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Review

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Oncologic, Endocrine & Metabolic

Emerging therapeutics for diabetic retinopathy: potential therapies for the new millennium

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1. Background

The Nobel Prize winning discovery of insulin by Frederick G. Banting, John J.R. MacLeod and Charles Best in 1921 saved the lives of many insulin-dependent (Type 1) diabetic patients. The prolongation of life with insulin therapy, however, did eventually reveal long-term complications of diabetes, including diabetic retinopathy, neuropathy, nephropathy and cardiovascular disease. The occurrence of these complications is attributed to the inability of conventional insulin therapy to maintain blood glucose in a euglycaemic range. For many years it was uncertain if putting a diabetic patient on intensive insulin therapy to achieve plasma glucose levels close to the non-diabetic range would produce a beneficial effect, by delaying or reducing the severity of diabetic complications. In 1993, the results from the ten-year Diabetes Control and Complications Trial (DCCT) in Type 1 diabetic patients demonstrated conclusively that intensive insulin therapy leads to a reduction in the development and progression of long-term complications [1]. The recent results from the 20-year United Kingdom Prospective Diabetes Study (UKPDS) [2] corroborated this conclusion with Type 2 diabetic patients. Intensive insulin therapy reduced the risk of developing retinopathy by 76% in the Type 1 patient (DCCT) [1,3], and by 25% in the Type 2 patient (UKPDS) [2,4]. Both studies also showed that retinopathy responds slowly to glycaemic corrections, and the amelioration of the retinopathy is influenced by the patient's prior history of glycaemia. Moreover, maintaining tight glycaemic control is difficult. The current therapies to control blood glucose (diet and exercise, oral euglycaemic agents and insulin) have inherent problems including compliance, ineffectiveness and hypoglycaemic episodes.

The prevalence of diabetes and diabetic retinopathy in the general population continues to grow and it remains a serious health problem throughout the world [5]. The number of people at risk of blindness from diabetic retinopathy in the United States alone is over 600,000 annually. Retinopathy is the leading cause of blindness in adults from 25 to 74 years of age in the United States [6]. To date, the most effective treatment for proliferative retinopathy is laser photocoagulation therapy [3,7]. However, this procedure is destructive by nature and is not always effective. There are no drugs that are specifically approved for the treatment of diabetic retinopathy. A therapy that directly intervenes to prevent the development of the

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complication, or slow its progression, would be of tremendous benefit to those afflicted with diabetes. This therapy would be given along with one of the current glucose lowering agents.

1.1 Clinical and pathological characteristics

Diabetic retinopathy is mainly a microvascular disease affecting the integrity of the retinal capillaries, involving vasoproliferation and characterised by a unique spectrum of microangiopathy resulting from vascular remodelling and aberrant blood vessel formation. Normally, retinal capillaries are composed of only two cell types: endothelial and (intramural) pericytes [8,9].

The clinical course of diabetic retinopathy can generally be classified into stages reflecting the progression of the disease and prognostic risk of blindness [10]. It is assumed that the full spectrum of retinal microangiopathy with diabetes arises from a continuum, and that one stage of the disease is likely to lead to another, more advanced stage with a greater risk for blindness [10]. The stage classification system to assess the severity of diabetic retinopathy has varied historically. The terms 'background', 'pre-proliferative' and 'proliferative' diabetic retinopathy have been used extensively [11]. More recently, the terms 'no retinopathy', 'non-proliferative' and (mild, moderate, severe) 'proliferative' are commonly used (**Figure 1**). The 'no retinopathy' stage signifies that the retina appears clinically normal by standard fundus examination; however early alterations in haemodynamic parameters and early histopathological changes are likely to be occurring. Alterations in retinal vascular reactivity have been reported to occur in the arteries and veins of people with diabetes [12]. The normal vasoconstrictor response of the retinal vasculature to increased oxygen is suppressed [12,13], suggesting a functional abnormality despite a paucity of visible changes by ophthalmic examination. Two of the earliest histopathological lesions, diffuse thickening of basement membrane and selective loss of pericytes [9], go undetected by routine fundus examination. The biological significance of basement membrane thickening is unclear but, despite the thickening, it has been reported that the basement membrane becomes more permeable and there are changes in its chemical composition, perhaps as a consequence of endothelial cell synthesis of matrix components [14,15]. The mechanism by which thickening of basement membranes occurs has not been clearly elucidated. It

has been hypothesised to be a proliferative response of vascular cells to chronic injury, perhaps as a result of increased vascular pressure and permeation [16]. It has not been determined unequivocally which lesion occurs first in humans, basement membrane thickening or pericyte loss. Normally, in humans, there is a 1:1 ratio of endothelial cells to pericytes [8], but in diabetes this ratio becomes 4:1. Simultaneously, there is the emergence of 'pericyte ghosts', remnants of basement membrane that circumscribe the degenerated pericyte [8]. The underlying mechanism determining why some pericytes degenerate while others persist remains enigmatic. Other early histopathological changes include increase in the number of dilated capillary channels, and an increase in capillary density [17,18].

The pericyte cell is believed to play a central role in the development of diabetic microangiopathy [19,20]. The pericyte has been ascribed a pivotal role in the regulation of the microvasculature [21]. It has been hypothesised that the loss of pericytes provides a 'permissive' environment for the subsequent proliferation of endothelial cells, giving rise to the characteristic and unique vasculopathy seen in diabetic retinopathy. Pericytes have contractile function [22], provide structural support to the vessel wall, and have thus been implicated in regulation of haemodynamic parameters. The synergism which occurs between pericytes and endothelial cells includes physical contact mediated by cell adhesion molecules, regulating the differentiation of precursor cells and the growth of microvascular endothelial cells by the release of soluble growth factors [21].

Diabetic vascular dysfunction and changes in blood flow appear to be an early event in the pathogenesis of diabetic complications [23,24,25,26]. It has been reported that blood flow is increased very early following the onset of diabetes, and that these haemodynamic alterations are important factors in the pathogenesis of diabetic microangiopathy [16]. Haemodynamic and vascular permeability changes have been implicated in capillary basement membrane thickening. Reductions in blood flow in advanced diabetic microangiopathy are believed to be a consequence of occlusive arterial and arteriolar disease.

The non-invasive measurement of retinal blood flow provides a monitor of early physiological changes in the retina. There are several clinical methods available for the measurement of retinal blood flow. These

Figure 1: Clinical course of diabetic retinopathy with proposed therapeutic targets.
 NO: nitric oxide; VEGF: vascular endothelial growth factor.

Disease classification	Clinical appearance	Mechanistic changes	Potential therapy
No retinopathy	Normal fundus exam Basement membrane thickening Blood flow alterations Pericyte loss (ghosts)	Protein glycation Polyol pathway activation Oxidative stress Ischaemia Early growth factor changes	Glycation inhibitor Aldose reductase inhibitor Protein kinase C inhibitor Anti-oxidant NO synthase inhibitor Adenosine antagonist
Non-proliferative	Increased vascular permeability Microaneurysms Haemorrhages Nerve fibre swelling Venous tortuosity Venous beading Macular oedema Capillary non-perfusion		
Proliferative	Neovascularisation Fibrovascular proliferation Vitreous haemorrhages Traction retinal detachment	Pronounced ischaemia Growth factor effects	VEGF antagonist Angiopoietin modulator Plasminogen activator inhibitor Thalidomide and analogue Angiostatin, endostatin Thrombospondin-1 Growth hormone antagonist

include cinefluorescein angiography [27], blue-light entoptic phenomenon [28] and laser-Doppler velocimetry [29,30]. The haemodynamic information acquired from the various methods differ and may include mean transit time (MTT), mean corpuscular velocity and regional volumetric blood flow. Each technique has distinct limitations. Not surprisingly, the interpretation of the results from the various methods makes it difficult to reach unanimity in characterising the retinal blood flow changes which occur in diabetic retinopathy. Additionally, there are discordant observations regarding the time-course of the haemodynamic changes. It is important to understand which haemodynamic parameter is being analysed, and how changes in rheology infringe on the generalisations that can be derived from each technique. The reader is referred to the cited references for an in-depth analysis of this area.

The earliest clinical manifestation of non-proliferative diabetic retinopathy is the occurrence of microaneurysms, usually confined to the region of the posterior pole [10]. Punctate 'dot and blot' haemorrhages, venous dilation/beading, hard exudates (extravasation of plasma proteins from leaky vessels), soft

exudates (cotton-wool spots: nerve fibre swelling/damage as a consequence of ischaemia) and intraretinal microvascular abnormalities (IRMA: irregular dilation and tortuous varicosities of capillaries) are characteristic lesions of the non-proliferative stage [10,11,31].

Diabetic patients with proliferative diabetic retinopathy develop focal capillary and arteriolar obstruction [32]. These areas of non-perfusion are demonstrable by fluorescein angiography. It is hypothesised that the resulting retinal ischaemia eventually promotes the production of vasoproliferative factors and subsequent new vessel formation, the hallmark of the proliferative or neovascular stage.

A high percentage of patients with long-term diabetes will eventually have some stages of non-proliferative retinopathy. After 15 years of known diabetes, the incidence is approximately 98% if they were diabetic before the age of 30, and 78% in people becoming diabetic after 30 years of age [10]. After 15 years of Type 1 diabetes, 50% of patients have progressed to proliferative retinopathy. This stage of retinopathy poses the greatest threat to visual loss [11]. The vessels that are formed in this advanced stage grow along the

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surface of the retina, particularly near the optic disc. The new vessels can invade the vitreous. The newly formed vessels, both intra- and extra-retinal, are fragile and prone to rupture. Pre-retinal and intra-retinal haemorrhages are a common complication. Repeated haemorrhagic episodes can cause macular oedema from increased vascular permeability, non-resolving vitreous haemorrhage requiring *pars plana* vitrectomy and fibrous proliferation, with traction retinal detachment causing irreparable blindness [10].

1.2 Current clinical management

The short- and long-term beneficial effects of photocoagulation have led to its wide clinical acceptance for the treatment of proliferative diabetic retinopathy [7,33]. Retinal hypoxia occurs in early diabetic retinopathy and is correlated with endothelial cell death, plugging of vessels and microaneurysm formation [34].

It has been hypothesised that panretinal photocoagulation improves retinal oxygenation and therefore, is an effective treatment for ischaemic retinopathies such as proliferative diabetic retinopathy. Laser photocoagulation therapy destroys the photoreceptor-pigment epithelium complex, including the mitochondria-rich inner segments of the photoreceptors, thereby decreasing the metabolic activity of the retina [23,35,36]. It is proposed that following photocoagulation, there is increased retinal oxygen tension due to diffusion of oxygen from the choroidal vasculature [23,37].

Photocoagulation may also exert its beneficial effect by destruction of retinal cells that produce angiogenic factors, or by altering the production of factors during the healing process [38,39,40]. Chorioretinal scarring which occurs following panretinal photocoagulation may be responsible for altering the balance of growth factors. The cell types that comprise the chorioretinal scars include fibroblasts, astrocytes and retinal pigment epithelial cells [38].

However, there are many patients in which laser therapy will not prevent vision loss due to obstructing vitreous haemorrhage or severe neovascularisation [41]. It has been reported that scatter photocoagulation may have a detrimental effect in patients with pre-existing severe ischaemic changes [32]. These patients must undergo surgical intervention with a high risk of complications. Therefore, pharmacotherapeutic intervention is needed in the clinical

management of a variety of ischaemic retinopathies which include proliferative diabetic retinopathy, retinal vein occlusion, retinopathy of prematurity (ROP) and other vasculopathies.

2. Prospective early intervention at the pre-proliferative stages

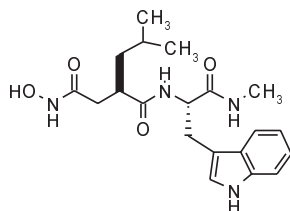
Data from the DCCT strongly suggests that treatment to prevent retinopathy needs to be instituted early, before the onset of clinically overt vascular disease. Histological findings in retinas from diabetic donors have disclosed the existence of microscopic lesions below the level of clinical detection. The presence of pre-existing damage can have a profound effect on the efficacy of the intervention therapy in animal models [42]. Documentation regarding the early manifestations of diabetic retinal microangiopathy has been very dependent on the limited number of reliable animal models [17,43,44], due to scarcity of human material. The few good animal models develop a diabetic-like microangiopathy essentially indistinguishable from that seen in humans.

The DCCT showed that patients receiving intensive insulin therapy, but already exhibiting diabetic retinopathy, had a 22% reduction in the ameliorative effect compared to those with no diabetic retinopathy [3]. The UKPDS results showed that 39% of male patients and 35% of female patients exhibited at least one microaneurysm by the time they were diagnosed to have non-insulin-dependent diabetes mellitus (NIDDM) [4]. More advanced lesions, characteristic of the non-proliferative stage of diabetic retinopathy, were observed in 8% of men and 4% of women at the time they were diagnosed to have NIDDM [4]. Thus, it appears that the extent of pre-existing retinal damage at the time of intervention is a primary factor in determining the outcome of therapy [42]. Both animal and clinical studies have indicated that early intervention affords the greatest beneficial effect.

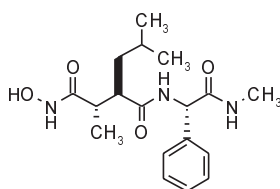
It is presumed that prevention of the early stages of retinopathy, such as pericyte loss, will also prevent the subsequent late stages of neovascularisation. The underlying mechanisms responsible for pericyte loss are very complex and not completely elucidated. Another early change in the retina that has been suggested to reflect aberrant pericyte function is an early alteration in retinal blood flow. As already discussed, the methodology to assess retinal blood flow varies, and so do the interpretations and

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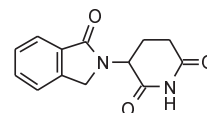
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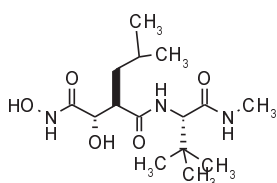
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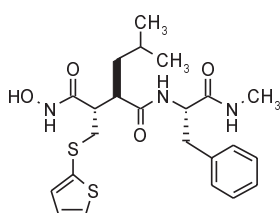
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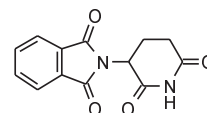
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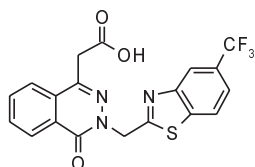
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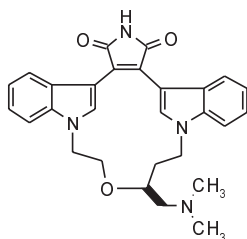
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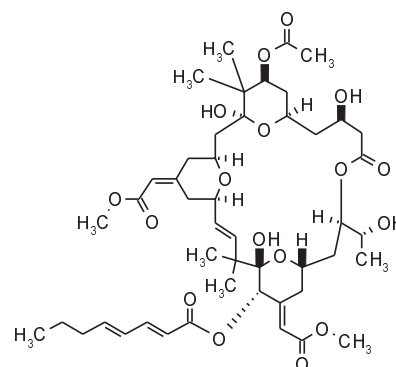
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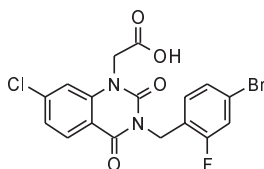
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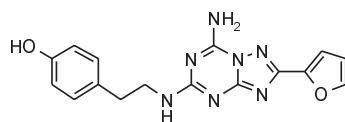
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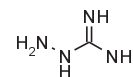
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aminoguanidine

microaneurysms. At the highest dose, 'dot and blot' haemorrhages were prevented [51] and all other retinal microangiopathies, including thickening of the retinal capillary basement membrane, were either prevented [50] or delayed [51]. Changes in blood flow and vascular permeability in affected tissues of diabetic animal models are preventable by ARIs [16]. Thus, animal data convincingly shows that AR plays an early role in the pathogenesis of the disease.

In 1983, sorbinil (Pfizer) was evaluated in man in a three-year trial to determine whether sorbinil could delay the onset, or slow the progression, of retinopathy from early to more advanced stages [53]. Four hundred and ninety-seven people with Type 1 diabetes with 1 to 15 years duration of the disease, and no more than five microaneurysms in either eye, were randomly assigned to either receive sorbinil at 250 mg/day dose, or to receive a placebo. Over the 4-year study, the progression of retinopathy was assessed by fundus photography. Sorbinil did not have a significant effect on the worsening of the disease however a slower progression rate in the number of microaneurysms was seen.

Lessons learned over the 15 years following the Sorbinil Trial suggest that patients were enrolled whose retinopathy was too advanced, there were too few patients, and the trial was not long enough to adequately assess the end-point [54]. The clinical outcome variable in both the DCCT and the UKPDS trials was a three-step worsening in the Early Treatment Diabetic Retinopathy Study (ETDRS) severity scale, as measured by fundus photography, while the Sorbinil Retinopathy Trial was limited to a two-step worsening. Currently, there are two ARIs in Phase III clinical trials for diabetic peripheral neuropathy; zenarestat (Warner-Lambert) [55] and zopolrestat (Alond, Pfizer) [56] (**Figure 3**). Pending a positive outcome in the diabetic neuropathy trials, ARIs may once again be considered for the treatment of diabetic retinopathy.

2.2 Non-enzymatic glycation

A relatively early alteration caused by diabetes is the non-enzymatic glycation of proteins. Glucose spontaneously forms a covalent bond with amino groups of a wide variety of proteins, which rearrange to form reactive molecules, such as 3-deoxyglucosone, which further react with protein and DNA to form covalent adducts and cross-links called advanced glycation end products (AGEs) [57,58,59,60]. Although there is

substantial protein glycation occurring within a few weeks of sustained hyperglycaemia, the formation of AGEs can take several years and is influenced by the long-term glycaemic state.

Numerous studies have shown a role for glycation and AGEs in the development of diabetic retinopathy [61,62,63]. It has been suggested that glycation of matrix proteins alters their structure and functional properties, and could be a contributing factor in retinal vessel basement membrane thickening [57]. Known sites of non-enzymatic glycation include lysine residues, as well as lysyl oxidase-mediated crosslink formation of Type IV collagen, in basement membranes [16]. Rodent studies have shown that basement membrane thickening is ameliorated by therapy with an antibody to glycated albumin [64], and by treatment with aminoguanidine, a small molecule inhibitor of AGE formation. Increased binding of albumin to basement membranes has been attributed to increased non-enzymatic glycation of basement membrane components [16].

AGEs are elevated in the vitreous of patients with proliferative diabetic retinopathy [65,66]. Additionally, it has been shown that there are receptors for the AGE (RAGE) [67,68] on monocytes, endothelial cells, pericytes, smooth muscle cells and the basement membrane of the retinal vasculature [61]. The activation of RAGE following AGE binding elicits a cytokine-induced inflammatory response [69].

The infusion of AGE can mimic some of the vascular abnormalities noted in diabetes, such as increased basement membrane thickening and alterations in vascular contractility. Intra-ocular injections of AGEs stimulate the expression of vascular endothelial growth factor (VEGF) mRNA in rat and rabbit retinas [70]. The effect of AGEs on VEGF expression was time- and dose-dependent, additive with hypoxia and preventable with anti-oxidants.

Current preclinical data suggests that the inhibition of AGE formation, or the inhibition of the activity of RAGE, are viable targets for the treatment of diabetic retinopathy. Aminoguanidine (Pimagedine, Alteon & Genetech), a hydrazine compound (**Figure 3**), reacts with early reactive sugar products generated in the glycation pathway, such as 3-deoxyglucosone, and prevents AGE formation [71]. In addition to the inhibition of retinal basement membrane thickening, aminoguanidine has been shown to prevent pericyte drop-out [44], and prevent increased vascular permeability in the retina [72].

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Aminoguanidine has been reported to function as an anti-oxidant by quenching hydroxyl radicals and lipid peroxidation *in vivo*, and preventing oxidant-induced apoptosis of retinal Muller cells [73]. Since glycation has been implicated as an underlying mechanism in the pathogenesis of most diabetic complications, including neuropathy and nephropathy, aminoguanidine is expected to have broad-based clinical utility. Currently, aminoguanidine is being evaluated in Phase III clinical trials for the treatment of diabetic nephropathy. The outcome of this trial will most likely govern the future clinical evaluation of aminoguanidine for the treatment of diabetic retinopathy.

2.3 Nitric oxide

Dynamic changes in retinal microcirculation have been associated with the development of diabetic microangiopathy. In early diabetic retinopathy, dilatation of microvessels may reduce peripheral vascular resistance resulting in increased blood flow [74]. The mechanisms governing the regulation of retinal blood flow have not been clearly elucidated, but appear to involve the interaction of intrinsic myogenic vessel tone and the effects of endogenous vasodilator agents [75]. The endothelium has been shown to be a metabolically active mediator of vascular smooth muscle tone. Endothelium-derived nitric oxide (NO) is synthesised from L-arginine by the cytosolic enzyme, nitric oxide synthase (NOS) [76]. NOS has been immunohistochemically localised in ocular tissues [77]. Endothelial regulation of vascular tone has been demonstrated in isolated human ophthalmic arterial rings [78]. Retinal arteriolar diameter, as determined from funduscopy images, suggest that arteriolar tone is controlled by NO [79]. The autoregulatory mechanism of retinal circulation under certain physiological conditions is, in part, regulated by NO [80]. These studies suggest that NO plays an important physiologic role in the regulation of ocular blood flow. The biological effects of vasodilation and increased albumin permeability induced by NO mirror the early vascular changes reported to occur in diabetic retinopathy [81]. L-arginine and its derivatives, or NO donors and precursors, augment recovery of b-wave amplitude and improve functional recovery following retinal ischaemia in animal models [82,83]. L-arginine and lipophilic derivatives have been shown to increase ocular blood flow and lower intraocular pressure in rabbits [84].

It has been proposed that vascular dysfunction in diabetes may reflect either relative or absolute

increases in NO production [81]. Increased production of NO as a result of hypoxia and diabetes may result from increased intracellular calcium activating NOS, and/or by increased PKC leading to phosphorylation and subsequent activation of NOS [85]. It has been suggested that diabetic vascular dysfunction could be attenuated by NOS inhibitors, such as L-NMMA and L-NAME [81]. Evidence for this hypothesis stems from the finding that two compounds with varied potency for inhibiting AGE formation, methylguanidine and aminoguanidine, exert their physiological effect, in part, as a consequence of inhibiting both the constitutive and inducible isoforms of NOS [81].

2.4 Adenosine

Tissue levels of the endogenous purine nucleoside, adenosine, are increased with hypoxia and depletion of cellular ATP. Adenosine is a potent vasodilator in certain vascular beds [86,87]. Intravitreal injection of adenosine causes dilation of the retinal vasculature, demonstrating the presence of functional adenosine receptors in the retina [88]. Adenosine has been shown to be involved in vasodilative responses to hypoxia [89], and in compensatory retinal blood flow increases in response to acute insulin-induced hypoglycaemia in newborn piglets [90].

Basal levels of adenosine may mediate the constitutive expression of VEGF mRNA. The binding of adenosine to A2a receptors activates intracellular adenylate cyclase, increases cyclic adenosine monophosphate levels, activates protein kinase A and results in VEGF synthesis [91]. It has been demonstrated that hypoxia induces the expression of VEGF mRNA by adenosine A2 subtype receptors in a lymphocytic cell line [92]. Hypoxic regulation of the VEGF receptor (VEGFR-2) in cultured bovine retinal endothelial cells is mediated through the A2 receptor [93]. There appears to be a biphasic regulation of VEGF receptors to hypoxia. Hypoxia induces an initial decline in VEGFR-2 mRNA levels mediated by adenosine. Adenosine analogues increase, and A2 receptor antagonists prevent the hypoxic decreases in VEGFR-2 mRNA expression [92,93]. Adenosine has been shown to stimulate bovine [94] and human [95] endothelial cell proliferation and chemotaxis *in vitro*, and increase the vascular density of the chick chorioallantoic membrane (CAM) assay in a dose-dependent manner [93,96]. Adenosine is capable of regulating hypoxia-inducible genes [97,98]. The glucose transport system (GLUT1) in cultured bovine retinal endothelial cells is regulated by

hypoxia. In this cell line, hypoxia stimulated production of GLUT1 mRNA, up-regulated protein expression and increased transport activity. Adenosine A2 receptor agonists increased, and A2 receptor antagonists inhibited, hypoxia-induced GLUT1 mRNA expression, respectively [98].

The modulation of adenosine action in the retina as a treatment for diabetic retinopathy has recently received increased attention. A number of small molecule A2a antagonists have been identified but their effect on diabetes-induced changes in the retinal vasculature has not been elucidated. One of the most potent and selective non-xanthine A2a antagonists is ZM 241385 (Zeneca Pharmaceuticals) (**Figure 3**).

2.5 Oxidative stress

Ischaemia is thought to underlie the early subclinical stages of retinopathy. Reactive oxygen species are believed to be increased in diabetes, leading to increased oxidative stress [99,100]. According to this hypothesis, hyperglycaemia can promote oxidative stress and produce adverse effects on the vasculature. Retinal blood flow is reduced in short-term diabetic rats, and the anti-oxidant, vitamin E (D- α -tocopherol), has been shown to normalise retinal blood flow in these animals. Diabetic patients show an increase in mean circulation time (MCT) and decreased retinal blood flow [99]. In a double-masked crossover clinical trial of 8 month duration, with crossover at 4 months, vitamin E treatment had a beneficial effect in normalising retinal haemodynamic parameters in diabetic patients with less than 10 years duration, and the benefit was maintained 4 months post-treatment. This study utilised 1,800 IU/day (1350 mg/day) of vitamin E. The beneficial effects appear to be restricted only to very high dosages. The effectiveness of vitamin E suggests that there may be a role for anti-oxidant therapy for diabetic retinopathy. However, vitamin E may have non-antioxidant functions that could, in part, account for the observed beneficial effects. Vitamin E has been shown to normalise hyperglycaemia-stimulated increases in diacylglycerol (DAG) levels and PKC activity in aorta and rat retinal blood vessels [101,102].

2.6 Protein kinase C

Several of the twelve known PKC isoforms have been found to reside in endothelial cells and smooth muscle cells of the microvasculature [103]. The PKC signal transduction pathway phosphorylates protein serine and threonine residues, inducing a cascade of

intracellular events that regulate a variety of vascular functions including contractility, haemodynamics and cell proliferation [104]. With hyperglycaemia, PKC activity increases in a tissue-specific manner. PKC activity is increased in the retina, aorta, heart and renal glomeruli, but not in the brain or the peripheral nervous system [105].

The PKC- β 2 isoform present in vascular cells is believed to play a key role in initiating the intracellular cascade that leads to the development of diabetic retinopathy [104]. PKC- β 2 activation in diabetic tissues results from increased DAG, a consequence of changes in glucose metabolism. Intracellular DAG is the endogenous activator of PKC- β 2; DAG induces PKC- β 2 translocation to the membrane, thus enabling downstream enzyme activation.

Vascular complications of diabetes have been correlated with activation of PKC- β 2 [104,106]. Endothelial cell permeability is increased by PKC- β 2 [107,108], suggesting that PKC- β 2 activation causes endothelial cell dysfunction. Moreover, a relationship between glucose-induced elevations in DAG and retinal blood flow changes has been demonstrated in diabetic rats [105] and diabetic patients [25].

The concept that diabetic retinopathy can be prevented with a potent, selective PKC- β 2 inhibitor raises some interesting possibilities. A selective inhibitor of PKC- β was identified by Lilly, LY333531 [106,109]. This macrocyclic bis(indolyl)maleimide molecule (**Figure 3**) is a competitive reversible inhibitor of both PKC- β splice variants, β 1 and β 2. The compound competes with ATP at its binding site, and it is 60-fold more selective for PKC- β than PKC- α .

Diabetic rats have shown an early decrease in retinal blood flow as assessed by MCT [27]. Presumably, this early increase in MCT arises from increased peripheral vascular resistance. Oral administration of LY333531 to diabetic rats at doses of 0.1-10 mg/kg/day normalised retinal MCT after two weeks of treatment [106]. More recently, the ability of the compound to prevent ischaemia-induced neovascularisation was assessed in a pig model of branch retinal vein occlusion (BVO) [110]. LY333531 was given orally to pigs at a dose of 0.5 mg/kg twice daily, commencing immediately after BVO. Twelve weeks of LY333531 treatment effectively inhibited the preretinal and optic nerve head neovascularisation typically observed in this model.

The preliminary results of an ongoing Phase I clinical trial with LY333531 show encouraging effects with apparently minimal side-effects [111]. A single dose escalation study in non-diabetics showed that the compound is well-tolerated and orally bioavailable. Additionally, a one-month study in diabetic patients was completed. The findings from this study show a dose-dependent normalisation of MCT and retinal blood flow, and no overt toxicity or excess of adverse effects. Reported adverse reactions in a small percentage of subjects include neutropenia, abdominal pain, a slight elevation in uric acid and a slight elevation in immunoglobulins (IgA). Thus, in humans, LY333531 is well-tolerated. It appears to reach therapeutic levels in the retina, and it normalises the diabetes-associated early alteration in retinal blood flow. Of course, longer term clinical trials must now be conducted to determine if LY333531 will ultimately halt the progression of early retinopathy, prevent the proliferative stages and prevent vision loss.

3. Prospective therapy for the proliferative stage: retinal vascularisation

The processes involved in the establishment of the retinal vasculature are highly complex, not fully elucidated, yet are central to the fundamental understanding of the aetiology of diabetic retinopathy. Although the processes of vasculogenesis and angiogenesis share common regulatory features, there are substantial fundamental differences which are of considerable importance for the implementation of novel therapeutic approaches aimed at modulating the process by which retinal blood vessels and/or angiopathies form.

Both vasculogenic (vascularisation by mesenchymal precursors) and angiogenic (new blood vessel formation [neovascularisation] resulting from budding, sprouting and remodelling of pre-existing vessels) mechanisms appear to operate in the formation of the retinal vasculature [112,113,114,115]. Vascularisation of the inner retina occurs early in gestation. Vasculogenesis commences with the aggregation of primordial vascular cells giving rise to blood islands in the yolk sac [21]. The blood islands contain endothelial cells arising from precursor cells, termed angioblasts. Fusiform-shaped vascular precursor cells (differentiated angioblasts), termed 'spindle cells', migrate into the retina from the optic

fissure and spread over the inner surface of the retina [114,116]. The origin of the spindle cells is poorly defined, but immunohistochemical data suggest that they are distinct from microglia and astrocytes [114,116]. Astrocytes have been ascribed a role in the development of retinal vessels and respond to hypoxia by secreting angiogenic factors [117,118,119].

Angioblasts become aligned to form a rudimentary tubular structure (tubulogenesis), giving rise to the initial pattern of the vasculature. A capillary network composed of endothelial cells and pericytes, derived presumably by transformation from spindle cells [114,115,116], is formed in the nerve fibre layer and ganglion cell layer of the retina. The larger calibre vessels found in the inner retina differentiate from the newly established capillary plexus [114].

The adult retinal capillary plexi consist of an inner and outer capillary bed. The inner capillary plexus is located within the nerve fibre layer and the ganglion cell layer of the retina. In humans, the outer capillary plexus resides almost exclusively in the inner nuclear layer [8]. It is believed that the pattern of the inner retinal vasculature is established by the migration of spindle cells (vasculogenic process), while the outer vascular network is established by budding from the inner vasculature (angiogenic process) in response to the metabolic needs of the developing retina [114,115].

The process of angiogenesis has been described as occurring in various phases [120,121]. The earliest phase is characterised by increased vasopermeability, when factors such as VEGF and PKC are suspected to play a role. Mediator molecules such as the angiopoietins (e.g., ANG-1 and ANG-2) potentiate the biological effect of angiogenic cytokines by permitting the vasculature to be more responsive to angiogenic stimuli [122]. Cellular interactions with the extracellular matrix (ECM) are mediated *via* receptors for adhesion molecules known as integrins [123]. Cell adhesion-dependent processes may lead to focal proteolytic activity by enzymes present in endothelial cells, such as matrix metalloproteinases (MMPs), which break down the ECM proteins of basement membranes, promoting the migration of endothelial cells [124]. Subsequently, mesenchymal cells such as pericytes and vascular smooth muscle cells are recruited to stabilise the vessel wall, possibly mediated by ANG-1 [120]. Ultimately, the maturation of the vessel requires *de novo* deposition of collagenous matrix [125].

3.1 Mediators of angiogenesis

The notion that a certain angiogenic factor(s) released by the retina might be responsible for pathological angiogenesis is not novel. The concept originated in 1948 with the British ophthalmologist, Isaac Michaelson [126,127]. He proposed that initiation and cessation of retinal blood vessel growth was dependent on the local concentration of a 'chemical factor' (termed 'factor X' in 1956 by G.N. Wise) in response to retinal hypoxia. This general concept has survived for half a century, and currently this notion remains central to many of the present theories of aberrant vasoproliferation in the retina. However, the characterisation of the vasoformative factors has remained elusive. The list of potential candidates for 'factor X' has expanded considerably in recent years, not necessarily from advances in ophthalmology but rather from advances in the area of cancer research.

The discovery that the growth and metastasis of many malignant neoplasms is dependent upon their vascular supply [128], and that tumour growth can be arrested by restricting its blood supply, has led to a revolution in the search for the identification of many anti-angiogenic agents. The recent advances in elucidating the mechanisms regulating the process of angiogenesis [120,121,129,130], and the supposition that these mechanisms are fundamentally similar to those governing the neovascularisation phase of diabetic retinopathy, suggest that there may be extensive overlap between the angiogenic process in cancer and diabetic retinopathy. Common factors between invasive tumour cells and the process which occurs in angiogenesis are the invasion by microvascular endothelial cells into the basal lamina of the vessels, and the degradation of the ECM proteins which activate and mobilise growth factors chemoattractive for endothelial cells [131]. However, unlike tumour cells, angiogenesis requires strict spatial and temporal modulation [131]. Nevertheless, this seemingly fundamental connection between cancer and diabetic retinopathy may provide a means for modulating the angiogenic 'switch' which appears to be functional in the more advanced stages of diabetic retinopathy.

Diabetic retinopathy is a progressive disease which continues despite good glycaemic control, and takes many years to develop [132,133]. Therefore, the progressive chronic nature of diabetic retinopathy is more consistent with the biological effect of having elevated levels of growth factors which are protracted

rather than transient. Whether alterations in the expression of growth factors occur late in the sequence of events leading to angiogenesis, or play an early or initiating role in the disease process, is an area of controversy and active research [134].

3.2 Tyrosine receptor kinases: vascular endothelial growth factor

The dimeric glycoprotein VEGF has emerged as a potentially exciting ischaemia-induced ocular angiogenic factor [126,135]. It is a secreted, endothelial cell-specific mitogen and is upregulated by hypoxia in cultured cells [97,136]. VEGF receptor (VEGFR) regulation demonstrates a biphasic response to hypoxia in bovine retinal endothelial cell cultures [93]. Four VEGF isoforms have been characterised which arise from alternative mRNA splicing [137]. Two high affinity VEGFRs have been characterised and expressed on the surface of endothelial cells:

- VEGFR-1/Flt-1 (*fms*-like tyrosine kinase-1), believed to have a function in quiescent endothelium of mature vessels not directly related to growth. It may play a role in endothelial organisation during development.
- VEGFR-2/Kdr/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1), a major regulator of vasculogenesis and angiogenesis required for the formation of blood islands and blood vessels [135,137].

VEGF induces the *in vitro* proliferation and migration of isolated bovine retinal endothelial cells that synthesise and respond to VEGF by an autocrine mechanism [138]. Vascular cells have also been shown to respond to VEGF by a paracrine mechanism [139]. There appears to be a constitutive level of VEGF mRNA expression in the retina [140]. *In situ* hybridisation studies have localised VEGF mRNA in retinal ganglion cells and in the inner nuclear layer, which could include Muller cells, amacrine cells, horizontal cells or bipolar cells. Increased VEGF immunoreactivity is detected in human retina and choroid from diabetic patients in the pre-proliferative stage of retinopathy [141]. Elevated levels of VEGF mRNA are detected within a few days of induced retinal ischaemia, confined to the region affected by impaired perfusion [142,143]. Ischaemic retinas predominantly produce VEGF mRNAs corresponding to the diffusing isoforms (VEGF₁₂₁ and VEGF₁₆₅), and low levels of the heparin-binding isoform (VEGF₁₈₉) [140].

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Elevated concentrations of immunoreactive VEGF have been reported in aqueous and vitreous humours of patients with active, proliferative diabetic retinopathy [144]. Since VEGF increases vascular permeability [145], it may enhance the permeability of the blood-retinal barrier. Blood-retinal barrier breakdown in streptozotocin-diabetic rats has been associated with vessels immunoreactive for VEGF [142,145]. VEGF activates phospholipase C γ and increases DAG, which activates PKC- β 1 and - β 2, leading to translocation of PKC isoenzymes and causing increased vasopermeability [108]. In advanced diabetic retinopathy there are elevations of various growth factors in the vitreous humour [134]. A compromised blood-retinal barrier, as a result of elevated VEGF, would promote extravascular deposition of plasma proteins, such as albumin, circulating growth factors and angiogenic cytokines. Extravasation of proteins and other growth factors in plasma could have an angiogenic potential, and this could be an indirect mechanism by which VEGF promotes neovascularisation [134,142]. Inflammatory cytokines like tumour necrosis factor- α (TNF- α) augment the expression and function of VEGFR-2 in endothelial cells and enhance the biological response of endothelial cells to VEGF₁₆₅ by increasing the number of binding sites [146].

Gene knock-out studies have provided a compelling body of evidence implicating VEGF as an essential growth factor early in embryonic vasculogenesis [137,147]. Substantial evidence links VEGF upregulation with the development of iris neovascularisation, ROP, and to the late stages of diabetic retinopathy in the proliferative phase [148].

The premise that VEGF is a causative agent in the development and early manifestation of diabetic retinopathy has not yet been demonstrated. Increased VEGF is more likely to be a late manifestation of the disease after the development of ischaemia, rather than an early event in the progression of the disease. Acute, transient hypoxia has demonstrated an initial decline in VEGFR-2 mRNA levels and VEGF binding sites in bovine retinal endothelial cells, suppressing VEGF-mediated angiogenesis. However, during protracted hypoxia, VEGF receptors are upregulated and the angiogenic actions of VEGF are facilitated [93]. It is postulated that overexpression of VEGF would offset the balance between angiogenic and angiostatic molecules [138]. Early loss of pericytes in diabetic retinopathy would lead to a reduced production of the angiostatic agent, transforming growth factor- β 1

(TGF- β 1), promoting a permissive environment in which the biological action of VEGF is not counterbalanced. The endothelial cells would undergo a transformation from a quiescent to a proliferative state, providing a conducive environment for the development of angiopathy.

Aqueous levels of VEGF are temporally and spatially correlated with iris neovascularisation [149]. In ischaemic retina, VEGF is maximally increased by 13 days of ischaemia and significantly reduced by 28 days [140]. In patients with quiescent neovascularisation, but already having been diagnosed with diabetic retinopathy, concentrations of VEGF are low or undetectable [144]. In an animal model of diabetic retinopathy, treatment with both aminoguanidine and an ARI prevented immunohistochemical expression of VEGF. Only the ARI prevented the diabetic-like retinopathy. Aminoguanidine, despite preventing expression of VEGF, did not prevent the development of microangiopathy [150], suggesting the existence of other critical modulating factors. However, if it is demonstrated that specific inhibition of selected growth factors such as VEGF is pharmacologically feasible, and proves to be non-toxic, it could provide a promising approach for therapeutic intervention against ischaemia-induced ocular neovascularisation.

Antisense oligonucleotides against human VEGF, to inhibit the expression of the VEGF gene in retinal cells, is being explored (Hybridon, Inc.). Specific sequences on the VEGF mRNA have been identified as targets for chemically-modified antisense oligonucleotides. In a hyperoxic mouse model of ROP, two different antisense phosphorothioate oligodeoxynucleotides reduced the level of retinal neovascularisation when injected intravitreally before the onset of proliferative retinopathy [151]. One of the active sequences is complementary to the 3' end of the transcript, including the translational termination codon, and the other oligo hybridises to the 5'-untranslated region of the mRNA. Both sequences are proposed to form a stem-loop structure, providing an advantageous site for oligonucleotide binding and inhibition of VEGF expression as a result of incomplete translation of VEGF. In addition to the studies in mice, there is evidence that the antisense oligos are active in a simian model of ischaemia-induced iris neovascularisation. The oligos appear to have good bioavailability in the retina, and low toxicity when given intravitreally.

Inhibitors of the VEGF/Flk-1 system may have potential therapeutic use in preventing the vasoproliferative phase of diabetic retinopathy [135]. Intravitreal injections of an anti-VEGF monoclonal antibody have been shown to prevent iris neovascularisation [152]. The possibility exists for inhibiting VEGF-induced endothelial mitogenesis by engineering truncated, soluble, high affinity receptors [137,153]. The mutated receptors act by forming inactive heterodimers with functional, endogenous VEGFRs and suppressing their biological effect [153]. VEGF-binding chimeric proteins have been reported to inhibit VEGF-stimulated and hypoxia-stimulated retinal endothelial cell growth *in vitro* by competitive binding of VEGF. Alternatively, a coupled heterodimer composed of a VEGF and placental growth factor subunits may be formed to produce a less biologically active VEGF with suppressed mitogenic activity.

Small molecule selective inhibitors of the VEGFR-1 tyrosine kinase domain have been identified (Sugen, Inc.) [154,155]. Currently, a Flk-1 angiogenesis inhibitor (SU5416) is undergoing Phase I/II clinical evaluation (i.v. administration) for the inhibition of solid tumour regrowth. Kinase inhibitors are being evaluated in models of proliferative ocular disease (Sugen & Allergan). A small molecule inhibitor of VEGF, TBC-1635 (Texas Biotechnology Corp.), has been identified and reported to inhibit glucose-induced vascular dysfunction. It is presently in preclinical evaluation for the treatment of diabetic retinopathy.

3.2.1 Basic fibroblast growth factor

Basic fibroblast growth factor (bFGF) stimulates the proliferation of endothelial cells, vascular smooth muscle cells and fibroblasts. It is also chemotactic for endothelial cells *in vitro*, inducing capillary tube formation. Recent evidence has demonstrated that bFGF stimulates the *in vitro* proliferation of bovine aortic endothelial cells, retinal capillary endothelial cells and pericytes [156].

bFGF has been proposed to play a role in many neovascular processes, and tissue injury or cell damage may be required for the release of bFGF [157,158,159]. Chronic systemic administration of bFGF to dogs with induced regional myocardial ischaemia resulted in an increase in collateral conductance, and an increase in collateral zone vascular density [160]. Although myocardial neovascularisation

would provide a distinct survival benefit in persons with myocardial ischaemia [161,162,163], such an increase in retinal vessels could lead to retinopathy [164].

Intravitreal injections of bFGF into rabbit eyes have produced sight-threatening vitreoretinal proliferative changes [165]. Co-polymer implants (EVAc) have been used as an intraocular delivery system for bFGF in retinectomised chick embryos [166]. In these embryos, retinal regeneration was induced in a dose-dependent manner by bFGF. A redistribution of immunoreactive bFGF from retinal vascular basement membranes to intracellular sites has been reported to coincide with the development of neovascularisation in proliferative diabetic retinopathy in human eyes [167]. Elevated levels of bFGF have also been found in the vitreous of patients with diabetic retinopathy [168,169].

Like the other tyrosine kinase receptors involved in angiogenesis, the FGF receptor is being pursued as a therapeutic target. A compound that inhibits multiple growth factor receptors and has minimal toxicity has been described recently. This agent (SU6668) selectively inhibits Flk-1 and platelet derived growth factor receptor, as well as the FGF receptor [170]. The compound will initially be evaluated for cancer.

3.2.2 Angiopoietins

ANG-1 is a novel angiogenic factor that is the major physiological ligand for the tyrosine kinase receptor Tie2/Tek. This receptor is expressed in early haematopoietic cells, endothelial progenitor cells, fully differentiated normal vascular endothelial cells, and the endothelium of vessels undergoing remodelling [171]. The binding of ANG-1 activates the Tie2 receptor by inducing autophosphorylation. Absence of ANG-1 or its receptor results in extensive vascular abnormalities in mouse embryos [233]. Mutations altering Tie2 activity in humans cause vascular malformations [172]. ANG-1 and Tie2 receptors may regulate the recruitment and maintenance of periendothelial support cells, such as smooth muscle cells and pericytes [122]. Because of this function, ANG-1 is believed to be the factor responsible for maturation and stabilisation of the *vascular plexus*. ANGs appear to play a role in vascular remodelling and maturation.

ANG-2 is a natural antagonist to ANG-1 and Tie2. ANG-2 functions as a natural competitive antagonist to ANG-1-induced activation of Tie2 in endothelial cells or pericytes. ANG-2 may modulate ANG-1

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function of vascular maturation and stabilisation. ANGs may potentiate the effects of other angiogenic cytokines. If co-administered with VEGF, they modulate the *in vivo* actions of VEGF [122]. In the presence of VEGF, the antagonistic action of ANG-2 on ANG-1 maturational effects leads to vascular sprouting, remodelling and neovascularisation. In the absence of VEGF, the inhibitory effects of ANG-2 on the biological effects of ANG-1 lead to vessel regression and vaso-obliteration. The ANGs may prove to provide a 'permissive' rather than an 'instructive' environment for vessel formation [147]. ANG-2 promotes vascular destabilisation and sprouting, providing a more responsive vasculature to the angiogenic stimulus of VEGF. Thus, it appears that the process of vascular formation is under the dual influence of both VEGF and the ANGs. Successful therapeutic intervention of angiogenic diseases will most likely require manipulation of these two systems. The possibility that periendothelial cell maintenance in neovascular development is ANG-1-dependent provides some intriguing possibilities; that pericyte loss observed in diabetic retinopathy may involve a reduction in ANG-1, an increased resistance to the biological actions of ANG-1, or an increase in bioactive ANG-2. Tie2 ligands are in preclinical phase development (Regeneron Pharmaceuticals) for the treatment of retinopathy.

3.3 Integrins

Endothelial and smooth muscle integrins are heterodimeric, transmembrane, glycoprotein cell surface receptors mediating cell-cell and cell-ECM adhesion and signal transduction [173,174,175]. The intracellular domains of these glycoprotein receptors are linked to the cytoskeleton of cells [176]. Integrins interact with the conserved tripeptide sequence Arg-Gly-Asp (RGD) in ECM proteins, such as vitronectin and fibrinogen [177,178], and with other plasma proteins [174,176,179]. RGD-containing peptides are known to inhibit the function of $\alpha\beta3$ integrin [174]. The $\alpha\beta3$ and $\alpha\beta5$ receptors are both members of the integrin superfamily, and play a role in the pathogenesis of retinopathy [176].

Integrins have a synergistic role with MMPs in promotion of angiogenesis [180]. There appears to be a complex interaction between the integrins and the proteolytic cascade essential for vascular remodelling [181,182]. Integrin $\alpha\beta3$ recruits matrix MMP-2 to the surface of endothelial cells and allows degradation of the ECM to occur [183]. Proteolytically active MMP-2

and $\alpha\beta3$ co-localise on the surface of cytokine-induced angiogenic blood vessels *in vivo*. The C-terminus of MMP-2 forms a stable complex with $\alpha\beta3$ facilitating localised proteolytic activity [183]. Integrin-mediated cell adhesion to the ECM in the presence of growth factors establishes a sequence of events that promotes survival and proliferation of endothelial cells, prevents apoptosis and facilitates localised collagen degradation by MMP activity [173,183,184]. Inhibitors of $\alpha\beta3$ prevent the maturation of newly sprouting vessels by promoting apoptosis [184,185].

It appears that nature has designed the regulation of the angiogenic process to be governed by multiple, overlapping mechanisms. This ensures sufficient biological redundancy in a process of fundamental importance to the survival of the organism. At least two angiogenic pathways appear to operate, based on *in vivo* corneal micropocket and CAM assays. The angiogenic pathways are induced by different cytokines but form a final common pathway, with a dependency on two functional homologues of α -v integrins [186,187]. Therefore, the process of angiogenesis is partly dependent on integrins, which form the final common pathway for angiogenesis irrespective of the inducing cytokine(s) [184,186,188]. One angiogenic pathway, initiated by either VEGF, TGF- α or phorbol ester, is dependent on $\alpha\beta5$ integrin and PKC activation. The other pathway is induced by either bFGF or TNF- α . It depends on integrin $\alpha\beta3$ and is PKC-independent [187]. In this pathway it appears that TNF- α also regulates the expression of $\alpha\beta3$ [146].

Bilateral rabbit corneal implant experiments have demonstrated that corneal angiogenesis is dependent on distinct integrins [187]. Implantation of a pellet containing bFGF and a monoclonal antibody (LM609) against $\alpha\beta3$ was performed in one cornea. The contralateral corneal implant contained VEGF and an antibody (P1F6) against $\alpha\beta5$. The bFGF-induced angiogenesis was inhibited by 86% with LM609 compared with P1F6. VEGF induced-angiogenesis was reduced by 60% with P1F6 compared with LM609. In a chick embryo CAM assay, the induction of angiogenesis by both cytokines was prevented with a cyclic peptide antagonist (RGDfV) to both integrins.

In the microvasculature of CNS tumours within hours of focal cerebral ischaemia, there is up-regulation of $\alpha\beta3$ [188]. This integrin is detected in smooth muscle of lenticuloatrial arterioles (30-50 μ m) confined to

the ischaemic region [174]. Integrin overexpression may reflect fibrin deposition or cytokine release. These events may contribute to increases in endothelial cell permeability, thrombin generation and intravascular fibrin deposition. This could promote microvascular occlusion events resulting in areas of non-perfusion. There are alterations in the expression of integrins in neutrophils from patients with diabetic retinopathy, and this has been proposed as a contributing factor to neutrophil-plugging of retinal capillaries giving rise to areas of non-perfusion [189].

Both $\alpha v\beta 3$ and $\alpha v\beta 5$ have been shown to be present on vascular cells in tissues from patients with proliferative diabetic retinopathy [186], and in retinal endothelial cells undergoing neovascularisation [41]. Immunohistochemical studies in retinal vasculature have demonstrated the absence of $\alpha v\beta 3$ in normal human retina and in non-vascular fibroproliferative tissue. However, this integrin is intensely expressed in endothelial cells of neovascular membranes, and choroidal tissue removed from patients with diabetic retinopathy [41]. In an murine model of oxygen-induced ischaemic retinopathy (ROP), endothelial cells positive for $\alpha v\beta 3$ localised to the neovascular region, but no staining was observed in normal retinal blood vessels. Intraperitoneal or periocular injections of synthetic peptides antagonise $\alpha v\beta 3$ -mediated retinal neovascularisation in this model [41]. Subcutaneous injections (20 μg 4 times/daily) of cyclic peptides 66203 (cyclo-RGDfV; Arg-Gly-Asp-D-Phe-Val) and 69601 (Arg- β -Ala-Asp-D-Phe-Val) (Merck KGaA) have been shown to specifically inhibit new blood vessel formation without affecting the pre-existing vasculature in a murine model of ROP [186]. No clinical or histopathological effects were observed in the eye following a two-month examination for toxic effects of cyclic RGDfV. An orally active $\alpha v\beta 3$ antagonist is currently being pursued (SmithKline Beecham Pharmaceuticals), and is in preclinical stages.

Peptides or antibodies antagonising the action of integrins may prove to be a powerful therapeutic modality for angiogenic diseases, because the mechanism of action appears to be at a final common pathway of angiogenesis. Furthermore, the recognition sites for these antagonists are well characterised, they are highly specific and have no apparent ill-effect on the pre-existing vasculature.

The murine antibody LM609 has been humanised utilising codon-based mutagenesis technologies

(IXSYS, Inc.). The humanised antibody VITAXIN has a high *in vitro* affinity for human $\alpha v\beta 3$ and demonstrates a similar biological activity to LM609. *In vivo* studies with VITAXIN have shown inhibition of angiogenesis induced by either growth factor. VITAXIN was filed as an investigational new drug (IND) for cancer in 1996 and has successfully completed Phase I. Its usefulness in diabetic retinopathy is being explored.

Recently, a 7.5 kDa cysteine-rich, trigramin-like, anti-platelet, RGD-containing polypeptide (disintegrin), named Triflavin, purified from *Trimersurus flavoviridis* snake venom, has been identified [190]. This peptide showed 20-30 times greater potency in inhibiting human umbilical vein endothelial cell adhesion and migration than a monoclonal antibody against $\alpha v\beta 3$. Triflavin was of equal potency compared to the antibody in inhibiting angiogenesis in the CAM assay.

3.4 Plasminogen activator inhibitor-1

Two plasminogen activators (PAs) are the endothelium-derived tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA). These enzymes convert the inactive proenzyme plasminogen to plasmin. Plasmin degrades ECM components such as fibronectin, laminin and proteoglycans. It has been reported that plasmin mediates the release of bFGF, and of VEGF₁₆₅ and VEGF₁₈₉ isoforms from the cell surface and ECM [131].

The tPA enzyme has been ascribed the function of fibrinolysis and proteolysis while uPA is primarily involved in ECM proteolysis [191]. The biological activity of these PA enzymes are tightly regulated by endogenous plasminogen activator inhibitor type-1 (PAI-1). PAI-1, a 45-kDa protein, is a member of the serpin (serine proteinase inhibitor) family produced by the liver. It is secreted by vascular endothelial cells, retinal endothelial cells and pericytes in monolayer cultures [15,192]. PAI-1 is a principal regulator in fibrinolysis, and a potent inhibitor of ECM proteolysis mediated by uPA [191]. Retinal microvascular cells secrete a higher level of PAI-1 than several other cell types [192].

In vitro activation of the potent anti-mitogenic factor, TGF- β 1, occurs *via* a plasmin-dependent mechanism upon endothelial-pericyte contact [21,193]. Ligation of uPA receptors by uPA activates latent TGF- β 1 in the presence of plasminogen [131]. It has been suggested

that matrix degradation by TGF- β 1 is prevented by PAI-1 [191]. Overexpression of PAI-1 can result in basement membrane thickening [15]. Both bFGF and TGF- β 1 regulate the synthesis of PAI-1 [192]. In capillary endothelial cells, TGF- β 1 stimulates the production of PAI-1 and suppresses uPA expression [131].

Certain growth factors induce functional expression of uPA, and its cell surface receptor appears to be required for α v β 3 integrin interaction with vitronectin [194]. PAI-1 binds to ECM, possibly through interaction with vitronectin. Competitive inhibitors of uPA and uPA receptor reduce α v β 3-dependent motility [194]. PAs allow the attainment of high tissue concentrations of plasmin, and activate several MMPs [131]. The PA-plasmin system appears to be central to the proteolytic cascade because PAIs will inhibit MMP activation, but inhibitors of MMPs will not inhibit plasmin [131].

In diabetic retinopathy, the lack of TGF- β 1 as a consequence of pericyte loss would render the microvasculature more receptive to angiogenic stimuli [193]. In the absence of active TGF- β 1 the mitogenic effects of bFGF on endothelial cells would not be counteracted, uPA and MMP expression would not be downregulated, and PAI-1 and TIMP synthesis would not be stimulated [131].

Quantitative analysis of colloidal gold-labelled immunohistochemistry for PAI-1 in retinal vessels of diabetic monkeys with NIDDM has shown a 3.6-fold increase in immunoreactive PAI-1 relative to non-diabetic controls. Increased immunolabelling for PAI-1 correlated with the extent of basement membrane thickening. In a diabetic monkey model [195] and a rabbit model [15] of retinopathy, PAI-1 overexpression was detected in the lumen of capillaries, cytoplasm of endothelial cells and in basement membranes.

Elevated serum levels of PAI-1 and increased PAI-1 mRNA levels in retinal microvasculature has been detected in non-insulin-dependent diabetic patients. Diabetic patients with non-proliferative retinopathy demonstrate increased immunoreactivity of PAI-1 in retinal capillaries [15]. uPA receptor expression is upregulated in endothelial cells by bFGF and VEGF. TGF- β 1 decreases endothelial cell uPA levels without affecting receptor expression and up-regulates PAI-1 [131]. PAI-1 over expression has been associated with microthrombi formation with the possible sequelae of capillary occlusion and regions of non-perfusion [15].

Potential therapeutic approaches include the use of soluble uPA receptors, antibodies directed against the active binding site of uPA-uPAR, and a 17-mer peptide which can neutralise anti-uPA antibody 394 [194]. Recently, a 38 kDa fragment of plasminogen, termed angiostatin, has demonstrated potent anti-angiogenic activity. Thus, the co-ordination of integrins, MMPs and the uPA system provides a mechanistic link between cell migration and proteolysis of ECM, essential in angiogenic processes.

3.5 Matrix metalloproteinases

MMPs are a family of at least 15 different secreted and membrane-bound, zinc-dependent endopeptidase enzymes present in various tissues and cell types including fibroblasts, chondrocytes, inflammatory phagocytes and endothelial cells. They are secreted as inactive proenzymes, requiring a 10 kDa N-terminus modification for enzymatic activity. MMPs are capable of degrading most components of the ECM. The *in vivo* activity of these enzymes is regulated at several levels to ensure an exquisite balance of proteolytic activity [196]. The regulation of MMP activity occurs by transcriptional control, activation of proenzymes by partial proteolytic cleavage, and by at least three endogenous tissue inhibitors of MMPs (TIMPs) [131,197,198].

Batimastat (BB-94) and marimastat (BB-2516) (**Figure 3**) are potent broad-spectrum prototype inhibitors (British Biotech, Inc.) of all major MMPs and are the best characterised MMP inhibitors in reference to preclinical and clinical development [198,199]. These compounds are competitive, reversible, synthetic inhibitors of MMPs. They compete with the natural physiologic substrate for MMPs by possessing a 'collagen-mimicking hydroxamate structure' which facilitates chelation of the required zinc ion in the active site of the MMPs (S1' pocket) [200,201,202]. Batimastat, with its peptide backbone, is highly insoluble and has poor bioavailability with oral administration [202]. Intraperitoneal administration to patients with advanced lung carcinoma demonstrated dose-related pain, and irritation at site of injection which required narcotic analgesia. In addition, vaso-vagal reactions were also noted [200]. Batimastat demonstrated a reduction in the amount of malignant pleural effusions without significant systemic toxicity [198,201]. Due to their mechanism of action, a sustained level of an MMP inhibitor is required for efficacy; therefore, clinical development is focused on oral bioavailability [203].

Substrate-based design of MMP inhibitors has resulted in a diversity of structural features which include: succinyl (marimastat) and sulfonamide hydroxamates, carboxylic acids, phosphorous- (as well as sulfur)-containing compounds, substituted 4-biarylbutric or 5-biarylpentanoic acids, and numerous other compounds with potential zinc ligating function [201,202,203]. The second generation MMP inhibitor, marimastat, has a favourable pharmacokinetic profile with oral administration due to a sterically bulky tert-butyl group, and α -hydroxy group which increases aqueous solubility [198,202]. Phase I/II clinical trials have been completed in North America and Europe. Marimastat treatment reduced the rate of rise of cancer specific antigens in a dose-dependent manner in patients with advanced cancers [201]. The drug was well-tolerated, with the exception of reversible musculoskeletal pain and stiffness reported in approximately 30% (10 mg bid) and 70% (25 mg bid) of patients after several months of treatment [198,203]. Marimastat is currently in Phase III clinical trials for cancer [202].

MMP transcription can be induced by plasmin, growth factors, phorbol esters, interleukin-1 (IL-1), TNF- α , PKC and tyrosine kinase signal transduction pathways [131]. Capillary endothelial cells have more collagenase activity and exhibit MMP induction by tPA, a unique feature which distinguishes them from endothelium of larger calibre vessels [196]. Migrating endothelial cells produce a variety of MMPs. TGF- β can block the induction of MMP expression in vascular endothelial cells by other cytokines, possibly by upregulating the expression of TIMPs [131]. TIMPs are able to regulate matrix degradation by inhibiting MMP activity, which is reported to be the rate-limiting step in ECM remodelling [196]. Pharmacologic application of TIMPs is difficult due to their short *in vivo* half-life, and low oral bioavailability. However, elucidation of particular functional domains of the TIMP molecule may render new synthetic and recombinant approaches possible [200,203].

Bryostatins (**Figure 3**) are naturally occurring macrocyclic lactones derived from marine bryozoans, which have been shown to have anti-cancer bioactivity [200,204]. The prototype drug, bryostatin-1, does not affect MMP activity directly but exhibits a biphasic regulation of PKC activity. Initially, it is a transient PKC activator, acting *via* tight binding of PKC and simulating the biological effects of the endogenous activator, DAG. With more prolonged use, bryostatin-1 is a PKC antagonist which rapidly

downregulates PKC [200]. Since elevated PKC levels have been implicated in diabetic ocular complications, bryostatin-1 may share similar physiological effects with another PKC inhibitor, LY333531 [25,104,106]. Clinical trials with bryostatin-1 showed minimal side-effects [200]. These effects were dose-dependent and included neutropenia, which was also seen with LY333531. Bryostatin-1 inhibits the production of certain MMPs *via* its inhibitory effects on PKC, and could also alter TIMP-1 levels which are regulated by a PKC-responsive gene [200]. More potent analogues of bryostatin-1 are currently being designed [204].

Other inhibitors of MMPs include α -2-macroglobulin (a 750 kDa protein produced in liver) tetracycline, and its semi-synthetic derivatives, doxycycline and minocycline (with IC₅₀ values for MMP inhibition in the micromolar range) [200]. However, not all MMP inhibitors are capable of preventing proliferation of microvascular endothelial cells [196].

The existence of MMPs in the vitreous humour from diabetic patients has been reported [205]. Glucose levels have been found to modulate MMP function in human retinal endothelial cells by interaction with fibronectin [206]. The MMP inhibitor, ilomastat (GM6001) (**Figure 3**) (Glycomed, Inc.), has completed Phase II/III trials for the treatment of corneal ulcers and is being pursued for the treatment of corneal transplant neovascularisation. The drug is in Phase II trials in Japan (Sankyo) for diabetic retinopathy, and has been reported to show activity in an animal model of ocular blood vessel proliferation [207].

MMP inhibitors may prove to be a new type of anti-diabetic agent [208]. The inflammatory cytokine TNF- α , produced predominantly by activated macrophages and monocytes, is a key mediator of insulin resistance. TNF- α -mediated insulin resistance may be due to reduced tyrosine autophosphorylation of the insulin receptor and insulin receptor substrate-1 [208]. Also, the formation of AGEs as a result of non-enzymatic glycation activates RAGE on inflammatory cells, leading to the release of cytokines such as TNF- α [68]. TNF- α is synthesised as an inactive, membrane-bound precursor protein (26 kDa), and is converted to its active form (17 kDa) by proteolytic cleavage *via* an MMP-like enzyme, termed TNF- α converting enzyme (TACE) [201,208]. A new class of oral 'TMI' drugs which are combined TNF and MMP inhibitors is being pursued (Glaxo Wellcome) [209].

One example of a TMI is the MMP inhibitor, KB-R7785 (**Figure 3**). KB-R7785 (200 mg/kg/day) decreased plasma glucose levels in KKA^y mice and inhibited LPS-induced increases in plasma TNF- α levels [208]. Presumably, this compound could inhibit AGE-RAGE-mediated TNF- α activation and also improve insulin sensitivity.

3.6 Thalidomide derivatives

Oral administration of thalidomide, a derivative of glutamic acid (**Figure 3**), has been shown to have anti-angiogenic activity in the rabbit cornea micropocket assay [210]. It is postulated that the well-known teratogenic effects of dysmelia (stunted limb growth) in infants from maternal use of thalidomide is secondary to the inhibition of blood vessel development in the undifferentiated fetal limb bud [210]. Thalidomide has no effect on cultured endothelial cells, or on the CAM assay, and is effective only when given systemically.

Ultrastructural analysis of thalidomide-induced changes in vessels reveals increased fenestrations, thinning of cell processes at junctional complexes and vesicular projections. All these changes are reported to be consistent with vascular remodelling [210]. The biological effects of thalidomide arise from the *in vivo* hepatic metabolism of the compound, giving rise to an epoxide. Several angiostatic agents have been known to contain an epoxide structure, which apparently is required for biological activity. Cytochalasin E is a naturally occurring inhibitor of actin, which differs from other cytochalasins in that it contains an epoxide moiety [211]. Cytochalasin E has been shown to inhibit bFGF- and VEGF-induced angiogenesis in the mouse corneal micropocket assay. Racemic thalidomide inhibits both bFGF- and VEGF-induced corneal neovascularisation in C57 black mice when given ip. Thalidomide is able to cause a 36% reduction in bFGF-induced angiogenesis in the rabbit corneal micropocket model. The (-) isomer is a more potent inhibitor of bFGF and VEGF than the (+) isomer, which is believed not to be teratogenic. However, because racemisation occurs rapidly with thalidomide, it is impossible to clearly attribute any biological effect of thalidomide to either one of its enantiomers [212]. This is a potential limitation from a drug development standpoint since there is a correlation between teratogenic potential and the anti-angiogenic effect of thalidomide and its analogues.

When thalidomide is used in combination with certain non-steroidal, anti-inflammatory drugs there is a potentiation of its anti-angiogenic effect without additional toxicity. Analogues of thalidomide that are more stable to hydrolysis (EM-12) (**Figure 3**) appear to have anti-angiogenic activity, but also retain teratogenicity. The fate of drugs like thalidomide or its analogues for the treatment of angiogenic diseases is dependent on the ability to develop analogues that have no teratogenic effects and retain anti-angiogenic bioactivity. The other principal factor limiting the use of thalidomide is the high incidence (20-50%) of peripheral neuropathy [213].

A derivative of thalidomide, phthalimidophthalimide, that lacks the glutarimide moiety has been shown to have little teratogenicity in a non-human primate, but its anti-angiogenic potential has not been documented [214]. The low teratogenicity of this compound may, in part, be attributable to the finding that it does not decrease the expression of integrin surface receptors on white blood cells. Marmosets treated with thalidomide or EM-12 have shown alterations in reactivity of the integrin receptors in T-lymphocytes, B-lymphocytes, monocytes and neutrophils [215]. Thalidomide and its analogues may suppress inflammatory cell adhesion to the endothelium by decreasing the expression of the β_3 subunit of integrins in leukocytes [216], and may exert its anti-angiogenic effect by downregulating β chains on integrins [216]. This could impede integrin-mediated cell migration necessary for angiogenesis.

Thalidomide has been shown to suppress the expression of inflammatory cytokine tumour necrosis factor α (TNF- α) from macrophages [210]. It also enhances degradation of TNF- α mRNA induced by lipopolysaccharide [217], but increases TPA-induced TNF- α in another cell line, suggesting cell-type specificity [218]. TNF- α has been shown to have angiogenic activity *in vivo*. Thalidomide, and analogues of thalidomide (EntreMed), are in Phase II clinical trials and in a preclinical stage for the treatment of retinopathy, respectively.

3.7 Angiostatin, endostatin and thrombospondin-1

Two novel and potent anti-angiogenic agents which are endogenous fragments of proteins have been identified. A 38 kDa fragment of human plasminogen, purified from the serum and urine of tumour-bearing mice, has been shown to completely inhibit

bFGF-induced corneal neovascularisation and suppress metastasis, suggesting that angiostatin could be useful in the management of both non-neoplastic and carcinomatous angiogenesis. The inhibitory fragment of plasminogen includes several triple loop structures (kringle regions) of plasminogen. Intact plasminogen, by contrast, does not have anti-angiogenic activity. The cleavage of plasminogen to form the active, anti-angiogenic fragment could be mediated by a macrophage-derived MMP [196]. A human plasminogen fragment which contains the first three kringle structures (lysine-binding site I) has been shown to inhibit endothelial cell proliferation *in vitro* and angiogenesis *in vivo* using the CAM assay. No toxicity has been detected with systemic administration of angiostatin (100 mg/kg) in experimental animals [219].

Endostatin is a 20 kDa C-terminal fragment of collagen XVIII endogenously produced by haemangioendotheliomas [220]. Both native and recombinant endostatin inhibit endothelial cell proliferation in a dose-dependent manner. Collagen XVIII is found predominantly around blood vessels. Therefore, its localisation is well suited to providing a blood vessel growth regulatory function [220]. The systemic administration of both angiostatin and endostatin has been accomplished by a sc. injection of recombinant non-refolded protein. This novel mode of administration allows for a controlled and sustained release method for *in vivo* therapeutics [220].

The biological activity of angiostatin may be comparable to that of another angiostatic glycoprotein, thrombospondin-1 (TSP-1). This factor is an adhesion antagonist secreted from tumour cells, endothelial cells, pericytes and platelets [192,221]. TSP-1 can modulate angiogenesis by either direct inhibition of endothelial cell proliferation, migration or the prevention of adhesion events to the ECM [219]. TSP-1 is responsible for activation of TGF- β 1 *in vivo* [221].

The three proteins described have tremendous potential as anti-angiogenic agents. To date, most of the information on these agents has been generated in cancer models so, the scientific community anxiously awaits the preclinical data showing their ability to prevent aberrant vessel growth in an animal model of retinopathy.

3.8 Growth hormone

One of the earliest suggestions that growth factors are involved in the progression of retinopathy evolved from an early treatment for diabetic retinopathy, pituitary ablation [222]. Although the loss of the pituitary affected a number of systemic factors, the post-surgery deficiency of growth hormone (GH), leading to a decline in plasma insulin-like growth factor-I (IGF-I), was thought to contribute to the observed reduction in retinopathy in these patients. Although GH has been shown to have direct effects in some tissues, the primary growth promoting effects of GH are due to the induction of IGF-I [223].

Vitreous IGF-I levels are elevated in patients with proliferative diabetic retinopathy [224], and the intravitreal injection of IGF-I induces retinal neovascularisation in rabbits [225]. Clinical studies employing somatostatin to regulate GH secretion have shown an association between plasma GH levels, plasma IGF-I levels and diabetic retinopathy [226].

Significant inhibition of ischaemia-associated retinal neovascularisation was recently observed in transgenic mice constitutively expressing a mutant GH gene (GH antagonist) [227,228], or with systemic suppression of GH release using an inhibitor of GH secretion (MK678) [229]. The extent of retinal neovascularisation was directly proportional to serum levels of IGF-I in the GH-mutant mice. Administration of exogenous IGF-I to the GH-mutant mice restored the normal vasoproliferative response to ischaemia. Receptors for IGF-I are widely distributed in the eye, including the vascular cells [230], but the mechanism of action of IGF-I with regard to retinal neovascularisation is unknown. Nonetheless, the data suggest that GH and/or IGF-I is an important component for ischaemia-induced retinal vasoproliferation, and systemic inhibition of the GH/IGF-I axis has therapeutic potential.

The GH antagonist approach discussed [227,228] is currently being examined in the clinic for the treatment of acromegaly. The GH antagonist Trovert (B2036-PEG, Sensus Corp) is nearing the completion of Phase III trials [231]. Trovert is a pegylated analogue of GH in which eight amino acids are mutated to increase binding affinity to the first receptor binding site, and one amino acid that binds to the second receptor is mutated to prevent binding [227,228]. This analogue displaces wild-type GH from its receptor. It binds only one receptor, prevents receptor

dimerisation and subsequent receptor activation. Trovert is expected to be evaluated for diabetic retinopathy in the near future.

4. Challenges ahead

Diabetic retinopathy continues to be a major health problem worldwide. The crisis is magnified by the continued escalating rate of newly diagnosed cases of diabetes combined with the lack of effective therapy. The pathogenesis of the disease is extremely complex (**Figure 2**). However, this complexity also provides a plethora of possible mechanistic targets for therapeutic intervention (**Figure 1**).

Preclinical animal data for the emerging therapies that target the early (non-proliferative) or late (proliferative) stages of the disease are very promising, but currently there are no 'proof of principle' data for any of these novel therapeutic targets in humans. Since the clinical manifestations of retinopathy require several years to develop, the evaluation of therapies is slow. Furthermore, future clinical trials need to be carefully designed with regard to disease stage, patient number and clinical end-point, as well as duration of the trial and follow-up. Currently the clinical outcome end-points that are typically evaluated by the ETDRS scale predicate clinical trials of 3-5 years duration. Ultimately, the design of the trial should depend on the mechanism of action of the therapy. Targeting an early event in the pathogenesis of retinopathy, such as high AR activity and early ischaemia, may require a much longer trial than targeting a later event, such as angiogenesis, if the same clinical outcome end-points are used. One possible way to shorten the length of the trial is to establish new surrogate end-points of clinical and prognostic significance [26]. Perhaps the ongoing novel and sophisticated preclinical research that is revealing potential new mechanistic targets for the treatment of retinopathy will also reveal new surrogate end-points that can be employed in future clinical trials.

In order to successfully implement anti-angiogenic compounds as a powerful therapeutic modality, a more comprehensive understanding of the mechanisms governing the vascularisation of the retina must evolve. The current concepts suggest that new anti-angiogenic agents for cancer will also have utility in the proliferative stages of retinopathy, since the mechanisms of angiogenesis appear to be similar. Many of the anti-angiogenic agents presented here are

in Phase I clinical trials for the treatment of cancer, and only in a preclinical stage for the treatment of diabetic retinopathy. It is presumptuous at this time to assume that these agents will have benefit in both diseases, since the cause of each disease is very different. However, the wealth of scientific information that is emerging from each area of research can not be overlooked, since it does provide numerous promising therapeutic avenues.

There are factors that are unique to the treatment of diabetic retinopathy. First, the selective permeability of the blood-retinal barrier provides a challenge. High blood levels of systemically administered drugs are required to achieve a therapeutic effect in the eye. This single factor can reduce the therapeutic index of a drug and severely limit its clinical usefulness. Second, and even more perplexing, is the possible unwanted side-effects from administering a systemic anti-angiogenic agent to patients with diabetes that are at risk of developing atherosclerotic vessels of the myocardium, and may require the growth of collateral microvessels to compensate. Alternatively, they may need angiogenesis to overcome peripheral ischaemia and common wound healing problems. The ideal therapy for the treatment of diabetic retinopathy is an anti-angiogenic agent that is specific for the aberrant retinal vasculature. This ideal agent may emerge from a retinal vessel-specific targeting molecule [232], or perhaps become clinically feasible by the implementation of a novel ophthalmic drug delivery mechanism.

Many of the approaches under investigation share common mechanisms of action, but some drugs still have a poorly defined mechanism of action which remains to be elucidated. It is likely that the neovascularisation of diabetic retinopathy is mediated by a complex and highly orchestrated biological action of several modulating factors, rather than by the single action of a particular growth factor [38]. Some of these factors and therapeutic targets may be found to synergise with one another, thus suggesting that a combination therapy may be most effective. Also, these novel therapeutic targets may prove to be effective in delaying the progression of the disease rather than in prevention. Nonetheless, if these agents and therapeutic approaches prove to have clinical benefit, we are truly at the crossroads in terms of the future direction and management of diabetic retinopathy.

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