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Current Understanding of the Molecular and Cellular Pathology of Diabetic Retinopathy

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Abstract

While diabetes has profound effects on multiple organ systems, the loss of vision caused by diabetic retinopathy may be of one of the most impactful in a patient's life. The retina is a highly metabolically active tissue that requires a complex interaction of cells spanning light sensing photoreceptors to neurons transferring the electrochemical signal to the brain with support by glia and vascular tissue. Neuronal function depends on a complex inter-dependency of retinal cells that includes the formation of a blood-retinal barrier (BRB). This dynamic system is negatively impacted by diabetes, which alters normal cell-cell interactions and leads to profound vascular abnormalities, loss of the blood-barriers and impaired neuronal function. Understanding the normal cell signaling interactions and how they are altered by diabetes has already led to novel therapies that have improved visual outcomes for many patients. Recent research highlighted in this review, has led to new understanding of retinal pathophysiology during diabetes and uncovered potential for new therapeutic avenues to treat this debilitating disease.

Introduction

Diabetic retinopathy (DR) is one of the most common complication of diabetes and remains a leading cause of visual loss and blindness globally[1]. Diabetes impacts many components of the eye, but the primary vision threatening pathology occurs in the retina. Research has revealed alterations to both neuronal and vascular cells of the retina in DR. While a complete understanding of disease etiology is needed, recent breakthroughs for treating DR that focus on targeting vascular endothelial growth factor A (VEGF-A) now provide effective treatment options in the clinic. However, anti-VEGF therapy is only effective in the late stages of DR, requires regular intravitreous injections and not all patients respond optimally. The increasing rate of diabetes globally, the need to prevent progression from the early stages of DR, patients that fail to respond to anti-VEGF therapy and patients with

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ischemic retinopathy for which anti-VEGF is inappropriate, collectively requires the need for the development of new therapeutic approaches for this disease.

This review focuses on the current understanding of the molecular and cellular pathology of DR with a primary focus on the cellular signaling between the neuronal and vascular retina that promote formation of the inner blood-retinal barrier (iBRB) of the retinal vasculature as an important point of intervention. Changes in visual function will be correlated with novel retinal biomarkers identified by clinical imaging modalities such as optical coherence tomography angiography and ultrawide field retinal imaging. New therapies under investigation that may complement current laser treatment and anti-VEGF therapy will be presented along with the mechanism of action. Finally, the translational potential of novel approaches such as the development of patient-derived cells and retinal organoids for experimental investigation and the potential of tissue restoration will be considered.

Classification of Disease Severity

Studies on pathogenesis and treatment of diabetic retinal disease rely on the use of accurate methods to classify DR that are reflective of its natural history. DR has been well described using the modified Airlie House classification scale as applied in the Early Treatment Diabetic Retinopathy Study (ETDRS)[2] and recently detailed in a position statement from the American Diabetes Association[3]. Altered retinal blood flow and vascular permeability[4], basement membrane thickening[5], loss of pericytes and acellular capillary formation[6] contribute to clinically visible nonproliferative DR (NPDR) lesions such as microaneurysms, venous beading and intraretinal microvascular abnormalities. As ischemia increases, patients may develop proliferative DR (PDR), which presents a substantial risk for visual loss due to neovascular complications such as vitreous hemorrhaging or retinal detachment as blood vessels grow into the vitreous[7] (figure 1).

Also linked to ischemia, diabetic macular edema (DME) develops as a result of abnormal permeability of retinal capillaries and from microaneurysms leading to the accumulation of extracellular fluid and thickening of the normally compact macular tissue. As the severity of DR increases, the risk of developing DME similarly increases[8]. Loss of vision from DME correlates with the location and extent of retinal thickening on optical coherence tomography (OCT) scans and, macular blood vessel permeability and perfusion as assessed by fluorescein angiography[9,10]. Data from the ETDRS evaluating eyes with DME have shown that thickening involving the center of the macula, termed center-involved DME, has a nearly ten-fold greater risk for developing moderate visual loss compared to eyes without center involvement[11].

The retinal pigment epithelium (RPE) and the underlying choroid are also compromised during diabetes. The RPE provides a barrier controlling exchange of metabolites from the rods and cones with the underlying choroidal vessels and imaging focused on the RPE reveal evidence of permeability in patients with DME[12] which may relate to breakdown of the outer BRB[13] and activation of inflammation-linked pathways that drive pathology in the photoreceptors[14]. The RPE also shows impaired regulation of fluid outflow during

diabetes that may be linked to dysfunction of the normal activity of Na/K ATPase pumps and aquaporin channels[15]. These outer retina changes occur concomitantly with what has been termed diabetic choroidopathy[16] which manifests as progressive non-perfusion of the choriocapillaris.

ETDRS severity levels have been used to guide clinical practice recommendations for patient follow-up and treatment. In the ETDRS, a total of 13 eye and 26 patient levels of severity have been described and have been used extensively in research and clinical trials. The American Academy of Ophthalmology formed a consensus panel and created a simplified classification called the International Clinical DR and DME Disease Severity Scale[17]. This scale simplified descriptions of the categories of DR but is not a replacement for ETDRS levels of DR in large-scale clinical trials or studies in which precise DR classification is necessary. Despite advances in retinal imaging, the current DR classification scales have not incorporated new approaches such as ultrawide field imaging for the retinal periphery or optical coherence tomography for macular edema or neuroretinal changes. The current grading scales are still largely based on clinically visible retinal microvascular lesions and do not include neurodegenerative changes that may occur early and distinct from vascular changes[18]. The evolution of DR classifications are inevitable and should include measures that will better prognosticate and predict patient outcomes. But until then, the ETDRS severity levels should remain the standard for determining disease severity in both clinical and research settings.

While the pathological changes that occur during DR are often considered a progression, it remains possible that environmental or genetic factors promote a specific pathology. Epidemiological studies have quantified the risks for developing DR or DME and have shown significant differences between type 1 (T1DM) and 2 (T2DM) diabetes mellitus[19]. Both glycemic control and diabetes duration have been found to be significant risk factors in the development to DR and DME[19]. However, the 25 year rate of developing some degree of DR is over 95% in T1DM and only 60% in T2DM[8]. Furthermore, the 10 year rate of developing DME is 20% in T1DM, 25% in T2DM taking insulin and 14% in T2DM not taking insulin[20]. In general, T1DM patients tend to develop more DR and PDR while T2DM patients taking insulin are more at risk for developing DME. Future research on understanding what causes patients to present with DME, PDR or aspects of inflammation and whether these represent a progression or separate pathologies are greatly needed.

Multiple large-scale clinical studies have shown that glycemic control is essential to preventing progression of diabetic complications and DR (reviewed in[21]). A meta-analysis of multiple population-based studies of DR reveals glycosylated hemoglobin, blood pressure, and serum total cholesterol associate with the incidence and progression of retinopathy but only explain 9% of DR progression and 10% of PDR development[22]. Therefore, additional factors likely contribute to disease pathology. A recent study has implied very long chain (VLC) fatty acids that incorporate into VLC ceramides affect endothelial barrier properties[23]. Diabetes leads to loss of elongases including ELOV4 and alters the retinal lipid profile[24]. Depletion of ELOV4 can reduce endothelial barrier properties while overexpression promotes barrier properties and reduces diabetes effect on permeability *in vivo*[23].

Markers for Disease Activity

The ETDRS standardized grading scale is based on 30° retinal images from 7 standard defined retinal fields and characterizes the extent of retinal lesions located in the posterior pole. However, ultrawide field imaging has demonstrated that retinal lesions can appear or develop outside of the ETDRS fields[25–30]. Predominantly peripheral lesions (PPL) describe eyes with DR lesions that are greater in extent or severity outside the ETDRS standard fields. Eyes with PPL were shown to have increased retinal nonperfusion compared to eyes without PPL. The cause of PPL is currently unknown and may involve loss of autoregulation in retinal arterioles or microvascular degeneration causing capillary nonperfusion and retinal ischemia[31]. PPL are present in ~50% of eyes with DR and identify a more severe level of DR in ~10% of eyes compared to standard ETDRS field imaging[25,27]. Moreover, the baseline presence of PPL in an eye suggests an increased risk of future DR worsening and the development of advanced, sight-threatening retinopathy over the subsequent 4 years by 3.2 and 4.7 fold, respectively[27]. These findings suggest that PPL may become a robust marker of DR progression. Another marker may be the presence of vitreous hyperreflective foci in OCT scans. In a study of 97 patients, these foci, presumed to represent inflammatory cells, were increased in patients with DME compared to control or diabetic patients without DME[32]. Future longitudinal analyses can reveal whether these scans provide true biomarkers for disease progression.

The advent of optical coherence tomography-angiography (OCT-A) is providing an unprecedented assessment of retinal vascular detail and may reveal important changes not previously observed by traditional methods available to ophthalmologists. OCT-A allows the noninvasive mapping of retinal vessels and blood flow allowing visualization of the retina and choroidal vasculature[33,34]. Both the superficial vessels and deep retinal vascular layers, can be readily differentiated with OCT-A enabling the identification of specific retinal capillary layers responsible for the underlying disease[35]. A deeper understanding of how capillaries change over the course of diabetes and in response to treatments for diabetic eye disease provided by OCT-A may provide novel insight into disease treatment approach. In addition, there has been recent interest in the use of metabolomics to identify biomarkers. Metabolomic analysis of vitreous and serum samples have identified dysregulation in pathways such as the pentose phosphate pathway, arginine to proline pathway, polyol pathway and ascorbic acidic pathways[36,37]. However, further research is necessary to establish causative and longitudinal associations with DR.

Diabetes Alters the Neural/Vascular Interaction in the Retina

The retinal neurovascular unit (NVU) refers to the inter-dependency of the vascular endothelial cells with pericytes, glia, neurons and retinal-resident immune cells. While the vasculature provides the required nutritional support for the neural tissue, the neural and glial cells along with pericytes, signal to the vascular endothelial cells creating the blood-retinal barrier (BRB) providing tight control of the neural environment (figure 2A). An early deficiency in the function of the NVU in diabetes is observed after short-term diabetes in animal models[38] and patients[39] and results in impaired neurovascular coupling, loss of autoregulation and control of blood flow as well as disruption of the iBRB. Diabetes

also impacts Müller glia leading to mis-localized active transport mechanisms of inwardly rectifying channels at the capillary:Müller glial interface that contributes to swelling of Müller glia in diabetic retina[40]. Changes to Kir4.1 and aquaporins on Müller glia are consistent findings in diabetic animal models[41] and these changes can be rectified by blocking the accumulation of lipoxidation end products[42]. Further, Müller glial response in diabetes may amplify inflammation by activating microglia through $P2X_7$ purinergic receptors leading to neuroinflammation and vascular damage, including leakage[43]. Indeed, microglial activation has a significant impact on the retinal NVU and neuroinflammation-driven breakdown in the inner BRB in DR[44].

Vascular endothelial changes have so far, represented the only successful therapeutic target for diabetic retinopathy. Laser photocoagulation has long provided an effective means of controlling proliferation and edema in many patients[45]. More recent success in treating DR has evolved in the clinic by targeting factors that drive microvascular abnormalities. Vascular changes in DR have been attributed in part, to elevated VEGF-A that signals to retinal endothelial cells altering the blood vessel permeability and promoting neovascularization (reviewed in[46,47]). Multiple, multi-center clinical trials have demonstrated targeting VEGF-A with antibodies or trap can effectively reduce DME, prevent further vison loss and, in some patients, improve vision[48–50]. Among patients with PDR, anti-VEGF-A therapy has been shown to prevent or reverse neovascularization with 43% of treated patients demonstrating resolution of neovascularization after 2 years and only 27% worsening since the previous visit[51]. However, for PDR and DME[52] clinical studies reveal that approximately half of patients receive benefit while others remain unresponsive to anti-VEGF-A therapy suggesting other factors may drive disease pathology in DR. Interestingly a recent study has shown significant correlations between inflammatory cytokines and VEGF and, in particular, that the iBRB is regulated by localization of the tight junction protein claudin-5 via rho-associated coiled-coil–containing protein kinase (ROCK) activation. Administration of ripasudil, a selective ROCK inhibitor, attenuated retinal inflammation and claudin-5 redistribution. When combined with an anti-VEGF agent, this ROCK inhibitor was synergistic in suppressing cytokine upregulation, monocyte/macrophage infiltration, macrophage/microglia activation, and claudin-5 redistribution, an effect that was demonstrated pre-clinically but also in patients resistant to anti-VEGF[53]. These data indicates that inflammation may be a key mechanism in the responsiveness to anti-VEGF therapy in DME.

Associated with VEGF-A, notch signaling may be altered in DR. In vascular angiogenesis during retinal development, VEGF-A signal stimulates an endothelial cell with the highest VEGFR2 response to become a tip cell that migrates toward the VEGF source and signal to neighboring cells to become the proliferating stalk cells of the angiogenic sprout. This cell to cell communication utilizes the notch signaling pathway with delta like canonical notch ligand 4 (DLL4) and notch receptor[54]. Recent studies suggest both DLL4 and the typical notch antagonist jagged-1, are increased in diabetic mouse models and in endothelial cells in a glucose dependent manner[55]. Intra-ocular injections of either ligand induced a modest increase in retinal permeability dependent on notch since conditional gene-deletion of notch prevented the permeability response. Further, a notch trap reduced permeability in a diabetic animal model[55].

Diabetes alters the normal pericyte endothelial interaction in the retina. Studies using targeted genetic deletion of pericytes reveal that pericyte coverage of retinal vessels is required for proper formation of the BRB[56]. Platelet derived growth factor (PDGF)-B signaling to pericytes controls vessel stabilization as deletion of PDGF-B retention signal, that localizes the growth factor to the pericellular space, also causes deterioration of retinal vessels[57] and PDGF receptor-β blocking antibody induces retinal hemorrhage and permeability in a FOX01 dependent manner[58]. Interestingly, this study also revealed that loss of retinal pericytes in adult mice using inducible, targeted diphtheria toxin expression, does not confer leaky retinal vessels as observed in other organs such as lung and skin. Instead, loss of pericytes make the retinal vasculature highly susceptible to VEGF-A signaling with a dramatic increase in hemorrhage and vascular permeability to dextran[58]. Pericytes control endothelial expression of angiopoietin 2 and VEGFR2 through transcription factor FOX01, with loss of pericytes dramatically promoting VEGF signaling. This heightened response of retinal vascular endothelial cells to VEGF-A after pericytes loss has stark implications for the well-established loss of retinal pericytes in diabetes. In addition, chronic hyperglycemia has been shown to reduce PDGF receptor tyrosine kinase signalling which promotes pericyte apoptosis and diabetic vasculopathy through activation of protein kinase C-δ (PKC-δ) and increased expression of the tyrosine phosphatase Src homology-2 domain–containing phosphatase-1 (SHP-1)[59,60]. These studies provide a mechanistic link to diabetes.

Glial cells provide Wnt signaling to retinal vascular endothelial cells required for formation of the BRB and may be a target for treating DR. The cytokine norrin is not a Wnt molecule but like Wnt, norrin signals through the frizzled 4 (FZD4) receptor complex[61]. Gene deletion studies of norrin, the receptor frizzled 4 or the co-receptors low density lipoprotein receptor-related protein 5/6 (LRP5/6), or tetraspanin (TSPAN)12 reveal that this signaling complex is required for both retinal angiogenesis[62] and BRB formation[63,64]. Importantly, norrin and FZD4 knockout mice show high retinal vascular permeability that correlates with reduced endothelial cell border immunostaining of the TJ protein claudin 5, and increased expression of the transcytosis marker and plasmalemma vesicle associated protein. Further, this phenotype can be reversed by the expression of a stabilized form of β-catenin revealing the role of canonical Wnt pathway. Studies have begun to explore whether norrin signaling may be used to restore vascular function in animal models. Norrin treatment may reduce avascular area and inhibit neovascularization in oxygen-induced retinopathy models[65] and transgenic expression of norrin may reduce vaso-obliteration and promote vascular growth[66,67]. Recent studies demonstrate that norrin can reverse VEGF induced permeability in cell culture and in animals after intravitreal injection of VEGF or in diabetes[68]. Interestingly, these studies reveal that VEGF actually promotes norrin signaling by increasing membrane content of the FZD4 co-receptor TSPAN12. The addition of norrin after VEGF then promotes barrier induction suggesting a potential novel approach to vascular restoration. It will be important to ascertain whether norrin expression, as well as other Wnt signaling mediators, changes during diabetes to determine whether neuronal changes in Wnt signaling alter BRB in diabetes.

A variety of studies suggest cell signaling through inflammatory factors may contribute to DR pathogenesis. Vitreous proteomic analyses have identified a host of altered inflammatory

factors in the vitreous or aqueous humor at varying stages of diabetic retinopathy (reviewed in[69] and[70]), many of which are highlighted here. Gene deletion and cytokine capture studies in animal models have provided strong evidence for a role of tumor necrosis factor-α (TNF-α)[71,72] in DR and evidence of leukostasis with a role for intercellular adhesion molecule-1 or its binding partner CD18[73]. Human studies of vitreous fluid have found an association of elevated interleukin IL-1β and TNF-α in PDR patients[74–76]. IL-6, IL-8 and chemokine, C-C motif, ligand (CCL)-2 were also identified as elevated in patients with diabetic macular edema and PDR[77]. Conversely, antiangiogenic mediators such as pigment epithelium-derived factor (PEDF) have been reported to be in low patients with diabetes and in patients with active PDR[78]. Studies demonstrate targeting inflammation by inhibiting atypical protein kinase C (aPKC) may control vascular permeability in the retina. The aPKC isoforms contribute to endothelial permeability from a variety of inflammatory factors and growth factors including VEGF and also contribute to NFκB activation[79,80]. Reducing aPKC activation with a small molecule inhibitor or conditional expression of a dominant negative form of the kinase reduced permeability and monocyte and granulocytes recruitment in models of retinal inflammation[81]. Beyond broad-spectrum, anti-inflammatory approaches such as corticosteroids already in clinical use, targeting specific cytokines based on measures of patient vitreous or aqueous cytokine profiles remains an exciting possibility to improve therapeutic options[82].

Given sufficient time the development of DR is nearly universal in patients with diabetes[8] but the development of PDR plateaus at 60%, even after more than 50 years of diabetes[8,83]. Therefore, there may be protective mechanisms that delay or prevent the progression to PDR[84]. Proteomic analysis identified elevated concentrations of photoreceptor-secreted retinol binding protein 3 (RBP3) in the retina and vitreous of patients protected from advanced DR despite diabetes durations of over 50 years[85], consistent with earlier findings that RBP3 was reduced in the general patient population with diabetic retinopathy[86]. Retinal cell based assays and rodent models have demonstrated that RBP3 can prevent diabetes induced vascular permeability and altered retinal function measured by electroretinogram (ERG)[85]. RBP3 may have a role in protection against the progression of DR by decreasing the expression and signalling of inflammatory cytokines and VEGF. Further this group provided evidence for RPB3 reducing glucose uptake into Müller cells by binding and inhibiting glucose transporter 1, thereby mitigating the effects of chronic hyperglycemia[85]. These studies require further exploration of the normal physiological role of RBP3 in mediating glucose uptake but provide novel insight into retinal metabolism and potential therapeutic approaches to treat DR.

Novel Pathogenic Pathways

Studies have identified a range of alternative neurovascular signaling pathways that lead to leakage and/or neovascularization in addition to VEGF. Amongst the most promising targets is the kinin–kallikrein system. Carbonic anhydrase I (CA-1) and activation of plasma kallikrein (PK) was identified in the vitreous of patients with advanced DR[87]. Subsequent studies established PK cleavage of kininogen generates bradykinin which acts through bradykinin receptors on the blood vessels to induce permeability. Inhibitors of PK can block or reduce retinal permeability in animal models of diabetes and in response to direct CA-1

and PK injection[88] but not VEGF-A, suggesting a distinct pathway of vessel permeability. Currently, a range of PK inhibitors are being tested in clinical trials for patients with DME.

Recent experimental studies have implicated angiopoietin like 4 (ANGPTL4) in DR. ANGPTL4 was initially found elevated in aqueous fluid from the anterior chamber of patients with DME and the level of ANGPTL4 correlated with the ability of the aqueous fluid to induce permeability in an endothelial cell culture assay[89]. ANGPTL4 is downstream of hypoxia inducible factor regulated gene transcription and can induce endothelial permeability[90]. Interestingly ANGPTL4 was shown to bind to neuropilin and activates the small G-protein RhoA[89]. Neuropilin is a co-receptor for VEGFR2; however, the ability of ANGPTL4 to induce permeability was independent of VEGFR2, demonstrated in knockdown studies in cell culture. A soluble form of neuropilin was able to block ANGPTL4 and VEGF-induced permeability in cell culture and mice. It should be noted that there are a number of conflicting reports of the role of ANGPTL4 in permeability. For example, studies reveal ANGPTL4 can reduce permeability in the brain in stroke and can specifically attenuate VEGF induced permeability by inhibiting Src phosphorylation and activation[91] and recent findings reveal ANGPTL4 can inhibit pro-inflammatory genes and promote anti-inflammatory genes in macrophages in cell culture and in a myocardial infarct model[92]. Clearly additional studies on the complex role of ANGPTL4 are needed. However, a previous study identified another neuropilin binding protein semaphorin 3A (Sema3A) also induces retinal permeability and conditional knockout of neuropilin prevented Sema3A induced permeability but not VEGF induced permeability[93]. Targeting neuropilin may provide a novel option to treat DR and may potentially prevent induction of permeability from multiple sources if no toxicity is associated with this therapy.

Gene expression studies of pathological angiogenesis identified elevated expression of leucine-rich alpha-2-glycoprotein 1 (Lrg1) that promotes neovascularization. Lrg1 modifies transforming growth factor β (TGF-β) signaling by binding to the accessory receptor endoglin and promoting a pro-angiogenic signaling pathway[94]. Lrg1 knockout mice have a modest delay in retinal vascular development but both the knock out mice or an anti-Lrg1 antibody could dramatically reduce pathological angiogenesis in animal models. Currently, a humanized monoclonal antibody to LRG1 called Magacizumab is undergoing phase I/IIa clinical trial and could potentially provide an additional therapeutic option for PDR.

Finally, the direct role of hyperglycemia on endothelial cells has been extensively studies. However, a recent study provides an intriguing model of hyperglycemia regulated epigenetic control of oxidative stress in endothelial cells. Using siRNA and pharmacological inhibition of DNA methylation and hydroxymethylation, the investigators provide evidence for hyperglycemia induced increase in 5-hydroxy methyl cytosine and NFkB induced gene activation of Ras-related C3 botulinum toxin substrate 1 (Rac1)[95]. Rac1 is an essential component of NADPH oxidase 2 (Nox2) promoting reactive oxygen species (ROS) production, which is activated early in hyperglycemia induced endothelial cell dysfunction and contributes to mitochondrial production of ROS. These studies provide a novel model to link hyperglycemia induced epigentic gene regulation to ROS production and mitochondrial dysfunction (reviewed in[96]).

Altered Neuronal Function

While most clinical focus has been on vascular pathology during DR, there is now wide recognition that the impact of diabetes more broadly affects the cells of the retina. In addition to the clinically visible vascular defects, evidence points to changes occurring in the neural retina as well. For example, apoptosis of non-vascular cells has been consistently identified in animal models of DR[97]. Longitudinal studies of patients with diabetes suggests retinal degeneration as observed by thinning of the nerve fiber and ganglion cell layer, termed retinal neurodegeneration, without evidence of vascular pathology[98]. A number of changes in retinal function have been characterized that may occur before clinically observable vascular pathology including reduced electrical response as measured by ERG and diminished contrast sensitivity (reviewed in[99,100]). A recent animal study found that apoptosis in the diabetic retina depended on protein regulated in development and DNA damage response 1 (REDD1)[101]. REDD1 promotes dephosphorylation and inhibition of Akt kinase activity allowing the transcription factor FOXO1 to promote cell death. Depletion of REDD1 in retinal neural cell culture prevented hyperglycemia induced apoptosis and deletion of REDD1 in mouse reduced diabetes induced retinal apoptosis and attenuated aspects of visual loss, most prominently loss of b-wave intensity in scotopic ERG and loss of contrast sensitivity. Some factors may directly impact both vascular and neural tissue such as endothelin which impacts vascular and neural tissue through different receptors subtypes. Recent data revealed topical administration of an endothelin antagonist in a diabetic mouse model prevented neurodegeneration[102].

While vascular and neuronal changes clearly both occur during DR, the question remains whether neural or vascular dysfunction initiates the disease process or whether the alterations are coincident but unrelated. The European Consortium for the Early Treatment of Diabetic Retinopathy (EUROCONDOR) recently studied 449 diabetic patients with no versus mild vascular defects as assessed by ETDRS scoring and measured alterations in retinal function using multifocal ERG (mfERG) or retinal structure measured by OCT. The study found 61% of patients without microvascular disease presented abnormalities related to neurodegeneration assessed by mfERG or OCT[103]. Conversely, 32% of patients with visible microvascular disease did not present any sign of neurodegeneration. It is important to note that the lack of observable vascular defects do not confirm unaltered vessel function. However, the authors raise the possibility of distinct disease etiology in DR. The use of conditional gene regulation targeting specific cell types is necessary to begin to elucidate the causal relationship between retinal vascular and neural changes observed in animal models of diabetes. Further, longitudinal studies of patient populations assessing vascular and neuronal alterations and retinal function are needed to clarify potential differences in disease progression that will inform therapeutic approaches.

There is growing clinical evidence that neurovascular changes occur in the brain of patients with diabetes, especially in the context of T2DM leading to increased risk of dementia[104] or Alzheimer disease[105]. There is also growing evidence of an association between retinal vessel abnormalities and cognitive impairment and dementia[106] with the possibility of retinal imaging as an effective biomarker for neurodegenerative diseases. Recent proteomic analysis of the vitreous has identified changes in proteins associated

with Alzheimer's and Parkinson's disease[107]. While these studies are intriguing, significant research with mechanistic detail is needed to explore a potential role for diabetes in brain neurodegeneration and similarities or differences with the retina.

Extensive studies have illuminated a role of oxidative stress in contributing to the pathology of DR (reviewed in[108]). NF-E2-related factor 2 (Nrf2) is a transcription factor that is a master regulator of a host of genes that act in a cytoprotective manner and provide cellular antioxidant gene products. Nrf2 is normally bound and inhibited by Keap1, targeting Nrf2 for degradation. Stress induced alteration in Keap1 binding stabilize Nrf2 and provide cellular protection. Gene deletion of Nrf2 exacerbates the degree of retinal ischemia and increases pre-retinal neovascularization in the oxygen induced retinopathy (OIR) model[109]. The OIR model takes advantage of the plasticity of the neonatal retina and creates a central retinal ischemia which drives pathological angiogenesis. By using broad neuronal conditional knockout, glial specific knockout and endothelial knockout, the investigators demonstrated this effect on vascular development, particularly the increased avascular area, was driven by neuronal cells. Loss of Nrf2 increases expression of semaphorin (Sema)6a that acts extracellularly on endothelial cells through notch signaling. The vascular pathologies in the Nrf2 knockout animals were reversed by lentiviral delivery of shRNA targeting Sema6a. A role of Nrf2 signaling in diabetes was demonstrated with increased vessel permeability and loss of visual acuity in Nrf2 gene deleted diabetic animals[110] and an ischemia reperfusion (IR) model was used to demonstrate a role of Nrf2 in retinal ganglion cell protection[111]. IR was previously shown to model aspects of VEGF-dependent vascular permeability, inflammation and retinal cell loss observed in diabetes but more dramatically and over a shorter time-frame[112,113]. Recent studies reveal an impressive protection of visual acuity with a Nrf2 activating drug in the IR model[114] suggesting this approach may provide therapeutic benefit for neurons in ischemic retinal diseases including DR.

Potential for Regenerative Medicine

Recent studies have begun to explore the potential for retinal vascular regeneration. In a clinical case study, spontaneous re-perfusion of ischemic retina followed by recovery of visual acuity has been reported following radiation retinopathy[115] thus suggesting that return of adequate blood flow can restore retinal function. Spontaneous re-perfusion of the ischemic diabetic retina has been reported[116 117] although this is generally a rare occurrence in DR and there is a general assertion that normal vascular reparative processes are defective in early diabetes and can, at least in part, account for the observed progressive vascular degeneration. The diabetes-related deficiencies in vascular repair processes are not well-understood although it is widely appreciated that diabetic patients suffer exacerbated cardiac and peripheral limb ischemia through reduced collateral vessel development and abnormal repair following infarct[118]. Interestingly, the Joslin Medalist T1DM cohort show normal levels of endothelial progenitor cells (EPC) and circulating progenitor cells when compared to other patient cohorts with diabetes suggesting endogenous, protective factors may serve to provide a protective effect in the Medalist[119]. Indeed, there is increasing evidence that diabetes suppresses resident progenitor cells that would normally be activated by injury[120,121]. This is especially true for the recently identified, side population cells that possess a progenitor phenotype in the endothelium[122,123]. Lineage tracing experiments in

mice show that these self-renewing progenitors can become activated by vessel damage after which they re-establish a viable endothelium and restore perfusion[123–127]. Although currently unknown, altered retinal progenitor cells could account for the observed deficits in repair in DR.

In view of diabetes-related damage to the retinal NVU, a strategy that could replace damaged endothelium is attractive and has clear translational potential. Cell therapy using vasoactive progenitors has received attention since such cells are recruited to sites of capillary loss where they promote re-perfusion[128]. Various cell-types including $CD34^+$ cells[129], Lin^- hematopoietic stem cells (HSCs)[130], $CD44^{hi}$ cells[131] and circulating angiogenic cells[132] have all been shown to enhance tissue repair of ischemic tissues in pre-clinical models, including the retina. Although described as endothelial progenitor cells (EPCs), many such populations are, in fact, heterogenous mixtures of myeloid cell types with no evidence of incorporation into the vasculature[133]. Unfortunately, the majority of clinical trials using the heterogenous and poorly defined EPCs have been disappointing[134]. However, an ongoing, retina-focused trial using CD34+ cells has demonstrated in Phase I that intravitreous delivery is safe[135] and clinical trial for various retinopathies, including DR, is currently ongoing (ClinicalTrials.gov Identifier: NCT01736059). This is encouraging, although in the context of DR there may need to be some caution since recent evidence suggests that the use of progenitors that carry myeloid markers may actively participate in pro-inflammatory responses[136].

Perhaps the most promising cell from a therapeutic perspective is the EPC-type called endothelial colony forming cells (ECFCs) isolated from peripheral adult blood or umbilical cord blood. These have proven to be homogenous and distinct from HSCs and cells sorted on $CD34^+$ [133]. ECFCs possess many endothelial and progenitor cell characteristics and lack the hematopoietic markers CD45 and CD14. They also possess *de novo* endothelial tube forming potential *in vitro* and *in vivo* and can form *de novo* vessels or directly incorporate into pre-existing capillaries[137–140]. *In vivo*, ECFCs appear to share properties with side population cells that are present in the vasculature[122] and can become activated by vessel damage upon which they incorporate into the endothelium[124–126]. Emerging pre-clinical studies validate the potential for ECFCs in diseases where vascular insufficiency is a cardinal feature such as stroke, peripheral artery disease, heart disease, and DR[141]. Intravitreous delivered ECFCs have been shown to migrate to ischemic retina and activate vascular repair[142–144]. In diabetic mice, ECFCs combined with recombinant angiopoietin 1 gene therapy, prevent barrier dysfunction and restores vision as measured by opto-kinetic functional readouts[145].

While most attention has been focused on replacement of damaged endothelial cells, in the diabetic retina there is also a need to restore other damaged cells. For example, replacing lost pericytes may be possible using mesenchymal stromal cells (MSCs) since their potential has been shown to reside adjacent to retinal vessels and adopt pericyte-like phenotype which maintains vascular integrity[146,147]. Although not yet studied in diabetic retina, there is likewise potential for replacement of defective Muller glia, RPE and perhaps even neurons[148].

Patient-Specific Cells and Organelles

The potential of using induced pluripotent stem cell (iPSC) technology to produce retinal organoids has already led to significant impacts in ophthalmology and vision science. So-called “retina in a dish” approaches have been developing apace in recent years and they have provided great insight into both developmental biology and retinal neurodegenerative diseases[149,150]. Indeed, iPS-derived, patient-linked cells are already advancing other ophthalmic disease fields such as informing the pathogenesis of drusen formation in iPSC-RPE from patients with macular degenerative disease[151]. DR research has been heavily reliant on animal models and while this has led to many important advances, there have always been limits in the clinical fidelity. Studies using retinal organoids for DR research may help circumvent this limitation. There are already findings on iPSC-RPE derived from T2DM patient donors revealing decreased barrier function and attenuated autophagic capacity when compared to iPSC-RPE from non-diabetic controls have been reported[152]. Depending on the pluripotency approach used, these iPSC-derived cells may carry an epigenetic imprint and harbor DNA methylation signatures characteristic of their somatic tissue of origin[153]. Use of iPSC has the potential to combine laboratory studies with clinically relevant cells to more fully understand DR phenotypes from a molecular perspective with the clear potential to develop more patient-specific therapeutic approaches.

Conclusions

Diabetes remains a leading cause of vision impairment worldwide. While the precise etiology of metabolic dysregulation contributing to loss of retinal functions remains to be fully elucidated, targeting VEGF-A cytokine signaling driving microvascular pathologies has proven effective in preventing disease progression and improving vision for many patients. Studies exploring the cellular communication of the NVU in the retina and the alterations that occur in diabetes may provide additional targets to treat those patients that fail to respond to current therapy. Disease management of DR may by further improved with the development of novel biomarkers that take advantage of the unique availability of retinal imaging. However, a better understanding of the disease etiology, what factors may drive DME or PDR, what specific cytokines or factors mediate specific disease processes and additional information on the genetic basis of susceptibility or protection to DR is needed. Accurate phenotypic description of patient populations coupled with analysis of altered cytokine profiles in vitreous or aqueous fluid may lead to precision medicine with improved patient outcomes. In basic research, studies that utilize conditional gene targeting to explore the cell communications in vivo are needed to elucidate the functional relationship of the cells in the neurovascular unit and the contribution to vascular and neuronal dysfunction in DR. Finally, regenerative and restorative approaches provide hope to restore retinal function lost by diabetes.

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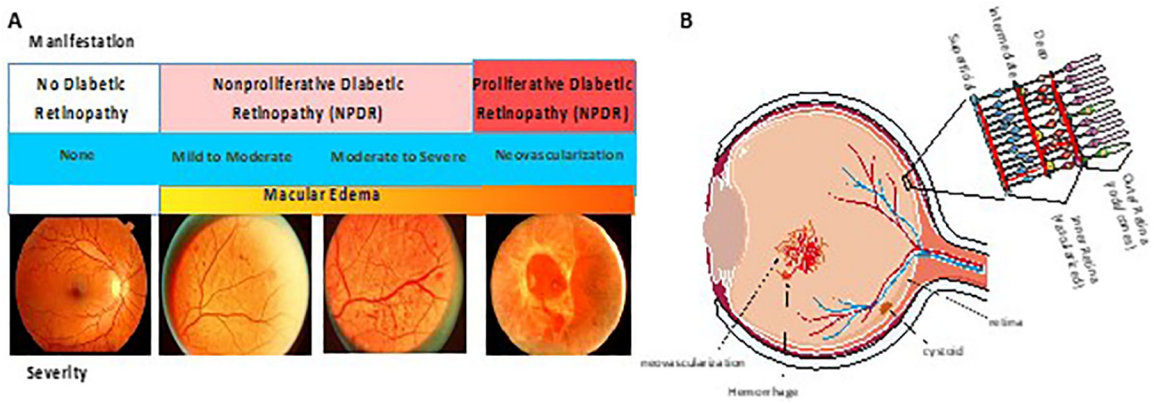

Figure 1. Diabetic Retinopathy (DR) Manifests with Multiple Pathologies.

A. Patients with diabetes may have no readily observable alterations to the retina as observed by fundus photography. Alternatively, microvascular abnormalities, hemorrhages, microaneurysms and venous beading reveal evidence of disease process that may range from mild to severe and occur in patients with non-proliferative DR (NPDR). Patients with proliferative DR (PDR) have neovascularization in the retina that may lead to retinal detachment. Diabetic macular edema (DME) may occur in both NPDR or PDR. (Adapted from[7]) B. Schematic diagram of a cross section of the eye. Vessel leak, neovascularization and cystoid formation due to DME are indicated. Cross section of retina indicating organization of ganglion cells and bipolar cells in the inner retina versus rods and cones in the outer retina. Blood vessels in the inner retina make the inner blood-retinal barrier and the retinal pigment epithelium (RPE) makes the outer blood-retinal barrier.

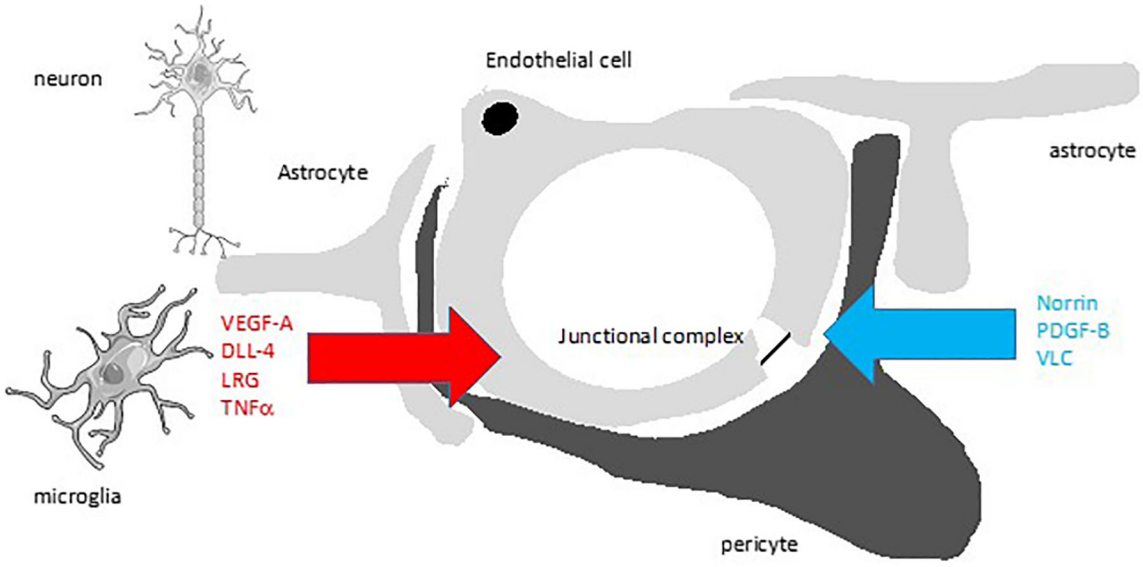

Figure 2. The Neurovascular Unit (NVU) and Cytokine Signaling in Diabetic Retinopathy (DR).

A) Proper retinal functions require an intimate relationship of the retinal blood vessels in the inner retina with neurons, glia (astrocytes and Müller cells), and pericytes. Glia provide norrin signaling required for BRB formation. Endothelial cells recruit pericytes by PDGF-B signaling and pericytes promote BRB by an unknown mechanism. B) In DR, glia have increased aquaporin and Kir4.1 channels contributing to swelling and now produce vasoactive substances such as VEGF-A and associated DLL-4, ANGPTL4 and LRG that promote permeability, angiogenesis or both. Loss of pericytes leads to hyper-responsiveness of endothelial cells to VEGF signaling. Further, inflammatory cytokines such as TNFα, IL1β and CCL2 among many others, are produced by microglia and other retinal cells as well as adherent inflammatory cells. In addition, hyperglycemia induces direct endothelial dysfunction through change in redox state (NAD(P)H and ROS). Not shown, RPE also undergo dysfunction with increased cytokine production. Collectively these changes disrupt the neurovascular unit and alter normal retinal function.