CHAPTER 3

THE BLOOD-RETINA BARRIER
Tight Junctions and Barrier Modulation

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Abstract: The blood-retina barrier (BRB) is composed of both an inner and an outer barrier. The outer BRB refers to the barrier formed at the retinal pigment epithelial (RPE) cell layer and functions, in part, to regulate the movement of solutes and nutrients from the choroid to the sub-retinal space. In contrast, the inner BRB, similar to the blood brain barrier (BBB) is located in the inner retinal microvasculature and comprises the microvascular endothelium which line these vessels. The tight junctions located between these cells mediate highly selective diffusion of molecules from the blood to the retina and the barrier is essential in maintaining retinal homeostasis. In this chapter, we summarize the key differences between the iBRB and oBRB and outline the molecular constituents of the tight junctions associated with the iBRB. We also describe a process for modulation of the iBRB to enhance systemic delivery of therapeutics to the retina, a technology which may pave the way for safer and more effective therapies for retinal diseases.

INTRODUCTION: GENERAL ANATOMY OF THE HUMAN EYE

The eye is one of the most specialized organs of the human body. It allows for light from the environment, mediated by specialized cells in the retina, to be converted into specific signals required for the process of vision. The retina, located at the back of the eyeball, contains photoreceptor cells (rods and cones) which receive the light, and the resulting neural signals subsequently undergo complex processing by other neurons of the retina, and are transformed into action potentials in retinal ganglion cells whose axons form the optic nerve. The optic nerve forms at the point of the retina where ganglion cell

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axons converge at the optic disk, and allows for the transmission of information to the brain, to be processed for the function of vision.

The retina is made up of a distinct group of neurons, including photoreceptor cells, bipolar cells, ganglion cells, Müller glial cells and various forms of interneurons. In general, the retina can be divided histologically into ten distinct layers, with each layer formed by certain cell types. The human retina has the highest oxygen consumption per weight of any tissue in the body. The high metabolic rate of the neural retina underlines the need for a distinct and regulated blood supply, and this is mediated via the Blood Retina Barrier (BRB), which will be discussed later in this chapter.

There are two sources of blood supply to the retina; the central retinal artery arises adjacent to the optic nerve, passing forward into the neural retina in the center of the optic nerve accompanied by the central retinal vein, and the choroid, which functions to nourish the outer layers of the retina. The choroid however is thin and loose compared with the highly regulated barrier properties of the vasculature associated with the inner retina, and is situated at the posterior portion of the eye, with the choriocapillaris providing nutritional requirements for outer retinal cells and the photoreceptors. At the inner retina, retinal capillaries arising from the central artery permeate the retina as far as the outer plexiform layer (OPL), with the outer segments of the retina remaining avascular.[1]

THE BLOOD-RETINA BARRIER (BRB)

Similar in structure and function to the Blood Brain Barrier (BBB), the Blood Retina Barrier (BRB) in the eye allows for the maintenance of neural tissue environments through the regulation of ion concentrations, water permeability, delivery of amino acids and sugars, and by preventing the exposure of the neural tissue to circulatory factors such as antibodies and immune cells.[2] In contrast to the BBB, however, the BRB consists of both an inner blood retina barrier (iBRB) and an outer blood retina barrier (oBRB). The iBRB comprises retinal endothelial cells, which line the micro-vessels allowing for the maintenance of blood vessel integrity and preserving the vessel's homeostasis. The oBRB is made up of Retinal Pigment Epithelial (RPE) cells and Bruch membrane, and it acts as a filter to restrict the passage of macromolecules to the outer segments of the photoreceptors. The RPE cells that comprise the oBRB allow for supporting functions essential to photoreceptor survival. These include phagocytosis of photoreceptor outer segments and transport of nutrients from the choroid to the sub-retinal space. The RPE also allows for the transport and processing of Vitamin A, general adhesion of the retina, and the absorption of scattered and out of focus light.[3] The apical surface of the RPE interacts with the photoreceptor outer segments in the outer nuclear layer of the retina, while the basolateral side interacts with the choroid, acting as a barrier to the highly perfused and permeable choriocapillaris.[4]

Both the iBRB and oBRB contain tight junctions that confer highly selective properties on barrier function. Tight junctions contain a unique assembly of proteins which constitute a highly selective barrier. Proteins making up the tight junction include Occludin and the Claudins, which span the plasma membrane and interact homotypically, while a series of peripheral cytoplasmic proteins function to anchor the transmembrane proteins to the actin cytoskeleton. Among others, these peripheral proteins include Zonula occludens-1 (ZO-1), Zonula occludens (ZO-2), and Zonula occludens-3 (ZO-3), which act through multiple protein-protein interaction domains, and are crucial for the distinct organization and initial formation of tight junctions.[5-8] Although the principle of the tight junctions is

inherently similar at the iBRB and the oBRB, i.e., the restriction of paracellular diffusion of molecules into the neural environment, the molecular composition of these junctions differs considerably.

TIGHT JUNCTIONS

Tight junctions were first identified in 1963 in epithelial cells, using electron microscopy.[9] Since then, our understanding of the tight junction and its composition and localization has increased dramatically. Tight junctions are formed at the apical periphery of endothelial cells of the iBRB and RPE cells of the oBRB. They perform the dual role of creating a primary barrier to the diffusion of solutes through the paracellular pathway, while also maintaining cell polarity as a boundary between the apical and basolateral plasma membrane domains.[10] Tight junctions are complex structures, which are composed of a series of integral and peripheral membrane proteins. The transmembrane proteins of the tight junction include occludin, Junctional Adhesion Molecule (JAM) and claudins, and they extend into the paracellular space, creating the seal characteristic of the tight junction.[11] Of the known integral membrane proteins of the tight junction, the proteins ZO-1, -2, -3 and cingulin have been shown to play an integral role in the scaffolding of transmembrane proteins, while also creating a link to the perijunctional actin cytoskeleton.[12] The 220 kDa phosphoprotein ZO-1 in particular has three PDZ (PSD-95, DLG, ZO-1) domains that could potentially bind to a wide variety of protein partners and allow for the control of tight junction assembly.[13,14] The PDZ domains associated with ZO-1 will be discussed in more detail later in this chapter.

At the ultra-structural level in freeze-fracture replicas, tight junctions have been shown to appear as an intricate network of fibrils encircling the apical end of the lateral membrane in cells expressing tight junctions. These fibrils have previously been identified as transmembrane proteins.[15,16]

Breakdown of the iBRB is a hallmark of many degenerative retinal diseases, including diabetic retinopathy, sickle-cell disease, and cystoid macular edema.[17-21]

TIGHT JUNCTION PROTEINS

Occludin

Occludin is an integral membrane protein located at the tight junction. It has been described as a phosphoprotein which can exist in many phosphorylated states, and normally migrates on SDS-PAGE between the molecular weights 60–100 kDa.[22]

Occludin has four transmembrane domains with both the N- and C-terminal located cytoplasmically. It contains two extracellular loops, which are involved in interactions between adjacent cells, regulating permeability and certain selectivity functions. The first loop contains approximately 60% Tyrosine and Glycine residues, which may possibly play an important role in cell-cell coupling through homotypic interactions with other extracellular occludin loops.[23]

The second loop has been implicated in the formation of the paracellular barrier characteristic of the tight junction. The C-terminal end of occludin interacts with ZO-1 and ZO-2 and the last 150 amino acids of the C-terminal interact with F-actin.[24-27]

It has been shown previously that overexpression of chicken occludin in Madin-Darby Canine Kidney cells (MDCK) increases the trans-epithelial/endothelial electrical resistance (TER), which correlates with decreases in paracellular permeability. However, the COOH-terminal truncated occludin in MDCK cells increases paracellular flux of small tracer molecules from the apical to basolateral domain. This increase in paracellular flux upon transfection of the COOH-terminal cytosolic domain of occludin suggests that this domain may be involved in regulating paracellular permeability.[28]

It has been suggested that occludin associates with ZO-1 at the tight junction upon recruitment by JAM. This conclusion was drawn after co-transfection of Chinese Hamster Ovary (CHO) cells with both JAM and occludin, increased the localization of occludin to the tight junction.[5]

With regard to occludin's association with the tight junction protein ZO-1, it has been reported that this interaction may play a fundamental role in modulating the function of occludin at the tight junction. It was reported that at low to normal levels of occludin, an optimum amount of ZO-1 would be present to interact with it, thus mediating an increase in TER and a decrease in paracellular flux. However, upon overexpression of occludin, the binding sites on ZO-1 may become saturated; leading to a surplus of occludin that may mediate the formation of pores as opposed to sealing the paracellular space.[29] This study only further highlighted the dynamics of this transmembrane protein's function at the tight junction, and its involvement in the maintenance of tight junction integrity.

Phosphorylation of Occludin as a Regulatory Mechanism

In Retinal Microvascular Endothelial cells (RMECs), occludin exists in multiple phosphorylation states.[30-32] In RMECs, occludin has been shown to migrate at molecular weights of 60 and 62 kDa on SDS-PAGE, with higher molecular weight bands frequently appearing at between 75 and 100 kDa. Higher molecular weight forms of occludin have previously been proposed to be richly phosphorylated on serine residues while also exhibiting phosphorylation on threonine residues.[10]

These post-translational modifications to occludin may have a significant bearing on the overall function of the tight junction protein and the overall integrity of the tight junction itself. When occludin was immunoprecipitated and treated with a phosphatase, the higher molecular weight bands disappeared. Also, the addition of a peptide constituting an extracellular domain of occludin decreased the TER of MDCK cells along with a decrease in the higher molecular weight forms of occludin.[33] This strongly suggests that hyper-phosphorylated occludin is essential in maintaining tight junction integrity.

The phosphorylation of occludin on tyrosine residues can increase paracellular permeability in endothelial and epithelial cells, further highlighting the importance of specific residue phosphorylation status in determining occludin localization at the tight junction of cells.[34] Interestingly, at its C-terminal tail, the tyrosine phosphorylation status of occludin is essential in mediating its binding properties to ZO-1, ZO-2 and ZO-3. It has also been shown that the binding properties of ZO-1,-2 and -3 to the C-terminal tail of occludin were significantly decreased compared with non-phosphorylated occludin.[35] Tyrosine phosphorylation of occludin has also been directly postulated as a mediator in disrupting the association of occludin and ZO-1, thus leading to an increase in paracellular permeability.[36] Shear stress has also been shown to decrease occludin content while increasing phosphorylation, allowing for increases in Bovine Aortic Endothelial cell (BAEC) permeability.

It is clear however that the post-translational phosphorylation status of occludin is of fundamental importance in mediating its cellular localization and the regulation of paracellular permeability, with threonine and serine phosphorylation occurring in tandem with maintenance of tight junction integrity, while tyrosine phosphorylation appears to occur concomitant with increases in paracellular permeability.

It is not sufficient however to say that occludin alone can determine tight junction integrity, as it has been shown that differentiated embryonic bodies isolated from embryonic stem cells in which the occludin gene was knocked out, still developed a normal network of tight junction fibrils between adjacent epithelial cells.[37] It is however possible that in cells with an established tight junction, occludin will mediate important and highly significant regulations of the junction via interactions with integral membrane proteins and adjacent tight junctions associated with neighboring cells.

Zonula Occludens-1 (ZO-1)

ZO-1 is a member of the Membrane Associated Guanylate Kinase (MAGUK) family of proteins. It is a tight junction phosphoprotein with a molecular weight of approximately 220 kDa, and it was among the first tight junction associated proteins to be identified.[38]

ZO-1 contains several PDZ domains. These domains are regions of sequence homology found in a diverse range of signaling proteins. The name "PDZ" derives from the first three proteins in which these domains were identified: PSD-95, a protein involved in signaling at the post-synaptic density; DLG, the Drosophila Discs Large protein; and ZO-1, the zonula occludens-1 protein. PDZ domains are also sometimes called DH domains or GLGF repeats.[39]

By recruiting downstream proteins in a signaling pathway, PDZ domains mediate assembly of specific multi-protein complexes, including those complexes necessary for tight junction formation. Proteins that contain PDZ domains play important roles in many key signaling pathways, including the maintenance of epithelial cell polarity and morphology, organizing the postsynaptic density in neuronal cells, and regulating the activity and trafficking of membrane proteins.

As well as the PDZ domains, ZO-1 also contains a Src homology (SH3) domain which mediates intracellular protein-protein interactions through the recognition of proline-rich sequence motifs on cellular proteins. A guanylate kinase (GK) domain adjacent to the SH3 domain facilitates interaction of ZO-1 with occludin.[40]

ZO-1 also contains a long carboxyl terminal region with an acidic module, a proline rich domain and several alternative-splicing sites, (Fig. 1). Due to this alternative splicing, ZO-1 can be found in two different isoforms ZO-1α^+ and ZO-1α^-. The two isoforms differ by an internal 80 amino acid domain.[41]

ZO-1 is a peripheral membrane protein enriched at tight junctions, however, cells with decreased cell-cell contact reportedly display a strong presence of ZO-1 staining in the nuclei, and therefore ZO-1 may mediate certain signaling mechanisms un-related to the tight junction.[42]

ZO-1 is found to associate initially with adherens junction components prior to final localization at the tight junction, and it has been shown to bind to the adherens junction protein α-Catenin and the gap junction associated protein connexin-43.[43-46] Only in certain conditions will it localize to the nucleus.[38] It has been proposed that in the nucleus, ZO-1 interacts via its SH3 domain with a novel transcription factor, namely, ZO-1 associated nucleic acid-binding protein (ZONAB), and regulates gene expression of components involved in epithelial cell proliferation and cell density,including ErbB-2.[47]

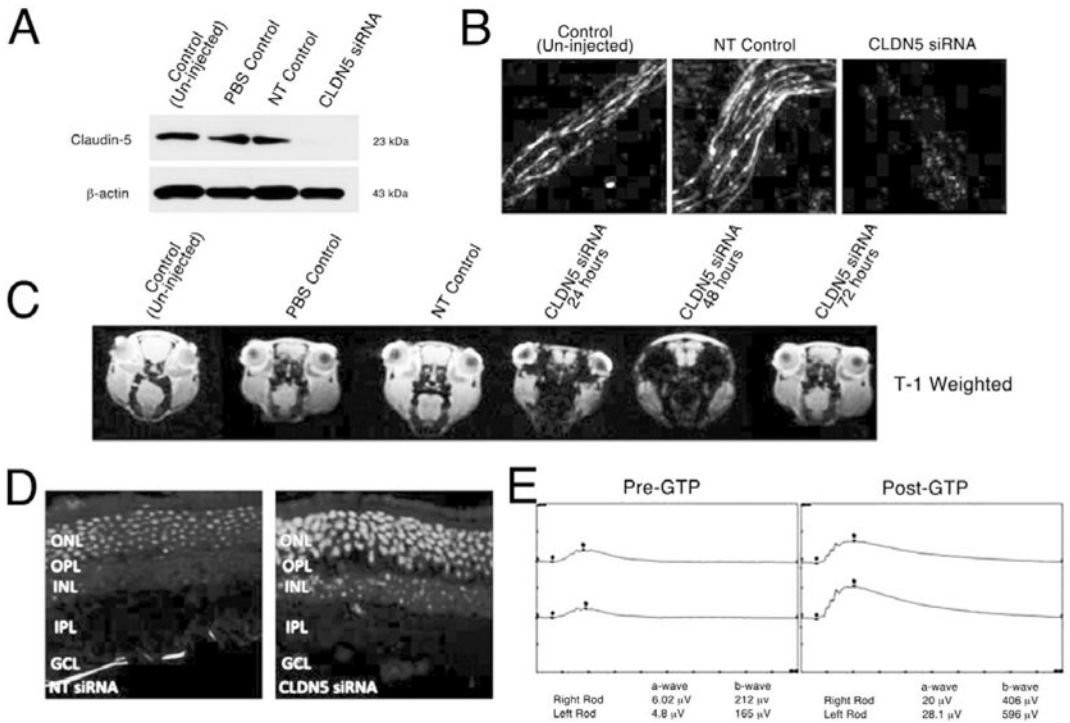

Figure 1. A) Systemic injection of siRNA targeting claudin-5 caused a transient decrease in expression of this tight junction protein in the brain and retina 48 h post injection. B) This suppression was manifested by a change in localization of claudin-5 from the microvascular endothelial cell tight junctions. C) Following injection of the contrast agent Gd-DTPA (742Da) however, intense contrasting was observed both 24 and 48 h post injection of claudin-5 siRNA when observed with T-1 weighted imaging. This was manifested as intense dark contrasting in the ocular region of the mouse. D) Immunohistochemical analysis of claudin-5 levels (red staining) in retinal cryosections, it was clear that levels were decreased in all retinal layers. Sections were counter-stained with DAPI (blue staining). E) Rod isolated ERG tracings in IMPDH1−/− mice pre- and post- injection of GTP with NT siRNA showed no distinct changes in waveform, however, upon analysis of ERG tracings pre- and post- GTP injection with claudin-5 siRNA, well formed a- and b- waves were observed in the retinas of mice. Reproduced from Campbell M et al. Proc Natl Acad Sci USA 2009; 106:17817-22;[73] A color version of this figure is available online at http://www.landesbioscience.com/curie.

The overall molecular structure of ZO-1 facilitates a capacity for multiple protein-protein interactions. At the tight junction, ZO-1 binds to the actin cytoskeleton through its carboxyl terminal end, and forms a bridge between the C-terminal sequences of occludin and β-actin. ZO-1 interacts with ZO-2 and ZO-3 through its second PDZ domain, while as previously discussed, associating with adherens junction proteins α-Catenin and gap junction proteins Connexins -43 and -45 at this domain.[48]

In a certain percentage of breast cancers, the putative expression of ZO-1 has been shown to be significantly decreased.[49] Therefore, as well as playing an important role in the regulation of the tight junction, ZO-1 and its respective family members may play a distinct and important role in the regulation of cell growth and differentiation.

ZO-1 Localization in the Mammalian Retina

Unlike the tight junction protein occludin, ZO-1 has been shown to localize to numerous regions and cell types of the mammalian retina, again highlighting its importance in mediating more cellular functions than solely barrier properties. ZO-1 has been shown to localize to retinal blood vessels and the RPE of the mammalian retina, however, it is also present at the Outer Limiting Membrane (OLM) mediating important interactions between the junctions of Müller cells and photoreceptor cells. ZO-1 is expressed in the blood vessels of the choroid and the core stroma of the ciliary process, however, as these vessels are highly permeable to solutes, it has been proposed that ZO-1 does not feature predominantly as a barrier related protein, but rather mediates important cell-cell interactions in these vessels.[50-52] ZO-1 is expressed at the borders of the anterior and the posterior epithelial cell layers in the iris and also in the epithelial layer of the ciliary body.[53] ZO-1 appears to be a dynamic protein whose functions are more diverse and complex than previously thought, and its wide distribution in the mammalian retina may be fundamental in the overall health and integrity of the eye.

Zonula Occludens-2 and -3 (ZO-2 and -3)

ZO-2 is a 160 kDa phosphoprotein located peripherally on the cytoplasmic side of the tight junction. It was originally identified through co-immunoprecipitation experiments with ZO-1. ZO-2 has 51% homology to ZO-1 most of which occurs in the MAGUK domain. ZO-1 and ZO-2 associate with each other through their second PDZ domains.[54] ZO-2 can interact with claudins through its first PDZ domain,[45] with occludin through its GK region and also with cingulin and catenin.[38,55]

The proline rich domain at the C-Terminal of ZO-2 binds to actin. It has also been proposed that ZO-2 interacts with transcription factors such as Fos, Jun and C/EBP (CCAAT/enhancer-binding protein),[56] and it has previously been shown to localize to the nucleus of epithelial cells.[57] This may imply that ZO-2 has a role to play in cell signaling and tight junction formation.

Our knowledge to date of the expression and localization of the tight junction protein ZO-3 is still at a very early stage, due mainly to a lack of antibodies that can specifically distinguish ZO-3 from the proteins ZO-1 and ZO-2. ZO-3 is a 130 kDa phosphoprotein that was initially identified as a protein that immunoprecipitated with ZO-and it has been shown to interact with both ZO-1 and occludin, but not ZO-2.[58] Interestingly, ZO-3 expression has not been detected at the OLM of the mammalian retina and appears to localize predominantly at the RPE.[59]

Both ZO-1 and ZO-2 have previously been reported to localize to the nucleus in dividing cells, which suggests a role for these proteins in distinct and specific signals necessary for tight junction formation.[60] As it is proposed that ZO-1 may interact with a transcription factor called Zonula-Occludens Associated Nucleic Acid-Binding protein (ZONAB) to regulate specific gene expression including that of cell division kinase (CDK) 4.

Claudins

Like occludin, the claudin family of proteins are transmembrane proteins that allow for the maintenance of tight junction integrity. Claudins, like occludin, span the membrane four times and interact with ZO-1 via their C-terminal domain, which all contain a tyrosine and a valine as the last two amino acids, allowing for interaction with ZO-1.[61] It has been shown that co-expression of occludin and claudin-1 in fibroblast cells causes co-localization of both proteins at the periphery of the cells in tight junction-like strands.[62] This suggests that, like occludin, claudins may play a role in regulating barrier function and the overall development of the tight junction.

Claudin-1 overexpression in Madin-Darby Canine Kidney (MDCK) cells has previously been shown to increase Trans-Epithelial Electrical Resistance (TER) and reduce Flourescien Isothiocyanate (FITC)-dextran flux across the monolayer, again suggesting it plays a role in regulating barrier function.[63] In 2003, claudin-5 knockout mice were first reported.[64] Although these mice died within hours of birth for reasons that have yet to be elucidated, they formed intact tight junctions at the blood-brain barrier (BBB). Following a series of tracer molecule experiments however, it was observed that the mice had a size-selective compromise to the BBB, where molecules below approximately 800 Da were able to freely diffuse form the blood to the brain while larger molecules were restricted to the blood. These findings offered clues to the function of claudin-5 at the neuronal barrier and it was proposed that claudin-5 played a fundamental role in the formation of paracellular pores at the tight junctions. Not only was this an important finding relating to the molecular biology of the neurovascular unit, but it also raised the possibility that levels of claudin-5 could be modulated to allow for the diffusion of low molecular weight drugs from the blood to the brain or retina.

A NOVEL THERAPEUTIC STRATEGY FOR RETINAL DISEASES

Globally, an estimated 161 million people are visually handicapped.[65] The most common causes of visual handicap are cataract and glaucoma, however in the developed world, age-related macular degeneration (AMD) is the most prevalent cause of registered blindness in the older population, closely followed by diabetic retinopathy. In the US, 1.75 million people are estimated to have advanced AMD defined as geographic atrophy or neovascularisation in at least one eye, with 7.3 million people at risk of developing the disease owing to the presence of drusen.[66] With regard to Diabetic retinopathy, in the US, for example, 4.1 million people over the age of 40 have the condition with almost 1 million people having sight-threatening disease.[67] Given that, in the overall, only limited therapies are available for these diseases, their negative social and economic impact is immense. The cost of AMD, involving diagnosis, monitoring, visual aids, habitation, accident treatment, rehabilitation, treatment of associated depression and anxiety, as well as direct treatment of the disease itself has been estimated to amount to approximately

€200,000 per patient in any five year period.[67] Costs associated with the management of diabetic retinopathy are also high.

In regard to therapeutic intervention in these major causes of visual handicap, there is currently no means of prevention of the dry form of AMD, apart from dietary supplementation and attempting to reduce the impact of environmental risk factors such as cigarette smoking. In regard to some wet cases of the disease, monoclonal antibodies targeting VEGF, Lucentis® and Avastin®, are now being used with increasing frequency, although the latter is licensed only for anti-tumor use and is therefore currently being used 'off label' for AMD.[68] These antibodies are introduced into the vitreous of the eye by direct intraocular injection at intervals of approximately one month to six weeks. While improvement in vision has been reported in numerous trials, each intraocular injection can result in the development of severe endophthalmitis, retinal hemorrhage, retinal detachment and cataract in up to 0.1% of patients. Other estimates in relation to the general intra-ocular injection of material into the eye put this figure at closer to 2%. The proliferative retinopathy associated with diabetes is treatable by scatter laser surgery, in which up to 2,000 burns are placed in areas of the retina away from the macula.[69] In addition, in cases of macular edema, focal laser surgery may be used to introduce burns around the macula. While treatments are generally useful, they do to an obvious extent, compromise retinal function and do not cure the disease and in addition, neovascular vessel leakage can occur post laser surgery in a significant proportion of cases.

Highly relevant to the advancement of preventive therapies for AMD and diabetic retinopathy, however, is the fact that an estimated 98% of drugs with established anti-neovascular, neuroprotective, anti-inflammatory or anti-apoptotic potential, do not easily cross the BBB or iBRB, rendering systemic delivery of such compounds either impractical or highly inefficient.[70] The endothelial cells of the microvasculature supplying the brain and inner retina contain tight junctions, composed of a variety of interacting proteins which form a tight seal, constituting the BBB and iBRB respectively.[71] Permanent and un-controlled opening of these barriers to large molecules such as anaphylatoxins, antibodies, other soluble proteins or pathogens would be disastrous for neuronal viability. However, controlled and transient modulation of these barriers for short periods of time to allow passive diffusion of very low molecular weight compounds could have substantial therapeutic potential, avoiding the necessity for regular invasive delivery to ocular tissues.

RNAi MEDIATED BARRIER MODULATION

In 2008, Campbell et al[72] reported for the first time on the use of barrier modulation technology to suppress levels of the tight junction protein claudin-5 at the BBB and iBRB. In this study, they employed the hydrodynamic delivery technique to introduce siRNA's to the microvasculature associated with the brain and retina. It was observed that 48 h post injection of the siRNA, the BBB and iBRB became transiently permeable to low molecular weight molecules similar to the phenotype observed on the claudin-5 knockout mouse. Molecules lower than approximately 1 kDa were able to freely passively diffuse across the BBB and iBRB, while molecules with molecular weights greater than this were restricted to the blood. This initial study was followed by a report in 2009 that barrier modulation could be used to enhance delivery of drugs with potential to prevent oxidative stress induced damage to the murine retina. Following modulation of the iBRB by suppression of claudin-5, Campbell et al,[73] demonstrated that the calpain inhibitor

ALLM could prevent light induced retinal degeneration in albino mice. Moreover, they also showed that mice lacking the enzyme IMPDH1, the rate limiting enzyme in the d novo synthesis of GTP, could be supplemented with systemically administered GTP and gain periods of functional retinal electrophysiology (Fig. 1). These studies provided the platform for the experimental use of barrier modulation as a means to enhance systemic drug delivery to neuronal tissues, however for chronic conditions such as those observed on retinal disease, repeated systemic injection of siRNA would not be a feasible approach and therefore alternative strategies were needed.

ADENO-ASSOCIATED VIRUS (AAV)-MEDIATED BARRIER MODULATION

In developing barrier modulation technology for general drug delivery in patients, it was necessary to develop a process for inducible, periodic and reversible modulation either of the BBB or the iBRB exclusively (i.e., modulating one, or other, but not both). In this regard, barrier-modulating machinery was incorporated into an AAV2/9 vector such that when sub-retinally introduced into the retina the virus accessed the vasculature, and reversible barrier modulation can periodically be induced by systemic treatment with a well tolerated inducing agent, doxycycline. It is of note that AAV infection of retinal tissues is long lasting or may even be permanent following a single sub-retinal inoculation and hence repeated ocular injections of this inducible barrier-modulating agent would not be required. Moreover, it is of note that AAV delivery systems have now been approved for use in a growing number of gene therapy trials, including those recently initiated for the congenital retinopathy, Leber congenital amaurosis (LCA).[74-76]

The objective was to develop a minimally invasive system, requiring only a once-off sub-retinal injection for periodic reversible modulation of the iBRB in mice, which could subsequently be used in human subjects. While AAV systems have been used extensively for the delivery of cDNAs and shRNAs to retinal tissues in mice a limitation of this form of delivery was that until recently, no viral serotype was available to allow efficient infection of retinal vascular endothelia. However, Foust et al[77] reported that AAV-2/9 preferentially targets neonatal neurons and adult astrocytes, and in so doing, traverses the blood-brain barrier. While no data were presented on transduction of ocular tissues, the similarities between brain and retinal vascular endothelia strongly suggested that this particular AAV serotype could also transfect the latter. This opened up the possibility of introducing an inducible barrier opening system into the genome of an AAV virus such that it could be delivered specifically to the retina. The iBRB could then be periodically and selectively opened by systemic administration of the inducing agent, doxycycline, which is required to traverse only a single lipid bilayer, that of the endothelial cells of the inner retinal vessels, in order to activate claudin-5 targeted shRNA (Fig. 2).

In 2011, it was reported for the first time that this AAV could allow for the enhanced systemic delivery of VEGF receptor antagonists systemically to prevent the development of laser induced choroidal neovascularisation (CNV), the hallmark pathology associated with wet AMD. The commercially available low molecular weight drugs Sunitinib malate (Sutent) and 17-AAG were used in his study and they were both shown to be highly efficacious in treating CNV development. Establishment of enhanced delivery of anti-neovascular compounds following reversible modulation of the iBRB provides a direct proof of principle for further development of an AAV-mediated system for

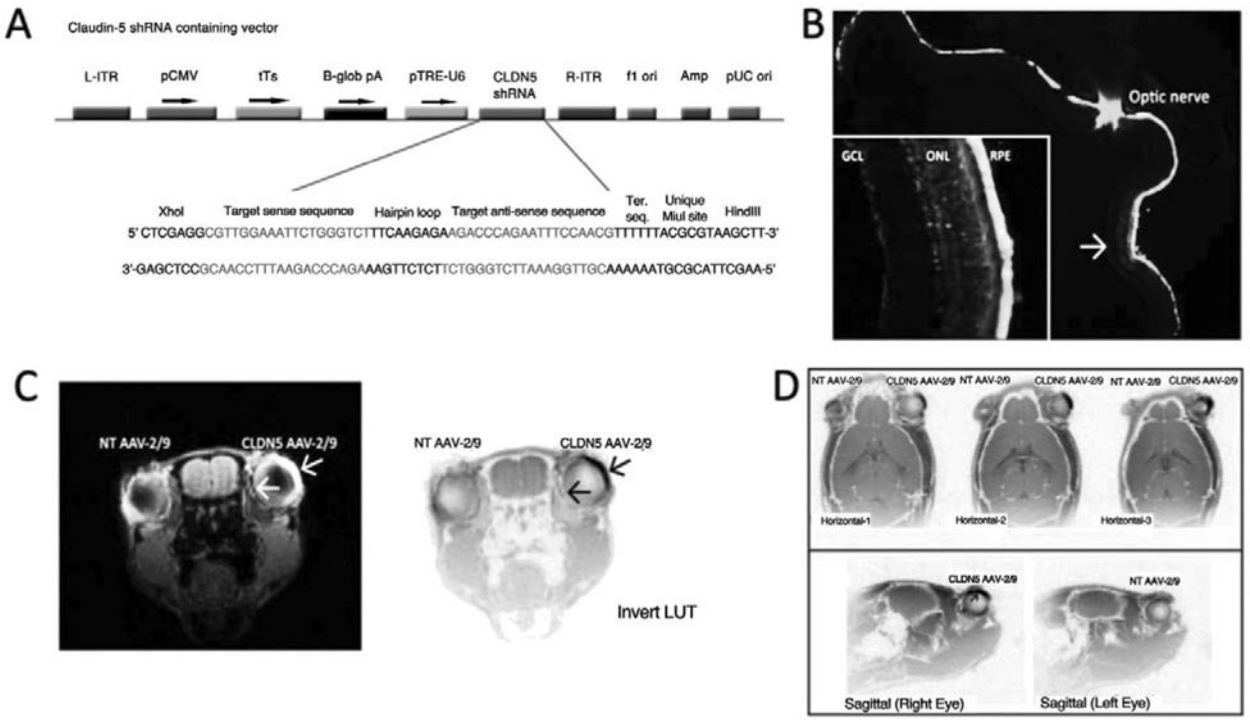

Figure 2. A) The plasmid incorporating the inducible system with claudin-5 shRNA or a non-targeting (NT) luciferase shRNA was cloned into the plasmid pAAV-MCS, such as to incorporate left and right AAV inverted terminal repeats. (L-ITR and R-ITR). AAV2/9 was then generated using a triple transfection system in a stably transfected HEK-293 cell line for the generation of high-titer viruses. B) An adeno-associated virus (AAV-2/9) with an eGFP reporter gene was injected sub-retinally in an adult C57/Bl6 mouse and 3 weeks post-injection, a retinal whole-mount showed the pattern of transduction to be widespread (green). C,D) Contrast enhanced magnetic resonance imaging (MRI) showed extravasation of the MRI contrasting agent Gd-DTPA (MW 742 Da) in mice injected in the right eye with CLDN5 AAV-2/9, but not in the left eye, injected with NT AAV-2/9 when animals were supplemented with the inducing agent doxycycline (2 mg/ml with 5% sucrose in their drinking water) for 3 weeks post-injection. Reproduced from Campbell M et al. EMBO Mol Med 2011; 3:235-45;[78] ©2011 with permission of John Wiley and Sons. A color version of this figure is available online at http://www.landesbioscience.com/curie.

inducible barrier opening, extending the use of this novel drug delivery system to an extremely important condition. Inducible suppression of claudin-5 in humans may pave the way for the controlled delivery of low molecular weight therapeutics currently deemed useless as they do not cross the iBRB.[78] In this regard, barrier modulation technology may have significant implications for the enhancement of drug delivery in general for a wide range of neurodegenerative conditions. In a clinical setting, a patient would require a "once-off" inoculation of the inducible AAV either by sub-retinal or intra-vitreal injection. Following this procedure, the iBRB could be induced to modulate by the administration of doxycycline via an oral tablet. Claudin-5 levels would then be sufficiently reduced in the retina 2–3 d post administration of doxycycline, at which time the active component could be taken by systemic or oral routes. This dosing regimen could be used in chronic conditions such as AMD or Retinitis pigmentosa and would negate the need for regular, risky and in many patients, stressful intra-ocular injection of monoclonal antibodies.

CONCLUSION

Barrier modulating technologies are now being tested for a range of neuronal conditions, not just limited to retina diseases. However a range of safety/toxicology studies are on-going so that AAV-mediated barrier modulation can be used in future human clinical trials associated with enhanced systemic delivery of therapeutics to the retina for AMD and Retinitis pigmentosa treatments. These non-invasive methods for chronic drug delivery should pave the way for a safer, more patent friendly option for treating the most common debilitating forms of blindness.

REFERENCES

1. Forrester JV, Dick AD, McMenamin PG, eds. The Eye. Philadelphia: Elsevier Health Sciences, 2001.
2. Antonetti DA, Lieth E, Barber AJ, Gardner TW. Molecular mechanisms of vascular permeability in diabetic retinopathy. Semin Ophthalmol 1999; 14:240-8.
3. Marmorstein AD, Finnemann SC, Bonilha VL, Rodriguez-Boulan E. Morphogenesis of the retinal pigment epithelium: toward understanding retinal degenerative diseases. Ann N Y Acad Sci 1998; 857:1-12.
4. Marneros AG, Fan J, Yokoyama Y et al. Vascular endothelial growth factor expression in the retinal pigment epithelium is essential for choriocapillaris development and visual function. Am J Pathol 2005; 167:1451-9.
5. Bazzoni G, Martinez-Estrada OM, Orsenigo F et al. Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. J Biol Chem 2000; 275:20520-6.
6. Bazzoni G, Martinez-Estrada OM, Mueller F et al. Homophilic interaction of junctional adhesion molecule. J Biol Chem 2000; 275:30970-6.
7. Bazzoni G. The JAM family of junctional adhesion molecules. Curr Opin Cell Biol 2003; 15:525-30.
8. Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiol Rev 2004; 84:869-901.
9. Farquhar MG, Palade GE. Junctional complexes in various epithelia. J Cell Biol 1963; 17:375-412.
10. Sakakibara A, Furuse M, Saitou M et al. Possible involvement of phosphorylation of occludin in tight junction formation. J Cell Biol 1997; 137:1393-401.
11. Fanning AS, Anderson JM. PDZ domains and the formation of protein networks at the plasma membrane. Curr Top Microbiol Immunol 1998; 228:209-33.
12. Riesen FK, Rothen-Rutishauser B, Wunderli-Allenspach H. A ZO1-GFP fusion protein to study the dynamics of tight junctions in living cells. Histochem Cell Biol 2002; 117:307-15.
13. Zahraoui A, Louvard D, Galli T. Tight junction, a platform for trafficking and signaling protein complexes. J Cell Biol 2000; 151:F31-6.

14. Zahraoui A. [Tight junctions, a platform regulating cell proliferation and polarity]. Med Sci (Paris) 2004; 20:580-5.
15. Balda MS, Matter K. The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. EMBO J 2000; 19:2024-33.
16. Balda MS, Garrett MD, Matter K. The ZO-1-associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density. J Cell Biol 2003; 160:423-32.
17. Vinores SA, Campochiaro PA, Lee A et al. Localization of blood-retinal barrier breakdown in human pathologic specimens by immunohistochemical staining for albumin. Lab Invest 1990; 62:742-50.
18. Vinores SA, Kuchle M, Derevjanik NL et al. Blood-retinal barrier breakdown in retinitis pigmentosa: light and electron microscopic immunolocalization. Histol Histopathol 1995; 10:913-23.
19. Dorchy H. Characterization of early stages of diabetic retinopathy. Importance of the breakdown of the blood-retinal barrier. Diabetes Care 1993; 16:1212-4.
20. Dorchy H, Toussaint D. Fluorescein leakage: First sign of juvenile diabetic retinopathy. Lancet 1978; 1:1200.
21. Carney MD, Paylor RR, Cunha-Vaz JG et al. Iatrogenic choroidal neovascularization in sickle cell retinopathy. Ophthalmology 1986; 93:1163-8.
22. Mitic LL, Schneeberger EE, Fanning AS, Anderson JM. Connexin-occludin chimeras containing the ZO-binding domain of occludin localize at MDCK tight junctions and NRK cell contacts. J Cell Biol 1999; 146:683-93.
23. Furuse M, Hirase T, Itoh M et al. Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 1993; 123:1777-88.
24. Furuse M, Itoh M, Hirase T et al. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. J Cell Biol 1994; 127:1617-26.
25. González-Mariscal L, Betanzos A, Avila-Flores A. MAGUK proteins: structure and role in the tight junction. Semin Cell Dev Biol 2000; 11:315-24.
26. Gonzalez-Mariscal L, Namorado MC, Martin D et al. Tight junction proteins ZO-1, ZO-2, and occludin along isolated renal tubules. Kidney Int 2000; 57:2386-402.
27. González-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. Prog Biophys Mol Biol 2003; 81:1-44.
28. Feldman GJ, Mullin JM, Ryan MP. Occludin: structure, function and regulation. Adv Drug Deliv Rev 2005; 57:883.
29. McCarthy KM, Skare IB, Stankewich MC et al. Occludin is a functional component of the tight junction. J Cell Sci 1996; 109:2287-98.
30. Brankin B, Campbell M, Canning P et al. Endostatin modulates VEGF-mediated barrier dysfunction in the retinal microvascular endothelium. Exp Eye Res 2005; 81:22-31.
31. Campbell M, Collery R, McEvoy A et al. Involvement of MAPKs in endostatin-mediated regulation of blood-retinal barrier function. Curr Eye Res 2006; 31:1033-45.
32. Antonetti DA, Barber AJ, Hollinger LA et al. Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zonula occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumors. J Biol Chem 1999; 274:23463-7.
33. Wong V. Phosphorylation of occludin correlates with occludin localization and function at the tight junction. Am J Physiol 1997; 273:C1859-67.
34. Staddon JM, Smales C, Schulze C et al. p120, a p120-related protein (p100), and the cadherin/catenin complex. J Cell Biol 1995; 130:369-81.
35. Kale G, Naren AP, Sheth P, Rao RK. Tyrosine phosphorylation of occludin attenuates its interactions with ZO-1, ZO-2, and ZO-3. Biochem Biophys Res Commun 2003; 302:324-9.
36. Rao RK, Baker RD, Baker SS et al. Oxidant-induced disruption of intestinal epithelial barrier function: role of protein tyrosine phosphorylation. Am J Physiol 1997; 273:G812-23.
37. Saitou M, Fujimoto K, Doi Y et al. Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J Cell Biol 1998; 141:397-408.
38. Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 1986; 103:755-66.
39. Woods DF, Bryant PJ. ZO-1, DlgA and PSD-95/SAP90: homologous proteins in tight, septate and synaptic cell junctions. Mech Dev 1993; 44:85-9.
40. Weng Z, Rickles RJ, Feng S et al. Structure-function analysis of SH3 domains: SH3 binding specificity altered by single amino acid substitutions. Mol Cell Biol 1995; 15:5627-34.
41. Balda MS, Anderson JM. Two classes of tight junctions are revealed by ZO-1 isoforms. Am J Physiol 1993; 264:C918-24.
42. Gottardi CJ, Arpin M, Fanning AS, Louvard D. The junction-associated protein, zonula occludens-1, localizes to the nucleus before the maturation and during the remodeling of cell-cell contacts. Proc Natl Acad Sci USA 1996; 93:10779-84.

43. Itoh M, Nagafuchi A, Moroi S, Tsukita S. Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. J Cell Biol 1997; 138:181-92.
44. Itoh M, Nagafuchi A, Yonemura S et al. The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. J Cell Biol 1993; 121:491-502.
45. Itoh M, Morita K, Tsukita S. Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and alpha catenin. J Biol Chem 1999; 274:5981-6.
46. Giepmans BN, Moolenaar WH. The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. Curr Biol 1998; 8:931-4.
47. Balda MS, Garrett MD, Matter K. The ZO-1-associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density. J Cell Biol 2003; 160:423-32.
48. Kausalya PJ, Reichert M, Hunziker W. Connexin45 directly binds to ZO-1 and localizes to the tight junction region in epithelial MDCK cells. FEBS Lett 2001; 505:92-6.
49. Hoover KB, Liao SY, Bryant PJ. Loss of the tight junction MAGUK ZO-1 in breast cancer: relationship to glandular differentiation and loss of heterozygosity. Am J Pathol 1998; 153:1767-73.
50. Tserentsoodol N, Shin BC, Suzuki T, Takata K. Colocalization of tight junction proteins, occludin and ZO-1, and glucose transporter GLUT1 in cells of the blood-ocular barrier in the mouse eye. Histochem Cell Biol 1998; 110:543-51.
51. Campbell M, Humphries M, Kennan A et al. Aberrant retinal tight junction and adherens junction protein expression in an animal model of autosomal recessive Retinitis pigmentosa: the Rho(-/-) mouse. Exp Eye Res 2006; 83:484-92.
52. Campbell M, Humphries M, Kenna P et al. Altered expression and interaction of adherens junction proteins in the developing OLM of the Rho(-/-) mouse. Exp Eye Res 2007; 85:714-20.
53. Freddo TF. Intercellular junctions of the iris epithelia in Macaca mulatta. Invest Ophthalmol Vis Sci 1984; 25:1094-104.
54. Wittchen ES, Haskins J, Stevenson BR. Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. J Biol Chem 1999; 274:35179-85.
55. D'Atri F, Nadalutti F, Citi S. Evidence for a functional interaction between cingulin and ZO-1 in cultured cells. J Biol Chem 2002; 277:27757-64.
56. Betanzos A, Huerta M, Lopez-Bayghen E et al. The tight junction protein ZO-2 associates with Jun, Fos and C/EBP transcription factors in epithelial cells. Exp Cell Res 2004; 292:51-66.
57. Jaramillo BE, Ponce A, Moreno J et al. Characterization of the tight junction protein ZO-2 localized at the nucleus of epithelial cells. Exp Cell Res 2004; 297:247-58.
58. Haskins J, Gu L, Wittchen ES et al. ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. J Cell Biol 1998; 141:199-208.
59. Inoko A, Itoh M, Tamura A et al. Expression and distribution of ZO-3, a tight junction MAGUK protein, in mouse tissues. Genes Cells 2003; 8:837-45.
60. Islas S, Vega J, Ponce L, Gonzalez-Mariscal L. Nuclear localization of the tight junction protein ZO-2 in epithelial cells. Exp Cell Res 2002; 274:138-48.
61. Furuse M, Fujita K, Hiiragi T et al. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 1998; 141:1539-50.
62. Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. J Cell Biol 1998; 143:391-401.
63. Inai T, Kobayashi J, Shibata Y. Claudin-1 contributes to the epithelial barrier function in MDCK cells. Eur J Cell Biol 1999; 78:849-55.
64. Nitta T, Hata M, Gotoh S et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 2003; 161:653-60.
65. Masland RH. The fundamental plan of the retina. Nat Neurosci 2001; 4:877-86.
66. Swaroop A, Chew EY, Rickman CB et al. Unraveling a multifactorial late-onset disease: from genetic susceptibility to diseasemechanisms for age-related macular degeneration. Annu Rev Genomics Hum Genet 2009, 10:19.43.
67. Resnikoff S, Pascolini D, Etya'ale D et al. Global data on visual impairment in the year 2002. Bull World Health Organ 2004; 82:844-51.
68. CATT Research Group. Martin DF, Maguire MG, Ying GS et al. Ranibizumab and bevacizumab for neovascular age-related macular degeneration. N Engl J Med 2011; 364:1897-908.
69. Fong DS, Girach A, Boney A. Visual side effects of successful scatter laser photocoagulation surgery for proliferative diabetic retinopathy: a literature review (2007) Retina. Sep;27(7):816-24.
70. Pardridge WM. Molecular Trojan horses for blood-brain barrier drug delivery. Curr Opin Pharmacol 2006; 6:494-500.
71. Piontek J, Winkler L, Wolburg H et al. Formation of tight junction: determinants of homophilic interaction between classic claudins. FASEB J 2008; 22:146-58.

72. Campbell M, Kiang AS, Kenna PF et al. RNAi-mediated reversible opening of the blood-brain barrier. 2008. J Gene Med. Aug;10(8):930-47.
73. Campbell M, Nguyen ATH, Kiang AS et al. An experimental platform for systemic drug delivery to the retina. Proc Natl Acad Sci USA 2009; 106:17817-22.
74. Maguire AM, Simonelli F, Pierce EA et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. N Engl J Med 2008; 358:2240-8.
75. Bainbridge JW, Smith AJ, Barker SS et al. Effect of gene therapy on visual function in Leber's congenital amaurosis. N Engl J Med 2008; 358:2231-9.
76. Hauswirth WW, Aleman TS, Kaushal S et al. Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. Hum Gene Ther 2008; 19:979-90.
77. Foust KD, Nurre E, Montgomery CL et al. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. Nat Biotechnol 2009; 27:59-65.
78. Campbell M, Humphries MM, Nguyen ATH et al. Systemic low molecular weight drug delivery to pre-selected neuronal regions. EMBO Mol Med 2011; 3:235-45.