0.396; Table 3). Consequently, the aggregate TJ length increased threefold with Dex treatment alone (1.86 vs. 6.12) or fivefold with both Dex and sense PONs (1.90 vs. 9.5; Table 3). In comparison, the aggregate length of SCE cells treated with Dex in the previous experiment (Table 1) increased less than twofold.

Intercellular gaps. The effect of a reduction of ZO-1 expression on the formation of intercellular gaps is shown in Table 4, where the number and size of widened openings in SCE cells treated with antisense PONs (CA and DA) were compared with those in cells treated with sense PONs (CS and DS). In the sense-treated cells, Dex induced a 5-fold decrease in the number of gaps (108 vs. 20) and a 4-fold decrease in their size (2.01 vs. 0.53), which resulted in a 20-fold reduction in the mean aggregate area of gaps. This finding is similar to that observed in cells treated with Dex alone, as shown in Table 2, where the SCE cells exhibited a 30-fold decrease in gaps. In cells treated with antisense PONs (CA and DA), Dex did not decrease significantly the number (134 vs. 94), size (2.3 vs. 2.2), or aggregate area (308 vs. 206) of gaps, suggesting that the antisense PONs effectively prevented the Dex-induced increase in gaps.

HC. The impact of inhibiting ZO-1 on the resistance of the SCE cells is shown in Fig. 8A. Dex induced a decrease in HC (Fig. 8, C and D) in these cells treated for 2 wk that was similar to the decrease observed in SCE cells treated for 7 wk (Fig. 2). The Dex effect on HC persisted in the presence of sense PONs (CS/DS; P < 0.002). However, in cells treated with both Dex and antisense PONs (DA), HC values were significantly higher than in cells treated with Dex alone or Dex and sense PONs (D and DS; P < 0.001). These results suggest that the addition of antisense PONs not only
GLUCOCORTICOIDS MODULATE JUNCTIONS AND FLOW RESISTANCE

DISCUSSION

The events identified in our experiments provide a rational paradigm for considering how glucocorticoids could increase the resistance presented by the SCE and TM cells to the outflow of aqueous humor in steroid glaucoma. The Dex-induced increase in fluid flow resistance was associated with an increased expression of the junction-associated protein ZO-1, a doubling of the number of TJs, and a marked reduction in the space occupied by intercellular gaps or preferential flow channels. The importance of ZO-1 in these glucocorticoid-mediated events was illustrated by experiments with antisense oligonucleotides in which ZO-1 expression was suppressed. This maneuver alone was sufficient to prevent the Dex-induced increase in resistance and attendant cellular alterations, suggesting that ZO-1 is essential for the glucocorticoid regulation of transendothelial fluid flow.

Dex has previously been shown to induce ZO-1 and to increase the electrical resistance across mammary epithelial cells (68, 79). However, this is the first report in which the reduction in fluid flow induced by Dex was related to the status of intercellular junctions, gaps, and ZO-1. The relationship between the expression of junction-associated proteins, formation of TJs, and flow resistance has also been studied by Balda et al. (10). These investigators observed that increasing the expression of occludin caused an increase in resistance but no detectable alterations in TJs. The reason we were able to detect an increase in TJs may be related to the different cell types used in our studies. The Madin-Darby canine kidney epithelial cells used by Balda et al. have very well-developed TJs that extend around the cell circumference forming a belt or zona occludens (10). In such cells, every profile of the ICS, viewed in cross section by TEM, would have a TJ, and our methods would not have been able to detect an increased representation of TJs with Dex treatment. In SCE cells, only a subset of ICS profiles contained TJs, and therefore an increase in their representation could be easily detected.

The time course and magnitude of the reduction in conductivity in our in vitro model were similar to those observed in steroid glaucoma. In our experiments the increase in resistance became well established after the cells had received 7 wk of Dex treatment. Similarly, steroid glaucoma usually develops after several weeks of topical glucocorticoid administration, and at this point in the disease course, a subset of TJs was noted to be associated with drug-resistant flow paths.

It is likely that similar phenomena may occur in the SCOC, although it is not known whether this tissue expresses ZO-1. In the SCOC, TJs are thought to form a network by which the cell circumference forms a belt or zona occludens (10). In such cells, every profile of the ICS, viewed in cross section by TEM, would have a TJ, and our methods would not have been able to detect an increased representation of TJs with Dex treatment. In SCE cells, only a subset of ICS profiles contained TJs, and therefore an increase in their representation could be easily detected.

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Table 3. Frequency of TJs assessed by determining number of ICS profiles in sense and antisense-treated SCE cells that contained TJs

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>P</th>
<th>CS</th>
<th>DS</th>
<th>P</th>
<th>CA</th>
<th>DA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TJ freq</td>
<td>10/19 (53)</td>
<td>18/20 (90)</td>
<td>&lt;0.01*</td>
<td>13/25 (52)</td>
<td>24/26 (92)</td>
<td>&lt;0.002*</td>
<td>7/25 (28)</td>
<td>6/21 (29)</td>
<td>&lt;0.34*</td>
</tr>
<tr>
<td>TJ length, µm</td>
<td>0.186 ± 0.210</td>
<td>0.34 ± 0.225</td>
<td>&lt;0.04†</td>
<td>0.147 ± 0.160</td>
<td>0.396 ± 0.196</td>
<td>&lt;0.001†</td>
<td>0.049 ± 0.086</td>
<td>0.093 ± 0.140</td>
<td>&lt;0.22†</td>
</tr>
<tr>
<td>TJ aggregate length, µm</td>
<td>1.86</td>
<td>6.12</td>
<td>1.90</td>
<td>9.50</td>
<td>0.34</td>
<td>0.56</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values for TJ length are means ± SD; values in parentheses are percentages. TJ length was determined by measuring and adding together the lengths of the individual segments where intercellular space was occluded by apposition of membrane outer leaflets within first 3 µm of apical side of cells. Cells were treated for 14 days; antisense and sense phosphorothioate oligonucleotides (PONs) were added after seeding. TJs were measured in transmission electron microscope at ×25,000. P values are for χ² (*) or unpaired t-tests (†) between means of treated and untreated groups.

Table 4. Comparison of gap size and number in SCE cells treated with sense and antisense PONs

<table>
<thead>
<tr>
<th></th>
<th>Sense PONs</th>
<th>Antisense PONs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>DS</td>
</tr>
<tr>
<td>Gap number</td>
<td>108</td>
<td>20</td>
</tr>
<tr>
<td>Gap size, µm²</td>
<td>2.01 ± 1.4</td>
<td>0.53 ± 0.30</td>
</tr>
<tr>
<td>Gap aggregate area, µm²</td>
<td>217</td>
<td>10.6</td>
</tr>
</tbody>
</table>

Values for gap size are means ± SD. Number and area of rounded openings or interendothelial "gaps" in ~2,800 µm of paracellular pathway were counted and measured. Results were normalized to a standard length (2,800 µm). P values are for χ² (*) and unpaired t-tests (†) between mean gap areas in treated and untreated groups. Treatment began 1 h after seeding and continued for 14 days.

inhibited TJs and increased gaps but also effectively inhibited the Dex-induced decrease in HC.

In TM cells the effect of blocking ZO-1 was similar to that observed in SCE cells (Fig. 8B). Again, Dex treatment was associated with a reduction in HC (Fig. 8, C and D), and in cells treated with sense or nonsense PONs (CS and DS, CN and DN) HC levels were approximately the same as in cells that received no PONs (Fig. 8, C and D). The addition of antisense PONs, on the other hand, resulted in an increase in HC compared with untreated or sense-treated controls (cf. CA with C or CS; P < 0.004 or 0.006, respectively). These findings suggest that the responses elicited by antisense treatment were sequence specific and not due to toxic oligonucleotide effects.

In summary, the antisense PONs inhibited the expression of ZO-1. This reduction in ZO-1 expression resulted in a significant decrease in resistance and the formation of TJs as well as an increase in the number of gaps. Moreover, the glucocorticoid-induced increase in resistance and alterations in TJs and interendothelial gaps were abrogated by the reduction in ZO-1 expression.

The importance of ZO-1 in these glucocorticoid-mediated events was illustrated by experiments with antisense oligonucleotides in which ZO-1 expression was suppressed. This maneuver alone was sufficient to prevent the Dex-induced increase in resistance and attendant cellular alterations, suggesting that ZO-1 is essential for the glucocorticoid regulation of transendothelial fluid flow.

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In conclusion, we propose that glucocorticoids cause resistance by increasing the expression of ZO-1, which then mediates a doubling of the number of TJs, a reduction in intercellular gaps, and an increase in resistance.
point the outflow facility is usually reduced by a factor of $>3$ (7, 15, 43, 44). The magnitude of the increase in resistance measured in the cultured endothelial cells treated with Dex was in the same three- to fivefold range. Furthermore, the concentration of Dex required to elicit these responses, 500 nM, is the same as that in the aqueous humor of patients who have received topical steroids (58).

Our observation that >10 cell lines tested in our laboratory responded to the administration of Dex with increased resistance suggests that glucocorticoids may affect SCE and TM cells from most individuals in a similar manner. If the development of steroid glaucoma was mediated by the cellular responses observed in our study, most individuals would exhibit increased intraocular pressure with the application of topical Dex. In fact, there is some clinical evidence that “The steroid-induced increase in aqueous outflow resistance is a dose-response phenomenon, perhaps in all individuals” (30). However, this notion is contrary to clinical evidence, which shows that only certain “susceptible” individuals develop glaucoma in response to Dex treatment (7, 15). These conflicting issues may be resolved when the genetic basis and the steroid susceptibility of patients with glaucoma are better understood.

Regulation of the permeability of endothelial and epithelial barriers by physiological concentrations of glucocorticoids circulating throughout the body may be an important activity of these hormones. Moreover, some of the well-established therapeutic effects of steroids could be at least partially related to alterations in vascular permeability. For instance, steroids, used as anti-inflammatory agents to reduce swelling after tissue trauma, may function at least in part by altering TJs, which in turn decrease vascular endothelial permeability and swelling.

To test this theory and to determine whether our findings are cell type specific, more studies on different types of endothelial and epithelial cells are required. Additional experiments using glucocorticoid inhibitors and other kinds of steroids and a determination of the glucocorticoid dose-flow resistance response would be required to ascertain the specificity and receptor dependency of such a Dex response.

Blocking the expression of ZO-1 with antisense oligonucleotides prevents the Dex-induced increase in resistance and in TJs and reduction in intercellular gaps. Antisense oligonucleotide technology offers important experimental strategies to enhance our understanding of the relationship between the expression of a specific gene and its function (50, 73). This approach is particularly useful when the proper safeguards are taken to avoid artifacts and toxic reactions to the application of the oligonucleotide (71). To minimize such nonspecific effects, we performed preliminary experiments to determine the lowest concentration of antisense PONs that could significantly increase HC (8 $\mu$M). The same concentration of sense and nonsense PONs had no effect on these biochemical, morphological, and physiological parameters. In addition, there appeared to be no overt toxic effects on the ultrastructural characteristics of cells treated with antisense PONs. These findings suggest that the induction of ZO-1 is required for glucocorticoids to increase the resistance to the flow of fluid across monolayers of the cultured SCE and TM cells.

Our data are part of an increasing body of evidence that supports an important role for ZO-1 and its isoforms in the regulation of fluid flow across cellular barriers (6, 25, 26). ZO-1 is localized to TJs, which in turn decrease vascular endothelial permeability. For instance, steroids, used as anti-inflammatory agents to reduce swelling after tissue trauma, may function at least in part by altering TJs, which in turn decrease vascular endothelial permeability and swelling.

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Thus ZO-1 appears to mediate the interaction of cytoskeletal elements and cell junctions. Our results support this model, since blocking the expression of ZO-1 decreased the formation of TJ in the apicalolateral membrane, which in turn was associated with an increase in intercellular gaps and fluid flow. The possibility that suppression of ZO-1 affected the expression of other TJ-associated proteins was not addressed in these experiments and requires further study.

The SCE and TM cells exhibited a difference in ZO-1 isoforms that correlated with HC. The more-resistant SCE cells contained both ZO-1α1 and ZO-1α2, whereas TM cells expressed only the smaller ZO-1α1. Balda and Anderson (8) described a similar relationship between isoform content and flow resistance which suggests that ZO-1α1 confers greater resistance than ZO-1α2. If this hypothesis is correct, then expression of ZO-1α1 in TM cells, which normally only express ZO-1α2, would increase resistance in this cell type.

Our in vitro model is similar to other models used to study barrier properties of a variety of endothelial and epithelial cells (1, 17, 18, 28, 34, 39, 48, 57, 67). The validity of the model is supported by experiments in which medications that alter aqueous humor outflow in vivo are administered and the cultures undergo congruous changes in resistance in vitro. For example, epinephrine, which increases aqueous humor outflow in vivo, also increases transendothelial fluid flow in vitro (4). The time course and magnitude of this response are similar to that observed in glaucoma patients (12, 45), in human eyes tested in an organ-culture system (31, 32), in freshly enucleated human eyes (2, 3), and in experimental animals (64). In the present study we found that Dex, a hormone that decreases aqueous humor outflow in vivo, induces congruous changes in transendothelial fluid flow in vitro. Altogether, these studies highlight a potential role for SCE and TM cells in the regulation of aqueous humor outflow.

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