Claudin-19 and the Barrier Properties of the Human Retinal Pigment Epithelium

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PURPOSE. The retinal pigment epithelium (RPE) separates photoreceptors from choroidal capillaries, but in age-related macular degeneration (AMD) capillaries breach the RPE barrier. Little is known about human RPE tight junctions or the effects of serum on the retinal side of the RPE.

METHODS. Cultured human fetal RPE (hfRPE) was assessed by the transepithelial electrical resistance (TER) and the transepithelial diffusion of methylated polyethylene glycol (mPEG). Claudins and occludin were monitored by quantitative RT-PCR, immunoblotting, and immunofluorescence.

RESULTS. Similar to freshly isolated hfRPE, claudin-19 mRNA was 25 times more abundant than claudin-3. Other detectable claudin mRNAs were found in even lesser amounts, as little as 3000 times less abundant than claudin-19. Claudin-1 and claudin-10b were detected only in subpopulations of cells, whereas others were undetectable. Knockdown of claudin-19 by small interfering RNA (siRNA) eliminated the TER. siRNAs for other claudins had minimal effects. Serum affected tight junctions only when presented to the retinal side of the RPE. The TER increased 2 times, and the conductance of K⁺ relative to Na⁺ decreased without affecting the permeability of mPEG. These effects correlated with increased steady-state levels of occludin.

CONCLUSIONS. Fetal human RPE is a claudin-19-dominant epithelium that has regional variations in claudin-expression. Apical serum decreases RPE permeability, which might be a defense mechanism that would retard the spread of edema due to AMD. (*Invest Ophthalmol Vis Sci.* 2011;52:1392-1403) DOI: 10.1167/iovs.10-5984

The retinal pigment epithelium (RPE) is an unusual epithelium from two perspectives. First, its apical surface abuts a solid tissue, the neural retina. Second, it collaborates with a fenestrated capillary bed to form a blood-tissue barrier, the outer blood-retinal barrier.¹ This barrier regulates the composition of the subretinal space to enable the photoreceptors to carry out their functions. The outer blood-retinal barrier is

disrupted in retinal diseases such as age-related macular degeneration. In the neovascular form of the disease, choroidal capillaries breach the RPE monolayer to invade the subretinal space, which separates the RPE from the photoreceptors. Since these capillaries are leaky, serum components gain access to the apical (subretinal) surface of the RPE. In lower vertebrates, serum on the apical side of the RPE makes the RPE monolayer leaky.^{2,3} Leakiness of RPE tight junctions might allow disease to spread laterally. As serum components leak across the tight junctions of one RPE cell, those components could lead to leakiness in the tight junctions of the neighboring RPE cell and so on. Fluorescein angiography is used clinically to detect breakdown of the RPE barrier, but clinical observations of age-related macular degeneration (AMD) provide limited information regarding the cellular basis of leakage.

There are two components to any tissue barrier. The first includes the membrane proteins that mediate the transcellular pathway for solutes that traverse the monolayer; these have been extensively studied in RPE; for review, see Strauss.⁴ Second is the circumferential band of tight junctions, which semiselectively retards the diffusion of solutes through the spaces between the cells of the monolayer. Tight junctions limit the dissipation of the electrochemical gradients established by the first component of the barrier. The permeability and selectivity of tight junctions are tissue specific and can be regulated physiologically.⁵⁻¹¹

The selectivity and permeability of tight junctions depends on their protein composition, especially which subset of claudins are included.⁹ Claudins are a family of transmembrane proteins. Twenty-four claudins have been described, and each is thought to have its own effect on selectivity and permeability. Permeability also appears to be regulated by a second transmembrane protein, occludin.¹²⁻¹⁵ Occludin has a half-life of 1.5 hours and rapidly associates and dissociates from the tight junction.^{16,17} These properties likely contribute to occludin's regulatory role and the dynamic nature of tight junctions. During normal chick development, the composition and functional properties of RPE tight junctions matures as the neural retina and choroid differentiate.¹⁸ In a culture model of chick development, retinal conditioned medium, serum, and extracellular matrix all affected the composition and functional properties of RPE tight junctions.^{3,19,20}

The relevance of chick RPE to human tissue may be limited. Claudin-19 is not expressed in chick, but it is an essential component of human RPE tight junctions as patients who lack claudin-19 suffer severe visual impairment.²¹ Besides claudin-19, we previously reported evidence of claudin-3 and claudin-10, which are also absent from chick RPE.²² The expression of other claudins was not resolved in that report. Most primary and secondary cultures of RPE fail to form robust junctions. A spontaneously transformed cell line, ARPE19, was able to form relatively leaky tight junctions when early passages of the cell line were properly maintained in specialized media.²³ The transepithelial electrical resistance (TER) had been reported as high as 90 $\Omega \times \text{cm}^2$, but this is low compared with the 206

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Supported in part by the National Eye Institute vision core grant EY000785 (Yale University), the International Retinal Research Foundation (IJR), Leir Foundation (RAA), Newman's Own Foundation (RAA), National Natural Science Foundation of China Grant No. 30772381 (SP), and the Yale Endowed Student's Research Fellowship (VSR).

Submitted for publication June 1, 2010; revised September 22, 2010; accepted October 8, 2010.

Disclosure: S. Peng, None; V.S. Rao, None; R.A. Adelman, None; L.J. Rizzolo, None

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$\Omega \times \text{cm}^2$ that has been estimated for human RPE in vivo.²⁴ Unfortunately as the passage number has increased, and in the culture conditions commonly used, the properties of ARPE19 are highly variable. The tight junctions they form in culture can be rudimentary, and the claudins that they express are variable.^{25,26} A more reliable model uses human fetal RPE (hFRPE). In the three culture models that have been developed for hFRPE, the TER ranges from 500 to 2000 $\Omega \times \text{cm}^2$. Media for these models contain 5% serum,²⁷ 1% serum,²⁸ or no serum.²⁹ Besides serum, the composition of the base media for these formulations also varied. It is unclear which model most closely resembles native RPE.

The present study uses hFRPE to examine claudin and occludin expression. We demonstrate that claudin-19 is the predominant claudin, but that nonuniform expression of the minor claudins might indicate regional variations in barrier properties around the globe of the eye. Further, we show that serum can increase the TER and modulate other aspects of barrier function when it comes in contact with the apical surface of the RPE.

METHODS

Cell Culture

The research followed the tenets of the Declaration of Helsinki, the ARVO animal statement, the institutional review board of the National Institutes of Health, and Yale School of Medicine guidelines. The primary cultures of human fetal RPE cells were supplied by the laboratory of Sheldon Miller (National Eye Institute, Bethesda, MD) and were reseeded, 1.3×10^5 cells per well, onto clear cell culture inserts (12-mm diameter inserts, 0.4- μm pores, polyester membranes; Costar Transwell, Corning, NY). Before seeding, the inserts were coated with human extracellular matrix (10 μg in 150 μL Hank's balanced salt solution per well; BD Biosciences, Franklin Lakes, NJ) and cured with UV light in a tissue culture hood for 2 hours. Growth medium was formulated by Mamanishkis et al.²⁷ and consisted of MEM α -modified medium, 1:100 (v/v) N1 supplement, 1:100 (v/v) glutamine-penicillin-streptomycin, 1:100 (v/v) nonessential amino acid solution, 20 $\mu\text{g/L}$ hydrocortisone, 250 mg/L taurine, and 0.013 $\mu\text{g/L}$ triiodo-thyronine (Sigma-Aldrich, St. Louis, MO), and 5% heat-inactivated fetal calf serum (FBS; Atlanta Biologicals, Norcross, GA). The cells were initially plated in growth medium supplemented with 15% FBS. The volume of medium in the apical chamber and the basolateral chamber were 0.5 and 1.5 mL, respectively. The cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After 24 hours, the plating medium was replaced with growth medium; the cells were fed 3 times per week. The cells reached confluence in 2 to 3 days, but the TER continued to rise over the next 6 to 8 weeks. Experiments were not performed until the TER became stable. For some experiments, the concentration of heat-inactivated FBS varied, as indicated in the figure legends.

For some experiments, cultures with a stable TER were transferred to a serum-free medium that has also been used to culture hFRPE.²⁹ The serum-free medium consisted of 70% DMEM containing 4.5 g/L D-glucose, 30% F12 nutrient mixture containing L-glutamine, and 1% antibiotic-antimycotic solution, supplemented with 2% B27 (Invitrogen Life Sciences, Camarillo, CA). The TER continued to decrease over the next 3 to 4 weeks. When TER was stable, the cultures were used for experimentation.

Measurement of TER and Ion Selectivity

TER of hFRPE monolayers was measured using electrodes (EndOhm; World Precision Instruments, Sarasota, FL) in growth medium or serum-free medium and reported as $\Omega \times \text{cm}^2$. The conductance (G) is the inverse of the TER. If the conductance is determined in the presence of different chloride salts, it becomes a measure of the selectivity for different cations.^{22,30} TER was measured in 150 mM XCl,

2 mM CaCl₂, 1 mM MgCl₂, 2 mM BaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4, where XCl was either NaCl or KCl. The osmolarity was measured and adjusted to 310–315 mOsm (the osmolarity of the culture medium). Cultures were incubated in each salt solution until the TER became stable. After the measurements, the cells were incubated in culture medium to demonstrate the measurements were reversible and the cells remained viable. The measurements could be reproduced in the same cultures the following day.

Paracellular Flux of Methylpolyethylene Glycol

The paracellular flux of methylpolyethylene glycol (mPEG) was measured in different media that were supplemented with 10 mM NH₄Cl after a 30 minute-preincubation. The mPEG (average molecular weight, 550 or 350; MP Biomedicals, Solon, OH) was added to the appropriate medium chamber to a final concentration of 50 $\mu\text{g/mL}$. The cultures were incubated for 1.5 hours at 37°C in a humidified chamber with 5% CO₂ and the medium from the opposite medium chamber collected for analysis. The concentration of mPEG was determined using a rabbit monoclonal ELISA packages (PEG ELISA Kit; Epitomics, Burlingame, CA) according to the manufacturer's protocol. The apparent permeation coefficient P_{app} was estimated as described previously.^{22,31} The P_{app} for mPEG₅₅₀ or mPEG₃₅₀ will be referenced as P_{550} or P_{350} , respectively.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

To screen claudin and occludin expression, reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described previously,²² using 35 cycles of PCR and the primers listed in Table 1. For a positive control human kidney total RNA was obtained from the Biochain Institute (Hayward, CA). Claudins detected by this technique were further examined by quantitative, real-time RT-PCR as follows. Total RNA was extracted (RNeasy Mini Kit; Qiagen, Valencia, CA), and 2 μg of total RNA was reverse-transcribed to cDNA (Quantitect Reverse Transcription Kit; Qiagen). Real-time RT-PCR was performed (iQ SYBR Green SuperMix and Bio-Rad CFX 96 thermal cycler; Bio-Rad, Hercules, CA) following the manufacturer's suggested protocol. Experiments were performed in triplicate with a minimum of two biological repeats. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control to normalize the data. The efficiency of the PCR reaction was estimated for each primer by using serial dilutions of the cDNA and analyzing the result with the accompanying software (Bio-Rad). All primers used had efficiency of $\geq 95\%$, except for claudin-10 and claudin-15 primers, whose results were adjusted for their lower efficiencies (88% and 87%, respectively). Relative expression of mRNA was calculated using the established $2^{-\Delta\Delta C_T}$ method.³² Using this method, data were normalized to GAPDH and compared with either claudin-19 mRNA expression or to the respective claudin's mRNA expression in a control, as noted in each figure.

Protein Electrophoresis and Immunoblotting

The cultured hFRPE were solubilized on ice in 200 μL of 25 mM Tris buffer, pH 8.0, containing 2% sodium dodecyl sulfate and 10 $\mu\text{L/mL}$ Protease Inhibitor Cocktail (Sigma-Aldrich). Melanin granules were removed by centrifugation. To prevent detergent-resistant multimers of claudin from forming, EDTA was added to 5 mM along with 50 μL of 5 $\times$ gel loading buffer. The samples were incubated for 10 minutes at 37°C and then for 5 minutes in a boiling water bath. Protein concentration was determined using protein assay kit (Micro BCA; Pierce, Rockford, IL). Equal amounts of protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted. The level of α -tubulin staining was used as an internal standard to normalize each sample. The following primary antibodies were used: rabbit polyclonal anti-claudin-1, rabbit polyclonal anti-claudin-2, rabbit anti-polyclonal claudin-3, mouse monoclonal anti-claudin-10, mouse monoclonal anti-claudin-15, rabbit polyclonal anti-claudin-15, mouse monoclonal anti-occludin, mouse

TABLE 1. Primers Used for RT-PCR and Real-Time RT-PCR

Gene	Upstream	Downstream	Location	Size (bp)
Claudin-1	CTGCCCCAGTGGAGGATTTA	CAATGACAGCCATCCACATC	107-392	285
Claudin-2	ACACACAGCACAGGCATCAC	TCTCCAATCTCAAATTCATGC	490-809	319
Claudin-3	AAGGTGTACGACTCGCTGCT	AGTCCCGGATAATGGTGTG	189-436	247
Claudin-4	TGGATGAATGCGTGCTGCAG	GAGGCGGCCCAGCCGACGTA	2753-3114	361
Claudin-5	ATGTCGTGCGTGCTGCAGAG	GGTGCAGACCCAGGCGCCGCA	1459-1872	413
Claudin-6	GATGCAGTGCAAGGTGTACG	CCTTGGAATCCTTCTCCTCC	183-343	160
Claudin-7	AGTGGCAGATGAGTCTCTATG	GTTATAAAAGTCTGTGACAATCT	404-768	364
Claudin-8	GAAGGACTGTGGATGAATTGC	GATGAAGATGATTCAGCCG	142-381	239
Claudin-9	TTTCATCGGCAACAGCATCGT	GCCCAGCCCAGGTAGAGGGA	954-1357	403
Claudin-10	TGTACCAAAGTCGGAGGCTC	GCATTTTATCAAACCTGTTTGAAGG	307-677	370
Claudin-10a	GCGGCGGCACATGTCCAGG	CGAGCTCTTTTAGACATAAGC	226-926	700
Claudin-10b	CCGGCGGCATGGCTAGCA	CGAGCTCTTTTAGACATAAGC	54-758	704
Claudin-11	TGGTGGACATCCTCATCC	AGAGAGCCAGCAGAATGAGC	203-397	194
Claudin-12	TTCTTCTCCTGTGTGGAATCG	GTTGCACATTCCAATCAGGC	181-475	294
Claudin-14	CTCATGGTCATCTCCTGCCTG	ACGTAGTCGTTTCAGCCTGTAC	1050-1519	469
Claudin-15	AGGAAGCAGAGAGACCCACA	AGAACCCTTAGGGAACCTGGA	50-200	155
Claudin-16	TTTGGATTCTCACCCTGCTC	TGTGCGAGGGGCTGAGTATGA	481-879	398
Claudin-17	TGCTTATTGGCATCTGTGGC	TTCTGACCTATGTGGATGGC	284-473	189
Claudin-18	GATGATCGTAGGCATCGTCC	ATGCCGGTGTACATGTTAGC	246-473	227
Claudin-19	CTCAGCGTAGTTGGCATGAA	GAAGAACTCCTGGGTACCA	289-447	159
Claudin-20	TCCCAGGCTTTGTTATTTGG	CCAGATAAGGCCAGGATGAA	271-431	160
GAPDH	TCACGAGGCTGCTTTTAAAC	GACAAGCTTCCGTTCTTCAG	51-204	153
Occludin	GAAGCCAAAACCTCTGRGAGC	GAAGACATCGTCTGGGGTGT	2094-2323	229

Location is numbered from the initiation codon. GenBank accession numbers: Claudin-1, NM_021101; Claudin-2, NM_020384; Claudin-3, NM_001306; Claudin-4, NM_001305; Claudin-5, NM_003277; Claudin-6, NM_021195; Claudin-7, NM_001307; Claudin-8, NM_199328; Claudin-9, NM_020982; Claudin-10, NM_006984; Claudin-11, NM_005602; Claudin-12, NM_012129; Claudin-14, NM_012130; Claudin-15, NM_014343; Claudin-16, NM_006580; Claudin-17, NM_006580; Claudin-18, NM_001002026; Claudin-19, NM_008993; Claudin-20, NM_001001346; GAPDH, NM_002046; occludin, NM_002538.2.

monoclonal anti-ZO-1, and mouse monoclonal anti- α -tubulin (Invitrogen), goat polyclonal anti-claudin-9 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-human claudin-12 (R&D Systems, Minneapolis, MN), rabbit polyclonal anti-claudin-16 (Abcam, Cambridge, MA), and rabbit polyclonal anti-claudin-19 (a kind gift from Mikio Furuse, Kobe University, Kobe, Japan). As a positive control, protein extracts of human kidney were obtained from G-Biosciences (St. Louis, MO). The immunoblots were developed using horseradish peroxidase conjugated secondary antibodies and chemiluminescence reagent (ECL Plus; Amersham Life Science, Arlington Heights, IL) and imaged (Molecular Imager ChemiDoc XRS System; Bio-Rad).

Immunofluorescence and Confocal Microscopy

The subcellular distribution of the claudins, ZO-1, and occludin was determined by indirect immunofluorescence. Cultures were fixed at 4°C in two changes of 100% ethanol (30 minutes total) and immunolabeled. In addition to the antibodies used for immunoblotting, samples were counterlabeled with mouse anti-occludin or mouse anti-ZO-1 (Invitrogen). The samples were then incubated with ML-grade secondary antibodies conjugated with Cy3 or Cy5 dyes (Jackson ImmunoResearch Laboratories, West Grove, PA). Alexa Fluor 488 phalloidin (Invitrogen) was used to label F-actin, and DAPI (4',6-diamidino-2-phenylindole) was used to label the nucleus. Fluorescence images were acquired with a spinning-disc confocal microscope and processed (Axioskop or LSM 410 microscope and AxioVision software; Carl Zeiss, Thornwood, NY).

Small Interfering RNA (siRNA)

To knock down the expression of claudin mRNA, we used a pool of four siRNAs specific for either claudin-2, claudin-3, claudin-4, claudin-10, or claudin-19 (siGENOME SMARTpool; Dharmacon, Lafayette, CO) according to the manufacturer's protocol. Briefly, cultures of hRPE were incubated in antibiotic-free, serum-free medium for 2 hours. Cells

were then transfected using an siRNA concentration of 0.025 μ M and transfection reagent (DharmaFECT 4; Dharmacon). Transfection with siRNAs specific for claudin-4 served as a negative control because claudin-4 is not expressed by hRPE. Cells were incubated with siRNA for 24 hours, after which they were switched back to either growth medium or serum-free medium for the duration of the experiment. Cells transfected with siRNA were harvested at 3 days, 5 days, 7 days, and 21 days post-transfection. In some claudin-19 siRNA experiments, we performed a sequential siRNA transfection, transfecting the cultures days 0 and 3 with the same siRNA.

RESULTS

Physiologic Responses of hRPE to Culture Conditions

As reported earlier, the TER of hRPE was low when they were first plated on filters and increased during the next 6 to 8 weeks.^{22,27} After this initial period, the TER ranged from 900 to 1400 $\Omega \times \text{cm}^2$ and was stable for months. Paralleling the maturation of junctions, the cells reformed melanin granules. After establishing the cultures in growth medium, it was possible to substitute an alternative, serum-free medium that was devised for quiescent monolayers of hRPE isolated from 13-week fetuses.²⁹ The serum-free media caused a gradual, month-long decrease in the TER to 250 to 400 $\Omega \times \text{cm}^2$, after which the TER was stable for months (Fig. 1A). This effect of serum-free medium could be reversed by returning the cultures to growth medium. After 2 weeks with growth medium restored, the TER was 85% of the original cultures (data not shown). To determine whether the reduction in TER was due to the lack of serum or to other differences in the base medium, the serum concentration of growth medium was reduced to 1%, and 1% serum was added to the serum-free medium formulation. Re-

FIGURE 1. Effect of culture medium on the TER. Cultures of hRPE were maintained in growth medium until the TER was stable with time. **(A)** On day 0, cultures were continued in growth medium or switched to the medium indicated on the graph. The percentage of serum contained in each medium is indicated on the graph. The presence of serum reduced the decline of TER in serum-free medium. **(B)** On day 35, cultures that had been maintained in serum-free medium were continued in serum-free medium or switched to: serum-free medium supplemented with 5% FBS in the apical chamber, serum-free medium in the basal chamber, serum-free medium in the apical chamber, serum-free medium supplemented with 5% FBS in the basal chamber, and serum-free medium supplemented with 5% FBS in both chambers. The TER increased only if serum was added to the apical medium chamber. The gray bar indicates the TER of native hRPE $\pm$ SEM.²⁴ Error bars, SEM for 4 to 6 filters. GM, growth medium; SFM, serum-free medium.

ducing the serum concentration of growth medium had no effect, but adding serum to the serum-free medium partially mitigated against the reduction in TER.

To test the role of serum, heat-inactivated FBS was added to the serum-free medium in the apical, basal, or both media chambers after the cells were adapted to serum-free medium (Fig. 1B). The effect of serum depended on which surface of the RPE was exposed to serum. Serum added to the basolateral medium chamber had no effect, but serum added to the apical chamber increased the TER to 50% of the TER for cultures maintained in growth medium. These data suggest other media components affected the barrier properties of RPE.

Transcellular and paracellular mechanisms contribute to the TER. To isolate the paracellular component, the cells were incubated in media that would inhibit or minimize transmem-

brane transport mechanisms. The TER was measured in NaCl or KCl solutions that contained BaCl₂ but lacked CO₂. The absence of Na⁺ or K⁺ would inhibit the Na,K-ATPase, which provides the energy for many of the coporters and antiporters. The barium would inhibit K⁺ transport, and the low concentration of CO₂ would inhibit bicarbonate co-transporters. Under these conditions the TER approximates the electrical resistance of the paracellular pathway.³³ In NaCl buffer, the TER for cells maintained in growth medium was ~1300–1600 Ω × cm², and for cells maintained in serum-free medium it was ~280–500 Ω × cm². The inverse of the TER, the conductance (G), reflected the relative selectivity for Na⁺ or K⁺ when the TER was measured in the NaCl and KCl solutions (Fig. 2). As reported previously $G_{Na^+}/G_{K^+} \approx 0.3$ in cultures that were maintained in growth medium,²² but in serum-free medium

FIGURE 2. Conductance in media that inhibit transport along the transcellular pathway. The TER was measured in buffered solutions that contained either NaCl or KCl. The solutions included BaCl₂ to inhibit K⁺ channels and lacked CO₂ to inhibit bicarbonate-coupled channels. The cultures were incubated first in NaCl buffer, followed by KCl buffer. Reversing the order of the buffers had no effect on the result. The TER was measured in growth medium before and after the experiment to confirm the cultures remained healthy throughout the experiment. The results are reported as the conductance, G, which is the inverse of the TER. Experiments were performed in triplicate. Similar results were obtained with independent cultures. Error bars, SD.

FIGURE 3. Effect of culture conditions on the apparent permeation coefficient for mPEG₅₅₀ relative to mPEG₃₅₀. Permeation was measured in the apical-to-basal (dark gray bars) or basal-to-apical (light gray bars) direction. P_{550} and P_{550}/P_{350} were greater in the apical-to-basal direction if there was no serum present in the apical medium chamber. Serum had minimal effect on diffusion in the basal-to-apical direction. Experiments were performed in triplicate. Similar results were obtained with independent cultures. Error bars, SD; pairs with a statistical difference ($P < 0.01$).

this ratio increased to ~0.9. The addition of serum to serum-free medium affected cation selectivity. Whereas addition of serum to the basolateral chamber had minimal effect on G_{Na+}/G_{K+} , serum in the apical chamber increased G_{Na+}/G_{K+} significantly. This effect was negated if serum was added to both chambers.

Conductance measurements reflect the interaction of ions with the pores of the tight junction. The transmonolayer diffusion of nonionic tracers monitors a distinct mechanism of paracellular transport.^{12,31,34,35} Because there are no membrane transporters for mPEG, mPEG should cross the monolayer via the paracellular pathway. Like ions, nonionic tracers with a Stoke's radius < 4 Å can diffuse through pores in the strands of tight junctions. In contrast, nonionic tracers with a Stoke's radius > 4 Å require strands to transiently break and reseal to cross the tight junction.³¹ mPEG₃₅₀ is a polydisperse mixture of PEG that contains predominately monomers and

oligomers with a Stoke's radius < 4 Å, whereas mPEG₅₅₀ contains predominately oligomers > 4 Å. Therefore, a change in the rate of strand breaking/resealing would have a greater effect on mPEG₅₅₀. Because diffusion across tight junctions is bidirectional, the ratio of P_{550}/P_{350} should be the same in the apical-to-basal and basal-to-apical directions.

In serum-free medium, the ratio of P_{550}/P_{350} depended on the direction of the mPEG concentration gradient. P_{550} was twice as high in the apical-to-basal direction (0.0038 ± 0.0003 cm/hr vs. 0.0018 ± 0.0002 cm/hr, $P < 0.01$). Consequently P_{550}/P_{350} was higher in the apical-to-basal direction (Fig. 3) compared with the basal-to-apical direction. In growth medium, P_{550}/P_{350} was nearly the same regardless of the direction of the concentration gradient. P_{550}/P_{350} in growth medium was statistically the same as the ratio found in the basal-to-apical direction with serum-free cultures. To further explore the effect of serum, serum-free cultures were adapted to serum in either the apical or basal medium chamber (Fig. 1B). Serum in the apical chamber decreased P_{550}/P_{350} , whereas basal serum increased it. With basal serum, P_{550} was 0.0060 ± 0.0007 cm/hr in the apical-to-basal direction.

An alternative mechanism for mPEG to cross the monolayer should be transcytosis. Receptor-mediated transcytosis would be inhibited by NH_4Cl , but this had no effect on the permeation coefficient (not shown). The data shown in Figure 3 were obtained in the presence of 10 mM NH_4Cl . The data are consistent with fluid-phase transcytosis that is primarily in the apical-to-basal direction. When serum is in the apical medium chamber, P_{550}/P_{350} is the same in both directions, which suggests P_{550}/P_{350} most closely reflects the properties of tight junctions in this circumstance. P_{550}/P_{350} in the basal-to-apical direction was unaffected by culture condition, despite a TER that varied as much as fourfold with culture condition.

Expression of Claudins and Occludin

The expression of claudins by RPE isolated from four fetuses (15–16 weeks) was screened using quantitative, real-time RT-PCR. The mRNA for claudin-19 was the most prominent, followed by claudin-3 (25% of claudin-19) and claudin-12 (7% of claudin-19). Each of the other claudin-mRNAs that were detected was ≥ 85 times less abundant than claudin-19. Notably, the mRNA for claudin-16 was > 3000 times less abundant than claudin-19 (Fig. 4A). Further, claudin-10b, but not claudin-10a, was expressed in vivo and in culture (Fig. 4B).

Qualitatively, the expression of claudins in cultured RPE was similar to expression in vivo. There were a few quantitative differences. When the data are normalized against GAPDH, the mRNA for most of the claudins appeared to be underexpressed in culture, with claudin-10 being the major exception

FIGURE 4. Expression of claudins and occludin in freshly isolated hRPE. (A) Total RNA was isolated from four fetuses and analyzed by quantitative, real-time RT-PCR. The data were normalized to GAPDH and expressed relative to claudin-19, as described in this article. (B) Claudin-10 was analyzed by 35 cycles of RT-PCR using primers that were specific for either claudin-10a or claudin-10b. RNA from human kidney was used as a positive control. Size markers in the left lane are from a *HaeIII* digest of Φ X174: 1353, 1078, 872, and 603 base pairs. Error bars, SD; cldn, claudin.

FIGURE 5. Expression of claudins and occludin in vitro. hRPE was isolated from two fetuses and cultured independently. Each isolate was maintained in the medium indicated in each panel. Total RNA was extracted from each culture and analyzed in triplicate. The amount of mRNA relative to claudin-19 was estimated by quantitative RT-PCR. The columns indicate the mean of the data obtained from the two sets of cultures, whereas the error bars indicate the range. (A) Comparison of growth medium and serum-free medium. Cultures were maintained, as described in Figure 1A. Data are expressed relative to the expression of claudin-19. For reference, horizontal bars indicate the level of expression in vivo (Fig. 4). (B) Effects of adding serum to the apical or basal side of the monolayer. Cultures maintained in serum-free medium were readapted to serum, as indicated in Figure 1B. Data were expressed relative to expression in serum-free medium. Generally, serum had minimal effect on the expression of the mRNAs tested. Error bars, SD; cldn, claudin.

(Fig. 5A). Nonetheless, claudin-19 remained the dominant claudin, with mRNA levels >20 times higher than any other mRNA. (Although claudin-12 mRNA was 3 times less than claudin-19, we could not confirm claudin-12 by immunoblotting or immunofluorescence.) Culture conditions had minimal effects on the expression of most claudin mRNA with the exceptions of claudin-1, claudin-2, and claudin-10. To distinguish the effects of serum from other differences between the culture media, the concentration of serum was varied in the growth medium, and serum was added to the serum-free medium. In growth medium, claudin-1 mRNA decreased further when the serum concentration was reduced. Claudin-2 and claudin-10 mRNA were unaffected by serum but were overexpressed in serum-free medium relative to native tissue. When added to serum-free media, serum had its largest effects on the mRNA of claudin-16, decreasing its expression to levels observed in vivo. The mRNA for occludin decreased when serum was reduced in growth medium.

Once cultures were adapted to serum-free medium, supplementing serum-free medium with FBS had minimal, or inconsistent, effects on mRNA expression except for claudin-10 and occludin. Regardless of which medium chamber received FBS, there was an increase in expression (Fig. 5B).

Immunoblotting indicated additional regulation of claudin protein levels. Although the claudin-1 mRNA expression level was unaffected by the addition of serum to serum-free medium, the steady-state level of the protein did increase. This increase in claudin-1 was observed when serum was present in the apical medium compartment, but not when serum was added only to the basolateral compartment (Fig. 6). With the exchange of serum-free medium for growth medium, there was an increase in claudin-10 mRNA, but the effect on protein levels was imperceptible. A correlation between mRNA and protein levels was observed for claudin-2 in serum-free medium. The effect on claudin-2 was greatest when serum was added only to the basal medium chamber. Claudin-3 steady-

state levels were highest when serum was added to the apical chamber with serum-free medium, but the mRNA levels were too variable to be conclusive. Like its mRNA, claudin-19 was unaffected by culture conditions. The other claudins were either not detected by immunoblotting or the background on the blots was too high for the results to be conclusive.

In contrast to the claudins, there was a large effect on the steady-state levels of occludin expression. Occludin was present in much lower amounts in serum-free medium (Fig. 7). Expression increased when serum was present on the apical side of the monolayer. This result was inconsistent with the effect on mRNA, where occludin mRNA increased even when serum was restricted to the basal side of the monolayer. Post-translational phosphorylation of occludin slows its migration on gel electrophoresis. Compared with occludin from human kidney, only slower migrating isoforms were observed in RPE. There was no evidence on immunoblots that culture conditions affected post-translational modifications.

Immunofluorescence localization of claudin-1 and claudin-10 indicated the cultures were heterogeneous and that heterogeneity was sensitive to culture conditions. As reported earlier,²² claudins 3 and 19 were expressed by all cells and concentrated in the position of tight junctions, as revealed by ZO-1 (Fig. 8). Claudin-2, claudin-12, claudin-15, claudin-16, and claudin-20 were below the threshold for detection by immunofluorescence. These findings were consistent from preparation to preparation. By contrast, the expression of claudin-1 was variable. RPE isolated from one fetus might exhibit claudin-1 in very few cells, whereas RPE isolated from a second fetus might show patches of RPE that were positive for claudin-1. Three-dimensional reconstructions demonstrated that claudin-1 was concentrated in the apical junctional complex of cells that expressed it (Fig. 9). In growth medium, the expression of claudin-10 was restricted to a subset of cells, but localized to the tight junctions.²² Consistent with the increased expression of its mRNA in serum-free medium, claudin-10 was

FIGURE 6. Effects of culture conditions on the steady-state levels of claudin expression. The samples for the *left three lanes* were cultured as described in Figure 1A. The samples for the *right four lanes* were cultured as described in Figure 1B. Protein from each culture was extracted and immunoblotted. The blots were representative of cultures derived from multiple fetuses. Serum in the apical chamber increased the expression of claudin-1 and claudin-3. GM, growth medium; SFM, serum-free medium.

detected in most of the cells in some preparations and in all cells in other preparations (Fig. 8).

Inhibition of Claudin Expression by siRNA

For cultures maintained in growth medium, tight junctions appeared to be disrupted when claudin-19 expression was reduced by siRNA (Fig. 10). Over 5 days, the TER decreased to $\sim 20\text{--}25 \Omega \times \text{cm}^2$, and remained that low after 21 days. There was a transient 5–10% decrease in TER after mock transfection or transfection with siRNA to an irrelevant mRNA (claudin-4) or to a minor claudin (claudin-2 or claudin-10, not shown). The expression of claudin-19 mRNA decreased 20–25 times after 5 days, which brought it to the same level of expression as claudin-3 mRNAs (Figs. 5, 10). A weak signal for claudin-19 was evident on immunoblots or by immunofluorescence (Figs. 11, 12). These signals could be eliminated by two rounds of transfection with siRNA, 2 days apart, but the data presented here were obtained with a single round of transfection. This procedure minimized the effects of exposure to the transfection reagent or nonspecific effects due to the siRNA. The transfection reagent, with or without an irrelevant siRNA (claudin-4), had <4 times effect on the expression of occludin or any claudin other than claudin-19 (Fig. 10). By contrast, the knockdown of claudin-3 had minimal effect on TER and ion selectivity, as protein levels dropped on days 5 and 7 (Fig. 11).

Transfection with the siRNA to claudin-19 caused a small compensatory increase in the steady-state expression levels of other claudin mRNAs and their proteins (Figs. 10, 11). These increases were insufficient to compensate for the loss of claudin-19. The 3.5 times increase in claudin-1 mRNA correlated with a slight increase in protein levels. The 4 times increase in claudin-2 mRNA correlated with an increase in protein levels observed by immunoblot but failed to result in an immunofluorescent signal for claudin-2.

Seven days post-transfection the level of claudin-19 mRNA recovered slightly to within 5 times of pre-transfection levels. The protein was detectable by immunofluorescence, but further increases were not evident during the next 2 weeks. Three weeks post-transfection, the TER remained very low. Nonetheless, examination of the cells 21 days post-transfection by immunofluorescence revealed an intact monolayer with cells that maintained a polygonal morphology. Tight junctional proteins, including claudin-3, claudin-19, actin, ZO-1, and occludin, remained concentrated in an apical junctional complex. Similar results were obtained with freshly plated, newly confluent cells in the weeks before the cultures developed a TER (data not shown).

For cells maintained in serum-free medium, the transfection efficiency appeared to be much less. The expression of claudin-19 mRNA was reduced only 2–3 times, and a decrease in expression was observed in only a subset of cells (data not shown). Nonetheless, this was sufficient to lower the TER to $\sim 50 \Omega \times \text{cm}^2$.

DISCUSSION

The Predominance of Claudin-19

Human RPE stands out among epithelia by using claudin-19 as its predominant claudin. The first indication that claudin-19 plays an essential role was that patients with a genetic defect of claudin-19 suffer profound ocular deficits.²¹ In a genomics study, claudin-19 was the only claudin that was identified as RPE-specific when RPE was compared with photoreceptors and choroid.³⁶ Besides claudin-19, the only other claudin that we could detect in all RPE cells was claudin-3, which was expressed in lesser amounts than claudin-19. Other claudins were expressed below the threshold for detection by immunofluorescence or were evident in only a subset of cells. Given the role of claudins in modulating function, heterogeneity suggests regional variation in the permeability and selectivity of tight junctions. With the switch from growth medium to serum-free medium, the most significant effects on claudins were increases in the expression of claudin-2 and claudin-10b. Instead of patches of immune-positive cells, claudin-10b became evident in nearly all the cells. The TER decreased and the Na^+ conductance increased more than the K^+ conductance.

FIGURE 7. Effects of culture conditions on the steady-state levels of occludin expression. The samples for the *left three lanes* were cultured as described in Figure 1A. The samples for the *right four lanes* were cultured as described in Figure 1B. Protein from each culture was extracted and immunoblotted. The blots were representative of cultures derived from multiple fetuses. Only high M_r isoforms were observed. Expression was highest when serum was present in the apical medium chamber. GM, growth medium; SFM, serum-free medium.

FIGURE 8. Effect of culture conditions on the distribution of tight junction proteins. hRPE was cultured in growth medium or serum-free medium and double labeled for claudin and either occludin, ZO-1, or actin, as indicated. Confocal images were captured in the plane of the tight junctions. ZO-1, actin, and occludin were uniformly expressed, but the undulating nature of the filter brought the tight junctions in and out of the confocal plane. The strips above and to the right of the x - y plane are the x - z and y - z planes, respectively. Regardless of the secondary antibody that was used, claudins appear *red*, the counterlabel is *green*, and the overlap of the two appears as shades of *yellow*. Nuclei were revealed by DAPI (*blue*). Claudin-3 and claudin-19 were evident in all cells. Claudin-1 and claudin-10 were evident only in subsets of cells, but more positive cells were observed in serum-free medium. Arrows: claudin-1-positive cells; bar, 20 μ m.

Each effect would be consistent with the properties of these two claudins.^{37–39} We were unable to test this hypothesis directly because the efficiency of transfection was low for cells maintained in serum-free medium. The heterogeneous expression of these minor claudins extends earlier observations that RPE exhibits regional variation in composition and function about the globe of the eye.^{40,41}

Knockdown of claudin-19 by siRNA demonstrated that the expressed claudin-3 was insufficient to form functional tight junctions across the monolayer, whereas knockdown of claudin-3 had no discernable effect. Despite a TER of only $\sim 20 \Omega \times \text{cm}^2$ after transfection with claudin-19 siRNA, the tight junction appeared to be intact as evidenced by the co-localization of actin, occludin, ZO-1, and the remaining claudins. There is

precedent for this phenomenon in studies of embryonic chick RPE.^{42,43} In a week-long process, the rudimentary tight junctions of early development transform into functional tight junctions. Early in the process circumferential bands of actin, occludin, ZO-1 and claudin were evident, but freeze-fracture electron microscopy revealed many discontinuities in the tight junctional network of strands. A medium conditioned by retinal organ culture could induce cultured RPE to fill in these discontinuities, reduce the permeation of nonionic tracers, and increase the TER. A similar phenomenon was observed with the ARPE19 cell line. This spontaneously transformed line of human RPE exhibits circumferential bands of ZO-1 and occludin, yet studies of function indicate the junctions range from leaky to rudimentary.^{23,26} Notably, genomics studies indicate

FIGURE 9. Co-localization of claudin-1 with occludin. hRPE was cultured in serum-free medium and double labeled for claudin and occludin. A three-dimensional reconstruction of a confocal image stack demonstrates that claudin-1 localized to tight junctions. Similar results were obtained with hRPE cultured in growth medium. *Red:* Claudin-1; *green:* occludin; *yellow to orange:* co-localized claudin-1 and occludin.

that ARPE19 cells do not express claudin-19 (Gene Expression Omnibus, National Center for Biotechnology Information, accession no. GSE18811).⁴⁴

Notably, function was slow to recover even 3 weeks after the exposure to claudin-19 siRNA. Slow remodeling of the apical junctional complex appears to be typical of human RPE cultures. We observed that tight junctions appear to be evident by immunofluorescence when newly plated cells become confluent (not shown), even though an additional 5 to 6 weeks are needed for mature tight junctions and melanin granules to reform.^{27–29} A similar time frame was required for the adherens junction to remodel in newly plated cultures of adult human RPE.⁴⁵

Human RPE appears to be unique, because the RPE of lower vertebrates express other claudins. In chick, claudin-19 is not expressed. Claudin-20 is the major claudin, with large amounts of claudin-1, claudin-4L2, and claudin-5 and minor expression of claudin-2, claudin-11, and claudin-12. In mouse, we could detect ZO-1 and occludin by immunofluorescence but not claudin-19 (Noriko Iwamoto and Mikio Furuse, Kobe University Graduate School of Medicine, Japan, personal communication, 2009, and our unpublished data). By contrast, claudin-19 is readily detected in mouse kidney using the same antibody preparations we used here. A proteomics study indicates that claudin-1 was the only detectable claudin in rats (Vera Bonilha, Cleveland Clinic, personal communication, 2010). The coordi-

nate regulation of claudins and membrane transporters by various epithelia suggest that transepithelial gradients are maintained by a collaboration of the paracellular and transcellular pathways.^{5,7,10} Accordingly, it would be informative to compare how different species regulate the subretinal space before extrapolating how studies of one species might relate to human physiology and disease.

Permeability and Selectivity

In the LLC-PK1 kidney cell line, exogenously expressed claudin-19 retards Cl^- diffusion across the tight junction.⁴⁶ This would be an important function in RPE and is consistent with our finding that the TER of hRPE is high. Apical-to-basal Cl^- transport drives the absorption of fluid from the subretinal space,⁴ which would be frustrated if Cl^- could leak backwards through the tight junctions. However, LLC-PK1 cells expresses other claudins, and it is unclear how they might modulate the function of claudin-19, because claudin-19 can dimerize with other claudins, for example, claudin-16 in the distal kidney tubules.⁴⁷ Dimer formation is needed for either their transport to or their stabilization in the tight junction. RPE provides an example where claudin-19 is expressed in the absence of any other claudin that is present in sufficient amount to form a tight junction on its own.

To say claudin-19 selectively restricts anions is another way of saying that claudin-19 is leakier to cations.⁴⁶ The RPE actively secretes Na^+ into the subretinal space and establishes a transepithelial electrical potential that is apical (subretinal) side positive.⁴ The Na,K-ATPase spends a lot of energy to create these electrical and chemical gradients for several purposes. They drive the Na^+ coporters and antiporters that mediate transport across the apical membrane and drive the absorption of Cl^- and water. Together with the basal secretion of K^+ , the leak of cations across the tight junctions helps balance the apical-to-basal Cl^- flux.

In RPE claudin-19 has a greater conductance for K^+ than for Na^+ , but this could be modulated by serum without dramatic effects on the expression of other claudins. A change in conductance signifies a change in the properties of the tight junction, but the functional significance is unclear. Conductance was measured with equal concentrations of ions on both sides of the monolayer and reflects the ability of ions to carry current down an electrical gradient. Besides an apical positive transepithelial electrical potential, RPE maintains chemical gradients of Na^+ , K^+ , and Cl^- . Because of the “sticky pore” problem,⁴⁸ the permeation coefficient for these ions needs to

FIGURE 10. Claudin and occludin mRNA expression after transfection with a siRNA cocktail that targets claudin-19. Expression levels of each claudin was expressed relative to its expression in control cells that were transfected with siRNA to claudin-4. (A) Time course for the effect of siRNA on claudin-19 expression. (B) On day 5, occludin and most claudins show <2 times change in expression with the exception of claudin-1. The columns indicate the mean of triplicate determinations obtained from each of two independent cultures of hRPE, whereas the error bars indicate the range.

be estimated from dilution and bi-ionic potentials with more sophisticated instrumentation than was available for this study.⁴⁹

Ions and organic tracers use different mechanisms to cross a tight junction, and these can be regulated semi-independently.^{9,31,34,35,50,51} Tracers with a Stokes radius less than ~4 Å can pass through putative pores in the tight junctional strands, but larger tracers depend on the rate at which tight junctional strands break apart and reseal. The polydisperse solutions of mPEG were used because mPEG₃₅₀ is predominantly smaller than 4 Å, whereas mPEG₅₅₀ is predominantly larger. The permeation coefficient, P_{app} , should be the same in both directions if only tight junctions are involved, but we found that it was greater in the apical-to-basal direction. Further, mPEG₅₅₀ was most sensitive to direction. These data suggest there is an apical-to-basal mechanism for fluid-phase transcytosis, consistent with our earlier study of VEGF.²² An apical-to-basal pathway for fluid-phase transcytosis would augment the absorption of water from subretinal space that is driven by the vectorial transport of Cl⁻.⁴ From the P_{550} data, fluid transport due to transcytosis would be estimated to be ~2 μL/cm²/hr in serum-free medium. By comparison, the total absorption of water, J_v , has been estimated for hRPE in culture to be ~10 μL/cm²/hr.⁵²

Effects of Ectopic Serum

As noted in the introduction, serum contacts the apical membrane only during disease. Apical serum increased the steady-state level of claudin-3 slightly but increased the expression of occludin substantially. There was no apparent effect on the amount of the respective mRNAs. Apical serum also caused an increase in the TER with a disproportionate effect on the K^+ conductance relative to the Na^+ conductance. Because the conductance measurements were made under conditions that inactivate transcellular transport, these data indicate that a significant contribution to the rise in TER comes from tightening the tight junction. Occludin is an important regulator of junction permeability that can rapidly respond to physiologic changes because of its short half-life and rapid association/dissociation rate constants.^{16,17,50,53} If we assume that P_{app} for mPEGs in the basal-to-apical direction reflected only diffusion across tight junctions, the data would indicate that the mechanisms for large and small organic tracers were unaffected by serum despite the large effects on TER. A similar dissociation of paracellular permeability and TER has been reported in MDCK cells and chick RPE.^{12,19}

Ectopic serum also had a big effect on transcytosis. When serum was presented only to the basal side of the monolayer, fluid transport due to transcytosis (estimated from P_{550}) was estimated as $\sim 2 \mu\text{L}/\text{cm}^2/\text{hr}$. When serum was presented only to the apical side of the monolayer there was little evident transcytosis. Therefore, serum in the subretinal space would aggravate edema.

The effect of apical serum on tight junctions was opposite that observed in lower vertebrates^{2,3} and may be an adaptation to limit the spread of a blood-retina barrier defect. If cells with leaky tight junctions could induce leaky tight junctions between neighboring cells, then the defect could spread. Instead our data suggest that in the human retina, serum that leaks into the subretinal space would induce tight junctions in the neighboring cells to become tighter. However, apical serum also presents a challenge by inhibiting apical-to-basal fluid-phase transcytosis, which would reduce the ability of RPE to absorb the subretinal fluid that leaks from invading capillaries.

The unique composition of human RPE tight junctions makes hRPE an interesting model for future study. The study of ion selectivity should be extended to other ions. Because hRPE expresses mainly claudin-19, exogenous gene expression can be used to explore the interaction of claudin-19 with other claudins. For example, by expressing a minor claudin in the entire culture one could explore how claudin-claudin interactions affect function from region to region within the retina. The sensitivity of occludin to culture conditions suggests it might be a target for managing changes in the environment of the RPE. An occludin-based mechanism might allow RPE to rapidly adapt to changes in the subretinal space that naturally occur during the transition between day and night.⁴ Finally, the unique composition of human RPE tight junctions makes it important to explore how the physiology of the subretinal space varies among species in a way that requires tight junctions with different permeability and selectivity.

Acknowledgments

The authors thank Sheldon Miller and Arvydas Maminishkis for helpful suggestions, sharing genomic data in advance of publication, and providing primary cultures of hRPE.

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