

## Diabetes and Retinal Vascular Dysfunction

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### Abstract

Diabetes predominantly affects the microvascular circulation of the retina resulting in a range of structural changes unique to this tissue. These changes ultimately lead to altered permeability, hyperproliferation of endothelial cells and edema, and abnormal vascularization of the retina with resulting loss of vision. Enhanced production of inflammatory mediators and oxidative stress are primary insults with significant contribution to the pathogenesis of diabetic retinopathy (DR). We have determined the identity of the retinal vascular cells affected by hyperglycemia, and have delineated the cell autonomous impact of high glucose on function of these cells. We discuss some of the high glucose specific changes in retinal vascular cells and their contribution to retinal vascular dysfunction. This knowledge provides novel insight into the molecular and cellular defects contributing to the development and progression of diabetic retinopathy, and will aid in the development of innovative, as well as target specific therapeutic approaches for prevention and treatment of DR.

**Keywords:** Diabetes; Retinal vasculature; Inflammation; Oxidative Stress; Thrombospondins

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### INTRODUCTION

The retina is a specialized tissue for vision, which converts visible light into the neuronal signals perceived by the brain. The retina's unique vascular system provides nutrients and oxygen to the inner and outer retina, whose integrity is essential for sensing light.<sup>[1]</sup> Thus, the retinal vascular structure acquires blood-retinal barrier (BRB) characteristics, which are crucial for the integrity of retinal vascular structure and the regulation of the retinal microenvironment. In the retina, BRB consists of inner and outer components. Inner BRB consists of tight junctions between endothelial cells, and outer BRB is formed by tight junctions between retinal pigment epithelial cells.<sup>[2]</sup> In inner BRB, endothelial cells are surrounded by pericytes and the foot processes of astrocytes.<sup>[3]</sup> Astro- and peri-cytes are crucial for maintaining the structure of inner BRB.<sup>[2]</sup> Dysfunction of vascular cells in inner BRB during disease states causes breakdown of BRB leading to serious impairment of

vision. In diabetic retinopathy (DR), the breakdown of BRB results in vascular leakage and subsequent macular edema, a major cause of vision impairment.<sup>[4]</sup> To better understand how the breakdown of BRB progresses, the cellular mechanisms responsible for the dysfunction of retinal vascular cells including endothelial cells, peri- and astro-cytes under pathological conditions need to be elucidated. The effects of high glucose conditions caused by diabetes on the function of retinal vascular cells, and the mechanisms for dysfunction of retinal vascular cells under high glucose conditions have been the subject of numerous studies. However, the detailed mechanisms involved, and the identity of the primary cellular target of diabetes have remained unknown. These gaps in our understanding of these mechanisms have hampered the development of effective therapies for DR. as one of the serious complications of diabetes and a leading cause of blindness among working-age people.<sup>[5]</sup> Delineating the identity of cellular and molecular mechanisms resulting

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in dysfunction of retinal vascular cells, and identification of protective mechanisms will provide the rationale for developing more specific and effective treatments.

## DIABETIC RETINOPATHY

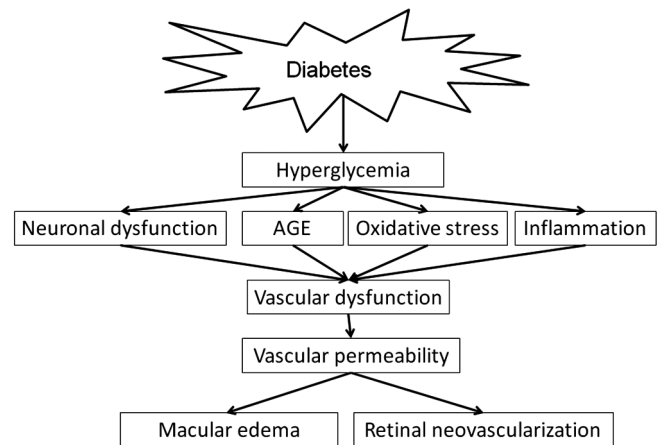
### Epidemiology of Diabetic Retinopathy

Diabetes mellitus is a serious worldwide health problem. In 2012, 371 million people were affected by diabetes and 4.7 million people died due to diabetes, including approximately 25.8 million patients in the United States.<sup>[6]</sup> Prevalence of diabetes increases the risk of serious diabetes complications. DR is one of the complications of diabetes and the main cause of blindness among working-age people. In the US, among patients with type 2 diabetes, estimated 40.3% of patients have DR and 8.2% of subjects have vision-threatening retinopathy.<sup>[7]</sup> For patients of type 1 diabetes, 86% and 42% of patients have retinopathy and vision-threatening stage of DR, respectively.<sup>[8]</sup> Studies based on 22,896 individuals with diabetes showed that 34.6% of patients have DR and the increasing risk was correlated with the duration of diabetes and inappropriate control of blood glucose and blood pressure. Vision-threatening stages of DR are proliferative DR and diabetic macular edema. Prevalence for proliferative DR was 6.96% and 6.81% for diabetic macular edema.<sup>[9]</sup> Impairment in vision due to DR remains a serious health issue worldwide. Tight blood glucose control and early detection and treatment of DR are effective to prevent vision loss and blindness caused by DR.<sup>[10]</sup>

### Biochemical Changes in Diabetic Retinopathy

The pathogenesis of DR is very complex, because many factors contribute to the pathophysiology of DR. Sustained hyperglycemia in retinal vasculature leads to accumulation of advanced glycation end-products (AGEs), inflammation, neuronal dysfunction and oxidative stress.<sup>[11]</sup> These biochemical changes under hyperglycemia are implicated in microvascular dysfunction leading to increased vascular permeability and capillary rarefaction. Consequently, these changes in retinal vasculature result in macular edema and neovascularization in the retina [Figure 1].<sup>[12]</sup>

Excess glucose in the blood can have nonenzymatic chemical reactions with amino groups of proteins, lipids, and nucleic acids to produce AGEs.<sup>[13]</sup> This modification by AGEs causes vascular dysfunction in the diabetic retina. Accumulation of AGEs induces retinal pericyte apoptosis. In the early phase of apoptosis, caspase-10 mediates apoptosis of pericytes.<sup>[14]</sup> Angiotensin II also induces apoptosis of pericytes by AGEs through elevating Receptor for AGEs (RAGE).<sup>[15]</sup> AGEs also increase vascular permeability in retinal endothelial cells. AGEs enhance translocation of protein kinase C- $\delta$  (PKC- $\delta$ )



**Figure 1.** Pathophysiology of diabetic retinopathy. Hyperglycemia instigates biochemical changes leading to vascular dysfunction which increases vascular permeability followed by macular edema and retinal neovascularization.

contributing to loss of tight junctions.<sup>[16]</sup> Thickening of basement membrane (BM) in capillaries is also observed in the diabetic retina. AGEs are also involved in the modification of BM. Accumulation of AGEs is associated with the expansion of BM and inhibition of the accumulation of AGE prevents BM thickening.<sup>[17]</sup> Furthermore, modified BM by AGEs prevents survival of retinal pericytes by regulating signaling pathway mediated by platelet-derived growth factor.<sup>[18]</sup> Thus, AGEs affect survival of pericytes by regulating signaling pathways and BM integrity of retinal blood vessels.

Reactive oxygen species (ROS) is generated from mitochondrial electron transport chain, cytochrome p450s, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase nitric oxide synthases (NOSs). ROS produced by cells is required to maintain normal cellular functions. However, excess production of ROS result in pathological conditions and oxidative stress.<sup>[19]</sup> Diabetes causes oxidative stress, and augmented level of oxidative stress contributes to the pathogenesis of diabetic vascular complications, including DR.<sup>[20]</sup> In the diabetic retina, ROS levels are elevated and related with vascular dysfunction, including loss of pericytes, formation of acellular capillaries, and vascular leakage and thickening of BM.<sup>[21,22]</sup> Oxidative stress in the diabetic retina has causal links with metabolic dysfunction such as vascular inflammation, RAGE activation, activation of PKCs, and activation of nuclear factor kappaB (NF- $\kappa$ B).<sup>[23-26]</sup> Photoreceptor cells have been recently shown to be an early source of oxidative stress and local inflammation in the diabetic retina. Diabetes increased generation of ROS by photoreceptor cells followed by induction of pro-inflammatory mediators, including inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule 1 (ICAM-1).<sup>[27]</sup>

Inflammation is one of the major factors induced during diabetes leading to macular edema, ischemia

and neovascularization.<sup>[10]</sup> Physiological changes in the diabetic retina are associated with inflammation, and anti-inflammatory therapy ameliorates development of DR in various animal models.<sup>[28]</sup> Several pro-inflammatory mediators are involved in the pathogenesis of DR. iNOS is an enzyme that catalyzes the reaction for nitric oxide (NO) generation. iNOS is up-regulated in the diabetic retina and plays a crucial role in the pathogenesis of vascular lesion during the early stage of DR.<sup>[29,30]</sup> Inhibition of iNOS by aminoguanidine inhibited the progression of retinal dysfunction caused by DR in the rodent model of diabetes.<sup>[31]</sup>

Eicosanoids, mediators of inflammation which are also affected by diabetic conditions have two major families; prostaglandins and leukotriens. Cyclooxygenase-2 (cox-2) is an enzyme that catalyzes the synthesis of prostaglandins. Cox-2 expression is up-regulated in the diabetic retina and treatment with cox-2 inhibitor prevents damage in the diabetic retina.<sup>[32,33]</sup> Production of leukotrienes by lipoxygenases (LOs), especially 5LO, by leukocytes promotes the retinal capillary degeneration in diabetic mice<sup>[34]</sup> and contributes to increased levels of pro-inflammatory cytokines. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) are increased in the retinas of diabetic animals.<sup>[35,36]</sup> Vascular endothelial growth factor (VEGF) also has a pro-inflammatory role and is elevated in the diabetic retina. The increase in VEGF level promotes neovascularization and damage in the retina,<sup>[37]</sup> and its inhibition ameliorates vascular leakage in the diabetic retina.<sup>[38]</sup>

Antiinflammatory molecules are also affected by diabetic conditions and further contribute to the severity of DR. Pigment epithelium-derived factor (PEDF) is a member of the super family of serine protease inhibitors. It has neuroprotective, antiangiogenic, and antiinflammatory features.<sup>[39]</sup> PEDF levels are attenuated in the diabetic retina and associated with up-regulation of VEGF levels.<sup>[40]</sup> PEDF and VEGF act reciprocally in regulating retinal inflammation under diabetic conditions. Although a role for PEDF in modulation of Wnt signaling has been demonstrated, the detailed mechanisms involved need further elucidation.<sup>[41]</sup>

Thrombospondin-1 (TSP-1) was one of the first endogenous inhibitors of angiogenesis identified, which is expressed at significantly high levels in vitreous and aqueous humor of various species, including humans.<sup>[42]</sup> We also showed that diabetes resulted in a significant down-regulation of TSP-1 in vitreous and aqueous humor samples prepared from rats which were made diabetic by streptozotocin (STZ). We later showed this is the case in vitreous samples prepared from humans with diabetes.<sup>[43]</sup> However, the vitreous level of PEDF was not significantly affected in these patients. What was interesting is that we observed two isoforms of PEDF in vitreous samples prepared from

humans with or without diabetes. The presence of higher molecular weight isoform was always associated with reduced levels of TSP-1, especially in diabetic patients. Furthermore, we recently showed that lack of TSP-1 and/or its selective down-regulation in the endothelium exacerbates the development and pathogenesis of DR.<sup>[44]</sup> These results, along with other published works from our laboratory and others regarding the antiangiogenic and antiinflammatory activities of TSP-1, support an important role for TSP-1 in the pathogenesis of DR.

The neuronal dysfunction in the retina is also an important effect of diabetes. Neuronal functional abnormalities of diabetic retina may precede the onset of vascular structural change in the retina.<sup>[45]</sup> Diabetes affects cells involved in neurosensory function of the retina through altered metabolisms and enhanced apoptosis.<sup>[5]</sup> Apoptosis in the neural retina was detected earlier than apoptosis in retinal vascular cells, and the rate of apoptosis in the neural retina was not affected by the duration of diabetes.<sup>[46]</sup> Neural apoptosis is associated with the breakdown of BRB and it is mediated by inflammation and oxidative stress.<sup>[47]</sup> Furthermore, extracellular glutamate is another factor that induces the apoptosis of neuronal cells. In the retina of rat, glutamate level was elevated after 2 months of diabetes.<sup>[48]</sup> At the early stages of DR, the ability of the Müller cells to remove glutamate from the extracellular space is attenuated, and glutamate homeostasis is altered. The increased level of glutamate in diabetic retina has toxic effects on retinal neurons by inducing apoptosis.<sup>[49]</sup> Oxidative stress caused by diabetic conditions also increases glutamate level in the diabetic retina.<sup>[48]</sup> Reducing glutamate levels in the diabetic retina prevents neuronal cell death and degeneration. Neuronal cell death is closely related with retinal vascular cell death. The vascular defects appear in the same region as neuronal dysfunction in DR.<sup>[50]</sup> These results imply that there are intimate interactions between neuronal cells and vascular cells in the retina in order to maintain the integrity of BRB and proper tissue perfusion. These interactions need further elucidation, and retinal glial cells may be a key player here.

Besides nonenzymatic protein modifications producing AGEs, diabetic conditions affect posttranslational modifications of proteins which may play a pivotal role in the pathogenesis of DR. Uridine diphosphate (UDP)-N-acetylglucosamine (GlcNAc) is an end product of the hexosamine biosynthetic pathway. This UDP-GlcNAc is a donor of O-linked GlcNAc (O-GlcNAc) for protein modification.<sup>[51]</sup> High glucose conditions elevate the levels of O-GlcNAcylated proteins in retinal vascular cells, more specifically in pericytes.<sup>[52]</sup>

Diabetic conditions also increase histone acetylation related with production of pro-inflammatory proteins including iNOS, ICAM-1 and VEGF in the retina.<sup>[53]</sup> Moreover, high glucose conditions in the retina affect glycosylation of proteins. Synaptophysin is a presynaptic

protein in synaptic vesicles and mediates release of neurotransmitters. Diabetic conditions increase mannose rich glycosylation of synaptophysin and enhances its degradation in the retina.<sup>[54]</sup> Thus, high glucose conditions modulate various posttranslational modifications of proteins leading to the pathogenesis of DR. However, the impact of diabetic conditions on posttranslational modifications of target proteins, and their identity in the retina needs further investigation.

### Vascular Changes in Diabetic Retinopathy

Alterations in vascular structure under diabetic conditions results in the breakdown of BRB and diabetic edema.<sup>[12]</sup> The vascular unit of the retina is composed of endothelial cells, peri- and astro-cytes. Diabetes affects the integrity of BRB by altering the structure of neurovascular unit of the retina. In the diabetic retina, VEGF level is increased contributing to vascular leakage.<sup>[55]</sup> VEGF activates PKC- $\beta$  which phosphorylates the tight junction protein, occludin. The phosphorylated occludin is then ubiquitinated and targeted for degradation leading to increased vascular permeability.<sup>[56]</sup>

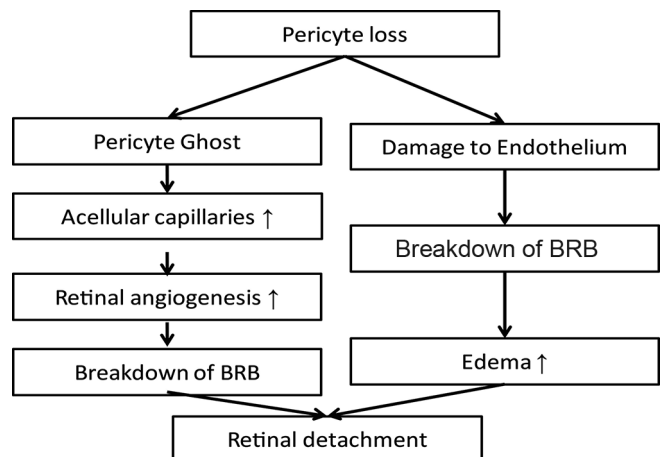
Pericytes have a crucial role in maintaining vascular stability and early depletion of pericytes is a hallmark of DR causing the formation of pericyte ghosts. The pericyte ghosts mark the space having pocket shape in BMs and is formed after pericytes.<sup>[57]</sup> While degeneration of pericytes leads to pericyte ghosts, the number of endothelial cells in retinal vessels is not affected. Pericyte loss also increases proliferation of endothelial cells contributing to microaneurysm formation in retinal vessels. The loss of pericytes in diabetic retinal vasculature can be detected by the formation of acellular capillaries. Acellular capillaries are BM tubes without cell nuclei that have at least one-fourth of the normal capillary diameter.<sup>[58]</sup> The density of pericytes inversely correlates with vascular abnormalities of the retina. Reduction of pericyte numbers causes vascular regression leading to retinopathy.<sup>[59]</sup> Loss and degeneration of pericytes affect the integrity and maintenance of BRB. Consequently, pericyte dysfunction leads to capillary dilation, microaneurysms and increased vascular permeability resulting in vascular leakage and macular edema.<sup>[12]</sup>

In the diabetic retina, nonperfused and obliterated microvessels are observed in the early stages of retinopathy. Vessel closure promotes the proliferative retinal neovascularization and can be histologically observed as the prevalence of acellular capillaries increases.<sup>[60]</sup> Adhesion of leukocyte to the retinal blood vessel induces endothelial cell death, pericyte loss and vascular closure leading to ischemia<sup>[12]</sup> which is the primary stimulus for neovascularization.<sup>[61]</sup> Leukocyte adhesion to retinal vessels is mediated by ICAM-1 and vascular cellular adhesion molecule-1.<sup>[62]</sup>

The activation of neovascularization is mediated by hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ ) which is a transcription factor and regulates the expression of various pro-angiogenic genes including VEGF, VEGF receptor-1, and angiopoietin-1 (Ang-1).<sup>[63]</sup> Aberrant activation of these pro-angiogenic factors under diabetic conditions results in pathological neovascularization. Thus, vascular changes in the diabetic retina promote the breakdown of BRB, macular edema, and proliferative neovascularization leading to retinal detachment and impairment of vision [Figure 2].

### Epigenetic Changes in Diabetic Retinopathy

Epigenetic modifications have important roles in regulation of gene expression and contribute to the pathogenesis of various diseases. The epigenetic changes associated with the pathological complications of diabetes including DR have been investigated.<sup>[64]</sup> In human umbilical vein endothelial cell, high glucose conditions elevate the level of p300, a transcriptional activator with histone acetyltransferase activity. The increased p300 protein promotes its binding to the promoter of genes whose altered expression occurs during diabetes including endothelin-1, VEGF and fibronectin. Furthermore, histone acetylation and phosphorylation of histone H2AX are up-regulated under high glucose conditions.<sup>[65]</sup> In the retina of diabetic rats, expression level and activity of histone deacetylases are augmented, while the activities of histone acetyltransferases are decreased, contributing to the attenuation of acetylated histone H3 level. These changes in histone acetylation are sustained even after termination of hyperglycemia and contribute to the "metabolic memory."<sup>[66]</sup> Moreover,



**Figure 2.** Vascular changes in diabetic retina. Pericytes control vessel stability and proliferation of endothelial cells. Pericyte loss contributes to breakdown of blood-retinal barrier (BRB) and damage to the endothelium, and formation of acellular capillaries. These vascular changes caused by pericyte loss lead to ischemia, proliferative vascularization of retina, and retinal detachment and loss of vision.

diabetic conditions are shown to regulate the expression of matrix metalloproteinase-9 (MMP-9) through epigenetic modifications. During diabetes, attenuated histone H3 dimethyl lysine (H3K9me2) and increased acetyl H3K9 (Ac-H3K9) are observed in the retina, which result in MMP-9 activation and mitochondrial damage.<sup>[67]</sup>

Diabetes also disrupts the expression pattern of microRNAs (miRNAs) contributing to the pathogenesis of DR.<sup>[68]</sup> In the retina of STZ-induced diabetic rats, expression of several miRNAs is altered. The expression of miR-200b was attenuated under high glucose conditions and was accompanied by increased VEGF levels. In addition, injection of miR-200b mimic prevents VEGF-mediated permeability and angiogenesis.<sup>[69]</sup> Furthermore, the level of miR-200b is up-regulated in retinas of Akita/+ mice, which spontaneously develop diabetes. miRNA-200b down-regulates oxidation resistance 1 gene expression leading to oxidative stress in human Müller cell.<sup>[70]</sup> When the expression profile of miRNAs was investigated in the retina of normal and diabetic rats, it was found that NF- $\kappa$ B, VEGF and p53-responsive miRNAs were up-regulated.<sup>[71]</sup> HIF-1 $\alpha$  and VEGF are regulated by common miRNAs. Silencing of HIF-1 $\alpha$  transcript decreases expression of VEGF and vice versa. Over expression of common miRNAs attenuated VEGF and HIF-1 $\alpha$  expression and inhibit vascular permeability in the retina of STZ-induced diabetic mice.<sup>[72]</sup> Thus, aberrant expression of miRNAs in diabetes has a significant impact on pathogenesis of DR and deserves further investigation.

## BLOOD-RETINAL BARRIER

### The Cellular Organization of Retinal Vasculature

To understand the function of BRB in maintaining the integrity of retinal structure, elucidation of the retinal vascular cell structure and organization is required. The retina is a tissue for perception of light, and it converts light into electrochemical signals, which are transmitted to brain through photoreceptor and retinal neuronal circuits for vision.<sup>[5]</sup> The retina is located in the posterior part of the eye and neural tissue lined between the vitreous body and the choroid for systemic circulation. The retina has four major cellular components which are affected by diseases such as diabetes. Blood vessels are the first cellular component consisting of endothelial cells and pericytes. Pericytes are smooth muscle cell-like cells enveloping capillaries. Endothelial cells line the interior surface of blood vessels for BRB. Glial cells, the second cellular component of the retina consist of Müller cells and astrocytes. Müller cells support neuronal cells and modulate function of neurons. Müller cells elongate from inner limiting membrane to the pigment epithelium. Astrocytes are associated with retinal blood

vessels by wrapping them with their processes. Glial cells are located at the interface between the neurons and retinal blood vessels, and incorporate vascular and neuronal activity of the retina. They may be the key mediators of neurovascular dysfunction associated with various pathological conditions of the central nervous system including DR. The third cellular component of the retina is the neurons. Neurons are involved in photo-transduction and convey electrochemical impulses to the brain for sensory functions. Retinal neurons consist of five primary cell types: Photoreceptors, horizontal cells, bipolar cells, amacrine cells, and ganglion cells. Microglia is the fourth cellular component of the retina whose. The microglial cells are involved in modulation of immune function in retina to maintain retinal homeostasis. They respond to stress and injury by release of cytokines, and phagocytosis and clearance of the dead cells.<sup>[5,73]</sup>

The inner BRB is formed by retinal endothelial cells associating with astrocytes and pericytes. Anatomically, endothelial cells are surrounded by basal lamina, which astrocytes and pericytes rest on. Pericytes envelop the outer side of the capillary wall and interact with endothelial cells through peg-and-socket contacts, which contain cell junction proteins. The end-foot of astrocytes encircles capillaries and basal lamina is located between the astrocytes and pericytes. Pericytes and astrocytes provide vascular integrity by interacting with endothelial cells.<sup>[74]</sup> Dysfunction in interactions among vascular cells can cause the breakdown of BRB resulting in retinal disease. An area of significant interest in our laboratory has been how hyperglycemia impacts the function of various cellular components of the retinal neurovascular system.

### Role of Endothelial Cells in Retinal Vasculature

In the inner BRB, retinal endothelial cells are the main components, which form the physical barriers between vascular lumen and the retina.<sup>[75]</sup> Transport of metabolites and nutrients between blood and the retina is selectively regulated by the physical barrier formed by endothelial cells to maintain homeostasis in the retina.<sup>[76]</sup> This physical barrier also regulates transport of ions and fluids providing an optimal condition, which is crucial for visual function. There are two routes for the selective transport in the retina: The paracellular aqueous pathway regulated by junctions between endothelial cells, and the transcellular pathway mediated by specialized vesicles including caveolae or transport proteins.<sup>[4]</sup> The junction between endothelial cells for the paracellular pathway consists of tight junctions, adherens junctions and gap junctions. These junctions at attachment sites between endothelial cells also regulate signaling for maintaining cell position, inhibit the growth by contact inhibition, and protect cells from apoptosis.<sup>[77]</sup>

Endothelial cells express cell-type specific transmembrane proteins for adhesion including vascular endothelial cadherin (VE-cadherin) at adherens junctions and claudins at tight junctions.<sup>[78,79]</sup> Transmembrane proteins at tight junctions include occludin, claudins and junctional adhesion molecules.<sup>[80]</sup> Occludin associates with zonula occludens (ZO)-1, ZO-2 and ZO-3, which directly bind to filamentous actin (F-actin).<sup>[81]</sup> Tight junctions act as a gate for paracellular transport, and a fence for maintaining cell polarity.<sup>[82]</sup> At adherens junctions, VE-cadherin is a main component associating with intracellular proteins including  $\beta$ -catenin, p120, and plakoglobin. Regulation of VE-cadherin expression and/or phosphorylation can affect the overall endothelial cell junctions and vascular stability through modulation of intracellular signaling pathways.<sup>[83]</sup>

At gap junctions, a hemi-channel on each of two neighboring endothelial cells makes contact. The hemi-channels consist of six connexins. Small molecules (<1000 dalton [Da]) pass freely through gap junctions. Gap junctions are involved in electrical or chemical communications.<sup>[4]</sup> Gap junctions are also involved in the regulation of barrier function by modulating expression and localization of tight junction proteins such as occludin, claudin-5 and ZO-1.<sup>[84]</sup> How hyperglycemia impacts the function of these proteins in endothelial cells are under intense investigation.

### Role of Pericytes in Retinal Vasculature

Pericytes play an essential role in maintaining BRB. Pericytes or mural cells envelope capillary walls and share basal lamina with endothelial cells. Pericytes have an elongated stellate shape and extend finger-like processes to cover the capillary wall formed by endothelial cells.<sup>[85]</sup> They directly interact with endothelial cells through N-cadherin and connexin-43 hemi-channels.<sup>[86,87]</sup> The ratio of pericyte-to-endothelial in the retina which is approximately 1:1, is higher than in any other organ (1:3 in the brain and 1:10 in lung).<sup>[88]</sup> This higher coverage by pericyte in retinal microvessels may imply the crucial role of pericytes in the retina in maintaining BRB structure and vascular integrity. Determining the pivotal role of pericytes in the regulation of retinal vascular functions has been the topic of numerous studies.

In pericyte-deficient mice, permeability of blood barrier was increased by up-regulation of endothelial transcytosis and abnormal polarization of astrocyte end-feet surrounding blood vessels.<sup>[75]</sup> Smooth muscle  $\alpha$ -actin (SMA) is expressed in smooth muscle cells and pericytes when activated, and related with contractile properties of these cells. In SMA null mice generated by global gene targeting, retinal structure, vascular pattern and covering of vessels by mural cells were normal. However, permeability in retinal vessels was significantly increased in SMA null mice.<sup>[89]</sup> These results suggest that pericytes regulate the maintenance of blood

barrier and structural integrity of blood vessels in the retina. Furthermore, pericytes regulate vessel stability by affecting survival of endothelial cells. VEGF produced by pericytes regulates the survival of endothelial cells.<sup>[90]</sup> Pericytes also promote the apoptosis of endothelial cells in selective vessel patterning by expressing endosialin, a type I transmembrane glycoprotein.<sup>[91]</sup> Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) signaling between endothelial cells and pericytes are also involved in the apoptosis of retinal endothelial cells. Systemic inhibition of TGF- $\beta$  signaling results in abnormalities in retinal vascular structure including impaired perfusion of the superficial vascular plexus and vascular leakage through enhanced apoptosis of endothelial cells.<sup>[92]</sup> Pericytes also have a significant role in the pathogenesis of diabetes complications including DR. In the diabetic retina, pericyte loss is one of the early hallmarks of DR and contributes to BRB disruption. Apoptosis of pericytes during diabetes leads to depletion of pericytes from retinal vasculature, formation of microaneurysms and acellular capillaries.<sup>[93]</sup> Thus, mechanisms which protect pericytes from cytotoxic effects of high glucose may be beneficial and important for development of new therapy.

### Role of Astrocytes in Retinal Vasculature

Astrocytes originate from the optic nerve head and migrate into the developing retina laying down the scaffolding for retinal vascularization.<sup>[94]</sup> Astrocytes are closely associated with the developing retinal vasculature with significant impact on retinal vessels function.<sup>[95]</sup> Pathological conditions such as diabetes and ischemia cause degeneration of astrocytes contributing to dysfunction of retinal vasculature.<sup>[96]</sup> Astrocytes are known for their interactions with blood vessels of central nervous system and help to establish and maintain barrier properties of the vasculature.<sup>[97]</sup> Retinal astrocytes help to enhance barrier function by releasing soluble proteins. Retinal astrocytes promote the integrity of blood barrier by increasing tight junction protein expression in retinal endothelial cells.<sup>[98]</sup> A-kinase anchor protein 12 (AKAP12) is derived by astrocytes. AKAP reduces VEGF level and increases TSP-1 levels leading to enhanced blood barrier through the inhibition of PKC- $\zeta$  phosphorylation and Rho kinase activity.<sup>[99]</sup>

Astrocytes also regulate the expression of glial-derived neurotrophic factor and VEGF in a reciprocal manner through stimulation of retinoic acid receptor  $\alpha$  to antagonize loss of tight junction for maintaining vascular integrity under hyperglycemic conditions.<sup>[100]</sup> In addition, astrocytes secrete sonic hedgehog to activate Hedgehog signaling pathway in endothelial cells, consequently decreasing permeability and pro-inflammatory mediators in endothelial cells.<sup>[101]</sup> Not only secretion of proteins by astrocytes, but also physical interactions between astrocytes and vascular cells in the retina is

important for the integrity of barrier structure. Astrocytes provide a scaffolding for endothelial cells organization into capillaries, and enhance the tight junction between endothelial cells.<sup>[102]</sup> Dysfunctions of retinal astrocytes in communication with endothelial cell and physical association with blood vessels contribute to BRB breakdown leading to pathological states.<sup>[74]</sup>

## PHYSIOLOGICAL CHANGES IN RETINAL VASCULAR CELLS UNDER HYPERGLYCEMIA

### Physiological Changes in Retinal Endothelial Cells

In hyperglycemic conditions, endothelial cells of retinal blood vessels are directly exposed to a microenvironment with high concentration of glucose. Therefore, effects of high glucose conditions on the physiology of retinal endothelial cells have been intensively studied. High glucose conditions cause loss of retinal endothelial cells or structural damage.<sup>[103]</sup> The cellular components affected by high glucose conditions are mitochondria. High concentration of glucose increased membrane potential and level of ROS in mitochondria of bovine retinal endothelial cells. Increase in ROS level and membrane potential was correlated with the rate of cell death.<sup>[104]</sup> In rat retinal endothelial cells, high glucose conditions increased mitochondrial fragmentation with concomitant increases in cytochrome c release leading to apoptosis.<sup>[105]</sup> Type 2A protein phosphatase (PP2A) is also involved in the apoptosis of retinal endothelial cells induced by hyperglycemic conditions which increase PP2A activity through up-regulation of methylation followed by the activation of apoptotic proteins, superoxide, and NF- $\kappa$ B.<sup>[106]</sup> However, apoptosis of retinal endothelial cells under high glucose is independent of caspase activity.<sup>[107]</sup>

High glucose conditions also affect junctional properties of retinal endothelial cells. Connexin 43 is one of the protein components of hemi-channels which allow neighboring cells to contact. Hyperglycemia reduces the expression of connexin 43 and decreases cell-cell communication triggering apoptosis.<sup>[108]</sup> Moreover, in human retinal endothelial cells, high glucose conditions disrupted tight junctions by decreasing occludin expression through the activation of VEGF and insulin-like growth factor-I receptors.<sup>[109]</sup> Hyperglycemic conditions also affect tight junctions by decreasing ZO-1 levels in the retina of diabetic mice.<sup>[110]</sup> Consequently, such conditions disturb junctional integrity leading to increased retinal endothelial cell permeability.<sup>[111]</sup> However, the majority of *in vitro* studies indicate the lack of apoptosis in microvascular endothelial cells exposed to high glucose, especially those from the retina.<sup>[112]</sup>

However, this may not be the case *in vivo*. The loss of endothelial cells *in vivo* may be the result of loss of pericytes leading to vascular dysfunction and formation of acellular capillaries.

Cell adhesive and migratory activities of retinal endothelial cells are critical for angiogenesis. High glucose conditions enhanced the migration of mouse and human retinal endothelial cells which this enhanced migration was mediated through activation of Src, PI3K/Akt1/eNOS and ERKs pathways<sup>[113,114]</sup> as well as through the up-regulation of heparanase. The activation of Akt and ERK phosphorylation are also involved in the up-regulation of heparanase.<sup>[115]</sup> Increased migration under high glucose conditions contributes to increased angiogenic activity of retinal endothelial cells and is consistent with down-regulation of TSP-1 in microvascular endothelial cells under hyperglycemia and enhanced migration of TSP-1-deficient retinal endothelial cells.<sup>[42,112,116]</sup>

### Physiological Changes in Retinal Pericytes

Depletion of pericytes is a hallmark of DR and the underlying mechanisms involved remain the subject of numerous investigations. Various mechanisms have been attributed to the progress of pericyte apoptosis under diabetic conditions. NF- $\kappa$ B is activated under high concentration of glucose leading to expression of proapoptotic proteins in retinal pericytes.<sup>[117]</sup> Under high glucose conditions, aldose reductase catalyzes synthesis of sugar polyol which accumulates resulting in the apoptosis of retinal pericytes.<sup>[118]</sup> PKC- $\delta$  and Src homology-2 domain-containing phosphatase-1 (SHP-1) are also activated by high glucose conditions leading to pericyte apoptosis in an NF- $\kappa$ B independent signaling pathway.<sup>[119]</sup> Furthermore, hyperglycemic conditions cause endoplasmic reticulum (ER) stress and oxidative stress contributing to apoptosis of pericytes.<sup>[120]</sup>

Up-regulation of oxidative stress in pericytes under hyperglycemia is regulated by thioredoxin interacting protein.<sup>[121]</sup> Apoptosis of pericytes is also mediated by pericytes-reactive antibodies. High glucose conditions render pericytes more susceptible to antibody-mediated attack leading to damage in pericytes and the damaged pericytes exhibit reduced activity in T-cell inhibition.<sup>[122]</sup> In addition, morphology and function of mitochondria in pericytes are affected by high glucose conditions which disrupted mitochondrial network and enhanced its fragmentation. Membrane potential is elevated, and oxygen consumption is attenuated in mitochondria under high glucose conditions. Changes in mitochondria morphology and metabolism causes apoptosis of retinal pericytes.<sup>[123]</sup>

High glucose conditions induce not only apoptosis, but also dysfunction of retinal pericytes. In bovine retinal pericytes, actin fibers were disassembled and lost

under high glucose conditions.<sup>[124]</sup> In diabetic mice with high blood glucose levels, pericytes in retinal vessels exhibit reduced adherence to endothelium. Ang-2 is involved in this reduced adherence of pericytes to endothelium.<sup>[125]</sup> Pericytes also protect retinal endothelial cells from inflammation-induced apoptosis by inhibiting proliferation of activated T-cell. Which this inhibition is attenuated under high glucose conditions.<sup>[126]</sup> Thus, hyperglycemia impair survival and function of retinal pericytes leading to diabetic complications in the retina.

Our recent work shows that increased production of inflammatory mediators including IL-1 $\beta$  and TNF- $\alpha$  results in sustained activity of signal transducer and activator of transcription 1 (STAT1) and increased expression of proapoptotic Bcl-2 family member, Bim. In addition, Bim expression was responsible for increased oxidative stress and loss of pericytes, since retinal pericytes prepared from Bim deficient mice were resistant to high glucose mediated oxidative stress and apoptosis. In addition, although the use of antioxidant N-acetylcysteine was protective, it did not affect Bim expression, thus suggesting that increased Bim expression by high glucose is up-stream of oxidative stress.<sup>[127]</sup> We showed that high glucose results in increased production of inflammatory mediators and activation of STAT1 transcription factor which drives Bim expression and death of pericytes.

### Physiological Changes in Retinal Astrocytes

Astrocytes have a crucial role in the structural integrity of BRB and high glucose conditions affect both BRB structures and astrocytes in the retinal vasculature. Diabetes contribute to the reduction of astrocytes in peripapillary and far peripheral retina and inhibit astrocytes from associating with superficial large vessels.<sup>[58,128]</sup> In addition to morphological changes in astrocytes, high concentration of glucose also affect cellular physiology of astrocytes. In the retina of diabetic mice, expression of glial fibrillary acidic protein, a marker of glial activation, is increased transiently in astrocytes, but not in Müller cells.<sup>[129]</sup> Aquaporins (AQP) are water channel proteins that regulate the water-electrolyte balance in the retina. High glucose conditions impact the distribution of AQP1 and AQP4 in retinal astrocytes. In normal retina, AQP4 is mainly expressed around retinal blood vessels, whereas under diabetic conditions, the mainly expressed protein is AQP1.<sup>[130]</sup>

Diabetes also affects gap junctions in astrocytes. Connexins are important proteins in the formation of gap junctions and cell-cell communication. Connexin-26 and -43 protein levels were attenuated in the astrocytes of diabetic rats. Decrease in connexin expression was followed by significant loss of astrocytes.<sup>[131]</sup> Furthermore, high glucose conditions activate defense mechanisms against ER stress in astrocytes. Cultured astrocytes under high glucose and retinal astrocytes from diabetic rat are

resistant to ER stress under hyperglycemic conditions.<sup>[132]</sup>

Our recent studies show that high level of glucose do not induce the apoptosis of astrocytes, but increase their proliferation and adhesion due to alteration in intracellular signaling pathways involved in cell survival, migration, and proliferation. High glucose conditions also resulted in production of inflammatory mediators in astrocytes. Under high glucose conditions, astrocytes migration was attenuated, which corresponded with their inability to undergo morphogenesis in Matrigel. In addition, the conditioned medium from astrocytes cultured under hyperglycemia was sufficient to block capillary morphogenesis of retinal endothelial cells. These activities were associated with increased oxidative stress in astrocytes culture under high glucose conditions and were reversed in the presence of the antioxidant N-acetylcysteine. The increased oxidative stress in astrocytes was concomitant with increased nuclear localization of NRF2, the oxidative stress activated transcription factor, and up-regulation of oxidative protective genes heme oxygenase-1 and periredoxin-2. These results are consistent with a protective role recently demonstrated for NRF2, mainly activated in the glial cells, in protection from DR.<sup>[133]</sup>

### CONCLUDING REMARKS AND FUTURE PERSPECTIVE

High glucose conditions affects morphology and physiology of retinal vascular cells including endothelial cells, pericytes, and astrocytes leading to dysfunction of retinal vasculature. The key factors in this dysfunction are inflammation and increased oxidative stress. However, these alterations have specific impacts on various retinal vascular cells. Although high glucose induces apoptosis of retinal pericytes, it had minimal impact on the apoptosis of retinal astrocytes or endothelial cells. Additionally, the studies on pericytes response to high glucose conditions indicated that inflammation is the primary cause of their demise since up-regulation of Bim by inflammatory mediators is responsible for increased oxidative stress and loss of pericytes. We have also observed that although pericytes are the major source of inflammatory mediators, retinal astrocytes and endothelial cells may respond to high glucose with increased production of inflammatory mediators, as well. However, the reason for selective up-regulation of Bim in pericytes and their loss in response to high glucose still needs further investigation. Our recent studies of proteasome activity and O-GlcNAC modification in these cells<sup>[134,135]</sup> suggest that inefficient proteasome activity and/or enhanced accumulation of O-GlcNAC modified proteins may be responsible for selective sensitivity of pericytes to increased concentrations of glucose. These possibilities are the subject of current investigation and will provide new insight into the mechanisms leading



to selective sensitivity of pericytes to hyperglycemia. Furthermore, our studies support the critical role for astroglial cells, as an intermediary between neuroretina and retinal circulation. We believe astroglial cells are the essential regulator of retinal neurovascular system and their role in the pathogenesis of central nervous system diseases including DR deserves further attention and careful evaluation.

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