



A Culture Model of Development Reveals Multiple Properties of RPE Tight Junctions

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Purpose: A culture model was used to examine the development of tight junctions in the retinal pigment epithelium (RPE).

Methods: Chick RPE was isolated on embryonic day 7 (E7), E10 or E14 and cultured on laminin-coated filters. Barrier properties were stimulated with E14 retinal conditioned medium. Morphology was characterized by confocal microscopy. Permeability was determined by measuring the flux of horseradish peroxidase (HRP), radiolabeled inulin and mannitol, and the transepithelial electrical resistance (TER). Changes in the expression of ZO-1 and a related protein, ZO-1LP, were determined by immunoblotting.

Results: RPE from each age formed epithelial monolayers of similar height, but the density of the cultures varied in parallel with density changes in vivo. The cultures appeared to regulate the permeability to ions and nonionic solutes independently. With embryonic age, there was a progressive decrease in permeability that first affected larger and then smaller tracers. Despite a small decrease in the permeability to mannitol, there was a large decrease in the permeability to ions. This suggests that in E14 cultures tight junctions discriminated by charge, as well as size. Although E14 retinal conditioned medium reduced the permeability to all solutes, it appeared to regulate size discrimination more than charge discrimination. Despite large effects on permeability, conditioned medium had no effect on the expression of ZO-1 or ZO-1LP.

Conclusions: The ability of tight junctions to discriminate on the basis of charge and size is regulated independently during development. The permeability of tight junctions cannot be predicted by the level of ZO-1 expression.

The retinal pigment epithelium (RPE) forms the outer blood-retinal barrier by regulating transport between the neural retina and the fenestrated capillaries of the choroid. Tight junctions are a critical feature of this barrier, because they retard diffusion through the paracellular spaces. Together with the adherens junction and associated actin filaments, tight junctions form an apical junctional complex that completely encircles each cell near the apical end of the lateral membranes. Like other regions of the blood-brain barrier, the barrier properties of the RPE develop gradually (1,2). An early step is the formation of high-permeability tight junctions. An intermediate step is a decrease in the permeability of the junctions to proteins. Although this has classically been taken as the formation of the blood-brain barrier, these junctions still have a relatively high permeability to smaller solutes. It is not until a late stage that the low permeability junctions typical of the blood-brain barrier develop (3). Studies of chick RPE are consistent with these three stages of development. Functional tight junctions were evident by embryonic day 7 (E7), but did not become impermeable to horseradish peroxidase (HRP) until E11-12 (4,5). The complexity of tight junction strands (a function of strand number and branching pattern) was examined by freeze fracture, and did not increase until E15-19 (6). This suggests a dramatic decrease in permeability during

the late stage (7,8).

The current study focuses on the intermediate stage (E7-E14 of chick development) when junction permeability first decreases. Despite the constancy of strand morphology, there was a change in the expression of the tight junction protein, ZO-1 (4). ZO-1 was the first tight junction protein to be described (9). Many studies correlate decreased permeability with increased expression of ZO-1 in junctional pools. For example, in mammary epithelia glucocorticoids increased transepithelial electrical resistance (TER) and localized ZO-1 to junctional complexes, but transforming growth factor- α decreased TER and shifted ZO-1 to a parajunctional pool (10,11). Studies of RPE and retinal endothelia correlated higher levels of ZO-1 with lower permeability under various culture conditions (12,13). Consequently, the presence of circumferential bands of ZO-1 and actin is commonly taken as evidence for tight junctions. However, ZO-1 is also present in other junction types (14-16). For example, circumferential bands of ZO-1 and actin were observed in the ependyma, which lacks tight junctions (1). Other studies show the level of ZO-1 expression often does not correlate with permeability (17-22). Similarly, permeability does not always correlate with the morphological complexity of tight junctional strands (7,8). Together, these data suggest that functional properties of junctional strands can differ among epithelia. Notably, there are at least eight isoforms of human ZO-1 that differ in tissue distribution and presumably serve different functions (9).

Two ZO-1 immunoreactive proteins were described in

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chicken (4). One appears to be ZO-1, because it binds several antibodies to ZO-1 and is present in mature tight junctions of the RPE. The second was named ZO-1LP (ZO-1-like protein). ZO-1LP bound some antibodies to ZO-1, had nearly the same mobility as ZO-1 on SDS gel electrophoresis, and was also found in apical junctional complexes. Unlike ZO-1, ZO-1LP was present in immature junctions of the RPE and the homologous junctions of the outer limiting membrane. Between E7 and E14, ZO-1LP gradually disappeared from the apical junctional complex of RPE. Meanwhile ZO-1 gradually appeared and its expression peaked on E10. ZO-1 expression then decreased when junction permeability presumably continued to decrease.

To explore the development of tight junctions, the current study refined a previous culture model of early RPE development (23). We demonstrated that permeability and cell density depend upon the embryonic age of the isolated RPE. Different mechanisms regulated the permeability to ionic and nonionic solutes without affecting the level of ZO-1 expression.

METHODS

Cell culture—RPE was isolated essentially as described earlier (24). Briefly, eyes were isolated from white Leghorn chicken

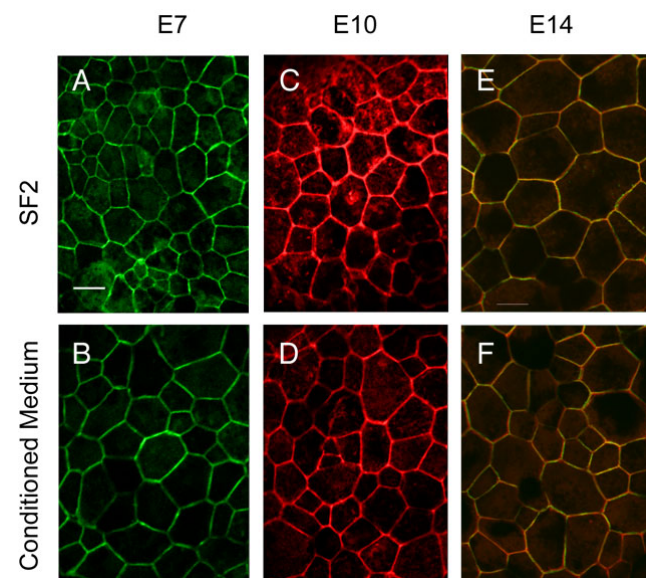


Figure 1. ZO-1 and actin colocalize at cell-cell junctions. Cultured RPE formed hexagonal arrays, and filamentous actin overlapped with ZO-1 at cell-cell junctions. RPE was isolated from E7, E10 or E14 embryos and cultured for 5 days on laminin-coated filters. Cultures were maintained in SF2 growth medium (A,C,E) or stimulated with E14 retinal conditioned medium (B,D,F). ZO-1 and actin were localized by indirect immunofluorescence using antibody R40.76 (ZO-1), or fluorescently tagged phalloidin (actin). Confocal images were obtained in an en face plane near the apical membrane. Similar results were obtained after 10 days in culture and with polyclonal antibody 7445 to ZO-1. Examples are shown for ZO-1 label (A and B), actin label (C and D) and double label (E and F). There was no apparent effect of conditioned medium. Bar, 10 μm .

embryos on embryonic days 7, 10, or 14 (E7, E10, or E14). After removing the anterior structures, vitreous body, and neural retina, the eyecups were transferred to phosphate buffered saline (PBS) containing 0.54 mM EDTA (E7 or E10) or 1.0 mM EDTA (E14) and incubated for 30 min at 37° C. The RPE was peeled from the choroid and incubated for 10 min at 37° C in PBS containing trypsin, collagenase and DNase. The cells were rinsed with DME containing 2.5% fetal calf serum and incubated for 2 h with periodic trituration in a serum-free medium (SF2). The cells were plated on coated 12 mm Transwell filters (Corning Costar Co., Cambridge, MA) at a density of $5\text{--}7 \times 10^5$ cells/cm². The filters were freshly coated with laminin (5 $\mu\text{g}/\text{cm}^2$) according to the manufacturer's directions (Collaborative Biomedical Products, Bedford, MA). Cultures failed to form on commercially coated filters or filters on which the laminin was allowed to dry. For some cultures, the medium in the apical medium chamber was replaced by E14 retinal conditioned medium (23). The conditioned medium was prepared by culturing neural retinas that were isolated from E14 embryos in SF2 (12–14 retinas/15 ml). The SF2 was supplemented with 1.0 mM glutathione and 50 μM ascorbic acid, and the cultures were maintained with gentle agitation for 5 h at 37° C in a humidified atmosphere of 10% CO₂ and 40% O₂.

Antibody preparations— The polyclonal antibody 7445 was raised against a 69 kDa peptide that corresponds to human sequences that flank the α region of ZO-1 (25). Affinity purified 7445 was obtained from ZYMED (San Francisco, CA). Antibody 7445 binds all known mammalian ZO-1's and to chick ZO-1 and ZO-1LP (4). The monoclonal antibody R40.76 was produced in rat against mouse ZO-1 (26). It binds chick ZO-1, but does not bind ZO-1LP. It is available from Chemicon (Temacula, CA).

Immunofluorescence labeling— Cultures were fixed for 10 min at room temperature with PBS containing 1.0 mM MgCl₂, 0.1 mM CaCl₂ and 3.3% formaldehyde. The cells were washed in PBS and permeabilized by incubation in PBS containing 0.1% Triton X-100 for 5 min. To label ZO-1, the cells were blocked by incubation in PBS containing 1% bovine serum albumin (BSA) and 5% goat serum for 30 min at 37° C. The cells were then incubated for 1 h at 37° C in blocking buffer containing the monoclonal antibody R40.76. The cells were washed in PBS containing 0.1% BSA, and incubated for 1 h at 37° C in PBS containing goat anti-Rat IgG. The cultures were washed as above and mounted in gelvatol with the cell side of the filter facing the coverslip. To label actin filaments, the cultures were fixed as above and labeled with BODIPY 581/591 phalloidin (Molecular Probes, Eugene, Oregon) according to the manufacturer's instructions and mounted as above. The cells were viewed with a Bio-Rad (Hercules, CA) MRC600 confocal microscope. Cell density was determined by counting cells in 20–30,000 μm^2 fields, 10 fields/filter, 4–6 filters/experiment in three experiments. Cell counts were performed using NIH Image software (V. 1.60). All tests of significance used the Student's t test with a minimum of three cultures per experiment.

Permeability assays— Assays were performed in triplicate

or quadruplicate after 9 days in culture, when the TER had reached a plateau. Radiolabeled [methoxy- ^3H]inulin (3.0 $\mu\text{Ci/ml}$; 405 mCi/g) or D-[1- ^3H (N)]mannitol (4.5 $\mu\text{Ci/ml}$; 19.7 Ci/mmol) in 0.6 ml of SF2 or E14 retinal conditioned medium was added to the apical medium chamber (Dupont New England Nuclear, Boston, MA). SF2 (1.5 ml) was added to the basal medium chamber. These volumes were used so that the height of the solution in each chamber was the same. The solutions were prewarmed to 37 $^\circ\text{C}$ and the cultures were maintained at 37 $^\circ\text{C}$ in a humidified incubator. Aliquots of 5 μl were removed from the apical and basal chambers of control filters that lacked cells at 5, 10 and 15 min. Aliquots were taken from cell cultures at 30, 60 and 90 min. Radiolabeled tracers were quantified by counting aliquots in a liquid scintillation counter (LKB Instruments, Inc., Gaithersburg, MD). For HRP, we observed a lag in its diffusion across control filters. This could be nearly eliminated by preincubating the filters in HRP (Type VI HRP, Sigma Chemical, St. Louis, MO). HRP (50 $\mu\text{g/ml}$) was added to the apical and basal media chambers for one hour. To start the experiment, the basal side of the filter was quickly rinsed 4-6 times by immersing it in SF2 and transferring the filters to wells with 1.5 ml SF2 in the basal chamber. Aliquots (5-10 μl) from the basal lateral chamber were assayed by adding freshly prepared substrate to 200 μl . The substrate was 400 $\mu\text{g/ml}$ o-phenylenediamine (Sigma Chemical, St. Louis, MO) and 0.012% H_2O_2 in 0.05 M citric acid and 0.1 M phosphate, pH 5.0. The reaction was terminated after 15 min by the addition of 50 μl of 0.3 M H_2SO_4 , and the absorbance at 492 nanometers was determined with a microplate reader (Bio-Rad Laboratories, Hercules, CA). Concentrations were calculated from a standard curve. To prevent transcytosis, the preincubation and incubation media contained 50 μM chloroquine.

To normalize the data, permeability was calculated from the flux as follows:

$$\text{Flux} = (X)_B / (Y)_i / A$$

where $(X)_B$ = counts per min (CPM) or micrograms in the basal chamber; $(Y)_i$ = the initial concentration in the apical chamber, expressed as CPM or micrograms per microliter, and A = the area of the filter in cm^2 . The final units become: microliter / cm^2 . Permeability was determined as the slope of the linear regression line of Flux versus time. Tests of significance used the Students t test.

The TER was measured every other day using endohm electrodes and EVOM voltohmmeter (World Precision Instruments, Sarasota, FL).

Immunoblotting—Filters were removed from their holders and the cells were scraped into 150 μl of PBS containing 100 μM EDTA, 10 U/ml aprotinin, 100 μM leupeptin and 4 μM pepstatin A. After adding 22 μl of 20% SDS, the samples were incubated in a boiling water bath for 2 min., and the melanin granules removed by centrifugation. Protein concentration was determined using the Micro BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (15 μg) were resolved by electrophoresis on 6% polyacrylamide gels for 2.5x the time required for bromophenol blue to reach the bottom of the gel. Immunoblots were prepared and quantified as described by Williams and Rizzolo (4).

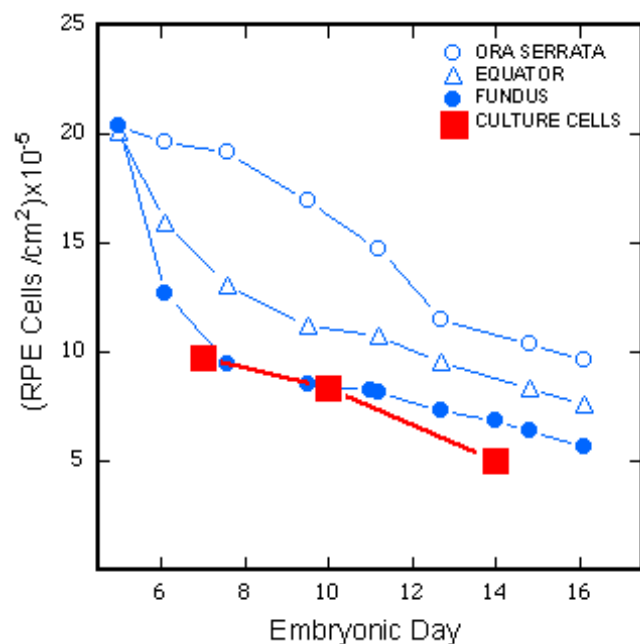


Figure 3. Cell density of cultured RPE decreases with embryonic age. Cell density of cultured RPE decreased with embryonic age in parallel with the decrease previously described *in vivo*. RPE was cultured and labeled as described in Figure 1. Cell density was determined as described in Methods (red line, squares). Standard error bars were smaller than the symbols. For comparison, the data of Coulombre (27) is replotted (blue lines).

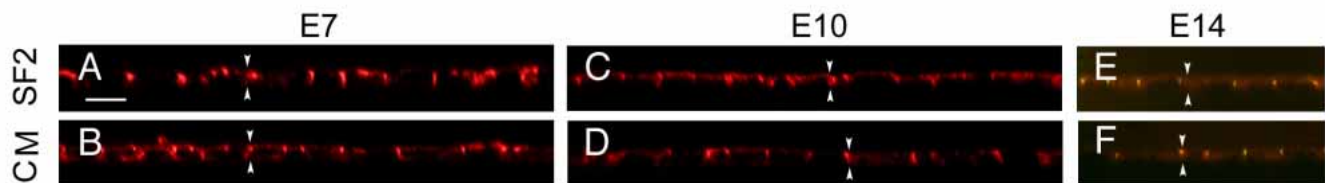


Figure 2. RPE forms monolayers with ZO-1 and actin in apical junctional complexes. Cultured RPE formed monolayers of similar height with ZO-1 and actin colocalized in apical junctional complexes. The cultures described in Figure 1 were examined by confocal microscopy in the transverse plane. In addition to apical, circumferential bands of actin, a small population of actin filaments was dispersed through out the cytoplasm. Actin staining (A,B,C,D) was used to determine cell height and for evidence of contaminating fibroblasts. In all fields examined (from triplicate filters, and from 4-6 experiments), there was no evidence of multilayering of RPE or an underlying layer of contaminating fibroblasts. ZO-1 colocalized with the circumferential bands of actin, as exemplified by double labeled cells on E14 (E and F). There was no significant difference in cell height between cultures. Downward arrows, apical membrane; Upward arrows, basal membrane; Bar, 10 μm .

RESULTS

The original culture system described by Rizzolo and Li (23) used Matrigel as a substrate, but Matrigel contains many growth factors that could interfere with these studies. Therefore, we tested purified laminin as a substrate for RPE that was isolated from E7, E10 and E14 embryos. After one day in culture, E14 retinal conditioned medium was added to the apical chambers of the indicated cultures to stimulate barrier properties. In each case, the cells proliferated until confluent monolayers formed by 3-5 days in culture. The total protein per filter and cell density did not increase after this time (data not shown). The morphology of the cultures was assessed by labeling actin filaments with fluorescently tagged phalloidin or ZO-1 by indirect immunofluorescence (Figure 1). Each label

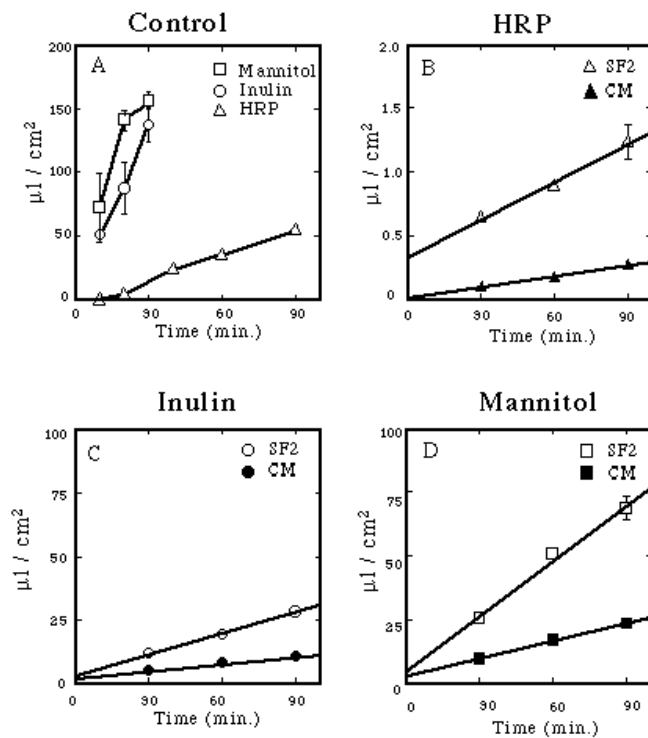


Figure 4. The flux of HRP, mannitol and inulin across the RPE monolayer was linear for 90 min. (A) Each solute diffused rapidly across control filters that lacked cells. The rate of diffusion decreased with increased size of the tracer. A lag was noted for the diffusion of HRP that was minimized by preincubating the filters in HRP, as described in Methods (B,C,D). Three to four cultures were established from E10 embryos, and maintained in SF2 (open symbols) or stimulated with E14 retinal conditioned medium (closed symbols). After 9 days in culture, the flux of HRP (B), [^3H]inulin (C) and [^3H]mannitol (D) was measured in the apical to basal direction. Standard error bars were sometimes smaller than the symbol, and linear regression lines are included in panels B, C and D. RPE monolayers retarded the diffusion into the basal chamber. Cultures stimulated with E14 retinal conditioned medium had lower permeability. Similar results were obtained with E7 and E14 cultures and are summarized in Figure 5.

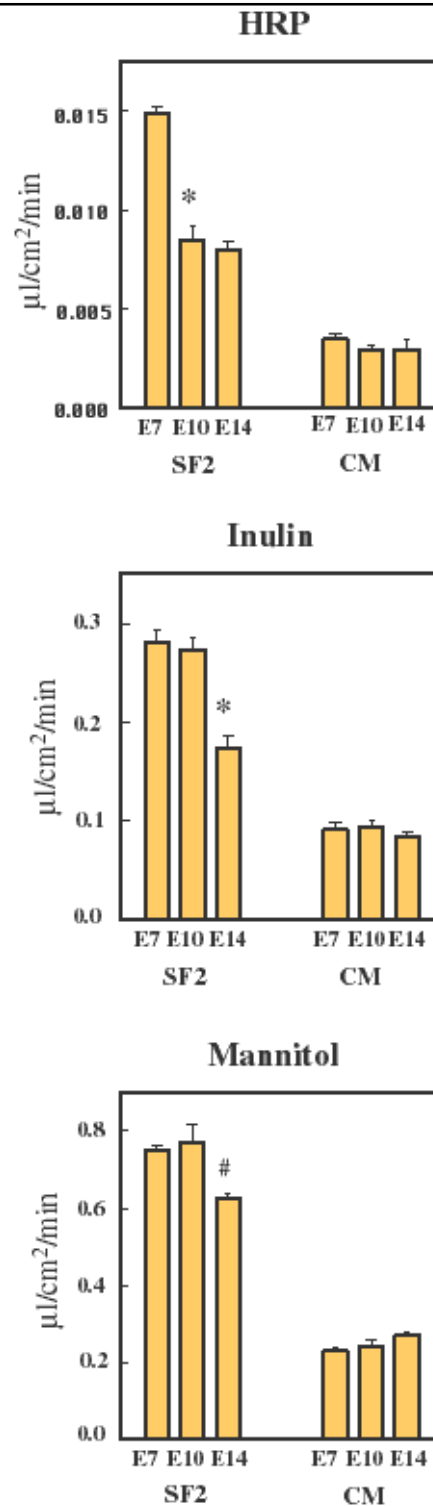


Figure 5. Effects of embryonic age and retinal conditioned medium on the permeability of RPE. The permeability of RPE was calculated from linear regression analysis of the data exemplified in Figure 4. Assays were performed on 3-4 cultures. In growth medium (SF2), there was a 40% decrease in the permeability to HRP between E7 and E10 with no difference for inulin or mannitol. Between E10 and E14, there was a 40% decrease in the permeability to inulin, but only a 15% decrease for mannitol. In E14 retinal conditioned medium (CM), the permeability of each tracer decreased, but the age related differences were eliminated. Error bars represent the standard error. Confidence levels: * $p < 0.01$; # $p < 0.05$.

yielded the same result for each culture. Examples are shown for actin in cultures of E10 and E14 cells (Figure 1C,D,E,F) and ZO-1 in cultures of E7 and E14 cells (Figure 1A,B,E,F). Both probes labeled circumferential bands that corresponded to the apical junctional complex. In each case, confluent monolayers were formed with the hexagonal packing typical of simple epithelia. In double label experiments, much of the filamentous actin colocalized with ZO-1 along the apical end of the lateral membranes (Figure 1E,F and Figure 2E,F). There was no evidence for non-junctional pools of ZO-1, but actin filaments were also present throughout the cytoplasm. This allowed us to use phalloidin staining to determine the height of the monolayer and search for multilayering or underlying fibroblasts (Figure 2). No statistical difference in height was observed between cultures established from different embryonic ages, or between cultures maintained in SF2 growth medium or retinal conditioned medium. Although many transverse sections were examined, there was no evidence of multilayering or fibroblast contamination.

The principal difference in morphology was the cell density. Density did not change with time in culture after day 5, but did depend upon the embryonic age of the RPE (Figure 3). Because no statistical difference was observed between cultures with or without conditioned medium, these data were pooled. For comparison, Figure 3 also presents the data from a developmental study by Coulombre (27). The density of the cultured RPE decreased in parallel with the decreased density of the parent cells. Although the RPE was isolated from the entire globe, the density of the cultures corresponded most closely with the density of the RPE in the fundus. This contrasts with RPE cultured on Matrigel (23; data not shown), where E7 and E14 RPE had nearly the same density (E7: $4.9 (\pm 0.5) \times 10^5$ cells/cm²; E14: $4.4 (\pm 0.3) \times 10^5$ cells/cm²). Because laminin had the least effect on age-dependent properties, laminin-coated filters were used for the studies of permeability.

The development of blood-brain barriers has been studied *in vivo* by examining the diffusion of injected HRP (2,28). In the current study, HRP was added to the apical medium chamber and its appearance in the basolateral chamber was monitored over time. An example is shown for E10-derived monolayers (Figure 4). Because of the need to preincubate the cultures with HRP (see Methods), the y-intercept was variable. However, permeability (the slope of the linear regression line) was very reproducible, as indicated in Figure 5. The monolayer retarded diffusion across the filter, as compared with coated filters that lacked RPE. Permeability was reduced nearly four-fold by maintaining the cultures in retinal conditioned medium.

Horseshoe peroxidase is large (hydrodynamic radius ~30 angstroms) and ionic. To separate the effects of size and charge on flux across the monolayer, we measured the flux of nonionic tracers and small ions. Nonionic tracers of different size were used to distinguish diffusion, which depends upon hydrodynamic radius, from transcytosis, which would be independent of size. Mannitol (hydrodynamic radius ~4 angstroms) and inulin (hydrodynamic radius ~10-14 angstroms) were selected, because there are no transmembrane

Figure 6. Effects of embryonic age and retinal conditioned medium on the TER of RPE. A small, but significant, increase of TER was observed between E7 and E10, but a large increase was observed between E10 and E14. These age-related differences were preserved after stimulation with conditioned medium.

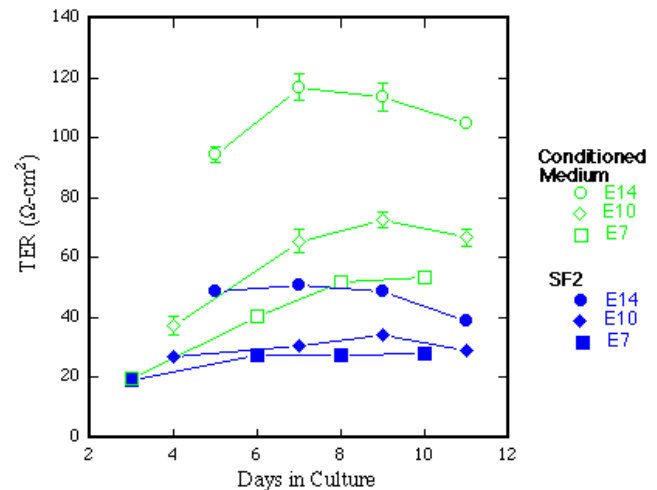


Figure 6a. The TER of RPE isolated on E7 (squares), E10 (diamonds) and E14 (circles) was graphed as a function of days in culture. Cultures were maintained in growth medium (closed symbols, blue lines) or stimulated with E14 retinal conditioned medium (open symbols, green lines). Standard error bars were sometimes smaller than the symbol.

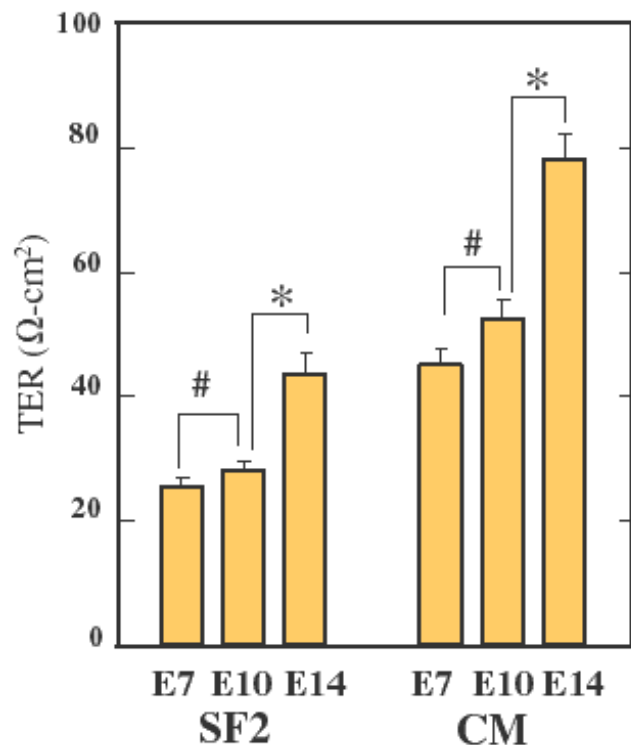


Figure 6b. Data is summarized for the cultures described in Figure 5. The measurements were acquired just before the permeability measurements. Conditioned medium from E14 retinas stimulated the TER of all ages about 2 fold. As illustrated by the two experiments in panels A and B, there was some variability in the strength of different preparations of conditioned medium. Confidence levels: # $p < 0.05$, * $p < 0.001$.

transport mechanisms for these compounds. Examples are shown for diffusion across E10 derived monolayers (Figure 4). For each tracer, the flux was much lower than for control filters that lacked a monolayer, and was linear over 90 minutes. As expected for a diffusion mediated process, the flux decreased with increased size of the tracer. Conditioned medium lowered the flux of each tracer.

To determine if the properties of the tight junctions change with age, similar measurements were made with cultures derived from E7 and E14 RPE. The results are summarized in Figure 5. The comparison between ages shows a selectivity based on size that changed with embryonic age. The largest tracer, HRP was affected first with an approximately 40% decrease in permeability between the E7 and E10 cultures. An effect on the other tracers was observed between the E10 and E14 cultures. The effect was greater for the intermediate-sized inulin (40% decreased permeability) than the smaller-sized mannitol (15% decreased permeability). For each culture, medium conditioned by E14 retinas reduced permeability, but the effect was greatest on the E7 and E10 cultures. Consequently, the permeability of these cultures became identical to the stimulated, E14 cultures.

Another measure of permeability is the TER, which measures resistance to ion flow across the monolayer. A stable TER was observed after 5-7 days in culture (Figure 6a). In those cultures that were stimulated by E14 retinal conditioned medium, the TER was consistently two-fold higher than unstimulated cultures. As expected for tight junctions, the TER was reversibly reduced by a 15 min incubation in medium containing 10 mM EDTA. For direct comparison, the TER of the cultures used for the non-ionic flux measurements in Figure 5 is summarized in Figure 6b. A small, but significant, increase in TER was observed between E7 and E10, and a large increase was observed between E10 and E14. Unlike the flux of non-ionic tracers, the increase of TER with embryonic age was preserved after stimulation with conditioned medium.

To determine if changes in ZO-1 expression observed *in vivo* can explain the concomitant decrease in tight junction permeability (4), we examined ZO-1 expression in culture (Figure 7). A polyclonal antibody was used to immunoblot ZO-1 after various times in culture. After one day, expression in E7 cultures was similar to E7 RPE *in vivo*. ZO-1LP was evident with very low levels of ZO-1 expression. By 3 days in culture, the cells became confluent, and by 5-6 days the TER of unstimulated cells reached a plateau of approximately 25 Ohm-cm². By 3 days in unstimulated cultures, ZO-1 expression spontaneously increased to nearly the same level as ZO-1LP and then remained constant. Just as there was no discernible effect on morphology or density, the expression of ZO-1 or ZO-1LP was unaffected by E14 retinal conditioned medium. Consequently, the decrease in permeability induced by conditioned medium was not accompanied by an increase of ZO-1 or a decrease of ZO-1LP.

The E14 cultures also required 3 days to become confluent and 5 days for the TER of unstimulated cultures to reach a plateau of approximately 45 Ohm-cm². Like native E14 RPE, the cultured cells expressed low amounts of ZO-1

immunoreactive protein, and longer exposures of the immunoblots were required to detect it. For the duration of the cultures, ZO-1 predominated over ZO-1LP. However, the expression of ZO-1 decreased 4-fold after 5 days without affecting the TER. Except for the increase of TER, E14 retinal conditioned medium had no effect on these events. There was no reproducible difference in the level of ZO-1 expression

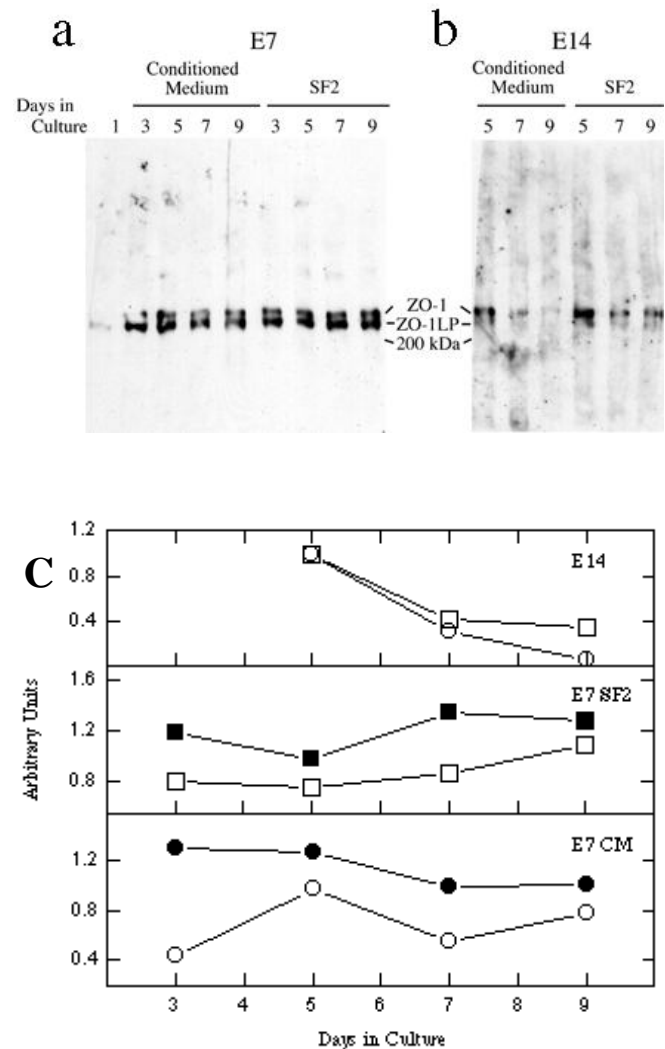


Figure 7. ZO-1 expression is unaffected by retinal conditioned medium. Changes in ZO-1 expression were unrelated to junction permeability and were unaffected by conditioned medium. Immunoblots of proteins from cultures of E7 (A) and E14 (B) embryos. E14 retinal conditioned medium was added on day 1 to the indicated cultures. Total protein was extracted on the indicated day of culture and 15 g per lane was immunoblotted with antibody 7445 to reveal ZO-1 and ZO-1 LP. The background is higher in (B), because longer exposures were required to detect a signal. The position of myosin (200 kDa) is indicated; beta-galactosidase (115 kDa) migrated off the bottom of the gel. (A) E7 RPE, 2 min exposure. (B) E14 RPE, 50 min exposure. (C) Densitometry of the immunoblots in panels (A and B). Qualitatively similar results were obtained in several experiments. Open symbols, ZO-1; closed symbols, ZO-1LP; squares, SF2; circles, conditioned medium.

between stimulated and unstimulated cultures. After 5 days in culture, the TER of the stimulated cultures was higher than that of the E7 cultures, and continued to rise slightly even as the level of ZO-1 decreased 4-fold. In summary, ZO-1 expression could change in culture without parallel changes in permeability, and E14 retinal conditioned medium could decrease permeability without altering the time-course or level of ZO-1 expression.

DISCUSSION

Diffusion across tight junctions can be regulated, because tight junctions discriminate on the basis of size and charge. Although variations in discrimination have been described for different epithelia (7,29), our study models changes in discrimination as a single epithelium differentiates. We modeled the intermediate phase of RPE development, because this is when permeability decreases without changes in the morphologic complexity of the tight junctions (2,4,6).

The validity of the culture model depends on whether *in vivo* properties are retained in primary cell culture. Several studies indicated that RPE can partially dedifferentiate in culture (30-33). We devised a serum free medium that helped preserve some of the native phenotype in culture and found that retinal conditioned medium further promoted differentiation (23,24). Differentiation was promoted more effectively by E14 than E7 retinal conditioned medium. These studies were complicated by culturing RPE on Matrigel, because Matrigel contains cytokines and growth factors that potentially modulate cell behavior. For example, transforming growth factor- α modulates the permeability of cultured mammary epithelia (10,11). To minimize this possibility, cultures were established on purified laminin. Monolayers of uniform height were formed. In contrast to cultures on Matrigel, the density of E14 cultures was two-fold lower than the density of E7 cultures. This parallels the decrease in density that occurs *in vivo* (27), and indicates that laminin allows density changes that mimic the *in vivo* condition. It thus should be used for subsequent mechanistic studies of cell growth as it is superior in this regard to other models. The permeability of the cultures also depended upon the embryonic age when the RPE was isolated. The permeability to HRP decreased between E7 and E14, as was observed in organ culture (4).

The presence of apical junctional complexes was indicated by the circumferential band of ZO-1 and filamentous actin. Four observations indicated that this complex contained functional tight junctions. The TER was reversibly reduced by EDTA, consistent with the dependence of tight junctions on extracellular calcium. The flux of nonionic solutes was size dependent, which is more consistent with diffusion across junctions than transcytosis. Permeability decreased in response to retinal conditioned medium, even if transcytosis was inhibited by chloroquine. In principal, conditioned medium could affect membrane transport. However, there are no transporters for inulin, mannitol or HRP, and the electrical resistance of biological membranes is much greater than the

reported TER. Consequently, more current flows across the junctions than through the cells, and the TER becomes insensitive to membrane effects (29). Conceivably, differences in cell density or the width and tortuosity of the paracellular path might account for small differences in permeability (34). However, these differences should affect all permeability assays the same way. The divergent changes in permeability described below are more compatible with effects on tight junctions.

There was a progressive change in size selectivity as the embryonic age of the RPE increased. First, a comparison of E7 and E10 cultures revealed that the older cultures were 40% less permeability to HRP, and yet there was no difference in permeability to inulin or mannitol. Although HRP is positively charged, this effect was unlikely due to charge. The TER indicated that the permeability of small ions decreased only slightly. Second, a comparison of E10 and E14 cultures revealed that the permeability to inulin and mannitol decreased by 40% and 15%, respectively. Even though there was little difference in the permeability to the large polyion, HRP, or the small monosaccharide, mannitol, the TER increased nearly 80%. Therefore, the permeation of large solutes was regulated according to their size, but the permeation of small solutes was regulated primarily by their charge.

E14 retinal conditioned medium revealed further differences between size and charge discrimination. Although E14 retinal conditioned medium reduced the permeability to ions and each of the tracers, the magnitude of the effect differed between cultures. For example, the permeability of E14 cultures to HRP was decreased 2.5 fold, but the permeability of E7 cultures decreased 4 fold. With respect to HRP, inulin and mannitol, the permeability of the E7, E10 and E14 cultures was identical after stimulation by E14 retinal conditioned medium. In contrast, the effect of conditioned medium on TER was approximately two fold, regardless of age, and the age-related differences were maintained. Conceivably, conditioned medium decreased permeability by decreasing the number of pores without affecting the charge of the pores. This would reduce the permeability to ionic and nonionic solutes alike, but would allow charge differences between cultures to remain. Regardless of mechanism, two laboratories provided a precedent for dissociating permeability to ionic from permeability to nonionic solutes. When occludin was overexpressed in Madin-Darby canine kidney cells, there were simultaneous increases in TER and permeability to mannitol (21,35).

In some respects the permeability of the stimulated cultures is comparable to other culture models of blood-retinal and blood-brain barriers, but the differences further demonstrate selectivity. The development of RPE is keyed to the development of the neural retina; E14 is just before photoreceptors extend outer segments (2). Rat RPE was cultured from a comparable stage of development (36). Rat and chick cultures had similar permeabilities to inulin (approximately $0.1 \mu\text{l}/\text{cm}^2/\text{min}$ for each), but different permeabilities to other solutes. In rat, the TER was 2-3 fold higher, which indicates lower permeability to ions, and the

permeability to HRP was 10 fold higher. Different means of regulating the permeability to small ionic and nonionic solutes explains a disparity between our studies and those on brain endothelia (20). Both studies measured similar fluxes (0.12 $\mu\text{l}/\text{cm}^2/\text{min}$ for sucrose in their study and 0.25 $\mu\text{l}/\text{cm}^2/\text{min}$ for mannitol in ours), but they reported a much higher TER (600 $\text{Ohm}\cdot\text{cm}^2$). These data are consistent with our hypothesis that distinct mechanisms regulate different aspects of junction permeability, and in chick RPE size discrimination is regulated by diffusible factors produced by the neural retina.

It is counterintuitive that the level of ZO-1 decreases in vivo when permeability also decreases (4). However, ZO-1 also functions in adherens junctions where it links cadherins to actin filaments (37). Further, Rajasekaran et al. (38) showed that when apical junctional complexes were dissociated and then reassembled, ZO-1 first participated in the formation of adherens junctions. Later ZO-1 became restricted to tight junctions, as the tight and adherens junctions were remodeled. Therefore, the changes in ZO-1 expression that accompany RPE differentiation could result from remodeling these two regions of the apical junctional complex. The current study shows the permeability of junctions can be regulated by E14 retinal conditioned medium without affecting ZO-1 expression. Although only ZO-1LP was evident in vivo, ZO-1 and ZO-1LP were expressed by E7 RPE in culture. The expression and distribution of each protein were unaffected by E14 retinal conditioned medium even though conditioned medium decreased permeability. As in vivo, cultures of E14 expressed more ZO-1 than ZO-1LP, and the level of ZO-1 was lower than in E7 cultures. (Note that much longer exposures of the immunoblots were required to detect ZO-1 in the E14 cultures.) The level of expression decreased further after 5 days in culture. Nonetheless, the permeability of E14 cultures was lower than the E7 cultures. Again, E14 retinal conditioned medium decreased permeability without affecting ZO-1 expression. Remarkably, the TER rose slightly between day 5 and 7, while the level of ZO-1 decreased nearly three-fold. Therefore, the level of ZO-1 expression cannot be used to infer junction tightness.

The transformation of RPE junctions into the low permeability form required by the outer blood-retinal barrier is a gradual, multifaceted process. We have evidence of at least two mechanisms that regulate the intermediate phase of the process. The roles of ZO-1 and ZO-1LP remain obscure, but since ZO-1 belongs to the MAGUK family of signaling proteins it could be a target for either of these mechanisms. ZO-1 includes PDZ, SH3 and guanylate kinase domains that help assemble regulatory complexes in other signal transduction pathways (39). If junction tightness is regulated by analogous complexes, relatively low levels of ZO-1 are required. The need for higher levels early in development remains under investigation.

ACKNOWLEDGEMENTS

The authors thank John Collins and Tammi James for expert

technical assistance, Chris Williams and Victoria Gross for help with initial experiments, and Colin Barnstable and Marvin Sears for critically reviewing the manuscript. James Anderson, members of his laboratory, and M.S. Mooseker provided helpful discussions, and antibodies to ZO-1.

This work was supported by National Eye Institute research grant EY08694 (LJR), ARVO/Alcon Research Fellowship Grant (YB) and CORE grant EY00785 to the Department of Ophthalmology and Visual Science, Yale University.

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