



Endothelial Dysfunction in Diabetic Retinopathy

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Diabetic retinopathy (DR) is a diabetic complication which affects retinal function and results in severe loss of vision and relevant retinal diseases. Retinal vascular dysfunction caused by multifactors, such as advanced glycosylation end products and receptors, pro-inflammatory cytokines and chemokines, proliferator-activated receptor- γ disruption, growth factors, oxidative stress, and microRNA. These factors promote retinal endothelial dysfunction, which results in the development of DR. In this review, we summarize the contributors in the pathophysiology of DR for a better understanding of the molecular and cellular mechanism in the development of DR with a special emphasis on retinal endothelial dysfunction.

Keywords: diabetes, diabetic retinopathy, endothelial cells, endothelial dysfunction, signaling pathways

INTRODUCTION

Diabetic retinopathy (DR) is one of the major complications of diabetes. In 2019, there were about 463 million adults with diabetes worldwide according to the International Diabetes Federation. Diabetes has been one of the most common causes for death in adults aged 20–74 years old (1). DR is resulted from long-term accumulated damages by hyperglycemia or other factors (such as hypertension) to the microvessels in the retina (2). It is a major cause of blindness and other relevant retinal diseases (such as diabetic macular edema and DME) and has received particular attention (3).

Although diagnosis and treatment at the early stage can reduce vision loss in some patients, DR remains a serious threat to vision and patients' quality of life. DR and relevant retinal diseases are related to retinal vascular dysfunction. Although DR now is more precisely defined as a neurovascular disease rather than a microvascular disease (4), retinal microvasculopathy remains the main pathological change of DR. Hyperglycemia causes retinal microvasculopathy, inflammation, and retinal neurodegeneration, all of which result in the breakdown of the blood–retinal barrier (BRB) and damages the endothelium to form acellular capillaries and edema in retinal vascular structure (5).

Diabetic retinopathy has two stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR is an early stage of DR which is characterized by loss of pericytes from retinal capillaries to form acellular capillaries, increase vascular permeability, and break down the inner endothelial BRB (6). It is usually asymptomatic. PDR is an advanced stage at which new, vulnerable, and tortuous blood vessels are formed in the retina. They can cause fibrovascular epiretinal membranes, vitreous hemorrhage, and retinal detachment, all of which contribute to vision loss (6).

The underlying molecular mechanisms associated with vascular dysfunction, especially endothelial dysfunction, in DR are multifactorial. Extensive studies have been performed to identify factors that are associated with endothelial dysfunction in DR, such as advanced glycosylation end

products (AGEs) and receptors (RAGE), disruption of peroxisome proliferator-activated receptor- γ (PPAR γ), chronic inflammation, leukostasis (7–10), oxidative stress, and dysregulated growth factors, cytokines, and microRNA (miRNA) networks (10–13). Here, we review the available data and summarize on AGE, PPAR γ , inflammation, miRNA, and signaling pathways that contribute to endothelial dysfunction in the development of retinal microvasculopathy and analyze the challenges in understanding the pathology of DR.

ADVANCED GLYCOSYLATION END PRODUCTS AND RECEPTORS IN ENDOTHELIAL DYSFUNCTION OF DR

AGEs are glycosylated proteins or lipids which are resulted from exposure to hyperglycemia over time. Hyperglycemia causes the activation of the polyol pathway to produce fructose, fructose-3-phosphate, and 3-deoxyglucosone, which are glycosylating agents (14). Glucose and the increased glycosylating agents form covalent bonds with the proteins or lipids to form AGEs.

AGEs are detrimental to vascular cells and have been shown to promote the development and progression of DR (Figure 1) (15, 16). A single dietary AGE can acutely impair endothelial function in diabetic and non-diabetic subjects (17). AGE accumulation in cells is a result of their generation from glucose-derived dicarbonyl precursors through non-enzymatic glycation reaction, which is called the “Maillard reaction” (18). Intracellular AGEs interfere with cell function by disrupting molecular conformation, altering enzyme activity, reducing degradation ability, and inhibiting receptor recognition (19).

Studies have shown that accumulation of AGEs in the retinal blood vessel walls is detrimental (10, 20, 21). It causes increased permeability of retinal endothelial cells (ECs) to induce vascular leakage (20). AGEs can upregulate AGE receptor (RAGE) gene expression levels in pericytes and microvascular ECs (21). Activation of RAGEs transduces multiple signals, leading to increased oxidative stress and synthesis of growth factors, adhesion molecules, and pro-inflammatory cytokines (22–24) and resulting in activation of nuclear transcription factors, such as NF- κ B (25, 26).

The interaction of AGEs and RAGE increases reactive oxygen species (ROS) product in ECs (22, 27, 28). Both nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the mitochondrial electron transport system are involved in ROS generation by AGE signal transduction in ECs (29) as the inhibition of both significantly reduced AGE-induced ROS production (29). Hyperglycemia-induced mitochondrial superoxide can be abrogated by inhibition of AGE-RAGE-mediated mitochondrial permeability transition *in vitro* (30). Similarly, lowering AGEs with alagebrium reduced mitochondrial superoxide generation. The AGE-mediated ROS generation is at least partly through NF- κ B activation and subsequent TNF- α production in ECs (31).

The interaction of AGEs and RAGE also promotes expressions of growth factors, proinflammatory cytokines and chemokines, and adhesion molecules through the mitogen-activated protein

kinase (MAPK) pathway, leading to NADPH oxidase-mediated ROS generation and translocation of NF- κ B (23, 32).

AGEs upregulate VEGF expression in retinal ECs (33). VEGF expression and PKC activation induced by AGEs in retinal ECs were inhibited by the PKC inhibitor and the antioxidant drug and compounds, but not compound that did not have antioxidant property. VEGF is known to stimulate angiogenesis and neovascularization, which are involved in the pathogenesis of proliferative retinopathy (15). The levels of VEGF in ocular fluid are associated with the breakdown of the BRB, which increases microvascular permeability (34).

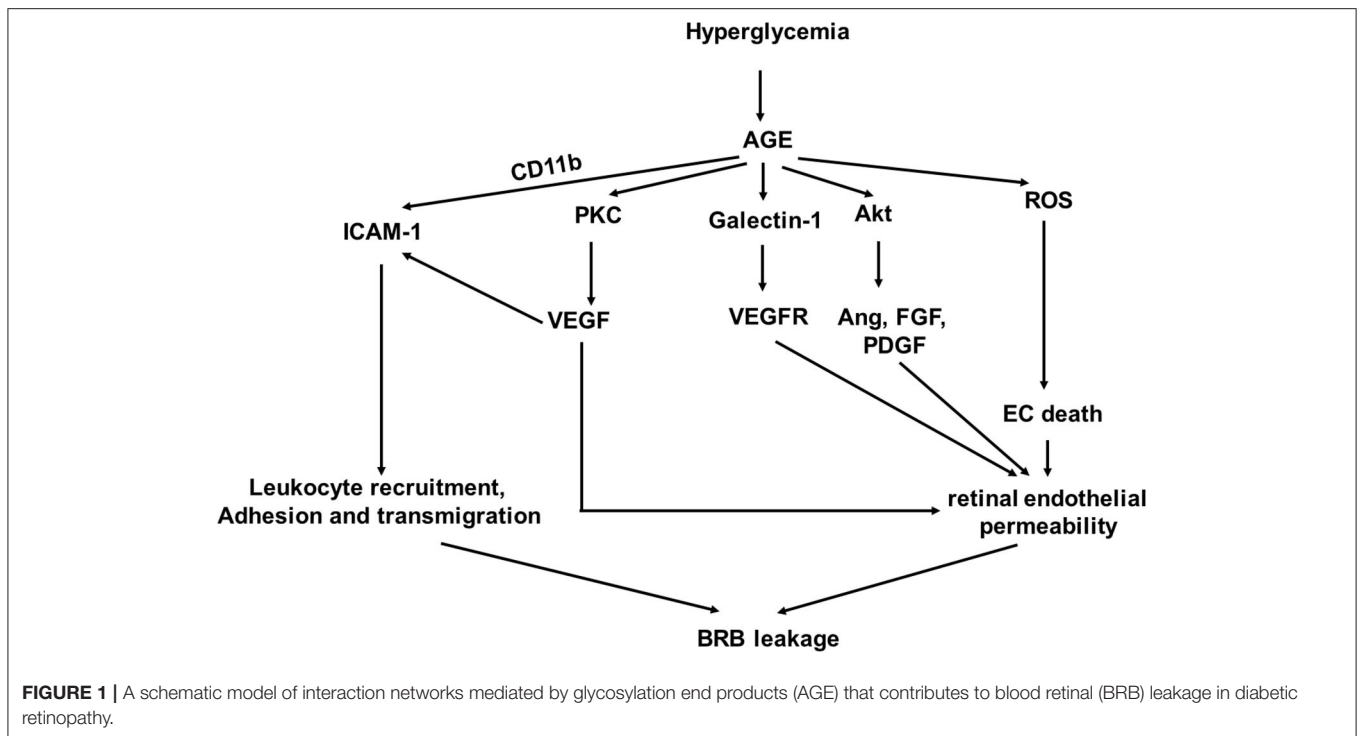
In addition to VEGF, other angiogenic factors, including angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), fibroblast growth factor (FGF), and platelet derived growth factor (PDGF), have been shown to be upregulated in retinal capillary ECs through Akt-mediated signaling activated by AGEs (15). AGEs can stimulate basic FGF expression in cultured Müller cells to affect pathogenesis of DR (35).

Endothelial cell-expressed RAGE can act as Mac-1 (CD11b) ligand and work cooperatively with Intercellular Adhesion Molecule-1 (ICAM-1) to mediate leukocyte adhesion during the acute inflammation *in vivo* (36). VEGF induces ICAM-1 expression on retinal ECs to promote monocyte adhesion (37). Increased ICAM-1 expression in the retinal ECs contributes to microvascular leukostasis, the adhesion, and transmigration of leukocytes to endothelium, in DR (38, 39). AGE induces specific galectin-1 expression, which may be correlated with disease activity in DR as galectin-1 can bind to VEGF receptors-1 and-2 in ECs, resulting in angiogenesis and vascular permeability, respectively (40, 41).

AGE upregulates PKC activation, increases ROS production, and promotes synthesis of growth factors, adhesion molecules, and pro-inflammatory cytokines. Understanding the underlying cellular and molecular pathogenesis mechanism of AGE-induced endothelial dysfunction in DR will facilitate early detection of DR and identify novel anti-AGE drugs, which can block the biological activity of AGEs.

DISRUPTION OF PPAR γ IN ENDOTHELIAL DYSFUNCTION OF DR

PPAR γ is a nutrient sensor that controls a variety of homeostatic functions. Its disruption leads to disorders of fatty acid/lipid metabolism, insulin resistance, and vascular pathology. Endothelial PPAR γ is essential for preventing endothelial dysfunction with aging (42, 43). Impaired endothelial PPAR γ causes age-related vascular dysfunction. PPAR γ activation mediates antioxidant response and nitric oxide (NO) product in ECs. It induces increased expression of nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor (I κ B), phosphatase and tensin homolog (PTEN), and Sirtuin 1 (SIRT1), all of which interfere with the activation of NF- κ B (44). PPAR γ promotes the expression of antioxidant enzymes, including catalase, heme oxygenase-1 (HO-1), and superoxide dismutase (SOD), which lead to a reduction of the ROS product (44). PPAR γ inhibits diabetes-induced retinal leukostasis and microvascular



leakage through its role on increasing expression of endothelial nitric oxide synthase (eNOS) activity, reducing oxidative stress, inhibiting apoptosis, inflammation, and angiogenesis (43). PPAR γ receptors have been shown to be downregulated in the diabetic eye, and their disruption is involved in the pathogenesis of DR (Figure 2) (45, 46).

Endothelial Nitric Oxide Synthase and Nitric Oxide

Nitric oxide produced by eNOS is a major medium which mediates relaxation and vasodilatation of the vessels. Production and bioavailability of NO are reduced in the early stages of DR (47), while PPAR γ activation increases production and bioavailability of NO. PPAR γ ligands, such as 15-deoxy- Δ (12, 14)-prostaglandin J₂ (15d-PGJ₂), rosiglitazone, and nitrooleate, are able to increase eNOS activity and NO release through increased interaction between heat shock protein 90 (HSP90) and eNOS (48, 49). Rudnicki et al. assessed the effect of 3 thiazolidinediones (TZDs), GQ-32, GQ-169, and LYSO-7, on NO, ROS, and adhesion molecules on ECs (50). Although all of three activated PPAR γ and enhanced the intracellular NO level, only LYSO-7 significantly increased the NO release from ECs. They all suppressed the adhesion molecule expressions induced by TNF- α . Additionally, GQ-169 and LYSO-7 inhibited ROS production in response to high glucose. PPAR γ activation decreases expressions of NADPH oxidase subunits and enhances the expression of superoxide dismutase (SOD), which result in enhanced NO bioavailability to reduce oxidative stress in the membrane of human umbilical vein endothelial cells (HUVECs) (51).

Aleglitazar, a dual-PPAR α/γ agonist, has been shown to increase eNOS, Akt, and telomerase activities in circulating angiogenic cells (52). Rosiglitazone increases eNOS and Akt activity and NO synthesized by endothelial progenitor cells (EPCs), which are reduced by AGEs. Its beneficial effect can be blocked by the eNOS inhibitor and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) inhibitor, indicating that rosiglitazone can improve AGE-induced EPC dysfunction by AGEs through upregulating the AKT/eNOS signal pathways in EPCs (53).

Oxidative Stress

Hyperglycemia induces oxidative stress in patients with diabetes. Oxidative stress, which resulted from increased NADPH oxidase, is a key factor involved in the development of DR (11, 12). It activates inhibitory redox-regulated transcription factors to attenuate PPAR γ expression and activity in vascular ECs (54). PPAR γ exerts its antioxidative function through transcriptional activation of a number of antioxidant genes (55–57). The major ROS produced in response to hyperglycemia is superoxide anion (O₂⁻) which combines with NO to produce peroxynitrite (ONOO⁻). This leads to decrease in NO bioavailability, which causes endothelial dysfunction (58).

PPAR γ can transcriptionally regulate HO-1 expression in vascular cells (56). Its activation induces the expression of glutathione peroxidase 3 (GPx3), which detoxifies the extracellular H₂O₂ level. The inhibition of GPx3 expression prevents the antioxidant effects of the PPAR γ ligand on oxidative stress in insulin-resistant cells (59). Troglitazone and pioglitazone increases Cu²⁺, Zn²⁺-superoxide dismutase (CuZn-SOD) gene expression and protein levels (60). 15d-PGJ₂ or

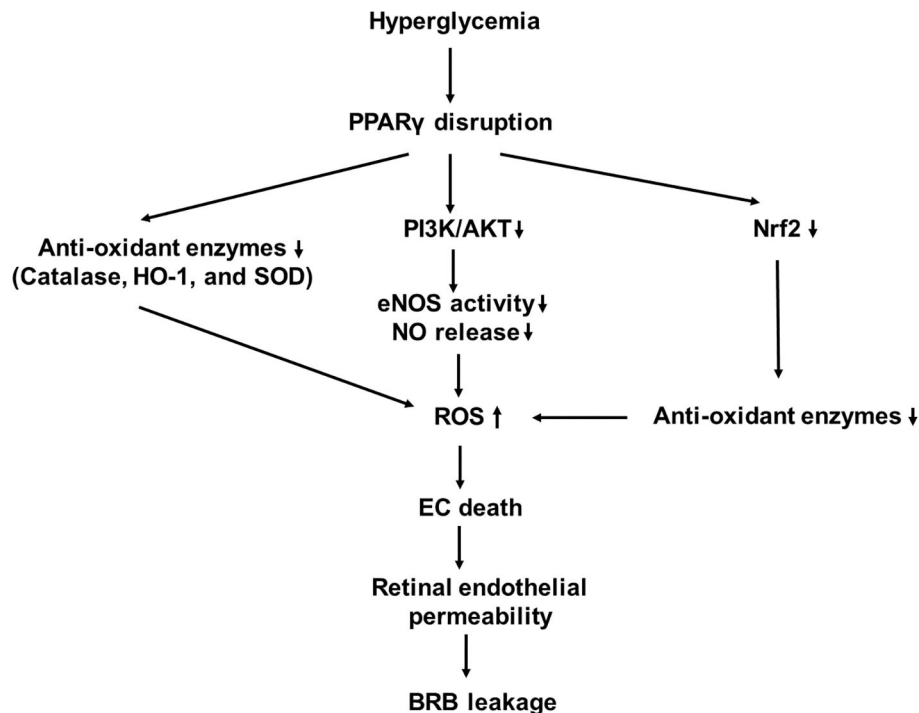


FIGURE 2 | A schematic model of interaction networks mediated by proliferator-activated receptor- γ (PPAR γ) disruption that contributes to blood retinal (BRB) leakage in diabetic retinopathy.

ciglitazone reduces gene and protein expressions of the NADPH oxidase subunits, such as nox-2 and nox-4, and stimulates protein expression and activity of Cu/Zn-SOD in HUVECs (51).

Oxidative stress also impairs reendothelialization ability of EPCs derived from patients with diabetes, while rosiglitazone improves reendothelialization EPC therapy potential by reduces NADPH oxidase activity (61). Pioglitazone can inhibit NADPH oxidase p22 (phox) and Rac1. The latter is responsible for producing ROS, which negatively regulates EPC migration, proliferation, and differentiation. Recently, Liu et al. have demonstrated that PPAR γ activation can transcriptionally upregulate the expression of long intergenic noncoding RNA 01230 (Linc01230), which reduces oxide low-density lipoprotein-induced endothelial dysfunction and affects the phosphorylation of AKT (62).

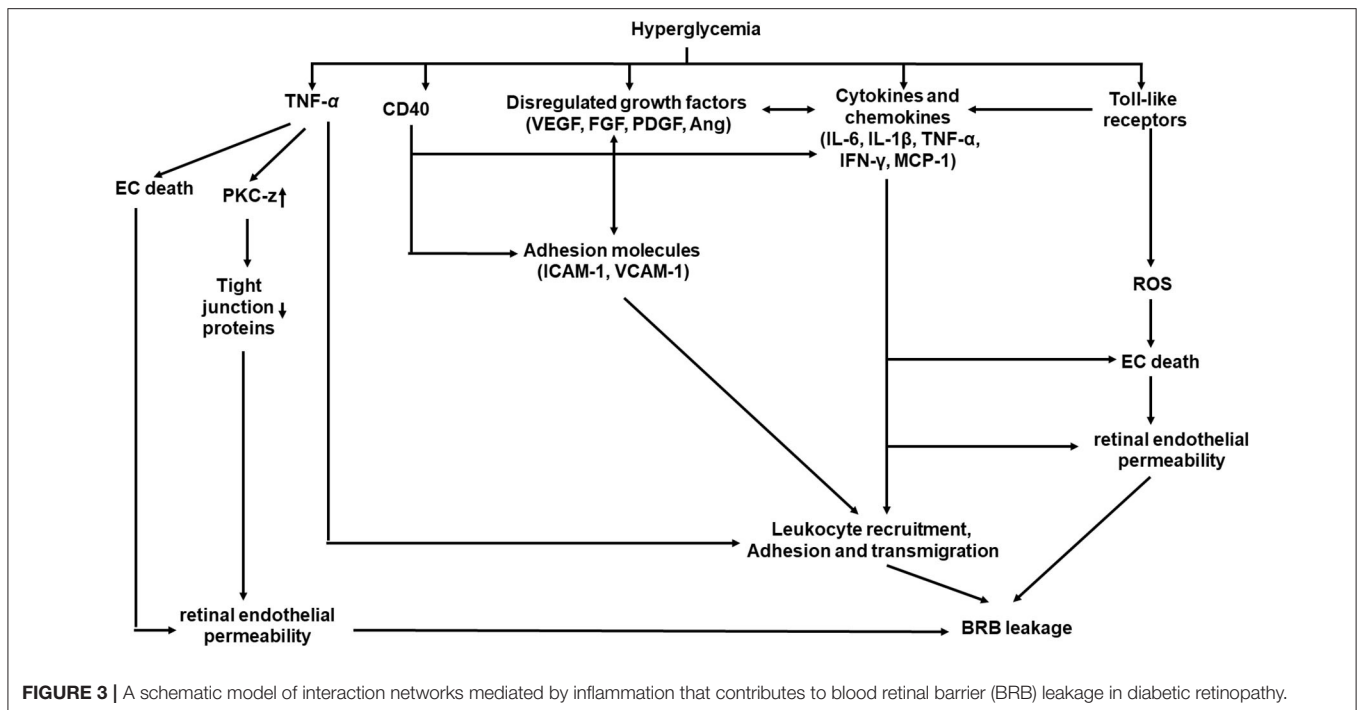
In addition to directly modulating oxidative stress response, PPAR γ can indirectly modulate through nuclear factor E2-related factor 2 (Nrf2) activation (63). Nrf2 is a transcription factor that regulates the expression of antioxidant proteins (64, 65). When transported inside the nucleus, Nrf2 works with other activators to form a protein complex. The latter binds to the antioxidant response elements (AREs) in the upstream promoter regions of cytoprotective and detoxifying genes to activate their transcription (64, 66). Studies have shown that there is a reciprocal transcriptional regulation between Nrf2 and PPAR γ pathways to enhance the expression of one another (57, 63). PPAR γ is upregulated in mice in which Nrf2 is increased and is downregulated in *Nrf2*^{-/-} mice (57, 67). ChIP assays have

shown that with cofactor Brg1, Nrf2 is coimmunoprecipitated on the ARE containing the upstream promoter region of PPAR γ (67). Nrf2 expression is reduced in mice with decreased PPAR γ (68). PPAR γ may act directly or through the upstream pathway to activate Nrf2 (57). A peroxisome proliferator response element, through which PPAR γ regulates Nrf2 expression, in the promoter region of Nrf2 gene has been proposed (57). Future studies are needed to prove a direct effect of PPAR γ on Nrf2.

Although PPAR γ activation promotes antioxidant response and promotes the expression of antioxidant enzymes and NO product in ECs, PPAR γ receptors are downregulated in the diabetic eye and their suppression is involved in the pathogenesis of DR (45, 46). Thus, it is not easy to fully reverse endothelial dysfunction using only PPAR γ ligands in DR. Strategies aiming to improve the sensitivity or upregulate PPAR γ receptor expression in ECs of DR are valuable therapeutic approaches.

INFLAMMATION AND ENDOTHELIAL DYSFUNCTION OF DR

Inflammation plays important roles in structural and molecular changes associated with DR (Figure 3) (69, 70). Systematically, hyperglycemia causes AGE formation and increases ROS product and plasma proinflammatory cytokines, including TNF- α and interleukin-6 (IL-6) (11, 15, 16, 71). Locally, retinal hypoxia leads to the release of many molecules in the vitreous, including proinflammatory cytokines [TNF- α , interleukin-1 β (IL-1 β), IL-6,



interleukin-8 (IL-8), and interferon- γ (IFN- γ), etc.), chemokines [monocyte chemoattractant protein-1 (MCP-1)], growth factor (VEGF, FGF, and PDGF etc.), adhesion molecules [ICAM-1 and vascular cellular adhesion molecules-1 (VCAM-1)], and receptors (CD40 and Toll-like receptors), from retinal vascular cells, inflammatory cells, and/or glial cells (72, 73).

Cytokines

Proinflammatory cytokines, such as TNF- α , IL-1, IL-6, IL-8, and IFN- γ , are the major players in inflammation in DR. Increased concentrations of TNF- α , IL-1, IL-6, IL-8, and IFN- γ have been found in the vitreous (74) or in aqueous humor (75) of patients with DR. Their concentrations may be associated with the severity of DR (75).

TNF- α

TNF- α can attract inflammatory cells, induce inflammatory cytokine release, and cause necroptosis on targeting cells and proliferation of immune cells (76). TNF- α can be synthesized and released by activated microglia, ECs, macroglia, Müller cells, and neurons (75). Increased levels of TNF- α have been found in PDR. A study of meta-analysis indicated that the level of TNF- α in DR patients was significantly different from that in healthy controls (77). There is a strong correlation between plasma TNF- α levels and the severity of (78). TNF- α concentration has been suggested as a biomarker for the severity of DR as TNF- α in tears increases and is highly correlated with DR severity (79). TNF- α increases retinal EC permeability by reducing the expression of tight-junction proteins through activation of protein kinase C zeta and NF- κ B (80). TNF- α is also a chemoattractant for leukocytes to stimulate leukocyte adhesion (78).

TNF- α is critical mediator for later complications in DR. In a TNF- α knockout mouse model, Huang et al. demonstrated that TNF- α is not required for early BRB breakdown in DR (81). However, the absence of TNF- α significantly suppressed BRB breakdown in 6-month-old mice with diabetes. Consistently, apoptosis of ECs, pericytes, and neurons was inhibited in TNF- α knockout mouse models with or without diabetes. However, recent studies showed that a higher level of TNF- α was observed in patient eyes with NPDR than with PDR (75) (82). The discrepancy may indicate the transit of NPDR into PDR.

IL-1 β

IL-1 β has been shown to be important in mediating innate immunity and contributing directly to several retinal degenerative diseases, including DR (83). IL-1 β can be produced by microglial cells, Müller cells, and astrocytes (9). A significantly high level of IL-1 β has been detected in the vitreous humor and serum of patients with PDR (83, 84). The aqueous concentrations of IL-1 β from the eye anterior chamber increased with the severity of DR (75). Intravitreal injection of IL-1 β caused apoptosis of capillary cells in retinal microvessels and acellular capillaries (85), which are probably mediated by NF- κ B and caspase-3 activation (86). IL-1 β is cytotoxic to choroidal blood vessels in the choroid, which may lead to the death of the retinal pigment epithelium and damage photoreceptor integrity (87). Hyperglycemia induces Müller cells to produce high levels IL-1 β , which induces the expression of pro-death cytokine IL-8 (88). Actually, IL-1 β is a more potent inducer of IL-8 expression than TNF- α , IL-6, VEGF, and IFN- γ in Müller cells through the p38MAPK and extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) pathways (88). IL-1 β is also

a stronger IL-6 inducer than TNF- α , IL-8, VEGF, and IFN- γ in Müller cells through upregulation of the p38MAPK/NF- κ B pathway (89). Hyperglycemia triggers retinal ECs to upregulate the expression of IL-1 β , which serves as an autocrine or paracrine to stimulate IL-1 β expression in ECs or microglial cells to a sustained overexpression of IL-1 β (90). IL-1 β leads to ROS release and promotes NF- κ B translocation to the nucleus to create a continuous inflammatory response (91).

IL-6

IL-6 is a member of the pro-inflammatory cytokines involved in expressing of a variety of proteins in regulating angiogenesis, immune responses, and vascular permeability (92). IL-6 can be produced by microglia and astrocytes (9). Significantly higher intravitreal IL-6 levels are found in patients with NPDR and PDR, (75, 82, 93, 94) and its intravitreal concentration correlates with the severity of PDR (95) and retinal macular thickness (96). Increased intravitreal concentration of IL-6 may be associated with the progression of DR from the NPDR to active PDR (95). The increased IL-6 levels may be independent of hypoxia-inducible factor-1 α (HIF-1 α) or NF- κ B activity in the vitreous of PDR (97). However, animal study suggests that HIF-1 α still regulates IL-6 expression in diabetic retina as increased HIF-1 α , IL-6, and TNF- α are found in diabetic retina of diabetic rats, which can be decreased by the HIF-1 α inhibitor (98). IL-6 promotes leukocyte adhesion, microvascular leakage, and TNF- α product in microglial cells in diabetes as these pathological phenotypes were dramatically reduced in the IL-6 knockout mice with diabetes (10).

IL-8

IL-8 is not only a potent angiogenic factor but also a chemoattractant for neutrophils and T lymphocytes (69). It can be produced by Müller glial cells, retinal ECs, and astrocytes. Although IL-8 has been detected both in the vitreous (9, 74) or aqueous humor (75, 99) of DR patients, it is higher in the eyes with NPDR than in the eyes with PDR (82). Elevated vitreous IL-8 level seems to correlate with poorer visual acuity in patients with diabetes, suggesting that IL-8 may cause visual acuity loss as DR progression (100). IL-8 has a strong correlation in vitreous and aqueous of patients with PDR (101). IL-8 is induced in Müller cells in response to IL-1 β or TNF- α (88), as well as VEGF in microvascular ECs (102).

IFN- γ

IFN- γ is an immunoregulatory cytokine which belongs to the Th-1 group lymphocytes. It signals innate immune system responses by recruiting and activating macrophage and cytotoxic T cells to produce a pro-inflammatory effect (103). Increased IFN- γ was observed in the vitreous or aqueous humor of patients with diabetes or with DR (104, 105). In contrast, increased aqueous IFN- γ was only observed in patients with NPDR or PDR (75). IFN- γ was increased in the retina of rats with diabetes (106). IFN- γ induced migration of microglial cells in the subretinal space to affect the ocular microenvironment in response to inflammation (107). Over-expressing IFN- γ in the retina caused intraocular cellular infiltration, photoreceptor death, corneal

clouding, cataract formation, and epithelial and microglial proliferation (108). IFN- γ -increased HUVEC permeability is, at least, partly related to its inhibition on NO production: IFN- γ significantly attenuates basal NO concentration and reduces NO increment in the presence of an NO donor in HUVECs (109). IFN- γ -induced disorganization of endothelial junctional integrity through a mechanism involving Rho-kinase mediated cytoskeletal contractions (110). IFN- γ together with TNF- α and IL- β downregulated the HSP27 expression, which led to apoptosis of retinal capillary ECs (111).

Chemokine: MCP-1

Monocyte chemoattractant protein-1 attracts and activates monocyte and macrophages (112, 113) and stimulates fibrosis and angiogenesis (114). MCP-1 is produced by Müller cells, microglia cells, astrocytes, retinal neurons, ECs, and retinal pigment epithelial cells in patients with diabetes (115). The migration of monocyte into the retina is mediated by MCP-1 coupling to its receptor CCR2 (116). Elevated MCP-1 has been observed in ocular tissues from patients with NPDR or PDR, (10, 82, 104, 117) and its level is higher in the vitreous than in the serum (74). The vitreous MCP-1 level has been shown to be associated with DR severity (100). Intravitreal increase in MCP-1 level may be associated with the progression of NPDR to active PDR (95). Through increasing vascular cell permeability and leukocytes' recruitment, MCP-1 affects BRB in animal eyes of DR (118). In response to IL-1 β or TNF- α , retinal ECs or microglial cells will express a high level of MCP-1 to attract macrophages (119), which may adhere to the retinal capillary endothelium, which leads to capillary occlusion and retinal ischemia (120). TNF- α and IL-6 produced by glial cells and microglial cells can stimulate ECs to release MCP-1, IL-6, and VEGF, all of which increase vascular permeability in NPDR (121). MCP-1 exerts its cytotoxic effect through oxidative stress produced by activated macrophage and microglia (122). Although MCP-1 is a potent inducer of angiogenesis, its angiogenic effect is achieved through induction of VEGF-A (123, 124). A significantly positive correlation has been observed between the MCP-1 and VEGF in PDR (125). Although lower levels of MCP-1 have been reported in the aqueous humor from NPDR and PDR patients (126, 127), the discrepancy may be due to different sample preservation and measurement techniques used.

Growth Factor: VEGF

Increased vitreous concentrations of the growth factors, such as VEGF, FGF (128), PDGF (129), placental growth factor (PlGF) (130), angiopoietin (131), insulin-like growth factor (IGF-1) (132), and hepatocyte growth factor (HGF), have been reported in patients with PDR. Among these, VEGF has received particular attention and will be summarized in this section.

Over the decades, VEGF has been recognized as a major angiogenic growth factor, which is responsible for pathologic retinal neovascularization in DR (133). VEGF is an angiogenic factor that not only induces new blood vessel sprouting from preexisting vessels but also increases vascular permeability. In addition to ECs, other retina cells, such as retinal pigment epithelial cells, pericytes, Müller cells, astrocytes, and glial cells,

are also able to produce VEGF upon activation or stimulated by long-term high glucose (9, 134–139).

Increased VEGF level has been observed in the vitreous humor and in fibrovascular tissues from eyes with PDR (140–142). Serum and vitreous VEGF levels have been found to correlate with glycemic control in patients with diabetes (143). A strong correlation between increased level of intravitreal VEGF and the development of DR has been detected (144, 145). Vitreous level of VEGF may be correlated with retinopathy activity (142, 146, 147). More recently, serum VEGF level in subjects with diabetes has been proposed to be a biomarker of severity of DR as it is associated with the severity of DR (148, 149).

VEGF is a key regulator of ocular angiogenesis and vascular permeability. It is involved in the pathogenesis of a number of complications of DR, such as DME and PDR (150). It has been shown that intraocular injection of VEGF alone produced many features of NPDR and PDR: areas of non-perfusion capillaries, vessel dilation, and tortuosity arterioles with endothelial hyperplasia and microaneurysm formation (151). A positive correlation has been found between the level of serum VEGF and the grade of the external limiting membrane (ELM) disruption, indicating that an increased level of VEGF is associated with DR severity and the grade of the external limiting membrane disruption (149).

VEGF modulates DR-associated inflammatory responses at the early stage of DR (13). It acts as a pro-inflammatory factor to promote the expressions of other proinflammatory cytokines, chemokines, and adhesion molecules, such as TNF- α , IL-1, IL-6, IL-8, IFN- γ , MCP-1, and ICAM-1 (102, 152, 153). Vice versa, activated glial cells, macrophages, and microglia cells will produce TNF- α , IL-6, and MCP-1, which in turn can stimulate VEGF release from retinal ECs (123). Increased activation of NF- κ B in NPDR and PDR subjects may be involved in increased upregulation of VEGF (154). VEGF induces MCP-1, IL-8, TNF- α , and ICAM-1 expression in retinal ECs by activating NF- κ B pathways (13, 102, 153). Müller glia-specific VEGF deletion resulted in 48% percent reduction in the NF- κ B activation in diabetic mouse retina (13). This was associated with 50% reduction of TNF α and ICAM-1 in retina and 75% reduction of leukocyte adhesion in the retinal microvasculature.

VEGF enhances the leukocyte adhesion to vessel walls through increasing ICAM-1 and VCAM-1 expressions on ECs (155). In addition, VEGF may initiate early diabetic retinal leukocyte adhesion in retinal arterioles through upregulated ICAM-1 expression (152, 156). Increased serum VEGF levels stimulate ROS generation, which causes endothelial activation (157).

Adhesion Molecules

Studies show that adhesion molecules play important roles in pathogenesis of vascular complications (158). Adhesion molecules participate in cell growth, differentiation, formation of cell junction, or cell polarity, as well as activation, circulation, or accumulation of white leukocytes at the inflammatory site (158). They participate in initiating the process of monocyte and lymphocyte adhesion to ECs and mediate their transmigration (158).

Numerous endothelial molecules, primarily located at junctions, such as ICAM-1, VCAM-1, platelet/endothelial-cell adhesion molecule-1 (PECAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1), are involved in leukocyte transmigration (158–160). ICAM-1 and VCAM-1 are upregulated in the conjunctiva of patients with DR (161). Blood serum soluble forms of VCAM-1, ICAM-1, and ELAM-1 are increased in patients with DR (162). ICAM-1 can act cooperatively with RAGE to mediate leukocyte recruitment during acute inflammation *in vivo* (36). The lymphocyte function-associated antigen-1 can distribute to form a ring-like structure to cocluster with endothelial ICAM-1 to mediate the neutrophil transmigration (130). A positive correlation is found between the level of serum ICAM-1 and the grade of the retinal ELM disruption (149), suggesting, in addition to VEGF, that serum ICAM-1 level is also associated with an increased DR severity and the grade of the external limiting membrane disruption (149). Thus, monitoring serum-soluble VCAM-1 levels in patients with diabetes may be clinically useful for assessing the severity and possibly the activity of diabetic retinopathy (163). The soluble ICAM-1 is a biomarker of endothelial injury and inflammation. ICAM-1, VCAM, and ELAM-1 expressions in ECs can be stimulated by IL-1, TNF- α , and VEGF through activation of the NF- κ B pathway (152, 164, 165).

PECAM-1 is important in maintaining vascular integrity (166). Although endothelial PECAM-1 homophilic interactions are required for the maintenance of EC barrier function (166), PECAM-1 also promotes leukocyte transmigration by undergoing homophilic interactions with PECAM-1 on monocytes to facilitate transmigration (167). More recently, soluble vascular adhesion protein-1/semicarbazide-sensitive amine oxidase has been shown to generate H₂O₂ and toxic aldehyde acrolein, which cause oxidative stress in eyes with PDR (168).

Receptors

CD40 and Toll-like receptors play roles in inflammation in the development of DR (169–172).

CD40

CD40, a member of the TNF receptor superfamily, is expressed not only on monocytes, dendritic cells, and ECs but also on Müller glia, microglial cells, and retinal pigment epithelial cells (169, 170). CD40 expression level is low at basal condition, and its upregulation results in downstream inflammatory response in diseases (173). Plasma-soluble CD40 ligand level is found to be positively associated with DR severity (173). Increased expression of CD40 was found in retinal Müller cells, ECs, and microglial cells of diabetic animals (174). CD40 upregulates ICAM-1, MCP-1, and VEGF expressions in ECs and Müller cells through TNF receptor-associated factors (175). CD40 knockout mice are protected from the development of DR: reduced retinal leukostasis, inhibited capillary degeneration, and diminished ICAM-1 upregulation (174). Diabetes upregulates P2X7 in the retina through CD40 to make retinal ECs susceptible to ATP/P2X7-mediated apoptosis (176).

Toll-Like Receptors

TLRs play an important role in innate immune responses and inflammation (177). TLRs promote proinflammatory cytokine expression, which in turn activate TLRs in immune cells to induce EC damage by the ROS product (171, 172). A high level of mobility group protein-1, a ligand of toll-like receptor (TLR)-4, has been found higher in active PDR than in inactive PDR (178). The agonist of TLR-3 can induce the retinal pigment epithelium to secrete MCP-1, IL-8, and ICAM-1 (179). High glucose significantly upregulates TLR-2 and TLR-4 expression and activates NF- κ B and increases expression of IL-1 β , IL-8, TNF- α , MCP-1, ICAM-1, VCAM-1, and adhesion of monocyte in human microvascular retinal ECs (180). TLR-4 or TLR-2 inhibitor and antioxidant treatment reduces the expressions of TLR-2 and TLR4 and associated downstream inflammatory markers. These suggest that activation of TLR-2 and TLR-4 and downstream signaling are involved in increased inflammation and ROS in DR. Furthermore, retinal photoreceptors are susceptible to mitochondrial oxidative stress and mitochondrial DNA damage in TLR4-mediated innate immune response, leading to visual impairment (181).

Although there is increasing evidence showing that inflammation is a critical contributor to the development of DR, some studies have also demonstrated that DR is not exclusively due to inflammation (182, 183). Thus, the exact underlying molecular mechanisms of inflammation in DR are not yet fully understood. In addition, inflammation is a complex cascade; thus, therapeutics targeting at one factor may be insufficient. Drugs that inhibit multiple factors in inflammation may help to control DR.

MIRNAS

Recent studies have shown that epigenetics also plays a key role in the development and progression of DR (184–186). Hyperglycemia affects the enzymatic machinery responsible for epigenetic modifications (187). The enzymes responsible for epigenetic modifications and non-coding RNA function may be aberrantly expressed (Figure 4). They have been shown to either promote or inhibit the development and progression of DR (187). miRNAs and long non-coding RNA, which are well-known for their regulatory functions, are gaining more attention. Several studies identified panels of miRNAs whose expressions are changed in the retinal ECs of diabetic rats (184–186). NF- κ B-responsive miRNA, such as *miR-21*, *miR-146*, *miR-155*, and *miR-132*, and VEGF-responsive miRNAs, such as *miR-17-5p*, *miR-18a*, *miR-20a*, *miR-21*, *miR-31*, and *miR-155*, have been identified in the retinal ECs (184). Wu et al. identified 11 increased miRNAs and 6 decreased miRNAs in the retinas of diabetic rats (185), while Xiong et al. identified 17 dysregulated miRNAs in the retinas of diabetic rats (186). Li et al. identified five differentially expressed miRNAs in serum between DR and non-DR patients (188). These miRNAs were found to regulate 55 target genes which were involved in controlling the vascular growth and morphogenesis.

Upregulated miRNAs in DR

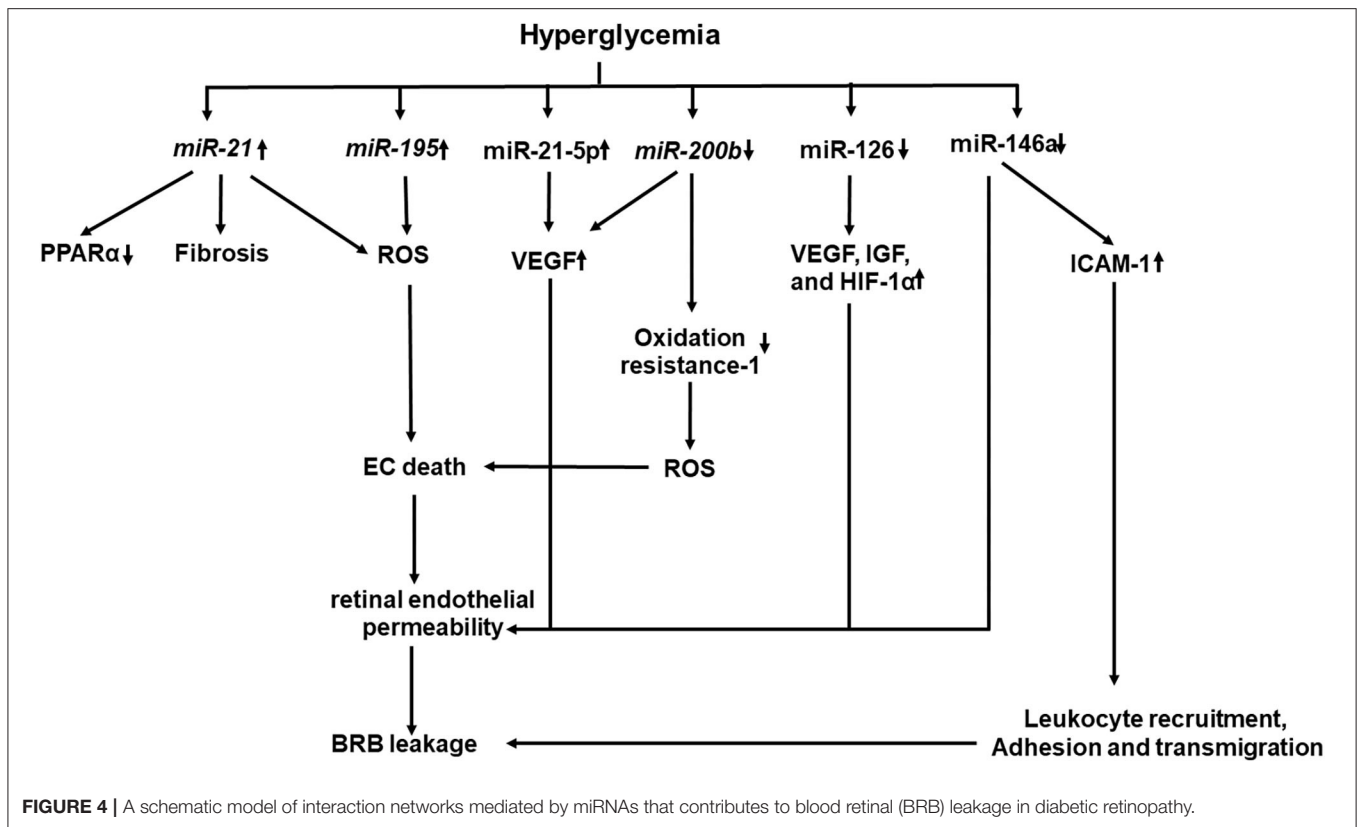
Increased miRNAs, such as *miR-21* and *miR-195*, have been demonstrated to be associated with fibrosis and oxidative stress in DR (189, 190). Increased *miR-21* level in the vitreous has been shown to be associated with retinal fibrosis in PDR (189). High glucose and TGF- β induce *miR-21* expression in retinal pigment epithelial cells. Furthermore, gain and loss of function studies have shown that *miR-21* promotes proliferation and migration of the human retinal pigment epithelium (189). *miR-21* affects PPAR α expression through inhibition of PPAR α mRNA translation (191). Intravitreal injection of the *miR-21* inhibitor attenuates PPAR α downregulation and ameliorates retinal inflammation in *db/db* mice (191). Knockout of *miR-21* prevents the reduction of PPAR α , which is associated with alleviated inflammation and microvascular damage in the retina of *db/db* mice. *miR-221* enhances retinal EC viability and angiogenesis through activation of PI3K/Akt/VEGF and inhibits the expression of PTEN (192). *miR-21* downregulates the expression of Krev interaction trapped protein 1 (KRIT1), Nrf2, and SOD2, all of which are involved in ROS homeostasis, while the *miR-21* inhibitor improves KRIT1 and SOD2 expressions, reduces ROS production, and ameliorates mitochondrial membrane potential in HUVECs treated with high glucose (193). More recently, plasma *miR-21* has been proposed to be an early marker for diagnosis and identification of diabetic nephropathy in type 1 diabetes mellitus (T1DM), as it starts to rise before microalbuminuria in patients with T1DM and has a greater sensitivity (94.1%) and specificity (100%) to identify DN than the urinary albumin/creatinine ratio at level 45 mg/gm with sensitivity of 88.2% and specificity of 89% (194).

High glucose stimulates *miR-21-5p* expression, in parallel with increased VEGF and VEGFR2 expressions and proliferation of human retinal microvascular ECs (195). Inhibition of *miR-21-5p* reduces proliferation, migration, and tube formation of human retinal microvascular ECs (HRMECs) through PI3K/AKT and ERK pathways (195).

Upregulated *miR-195* and downregulated SIRT1 have been observed in human retinal ECs exposed to high glucose and in the retinas of diabetic rats (190). Inhibition of *miR-195* recovers SIRT1 expression and decreases retinal damage in DR (190). In addition, oxidative stress induces overexpression of *miR-195* which downregulates mitofusin two expression in human retinal ECs and diabetic retinas, leading to increased permeability of retinal BRB (196).

Downregulated miRNAs in DR

Decreased miRNAs, such as *miR-126*, *miR-146a*, and *miR-200b*, have been shown to increase the angiogenic factor product, promote the NF- κ B pathway, and enhance VEGF-A expression and oxidative stress in DR, respectively. *miR-126* is involved in the production of angiogenic factors to mediate retinal neovascularization (197, 198). A significant reduction of *miR-126* in the serum is detected in patients with diabetes and macrovascular complications (199) or PDR (200). Downregulated *miR-126* is observed in the retinas of oxygen-induced retinopathy, while restoring its level



reduces high expression levels of VEGF, IGF, and HIF-1 α , which limits retinal neovascularization through p38MAPK and ERK pathways (197). *miR-126* is downregulated in hypoxia-treated rhesus retinal ECs and in retinas of diabetic rats, while restoring *miR-126* expression inhibits the hypoxia-induced neovascularization by inhibiting cell-cycle progression and the expression of VEGF and matrix metalloproteinase nine. Interestingly, hyperglycemic/hypoxia-treated mesenchymal stem cell-derived extracellular vesicles downregulate *miR-126* in pericytes, which express more VEGF and HIF-1 α (201).

miR-146a has a regulatory role in the NF- κ B-mediated inflammatory pathway. It binds to the 3'-UTR of I IL-1 receptor-associated kinase 1 to reduce the expression of NF- κ B-responsive ICAM-1 in both human retinal ECs and retinas of diabetic rats (202). Intravitreal delivery of *miR-146a* inhibits the hyperglycemia-induced upregulation of ICAM1 and reduces microvascular leakage and retinal functional defects. Increased *miR-146a* protects human retinal ECs from high glucose-induced apoptosis through suppressing the STAT3/VEGF pathway (203). Decreased *miR-146a* expression has been shown to be associated with the overexpression of fibronectin in high glucose-treated ECs and retinas of diabetic rats (204). Decreased *miR-146b-3p* has been shown to be associated with increased adenosine deaminase-2 (ADA-2) activity in the vitreous of patients with diabetes, while elevated expression of *miR-146b-3p* suppresses the ADA2 activity and TNF- α release in amadori-glycated albumin (AGA)-treated human macrophages (205) and decreases human

retinal EC permeability and leukocyte adhesion by upregulating ICAM-1 (205).

Decreased *miR-200b* and increased VEGF-A gene expression were observed in the sera of patients with DR (206). Decreased *miR-200b* is observed in high glucose-treated human retinal ECs and is accompanied with increased expressions of VEGF and transforming growth factor β (206). Increased *miR-200b* expression inhibits the oxidation resistance one expression, which enhances resistance to apoptosis and oxidative stress (207).

A number of miRNAs have been investigated and are considered as a therapeutic target of DR. However, as a single miRNA can regulate several target genes that modulate different signaling pathways, miRNA-based therapy should be more refined and controlled for its targeting genes. The systematic understanding miRNA action mechanism may help for the early diagnosis and improved therapeutics for DR.

OTHER FACTORS CONTRIBUTING TO OR ASSOCIATED WITH DR

In addition to the above discussed factors, recently studies identified new factors which may contribute to DR. Hyperglycemia induced circulating mitochondrial DNA change in parallel with increased circulating interleukin-4 and TNF- α in patients with DR, suggesting that mitochondrial DNA change in early diabetes may be an indicator of inflammation

and progression of DR (208). Loukovaara et al. have found that the nucleotide-binding domain and leucine-rich repeat receptor containing pyrin domain 3 (NLRP3) inflammasome activation is associated with the vitreous pathogenesis of PDR (209).

Monosodium urate (MSU) has been found in human retinas and vitreous (210). Its level is correlated with inflammatory biomarkers and increased expression of xanthine oxidase (210). The MSU level is also detected in the serum and vitreous of diabetic rats and associated with NLRP3 inflammasome, suggesting a role of MSU in diabetes-induced retinal inflammation.

Shalaby et al. showed that a disintegrin and metalloprotease domain-17 (ADAM-17) might be involved in the development of DR (211). ADAM-17 activity is upregulated in human diabetic retinas and diabetic mouse. Loss of ADAM17 in ECs significantly reduces oxidative stress and decreases leukocyte adhesion *in vivo* and *in vitro*. Reduction in oxidative stress in retinal ECs is mediated by downregulation of NADPH oxidase 4 expression, while reduced leukostasis is achieved through downregulation of ICAM-1.

Asymmetric dimethylarginine level has elevated in both aqueous from diabetic rats and culture medium in rhesus retinal ECs pretreated with hypoxia (212). ADMA promotes proliferation, migration, adhesion, and tube formation of rhesus retinal ECs through the ephrin-B2 pathway.

CHALLENGES

Although we know that diabetes causes DR, not all patients after many years with diabetes develop DR, even in patients with poor glycemia. In contrast, some patients under good glycemic control still develop vision-threatening DR complications, indicating that we are still unclear what is the master regulator that initiates

and controls the progress of diabetic retinopathy. VEGF has been proposed; however, a substantial proportion (40–50%) of patients with DME do not respond satisfactorily to anti-VEGF treatment (2).

Almost all cells in the retina can serve as effectors or donors of pro-inflammatory cytokines, VEGF, and ROS, through which to affect each other. Thus, it is difficult to dissect which cell type plays roles at which stage of DR. Knowing this is important and, as it may decide the outcome of treatment as various responses from different retinal cells, may abolish therapeutic effect.

The complex interactions among multiple contributors indicate that DR is a much more complicated disease situation. Treatment targeting at a single factor will be insufficient to reverse the progression of DR.

FUTURE DIRECTION

Diabetes is a metabolic disease which is associated with lifestyle, environment, and genetics. Genetic factors may determine the discrepancy of DR morbidity and severity seen in patients with diabetes. A genome-wide association study associated, including RNA-seq, with metabolomics may be needed to identify signature genetic information associated with phenotypes seen in DR and metabolites. These techniques will not only enrich our understanding of the molecular mechanism in the initiation and progression of DR but also provide new molecular targets.

AUTHOR CONTRIBUTIONS

FG: conception and first draft and revision. ZY, SF, and HW: first draft of manuscript. YZ: revision. All authors: contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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