Partial Characterization of the Human Retinal Endothelial Cell Tight and Adherens Junction Complexes

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PURPOSE. In diabetic retinopathy and macular edema, the blood-retinal barrier fails to function properly, and there is transvascular leakage of proteins and solutes. The tight junction protein occludin and the adherens junction protein cadherin-5 have been shown to be critical to maintaining the endothelial barrier and regulating paracellular transport of large vessel endothelia. However, the expression and distribution of these junction proteins in the retinal endothelium is not well characterized.

METHODS. Human and bovine retinal endothelial cells were isolated as described previously. Western blot analysis and flow cytometry techniques were used to assay for the presence of occludin, zona occludens-1 (ZO-1), cadherin-5, and β-catenin. The subcellular localization of the proteins was visualized by immunohistochemistry performed on cultured human retinal endothelial cells and cryosections of bovine retina.

RESULTS. Western blot analysis and flow cytometry techniques found occludin, ZO-1, cadherin-5, and β-catenin in cultured human retinal endothelial cells. Immunofluorescence staining of cultured retinal endothelial cells and cryosections of bovine retina showed junctional localization of occludin, ZO-1, cadherin-5, and β-catenin.

CONCLUSIONS. This report demonstrates the expression of occludin and cadherin-5 in retinal endothelial cells and their localization to sites of cell-cell contact. Expression of their respective regulatory proteins, ZO-1 and β-catenin, at sites of cell-cell contact suggests that occludin and cadherin-5 play a role in maintaining the retinal endothelial barrier.

In ocular diseases such as diabetic retinopathy and macular edema, the blood-retinal barrier exhibits an increased permeability to fluid and solutes, suggesting dysfunction of the retinal endothelial barrier. Some physiologic increases in permeability are associated with the formation of interendothelial gaps, implicating modification of the interendothelial junctions. Recent data suggest that cadherin-5 (VE-cadherin), an endothelial cell-specific adherens junction protein, plays a key role in the modulation of large vessel permeability. We are currently exploring the hypothesis that the retinal endothelial junction structure is critical to the loss of barrier function observed in retinal disease.

The relationship between the endothelial barrier and endothelial junction structures has been investigated in a number of organs, but it is difficult to extrapolate these results to the retinal vascular bed. Studies of specific endothelia are necessary, because the permeability of the endothelial barrier varies with the location of the vascular bed. For example, the blood-brain barrier is considered one of the tightest capillary barriers, whereas the fenestrated capillaries of the kidney are relatively permeable. Endothelial cell junctions have also been found to vary with the location of the vascular bed. A comparison of large- and small-vessel pulmonary endothelia reveals that the smaller vessels have a lower permeability and more complex junction structures.

Although relatively little is known about the identity and role of specific junction molecules in retinal endothelial cells, there are a number of studies of junction proteins in other barrier-forming cells. Adherens and tight junctions have been studied in epithelial and endothelial systems and have proven to be critical to the maintenance of barrier function. Two well-characterized components of the tight junction are occludin and zona occludens-1 (ZO-1). Occludin is the only identified transmembrane component of the tight junction, and it appears to be critical for an impermeable barrier. Furthermore, cell-cell interactions can be disrupted by the addition of competing peptides to the occludin homophilic binding site.

It has been shown that the carboxyl-terminus of occludin, which binds ZO-1, is necessary for the formation of tight junction barriers during development. ZO-1 is a cytoplasmic protein that is proposed to link occludin to the other intracellular junction structures. The level of ZO-1 expression is inversely related to permeability, since it has been shown that increased ZO-1 expression increases electrical resistance across monolayers of rat brain capillary endothelial cells.

Adherens junctions contain cadherins, a family of calcium-dependent transmembrane proteins that participate in homophilic binding (e.g., cadherin-5 binds cadherin-5). Cadherin-5 is an endothelial-specific cadherin. Blocking the homophilic binding of cadherin-5 with a monoclonal antibody blocks the formation of endothelial tube structures in vitro and the reformation of the endothelial barrier after low calcium disruption of the endothelial junctions. Targeted null-mutations in the cadherin-5 gene show that it is necessary for vessel formation in vivo. β-Catenin is a cytoplasmic protein that binds to the cytoplasmic domain of cadherins and contributes to adherens junction formation. Adherens junctions, in addition to providing adhesion between adjacent cells, may also mediate the formation of tight junctions, because formation of the tight junction complex is calcium dependent. In this study we characterize the expression and distribution of occludin, ZO-1, cadherin-5, and β-catenin in the retinal endothelial barrier.
MATERIALS AND METHODS

Isolation and Culture of Retinal Microvessel Endothelium

Human eyes were obtained from the Lions' Eye Bank (Vanderbilt University Medical Center, Nashville, TN). Human and bovine retinal endothelial cells were isolated as described previously. Briefly, retinal microvessel endothelium was isolated by mechanical and enzymatic dissociation of the retina with 0.25% collagenase (Boehringer Mannheim, Indianapolis, IN). Retinal microvessel fragments were filtered across a 100-μm nylon mesh, collected on 20-μm nylon mesh screen, and seeded onto fibronectin-coated tissue culture ware (10-μg/cm²). Culture medium consisted of D-valine supplemented-minimum essential medium (GIBCO, Grand Island, NY), supplemented with 20% fetal bovine serum (Intergen, Purchase, NY) plus 100 μg/ml brain-derived growth factor, 90 μg/ml heparin, and 0.1% penicillin/streptomycin and amphotericin. Retinal endothelial cells were designated "endothelial" by "cobblestone" morphology, uptake of dil-labeled low-density lipoprotein (Biomedical Technologies, Stoughton, MA), and the presence of factor VIII antigen (MAB 037-68/3; Chemicon, Temecula, CA).

Antibodies

Monoclonal antibodies specific for cadherin-4 (120A) and cadherin-5 (5Q8A) were obtained from ICOS Corp. (Bothell, WA) as hybridoma supernatants. A rabbit polyclonal antibody specific for β-catenin was also obtained from ICOS. Rabbit polyclonal anti-occludin and anti-ZO-1 were purchased from Zymed (San Francisco, CA). The same primary antibodies were used in western blot analysis, immunohistochemistry, and flow cytometry analysis. Horseradish peroxidase-conjugated secondary antibodies used in western blot analysis were obtained from Sigma Chemical Co. (St. Louis, MO). Biotinylated secondary antibodies and streptavidin-Cy3 conjugates used in immunohistochemistry were from Jackson Immuno Research Laboratories (West Grove, PA). Rabbit and mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, also from Jackson, and a rat FITC-conjugated secondary antibody from Tago (Burlingame, CA) were used in flow cytometry.

Western Blot Analysis

Lysates were prepared from cultured retinal endothelial cells. The cells were rinsed twice with cold (4°C) phosphate-buffered saline (PBS) without calcium and magnesium (CMF-PBS) and were lysed by the addition of TX buffer (consisting of 150

![Flow cytometry](https://example.com/flow_cytometry_graph.png)

**Figure 1.** Flow cytometry shows increased fluorescence staining for the junction proteins occludin (A), ZO-1 (B), cadherin-5 (C), and β-catenin (D) compared with controls.
FIGURE 2. Western blot analysis of retinal lysates probed for the tight junction proteins occludin (A) and ZO-1 (B) and the adherens junction proteins cadherin-5 (C) and β-catenin (D). (A) Lanes 1 and 2 are retinal capillary endothelial (RCE) lysates; lanes 3 and 4 are Madin-Darby canine kidney (MDCK) and L-cell lysates, respectively. (B) Lane 1 is a retinal lysate, lane 2 is an L-cell lysate, and lane 3 is a MDCK-positive control lysate. In (C) and (D), lanes 1 and 2 are RCE lysates. (C) Lane 3 is an MDCK-negative control, and lane 4 is a human umbilical vein endothelial cell (HUVEC)-positive control. (D) Lane 3 is also a HUVEC-positive control. Positions of molecular weight markers are shown at left.

mM NaCl, 10 mM HEPES, 2 mM EDTA, 0.5% Triton X-100, 1 mM phenanthroline, 50 mg/ml benzamidine) plus protease inhibitors (0.1 mM 4-(2-aminoethyl)benzenesulfonyl, 10 μg/ml each aprotinin, leupeptin, pepstatin A, antipain, soybean trypsin inhibitor, 40 μg/ml Na-p-tosyl-L-lysine chloromethylketone, 40 μg/ml bestatin, and 100 μg/ml each chymostatin, iodoacetamide, and N-tosyl-L-phenylalanine chloromethylketone) for 30 minutes. The lysates were then transferred to 1.5 ml Eppendorf tubes and centrifuged for 10 minutes at 4°C. The supernatant was removed and protein concentration assayed. The lysates were then frozen in 50 ml aliquots to minimize repeated freeze-thawing.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the polypeptides, which were transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C in blocking buffer (5% nonfat powdered milk in Tris-buffered saline containing 0.5% Tween). Primary antibodies were diluted in blocking buffer and applied to membranes for 1 hour at room temperature. The membranes were probed with cadherin-5 antibody diluted 1:200 or with anti-β-catenin diluted 1:100. The membranes were also probed with anti-occludin diluted 1:1000 or with anti-ZO-1 diluted 1:2000. The blots were washed in Tris-buffered saline with 0.5% Tween-20. Horseradish peroxidase-conjugated secondary antibodies were diluted in blocking buffer and applied to membranes for 1 hour at room temperature. The blots were washed in Tris-buffered saline with 0.5% Tween-20. Chemiluminescent substrate (Pierce, Rockford, IL) was added, and blots were processed according to manufacturer’s instructions. Film exposure times ranged between 5 seconds and 4 minutes.

Immunohistochemistry

Retinal endothelial cells were grown to confluence in eight-chamber slides. The cells were washed twice with warm (37°C) CMF-PBS. Three percent paraformaldehyde warmed to 37°C was added for 5 minutes; then the cells were washed twice with CMF-PBS. The cells were permeabilized by the addition of cold (4°C) CSK buffer (250 mM ammonium sulfate, 10 mM PIPES [pH 6.8], 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 0.5% Triton X-100) for 2 minutes; then the cells were washed twice with CMF-PBS.

Bovine retinas were frozen, and 6-μm cryosections were fixed in acetone. The cells and sections were blocked with 2% bovine serum albumin and 5% horse serum for 1 hour at room temperature. Primary antibodies specific for cadherin-4 or cadherin-5 were diluted 1:50 in blocking solution and applied for 1 hour. Anti-β-catenin and anti-occludin were diluted 1:100. Anti-ZO-1 was diluted 1:500. Secondary antibodies conjugated to biotin were applied for 30 minutes followed by streptavidin conjugated to Cy3 for 30 minutes. The cells and cryosections were mounted in Vectashield (Vector, Burlingame, CA). The staining was visualized using a Zeiss Axiosplan microscope with
fluorescence optics (Surgical Sciences Analytical Biochemistry and Cell Imagery Core Laboratory). Images were captured by an attached digital camera using Zeiss Image Pro Plus software (Media Cybernetics, Silver Spring, MD) or on 35 mm film.

**Flow Cytometry**

Retinal endothelial cells were removed from culture in 100-mm dishes by a 30-minute incubation at 37° with 3 ml Dispase (Becton-Dickinson, Bedford, MA). EDTA was added to make a 5 mM solution, and the cells were incubated for 30 minutes at room temperature. Cells were then washed once with CMF-PBS. Three percent paraformaldehyde was added for 5 minutes, and afterward the cells were washed. Cells were then permeabilized by the addition of CSK buffer for approximately 3 minutes followed by a wash step. At each step, the cells were gently pipetted to improve mixing with the solution.

After the cells had been fixed and permeabilized, they were blocked in a 2% bovine serum albumin solution in CMF-PBS for 1 hour. Cells were stained with antibodies specific for cadherin-4, cadherin-5, β-catenin, ZO-1, or occludin according to the immunohistochemistry protocol above. The cells were washed twice with CMF-PBS, and FITC-conjugated secondary antibodies were added at a dilution of 1:1000 for 30 minutes at room temperature. The cells were again washed twice with CMF-PBS. A Becton-Dickinson FACSCalibur flow cytometer was used to analyze the cells. Excitation at 488 nm was achieved with an argon laser, and the fluorescence was measured at 530 nm. Data consisting of side scatter, forward scatter, and fluorescence intensity were collected for 20,000 gated cells per sample. Winlist software (Verity Software House, Topsham, ME) was used to analyze the list-mode data.

**RESULTS**

**Flow Cytometry**

Flow cytometric analysis indicates that adherens and tight junction proteins are expressed in retinal capillary endothelial (RCE) cells. Occludin, ZO-1, cadherin-5, and β-catenin are expressed in the entire cell sample, not a subpopulation (Fig. 1). The histograms indicate the fluorescence intensity of two cell samples. The fluorescence of the cells immunostained with primary antibody and FITC-conjugated secondary antibody is shown by the histograms labeled with the indicated junction protein. Control histograms represent cells that received fluorophore-conjugated secondary antibody only. The control histograms reflect the background fluorescence of the cells and nonspecific binding of the secondary antibody. The protein-labeled histograms show a shift to the right, indicating the presence of junction protein in the immunostained cell population.

The histograms representing occludin-, ZO-1-, cadherin-5-, and β-catenin-stained cells are shifted to the right...
of their respective controls. As the fluorescence intensity is plotted on a log scale, the shift in the histograms indicates a significant increase in fluorescence. These results show the distribution of the junction proteins in all cells of the cultured cell population.

**Western Blot Analysis**

Western blot analysis demonstrates the expression of the tight junction proteins occludin and ZO-1. In Figure 2A, occludin protein is detected in RCE cell lysates (lanes 1 and 2) as several bands with apparent molecular weights between ~51 kDa and 55 kDa; there is also a faint band at ~44 kDa. The detection of several bands is characteristic of the different phosphorylation states of occludin.19 The Madin–Darby canine kidney (MDCK)-positive control lysate (lane 3) yields such intense staining that distinct bands are not detectable, but the protein is in the range of ~47 kDa to 62 kDa. L-cells (murine fibroblasts, lane 4), which do not express the adhesion proteins assayed for here, serve as a negative control. In Figure 2B, ZO-1 staining of the retinal lysate (lane 1) is seen in a dark band at ~195 kDa and lighter bands at ~175 kDa and 210 kDa. The MDCK-positive control (lane 3) has a band with an apparent molecular weight of ~230 kDa. The smaller molecular weight of the RCE lysate may be explained by differences in phosphorylation or by partial degradation of the RCE lysate.

Western blot analysis also indicates that the two adherens junction proteins cadherin-5 and β-catenin are present in RCE cells (Figs. 2C and 2D). Figure 2C shows cadherin-5-staining of RCE lysates (lanes 1 and 2) by a predominant band with an apparent molecular weight of ~120 kDa. A less intense band of ~95 kDa is also seen. A human umbilical vein endothelial cell (HUVEC) lysate (lane 4) serves as a positive control with a prominent band at ~135 kDa. The lower molecular weight obtained for the RCE cell (~120 kDa) is seen consistently and reproducibly with multiple RCE lysates (n = 5). The difference in molecular weight between HUVEC and RCE may be accounted for by differences in glycosylation between the two cell types11 or by degradation of the RCE lysate. Western blot
analysis for β-catenin produces a single band at ~95 kDa in both retinal lysates (Fig. 2D, lanes 1 and 2). The HUVEC control lysate has a band at ~90 kDa (lane 3).

**Immunohistochemistry**

Subcellular localization of the junction proteins was examined by immunofluorescence staining (Fig. 3). Confluent monolayers of human retinal endothelial cells were stained for occludin, ZO-1, cadherin-5, and β-catenin. All four junction proteins demonstrate clear staining of cell-cell contacts in cultured RCE cells.

Different secondary antibodies were used to stain for cadherin-5 and β-catenin. The nuclear staining of cadherin-5 is background staining seen with the mouse secondary antibody alone (data not shown). The nuclear staining of β-catenin is not seen with the rabbit secondary antibody alone (data not shown). The nuclear staining presumably indicates the presence of β-catenin in the nucleus, where it has been shown to function as a signaling molecule.

Immunostaining of bovine retinal cryosections demonstrates the expression of occludin, ZO-1, cadherin-5, and β-catenin in large retinal vessels (Fig. 4) and retinal capillaries (data not shown) in vivo, and confirms the in vitro results (Fig. 3). Punctate localization of all four junction proteins in retinal blood vessel endothelium indicates their restriction to sites of endothelial cell-cell contacts.

**DISCUSSION**

The junction proteins occludin and cadherin-5 are thought to be critical for regulation of paracellular permeability. This is the first report demonstrating the presence of these proteins in retinal endothelial cells. The localization of the transmembrane proteins and of the cytoplasmic binding proteins ZO-1 and β-catenin to sites of cell-cell contact suggests that occludin and cadherin-5 play a role in maintaining the endothelial permeability barrier.

The tight junction protein occludin is found in retinal endothelial cells in vitro (Figs. 1A, 2A, 3A) and in vivo (Fig. 4A). Both occludin and ZO-1 are found localized to sites of cell-cell contact (Figs. 3A, 3B, 4A, 4B). In epithelial cells, it has been shown that occludin requires ZO-1 for localization to tight junctions. However, recent studies have also examined occludin and ZO-1 in endothelial cells. Blood-brain barrier endothelial cells, which provide a tight barrier similar to the blood-retinal barrier, express greater amounts of occludin than nonneural endothelial cells.

These findings suggest that the study of occludin and ZO-1 provides the basis for future studies examining the contribution of each of these junction proteins to the retinal-endothelial barrier under normal and diabetic conditions.

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References

Intraocular Pressure in Rabbits by Telemetry II: Effects of Animal Handling and Drugs

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PURPOSE. To measure under carefully controlled conditions the effects in the rabbit eye of commonly used therapeutic agents for glaucoma.

METHODS. Rabbits were outfitted in one eye with an implantable telemetric pressure transducer and monitored for several months under controlled conditions of light/dark and handling. Effects of tonometry, handling, water drinking, and instillation of topical ophthalmic medications on intraocular pressure were recorded during each 24-hour day/night cycle.

RESULTS. Pneumatonometry, animal handling, and water drinking all had an effect on intraocular pressure that in many instances was of the same magnitude as the effects of pharmacologic agents. Dorzolamide and timolol caused a sustained reduction of intraocular pressure during the nocturnal period. Epinephrine had a biphasic effect, causing an immediate pressure elevation followed by a prolonged depression. Apraclonidine, latanoprost, and pilocarpine had no measurable effect.

CONCLUSIONS. Continuous telemetric measurement of intraocular pressure in rabbits permits the measurement of uncontrollable artifacts that occur with tonometric measurements and animal handling. If environmental conditions are rigidly controlled, this method is very sensitive for detecting therapeutic effects of candidates for ocular hypotensive drugs. When healthy animals are used, the method appears to be more sensitive for drugs that affect aqueous humor formation than for drugs that affect aqueous humor outflow resistance. (Invest Ophthalmol Vis Sci. 1998;39:2485–2489)

A considerable body of information about mammalian aqueous humor physiology has been acquired from experimental studies of rabbits. Until recently, most of the measurements of intraocular pressure in this animal were carried out by manometry or by tonometry. With these methods, it has always been necessary to disturb the animal in some way at the moment instantaneous intraocular pressure is measured.

McLaren and coworkers1,2 and Schnell and coworkers3,4 used a telemetric transducer system to permit continuous measurement of intraocular pressure over long periods of time in conscious and undisturbed animals. This method permits study under controlled experimental conditions without interference by the tonometric procedure itself. Using this system, these investigators confirmed the circadian rhythm of intraocular pressure in this species and demonstrated that the telemetric system is capable of measuring intraocular pressure effects of a variety of disturbances such as application of tonometry, intravenous infusion of osmotic agents, and topical application of timolol. The method is generally applicable to measurements of phenomena that occur in seconds or trends that extend over many months.

The present study explores additional advantages of the telemetric system as a screening tool for the intraocular pressure effects of pharmacologic agents. The study shows that artifacts caused by handling, tonometry, circadian cycle, and water drinking can be eliminated and that pharmacologic effects can be detected, even with the use of very small numbers of animals.

METHODS

Five pigmented rabbits weighing between 2 kg and 2.5 kg were studied in a protocol approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and in conformance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Each animal was outfitted with an implantable pressure monitor (model TA11PA-C40; Data Sciences International, St. Paul, MN) according to the surgical procedure described previously.2 Briefly, the body of the transducer was implanted in a musculocutaneous pocket in the nape of the animal's neck, the connecting tubing was tunneled beneath the skin into the orbit, and the tip of the tubing was inserted into the anterior chamber through a beveled corneal incision. After a period of healing and stabilization, each animal was ready for pressure studies using the caging, telemetric, and computer system described previously.2

References: