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Introduction

The blood–retinal barrier (BRB), which is analogous to the blood–brain barrier, maintains homeostasis in the retina by restricting the entry of blood-borne proteins from the retina and by maintaining strict ionic and metabolic gradients. When this barrier breaks down, excess fluid accumulates in the retina and this can result in macular edema, which is associated with ischemic retinopathies, including diabetic retinopathy (DR) and retinopathy of prematurity (ROP), ocular inflammatory diseases, retinal degenerative diseases, and a variety of other ocular disorders, or following ocular surgery. BRB breakdown can occur at the inner BRB, which is established at the retinal vasculature, at the outer BRB, which consists of the retinal pigment epithelial (RPE) cells, or at both sites. The BRB is established by the formation of tight junctions between the retinal vascular endothelial (RVE) cells and the RPE cells and a paucity of endocytic vesicles within these cells. The establishment and maintenance of the BRB is regulated by the perivascular astrocytes and pericytes, but the mechanism for this regulation is not entirely clear. Some studies have shown that cell to cell contact is necessary to establish and maintain the BRB, while others provide evidence that a soluble mediator is sufficient. BRB breakdown can result from a disruption of the tight junctions, which are composed of a complex network of junctional proteins, an upregulation of vesicular transport across the RVE or RPE, or by degenerative changes to the barrier-forming cells or to the regulatory cells, the pericytes and glia. In some cases, BRB breakdown is related to identifiable structural defects, such as loss of pericytes, astrocytes, or RPE cells or changes to the vascular endothelial cells, as would be caused by microaneurysm formation. In other cases, where retinal vascular leakage is diffuse, such as in uveitis, or when the leakage is remote from a lesion, such as a surgical wound or tumor, it is clear that diffusible factors are involved. Blood–tissue barriers exist only in the retina, brain, and nerve. Vascular endothelial cells in the choroid and in other tissues are fenestrated (Figure 1(a)), allowing large molecular weight molecules to freely pass from the blood to the tissue, and thus do not have a barrier function.

Tight Junctions

Tight junctions or zonula occludens consist of complex arrangements of over 40 proteins in the peripheral cytoplasm and apical plasma membrane that connect RVE or RPE cells and restrict flow between them (Figure 1(b)). Occludin and the claudins (over 24 isoforms), which form the junctional strands and are believed to constitute the backbone of the tight junction, span the plasma membrane and bind junctional proteins in adjacent cells. Zonula occludens proteins 1, 2, and 3 (ZO-1, -2, and -3) are intracellular proteins that associate with the cytoplasmic surface of the tight junctions and organize the complex. The binding of ZO-1 to occludin establishes the tight junction. Other integral components of the junctional complex are the junctional adhesion molecules, tricellulin, cingulin, 7H6, and symplekin. A breach of the tight junctions (Figure 1(c)) can result from an alteration in the content of the junctional proteins, their redistribution, or their
phosphorylation. Occludin content at the tight junction is higher in cells that have a tighter barrier and decreased occludin correlates with increased BRB permeability, but occludin knockout mice appear to form functional tight junctions, so the association is complex and not simply regulated by occludin. Increased occludin phosphorylation is also associated with increased BRB permeability. Altered expression of claudins can lead to changes in selectivity of the junctions and claudin-5 appears to be particularly important for maintenance of a functional tight junction.

Adenosine, prostaglandin E1 (PGE1), interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), and vascular endothelial growth factor (VEGF) appear to be capable of causing a morphological and functional opening of the RVE tight junctions. A significant number of interendothelial cell tight junctions appeared open along their entire length within 6 h of intravitreal injection of each agent into rabbits with TNFα showing the greatest effect (35.6% of the interendothelial cell junctions appeared open, morphologically). The effect of PGE1 on tight junctions appeared to be transient, that of VEGF and IL-1β were partially reversible by 24 h, and the effect of the adenosine agonist, N-ethylcarboxamidoadenosine was not reversible by 48 h. The demonstration of immunoreactive albumin, which would normally be confined to the lumens of vessels with a blood–tissue barrier, along the entire length of these junctions, from the luminal to the abluminal surface, suggests that they are also functionally open (Figure 2(c)).

**Vesicular Transport**

Since the tight junctions restrict the flow of molecules across the BRB, a series of pumps, channels, and transporter molecules are necessary to transport specific essential molecules from the blood to the retina. The nonspecific transport of high molecular weight molecules and fluids across the RVE by way of pinocytotic vesicles (Figure 2(a)) or caveolae is referred to as vesicular transport (Figure 2(b)) and serves as a transcellular means of BRB breakdown. This mechanism appears to be the predominant means for BRB compromise associated with VEGF-A-induced hyperpermeability in monkeys and in DR in humans, rats, and rabbits.

In addition to causing the opening of interendothelial cell tight junctions in the retina, adenosine, PGE1, IL-1β, TNFα, and VEGF also promote the formation of pinocytic vesicles in RVE cells and the distribution of albumin-containing intraendothelial vesicles across the entire RVE cell and at both the luminal and abluminal surfaces suggests that active vesicular transport is occurring. Although infrequently seen, the vesiculo-vacuolar organelle, which is associated with VEGF in the vascular endothelium of tumors, was also evident in the RVE of VEGF-treated rabbits, but not monkeys, and is likely to play a role in VEGF-mediated vascular permeability. The effect of these mediators on the outer BRB is less clear.

**Role of Inflammation**

Inflammation has been associated with BRB breakdown in DR, choroidal neovascularization (CNV) associated with age-related macular degeneration, aging, ocular
inflammatory disease, and the administration of pro-inflammatory molecules. The increased adhesion of leukocytes to endothelial cells in the retina is associated with increased expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), CD18, and other adhesion molecules, which are upregulated by VEGF and other pro-inflammatory molecules in DR and other ocular disorders, and appear to be regulated, at least in part, by protein kinase C (PKC). Diabetic CD18 and ICAM-1 knockout mice have significantly fewer adherent leukocytes than diabetic mice with normal CD18 and ICAM-1 and the decreased leukostasis is associated with fewer damaged endothelial cells and reduced BRB breakdown, supporting the role of adhesion molecules in increased inflammation and the correlation of an inflammatory response with endothelial cell damage and permeability. It is not clear whether the same molecules that facilitate leukostasis also mediate BRB breakdown or if this is attributable to molecules secreted by the recruited leukocytes, or both, but there appears to be a direct correlation between increased leukostasis and vascular permeability in the retina and pro-inflammatory molecules, such as TNFα and IL-1β, are among the most potent inducers of BRB breakdown. Leukocyte adhesion to the diabetic vascular endothelium can promote endothelial apoptosis and inhibition of leukocyte adhesion to the retinal vessels can not only prevent endothelial degeneration, but also reduce the diabetes-associated loss of pericytes, which support the vascular endothelium and help to confer BRB integrity. Inflammation can also alter the distribution of astrocytes and their ensheathment of retinal vessels, leading to alterations in BRB integrity. Leukocytes have also been shown to cause a downregulation and redistribution of tight junctional proteins, which leads to a disruption of tight junctions and a transient breakdown of the BRB during retinal inflammation.

**Molecular Mechanisms**

The induction of BRB breakdown is a complex process that is mediated, not by a single factor, but by the interaction of multiple factors operating through different receptors and signaling pathways. The list of molecules that have been identified as playing a role in BRB breakdown, which is by no means all-inclusive, includes VEGF, hypoxia-inducible factors-1 and -2 (HIF-1 and -2), placental growth factor (PIGF), TNFα, IL-1β, platelet-activating factor, adenosine, histamine, prostaglandins (PGE1, PGE2, and PGF2α), platelet-derived growth factors A and B (PDGF-A and -B), insulin-like growth factor-1 (IGF-1), ICAM-1, VCAM-1, P-selectin, and E-selectin. The key will be to determine what the initiating event is and which events are parts of the
resulting cascade. By targeting the appropriate molecules, subsequent events leading to BRB failure may be blocked.

The various isoforms of VEGF and PGF are members of the VEGF family. VEGF-A, a potent inducer of vascular permeability, binds to both VEGF type-1 (fms-like tyrosine kinase-1 or Flt-1) and type-2 (kinase insert domain-containing receptor, referred to as KDR in humans or Flk-1 in other species) receptors (VEGFR1 and VEGFR2), whereas PGF binds only to VEGFR1, so comparing their activities may be a means of dissecting the respective roles of the VEGF receptor isoforms, since both receptors have been implicated in BRB breakdown. VEGFR1-mediated signaling appears to operate primarily through p38 MAPK (mitogen-activated protein kinase), while VEGFR2 signaling may be mediated through RAS, phosphoinositide 3-kinases (PI3K)/Akt, or phospholipase C (PLC)γ1, but the interaction of VEGFR1 and VEGFR2 is complex and much remains to be learned about this interaction. Both receptors are associated with vascular permeability and angiogenesis, but in some circumstances, VEGFR1 can act as a negative regulator for VEGFR2.

VEGF is a key molecule in promoting increased retinal vascular permeability. This activity may be mediated, at least in part, by an upregulation of ICAM-1, E-selectin, and P-selectin as a means of facilitating its pro-inflammatory activity. VEGF-induced permeability showed a biphase pattern with a rapid and transient phase followed by a delayed and sustained phase, the latter of which was blocked by antibodies to urokinase plasminogen activator or its receptor. VEGF receptor kinase inhibitors can suppress VEGF-mediated BRB breakdown, but this strategy shows that TNFα, IL-1β, and IGF-1 do not induce BRB leakage through an induction of VEGF, indicating that these mediators operate through distinct pathways that may also be targeted. Endothelial nitric oxide synthase activation and NO formation also appear to be implicated in VEGF-mediated vascular permeability, probably through activation of the serine/threonine protein kinase AKT/PKB, which can lead to an increase in nitric oxide production and ICAM-1 upregulation. Deletion of the hypoxia response element of the Vegf promoter also suppresses BRB breakdown in oxygen-induced retinopathy, demonstrating that HIF-induced VEGF is critical in this process.

TNFα and IL-1β are upregulated in DR and other ischemic retinopathies, as well as ocular inflammatory disease, and both molecules are associated with increased leukostasis and BRB breakdown. IL-1β has been shown to accelerate apoptosis of retinal capillary endothelial cells through activation of nuclear factor kappa light-chain enhancer of activated B cells and this is exacerbated in high glucose. IL-1β can also stimulate the production of reactive oxygen species, which in turn can induce the release of additional cytokines. Aspirin and etanercept are inhibitors of TNFα and each can reduce ICAM-1 levels, diabetes-related leukostasis, and BRB breakdown in diabetic rats without altering VEGF levels, showing that TNFα is involved in this process and that it operates through a distinct pathway from VEGF.

These data show that there are a number of potential target molecules for inhibitors to suppress BRB breakdown. The challenge will be to identify the best target or targets and develop the most effective therapeutic strategy.

Assessing BRB Breakdown

A variety of methods exist for the quantitative and qualitative assessment of the BRB, but each method has its particular limitations and sensitivity, so the choice of methods will largely depend on whether quantitative or qualitative data are desired and on the nature of the tissue being evaluated, whether it be fixed tissue, patients in a clinical setting, or experimental animal models. Since no single method can provide a quantitative assessment with precise localization of the site of BRB breakdown, multiple approaches may be necessary to provide an overall perspective. In addition, some methods can produce precise data in experimental models or on tissue specimens, but are not appropriate for use in the clinic.

To identify and compare factors that cause BRB breakdown and to evaluate the efficacy of new treatments designed to prevent or reduce macular edema, a reliable quantitative assay for assessing BRB function is essential. The most widely used protocols for the quantitative assessment of BRB breakdown utilize Evans blue or 3H-mannitol as tracers. With the Evans blue assay, the extracted dye is quantified in the retina following intravenous injection of the dye and subsequent perfusion with saline. A spectrophotometer, set at 620 nm, is used to quantify the leakage of dye into the retina. With the 3H-mannitol assay, a scintillation counter is used to determine the CPM/mg tissue, 1 h after an intraperitoneal injection of the tracer, and the data are expressed as a ratio of retina/lung or retina/kidney. Since the lung and kidney do not have a blood–tissue barrier, the ratio corrects for any variation in the amount of isotope injected or absorbed. These methods have been used to assess the BRB in several models of ocular disease and to determine the effect of various factors, agents, and genetic manipulations on the integrity of the BRB. Thus, these methods have been useful in identifying factors that initiate BRB compromise and for determining the relative efficacy of various agents at preventing or reducing BRB failure.

Both methods produce highly reproducible results in an experimental setting, with the 3H-mannitol assay possibly being somewhat more sensitive due to the lower molecular weight of mannitol than Evans blue dye, but neither is applicable to the clinic. Vitreous fluorophotometry (VFP) is a more appropriate means of assessing BRB failure in
a clinical setting. Although these methods can provide a quantitative assessment of the BRB, they cannot localize the site of leakage or provide any insight into the mechanism, so alternative techniques are required to provide this information.

Fluorescein angiography has been used extensively in the clinic to visualize BRB breakdown, but it does not allow resolution at the cellular level. Magnetic resonance imaging (MRI) enhanced by the paramagnetic contrast agent gandoliniumdiethylene-triaminetetraacetic acid has been used to localize and quantify BRB breakdown in living animals. MRI is not subject to the optical limitations of VFP and allows the investigator to distinguish between inner and outer BRB failure in the rabbit. Its resolution is not as great as that resulting from microscopic evaluation of fixed tissue, but MRI allows in vivo analysis, thus enabling the investigator to monitor progressive changes in BRB integrity within the same animal. The use of exogenous tracer substances can provide a higher resolution, but several limitations are associated with their use. The use of tracers is impractical for clinical studies, the introduction of exogenous material may alter BRB integrity, and retrospective studies cannot be done on archival tissues. The immunolocalization of endogenous albumin (Figure 3) or IgG can circumvent most of these limitations and offers the following advantages for BRB assessment. The technique can be used with fixed surgical, autopsy, or archival specimens, no exogenous substance is introduced, and it can be used at the light and electron microscopic (EM) levels. Although, by nature, this is not a quantitative method, it can show the location and extent of BRB breakdown and, if used at the EM level, it can demonstrate the means by which serum proteins are extravasated from the retinal vessels or may transverse the RPE layer. This technique has been used to assess the BRB in a variety of human and experimental ocular disorders, including DR, retinitis pigmentosa, vascular occlusive disease, neoplastic disease, ocular inflammation or infection, and other diseases that develop macular edema, but for which pathological defects do not reveal a cause for BRB breakdown. EM immunocytochemical staining for albumin reveals that BRB breakdown can occur by the opening of tight junctions between RVE or RPE cells (Figure 2(c)), by an upregulation of trans-endothelial vesicular transport (Figures 2(a) and 2(b)), or by increased surface membrane permeability of RVE or RPE cells resulting from degenerative changes associated with the disease process. It has also provided insights into how various factors, such as VEGF, TNFα, IL-1β, prostaglandins, adenosine, and others promote BRB breakdown. VEGF transiently opens some tight junctions and some leakage through the interendothelial cell tight junctions is induced by VEGF, but electron microscopy has revealed that the predominant mechanism for VEGF-A-induced hyperpermeability of the

Figure 3  (a) In a normal mouse, immunohistochemical staining for albumin shows that, within the retina, albumin is confined to the vessels, indicating an intact BRB, but diffuse staining is demonstrated in the choroid (bottom) due to the fenestrated vessels and the absence of a blood–tissue barrier. (b) In a mouse infected with coronavirus, vascular leakage is demonstrated from a retinal vessel by immunohistochemical staining for albumin. (c) Immunohistochemical staining (red) shows that albumin has leaked from retinal vessels in a VEGF transgenic mouse. Vinores, S. A., et al. (2001). Blood–retinal barrier breakdown in experimental coronavirus retinopathy: Association with viral antigen, inflammation, and VEGF in sensitive and resistant strains. *Journal of Neuroimmunology* 119: 175–182, with permission from Elsevier.

RVE in monkeys and diabetes-related BRB breakdown in humans, rabbits, and rats is an upregulation of pinocytic vesicular transport.
Inhibiting BRB Breakdown

A variety of therapeutic approaches have shown success at inhibiting BRB breakdown, but generally not preventing it. Most of the agents currently in clinical trials target inflammatory processes or VEGF. Antibodies to key molecules, such as VEGF, PlGF, or TNFα, have been effective at suppressing BRB breakdown, as have inhibitors of these molecules. Drugs that block histamine receptors also reduce retinal vascular leakage in diabetic rats and humans. Bevacizumab (avastin), an anti-VEGF IgG1 antibody, Ranibizumab (lucentis), the Fab fragment of a humanized anti-VEGF antibody, Pegaptanib sodium (macugen), a VEGF aptamer, and VEGF trap, in which the binding domains of VEGFR1 and VEGFR2 are combined with the Fc portion of IgG to neutralize all VEGF family members, have all shown varying degrees of success in clinical trials for reducing macular edema by targeting VEGF. Corticosteroids inhibit BRB breakdown, but it is not clear whether this activity is mediated by their anti-inflammatory effect, which occurs, at least in part, through a downregulation of ICAM-1, their inhibition of VEGF expression, their induction of occludin and ZO-1 expression, their reversal of occludin phosphorylation, or a combination of these activities. Even though steroids may improve visual acuity, they carry a high risk of cataracts and glaucoma. The involvement of PKC in vascular permeability has been established and a PKC activator can promote BRB breakdown. PKC inhibitors can reduce retinal vascular permeability, particularly that mediated by VEGF or prostaglandins, but generalized inhibition of PKC is likely to have serious systemic consequences. A PKCβ inhibitor (LY333531) was also effective at suppressing retinal vascular permeability and may have fewer complications.

Prospects for the Future

As more studies are conducted, the complexity of BRB breakdown leading to macular edema becomes increasingly apparent. This process is not attributable to a single factor or event, but the interaction of an undetermined number of initiating events that generates a cascade of subsequent events, ultimately leading to BRB failure. Since this is a multifactorial process, a multifaceted or pleiotropic approach that restores the homeostatic balance is more likely to suppress BRB breakdown than targeting a single pathway with an inhibitor. That would explain why the currently used monotherapies may lead to a reduction of macular edema and improved visual acuity, but generally not a total resolution of the disorder. To develop more effective therapeutic strategies, a better understanding of the basic mechanisms in the pathogenesis of BRB breakdown is imperative. In addition, the frequent injections, high cost, and the occasional side effects associated with current therapeutic approaches emphasize the need for more effective treatment that is less invasive, less costly, and has little or no side effects.

Conclusions

BRB breakdown, leading to macular edema, occurs in a number of ocular disorders and can be due to structural changes or soluble mediators. Alteration in the content, distribution, or phosphorylation of junctional proteins can result in vascular leakage through the tight junctions. BRB breakdown can also result from an upregulation of trans-endothelial vesicular transport, which has been shown to be a major contributor to BRB failure caused by several mediators and in ocular disease models. Many of the same mediators can simultaneously promote opening of the tight junctions and upregulation of vesicular transport. Degenerative or structural changes to the RPE or RVE cells or to the pericytes and perivascular astrocytes that regulate the inner BRB can also lead to BRB breakdown. Inflammation promotes BRB breakdown, so the use of anti-inflammatory agents may be beneficial. BRB breakdown is not due to a single factor, but is a complex process involving multiple factors, receptors, and signaling pathways. Information on the molecular mechanisms is being revealed, but much remains to be learned. The complexity of the pathogenesis of BRB breakdown makes it likely that the greatest chance for success in preventing macular edema would be in targeting multiple molecules or pathways and a sensitive method for assessing the integrity of the BRB is necessary to monitor the efficacy of different therapeutic strategies.

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See also: Blood–Retinal Barrier; Breakdown of the Retinal Pigmented Epithelium Blood–Retinal Barrier; Macular Edema; Retinal Pigmented Epithelium Barrier; Retinal Vasculopathies: Diabetic Retinopathy; Retinopathy of Prematurity; Secondary Photoreceptor Degenerations: Age-Related Macular Degeneration.

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