Advances in our understanding of diabetic retinopathy

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Abstract
Diabetic retinopathy remains the most common complication of diabetes mellitus and is a leading cause of visual loss in industrialized nations. The clinicopathology of the diabetic retina has been extensively studied, although the precise pathogenesis and cellular and molecular defects that lead to retinal vascular, neural and glial cell dysfunction remain somewhat elusive. This lack of understanding has seriously limited the therapeutic options available for the ophthalmologist and there is a need to identify the definitive pathways that initiate retinal cell damage and drive progression to overt retinopathy. The present review begins by outlining the natural history of diabetic retinopathy, the clinical features and risk factors. Reviewing the histopathological data from clinical specimens and animal models, the recent paradigm that neuroretinal dysfunction may play an important role in the early development of the disease is discussed. The review then focuses on the molecular pathogenesis of diabetic retinopathy with perspective provided on new advances that have furthered our understanding of the key mechanisms underlying early changes in the diabetic retina. Studies have also emerged in the past year suggesting that defective repair of injured retinal vessels by endothelial progenitor cells may contribute to the pathogenesis of diabetic retinopathy. We assess these findings and discuss how they could eventually lead to new therapeutic options for diabetic retinopathy.

Key words: diabetes, diabetic retinopathy, retina, visual loss

EPIDEMIOLOGY OF DIABETIC RETINOPATHY

The worldwide incidence of diabetes is set to rise dramatically from 171 million people to an estimated 366 million in 2030 [1,2]. Type 1 diabetes is due primarily to autoimmune-mediated destruction of pancreatic β-cells, which leads to insulin deficiency. The frequency of Type 1 diabetes is low relative to Type 2 diabetes, which accounts for approximately 90% of diabetes worldwide. The phrase ‘diabetes epidemic’ refers predominantly to Type 2 diabetes, which is continuing to increase in both developed and developing countries [3]. This increase in Type 2 diabetes is mainly a consequence of increasing sedentary lifestyles, poor diet and obesity. Indeed, it has been estimated that the prevalence of diabetes among people aged >16 years will rise by 28.3% between 2010 and 2030, with 54.5% of this increase being attributed to increased obesity [4]. The health issues that diabetes imposes on societal healthcare are daunting, not least the range of debilitating complications associated with this condition. The macro- and micro-vascular complications arising from diabetes impose an ever-increasing burden on healthcare authorities globally.

Diabetic retinopathy is the most frequently occurring microvascular complication of diabetes and, although not all patients will suffer appreciable vision loss, this condition remains a leading cause of blindness. Following 20 years of diabetes, nearly all patients with Type 1 diabetes will have at least some retinopathy. Moreover, ~80% of insulin-dependent Type 2 diabetic patients and 50% of Type 2 diabetic patients not requiring exogenous insulin will have retinopathy after 20 years of diabetes [5,6]. In Europe and the U.S. alone, the WHO (World Health

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Organisation) has estimated that diabetic retinopathy accounts for approximately 15–17% of total blindness [7]. Worldwide, diabetic retinopathy is an even bigger problem, and a comprehensive study by Yau et al. [8] based on 22,896 individuals from 35 studies in the U.S., Australia, Europe and Asia demonstrated that the prevalence of diabetic retinopathy was almost 35% with increasing risk associated with diabetes duration, higher HbA1c (glycated haemoglobin) and hypertension. The prevalence of the sight-threatening stages typified by PDR (proliferative diabetic retinopathy) and DMO (diabetic macular oedema) was \( \sim 7\% \) [8].

DMO causes more vision loss than PDR and its effective treatment remains a major issue for healthcare authorities. For example, it has been shown recently [9] that the number of people with diabetes in England in 2010 was estimated to be 2,342,951 of which 2,334,550 were aged \( \geq 12 \) years. An estimated 166,325 (7.12%) had DMO in one or both eyes and, of these, 64,725 individuals had clinically significant reductions in visual acuity to poorer than 6/6 in at least one eye [9]. The overall health and social care costs in 2010, on the pathway from screening to rehabilitation and care in the home, were estimated to be £116,296,038 [9].

**CLASSIFICATION OF DIABETIC RETINOPATHY**

Diabetic retinopathy is largely asymptomatic and, by the time worsening vision is experienced, pathology may be significantly advanced. Therefore there is a need for screening to assess the presence and progression of the condition [10]. Various classifications are used to grade the severity of diabetic retinopathy, although traditionally it is classified into two main clinical forms: NPDR (non-PDR) and PDR. The PDR stage involves the formation of neovascularization that develops from the venous side of the retinal circulation and may penetrate the inner limiting membrane into the vitreous (Figure 1). Typically these new blood vessels are fragile and leaky and, if left untreated, can become enveloped by fibrous connective tissue. Adhesion often occurs between this fibrous tissue and the posterior hyaloid and resultant traction can lead to vitreous haemorrhage and/or tractional retinal detachment. Proliferative retinopathy occurs in approximately 50% of patients with Type 1 diabetes and in approximately 15% of patients with Type 2 diabetes who have the disease for 25 years [11].

The most commonly used grading system in clinical and epidemiological studies of diabetic retinopathy is the ETDRS (Early Treatment of Diabetic Retinopathy Study) scale [12], which relies upon a number of photographically detectable microvascular lesions as indicators of disease progression. Levels of NPDR are characterized by the number and severity of microaneurysms, dot and blot haemorrhages, cotton wool spots (nerve fibre layer infarcts), venous abnormalities (beading and looping) and IRMAs (intraretinal microvascular anomalies), which are large-calibre shunt vessels within non-perfused regions of the capillary bed. PDR involves the formation of new blood vessels and is graded according to the extent and location of new vessels at the optic disc or elsewhere on the fundus and the presence or absence of vitreous haemorrhage (Figure 1).

When diabetic retinopathy affects the macula and central visual acuity is threatened, it is termed diabetic maculopathy. In this area of the retina, excessive vasopermeability and oedematous damage is referred to as DMO and is the commonest cause of blindness in diabetes [13]. Although DMO can occur at virtually any stage of retinopathic development, it is most prevalent during the later phases of the disease [5]. Diabetic maculopathy is classified as (i) central or non-central, depending on whether the oedema affects the centre of the fovea, (ii) focal or diffuse, based on the extension of the area affected by the oedema, (iii) ischaemic or non-ischaemic, based on the preservation or involvement of the perifoveal capillary network, or (iv) mixed, when a combination of the above exists (Figure 2). Furthermore,
diabetic maculopathy has also been classified as tractional or not tractional depending on whether the presence of vitreoretinal traction plays a role in its occurrence.

RISK FACTORS FOR DIABETIC RETINOPATHY

Systemic factors and retinopathy

Identifying robust risk factors for diabetic retinopathy is difficult. At present, the best advice for diabetic patients is to maintain their glycaemic control, as indicated by HbA1c, as close to normal limits as possible. This was proven by DCCT (Diabetes Control and Complications Trial) for Type 1 diabetes conducted from 1983 to 1993 [14], although the benefits of so-called ‘tight’ glycaemic control took several years to manifest. Similarly for Type 2 diabetes, UKPDS (UK Prospective Diabetes Study) demonstrated that maintaining near normoglycaemia can significantly protect against the progression of diabetic retinopathy [15].

DCCT revealed that diabetic retinopathy can be offset with improved glycaemic control if treatment is commenced as soon as possible after diagnosis of Type 1 diabetes. However, previous periods of poor control are critical, as demonstrated in over 200 patients from the original DCCT who were followed for a further 10 years under the auspices of the EDIC (Epidemiology of Diabetes Interventions and Complications) trial [16]. This study revealed that the incidence of diabetic retinopathy was significantly less in the group initially maintained under tight glycaemic control and that these benefits extended beyond the period of intensified insulin therapy [16]. The patients under ‘conventional’ control for the first 10 years maintained a so-called ‘hyperglycaemic or metabolic memory’ and retained a strong association with retinopathic progression. Interestingly, the same memory phenomenon for retinopathy was also shown nearly 20 years previously by Engerman and Kern [17] in variously (insulin) controlled diabetic dogs.

Hyperglycaemia has been a focus for many studies on diabetic retinopathy, although it is clear that dyslipidaemia is also an important risk factor and that regulation of blood lipids should be considered as an effective means to offset progression of this complication. The seminal Hoorn Study identified dyslipidaemia as a clear additional risk factor for diabetic patients [18]. This is also shown by clinical trials such as FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) [19] and ACCORD (Action to Control Cardiovascular Risk in Diabetes) [20], which evaluated the role of fenofibrate and simvastatin + fenofibrate respectively. These drugs with proven lipidemic control modes of action are effective in ameliorating the progression of diabetic retinopathy, although it should be acknowledged that HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase inhibitors (statins) and fenofibrates may have an impact on a range of pathogenic pathways that are independent of lipids [21,22].

UKPDS served to highlight the importance of BP (blood pressure) as well as hyperglycaemia in diabetic retinopathy [15,23]. More recent studies, such as the META-EYE (Meta-Analysis for Eye Disease) study, have demonstrated a prevalence of diabetic retinopathy among people with normal BP of 5.5% compared with 10.6% in those with hypertension (BP >140/90mmHg or already on antihypertensive medication) [8]. It is now established that patients with diabetes should avoid systemic hypertension, which exacerbates initiation and progression of retinopathy [24].

Recent data suggests that controlling systemic modifiable risk factors could reverse DMO. In the RISE-RIDE trial, for instance, ∼18% of patients in the sham group had visual improvement of 15 letters and ∼30% of patients in the sham arm never received ‘rescue’ laser. The RISE and RIDE were parallel Phase III multi-centre double-masked sham-injection-controlled randomized studies that aimed to evaluate the efficacy and safety of intravitreal ranibizumab in DMO [25]. Patients (n = 759) were randomized to receive monthly sham injections or 0.3 mg or 0.5 mg intravitreal injections of ranibizumab. From month 3 onwards, patients were allowed to receive laser if the central foveal thickness was >250 μm with <50 μm change from a prior month, provided that macular laser had not been done in the previous 3 months and that it was considered by the evaluating physician that laser would be beneficial. The primary outcome was the proportion of patients gaining >15 ETDRS letter score from baseline at month 24. Only 70–74% of patients in the sham group received laser during the study period. As DMO was present in all patients entering the study and laser treatment was the standard of care at the time the RISE and RIDE studies were initiated, it would be expected that laser treatment would have been offered and applied to all patients in the sham group. Although other reasons may have accounted for the low rate of laser treatment performed in the sham arm, including spontaneous resolution of DMO [26], macular ischaemia (which would be a
contraindication for macular laser) or end-stage disease, it is possible that DMO may have improved or resolved in a large proportion of patients as a result of a tighter metabolic and BP control that could have been achieved as a result of patients being enrolled in RCTs (randomized control trials) in which monthly check ups were being undertaken [25].

**Genetic linkage to retinopathy?**
There have been many assessments of the role of hereditary factors in diabetic retinopathy (reviewed by [27]), but as yet there is no robust indication that this complication has a genetic component. This contrasts with diabetic nephropathy where some important associations have been highlighted in recent years [28]. Candidate gene studies and GWAS (genome-wide association studies) may yet find genetic linkage to particular retinopathy phenotypes and onset of pathology in relation to risk factors.

On the basis of the assumption that interactions between environmental factors and genetic predisposition could provide a powerful risk association to diabetic retinopathy, epigenetics has recently had an impact on this field. Studies on the direct and indirect effects of epigenetics and diabetic retinopathy are a fruitful area of investigation, although they add a further layer of complexity to the comparatively static genome. Chromatin alterations can persist in cells for their entire lifespan, controlling cell function/dysfunction without alteration to the encoding DNA sequence. Such epigenetic changes may play a key role in retinopathy initiation and progression. Various covalent modifications can occur in histones such as acetylation, phosphorylation or ubiquitination, but methylation via HMT (histone methyltransferase) enzymes at lysine residues is probably most important in the context of epigenetics [29]. Histone methylation at various promoter regions can significantly alter transcriptional regulation at defined promoters and lead to suppression or inappropriately sustained activation of a gene sequence. With applicability to diabetic retinopathy, it has been shown that exposure of vascular endothelial cells to high glucose, akin to the excursions in glycaemia observed in diabetes, induces significant epigenetic pathology [30]. Endothelial cells exposed to high glucose for 16 h followed by a return to normal glucose levels for 6 days leaves an ‘epigenetic mark’ and these cells displayed a sustained activation of NF-κB (nuclear factor κB) at the P65 promoter and oxidative stress responses akin to a memory phenomenon. Such transcriptional activation controls a range of oxidative and pro-inflammatory-linked cell responses. This has far-reaching implications and demonstrates that relatively short-term high glucose exposure induces a harmful chromatin remodelling and epigenetic changes that could be linked to diabetic complications in general and retinopathy in particular [31–33].

**RETINAL VASCULAR, NEURONAL AND GLIAL PATHOLOGY IN DIABETES**

**Pathogenic features of microvascular dysfunction**
Most investigations relating to the pathogenesis of diabetic retinopathy have focused on the vasodegenerative phase of the disease which leads directly to areas of retinal non-perfusion and hypoxia. This often precipitates breakdown of the BRB (blood–retinal barrier), oedema and/or the proliferative phase of the disease. A range of retinal microvascular abnormalities have been shown in diabetic animals (∼6 months to 4 years of diabetes duration) [34–37], but how closely these relatively short-term changes relate to human disease is a matter of debate.

One of the earliest histopathological changes observed in diabetic patients (and diabetic models) is thickening of the capillary BM (basement membrane) [38]. This is thought to be a consequence of increased synthesis of BM components (for example, collagen IV, fibronectin and laminin) and/or reduced degradation by catabolic enzymes [34,39–41]. It remains uncertain whether BM thickening is of primary or secondary importance in the development of diabetic retinopathy, but it has been speculated that such matrix modifications may contribute to impaired endothelial-pericyte communication, defects in capillary autoregulation or inappropriate cell interaction with constituent BM proteins [42–45]. AGEs (advanced glycation end-products) accumulate in diabetes and these adducts precipitate enhanced cross-linking in vascular BMs with associated enhanced component gene expression, parallel reductions in BM degradability, abnormal vessel elasticity and vascular cell death [44,46–48]. Inhibition of AGE formation during diabetes has been shown to prevent BM thickening in diabetic rats [34,49].

Pericytes and smooth muscle cells are among the first vascular components to die during diabetic retinopathy and this occurs via an apoptotic death mechanism [50,51]. Endothelial cells are also dysfunctional at a similar time point, although they may have the ability to make good the deficit, in the short-term at least. However, with prolonged exposure to the diabetic milieu, it is likely that the replicative potential of the endothelial cells will be exhausted, as occurs in all somatic cells when their Hayflick limit is exceeded [52,53]. Indeed, it has been shown that exposure to AGE-modified matrix proteins can induce a senescent phenotype prematurely so that the normal Hayflick limit may not be attainable under diabetic conditions [54]. Therefore it has been proposed that the primary defect in diabetic retinopathy lies with the vascular endothelium and that the complication could be considered to be, at least in part, an ‘endotheliopathy’ [55]. The endothelium is critical to normal function of other cells in the capillary complex [38]. The precise basis of endothelial, pericyte and smooth muscle dysfunction in the diabetic retinal microvascular in vivo remains obscure, but is most likely related to an array of cumulative biochemical insults coupled with impaired ability of the cells to repair and renew themselves. Retinal pericytes and smooth muscle cells are reliant on key growth/survival factors, such as PDGF (platelet-derived growth factor)-B [56,57], and there is selective depletion of this peptide during diabetes [58], which is closely related to acellular capillary formation [59].

Microaneurysms are hallmark lesions of diabetic retinopathy. Ophthalmoscopically, microaneurysms may appear as dark red or white spots in the fundus, whereas fluorescein angiography reveals perfused microaneurysms as discrete hyperfluorescent spots. Clinically, many microaneurysms are sclerosed and non-perfused, whereas others can be observed as fully or partially
perfusion during fluorescein angiography [60, 61]. Clinicopathological studies of diabetic retinopathy in which associations were made between fluorescein angiograms and trypsin digest preparations have confirmed that regions of capillary acellularity corresponded to non-perfused microvasculature angiographically often downstream from areas where microaneurysms were abundant [62]. Microaneurysms do not occur in diabetic rodents and therefore histological studies have been limited, but it is apparent that hypertension, microvascular BM thickening, endothelial proliferation, thrombus formation and pericyte cell death have all been implicated as causal or contributory factors to formation of microaneurysms [63–67]. Ultrastructurally, one of the earliest features of microaneurysms in the human diabetic retina is pericyte loss [67, 68], and uncontrolled hydrostatic pressure in the capillary beds may also result from selective loss of smooth muscle cells in the arteries and precapillary arterioles. Our group has demonstrated that microaneurysms occur largely on the arteriolar side of the circulation where they often occur immediately upstream of large areas of capillary acellularity [38]. Early-stage microaneurysms may also contain large numbers of monocyte and polymorphonuclear cells [67], perhaps reflecting the pro-inflammatory state of diabetes and the role of leucocyte-mediated capillary occlusion in diabetic retinopathy [69].

Capillary degeneration is a central tenet of progressive ischaemia during diabetic retinopathy and degenerative vessels are a universal finding in long-term diabetic animal models and post-mortem specimens [38]. On trypsin digest preparations, these acellular capillaries appear as non-perfused naked BM tubes where the endothelial cells have disappeared [38]. Increasing closure of capillaries may be linked with cotton wool spots in the neural retina and also the occurrence of IRMAs, which are represented in trypsin digests by wide-calibre multilayer channels within the capillary bed [60, 62]. Although there have been few studies conducted on the nature of IRMAs in the diabetic retina, it can be observed that these structures contain large numbers of endothelial-like cells and occur in association with acellular capillaries close to the arterial side of the circulation. IRMAs show large-calibre vessels traversing ischaemic retina with direct communication between pre-capillary arterioles and post-capillary venules and probably represent shunt vessels and an attempt to re-vascularize the hypoxic neuropile [60, 62].

**Retinal neuron and glial dysfunction during diabetes**

Although vascular dysfunction and loss of perfusion remain hallmarks of diabetic retinopathy, a growing body of evidence suggests that neuroretinal function is also compromised during this disease, perhaps even before overt vessel changes [70]. Electrophysiological studies of patients with diabetes suggest alterations in the neural retina, including loss of colour vision [71] and contrast sensitivity [72], and abnormalities in the ERG (electroretinogram) at diabetes onset [73]. Early neuronal alterations are also evident in experimental diabetes in rodents, including perturbation of the retinal histaminergic system leading to increased apoptosis of retinal ganglion cells and cholinergic and dopaminergic amacrine cells [74–76]. Apoptotic loss of photoreceptors has also been reported [77], corresponding to decreases in the rod and cone components of the ERG after ~12 weeks of diabetes-induction in animals [78]. Glia also show abnormalities during hyperglycaemia; in particular the Müller cells which demonstrate increased expression of GFAP (glial fibrillary acidic protein) [79] and concomitant synthesis of glutamate (as a function of disruption of the glutamate transporter [80]). This may contribute to excitotoxicity and eventual depletion of retinal neurons as a component part of diabetic retinopathy [81]. Taken together, the retinopathy literature suggests that neurodegeneration could constitute a significant pathophysiological defect in diabetes. It is clear that more research is required, especially to understand the complex interplay between neurons, glia and vascular components of the retina and key elements of disease-related dysfunction on this axis. Diabetic retinopathy may therefore be more accurately conceived as a disease of the neurovascular unit, resulting in dysfunction and eventual death of several of the key cells that maintain the BRB, namely pericytes, vascular endothelial cells, Müller glia and neurons.

**KEY PATHOGENIC PATHWAYS IN DIABETIC RETINOPATHY**

The pathogenesis of diabetic retinopathy is complex and a range of independent hypotheses have been proposed to explain the adverse effects of hyperglycaemia and dyslipidaemia, including increased polyol pathway flux, increased hexosamine pathway flux, increased formation of AGEs and overactivation of PKC (protein kinase C) [82]. Although detailed discussion of these pathways is beyond the scope of the present review, it is important to emphasize that these mechanisms should not necessarily be regarded as independent phenomena. Indeed, there is some evidence that they are linked as a direct result of hyperglycaemia-mediated overproduction of superoxide by the mitochondrial-localized electron transport chain [82]. This superoxide partially inhibits the glycolytic enzyme GAPDH (glyceraldehyde phosphate dehydrogenase), thereby diverting upstream metabolites from glycolysis into the four glucose-driven signalling pathways above. Importantly, the transketolase activator benfotiamine, which shunts hexose metabolism to the pentose pathway, appears to block all the major pathways of hyperglycaemic damage and effectively prevents the microvascular lesions of retinopathy in diabetic animals [83]. Recent clinical studies to determine whether benfotiamine might be useful in preventing plasma or urinary AGEs, however, have shown no benefit [84]. In addition, long-term (24 month) oral benfotiamine supplementation in Type 1 diabetic patients showed no change in peripheral nerve function or soluble inflammatory biomarkers [85], although the benefits of this drug on clinical diabetic retinopathy have yet to be determined.

**Changes in retinal blood flow**

The earliest functional changes in NPDR which cannot be visualized photographically include alterations in the rate of retinal blood flow and loss of autoregulatory mechanisms for adjusting retinal capillary perfusion to local metabolic demand [86].
The retinal vasculature lacks autonomic innervation and modulation of blood flow through the neuropile is dependent on local signalling mechanisms [87]. As early as the 1930s it was suggested that retinal blood flow was markedly altered in diabetic patients [88]. As technology evolved there were further indications that retinal vessel calibre consistently increased during diabetes [89,90]. Since the 1970s it has been recognized that haemodynamic changes in the retinal vasculature could play a role in pathology and perhaps also provide an early indicator of diabetes-related dysfunction in the retina [91,92].

Since then various patient-based studies have shown that retinal haemodynamic abnormalities occur before the onset of clinical diabetic retinopathy [93] and progression of the disease [94–97]. For example, in diabetic patients without retinopathy, retinal arteriolar vasoconstriction and decreased total retinal blood flow has been reported [93,98,99]. Decreased retinal blood flow during early diabetes may reduce O₂ and nutrient delivery to the retinal neuropile and thereby contribute to the initial neuroglial abnormalities observed in the diabetic retina.

As the disease progresses, retinal arterioles begin to dilate and bulk retinal blood flow increases in proportion to the severity of retinopathy [100]. Indeed, enhanced blood flow may hasten the progression of diabetic retinopathy by causing shear-stress-induced endothelial cell damage and death [86], and there has been a recent trial suggesting that these changes could be regarded as robust risk factors for retinopathy [101]. Significantly, patients with increased retinal blood flow who fail to demonstrate improvement of haemodynamic indices through normalisation of blood glucose tend to show a more rapid progression of the disease on follow-up [102]. Ongoing research into what causes changes in blood flow during various stages of diabetic retinopathy will be central to devising new ways to regulate this abnormality. This is critical because, although blood flow changes in diabetes are widely recognized, at present there is little direct evidence in the literature that such alterations contribute to pathology. Moreover, understanding the basic pathogenic mechanisms of what alters retinal blood flow will help link chronic hyperglycaemia to retinal vasoconstriction and flow changes prior to the onset of overt diabetic retinopathy.

**Inflammation and diabetic retinopathy**

Over recent years there has been considerable focus on the role of inflammation in diabetic retinopathy [103]. The aberrant expression of pro-inflammatory cytokines within the neural retina and up-regulation of adhesion molecules on the microvasculature leading to leukostatic responses have been linked to both the neurovascular dysfunction and formation of acellular capillaries [104]. Recent evidence suggests that leukocytes may actively damage the retinal vascular endothelium [105,106]. Global mRNA expression profiling has highlighted altered expression of pro-inflammatory cytokines and inter-related pathways not only in the retinal vessels [107], but also in the neuroglia [108]. There is undoubtedly a complex milieu of dysregulated pro-inflammatory factors evident in diabetic retina such as IL (interleukin)-1α, IL-1β, IL-6 and TNFα (tumour necrosis factor α) [103].

Although microglia and infiltrating monocytes are recognized as central to inflammatory CNS (central nervous system) pathology, the role of these cells in diabetic retinopathy is considerably less well understood. A number of *in vitro* studies and *in vivo* investigations of animal models and human post-mortem specimens indicate that activation of retinal microglia could play an important regulatory role in diabetes-mediated retinal inflammation [109] by modulating cytokine expression [110] and other pathologic responses [111]. Monocytes that infiltrate the retina and are distinct from microglia have not been adequately studied in diabetic retinopathy even though they reside in proximity to blood vessels (perivascular macrophages) or within various layers of the neuropile [112]. Monocytes and microglia have important roles in retinal homeostasis, but they are also central to neuroinflammation and these have been shown to increase in diabetes, in both humans and animal models [113–115].

Our group has studied the role of RAGE (receptor for AGEs) in the context of diabetic retinopathy and inflammation. RAGE is a component of the innate immune system and is a signalling receptor for various ligands, including AGEs, S100B, HMGB1 (high-mobility group box-1), amyloid-β and Mac-1 [116]. Ligand-binding and signal transduction activates transcription of NF-κB and induction of adhesion molecules, cytokines and/or oxidative stress. In the retina, RAGE is expressed by many cells, although the highest expression levels are in Müller glia [117]. S100B is neurotrophic at low levels [118], although up-regulation occurs in the Müller glia of diabetic animal models where it can induce inflammatory cytokine expression [119]. Blockade of RAGE may be a useful therapeutic strategy. For example, it has been shown that the soluble RAGE fragment (known as sRAGE) can prevent Muller cell dysfunction [117] during diabetes and retinal capillary leukostasis in AGE-infused (non-diabetic) mice [120]. Importantly, RAGE antagonists can prevent acellular capillary formation in diabetic mice [121], underscoring the potential for this receptor as a target for preventing inflammatory pathology in diabetic retinopathy.

**VEGF (vascular endothelial growth factor) and diabetic retinopathy**

Vascular permeability in the retina is controlled by the inner BRB, formed by the intra-retinal microvasculature and the outer BRB formed by the RPE (retinal pigment epithelium). The RPE acts as barrier limiting the passage of solutes and fluids between the choroidal stroma and the outer retina, while the retinal vasculature directly regulates flux into and out of the inner retina. The BRB is formed by tight junctions between adjacent cells which effectively blocks paracellular vascular permeability (i.e. the transport of substances between cells). Several tight junction proteins, including transmembrane proteins, such as occludin and the claudin family, and the membrane-associated protein zonula occludens, contribute to the formation of the BRB [122].

In diabetes, the inner BRB is compromised and macromolecules leak from the intraretinal vasculature into the interstitial spaces of the surrounding retina. In humans, MRI (magnetic resonance imaging) assessment of the retina shows an early stage permeability that occurs in advance of clinically recognizable diabetic retinopathy [123,124]. In animal models, increased retinal vascular permeability occurs shortly after diabetes onset, with a >60% increase in permeability to fluorescein-conjugated
albumin after only 4 weeks of diabetes [122]. It has also been recognized that the outer BRB is dysfunctional in diabetic retinopathy and it seems likely that leakage from the choriocapillaris through the RPE [125], perhaps in unison with impaired fluid clearance by this layer, may also contribute to oedematous change in the retina as diabetes progresses [126]. Fluid normally moves from the retina to the choroid largely due to the osmotic pressure exerted by the proteins in the choroidal stroma. In diabetes, breakdown of the outer BRB allows protein to leak into the subretinal space and thereby reduces the osmotic pressure gradient. Leakage and impaired fluid clearance are therefore most likely intimately linked to one another. Vascular leakage leads to DMO with or without cystoid degenerative changes, photoreceptor atrophy and an irreversible loss of central vision, although it is unclear whether this is related to a persistent vascular leakage or rather an acute wholesale breakdown of the BRB. It has also been suggested that Müller cell swelling could contribute significantly to oedematous changes in the diabetic retina [127].

CURRENT TREATMENTS OPTIONS FOR DIABETIC RETINOPATHY

At present there are few measures available to prevent diabetic retinopathy beyond maintenance of tight glycemic control [14], cessation of smoking [128], BP control [86] and correction of dyslipidaemia [129]. Beyond control of systemic factors, laser photocoagulation, intravitreal anti-VEGF drugs and intravitreal corticosteroids are the principal therapies to reduce sight-threatening DMO. Laser photocoagulation and vitreoretinal surgery are the main treatments for PDR.

Treatments for DMO

Macular laser photocoagulation

ETDRS demonstrated the value of laser photocoagulation in the treatment of clinically significant diabetic macular oedema [12]. Although laser reduced the risk of moderate visual loss by 50%, after 3 years of follow-up <3% of patients had visual acuity improvement by >15 letters. In ETDRS, although the diagnosis of CSMO (clinically significant macular oedema) was made based on clinical examination, laser treatment was guided by fluorescein angiography. Hence, traditionally, laser was applied with a view to preventing or slowing visual acuity deterioration rather than an attempt to restore vision. More recent data from RCTs suggests that laser treatment can actually improve vision in a relatively high percentage of patients. Thus, in the DRCR.net (Diabetic Retinopathy Clinical Research network) study comparing triamcinolone with laser in patients with DMO, an improvement of >10 letters was achieved in 32% of patients at 2 years in the laser group; the probability of improvement was similar in naïve eyes and in those that had received ≥3 bouts of laser therapy [130]. Interestingly, FFA (fundus fluorescein angiography) was not undertaken to guide laser treatment. Very similar results were obtained in a more recent study also conducted by the DRCR.net study and which compared ranibizumab combined with prompt or deferred laser with prompt laser alone and with prompt laser combined with triamcinolone in patients with centre-involved DMO [131]. In this study, a visual acuity improvement of >10 letters was achieved in 35% of patients at 2 years in the laser arm. The above data suggests that laser treatment can successfully improve vision in a proportion of patients with DMO.

Anti-VEGF therapies

BRB compromise and overt DMO during diabetic retinopathy has been closely linked to VEGF overexpression within the retina. This peptide is elevated in the ocular fluid of diabetic patients [132] and in the retina of diabetic animals before the appearance of observable retinopathy [133]. Laser photocoagulation reduces retinal VEGF [134] and is an effective treatment [130,135]. Furthermore, recently conducted RCTs have demonstrated that anti-VEGF drugs can reduce/eliminate DMO and improve vision [25,131,136,137]. It should be noted, however, that in most RCTs only around 50% of patients experienced clinically meaningful improvements in vision and in a similar number retinal thickness returns to normal levels [131,136]. Interestingly, not all patients with DMO respond to VEGF blockade and it is worth noting that a recent pharmacogenetics study in AMD (age-related macular degeneration) patients suggested that variation in nucleotide polymorphisms [tSNPs (tagging single nucleotide polymorphisms)] in the VEGFA gene were associated with responsiveness to anti-VEGF therapy [138]. It remains unknown whether this also applies to patients with DMO.

Several RCTs have demonstrated the clinical efficacy of intravitreal anti-VEGF treatment in DMO. The DRCR.net study (n = 854 eyes; n = 691 patients) [131] showed superiority in the primary outcome [mean change in BCVA (best correct visual acuity) at 1 year] in patients treated with ranibizumab and prompt (3–10 days after ranibizumab treatment) or deferred (>24 weeks following ranibizumab) laser compared with those treated with prompt laser + sham injection or prompt laser + triamcinolone injection. In this study, the percentage of patients receiving at least one session of focal/grid laser treatment in the ranibizumab + deferred laser group increased from 28% in the first year to 42% in the second year (extension of the trial) [131], indicating that laser treatment was still required to control patients longer term. The READ-2 (Ranibizumab for Edema of the mAcula in Diabetes) (n = 126 patients; primary outcome, mean change in BCVA at 6 months) [139] and RESTORE (n = 354 patients; primary outcome, mean average change in BCVA from baseline to months 1–12) [136] randomized patients to ranibizumab alone, ranibizumab + laser or laser alone and found greater visual acuity improvement in patients in the ranibizumab groups. The RESOLVE (n = 151 patients; primary outcome, mean average change in BCVA from baseline to months 1–12) [140] and RISE-RIDE (n = 377 and n = 382 for RISE and RIDE respectively; primary outcome, proportion of patients with ≥15 letters at month 24) [139] randomized patients to ranibizumab or sham and, similarly, found better visual acuity outcomes in patients treated with the former. Rescue laser was allowed in these trials in ranibizumab and sham groups and was used in ~35% and ~5% of patients in sham and ranibizumab groups in RESOLVE and in 70–74% and 20–40% of patients in sham and ranibizumab groups in RISE-RIDE. It is important to note that in
Steroid treatment

Triamcinolone, dexamethasone and flucinolone have been used in the treatment of patients with DMO. Several RCTs have compared triamcinolone with laser photocoagulation and laser has been shown to be superior. DRCR.net, however, found that, in pseudophakic eyes, visual acuity improvement in the triamcinolone + prompt laser group appeared comparable with that in ranibizumab groups (also see the section on anti-VEGF therapies above) [131]. Triamcinolone + prompt laser appeared also more cost-effective than ranibizumab + prompt or deferred laser for pseudophakic patients [143].

Intravitreal DXM (dexamethasone), at a dose of 700 μg, using an intravitreal drug delivery system was found to be superior to no treatment short-term in an RCT that randomized patients to 350 μg, 700 μg or no treatment (n = 171 patients; primary outcome, proportion of eyes that achieved an improvement in BCVA of ≥10 letters or more from baseline at day 90) [144].

The FAME (Fluocinolone Acetonide in Diabetic Macular Edema) trial compared fluocinolone (0.2 μg/day or 0.5 μg/day) with sham treatment (n = 956; main outcome, percentage of patients with ≥15 letter improvement at 2 years) [145] in patients with DMO. Rescue laser was allowed in all groups. Fluocinolone was found to be superior to sham, but side effects including cataract, increased intraocular pressure and glaucoma, occurred as a result of the treatment with fluocinolone. Rescue laser was performed in ~37% and 59% of fluocinolone-treated and sham-treated eyes respectively.

Treatments for PDR

In contrast with DMO, treatment advancements in the management of PDR have been more restricted. PRP (panretinal photocoagulation) remains the mainstay treatment for PDR, although vitreoretinal surgery is used now in earlier stages (rather than as a treatment of last resort). Indeed, recent data supports better visual and anatomical outcomes and decreased complications of vitrectomy in patients with diabetic retinopathy (DMO/PDR) compared with earlier data published by DRVS (Diabetic Retinopathy Vitrectomy Study) [146]. RCTs have suggested less retinopathy progression in patients receiving intravitreal anti-VEGF or triamcinolone with prompt/deferred macular laser [131], intravitreal anti-VEGF alone [25,140], or oral fenofibrate [147] and, thus, these treatments may reduce the number of patients reaching the proliferative stage. Further management options for established PDR are, nonetheless, very much needed.

SHORTCOMINGS OF CURRENT THERAPY AND POTENTIAL NEW AVENUES

The approaches described above are focused on end-stage disease and carry significant sight-threatening side effects. Importantly, they do not address the early and potentially reversible failure of retinal perfusion [148]. The use of VEGF-neutralizing antibodies, crossing over from success in treating wet AMD, has shown significant benefit, although the effects are short-lived and require multiple intravitreal injections [131,136,149]. Although hopeful, inhibition of VEGF bioactivity is end-stage and is not without drawbacks. For example, there have been concerns raised that such therapy could compromise retinal neuroglial and resident microvascular survival [150–154]. In addition, there have been clinical reports that some patients with diabetic retinopathy may respond poorly to VEGF inhibition and it could even be associated with poor visual outcomes [155,156]. Although anti-VEGF therapy has revolutionized ophthalmic care, there is a need for caution surrounding long-term use of these approaches [157]. In the context of diabetic retinopathy, this is an increasing concern. For this reason, the development of therapies capable of preventing or slowing the onset and progression of diabetic retinopathy remain a priority.

It is clear that many other pathogenic mechanisms besides VEGF are involved and play an important role in diabetic retinopathy, including PDR and DMO. A multitude of new approaches based on retinal glial, neuronal and vascular pathophysiology during diabetes are being developed. However, many therapeutic strategies are simply attempting more convenient or better-tolerated ways of having an impact on the VEGF pathway. Consequently therapeutics targeted at non-VEGF mechanisms are perhaps the more interesting and offer the greatest hope for a wider treatment response in DMO patients or additional therapeutic benefit in an adjunctive treatment setting with anti-VEGFs (see below).

Therapeutics with proven efficacy in other diseases may prove useful in diabetic retinopathy. One such cellular mechanism of significant interest in diabetes is oxidative stress. Nrf2 (nuclear factor erythroid 2-related factor 2) is a transcription factor that plays key roles in controlling the expression of antioxidant and detoxification genes, and mice deficient in Nrf2 are more vulnerable to oxidative damage especially in tissues with high oxygen consumption, such as the retina. Nrf2 has been reported to be associated with the altered inflammatory status present in chronic obstructive airway disease [158–161] and, indeed, bardoxolone (Reata), an Nrf-2 activator which progressed to a significant stage of development, but was later terminated, was shown to improve glomerular filtration in type 3b-4 chronic kidney disease associated with Type 2 diabetes in Phase II clinical studies [162]. An additional approach which is currently being targeted at the treatment of diabetic retinopathy is NOX-2 inhibitors (part of the
NADPH oxidase system), whose major function is to generate ROS (reactive oxygen species) as part of the respiratory burst. In spite of the fact that nephritic vasculopathy is the targeted indication, there is significant pre-clinical evidence to support the utility of these molecules in the treatment of diabetic retinopathy. ROS formation is correlated with diabetes-induced retinal vascular injury and overexpression of VEGF [163] and can lead to activation of the polyol pathway, AGE formation, RAGE induction and activation of the PKC and hexosamine pathways [31]. Up-regulation of NADPH oxidase activity or ROS production also correlates with increases in VEGF in rat models of Type 2 diabetes [164–166]. Inhibiting NADPH oxidase activity can limit retinal leukostasis [167] and reduced BRB integrity secondary to hyperglycaemia [168]. These approaches may have an ability to have an impact on an diabetic retinopathy at all stages. There is also a considerable body of emerging evidence that local inflammation has a significant impact on disease and a myriad of cytokines, including TNFα, have been implicated in PDR and DMO. However, small experimental medicine studies using intravitreal neutralizing anti-TNFα antibodies have shown little benefit in DMO patients refractory to anti-VEGF therapy.

Other therapeutics may arise from novel non-biased genomic and proteomic screens in the context of diabetic retinopathy. One such example focuses on the involvement of extracellular CA (carbonic anhydrase), which is up to 15-fold higher in the vitreous of individuals with PDR compared with that in vitreous of non-diabetic individuals [169]. Intravitreal injection of CA-I stimulated an increase in retinal vascular permeability to a similar magnitude to that shown for VEGF. CA-I increases retinal vascular permeability by elevating the vitreal pH, leading to activation of the PKK (prekallikrein/kinin) pathway, and this has opened up therapeutic opportunities for inhibitors or antagonists of the kallikrein system which could be useful as an adjunct to laser photocoagulation or anti-VEGF agents. Indeed, in a pilot study, the CA-1 inhibitor acetazolamide has been shown to improve visual outcomes in patients with DMO [170]. Inhibitors of PKK are being developed which will be initially targeted at intravitreal use, but could probably be developed further as oral medications if initial studies show promising results.

THE DIFFICULTY OF EXPLORING NEW AVENUES

Despite the possibility of significant drawbacks associated with long-term intravitreal anti-VEGF use in diabetic retinopathy, these agents now represent the effective standard of care for regulatory authorities. Consequently, when considering a new therapeutic strategy for diabetic retinopathy, it is important to understand whether the targeted mechanism is likely to be independent of VEGF, or simply work via a mechanism which ultimately suppresses VEGF. If the action of the therapeutic is to simply have an impact on the VEGF pathway or VEGF levels, then it is unlikely that this treatment, however dosed, will be superior to intravitreal anti-VEGF therapies in responsive patients. This situation will significantly have an impact on the likelihood of successful development since reduction of treatment burden is often not an acceptable regulatory endpoint in some regions. Consequently, the new therapeutic has to demonstrate non-inferiority to intravitreal anti-VEGF therapy or increased efficacy in an adjunctive treatment setting. The situation is easier when the targeted therapeutic is clearly independent of VEGF since all marketed anti-VEGF therapeutics show similar efficacy, suggesting that additional suppression of this pathway is incapable of translating into greater efficacy and only additional independent mechanisms can probably deliver improved efficacy. An example of this situation has been demonstrated with PKK inhibitors. Vitreal samples from DMO patients were observed to have high PKK or high VEGF levels or a combination of both. Interestingly, human vitreous with either high PKK or high VEGF were both able to increase retinal vascular permeability when injected into mouse eyes and such effects were inhibited by bradykinin antagonists or anti-VEGF antibodies respectively. When the opposite experiment was performed attempting to block PKK samples with anti-VEGF antibodies and VEGF samples with bradykinin antagonists, there was no impact on the ability of the human vitreal samples to stimulate retinal vascular permeability in the mouse [171]. This was a clear demonstration of a VEGF-independent pathway and may explain why a significant proportion of DMO patients do not respond adequately to anti-VEGF therapeutics and require additional treatment strategies to be conceived.

REGENERATIVE MEDICINE AND DIABETIC RETINOPATHY

Our own group and others have recently focused on the possibility that vascular regeneration could be beneficial for diabetic retinopathy. Indeed, if the ischaemia that drives DMO and PDR can be contained or reversed, then this could become an exciting and viable therapeutic option. Such an approach is commonplace in other non-retinal systems where the growth of new blood vessels can be stimulated to promote regeneration in ischaemic tissues [172]. Reparative angiogenesis in the retina is also a realistic option. Most of these studies have used OIR (oxygen-induced retinopathy) [173] in neonatal mice as a model system for regulating intra- and pre-retinal neovascularization. Our laboratory has used several approaches to achieve effective re-vascularization of the ischaemic retina, including treatment with an agonist peptide that promotes vascular endothelial cell substrate attachment [174–176], genetic knockout or pharmacological inhibition of TNFα [177], treatment with low-dose simvastatin [178] or use of a human cell-derived recombinant EPO (erythropoietin) [179]. The Smith laboratory has also demonstrated that early-stage delivery of recombinant EPO to the OIR model can prevent vascular and neural degeneration and hypoxia-induced neovascularization [180]. With respect to EPO, we have also recently shown that an EPO-receptor analogue can effectively regenerate defunct acellular capillaries in the diabetic retina with a suggestion that this could be linked to increased mobilization and incorporation of vascular progenitor cells [181].
Figure 3  Schematic diagram highlighting the complex pathogenesis of diabetic retinopathy and a range of points that may be addressed by novel therapeutic intervention

ACE, angiotensin-converting enzyme; Ang, angiotensin; DME, DMO; PEDF, pigment epithelium-derived factor.

Possibilities for cell therapy

EPCs (endothelial progenitor cells) are circulating bone-marrow-derived cells with the ability to differentiate into mature functional endothelial cells and repair vessel integrity [182]. This is particularly relevant for diabetic retinopathy which is associated with an accelerated loss of endothelial cells and capillary occlusion. Although there is no consensus as to a definite vasoreparative or antigenic phenotype for EPCs, it is generally accepted that these cells are capable of differentiating into endothelial cells and integrating into vascular structures [183]. EPCs normally only constitute 0.01% of circulating cells, but, in response to growth factors and cytokine gradients secreted from sites of injury, inflammation or ischaemia, they are recruited to damaged vasculature [183–185].

Diabetes alters EPC number and function and poor glycaemic control is associated with lower circulating levels of EPCs [186,187], and cells isolated from diabetic donors show significantly impaired vasoreparative potential [188] and are susceptible to premature senescence [189]. Such EPC dysfunction has been reported in patients with Type 1 diabetes [190] and also Type 2 diabetes [191]. The depressed EPC function is negatively associated with severity of vascular complications. Indeed, EPC dysfunction has been implicated in a range of cardiovascular disorders, including coronary artery disease, myocardial infarction and microvasculopathies [192].

In contrast with many other vascular beds, EPC therapy for the retina has received comparatively less attention. Beginning with the first report that adult HSCs (haematopoietic stem cells)
contribute significantly to neovascularization in the ischaemic retina [193], other reports have demonstrated that intravitreal delivery of EPCs (and related cells) can repair the ischaemic retina [194–198]. These studies show the potential of EPC therapy for ischaemic retinopathies, but they also highlight the need for a more thorough characterization of EPC subsets so that the precise fate and utility of delivered cells can be determined without the potential to evoke unwanted responses. This is especially important in the context of a complex milieu, such as diabetes, which is known to alter EPC phenotype [199]. Nevertheless, when the molecular nature and precise function of EPCs is fully researched, vascular cell therapy has potential to translate to the clinical realm and patients with diabetic retinopathy could benefit from the remarkable regenerative potential of these cells.

The differentiation potential of MSCs (mesenchymal stem cells) and multipotent adult progenitor cells into mural cells such as smooth muscle and pericytes has been reported [200,201]. Pericytes are crucial for capillary integrity, so if EPC therapy is ever to be used for diabetic patients it will be essential to also achieve adequate pericyte coverage on intra-retinal vessels. It has been reported that human pluripotent stem cells differentiate to mesodermal precursors that can be expanded to produce cells with a pericyte phenotype expressing CD146/NG2/PDGFR-β (PDGF-receptor-β) [202]. Implantation of these cells into a murine model of ischaemic limb injury induces vascular regeneration [202]. It has also been shown that human saphenous-vein-derived pericyte precursors have prolonged therapeutic benefit in a model of myocardial infarction [203]. This is an interesting and important area for diabetic retinopathy research, since pericyte death is a hallmark lesion of this condition. The ability to regenerate these cells in the early stages of disease would be a signal advance and this is being actively pursued by our group.

**SUMMARY**

Diabetic retinopathy is a multifactorial condition arising from the complex interplay between biochemical and metabolic abnormalities occurring in all cells of the retina. Identification of a precise pathogenesis that links the progressive neuroglial and microvascular damage occurring in the diabetic retina remains a valid but somewhat elusive goal. Nevertheless, over the coming years continued progress is anticipated in our understanding of the molecular and cellular basis of diabetic retinopathy. We are also entering a new exciting era in which the first pharmacological treatments based on an understanding of the causative mechanisms of diabetic retinopathy may soon become available (Figure 3). These will include vascular and neuroprotection therapies that can halt the progression of retinopathy in the early stages. There are also exciting possibilities of harnessing reparative cells and thereby regenerating retina after retinopathy has already become established. In view of the complexity of diabetic retinopathy, it seems likely that combinatory therapy will be required with pathways targeted in different cell types at different stages of the disease process. With genome-wide assessments for patients becoming a possibility, the likelihood is that highly tailored pharmacogenetic approaches will be most efficacious.

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