Endothelia of Schlemm’s canal and trabecular meshwork: distinct molecular, functional, and anatomic features

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The purpose of this study was to compare human endothelial cells from Schlemm’s canal (SCEs) and the trabecular meshwork (TMEs) in terms of ZO-1 isoform expression, hydraulic conductivity (HC) properties, and “giant” vacuole (GV) formation. The principal study methods were Western blot, RT-PCR, immunofluorescence, and perfusion chambers. Blot signals for α+- and α−-isoforms were similar in SCEs but less intense for the α+ relative to the α−signal in TMEs. With the anti-α+ antibody used at 1/50 dilution, binding occurred at cell borders of both cell types, but only to SCEs when used at a 1/120 dilution in vitro and in vivo. SCEs were more resistive than TMEs (HC = 0.66 vs. 1.32 μl·min⁻¹·mmHg⁻¹·cm⁻²; P < 0.001) when perfused from apex to base. When perfused in the other direction, SCEs were again more resistive (5.23 vs. 9.04 μl·min⁻¹·mmHg⁻¹·cm⁻²; P < 0.01). GV formation occurred only in SCEs as a function of flow direction, perfusion pressure, and time. We conclude that SCEs and TMEs have distinctive phenotypic properties involving their content of ZO-1 isoforms, barrier function, and GV formation.

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scope and cut with an I-Knife (Alcon Surgical, Fort Worth, TX). A 9-0 nylon suture was passed through the canal lumen to identify its location. Trabecular meshwork (TM) tissues attached to the canal were then teased away from the inner canal wall with sharpened jeweler’s forceps. When the ends of the 9-0 nylon suture were pulled anteriorly, the inner canal wall was torn longitudinally, remaining attached along its posterior boundary. The inner wall of the canal was then freed by cutting this attachment with the I-Knife (53).

Explant were placed on dishes that had been coated with an ECM synthesized by cultured corneal endothelial cells. This ECM has been determined empirically to encourage the formation of endothelial cells including human SCEs and corneal endothelial cells (26). Typically, SCEs grow out from explants as cohesive sheets, instead of single cells or clumps of cells as occurs with TMEs (5). When these sheets covered an area of ~100 μm in width, they were separated from the explant with a cloning ring. Isolated clones were plated on ECM-coated dishes containing culture medium 199 (M199, GIBCO, Buffalo, NY), supplemented with 15% fetal calf serum (FCS) and 250 ng/ml of fibroblast growth factor (FGF). To expand stocks, these cells were subcultured four times, and at the fourth passage they were frozen and stored in liquid nitrogen.

TMEs were obtained from explants of the corneoscleral and uveoscleral meshworks also obtained from the freshly enucleated eye of the 12- to 18-month old mongrel-dog donors and other TMEs available in our laboratory that had been cultured according to methods previously described (3, 5, 6, 42, 47, 53, 56). TM explants were dissected with an operating microscope and sharpened jeweler’s forceps. Tissue explants were placed on plain plastic culture dishes, and at the fourth passage the cultured TMEs were stored as frozen stocks for later use.

Stocks of SCEs and TMEs were defrosted and grown in 10-cm culture dishes containing Dulbecco’s modified Eagle’s medium supplemented with 15% FCS, 2 μM glutamine, and 50 μg/ml of gentamicin. These fifth-passage cells were grown until nearly confluent, when the cells were dissociated with 0.05% trypsin, 0.02% EDTA, and 0.58 g/l NaHCO₃, and 5 × 10⁵ cells/cm² were seeded onto four-well plastic slides for immunofluorescence, 6-cm dishes for Western blots, or methylcellulose filters (0.45-μm pores, catalog no. PIHA01250, Millipore, Bedford, MA) for perfusion experiments. The cells were fed every other day with the DME medium described above and maintained in 8% CO₂ in a humidified incubator at 37°C. After the cells had remained confluent, the serum concentration was reduced to 10% and the preparations were used 20–30 days later for the various experiments.

Informed consent was obtained from patients and tissue donors according to standard procedures [approval no. H111-00511-19 from the University of California, San Francisco (UCSF) Committee on Human Research].

**Antibodies for ZO-1 and its three isoforms.** An extensive battery of antibodies was used to determine the localization of ZO-1 and its isoforms in the cultured cells and frozen tissue sections. A commercially available rabbit anti-human ZO-1 antibody (no. 61–7300, Zymed, South San Francisco, CA) was used for baseline studies. This antibody was designed to recognize the middle portion of the ZO-1 protein (amino acids 463–1109 of the human ZO-1 cDNA) and therefore is capable of reacting with ZO-1 α- and α-isoforms.

The first splicing event identified for the ZO-1 protein involves its α-domain, yielding α'- and α'-isoforms. An affinity-purified rabbit polyclonal antibody against the α'-isoform of ZO-1 has been raised with a recombinant human fusion protein (antibody 8040), and this antibody was kindly provided to us through the courtesy of Dr. J. M. Anderson (Yale University, New Haven, CT; Ref. 9). The anti-α' antibody does not recognize the α-isoform because it was specifically affinity-purified against the α-domain.

Two other splicing domains, designated as β and γ, have been identified at the proline-rich carboxy terminal region of ZO-1 (25). The resulting β₂-isoform contains a 60-nt insertion (bp 5170–5229 of canine ZO-1; GenBank accession no. U55935), and the β₁-isoform comprises the first 21 nt of the β₂-insert (from bp 5170 to 5190 of canine ZO-1). The γ-domain has an insertion of 135 nt (from bp 4255 to 4389 of canine ZO-1). Polyclonal antibodies have been generated against synthetic peptides of β- and γ-sequences as previously described (25). The antibody against β₁ was generated with the first seven amino acids contained in both β₁- and β₂-isoforms; consequently, this antibody cannot distinguish between the two β-isoforms. The polyclonal anti-β₂ was raised with a peptide corresponding to the last 10 amino acids of the β₂-isoform; consequently, recognition by this antibody of β₂-reporter constructs was dependent on the α-domain. The polyclonal antibodies against γ₁₁- and γ₁- were made with peptides that correspond to the first 16 and the last 12 amino acids of the γ-sequence, respectively.

**Protein extraction, immunoblot analysis, and RT-PCR.** To detect protein expression by immunoblotting, the SCEs and TMEs were washed four times for 5 min each with ice-cold PBS, followed by 30 min of continuous and vigorous shaking in RIPA buffer (150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS), the protease inhibitor cocktail Complete (catalog no. 1697498, Roche Diagnostics, Mannheim, Germany), and PMSF (20 μg/ml). The extract was collected with a rubber policeman, sonicated for 6 s, and centrifuged for 20 min at 14,000 rpm to remove debris. Total RNA was extracted from the supernatant, samples were separated in a 6% SDS-PAGE (50 μg/well) and transferred to polyvinylidene difluoride (PVDF) membranes (Amer sham, Arlington Heights, IL).

Blotting was performed with polyclonal antibodies against ZO-1 and α', β₁, β₂, γ₁₁-, and γ₁-isoforms, followed by the alkaline phosphatase conjugate substrate kit with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) solutions (170–6432, Bio-Rad) or the chemiluminescent ECL plus system (RPN2132, Pharmacia Biotech).

Direct isolation of polyadenylated RNA from TMEs and Madin-Darby canine kidney (MDCK) cells was performed with a QuickPrep Micro mRNA Purification Kit (catalog no. 27–9255–01, Pharmacia Biotech). CDNA was reverse-transcribed from RNA with SuperScript II reverse transcriptase (catalog no. 18064-D14, GIBCO-BRL) with the 3’- primer (AGTTCCATTATTTAAGTTCCTA) spanning nt 6472–6449 of the human ZO-1 sequence (GenInfo identifier: L14837). The following primers were used for PCR amplification: primer 1, GAAAGTTTCATCTCCAGTCCCT (from 3960 to 3980 bp of the human ZO-1 sequence), which localizes in the α-domain of ZO-1, and primer 2, TGGGTAGGGCTTGTGGTACATCA (from 4493 to 4470 bp of the human ZO-1 sequence), which is localized at the Pro-rich region of ZO-1, between its α- and γ-splicing domains. PCR amplification was performed according to standard procedures with agarose gel electrophoresis and ethidium bromide staining to detect reaction products. A sample with the TME template but with no primers served as a negative control.

**Use of cyanine 3 fluorochrome and 3-amino-9-ethylcarbazole chromagen methods for immunolocalization of ZO-1 and its three isoforms.** Cells were cultured in four-well plastic slides and fixed for 1 min in 100% methanol chilled to −20°C followed by three “standard” rinses. A standard rinse consisted of immersion of the slides in PBS three times, each lasting 5 min. This rinse was performed after each of the steps described below. The first step involved blocking for nonspecific antibody binding by exposing the cells to 3% Carnation nonfat dry milk in PBS and 0.5% BSA for 0.5 h. Next, the primary antibody was applied for 1 h, followed by a secondary blocking step with 5% donkey serum for 0.5 h. A biotin spacer was applied for 1 h, and then the cyanine 3 fluorophore (Cy3) was applied for 1 h in the dark. The slides were then maintained in the dark until being photographed. Each positive background reaction can be blocked by preincubation of the Cy3 technique is used. To eliminate these, a second method based on the application of the chromagen 3-amino-9-ethylcarbazole (AEC; Zymogen, South San Francisco, CA) was used. As in the former method,
before and after each step, slides received the described standard PBS rinse. Exposure to the primary antibody lasted for 1 h, followed by application of a biotinylated secondary antibody for 10 min. A streptavidin-peroxidase conjugate was added for 10 min, followed by the addition of the chromagen AEC for 10 min. A final rinse with distilled water twice for 3 min each was done, followed by the application of a glycerol vinyl alcohol (GVA; Zymed) mounting medium, placement of a coverslip, and photography.

The frozen sections were cut from an eye donated for corneal transplantation, using remaining tissues that included the peripheral cornea and aqueous outflow pathway. These peripheral corneal “rims” were cut into 3 × 4-mm blocks, cryoprotected overnight in 2.6 M sucrose in 0.07 M cacodylate buffer, frozen and cut into 3-μm sections with a Leica Frigocut (model 2800N) cryostat, and stored at −20°C until exposed to the various antibodies with the same protocols used with the cultured cells described above.

Hydraulic conductivity and electrical resistance measurements. For these perfusion experiments, cultured cells were seeded onto 0.45-μm Millipore filters (no. PHA01250; Bedford, MA) according to previously described protocols (5, 42, 53). Once the cells had become confluent, hydraulic conductivity (HC) measurements were performed by first perfusing the monolayer from the apical toward the basal cell surface. The HC was measured in microliters per minute per millimeter of mercury per square centimeter with a computer-controlled flowmeter apparatus previously described (5, 42, 53). Twenty-two preparations per experiment per cell type were placed on the apparatus and perfused with cultured medium at a transendothelial pressure (TEP) of 4.5 mmHg until stable measurements were recorded for 30 min. At this point, the direction of flow was reversed and the monolayers were perfused from the basal to the apical cell surface until a stable reading was obtained for 30 min.

Transendothelial electrical resistance (TER) measurements across confluent SCE and TM monolayers grown on Millicells were performed at 25°C with STX-2 Ag/AgCl electrodes and an EVOM Voltohmeter (World Precision Instruments). The TER generated by the culture medium and filter support was subtracted from that of the cultured cell layer, and the result was expressed in units of ohms times square centimeters. Five cultures of each cell type were measured.

GV formation in vitro. Millicell preparations and perfusion experiments were also used to study the process of GV formation. GVs were formed only when perfusion is from the basal to the apical cellular surface; therefore, monolayers were perfused with culture medium flowing in this direction at TEPs of 0.5, 1.5, 3.0, and 4.5 mmHg. We measured GV formation as a function of TEP when the preparations were perfused for 15 min (53) and as a function of perfusion time when TEP remained constant at 2 mmHg. At the end of each experiment, the perfusate was switched to a fixative solution and perfused for 30 min, under the same conditions that existed at the end of the experiment. The fixed preparations were then removed from the perfusion apparatus and immersed in the same fixative solution for an additional hour. The fixative contained 0.5% paraformaldehyde and 1% glutaraldehyde, at a pH of 7.2, in 0.1 M Na-cacodylate buffer. Filters were cut into quadrants and postfixed in 1% OsO4, dehydrated in ethanol, critical point dried, sputter coated with Au/Pd, and examined by scanning electron microscopy (SEM; JEOL JSM-840A).

Some preparations were also examined by transmission electron microscopy (TEM) after thin sectioning and embedding in Araldite. A second set of experiments was also carried out during which the perfusion time was varied at 15-min intervals from 15 to 90 min. These filters were then prepared as described above.

SEM was used to photograph the apical cell surface of cells coating each filter at ×100. All protuberances present on the surface of the monolayer were rerephotographed at ×2,300 for further evaluation and morphometry. Areas occupied by these protuberances and any openings therein were measured with a Sigma Scan (Jandel Scientific, Corte Madera, CA) digitizing program. For data analysis, GVs were defined as protuberances of the cell membrane ≥100 μm² in area. Ninety ×100 fields each were evaluated for SCEs and TMEs.

RESULTS

TMEs express α⁺-isoform of ZO-1. Protein extracts from SCE, TME, and MDCK cells, the latter renal epithelial cell line employed as a positive control, were exposed to the commercially available polyclonal ZO-1 antibody in the Western blot in Fig. 1A. Binding occurred within two bands for all cell

Fig. 1. Western blots and PCR reveal the presence of the α⁺-isoform of ZO-1 in trabecular meshwork endothelial cells (TMEs). Western blots for the α⁺- and α⁻-isoforms used 2 antibodies and protein extracts obtained with a high-stringency buffer containing 1% Triton X-100 and 0.2% SDS. Arrows on left indicate the molecular weight of the isoforms, and the horizontal lines on right mark the position of the SDS-PAGE standards. A: Western blot made with the commercially available polyclonal ZO-1 antibody. Bands of molecular weight 228 and 205, corresponding to those of the α⁺- and α⁻-isoforms, are present in both TMEs and endothelial cells from Schlemm’s canal (SCEs), as well as in Madin-Darby canine kidney (MDCK) cells used here as a positive control. B: Western blot made with the antibody recognizing the α⁺-domain of ZO-1 and the protein extract from TMEs. Expression of the α⁺-domain is shown in TMEs under baseline conditions (left lane). This expression level was augmented with the administration of 500 nM dexamethasone (Dex) to TMEs for 8 wk (right lane). C: RT-PCR of the α-domain of ZO-1 in TMEs and MDCK cells, which were used as a positive control. Primers were used that allow PCR amplification only when the ZO-1 α-domain is present. A band of 533 bp was amplified in TMEs (lane 3) and in MDCK cells (lane 4), which corresponds in size to the α-domain of ZO-1. A sample with the TME template but no primers served as a negative control (lane 2). A 1-kb DNA ladder is shown in lane 1.
types, with the first band having a molecular weight (MW) of 228 and the other of 205, which correspond to those of the $\alpha^+$- and $\alpha^-$-isoforms, respectively. The three cell types expressed the $\alpha^+$- and $\alpha^-$-isoforms, but TMEs displayed a less intense signal for the higher-MW band relative to that for the lower-MW band whereas SCEs displayed signals of similar strength for both isoforms. MDCK cells showed a slightly higher expression of the $\alpha^-$-isoform.

We proceeded to confirm that TMEs do express the $\alpha^+$-isoform in two experiments. First, we exposed the TME protein extract to the antibody specific for the $\alpha^+$-isoform (9), as shown in Fig. 1B (left lane), and found binding to the higher-MW band. This signal could be greatly amplified by treating the TMEs for 8 wk with 500 nM Dex (Fig. 1B, right lane). The finding that glucocorticoids augmented the expression of the $\alpha^+$-isoform in TMEs is complementary to our previous observation (53) that this steroid treatment had a similar effect on the ZO-1 $\alpha^+$-isoform in SCEs.

In the second experiment, RT-PCR was used to learn whether mRNA for the $\alpha^+$-isoform could be amplified from TMEs. As shown in Fig. 1C, a 533-bp band could be amplified from TME (lane 3) and MDCK cells (lane 4), which were used as a positive control (9). The amplified bands were sequenced and proved to correspond to the $\alpha^+$-domain of ZO-1.

Binding by SCEs and TMEs of polyclonal ZO-1 antibody and its correlation with ultrastructural features of apical cell border. The apical cell border of SCEs and TMEs is shown in Fig. 2 with the polyclonal ZO-1 antibody, Cy3 immunofluorescence, the AEC chromagen method, and ultrastructural approaches. Results in controls are shown in Fig. 3, A, C, and D, demonstrating some background with the Cy3 method (Fig. 3A) and none with the AEC method (Fig. 3, C and D). However, the intercellular space is devoid of any staining that could be related to intercellular junctions in any of the controls regardless of the methods used. The appearance of the intercellular border in SCEs is shown in Fig. 2, A and C, and compared with that of TMEs in Fig. 2, B and D, at the same magnification. It should be noted that ZO-1 deposition was sharply confined to cell borders in both cell types, making differences in cell size and shape readily apparent. SCEs are larger and have a more regular hexagonal shape in Fig. 2, A and C, than TMEs in Fig. 2, B and D, which have a variable size and an irregular shape. In Fig. 2C the profile of one SCE cell is shown, which measures $2,400 \mu m^2$. The complete outline for several TMEs is observed in Fig. 2, B and D. No complete cell outlines are observed for the larger SCEs in Fig. 2A and a single complete cell outline in Fig. 2C despite the fact that all these figures represent areas of identical size. The TMEs have an average size of $\sim300 \mu m^2$ and vary in size from 90 to 650 $\mu m^2$, with a standard deviation of 200 $\mu m^2$ ($n = 18$).

The antibody deposition pattern is unusual in that instead of forming a continuous band around each cell border, it consists of many short, linear structures, extending perpendicularly and across the cell border as pointed out by arrows in Fig. 2, A–C. At higher magnification (Fig. 2E), one can easily appreciate the palisade arrangement of the antibody deposition pattern. This arrangement is caused by the formation of discrete bands of fluorescence, along short, thin structures disposed in parallel to each other, oriented perpendicular to and extending across the cell border.

SEM shows that the cell border along the apical cell surface consists of oval openings referred to as “gaps” (1, 11, 12, 37, 52, 55), which are indicated by arrowheads in Fig. 2F, and fingerlike structures or filopodia that extend from one cell, across the cell border, and onto the surface of its neighbor in both directions, as indicated by arrows in Fig. 2F. This arrangement is reminiscent of fingers in hands that are clasped together. The fingers form a broad, overlapping area between cells, which has a width of 1–3 $\mu m$. By TEM, the filopodia have the appearance shown in Fig. 2G. Each finger is tightly apposed to the underlying cell surface by intercellular junctions, as shown by white arrows in Fig. 2G. An electron-dense material is also present in the cytoplasm of fingers (Fig. 2G, black arrows) as well as in the underlying cell (Fig. 2G, white arrows). The ZO-1 accessory proteins have also been shown to have a location similar to that of these electron-dense deposits (25).

In vitro $\alpha^+$ antibody binds with greater intensity to SCE cells than to TME cells. The intensity of ZO-1 $\alpha^+$-isoform expression in SCEs was compared with that in TMEs with the specific $\alpha^+$ antibody, as shown in Fig. 4. The controls for this figure are those shown in Fig. 3, A, C, and D. To establish threshold conditions for each cell type the $\alpha^+$ antibody was used at various concentrations. At a dilution of 1:50, SCEs displayed more intense binding for the $\alpha^+$ antibody than TMEs with either the immunofluorescence-Cy3 method (Fig. 4, A and B) or the AEC chromagen method (Fig. 4, C and D). The latter method, because of its lack of background binding, as shown in Fig. 3, C and D, showed the difference more clearly. When the $\alpha^+$ antibody was diluted fourfold and used at a 1:200 dilution, threshold conditions were reached and binding by intercellular junctions of TMEs was no longer perceptible (Fig. 4F), although it is apparent that the background binding is very similar to that shown in Fig. 3A. However, intercellular junction binding remained clearly visible for SCEs (Fig. 4E). A more striking difference in binding intensity between the two cell types became apparent with the AEC chromagen method, which showed a lack of nonspecific binding (Fig. 3, G and H). Regarding cell size differences, these panels showed that the SCEs shown in Fig. 4, A, C, E, and G, have an average size of 1,100 $\mu m^2$ (SD 550 $\mu m^2$; $n = 10$) compared with 440 $\mu m^2$ for TMEs (SD 450 $\mu m^2$; $n = 9$). Of some interest is the finding that the nuclei of SCEs measured 175 $\mu m^2$ in average size compared with 120 $\mu m^2$ for the TMEs seen in Fig. 2, C and D. When the cell sample in Figs. 2 and 4 are considered together, the SCEs measured 1,250 $\mu m^2$ (SD 650 $\mu m^2$; $n = 11$) in average size and the TMEs measured 350 $\mu m^2$ (SD 300 $\mu m^2$; $n = 27$) in average size.

Both cell types expressed the $\alpha^+$-isoform in these in vitro antibody-binding experiments, confirming results obtained with Western blots and RT-PCR as shown in Fig. 1. The immunoblot observations were extended in these experiments by demonstrating that antibody binding for the $\alpha^+$-isoform was significantly more intense in SCEs than in TMEs.

In vivo $\alpha^+$ antibody also binds with greater intensity to SCEs than to TMEs. Unfixed frozen sections cut from the aqueous outflow pathway of a normal human eye were used for in vivo antibody binding experiments. When the polyclonal antibody was used at a dilution of 1:200, binding to cells lining the TM tissues and the lumen of SC was observed as shown in
Fig. 5, A and B. The control for this experiment is shown in
Fig. 3, E and F, where minimal background is apparent with
the immunofluorescence method and no background is discern-
ible with the AEC chromagen method. When the anti-α⁺
antibody was used at the same 1:200 dilution, as shown in Fig.
5C, staining of both SCEs and TMEs was also apparent, in
agreement with the above in vitro studies. However, when the
anti-α⁺ antibody was diluted to a 1:400 concentration, fluo-
rescence was detectable only in the SCEs and no antibody
binding was discernible in the TMEs (Fig. 5D). These results
showed that SCEs bound the α⁺ antibody at a lower concen-
tration in vitro and in vivo, suggesting that the ZO-1 α⁺
isoform is expressed with greater intensity in SCEs compared
with TMEs.

Fig. 2. ZO-1 binding pattern on the cell
border of SCEs and TMEs is correlated with
the ultrastructural features of this paracellu-
lar route along the apical cell surface. The 2
cell types were exposed to the polyclonal
antibody at a 1:200 dilution; for controls,
shown in Fig. 3, A, C, and D, application of
the primary antibody was omitted. A: SCE
binding pattern shown with the cyanine 3
(Cy3) immunofluorescence method. White
arrow indicates antibody deposition along
the cell border at a location where the pali-
sade arrangement is better appreciated (×730).
B: TMEs treated as in A are shown for com-
parison. Arrow indicates the palisade ar-
rangement (×730). C: SCEs shown with the
3-amino-9-ethylcarbazole (AEC) chromagen
method. Note the fingers or filopodia along
cell borders (arrow) and the larger size and
more regular shape of these cells. The entire
outline of only 1 cell can be appreciated at
this magnification for the larger SCE cell
type (×730). D: TMEs treated as in C. Ar-
row indicates filopodia along cell border.
Note that, in contrast to the situation with
SCEs, many TMEs can be appreciated in this
field, which includes an area equal in size to
that in C. TMEs are smaller and have a more
irregular shape than SCEs (×730). E: higher
magnification to show the antibody deposi-
tion pattern with greater clarity in a cultured
SCE. The apical cell border extends horizon-
tally. The filopodia consist of short, thin,
linear structures disposed in parallel to each
other and extending across the cell border in
a manner reminiscent of ties across a railroad
track (×1,650). F: Scanning electron micros-
copy view of a portion of the apical border in
a TME cell showing a area similar to that in
E. The intercellular space consists of multi-
ple gaps indicated by arrowheads. On either
side of these gaps, fingers are seen extending
across the intercellular space from 1 cell onto
the apical surface of its neighbor (arrows)
(×1,800). G: Transmission electron micro-
graph of fingers cut along their long axis of a
TME cell. Note that the fingers have a uni-
form thinness and are associated with inter-
cellular junctions extending along and bind-
their underside to the apical surface of neighboring cells. The intercellular junction
contains an electron-dense material within
fingers (black arrows) and within the under-
lying cell (white arrows), which presumably
depends on the sites of ZO-1 deposition
shown in E (×1,800).
Fig. 3. Controls consisted of preparations in which all of the ingredients employed for experimental cells were used except that the primary antibody was omitted. A: control for experiment shown in Fig. 2 using the Cy3 immunofluorescence method and SCEs. Note in particular the absence of any fluorescence along the cell border (×730). B: control for experiment shown in Fig. 7, showing some diffuse cytoplasmic and perinuclear staining; however, no nuclear staining is apparent, such as the dots seen in Fig. 7. TMEs are shown (×730). C: control for experiments in which the AEC chromagen method was used, showing SCEs. This method was used in all of our experiments because it shows no background that can be interpreted as nonspecific fluorescence (×730). D: similar control as in C but showing TMEs and no background staining (×730). E: control for experiment shown in Fig. 5, showing a tissue section having Schlemm’s canal (SC) near the center of the panel. Essentially no background staining is apparent here, where the Cy3 immunofluorescence method was used (×1,050). F: control for experiment in Fig. 5 using the AEC chromagen method and a tissue section containing Schlemm’s canal at SC and the overlying corneoscleral and uveoscleral trabecular meshwork (TM) elements (×1,050).

TMEs and SCEs also express β- and γ-isoforms of ZO-1. Polyclonal antibodies generated against synthetic peptides for β- and γ-isoforms were used to examine their expression in vitro with immunoblots. Figure 6 shows that the β1- and β2-isoforms are expressed in both TM and SCE cells, but only within the MW 228 band, which was shown to correspond to the α13 isoform in Fig. 1A. In contrast, the γ-insertion is present with a similar intensity in the higher- and lower-MW bands of ZO-1 in both TMEs and SCEs, being slightly more intense in the higher-MW band. The TME result differs from that shown in the Western blot in Fig. 1A, where the lower-MW band in TMEs is more intense than the higher-MW band. This discrepancy can be explained when one considers that the contribution of the γ-isoform to the MW 205 band might be low relative to that of other isoforms.

Antibodies for ZO-1 β- and γ-isoforms bind more intensely to cell borders of SCEs than TMEs. The distribution of ZO-1 β- and γ-isoforms in SCEs and TMEs was analyzed by immunofluorescence in vitro. The control for this experiment appears in Fig. 2B, showing some background, in particular around the nucleus and cytoplasm. SCEs are shown in Fig. 7, A, C, E, G, and TMEs are shown in Fig. 7, B, D, F, and H. In SCEs the β1 (Fig. 7A)-, β2 (Fig. 7C)-, and γ (Fig. 7, E and G)-isoforms are present at the cell borders, whereas in TMEs β1 (Fig. 7B) and β2 (Fig. 7D) binding is restricted to multiple, uniformly spaced dots within the nucleus. Such dots were not present in controls as shown in Fig. 3B. The nuclear staining pattern differs between cell types; in SCEs there is also a diffuse background nuclear staining (Fig. 6, A and C) that is not present in TMEs (note the marked difference in nuclear size between the 2 cell types). The β2-antibody also binds to nuclear sites, which is more intense in SCEs, where one can observe punctate nuclear as well as diffuse staining. Punctate nuclear staining is also present in TMEs, but the dots are less distinct. Application of the antibody for the γ-isoform results instead in a strong perinuclear and cytoplasmic staining in TM cells (Fig. 5, A and C) that is not present in TMEs (note the marked difference in nuclear size between the 2 cell types). The β2-antibody also binds to nuclear sites, which is more intense in SCEs, where one can observe punctate nuclear as well as diffuse staining. Punctate nuclear staining is also present in TMEs, but the dots are less distinct. Application of the antibody for the γ-isoform results instead in a strong perinuclear and cytoplasmic staining in TM cells (Fig. 5, A and C) that is not present in TMEs (note the marked difference in nuclear size between the 2 cell types). The β2-antibody also binds to nuclear sites, which is more intense in SCEs, where one can observe punctate nuclear as well as diffuse staining. Punctate nuclear staining is also present in TMEs, but the dots are less distinct. Application of the antibody for the γ-isoform results instead in a strong perinuclear and cytoplasmic staining in TM cells (Fig. 5, A and C) that is not present in TMEs (note the marked difference in nuclear size between the 2 cell types). The β2-antibody also binds to nuclear sites, which is more intense in SCEs, where one can observe punctate nuclear as well as diffuse staining. Punctate nuclear staining is also present in TMEs, but the dots are less distinct. The γ-antibody may be related to this general difference in ZO-1 content between SCEs and TMEs.
SCEs offer greater resistance to transendothelial fluid flow than TMEs. The preparations used for these experiments were shown to remain stable when perfused at a TEP of 4.5 mmHg for 30 min. Subsequently, the preparations were perfused for 15 min in either one of two flow directions. When fluid was flowing from the apical toward the basal direction, HC in SCEs measured 0.66 µl·min⁻¹·mmHg⁻¹·cm⁻² (SD = 0.26 µl·min⁻¹·mmHg⁻¹·cm⁻²; n = 22) whereas in TMEs it measured 1.32 µl·min⁻¹·mmHg⁻¹·cm⁻² (SD = 0.27 µl·min⁻¹·mmHg⁻¹·cm⁻²; n = 22) as shown in Fig. 8A. These differences are highly significant (P < 0.001). The TER results were similar, measuring 33.5 Ω·cm² (SD = 1.34 Ω·cm²; n = 5) for SCEs and 21.5 Ω·cm² (SD = 0.41 Ω·cm²; n = 5) for TMEs. This difference is also highly significant (P = 0.0028).

When perfused with fluid flowing from the basal to the apical surface (i.e., in the opposite direction), the conductivity in SCEs measured 5.23 µl·min⁻¹·mmHg⁻¹·cm⁻² (SD = 0.80 µl·min⁻¹·mmHg⁻¹·cm⁻²; n = 22) compared with 9.04 µl·min⁻¹·mmHg⁻¹·cm⁻² (SD = 3.61 µl·min⁻¹·mmHg⁻¹·cm⁻²; n = 22) for TMEs. Thus, regardless of the perfusion direction, the SCEs behaved as the more resistive cell type.

SCEs but not TMEs form GVs. Monolayers of SCEs and TMEs perfused in both directions were evaluated for the presence of GVs, and this process was quantified with SEM-based morphometry. GV formation was unidirectional, occurring only when flow was from the basal toward the apical surface. When perfusion was carried out in the opposite direction, no GV formation occurred and the monolayer maintained a relatively smooth and flattened appearance, as shown in Fig. 9A for SCEs and Fig. 9E for TMEs (see also Fig. 10C). When the perfusion proceeded from the basal toward the apical cell surface, GVs were formed only by SCEs. GVs, as shown in...
Fig. 9. **B**–**D** are large balloon-like structures, which were observed near the cell border (Fig. 9 **B**) or more centrally near the nucleus (Fig. 9 **D**; see also Fig. 10, **A** and **B**). Approximately 16 GVs could be identified per filter, which measures 0.6 cm² in area. Each GV measured 200 µm² in average size (SD = 98.1 µm²), with a few of them covering >500 µm² each. Some GVs contain a central pore (Fig. 10, **C** and **D**), suggesting that these structures might be transcellular fluid pathways. In Fig. 10, **A** and **B**, one GV is shown both in profile and from the top. Viewed from its side, the GV can be seen to arise from a platform indicated by arrows near the center of this cell. Viewed from the top, the GV acquires a white appearance and the platform is observed extending onto the cell margin. TMEs were not observed to undergo GV formation. Instead, numerous, tiny nodular structures measuring 1–5 µm², with a single cell containing as many as 50–100 such structures (Fig. 9 **F**), were observed in TMEs. These nodules did not have pores, as observed in the GVs of SCEs, and as such they are unlikely to represent transcellular pathways.

Examination of the monolayer by light microscopy allowed for a different appreciation of the GV formation process as Fig. 5. Unfixed tissues of the conventional aqueous outflow pathway of normal human specimens exposed to 2 antibodies at 2 dilutions to examine differential antibody binding to TMEs and SCEs in situ. Fig. 3, **E** and **F**, shows controls for these experiments. **A**: commercially available polyclonal ZO-1 antibody was used at a 1:200 dilution. Strong binding occurred to TMEs lining aqueous channels in the trabecular meshwork (white arrow) and to SCEs lining the lumen of Schlemm’s canal (SC), which is seen near the bottom (**×670**). **B**: comparison using the AEC chromagen method and the same conditions as in **A**. Antibody binding is sharply confined to the lining TMEs (arrow at top), with the trabecular beams remaining devoid of any reaction product. The canal lumen is shown at the bottom, and an arrow indicates the reddish-brown reaction product on SCEs. Controls show no positive reaction product as shown in Fig. 3 **F** (**×670**). **C**: the α⁺-isoform antibody was used at a 1:200 dilution in this experiment, which shows binding to TMEs (**top**) and to SCEs lining the canal lumen, as had been observed in vitro with the cultured cells (**×1,050**). **D**: when the α⁺-specific antibody was diluted to a 1:400 concentration, binding was no longer discernible by TMEs. SCEs are the only cells binding the antibody, which is better appreciated along the inner canal wall. This result is similar to that obtained in vitro. Controls, as shown in Fig. 3 **F**, showed little to no discernible nonspecific reaction product (**×1,050**). Arrows point out location of junctions with cells lining Schlemm’s canal.

Fig. 6. Western blots using extracts from SCEs and TMEs run in an SDS-PAGE and blotted with polyclonal antibodies against ZO-1 β₁, β₂, and γ-isomers. In **A**–**C**, the arrows on the left indicate the molecular weight of the isoforms and the horizontal lines on the right mark the position of the SDS-PAGE standards. **A**: ZO-1 β₁-isomer was present in both TMEs and SCEs, but only within the same molecular weight 228 bands. **B**: ZO-1 β₂-isomer was also present in both TMEs and SCEs within the same bands. **C**: γ-isomser was present in both the upper and lower bands of molecular weight 228 and 205, which correspond to the α⁺- and α⁻-isomers of ZO-1, respectively.
shown in Fig. 10, C–G. In Fig. 10C a control SCE monolayer perfused from the apex toward the base is shown to be devoid of any vacuolar structures. In Fig. 10D the SCE monolayer perfused in the opposite direction is shown to contain two prominent vacuoles. The vacuole in Fig. 10G represents a vacuole that is larger in size than the cell nucleus, which is pointed out with arrows. GVs are derived from smaller vacuoles that begin to form along the basal surface in the majority of cells in the monolayer. However, these vacuoles reached a giant status in only a few cells, and when present there was usually one GV per cell.

The relationship between the GV formation process and TEP was assessed by measuring the aggregate area occupied by GVs as a function of TEP when flow was maintained steady for 15 min. The response observed is graphed in Fig. 8A, where it is shown that GV formation did not occur at a TEP of 0.5 mmHg. However, GVs did form at a TEP of 1.5 mmHg, increasing approximately sixfold when the TEP was doubled to 3.0 mmHg. At a TEP of 4.5 mmHg, a marked decline in the GV formation rate to one-third of that measured at 3.0 mmHg was observed. The flow rates maintained at each of these TEPs are displayed in Fig. 8C. Flow rates doubled progressively as the perfusion pressure increased in 1.5-mmHg steps, measuring 4 μl/min at 1.5 mmHg and 8 μl/min at 3.0 mmHg and reaching 16 μl/min at 4.5 mmHg. Although the GV formation process was disrupted dramatically at 4.5 mmHg, the increment in flow rates remained unchanged.

The pore diameter of GVs was likewise measured as a function of TEP, as shown in Fig. 8D. The pore diameter of each GV increased progressively with increasing TEP, being much less than 1 μm when perfused at both 1.5 and 3.0 mmHg.
TEP but reaching 2 \( \mu \text{m} \) at 4.5 mmHg, when we measured a large fluctuation in pore diameter (Fig. 8D). These findings are helpful in analyzing the decline in GV frequency at 4.5 mmHg shown in Fig. 8B. We propose that this is likely related to the simultaneous widening of the transcellular pathways because of a fourfold enlargement in pore diameter, as well as widening of the paracellular pathway (Fig. 10, A and B), as observed by SEM. Furthermore, we propose that GV formation decreased at a TEP of 4.5 mmHg because of the presence of widely dilated paracellular and transcellular pathways with a concomitant generation of less tension along the monolayer of SCEs.

We measured the effect of perfusion duration by maintaining TEP at 2 mmHg while increasing perfusion time in 10-min steps (Fig. 8E). GV formation increased in a stepwise fashion with perfusion duration up to the 30-min perfusion time. As in the experiment shown in Fig. 8B, there was an abrupt decline in the GV formation at the 40-min perfusion time. Therefore, we conclude that SCE monolayer integrity has a tolerance that can be surpassed by increasing either perfusion pressure or its duration. The monolayer was disrupted when the perfusion pressure reached 4.5 mmHg, or when the perfusion duration measured 40 min at a TEP of 2 mmHg. It should be noted that GV formation was similar when the monolayer was perfused for 15 min at 3 mmHg and for 40 min at 2 mmHg (\( \sim 2,500 \mu \text{m}^2 \)).

GV formation was another distinct feature of SCEs, which occurred as a function of the direction of fluid flow, the driving TEP head, and the perfusion time. GVIs disappeared rapidly after reduction of the driving force to a pressure \( \leq 1.0 \) mmHg. The monolayer of cultured SCEs was not able to maintain its integrity above a certain driving pressure and perfusion time, resulting in a decline in the number of GVs formed and a breakdown of its barrier function.

**DISCUSSION**

We have uncovered three major differences in the phenotypes of cultured SCEs and TMEs: 1) the expression of ZO-1 isoforms is different for each cell type; 2) the resistance presented to fluid flow across SCEs is greater than that across TMEs; and 3) the formation of GVs is a unique characteristic of the SCE cell type.

Previously we showed (53) that SCEs express the \( \alpha^+ \)- and \( \alpha^- \)-isoforms of ZO-1 whereas TMEs express only the \( \alpha^- \)-isoform; this led us to propose that the \( \alpha^+ \)-protein might be a molecular marker for the SCE cell type. Because most other
endothelial cells tested have expressed both isoforms (9), with the exception of the peritubular renal capillary (24, 35), we decided to revisit this issue. We have now succeeded in extracting the \(\alpha^+\)-isoform of ZO-1 from TMEs and have shown that the difference in ZO-1 expression between SCEs and TMEs is quantitative. Both cell types express the \(\alpha^+\)- and \(\alpha^-\)-isoforms; however, TMEs have a lower signal strength for the \(\alpha^+\)- than the \(\alpha^-\)-isoform whereas SCEs display signals of similar strength for both (Fig. 1). Therefore, measuring the \(\alpha^+\)-to-\(\alpha^-\) ratios with the commercially available polyclonal antibody is differentiating for both cell types.

The proteins making up ZO-1-containing junctions, such as the tight (TJ) and adherens (AJ) junctions present in SCEs and TMEs (53), have a tripartite arrangement of extra- and intracellular proteins linked in series. We compare this mechanism to one containing a gate, a pulley, and a chain linked in series. Integral transmembrane proteins, such as occludin, claudins, junctional adhesion molecule (JAM), and others, are located within the paracellular pathway forming the intercellular junction proper or gate (29, 51). Membrane-associated guanylate kinase (MAGUK) accessory proteins, which include ZO-1, ZO-2, and ZO-3, are located just inside the cell membrane at the site of TJs and AJs, and the accessory proteins form the pulley, which is linked to transmembrane proteins (24, 25, 31, 41). F-actin, a modified fibrillar form of actin, is located just distal to the MAGUK proteins with which ZO-1 is also linked, forming the third element of the chain (29, 51). Thus ZO-1 represents a bridge linking the other two components of the tripartite system. Moreover, ZO-1 is at the crossroads of important transduction pathways (57), acting as a scaffold that holds transcription factors (7, 8, 10, 41) and signaling molecules (20, 44, 45, 57) in a close spatial relationship. ZO-1 is also involved in the biogenesis, disassembly, and reassembly of TJs and AJs (31, 46).

In view of this extensive role of ZO-1 in the function of cellular barriers, it was important to examine its distribution in SCEs and TMEs. The barrier formed by SCEs and TMEs differs in several respects from that of typical vascular endothelial (VECs) and epithelial cells. In typical VECs ZO-1 is deposited in a continuous pattern encircling each cell, much like caulking around tiles (15–17). In contrast, in SCEs and TMEs, ZO-1 is deposited in a discontinuous palisading pattern, underneath short filopodia that extend across the apical cell surface (Fig. 2). Thus, in SCEs and TMEs, ZO-1 and associ-
Fig. 10. GV formation process. A: 1 GV viewed from the side is shown. The cell within which this vacuole arises is appreciated at the forefront of this image. The vacuole itself arises from a platform formed near the center of this cell, which is indicated by white arrows. The cell border, which was artifactually disrupted during the critical point drying process, is apparent at the site of black arrows (×2,607). B: same cell seen in A photographed from the top. White arrows indicate the surrounding platform, and the cell border is shown with black arrows along the top (×3,000). C: SCE monolayer perfused from the top (apex) toward the bottom (base) is shown to illustrate the fact that no vacuolar structures are apparent with flow in this direction (i.e., control condition) (×480). D: when perfused from the bottom (base) toward the top (apex) vacuoles are formed in the SCE monolayer, 2 of which can be appreciated at this slightly higher magnification (×575). E: higher-magnification view of 1 vacuole, which protrudes toward the basal surface of the SCE monolayer (×740). F: this vacuole appears to be protruding toward the apical cell surface (×920). G: the largest vacuole we observed with light microscopy. This vacuole protrudes into the apical cell surface. The nucleus of this cell is indicated by arrows (×2,200).

ated junctions are located outside of the paracellular pathway proper. This arrangement may be particularly well suited to withstand tension generated across the inner wall of SC. Forces acting on this wall include the intraocular pressure (~16 mmHg), which is counteracted by pressure in the lumen of SC and episcleral veins (i.e., ~4–8 mmHg) (13, 14, 36). In addition, ciliary muscle contraction is transmitted by tendons and their connections to the scleral spur onto the TM and SC during accommodation, producing other significant mechanical effects (30, 33). It is interesting to note that the lung, which is subject to continuous and major changes in conformation under the influence of glucocorticoids, adrenergic agonists, and other vasoactive agents (4, 5, 12, 53).

The role of ZO-1 in the biogenesis, assembly, and disassembly of SCE and TME junctions has begun to be examined (3, 5, 53). The use of antisense oligonucleotides to abrogate ZO-1 expression is associated with a reduction in the number of ZO-1-containing junctions (30). This alteration results in widening of the paracellular route and in a congruous reduction in transendothelial fluid flow resistance (53). Similarly, exposure to β-adrenergic agonists, such as those used to increase aqueous outflow during glaucoma therapy, results in widening of the intercellular space and disassembly of intercellular junctions (3, 5, 53). Exposing SCEs and TMEs to glucocorticoids, which decrease aqueous outflow and induce steroid glaucoma, is accompanied by narrowing of the paracellular pathway, increases in ZO-1 expression, TJ assembly formation, and fluid flow resistance (53). The transmission of cytoskeletal effects onto ZO-1 and integral transmembrane proteins has also begun to be studied. Inactivation of the GTPase RhoA, which regulates actin-myosin phosphorylation and contraction (28, 34), is associated with disassembly of ZO-1-containing junctions in SCEs. Furthermore, the disassembly of such junctions was associated with an increase in outflow facility measured in human outflow pathway tissues maintained in organ culture (54).

Use of the specific anti-α+ antibody permitted us to detect clear-cut differences in binding affinity when using extracts from either cell type and Western blots (Fig. 1), cultured cells in vitro (Figs. 2 and 3), or unfixed frozen sections in vivo (Fig. 4). In testing the two cell types for the presence of the α-, β-, and γ-isoforms we find no qualitative differences in ZO-1 isoform expression, although their binding along cell borders is more intense in SCE cells. The antibody for β-isoforms of ZO-1 is bound at multiple nuclear sites of SCEs and TMEs, in agreement with studies in other cell types showing ZO-1 in nuclei of subconfluent monolayers (27) and ZO-2 at speckles colocalizing with the splicing protein SC35 (32). This nuclear location of some ZO-1 isoforms has raised the possibility that ZO-1 has a role in regulating transcription (8, 57).

The two α-isoforms of ZO-1 might perform different roles in the assembly of TJs. In embryos, the α−-isoform is present at the blastomere stage, even before TJs are assembled, whereas the α+ -isoform appears later, before the formation of the nascent blastocele and the appearance of TJs (46). This distribution suggests that the α+ -isoform is expressed in high-resistance junctions whereas the α−-isoform is present in relatively leaky adherens-type junctions. We found that SCEs, which express the α−-isoform to a greater extent than TMEs, offer greater resistance to transendothelial fluid flow and higher
TERs (33.5 Ω·cm² compared with 21.5 Ω·cm² for TMEs). Overall, in vitro HC and electrical resistance data indicate that, compared with other endothelia, the SCEs and TMEs form relatively leaky barriers similar to those containing AJs. By way of comparison, endothelial cells of the blood-brain barrier have a TER of 1,500–2,000 Ω·cm², whereas leaky human placental endothelial cells have a TER of 22–52 Ω·cm² (19, 31, 43). We propose that the relatively leaky SCEs and TMEs are particularly well suited to facilitate the outflow of aqueous humor.

We emphasized in the introduction to this article that SCEs play a critical role in the regulation of aqueous outflow. However, we would also like to point out that TMEs face aqueous outflow in the direction in which they are more resistive (i.e., apical to basal) whereas SCEs face outflow in the direction in which they are less resistive (i.e., basal to apical) (30). The net effect should be that TMEs present a greater resistance to transendothelial fluid flow compared with SCEs. The significance of this relationship vis-à-vis aqueous outflow must be investigated. We suggest that one function of the TMEs is to lessen the impact of forces generated by aqueous outflow and any damage due to shearing and other effects on the basal surface of SCEs. Another role of TMEs may involve the release of autacoids and other factors that can flow downstream with aqueous humor to influence the barrier function of SCEs so as to regulate the egress of aqueous humor. The ZO-1-containing junctions in SCEs and TMEs are disposed like ties in a railroad or fingers in hands clasped together. This arrangement should help to prevent movement and blockage of the ocular vessels, whereas other agents, like glucocorticoids, which induce polymerization of cytoskeletal proteins (18), might reduce GV formation and still others, such as adrenergic agonists/antagonists (4) and GTPases (54), which alter the phosphorylation state of cytoskeletal, accessory, and transmembrane junction proteins, might be expected to have an effect as well on GV formation and the regulation of the intraocular pressure.

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