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Invited Review

Polarity and the development of the outer blood-retinal barrier

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Summary. The retinal pigment epithelium (RPE) is a monolayer that separates the outer surface of the neural retina from the choriocapillaris. Because the choriocapillaris is fenestrated, it is the RPE that forms the outer blood-retinal barrier and regulates the environment of the outer retina. Like all epithelia and endothelia, the ability of RPE to regulate transepithelial transport depends upon two properties: apical tight junctions to retard diffusion through the paracellular spaces of the monolayer, and an asymmetric distribution of proteins to regulate vectorial transport across the monolayer. During development, these properties form gradually. Initially, the tight junctions are leaky, and the RPE exhibits only partial polarity. As the neural retina and choriocapillaris develop, there are progressive changes in the composition of the apical junctional complexes, the expression of cell adhesion proteins, and the distribution of membrane and cytoskeletal proteins. Development can be used to dissect the multiple mechanisms that establish and maintain polarity and barrier function. These mechanisms are regulated by the interactions that develop between the RPE and its neighboring tissues. This review discusses the remodeling of the apical, lateral and basal plasma membranes of RPE that occurs during normal development, and establishes a framework to integrate the data obtained from multiple species. It examines the progress in understanding how environmental interactions regulate this development.

Key words: Blood-retinal barrier, Blood-brain barrier, Retinal pigment epithelium, Epithelial cell polarity, Tight junctions

Introduction

The blood-brain barrier regulates transport between the blood and the brain or cerebrospinal fluid. Generally, the barrier is formed by capillary endothelia, but in some regions the capillaries are fenestrated. There, an adjacent epithelium forms the barrier. In the retina, there is an example of each type. In many vertebrates, an inner blood-retinal barrier is formed by capillary beds near the inner surface of the retina. An outer blood-retinal barrier is formed by the retinal pigment epithelium (RPE), which separates the outer surface of the retina from the fenestrated choriocapillaris. The degeneration of these barriers is a primary or secondary event in many retinopathies (Vinores, 1995; Gariano et al., 1996). In response to injury, RPE will proliferate (or can be transplanted) to close a wound or replace damaged RPE. Unfortunately for reasons that are not understood, the RPE often does not redifferentiate into a fully polarized epithelium with restored barrier properties (Grierson et al., 1994). This review examines our current understanding of polarization and barrier formation during the embryonic development of the RPE. Studies on RPE proliferation and regulation of gene expression during development have been reviewed elsewhere (Zimmerman, 1975; Stroeva and Mitashov, 1983; Zhao et al., 1997).

There are two aspects to barrier function. The first is an occluding seal that encircles the endothelial or epithelial cell, binding it to each neighbor in the monolayer. This seal, the tight junction, retards diffusion across the monolayer through the spaces between cells (Anderson and Van Itallie, 1995). The permeability of tight junctions varies considerably among tissues (Frömter and Diamond, 1972). They are dynamic structures whose permeability can be regulated both physiologically and pharmacologically (Balda et al., 1991; González-Mariscal, 1992). During development, the tight junctions of the brain microvasculature are converted from a high permeability to a low permeability state. This conversion is induced by astrocytes (Stewart and Wiley, 1981; Janzer and Raff, 1987; Rubin et al., 1991; Chan-Ling and Stone, 1992; Jiang et al., 1994; Gariano et al., 1996). The second aspect of barrier function is the ability of the monolayer to regulate vectorial, transepithelial transport of ions and solutes. This property requires an asymmetric or polarized distribution of pumps, channels, transporters and other proteins (Rodriguez-Boulan and Nelson, 1989).

The embryonic development of RPE polarity and

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barrier function has been studied primarily in chick. To generalize these studies, RPE development will be related to stages in retinal development. Retinal and choroidal development appear fairly similar among vertebrate species, when various events are timed as a percentage of total developmental time. This allows key events to be used as landmarks to correlate data from different species. Two key events are the extension of photoreceptor inner segments beyond the outer limiting membrane and the formation and elongation of photoreceptor outer segments. These events were used to compare the development of Bruch's membrane between species (Braekevelt and Hollenberg, 1970; Greiner and Weidman, 1991). Bruch's membrane is composed of three fiber layers sandwiched between the basal laminae of the RPE and choriocapillaris. The basal lamina of the neuroepithelium becomes the basal lamina of the RPE, and consequently it is the first layer of Bruch's membrane to appear (Fig. 1). The basal lamina of the choriocapillaris develops about the time that inner segments appear, and the pentilaminar structure is completed about the time outer segments form. Similarly, the walls of the choriocapillaris begin to thin about the time that inner segments form, but fenestrae do not become numerous until outer segments form. This generalization is further justified, because epithelial polarity is induced by the environment (Eaton and Simons, 1995; Drubin and Nelson, 1996). Since the RPE monolayer is established before the neural retina and choriocapillaris differentiate, this suggests that these neighboring tissues regulate the further development of the RPE.

Origins and structure of the RPE

The RPE derives from the neuroepithelium that formed the neural tube. The apical membrane faces the lumen of the tube, while the basal membrane faces the mesoderm (Fig. 1). Neighboring cells are joined by apical junctional complexes. The complexes contain tight junctions that retard diffusion across the epithelium, and adherence junctions that provide mechanical strength. It is the fate of these junctions that is important here. In most regions, cells at the apical surface proliferate to generate layers of neurons and glia, and the lumen becomes the ventricular system. The cell layer that lines the ventricles becomes the ependyma, an epitheliod monolayer that retains the adherence junctions, but loses the tight junctions. A variation of this theme occurs in the neural retina, where the ventricular surface is lined by photoreceptors and Müller cells. Like the ependyma, only the adherence junctions are retained to form a structure named the outer limiting membrane. There are two exceptions to this general pattern, the epithelium of the choroid plexus and the RPE. Here, the neuroepithelium stops proliferating, forms a monolayer, and converts the tight junctions from a high permeability to a low permeability form.

Epithelial polarity is plastic and depends upon interactions with the environment (Eaton and Simons, 1995; Drubin and Nelson, 1996). The environment of the presumptive RPE is altered by rearrangements of the neural tube (Fig. 1). A diverticulum of the neural tube forms the optic vesicle, which then invaginates to form a two layered cup. This brings the apical membrane of the RPE into direct contact with the apical surface of the presumptive neural retina. Soon after this optic cup forms, the outer layer slows proliferation, manufactures pigment (melanin) granules, and converts from a pseudostratified to a simple monolayer (Zimmerman, 1975; Stroeva and Mitashov, 1983). I will define the formation of the RPE as the time when this pigmented monolayer appears. By contrast, proliferation and differentiation is just beginning for the neighboring neural retina and choriocapillaris. As the apical and basal environments gradually differentiate, there are progressive changes in the polarity and barrier function of the RPE. Different components polarize at different times, which suggests that multiple mechanisms regulate the differentiation of the apical, lateral and basal plasma membranes.

A schematic representation of the mature RPE is

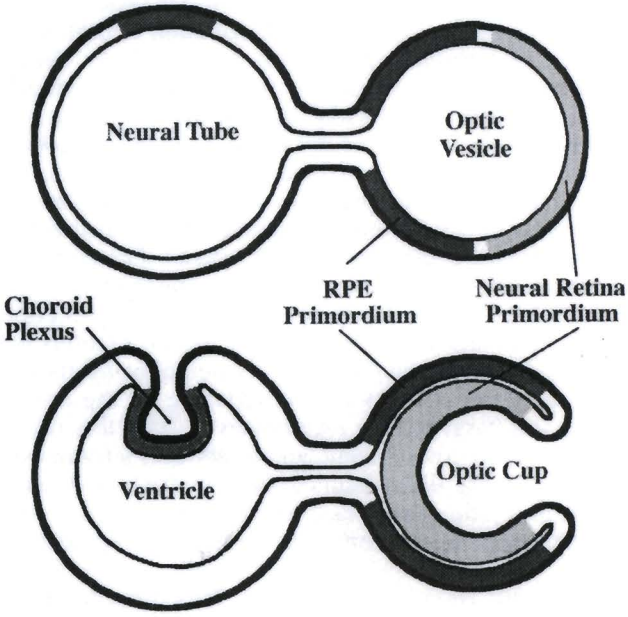

Fig. 1. The RPE and the neural retina are derived from neuroepithelium. The neural tube is a pseudostratified epithelium with an apical aspect (thin line) that faces a central lumen and a basal aspect (thick line) that lies on a basal lamina and faces the mesoderm. The optic vesicle forms from a diverticulum of the neural tube early in development. The vesicle invaginates to form a double layered cup such that the apical surfaces of the RPE and neural retinal primordia become apposed. The space between these surfaces becomes the interphotoreceptor or subretinal space. In some regions, the neuroepithelium invaginates into the forming ventricles to become the epithelium of the choroid plexus. The neural epithelium proliferates and thickens to form the neurons and glia of the central nervous system and retina. The RPE and choroid plexus form next to a fenestrated capillary bed. In contrast to most regions, where an endothelium forms the BBB, an epithelium forms the BBB in the ventricles where the cerebro-spinal fluid (CSF) forms (choroid plexus) and in the outer retina.

presented in Fig. 2. At the apical pole, elongated microvilli extend into the interphotoreceptor space where they interdigitate with the outer segments of photoreceptors. Additionally, RPE microvilli face the microvilli of Müller cells, which also extend beyond the outer limiting membrane. At the basal pole, numerous infoldings appose Bruch's membrane. The underlying choriocapillaris is fenestrated. The lateral membranes of the RPE are often infolded and lateral interactions are confined to junctional complexes near the apical pole. The apical junctional complex forms a band that completely encircles each cell in the epithelium. In most epithelia, this complex contains tight junctions that lie apical to the adherence junctions. The complexes are unusual in RPE, because the tight and adherence junctions partially overlap, and a third type of junction, the gap junction, is also present (Hudspeth and Yee, 1973).

Development of lateral junctions and barrier function

Early studies described morphological changes in the apical junctional complexes of chick RPE. Gap junctions were evident when the RPE monolayer first appeared on E3 and rapidly increased in number (Fujisawa et al., 1976). Before E7, these authors found rudimentary tight junctions that were evident by transmission electron microscopy, but not by freeze-fracture. Kniesel and Wolburg (1993) observed that the complexity of thight junction strands was constant from E10 to E15, but that complexity increased between E15 and E19. Sandig and Kalnins (1988, 1990) described progressive morphological changes in the adherence junctions between E7 and hatching (E21).

These morphological changes are accompanied by a decrease in the permeability of tight junctions. Functional tight junctions were demonstrated in E7 RPE by injecting horseradish peroxidase (HRP) into the systemic circulation (Latker and Beebe, 1984). When exposed to low concentrations of HRP for 5 minutes, the RPE prevented the HRP from entering the subretinal (ventricular) space. The results of this experiment are ambiguous if the RPE is exposed to HRP for longer times in vivo, because HRP is toxic to the eye. It and can also have systemic effects (e.g., release serotonin or histamine) that decrease permeability (Vinores, 1995). Rizzolo and Williams (1996) circumvented this problem by isolating chick RPE together with the underlying choroid. The tissue was incubated in a medium containing HRP from 20 to 45 min at 37 °C. Under these conditions HRP was unable to cross the choroid to the basal surface of the RPE, but had immediate access to the apical surface. HRP readily diffused across the junctional complex of tissues isolated on or before E10, but failed to cross the junctions of tissue older than E11. Although Latker and Beebe (1984) showed that RPE

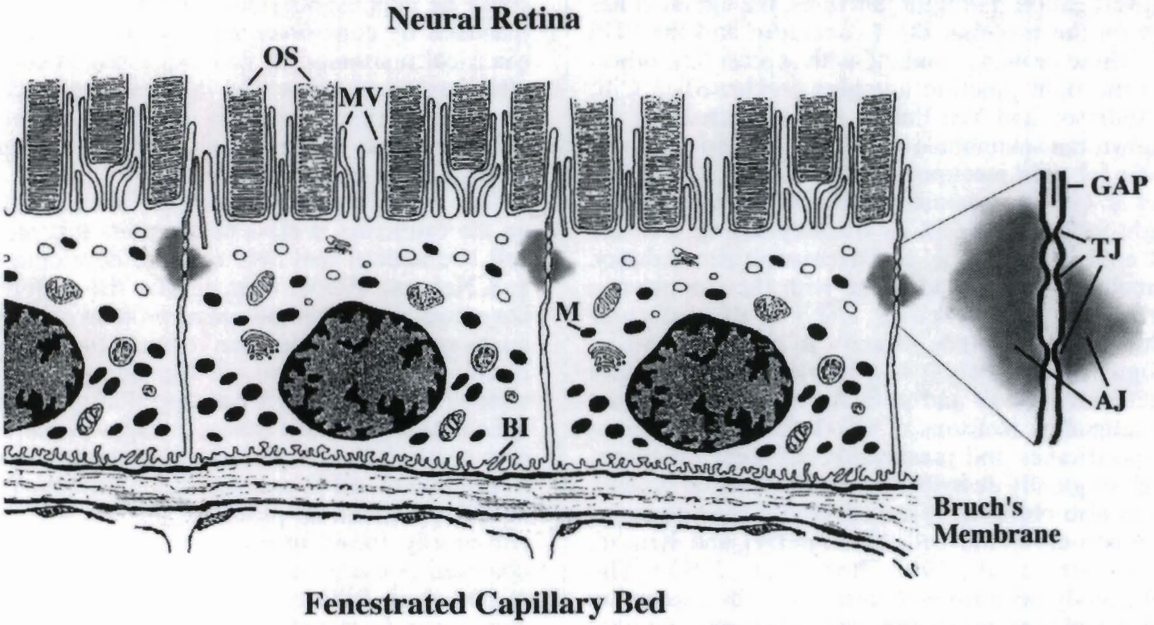

Fig. 2. A schematic drawing of the outer blood-retinal barrier (adapted from Clark, 1986). The apex of the RPE is modified into microvilli (MV) which envelope the impinging tips of photoreceptor outer segments (OS). Melanin granules (M) fill the cell and absorb stray light. Basal infoldings (BI) form above the multilaminar Bruch's membrane which lies between the RPE and a fenestrated capillary bed. On the lateral surface of the RPE an apical junctional complex develops to seal the lateral space. This complex is shown magnified to the right of the drawing to highlight the junction complex. This complex is formed by partially overlapping junctions including gap junctions (GAP), tight junctions (TJ), and adherence junctions (AJ). Gap junctions serve an important role in intercellular communication. Tight junctions (TJ) form a band that completely encircles each cell. Adherence junctions (AJ) also form a band around each cell but their role is more structural, relating to the cytoskeleton, whereas the tight junctions (TJ) play a role that is more selective in what passes between the apical and basolateral environments.

tight junctions function at some level on E7, the tight junctions are unable to withstand prolonged exposure to high concentrations of HRP until E11. This approximates the time when the epithelium of the choroid plexus becomes tight, as measured with HRP in vivo (Wakai and Hirokawa, 1981). Similarly, other regions of chick blood-brain barrier become tight between E12-E15 (Delorme et al., 1970; Wakai and Hirokawa, 1978; Beebe et al., 1986).

The decrease in permeability is regulated by diffusible factors produced by the neural retinal (Rizzolo and Li, 1993). As predicted by the studies of HRP diffusion, primary cell cultures of E7 RPE were leaky. This was determined by culturing the RPE on filters and measuring the transepithelial electrical resistance. The permeability of the cultures decreased when they were maintained in medium that was conditioned by organ cultures of neural retina. The effect was greatest when the retinas were isolated from E14 or E16 embryos, after the junctions of native RPE became impermeable to HRP. The effectiveness of the conditioned medium depended upon when it was first presented to the RPE. After several days in culture without conditioned medium, the RPE lost the ability to respond to conditioned medium. The active factors had a molecular mass less than 10 kDa and were protease resistant.

The molecular basis for these changes in morphology and function is not understood, but some of the key components of the adherence and tight junction are under investigation. For tight junctions, the attention has focused on the proteins ZO-1, occludin and the 7H6 antigen. These proteins combine with at least four others to form the tight junction complex (reviewed in Citi, 1993; Anderson and Van Itallie, 1995). Occludin is the only known transmembrane protein of the complex, and is specific for tight junctions (Furuse et al., 1993, 1994). Balda et al. (1996) demonstrated that occludin functions as a tight junction protein by overexpressing it in the MDCK epithelial cell line, and decreasing permeability. The remaining proteins associate with the cytoplasmic plaque of the tight junction. ZO-1 is of particular interest, because it binds directly to occludin and its homologue in *Drosophila* is a junctional protein that regulates cell growth and polarity. There are at least eight mammalian isoforms of ZO-1 that have different tissue specificities and presumably different functions. Although originally described as a tight junction protein, ZO-1 was also observed in the adherence-type junctions of some non-epithelial cells (Dermietzel and Krause, 1991; Howarth et al., 1992; Itoh et al., 1993). The following study demonstrated changes in the expression of ZO-1's, and their association with adherence and tight junctions, during the development of chick RPE.

Two putative isoforms of ZO-1 were observed in chick, termed ZO-1' and ZO-1" (Rizzolo and Williams, 1996). Immunologically, ZO-1' is the most closely related to mammalian isoforms, but sequence data will be required to relate the chick proteins to their mammalian counterparts. On E3, the entire neuro-epithelium of the optic cup expressed ZO-1" and occludin. The presence of occludin suggests the presence of tight junctions in the undifferentiated neuro-epithelium. If so, the junctions are rudimentary, because they cannot be demonstrated by freeze fracture (Fujisawa et al., 1976). In the presumptive neural retina, the apical junctional complex becomes the outer limiting membrane. It contained occludin until E7-E8, just before the inner segments of photoreceptors extend beyond the outer limiting membrane on E9 (Olson, 1979). ZO-1" continued to be expressed in the adherence junctions that comprise the outer limiting membrane. In the RPE, ZO-1' gradually replaced ZO-1" between E7 and E16, but expression of total ZO-1 decreased 10x. Because occludin expression was constant or increased slightly, the ratio of ZO-1/occludin also decreased 10x. Conceivably, ZO-1 is part of the adherence junction early in development, but as the adherence and tight junctions mature ZO-1 becomes confined to the tight junction. Together with the data from the neural retina, this suggests that ZO-1" preferentially associates with the adherence junction, but ZO-1' preferentially associates with the tight junction. The functional significance of these changes is uncertain, but clearly, the decrease in permeability must be attributed to factors other than increased occludin expression.

The 7H6 antigen has been related to junction tightness. Using the Madin-Darby canine kidney cell line, Zhong et al. (1994) showed that the 7H6 antigen could be dephosphorylated and dissociated from tight junctions by conditions that lowered the transepithelial electrical resistance. It was expressed in cultured RPE after barrier function was induced pharmacologically. The 7H6 antigen has been demonstrated in E17-E19 chick embryos (Konari et al., 1995), however younger embryos were unexamined.

For the adherence junction, the attention has focused on the cadherins, a class of receptors that mediate cell-cell interactions and help regulate development (Marrs and Nelson, 1996). Classically, the cadherins were described as cell-cell adhesion proteins. However, there are many more of them than required for adhesion, and it is now clear that these are signalling molecules. The various isoforms have tissue specificity, and changes in their expression effect multiple stages of morphogenesis and epithelial cell polarity. At different stages of development, RPE expresses N-cadherin, B-cadherin and R-cadherin in place of the E-cadherin that is commonly found in epithelia. Huotari et al. (1995) observed N-cadherin along the entire lateral membrane of E10 chick RPE, but by E17 the immunofluorescence signal was faint and confined to the apical junctional complexes. Capper et al. (1995) observed that R-cadherin first appears in E9 RPE. In cultures of E10 chick RPE, B-cadherin was prominent in proliferating cells and loosely packed cells, but R-cadherin was the predominant isoform in tightly packed cells. Both laboratories observed that N-cadherin increased or was re-expressed when RPE was isolated and placed in

primary culture. These data are consistent with other studies in which cultured RPE reverted to an earlier embryonic state and partially redifferentiated in culture (Zhao et al., 1997). These changes in cadherin expression are intriguing, but it is unclear how the signaling pathway of each cadherin differs to effect development or polarity. Our current understanding is discussed below.

Development of polarity

Epithelia use cell adhesion proteins to convey information from the environment into the cell and regulate polarity. The cadherin, CAM and integrin families share this function (Eaton and Simons, 1995; Drubin and Nelson, 1996). These proteins transmit signals from the environment into the cell, in part by rearranging or chemically modifying the cytoskeleton. Although closely related, E-cadherin and B-cadherin have different effects on the expression of junctional proteins and cell polarity (Gundersen et al., 1991; Marrs et al., 1993, 1995). Epithelia that express E-cadherin distribute the Na^+,K^+-ATPase to the basolateral membrane, but the RPE and choroid plexus express B-cadherin and distribute this pump protein to the apical membrane. When exogenous E-cadherin was expressed in non-polarized cells, it redistributed the cortical cytoskeleton to regions of cell-cell contact. Because the Na^+,K^+-ATPase is linked by ankyrin to the cortical cytoskeleton, it was redistributed along with the cytoskeleton (McNeill et al., 1990). Besides cytoskeletal effects, E-cadherin induced the expression of a number of proteins that might effect polarity. This effect was observed in the RPE-J cell line, a transformed line that exhibits partial polarity (Marrs et al., 1995). In the parent cell line, the cortical cytoskeleton was found along the lateral membranes, but the Na^+,K^+-ATPase was not polarized. When exogenous E-cadherin was expressed, it induced the expression of the ankyrin 1/2 isoform, and the Na^+,K^+-ATPase was polarized to lateral membranes. This suggested that only certain isoforms of ankyrin link the Na^+,K^+-ATPase to the cytoskeleton, however E-cadherin also induced other proteins that might also effect polarity. It is unknown if exogenous E-cadherin could reverse the polarity of the Na^+,K^+-ATPase in fully polarized RPE cells. Most studies focus on the basal and lateral membrane interactions that induce polarity, but in the RPE, apical interactions modify that polarity. The following sections describe the plasticity of RPE during development and identify candidates that may mediate the effects of the apical and basal environments.

Development of apical polarity

The apical membrane of RPE is enriched in the various proteins that support its interactions with photoreceptors (Zhao et al., 1997). Apical microvilli are present as soon as the RPE monolayer forms. In rat, these microvilli begin to elongate when inner segments begin to appear near birth until postnatal day 14 when outer segments are complete (Braekevelt and Hollenberg, 1970). In chick, this elongation is temporally related to the extension of the outer segments (Coulombre, 1955). If the retina is removed, as in retinal detachment, the microvilli degenerate (Anderson et al., 1983). Various cell adhesion proteins have been proposed to mediate photoreceptor/RPE interactions, and their expression changes during development. In mature RPE, a cadherin-like protein and an integrin, the vitronectin receptor, are present in the apical membrane (Rizzolo et al., 1994; Anderson et al., 1995; Huotari et al., 1995). Other proteins are present only early in development, and their distribution to the apical membrane depends upon the apposition of the neural retina.

The first example of an early adhesion protein is N-CAM (Neill and Barnstable, 1990). In developing rat RPE, N-CAM was observed in the apical membrane in vivo and in explant cultures (Gundersen et al., 1993). N-CAM was only basolateral in cells that grew out of the explant. In other words, cells that dedifferentiated, migrated and redifferentiated in the absence of the neural retina failed to reestablish an apical pool of N-CAM. In this case, cells in the center of the explant maintained polarity even though the neural retina was absent.

The second example is a subset of integrins. Integrins mediate cell-cell and cell-basal lamina interactions, and are commonly distributed to the basal or basolateral membranes. In chick, the integrin $\alpha 3\beta 1$ adopts a basal distribution as soon as the RPE monolayer forms. This might be expected, because the basal lamina is present by this time (Turksen et al., 1985). However, other members of the $\beta 1$ family of integrins, including $\alpha 6\beta 1$, are initially non-polarized, but gradually become basally polarized between E9 and E14 (Rizzolo and Heiges, 1991; Rizzolo et al., 1994). A basal distribution of these other integrins can be induced by interactions at the basal membrane. This was demonstrated by culturing E7 RPE on laminin, collagen IV or Bruch's membrane (Rizzolo, 1991). Specific interactions were required, because fibronectin and other collagens were unable to induce polarity. To explain the apical distribution of certain integrins on E7, organ culture experiments were performed in which the RPE/retinal interface was disrupted and reconstituted (Rizzolo et al., 1994). These experiments demonstrated that interactions with the developing neural retina modified the polarity that would have been induced by the basal lamina alone. Unless the apical surface of the E7 neural retina was directly apposed to the RPE, the distribution of $\alpha 6\beta 1$ was basal. As the neural retina matures, the interactions responsible for the apical distribution disappear. Because integrins transmit signals across the plasma membrane, this might provide the RPE one mechanism to sense changes in the developing neural retina and coordinate the differentiation of the two tissues.

An early signal induces a shift in the distribution of the Na^+,K^+-ATPase from the apicolateral to the apical

plasma membrane (Rizzolo and Heiges, 1991; Rizzolo and Zhou, 1995). Although the polarity of many membrane proteins in different epithelium is regulated by basal and lateral interactions, that is not the case here. In posterior pole of chick RPE, the shift occurs on E6, when the tight and adherence junctions are poorly differentiated. Further, the apical distribution could not be induced by Bruch's membrane, or individual components of the basal lamina (Rizzolo, 1991). The shift in distribution may reflect changes in interactions with the cytoskeleton. In most epithelia, the Na^+,K^+-ATPase is linked via ankyrin to spectrin along the lateral or basolateral membranes. This led to the hypothesis that the cortical cytoskeleton helps maintain the polarized distribution of membrane proteins (Rodriguez-Boulan and Nelson, 1989; Hammerton et al., 1991; Drubin and Nelson, 1996). However in chick RPE, several laboratories demonstrated that the Na^+,K^+-ATPase is segregated from spectrin. The Na^+,K^+-ATPase is found in the microvilli, but the apical pool of spectrin is confined to a terminal web-like region at the base of the microvilli (Huotari et al., 1995; Rizzolo and Zhou, 1995). The remainder of the spectrin and ankyrin was distributed along the lateral and basal membrane, as discussed in detail in the following section. Therefore, the distribution of spectrin is similar to that of intestinal or proximal kidney tubules, where the Na^+,K^+-ATPase has a basolateral polarity. This suggests that RPE microvilli sequester the Na^+,K^+-ATPase by a specialized mechanism that supersedes the house-keeping mechanism common to all epithelia. Such a mechanism likely includes ankyrin, or an ankyrin-like peptide, that may link the enzyme to the microvillar cytoskeleton. Besides its colocalization with spectrin, ankyrin immunoreactivity is also distributed throughout the microvilli (Rizzolo and Zhou, 1995). This is consistent with data obtained in rat, where the Na^+,K^+-ATPase was readily cross-linked to ankyrin, but poorly cross-linked to a complex with spectrin (Gundersen et al., 1991). The presence of ankyrin in microvilli is unusual and may be due to a specialized isoform.

The microvilli of RPE differ from those of other epithelia in other ways. Although they contain common skeletal elements such as ezrin and bundled actin, they lack villin (Höfer and Drenckhahn, 1993). Unlike most other epithelia, they contain myosin VIIa (Hasson et al., 1995). The myosins are thought to shuttle proteins and vesicles along actin filaments, and the presence of this tissue-specific isoform may be important for regulating transport into the microvilli. The importance of the

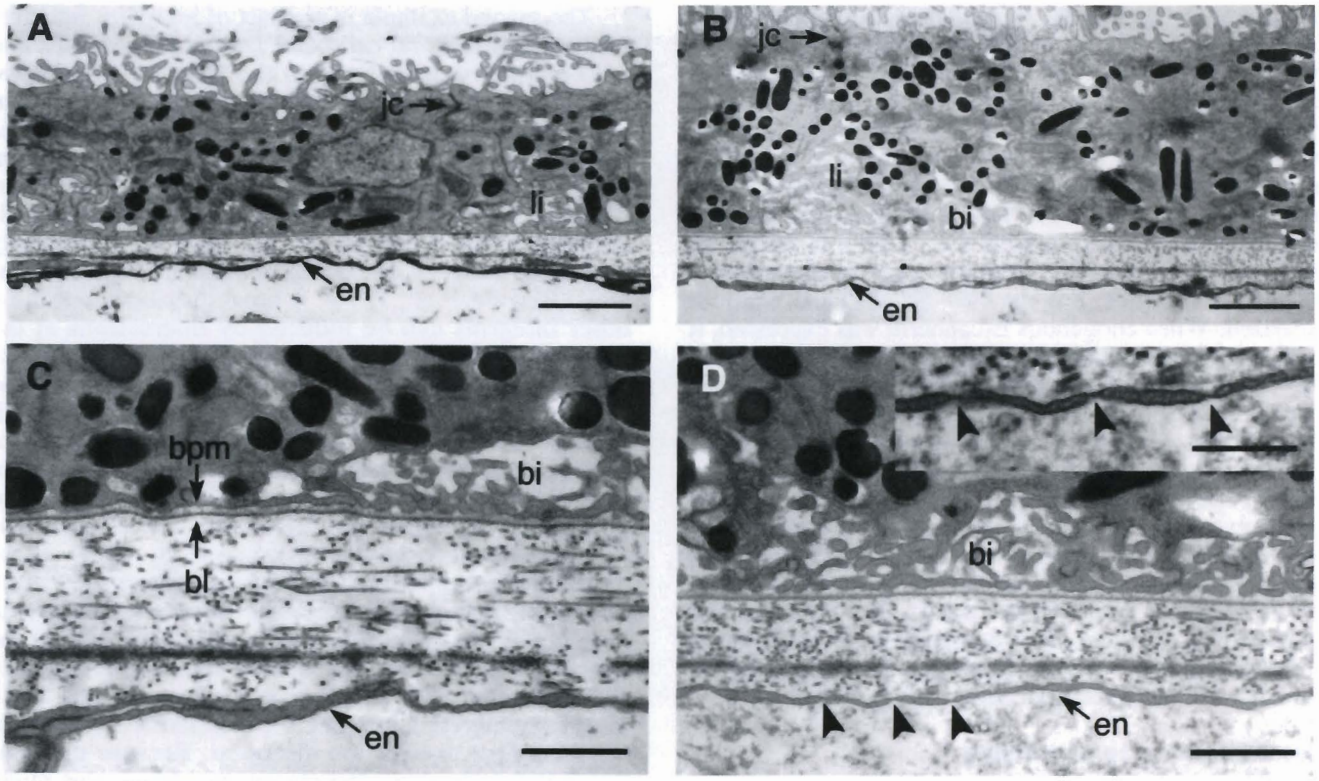

Fig. 3. The development of basal infoldings in the RPE and fenestra in the choriocapillaris. The apical membrane faces the top of the micrograph, and capillary endothelial cells face the bottom. **A.** On E10, lateral infoldings (li) were observed beneath junctional complexes (jc), but the basal plasma membranes were flat. **B.** On E12, patches of basal infoldings (bi) became evident. **C.** On E14, most of the basal membrane was infolded, but flat regions were also observed. Fenestra in the capillary endothelial cells (en) were rare. **D.** On E16, virtually all of the basal membrane was infolded and fenestra (arrowheads) were common in the capillary walls. bpm: basal plasma membrane; bl: basal lamina of the RPE: Bars: 2.0 μm (A,B), 1.0 μm (C,D) and 0.5 μm (inset).

microvillar cytoskeleton for apical polarization was demonstrated in intestinal epithelia. Disruption of this region of the cytoskeleton disrupted the apical polarization of sucrase-isomaltase (Costa de Beauregard et al., 1995). In RPE, the formation of an appropriate microvillar cytoskeleton may depend upon the absence of E-cadherin signaling on the lateral membrane and the presence of apical signaling from other adhesion proteins.

Development of basal polarity

The basal plasma membrane was initially flat in rat RPE, but became infolded in focal regions when inner segments appeared. Infoldings encompassed the entire basal membrane by the time outer segments were apparent (Braekevelt and Hollenberg, 1970). Similar observations for chick are shown in Fig. 3. Lateral infoldings were prominent before E9-10, when inner segments extended beyond the outer limiting membrane. By E12, some lateral infoldings were continuous with short segments of basal infoldings. By E14, most of the basal membrane was infolded, but flat regions were still evident. The process was complete by E16, when outer segments were evident. The development of the basal infoldings preceded the fenestration of the choriocapillaris. Occasional fenestrae were evident on E14, but were numerous on E16 (Fig. 3).

These events are coordinated by interactions between the RPE and choriocapillaris (Korte et al., 1989). Several lines of evidence indicate that epithelia induce the formation of fenestrae. Burns and Hartz (1992) examined retinal neovascularization where new vessels were encapsulated by RPE. They observed a correlation between time of encapsulation and the number of fenestrae formed. In studies with a kidney epithelial cell line (MDCK), the extracellular matrix deposited by the epithelium induced fenestrations in endothelial cells (Milici et al., 1985; Carley et al., 1988). Roberts and Palade (1995) demonstrated that vascular endothelial growth factor induces fenestrations and Adamis et al. (1993) showed that mature RPE can secrete this growth factor. Together these data suggest that the RPE induces the formation of fenestrae, but only after remodeling the basal plasma membrane.

The increased surface area that results from infolding the basal membrane is accompanied by a change of protein composition. For example, REMP, a putative transport protein, lies in the apicolateral membranes until basal infoldings form (Philp et al., 1995). The infoldings may be formed or stabilized by rearrangement of the cortical cytoskeleton. In chick, the cortical cytoskeleton changed its distribution beginning on E12. Earlier in development, two components of this cytoskeleton, spectrin and ankyrin, localize to the base of the microvilli and along lateral membrane (Huotari et al., 1995; Rizzolo and Zhou, 1995). On E12, patches of spectrin and ankyrin appear on the basal membrane. These patches grow in size and number to form a continuous distribution along the basal membrane by E16. This shift in distribution might correspond to the formation of basal infoldings during this time (Fig. 3). This correlation needs to be verified by immunoelectron microscopy.

A membrane protein that appeared in the basal plasma membrane together with the ankyrin and spectrin is also a marker for the formation of the blood-brain barrier. The 5A11 antigen is the same as, or very closely related to, neurothelin or HT7, which appears in capillaries of the central nervous system when they become impermeable to HRP (Seulberger et al., 1990; 1992; Fadool and Linser, 1993; Schlosshauer et al., 1995). This protein belongs to the immunoglobulin superfamily of surface receptors, and the 5A11 antibody interferes with Müller cell-neuronal interaction in culture (Linser and Perkins, 1987). Early in development this protein was observed in the neuroepithelium and its derivatives. Long before the blood-brain barrier forms, its expression was suppressed in neurons and glia. However, its continued expression in Müller cells is controversial. 5A11 antibodies label Müller cells, but neurothelin antibodies do not. It is unclear whether 5A11 antibodies are more sensitive, or whether neurothelin and 5A11 are different, closely-related proteins. Early in RPE development, 5A11 is in the apical and lateral membranes. About the time RPE becomes impermeable to HRP, 5A11 also appears in the basal plasma membrane where it always colocalizes with patches of spectrin and ankyrin (Fadool and Linser, 1994; Rizzolo and Zhou, 1995). Clearly, 5A11 cannot interact with neurons in this location, but may have a second function related to barrier formation. It is unknown if 5A11 complexes with spectrin and ankyrin, or if it colocalizes by another mechanism. An intriguing possibility is that 5A11 helps form or stabilize basal infolds by mediating an interaction between neighboring segments of basal membrane.

Parallels to blood-brain barrier development

The development the blood-brain barrier was recently reviewed (Dermietzel and Krause, 1991; Schlosshauer, 1994). The capillaries that initially form in the brain are as permeable as those in the systemic circulation. During development, capillary permeability decreases several orders of magnitude. This is a gradual process that can be dissected by monitoring the expression of certain proteins, blood-brain barrier specific antigens and the diffusion of tracers such as Evans blue or HRP. The process can be subdivided into three phases. In the early phase, there are leaky tight junctions that contain ZO-1. In the intermediate phase, astrocytes appear and capillary tight junctions decrease their permeability to HRP. Among the changes in protein expression at this stage is the appearance of (neurothelin, HT7, Seulberger et al., 1990, 1992; Fadool and Linser, 1993; Schlosshauer et al., 1995). In the late phase, there are further changes in protein expression and decreases in

permeability.

Neural tissue induces the development of the blood-brain barrier, especially if astrocytes are present (Stewart and Wiley, 1981; Janzer and Raff, 1987; Chan-Ling and Stone, 1992; Jiang et al., 1994; Gariano et al., 1996). In part, the induction is caused by diffusible factors produced by astrocytes (Rubin et al., 1991). Although neural retina-derived factors increased RPE barrier function, the chick retina is avascular and lacks astrocytes. A clue to the source of the retinal factors comes from the study of vascularized retinas. The inner blood-retinal barrier contains inner and outer vascular plexi, but only the inner plexus associates with astrocytes. Tout et al. (1993) demonstrated that Müller cells are able to induce barrier properties and may serve this role in the outer vascular plexus. Because the apical processes of Müller cells face the apical surface of the RPE, and they are among the last retinal cells to differentiate, Müller cells are a potential source of factors that induce the barrier function of RPE.

The development of RPE polarity and barrier properties is summarized in Fig. 4. To compare this process to the development of endothelial regions of the blood-brain barrier, the intermediate phase is defined as the decrease in permeability to HRP and the initial shift in the distribution of the 5A11 antigen. In each case, development begins with the presence of apical junctional complexes that contain ZO-1, and proceeds by a gradual, multistep process. Studies with RPE suggest that different isoforms of ZO-1, or ZO-1-related proteins, are expressed when the junctions are converted to less permeable forms, and suggest that different isoforms may associate with different elements of the apical junctional complex. Notably, the commonly used antibodies to ZO-1 recognize multiple proteins that comigrate in commonly used gel electrophoresis systems. Therefore, it may be necessary to reexamine the issue of ZO-1 expression during development of brain capillaries. While studies with endothelial cells focused on changes in protein expression, the morphology of the RPE also lends itself to examining the shifts in protein distribution required for vectorial, transepithelial transport and cell-cell interaction. Figure 4 describes the changes in RPE structure that must occur before the opening of fenestrae reduces the barrier function of the choriocapillaris.

Future directions

Like all epithelia, RPE polarity is plastic and depends upon interactions with its neighbors. Although considerable attention has been given to the effects of RPE on its neighbors, studies on the effects of its neighbors on the RPE are just emerging and are necessarily descriptive. Now that some of these interactions and the relevant proteins have been identified, the challenge is to investigate the underlying mechanisms. Among the key issues is how the permeability of tight junctions is regulated. The neural retina signals the junctions to convert from a leaky to a tight form, but it is unclear what the signals are, how they are received, and how changes in composition effect changes in the function. Multiple cadherins regulate RPE development, but the differences between their signaling pathways are unknown. The composition and function of RPE microvilli are unique, but it is unknown how the neural retina induces these properties. It is unclear how unique elements in the microvillar cytoskeleton sequester a common protein, the Na^+,K^+-ATPase, into this uncommon location. There is a gradual remodeling

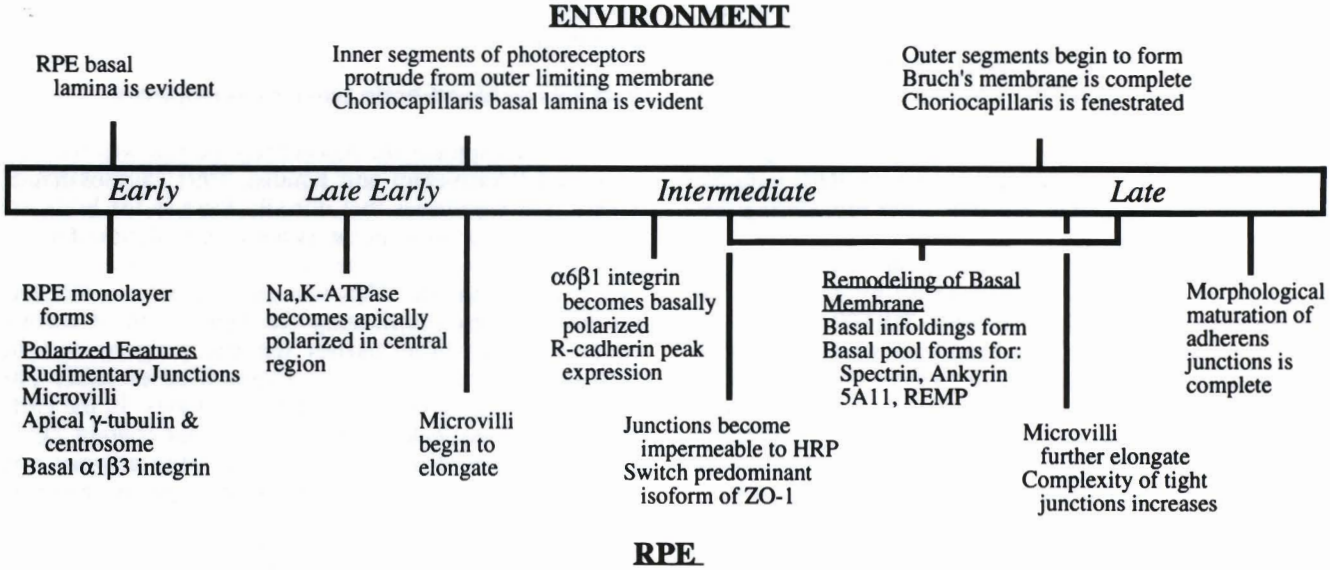

Fig. 4. Time line for RPE development. By analogy to the development of the blood-brain barrier, the intermediate phase is defined by the change in permeability to HRP (see text). Changes in the environment are indicated above the line, while changes in the RPE are indicated below the line.

of the basal plasma membrane that includes changes in structure and composition. Although RPE likely induces the formation of fenestrae in the choriocapillaris, it is unknown what induces the basal membrane of RPE to infold before this event. It is unknown what role the cytoskeleton and membrane proteins play in forming and maintaining the infoldings. These issues are central to epithelial cell biology. They are also critical to understanding proliferative retinopathies, wound healing and transplantation of RPE. Although RPE has a limited capacity to re-epithelialize wounds and transplanted RPE benefits nearby photo-receptors in several ways, the ultimate criterion for the success of these processes is the re-establishment of a functional blood-retinal barrier.

Note added in proof: The preliminary report of Rizzolo and Williams (1996) has been accepted for publication (Williams C.D. and Rizzolo L.J. (1997). Remodeling of juntional complexes during the development of the outer blood-retinal barrier. Anat. Rec. in press) with the following alterations. ZO-1' is termed ZO-1 and ZO-1" is termed ZO-1LP (like protein).

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