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REVIEW

The pathogenesis of diabetic retinopathy: old concepts and new questions

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Abstract

Hyperglycaemia appears to be a critical factor in the aetiology of diabetic retinopathy and initiates downstream events including: basement membrane thickening, pericyte drop out and retinal capillary non-perfusion. More recently, focus has been directed to the molecular basis of the disease process in diabetic retinopathy. Of particular importance in the development and progression of diabetic retinopathy is the role of growth factors (eg vascular endothelial growth factor, placenta growth factor and pigment epithelium-derived factor) together with specific receptors and obligate components of the signal transduction pathway needed to support them. Despite these advances there are still a number of important questions that remain to be answered before we can confidently target pathological signals. How does hyperglycaemia regulate retinal vessels? Which growth factors are most important and at what stage of retinopathy do they operate? What is the preferred point in the growth factor signalling cascade for therapeutic intervention? Answers to these questions will provide the basis for new therapeutic interventions in a debilitating ocular condition.
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Diabetic retinopathy, a major cause of blindness in developed countries, is characterised by hyperglycaemia, basement membrane thickening, pericyte loss, microaneurysms, IRMA and preretinal neovascularisation which can eventually lead to blindness through haemorrhage and tractional retinal detachment. Since it was first described in 1977[1] there has been much debate about the initiating factor(s) in diabetic retinopathy with lack of glucose control considered of major importance. This review aims to consider the role of hyperglycaemia in retinopathy and how this contributes to a change in the balance of regulators of the retinal vasculature.

Can glucose control in patients with diabetes prevent the onset of retinopathy?

The answer appears to be 'yes' from three hallmark trials undertaken on different continents: the Diabetes Control and Complications Trial (DCCT) undertaken in the USA in 1993,[2] the United Kingdom Prospective Diabetes Study (UKPDS) in 1998[3] and a Japanese trial.[4] DCCT highlighted that intensive glycaemic control can prevent or delay the development or progress of diabetic retinopathy by 76% in patients with type 1 diabetes within a primary prevention group over an average of 6.5 years. The UKPDS came to a similar conclusion when assessing glucose control and disease progression in patients with type 2 diabetes which supported the observations from the Japanese study.[4] These trials demonstrated that blood glucose levels of diabetic patients at the first doctors visit indicated the outcome of their retinopathy and that glucose control early in the condition is probably vital to prevent or delay the onset of retinopathy.

How does hyperglycaemia regulate the progression of retinopathy?

Hyperglycaemia is associated with a variety of biological events identified in the progression

of diabetic retinopathy (eg glucose transport, basement membrane thickening, pericyte loss, blood characteristics). Animal models such as the streptozotocin rat suggest that long-term hyperglycaemia is necessary to elicit changes to the retinal vasculature.[5] Hyperglycaemia does not result in pathological changes in the retinal vasculature within the first 6 weeks. However, after this period proliferation of endothelial cells and swollen retinal vessels are observed. Interestingly, the retinal vessel lesions persisted even after the blood glucose levels have returned to normal. These abnormalities extended to include loss of pericytes and endothelial cells from the capillary beds and the appearance of microaneurysms. It is clear that there is a time point after which the progress of diabetic retinopathy is inevitable and reinforces that it is crucial to elicit preventative measures such as intensive blood glucose control at the very early stages of diabetes to prevent or slow progression of retinopathies. As will be discussed below, glucose can have a detrimental effect on a variety of biological processes.

Retinal endothelial cell glucose transport

To date, several possible mechanisms, including the polyol pathway,[6] non-enzymatic glycation,[7] oxidative stress[8] and activation of protein kinase C (PKC)[9] have been implicated in the development of retinopathy. These mechanisms are mostly dependent on excessive transport of glucose into retinal cells resulting in increased intracellular glucose levels. GLUT1, one of a family of glucose transporters, is exclusively responsible for glucose crossing the inner retinal-blood barrier.[10,11] Surprisingly, the early stage of diabetic retinopathy exhibits a decrease in expression of GLUT1 in retinal endothelial cells, inferring that GLUT1 is not strongly linked with the development of retinopathy. Since expression of GLUT1 in the retinal pigment epithelium (RPE) is not affected by diabetes, it is likely that glucose entering the retina is greater across the RPE than across retinal endothelial cells.[10] However, it has been proposed that an increase in density of relocalized GLUT1 in the inner blood-retinal barrier is enhanced by vascular endothelial growth factor (VEGF),[12] a factor upregulated in retinopathy.

Retinal blood control

It is quite evident that there are numerous changes taking place in the vasculature that are associated with the early stages of diabetic retinopathy prior to the appearance of pathological changes. Furthermore, a duration of years or decades elapse before subtle changes lead to the observation of retinopathy. A typical example is the functional changes in the retinal vasculature[13,14] resulting in a change of retinal tissue blood flow pattern including an increase in retinal blood flow and heterogeneity of distribution of retinal blood flow.[15] The retinal vasculature relies on local mechanisms to regulate appropriate blood flow.[16] To date at least 10 regulating factors have been proposed for control of blood flow.[17] They fall into two main classes: endothelium-derived relaxing factors (nitric oxide, prostacyclin, endothelium-derived hyperpolarizing factor) and endothelium-derived contracting factors (endothelin, cycloxygenase products) which inhibit or stimulate respectively the underlying smooth muscle cells and pericytes. Importantly, many of these factors are regulated by local glucose levels.

Endothelium-derived relaxing factors

Nitric oxide Nitric oxide (NO) acts to maintain appropriate arteriolar vasodilation as well as stablilizing platelets.[18–25] It is synthesized in cells from L-arginine to L-citrulline via activation of a calcium-dependent nitric oxide synthase. In response to platelet-derived products, hormones and mechanical changes such as transmural pressure,[26] endothelial cells release NO into the surrounding milieu. NO enters smooth muscle cells and activates soluble guanylate cyclase. This results in increased cyclic guanosine 3′,5′-monophosphate (cGMP), which is responsible for subsequent relaxation of the smooth muscle cells through a decrease in Ca^{2+} and dephosphorylation of myosin light chains.[27] In the retinal vascular bed there is a constant basal release of NO which maintains the retinal circulation in a constant state of vasodilation.

Evidence indicates that there are at least three mechanisms for diminishing production and/or increasing quenching of NO by hyperglycaemia. First, hyperglycaemia causes *de novo* synthesis of diacylglycerol (DAG), leading to activation of PKC. The consequence of PKC activation is that PKC reduces the capacity of a number of agonists to increase intracellular Ca^{2+} and stimulate NO synthesis.[28–30] Furthermore, PKC may provoke expression of superoxide in endothelial cells which quenches NO.[31] Second, hyperglycaemia activates the polyol pathway by increasing substrate-glucose for endothelial aldose reductase.[32] This enzyme converts glucose to sorbitol by a reaction that oxidizes NADPH and reduces its availability (NADPH is one of the cofactors for NO synthesis).[33] Third, hyperglycaemia generates non-enzymatic glycated proteins[34] which lead to subsequent superoxide generation resulting in

inactivation of NO. Additionally, glycated proteins can directly quench NO.[35]

Prostacyclin (PGI2) PGI2 is generated from the metabolism of arachidonic acid via cyclooxygenase[36] and is complementary to NO. PGI2 stimulates enhanced production of cyclic adenosine 3′,5′-monophspate (cAMP) through adenylcyclase in smooth muscle cells and pericytes, leading to relaxation of those cells.[37] In endothelial cells PG12 synthesis is mediated by prostacylin-stimulating factor (PSF), which is constitutively expressed in retinal pericytes.[38] However, early hyperglycaemia results in a transient decrease in PSF production, causing a decrease in PGI2 synthesis.[39] In animal models it has been shown that after the induction of diabetes an early decrease of PSF in the retina is followed by an increase in PSF levels. Hyperglycaemia also inhibits PGI2 synthesis through generating lipid peroxide and arachidonic acid via microsomal desaturase.[39] Intriguingly, normal levels or even increased of PGI2 in diabetes have been reported.[39] This might be due to upregulation of phospholipase A_2 (PLA_2) providing increased substrate for PGI2 synthesis.

Endothelium-derived hyperpolarizing factor (EDHF) EDHFs are substances that are distinct from nitric oxide (NO) or prostacyclin (PGI2). EDHFs are thought to mediate endothelium-dependent hyperpolarization of vascular smooth muscle cells or pericytes. EDHF contributes greatly to vascular control of small-diameter vessels and microvessels involved in the local regulation of peripheral vascular resistance and thus in the distribution of blood flow.[40] By contrast, NO and PGI2 are more committed to regulation of large-diameter vessels. A list of potential agents/cellular events which could function as EDHF include eoxyeicosatrienoic acid (EET),[41–46] hydrogen peroxide,[47] potassium efflux,[48] and gap junction communication between endothelial cells and smooth muscle cells.[49] For example, EET is released from endothelial cells in response to acetylcholine (ACh) and hyperpolarizes smooth muscle cells by opening Ca^{2+}-activated K^+ channels and causing vasodilatation.[47] A line of evidence for a role of EDHF in diabetic retinal vascular dysfunction is inferred from data indicating that in diabetes, endothelium-dependent hyperpolarizations are diminished by hyperglycaemia largely due to a defective vascular response to EDHF.[50]

Endothelium-derived contracting factors

Endothelin-1 (ET-1) ET-1 is a powerful vasoconstrictor peptide. The circulation levels of ET-1 are low under normal conditions suggesting that ET-1 acts as a local regulatory factor.[51] ET-1 causes vasodilation at low concentrations and a constrictive response at high concentrations via the interaction with endothelin receptors (ET_A-, and ET_B) on smooth muscle cells and pericytes.[52–54] ET-1-induced activation of endothelin receptors, linked to voltage-operated Ca^{2+} channels, either opens the gates of the Ca^{2+} channel leading to influx of Ca^{2+},[55] or induces activation of phospholipase C (PLC) by the formation of diacylglycerol (DAG), shifting Ca^{2+} from intracellular stores and increasing intracellular Ca^{2+}.[56,57] Increased intracellular Ca^{2+} in smooth muscle cells in turn induces lasting contractile effects. Hyperglycaemia is most likely to induce an increase in ET-1 levels in retinal vascular cells.[58] PKC and mitogen-activated protein kinase (MAPK) are activated in retinal microvascular cells by the elevation of glucose levels.[59,60] Thus, PKC and MAPK pathways enhance the ET-1 transcription rate.

Cyclooxygenase products Cyclooxygenase products induce vasoconstriction and include thromboxane (TX),[61] prostaglandin (PG)[62] and lipid peroxides (LPO)[63] which can be found in endothelial cells and platelets. However, overproduction of these factors has been detected in diabetic retinopathy. For instance, PKC, enhanced by hyperglycaemia, activates phospholipase A_2 (PLA_2) which sequesters arachidonic acid from membrane phospholipids.[64] Arachidonic acid is a substrate that is catalyzed by cyclooxygenase and lipoxygenase promoting LPO generation.[65]

Retinal capillary cell death

Histological analyses of diabetic retina demonstrate localised regions of non-perfused acellular 'vessels' consisting solely of basement membrane.[66] The early and progressive loss of retinal capillary cells, including pericytes and endothelial cells, inevitably leads to microaneurysms and vascular obstruction. Retinal capillary cell death unquestionably has a major impact on retinal vessels in diabetes and in the case of pericyte loss occurs long before the onset of proliferative diabetic retinopathy (PDR). However, capillary cell death, specifically pericytes, has been found to be rare or absent from capillaries of the optic nerve and cerebrum.[67] Perhaps this is evidence that disappearance of retinal capillary cells may be due to a local disorder rather than systemic abnormalities such as hyperglycaemia? However, hyperglycaemia has been shown to induce pericyte apoptosis both *in vivo* and in *vitro*,[67,68] with *in vitro* evidence that cell death is exacerbated when glucose levels fluctuate between hyper- and normoglycaemia as often occurs in poorly controlled diabetes.[68]

Polyol pathway There is limited evidence that hyperglycaemia can, in tissues such as the retina that do not require insulin for cellular glucose uptake, induce polyol pathway hyperactivity and aldose reductase expression.[69] In addition to its well-documented glucose metabolism role, the polyol pathway has been found to cause a loss of retinal capillary cells and that this involves aldose reductase, a rate-limiting enzyme of the polyol pathway that reduces glucose to sorbitol.[70,71] Aldose reductase inhibitor has been reported to inhibit the high glucose-induced death of retinal capillary cells.[72,73] Sorbitol, a common organic osmolyte in many cells, accumulates in retinal capillary cells in response to hyperglycaemia and causes hyperosmolality of the cells.[74,75] Thus, hyperosmolality induces an increase in intracellular water and lactate production, and a decrease in oxygen uptake. The other part of the polyol pathway involves glutathione reductase reducing NADPH to NAD, in which aldose reductase competes with NADPH.[76,77] NADPH is required not only by glutathione reductase for the reduction of oxidized glutathione (GSSG) to glutathione (GSH), but also by aldose reductase for conversion of glucose to sorbitol. Reduced NADPH may also be responsible for dysfunction of endothelial enzymes, for example eNOS.[33] In addition, hyperactivity of the polyol pathway requires large quantities of ATP[78] and may consume the energy required for production of endothelium-dependent relaxation factors.

Glycation pathway During normal ageing, glucose binds non-enzymatically to free amino groups in proteins and forms Amadori adducts through a series of oxidative and non-oxidative reactions.[79] Hyperglycaemia and oxidative stress probably confer on Amadori adducts the opportunity to continue to rearrange and generate irreversible advanced glycation end products (AGEs) in diabetes.[80] The impact of AGEs on retinal capillary cells is related to their capacity to accumulate in tissues over time, to form cross-links and to generate oxygen-derived free radicals.[81–84] Additionally, binding of AGEs with their receptors may provoke sustained cell activation and further oxidative stress.

Oxidative stress Oxidative stress is defined as an increase in the steady-state levels of reactive oxygen species. There are several endogenous enzyme systems that protect the cell and tissue from oxidative stress, for example superoxide dismutase (SOD),[85] catalase,[86] and glutathione peroxidase (GSH-Px).[87] Although there is controversy about the antioxidant status in diabetes, several studies report decreased levels of SOD and GSH-Px in both clinical and experimental diabetes,[83,84,88,89] indicating an impaired defence system for free radical scavenging. Sources of reactive oxygen species in diabetes may include autoxidation of glucose, AGE-formation and the binding of AGE to AGE receptors, increased substrate flux through the polyol pathway and stimulation of eicosanoid metabolism. 8-epi-PGF(2alpha), one of the prostaglandin-F(2)(PGF(2))-like compounds produced during peroxidation of arachidonic acid (AA) by a mechanism independent of the cyclo-oxygenase,[90] has recently been detected in the retina during diabetes. This provides direct evidence that oxygen-derived radicals produced during prostanoid synthesis rather than the prostanoids themselves are responsible for endothelial dysfunction in diabetes mellitus.[91] Oxygen-derived free radicals may impair endothelium-dependent vasodilation through inactivation of NO.[92] In addition, oxidative stress can cause an increase in the conversion of deoxyguanosine to 8-oxo, 2′-deoxyguanosine in DNA.[93] Both the altered gene profile of scavenging enzymes[94] and overexpression of the cell death protease gene[95] are believed to increase apoptosis of retinal capillary cells in diabetic retinopathy.

What causes retinal ischaemia?

Retinal ischaemia is generally believed to result from structural and functional derangement of the retinal microcirculation. The formation of acellular capillaries is a major histological feature of the ischaemic retina.[96,97] The capillary basement membrane tubes without endothelial cells and pericyte nuclei firstly only occur singly or as small groups scattered about the retinal. Later they are found in large clusters with atrophic arterioles.[98,99]

Several possible mechanisms have been proposed for the appearance of retinal ischaemia in diabetes. These include thickened basement membranes, platelet aggregation, leukocyte activation/adherence or a combination thereof. Furthermore, hyperglycaemia is likely to be a major risk factor.

Retinal basement membrane thickening

In diabetes, early hyperglycaemia is sufficient to increase the synthesis of basement membrane components in the retina[100] which in turn may contribute to the closure of capillaries. For example, mRNA for fibronectin and collagen types I, III, IV(α1, α2), and V are found to be upregulated in the retinal basement membrane of diabetic retinopathy. Furthermore, in the retina of diabetic patients increased

immunostaining is observed compared to normals for vitronectin in the arterioles,[101] collagen types I, II, III, IV in the venules[102] and laminin and fibronectin in both arterioles and venules.[103] Animal models also show that retinal expression of collagen type IV and fibronectin increases in hyperglycaemic rats.[104–106] Diabetic basement membrane thickening appears to involve qualitative alterations of specific basement membrane markers at an advanced disease stage, with the appearance of diabetic retinopathy. For instance, abnormal accumulation of several extracellular matrix components in retinal basement membranes may trigger the deposition of small tenascin-C isoforms in the blood vessel walls.[107] The expression of tenascin,[108] an extracellular matrix glycoprotein, originally found to modulate organogenesis in tendinous and glial tissue, suggests that this glycoprotein may promote retinal basement membrane thickening.

Platelet aggregation

Diabetic retinopathy is associated with an increased number and size of platelet-fibrin thrombi in the retinal capillaries compared to normal.[109] These thrombi can contribute to capillary obliteration and retinal ischemia. It has been reported that chronic hyperglycemia causes an increase in diacylglycerol (DAG) levels in the retina, which may activate PKC.[110] Through increased intracellular Ca^{2+}, PKC stimulates endothelial cells, leuckocytes and platelets to produce platelet-activating factor (PAF).[111–113] PAF, confined to membranes, stimulates PAF receptors[114] on platelets, inducing activation of these platelets. Activated platelets produce a number of platelet-derived microparticles,[115,116] which contribute to thrombus formation by providing and expanding a catalytic surface for the coagulation cascade. Pathological levels of fluid shear stress in abnormal retinal blood vessels affected by hyperglycaemia may cause both further platelet aggregation and shedding of more microparticles from the platelet plasma membrane.[117] In addition, elevated sorbitol in the retina and erythrocytes can reduce vascular prostacyclin accompanied by an increased synthesis of thromboxane via induction of adenosine diphosphate (ADP)[118] or collagen[119] in whole blood. The imbalance of thromboxane and prostacylin enhances platelet hyperactivity.[120] Adhesion proteins[121] that are cofactors in the aggregation of human platelets and mediating the adenosine diphosphate (ADP)-induced response of these cells are also increased significantly.

Leukocyte activation and adherence

Although diabetic retinopathy generally is not considered as an inflammatory disease, leukocytes adhere to the retinal vascular endothelium early in experimental diabetic retinopathy.[122] Excess activation of endothelial PKC promotes PAF synthesis in diabetes.[110,123,124] PAF stimulates PAF receptors on peripheral leukocytes rolling on the lumenal endothelial membrane leading to their activation.[125] Activated leukocytes also synthesise PAF[126] and leukotriene B4 (LTB4),[127,128] which further enhance activation of leukocytes via autocrine action. β2 integrins[129,130] on activated leukocytes enable the leukocytes to adhere tightly to the endothelial cell via binding intercellular adhesion molecule-1 (ICAM-1).[131] ICAM-1 is upregulated by endothelial PKC which normally acts to stabilize mRNA of β2 integrins.[132,133] Physiologically, nitric oxide (NO) plays a role in modulating leukocyte activation and adherence. Presumably, NO deficiency can allow leuckocytes to escape from NO control, leading to leukocyte activation and adherence.[134,135] Furthermore, leukocytes in diabetes have been reported to be less deformable due to actin polymerization and increase in their viscosity.[136] Alteration in retinal blood flow could reduce pressure gradients across retinal capillaries owing to stenotic or constricted arterioles resulting in activated leukocytes becoming wedged in capillaries and postcapillaries and obstructing retinal microvessels.[137,138]

What is the importance of retinal hypoxia?

The retinal vasculature is relatively sparse in order to minimise optical interference in the light path. This results in a large oxygen tension difference between retinal arteries and veins which can easily be compromised if damage occurs to the vascular bed. Capillary nonperfusion, loss of retinal capillaries, AGEs and/or oxidative stress can lead to progressive retinal hypoxia.[139–143]

Acute hypoxia rapidly activates retinal vascular endothelial cells to release inflammatory cytokines.[144] These inflammatory mediators are able to recruit and promote the activation and adherence of leukocytes,[145] which contribute to the obstruction of retinal capillaries, leading to further hypoxia. Chronic hypoxia is, at least in the retina, sufficient to induce the expression of angiogenic growth factors,[146,147] resulting in the characteristic retinal neovascularization associated with proliferative diabetic retinopathy (PDR). The observation that retinal neovascularization occurs adjacent to the nonperfused area[148,149] supports

the hypothesis that angiogenic factors are released from hypoxic tissue. In the majority of instances regression of preretinal new vessels can be achieved through the use of scatter laser photocoagulation.[150,151] While the mechanism of action of scatter laser photocoagulation remains elusive, there is support for the hypothesis that destruction of retinal tissue makes more oxygen available for the retina and returns it to normoxia.[152]

The whole picture of how hypoxia induces and increases the expression of angiogenic factors is not clear, but parts of the puzzle are beginning to emerge. A cytosolic flavoheme protein acts as an oxygen sensor that detects decreased oxygen tension and activates transcription factors through signal transduction pathways.[153,154] Hypoxia inducible factor 1 (HIF-1) is a major transcription factor.[155] The activation of HIF-1 depends upon signaling-dependent rescue of its alpha-subunit from oxygen-dependent degradation in the proteasome and formation of a heterodimer with HIF-1beta, which then translocates to the nucleus and impacts on the transcription of genes that are upregulated by hypoxia[156–158] Activation of HIF-1 has been shown to increase production of a variety of factors implicated in the pathogenesis of diabetic retinopathy (eg ischaemic retina[159]).

How do growth factors play a pivotal role in diabetic retinopathy?

It is now quite evident that there is a plethora of growth factors which regulate the retinal vasculature and are involved in the development and progression of diabetic retinopathy. However, identifying the role of each growth factor is difficult since growth factors can act alone or, as appears to be more often the case, interact with each other. Examples include: one growth factor inducing the synthesis of a more potent growth factor, synergy between growth factors and commonality in the downstream transduction cascade. In addition, knockout studies have shown that if the action of a growth factor is negated other growth factors are synthesised to overcome this deficit.

The last three decades have seen the discovery of a large number of growth factors, most of which have been implicated to a greater or lesser extent in diabetic retinopathy. Of these vascular endothelial growth factor has received considerable attention of late due to its potent angiogenic activity.[160]

Vascular endothelial growth factor (VEGF)

VEGF is a potent angiogenic factor capable of stimulating endothelial cells to degrade extracellular matrix, migrate, proliferate and form tubes.[161–163] Recently, it also has been found to act as a survival factor for newly formed vessels.[164] VEGF exerts its functions on endothelial cells via interaction with cellular receptors Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2),[165–171] both receptor tyrosine kinases. Interaction between VEGF and its receptors initiates a signal transduction pathway, and thus induces phosphorylation of proteins downstream in endothelial cells, including phospholipase Cγ (PLCγ),[172,173] phosphatidylinositol 3-kinase (PI3-Kinase)[174,175] and guanine 5′ triphosphate (GTP)ase-activating protein.[176] Phosphorylation of PLCγ may be more important for retinal endothelial cells. Phosphorylated PLCγ converts inositol phosphate into diacylglycerol (DAG), causing activation and translocation of PKC that engages subsequent changes in endothelial cells.[177,178] It is generally considered that activation of the Flt-1 receptor regulates the metabolism of a range of vascular and non-vascular cells while KDR which is relatively specific for vascular endothelial cells promotes migration and proliferation.

Increased levels of VEGF have been identified in the vitreous and the retina of patients with diabetes.[162,179–181] This increase is likely to be hypoxia-induced since elevated levels of VEGF protein and mRNA are present in the ischaemic retina adjacent to the areas of neovascularization in diabetic animals and human pathology specimens.[182–184] Moreover, *in vitro* hypoxia also induces expression of VEGF mRNA in retinal cells.[185] Hypoxia is also reported to induce expression of VEGF receptors in endothelial cells indicating that sensitivity to VEGF is enhanced in the ischaemic retina.[186,187]

VEGF also appears to play an early role in the development of diabetic retinopathy. VEGF is clearly elevated in diabetic retinal tissue without overt retinopathy and is likely to initiate the increased permeability associated with the retinal vasculature in diabetes.[186,188–190] Animal experiments have convincingly produced clinical features of nonproliferative diabetic retinopathy by repeated intravitreal injections of VEGF.[191] VEGF has long been known to increase the permeability of vascular endothelium, which may involve rearrangement of interendothelial junctional proteins,[192,193] including VE-cadherin, tight junction proteins (eg occludin and zonula occluden 1) in retinal endothelium. Such effects presumably underlie the increasd risks of vessel leakage and macular edema in diabetic retinopathy. Clinical experience suggests that there is fluctuation of retinal blood flow in patients with diabetes, who have a decrease in retinal blood flow at the early stage of retinopathy, with a progressive increase in retinal

blood flow in more advanced stages.[190,194] Similar changes in retinal blood flow are observed in diabetic animals following intravitreal injections of VEGF.[191] Such observations support the concept that VEGF not only contributes to retinal neovascularizton, but also produces earlier changes in diabetic retinopathy. Interestingly, inhibition of VEGF activity by specific antisense oligonucleotides,[195] VEGF-neutralizing antibodies[196] or soluble receptors[197] is insufficient to completely prevent neovascularization. The incomplete inhibition of neovascularization indicates that retinal neovascularization may be driven at least in part by alternative angiogenic factors.

Alternative angiogenic factors

A plethora of other angiogenic factors including insulin-like growth factor-I (IGF-I), basic fibroblast growth factors (bFGF or FGF2), platelet-derived growth factor (PDGF), hepatocyte growth factor/scatter factor (HGF/SF), placenta growth factor (PIGF) and angiopoietin2 (Ang2) have been implicated in retinal neovascularization.

IGF-I A role for a pituitary associated factor was hypothesised over 40 years ago when retinal neovascularisation was found to regress after pituitary infarction.[198] Subsequently the pituitary factor has been identified as growth hormone and the mitogenic mediator of growth hormone action is Insulin-like growth factor-I (IGF-I).[199] IGF-I was one of the first growth factors to be directly linked with diabetic retinopathy.[200] Initial reports demonstrated an acute increase in serum levels of IGF-I preceded the onset of proliferative diabetic retinopathy (PDR) in animal models.[201,202] Subsequently, increased IGF-I levels were measured in the vitreous of patients with PDR,[203] indicating that IGF-I may play a role in retinal neovascularization and that the localised effect of IGF-I may be more important than its systemic role in the development of neovascularization. It has been proposed that leakage across the blood–retina barrier and high serum levels of IGF might be the major source for vitreous IGF levels. Confirmation has come from *in vitro* studies[204–206] showing that IGF-I can induce almost all steps of the angiogenesis process including endothelial cell proliferation, migration and basement membrane degradation. *In vivo*, retinal angiogenesis has been confirmed following application of IGF-I to the retina of rabbits.[207] IGF-I exerts its effect on endothelial cells via coupling with the IGF-I receptor (IGF-IR). Two pathways are prominent in IGF-I signalling, the Ras/Raf/MAPK cascade[208] and the PI 3-kinase system,[209] both of which promote cell survival and proliferation.[210,211] IGF-I is regulated by a family of insulin-like growth factor binding proteins (IGFBPs)[212] which can inhibit or potentiate IGF-I activity depending on a number of parameters such as their affinity for IGF-I, the biological system in question and post-translational modifications.[213] IGFBPs 1, 2 and 3 have been reported to be significantly increased in vitreous from patients with PDR but not in non-ischaemic eye disease.[214–216] The contribution of leakage of the blood retinal barrier and local synthesis to vitreous levels of these proteins is unclear, however, local synthesis will certainly contribute. Comparison of cultured retinal endothelial cells from normal and diabetic donors demonstrates a decrease in IGF-1 and an increase in the IGFBP 1, 2 and 5 message for diabetic cultures compared to normals.[217] An important link between IGF-1 and IGFBP expression and diabetic retinopathy is hypoxia. Several studies *in vitro* have shown that IGF-I and IGFBPs are subject to regulation by hypoxia.[218–220]

bFGF Whether basic fibroblast growth factor (bFGF) participates in the stimulation of retinal neovascularization has been a matter of considerable controversy. bFGF is stored at high concentration within the extracellular matrix (ECM) as an inactive complex, and released when endothelial cells dissolve ECM via the release of proteases.[221–223] bFGF and hypoxia act synergistically to not only induce mitogenesis in endothelial cells, but also to upregulate VEGF in smooth muscle cells and endothelial cells, resulting in retinal angiogenesis.[224] However, the fact that bFGF-deficient animal models develop the same degree of retinal neovascularization as wild-type animals argues against a major angiogenic role for bFGF in diabetic retinopathy.[225] Although bFGF may not directly induce retinal neovascularization, it can regulate VEGF expression in retinal vascular cells.[226]

PDGF The platelet-derived growth factor (PDGF) family comprises three isoforms, PDGF AA, BB and AB, which act via PDGF receptor subunits (α- and β-). PDGF is widely expressed upon tissue injury and repair[227,228] and the PDGF BB isoform is induced by hypoxia.[229,230] Additionally, significantly elevated concentrations of PDGF AB are found in the vitreous and preretinal membranes of patients with proliferative diabetic retinopathy (PDR).[231] PDGF may act directly on endothelial cells[232] that are engaged in angiogenesis or that express PDGF receptor beta-subunits.[233,234] However, it is reported that PDGF AB is also elevated in ischemic non-diabetic retinopathy, indicating that ischemia rather than diabetes *per se* might be a strong stimulator of PDGF production in the retina.[231] Since

PDGF is known to induce the generation of a vascularized connective tissue stroma in many angiogenic and proliferative processes,[235] retinal neovascularization in response to PDGF-BB may be partially due to its direct effects on the formation of fibrovascular retinal membranes.

HGF/SF Hepatocyte growth factor/scatter factor (HGF/SF)[236] is a most potent mitogenic factor for a number of cell types, including hepatocytes,[237] myeloid precursor cells,[238] and various epithelial[239] and endothelial cells.[240] HGF/SF also promotes epithelial and endothelial cell motility in addition to regulating tube morphogenesis and tube branching.[241,242] HGF/SF binds to the c-Met receptor[243] and initiates signaling via activation of both protein kinase C (PKC)[244] and phosphatidylinositol 3-kinase (PI3-Kinase),[245,246] inducing MAPK phosphorylation that is critical for migration and growth. HGF/SF and its receptor levels have been shown to significantly increase in the vitreous of diabetic patients compared to normal control groups.[247] HGF/SF can also induce VEGF production by a variety of cells and tissues.[248–250] Since VEGF does not appear to mediate these initial HGF effects it suggests that HGF/SF acts as a co-factor promoting retinal neovascularization.

PIGF PIGF is a member of the VEGF family and shares 35% primary sequence homology with VEGF.[251] However, unlike VEGF which binds to both VEGFR-1 and VEGFR-2, PIGF binds only to VEGFR-1.[252] Furthermore, PIGF can form a heterodimer with VEGF presumably regulating VEGF-receptor binding through both VEGFR-1 and VEGFR-2. The different affinity for VEGFRs has been shown to influence endothelial cell behaviour. Generally, the ligand-receptor interaction is reflected in the different signal transduction pathways that involve different signal components. For example, as a marker for DNA synthesis, mitogen-activated protein kinase (MAP kinase) is activated in endothelial cells stimulated both by VEGF and PIGF,[253] whereas phospholipase C-γ (PLC-γ) which plays a role in cell migration is only tyrosine phosphorylated by VEGF stimulated cells.[254] *In vitro* studies indicate that VEGF essentially has no effect on Flt-1 expressing cells while PIGF can induce DNA synthesis in Flt-1 expressing cells, leading to mitogenesis but not migration. However, PIGF appears to be different *in vivo* and not only stimulates proliferation of endothelial cells, but also induces angiogenesis.[255] Furthermore, the PIGF/VEGF heterodimer induces angiogenesis more effectively than the PIGF homodimer alone.[256]

There is considerable evidence to support a role for PIGF in diabetic retinopathy. PIGF levels are upregulated in the vitreous of patients with PDR[257,258] and PIGF protein localises to areas of active retinal neovascularisation.[258] Interestingly PIGF was found to induce secretion of VEGF and was co-expressed with VEGF.[257] The PIGF gene has also been shown to be elevated in PDR retinas[259] and in retinas in a mouse model for retinopathy of prematurity.[260] While the origin of PIGF *in vivo* remains to be determined, cell culture studies demonstrate that both endothelial cells and pericytes express PIGF mRNA.[261] PIGF was more highly expressed in endothelial cells compared to pericytes. It is likely that in PDR PIGF potentiates the effect of VEGF either via enhancing expression of VEGF or the formation of a heterodimer with VEGF.[255]

Angiopoietin Another family of tyrosine kinase receptors which play an important role in angiogenesis are the Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) receptors.[262] To date two receptors have been identified, Tie1 and Tie2. No ligand has been identified for Tie1 while the best characterized ligands for Tie2 are angiopoietin1 (Ang1)[263] and angiopoietin2 (Ang2),[264] both sharing 60% amino acid homology. Ang1 and Ang2 appear to have different effects on endothelial cells. Ang1 induces tyrosine phosphorylation of Tie2 and activates the downstream signalling pathway to promote vascular maturation,[265] whereas Ang2 acts as a naturally occurring antagonist of Ang1 by competing for binding to Tie2 and blocking Ang1 induced Tie2 phosphorylation.[264] Ang1 has been reported to induce sprouting and chemotaxis in endothelial cells *in vitro*,[266,267] whereas Ang2 appears to play a critical role in vascular remodelling.[268,269] It has been shown that Ang2 is upregulated during angiogenesis in retinal development[270] and in mouse models of ischaemia-induced retinal neovascularisation.[270,271] A recent study has determined the spatial and temporal expression of Ang1, Ang2 and the Tie2 receptor during the pathogenesis of diabetic retinopathy (Smith, personal communication). Interestingly, Ang1 protein was upregulated in PDR while Ang2 was downregulated suggesting that while angiopoietins play a key role in the pathogenesis of diabetic retinopathy their action may differ depending on the development stage of the vasculature and the type of disease.

Pigment epithelium-derived factor

Pigment epithelium-derived factor (PEDF) is a 50-kDa glycoprotein originally identified in RPE cells.[272] Subsequently, PEDF mRNA has been found in most cell types and appears to have a wide variety of

functions, eg neural development, neuronal protection and as a regulator of neovascularisation.[272,273]

PEDF is seen to be downregulated in eyes with active PDR[274] suggesting that PEDF is a negative regulator of angiogenesis. Support for this comes from studies which have shown that: (a) PEDF regulates the development of the retinal vasculature;[275] (b) is downregulated by hypoxia; and (c) PEDF inhibits retinal and choroidal neovascularisation in a number of animal models.[276,277] That the latter was achieved by adenoviral transfection opens the possibility for gene therapy to upregulate PEDF and inhibit abherrant angiogenesis. It is unclear how PEDF exerts its effect but *in vitro* experiments show that PEDF may inhibit neovascularisation by promoting apoptosis of endothelial cells.[277] The mode of action of PEDF will be clarified once its receptor(s) and downstream transduction pathway(s) have been identified.

Concluding comments

Research over the past few decades has provided ample evidence that hyperglycaemia is one of the main forces driving the onset and progression of diabetic retinopathy. Several mechanisms, by which hyperglyaemia causes retinal capillary damage include increased polyol pathway, activation of protein kinase C, increased non-enzymatic glycation and generation of reactive oxygen species (Figure 1). Furthermore, hyperglycaemia-induced events regulate the synthesis of a variety of growth factors implicated in retinopathy. A number of key growth factors have emerged of which the VEGF and PEDF families are critically important. The question we now ask is can the therapeutic modulation of growth factor pathways prove efficacious in the intervention in diabetic retinopathy at clinic level?

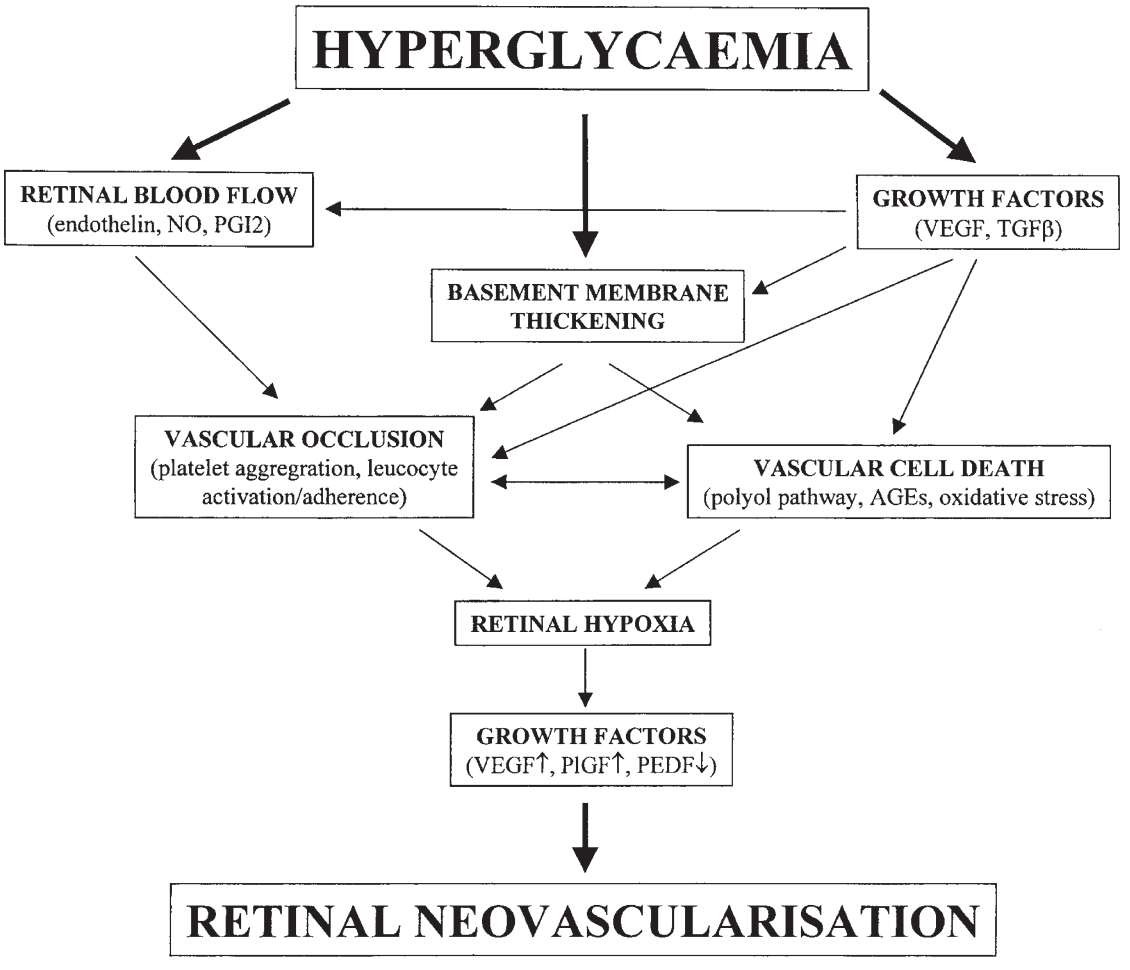

Figure 1 Schematic diagram of the pathogenesis of diabetic retinopathy. Abbreviations: NO, nitric oxide; PGI2, prostacyclin; VEGF, vascular endothelial growth factor; TGFβ, transforming growth factor beta; AGEs, advanced glycation endproducts; PIGF, placenta growth factor; PEDF, pigment epithelium-derived factor.

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